Establishment of Perineural Invasion Models and Analysis of Gene Expression Revealed an Invariant Chain (CD74) as a Possible Molecule Involved in Perineural Invasion in Pancreatic Cancer

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Abstract

Purpose: Perineural invasion causes frequent local recurrence even after resection and a poor prognosis for pancreatic cancer. We established perineural invasion models and analyzed the molecular mechanism of perineural invasion in pancreatic cancer.

Experimental Design: Seven pancreatic cancer cell lines with or without human peripheral nerves were s.c. implanted in nonobese diabetes/severe combined immunodeficient mice. We compared expression profiles among high and low perineural invasion cell lines by using an oligonucleotide microarray. We examined up-regulation of the invariant chain (CD74) in high perineural invasion cell lines in mRNA and protein levels and surgical cases immunohistochemically.

Results: Four of seven pancreatic cancer cell lines (CaPan1, CaPan2, CFPAC, and MPanc96) showed perineural invasion to s.c. transplanted human peripheral nerves. Moreover, CaPan1 and CaPan2 (high perineural invasion group) also resulted in a high frequency of perineural invasion to mouse s.c. peripheral nerves, whereas three pancreatic cancer cell lines HPAFII, AsPC1, and Panc1 (low perineural invasion group) did not show perineural invasion to either human or mouse nerves. We identified 37 up-regulated genes and 12 down-regulated genes in the high perineural invasion group compared with the low perineural invasion group. Among them, CD74 was up-regulated in the high perineural invasion group in mRNA and protein levels. Furthermore, immunohistochemical expression of CD74 in clinical cases revealed its significant overexpression in pancreatic cancer with perineural invasion ($P < 0.008$).

Conclusions: This is the first report of perineural invasion models using human pancreatic cancer cell lines. In combination with gene expression profiling, it was indicated that CD74 could be a candidate molecule involved in perineural invasion. These models provide new approaches for study of perineural invasion in pancreatic cancer.

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States (1). Worldwide pancreatic cancer causes an estimated 213,000 deaths a year (2). In the United States, ~32,180 patients are diagnosed with pancreatic cancer annually, and nearly an equal number will die from the disease (1). When first diagnosed with pancreatic cancer, about 80% of all patients receive palliative therapy instead of surgery because of locally advanced disease, depending on perineural invasion or metastasis. The remaining 20% of patients receiving surgery still have a poor prognosis due to high incidence and early occurrence of local recurrence and hepatic and lymph node metastasis, even after pathologically curative surgery (3–5). It is suspected that microscopic hepatic metastasis is already present (6). Cancer cells spreading in the perineural space even at an early clinical stage also cause local recurrence in the retroperitoneum because of residual tumor cells in the perineural space after surgical resection (7). Moreover, many previous clinicopathologic reports showed that perineural invasion in pancreatic cancer was one of the most significant poor prognostic factors (8, 9).

Genetic alternations seem to be responsible for the development of pancreatic cancer (10). Recently, pancreatic cancer–specific expression profiles using cDNA microarrays (11–13), Affymetrix gene chip (14, 15), and serial analysis of gene expression (16) have been used by many investigators. However, the molecular mechanisms of hepatic metastasis and perineural invasion in pancreatic cancer are far from clear. Some studies reported metastasis-related genes by analyzing gene expression profiles between a highly metastatic variant and a parental pancreatic cancer cell line in an orthotopic

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transplanted nude mouse model (17, 18). However, there are few reports about the molecular mechanisms of perineural invasion of pancreatic cancer due to the lack of good disease models. Understanding perineural invasion at the molecular level is an important step towards the identification of prognostic markers and therapeutic targets for pancreatic cancer treatment.

To analyze the mechanisms of perineural invasion in pancreatic cancer, we constructed perineural invasion models using s.c. implantation of human pancreatic cancer cell lines. CaPan1 and CaPan2 (high perineural invasion group) implanted s.c. into nonobese diabetes/severe combined immunodeficient (NOD/SCID) mice were frequently found to form perineural invasion to the mouse s.c. nerves and transplanted human nerves, whereas three pancreatic cancer cell lines HPAFII, AsPC1, and Panc1 (low perineural invasion group) did not show perineural invasion to either human or mouse nerves. Next, we compared gene expression profiles between the high and low perineural invasion groups using a microarray technique. Of these genes, we further investigated whether *invariant chain* (*CD74*) expression was associated with perineural invasion.

Materials and Methods

Cell culture. The human pancreatic cancer cell lines CaPan1, CaPan2, HPAFII, AsPC1, Panc1, CFPAC, and MPanc96 were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were cultured in RPMI 1640 (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum, 100 μg/mL ampicillin, and 100 μg/mL streptomycin. All cell cultures were done at 37°C under 5% CO_2.

Perineural invasion models in mice. NOD/SCID (NOD/LtSz-scid) mice were maintained in a specific pathogen-free environment. Eight- to 12-week-old mice were used in this experiment. The studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

The human nerve perineural invasion models were prepared as follows. The human nerve plexus around the celiac axis or superior mesenteric artery were obtained from autopsies done until about 6 hours after death in sterile conditions, immediately placed in RPMI 1640 with antibiotics, divided into 1-cm pieces (about 0.25 cm^3), and kept in the medium for a brief period until transplantation. The divided tissue was placed in the s.c. space of NOD/SCID mice under anesthesia. After 4 weeks, each mouse received an injection of a pancreatic cancer cell line near the s.c. transplanted tissue with 7×10^6 viable tumor cells, which were harvested from subconfluent cultures, under the appropriate anesthetic procedure. Five to 8 weeks later, when the tumor grew up to about 1.5 cm in long diameter, after inoculation, mice were sacrificed, and autopsies were done immediately. The s.c. tissue was removed, fixed in 10% formalin, cut into 2- to 3-mm-thick slices, and embedded in paraffin. The samples were processed for histologic examination.

The mouse nerve perineural invasion models were prepared as follows. Each mouse received a s.c. injection of 7×10^6 viable pancreatic cancer cells on the midline of the mouse's back and processed by the same protocol as above. All animals tolerated each procedure well.

RNA preparation and oligonucleotide array. Total RNA was extracted from cells with an RNAeasy Mini kit (Qiagen, Hilden, Germany). According to the manufacturer's protocol (Affymetrix, Santa Clara, CA), biotin-labeled cRNA was synthesized from 5 mg of total RNA, hybridized to the GeneChip HG-U133A, stained with streptavidin-phycoerythrin, and then detected by scanning. Each of the scanned images was normalized as mean signal intensity of all probe sets at 1,000. Two-dimensional clustering analysis using the standard correlation as a similarity measure was done by the software, GeneSpring version 6.2 (Silicon Genetics, Redwood City, CA).

Real-time quantitative reverse transcription-PCR analysis. Real-time quantitative reverse transcription-PCR (RT-PCR) analysis was done as follows. The primer set 5′-GACCTTATCTCCAACAATGAGCAAC-3′ (forward) and 5′-AGCAGAGTCACCAGGATGGAA-3′ (reverse) was used for CD74. To standardize the amount of RNA, expression of

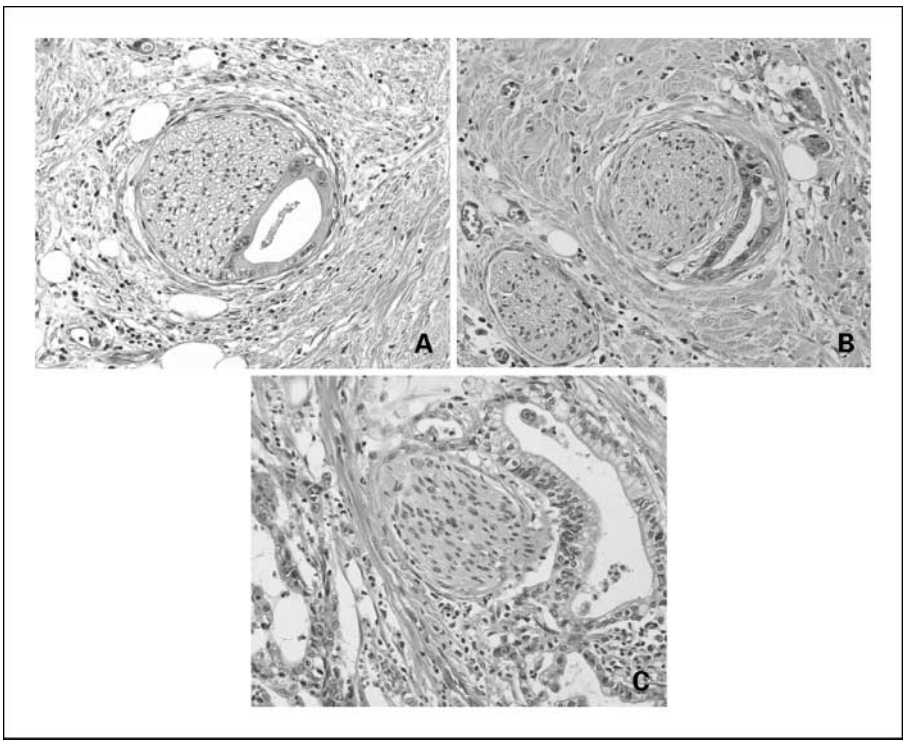

Fig. 1. Three patterns of relationships between cancer cells and peripheral nerves. Perineural invasion: cancer cells invaded to the perineural space between the perineurium and endoneurium of the peripheral nerve with direct contact with the endoneurium (*A*). Epineural invasion: cancer cell invaded along the perineurium without contact with the endoneurium (*B*). Nerve involvement: cancer nests included nerves without direct mutual contact (*C*). H&E; magnification × 200.

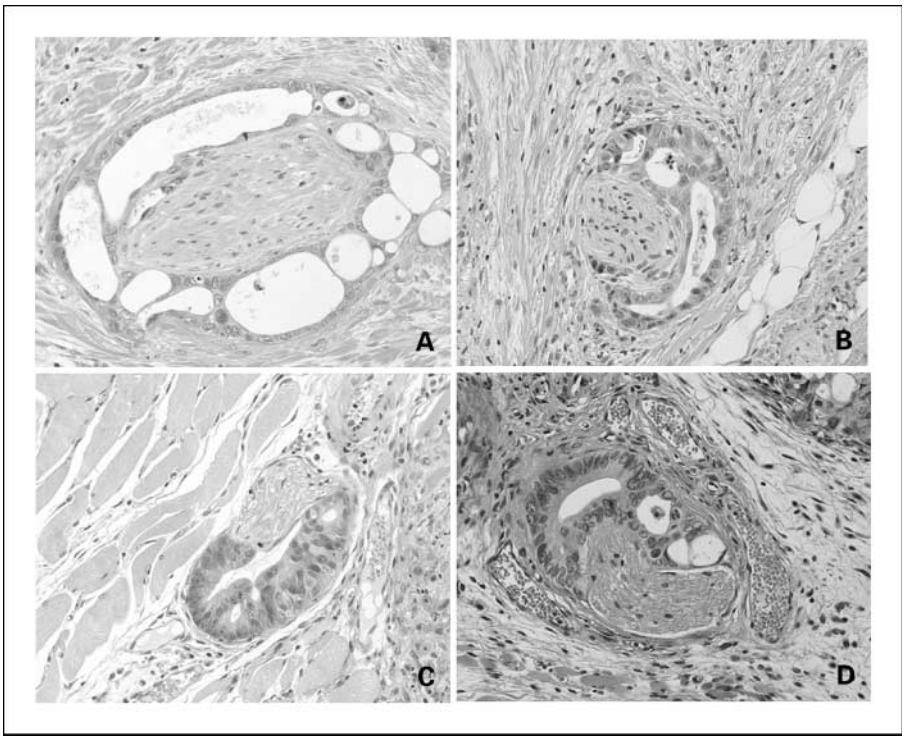

Fig. 2. Perineural invasion to human and mouse nerves. Perineural invasion in a human nerve perineural invasion model (*A,* CaPan2; *B,* CFPAC) and perineural invasion in a mouse nerve perineural invasion model (*C,* CaPan1; *D,* CaPan2). H&E; magnification × 200.

glyceraldehyde-3-phosphate dehydrogenase in each sample was quantified by using the primer set 5′-GCACCGTCAAGGCTGAGAAC-3′ (forward) and 5′-ATGGTGGTGAAGACGCCAGT-3′ (reverse), and the amount of CD74 expression was divided by that of the glyceraldehyde-3-phosphate dehydrogenase in each sample. All PCR reactions were done under the following conditions: one cycle at 95°C for 10 seconds and then 40 cycles at 95°C for 5 seconds and 60°C for 30 seconds. Real-time detection of the emission intensity of SYBR Green (Qiagen) was done using an ABI prism 7000 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster city, CA), using a previously reported method (19). Quantitative RT-PCR was done at least thrice.

Western blotting. All 20-μg cell lysates were subjected to 10% SDS-PAGE and then separated proteins were transferred to Hybond-P (Amersham Biosciences, Buckinghamshire, United Kingdom). After blocking, an anti-CD74 mouse monoclonal antibody (LN 2; DAKO, Glostrup, Denmark) was used at a dilution of 1:1,000 overnight at 4°C. The membrane was incubated with a horseradish peroxidase–conjugated secondary antibody (DAKO) and visualized by using an enhanced chemiluminescence kit (Amersham Biosciences). A Burkitt's lymphoma (Raji) cell line was used as a positive control for CD74.

Immunofluorescence. CaPan2 cell lines were grown on glasses and fixed in 8% paraformaldehyde for 20 minutes on ice. They were incubated with an anti-CD74 antibody for 1 hour followed by a FITC-labeled secondary antibody (DAKO). Texas Red-X phalloidin (Molecule Probes, Eugene, OR) was used to visualize filamentous-actin. Slides were examined using an Olympus fluorescence microscope with a MRC-600 scanning laser confocal apparatus (Olympus, Tokyo, Japan).

Patients and tissue samples. For immunohistochemical analysis, 67 invasive ductal pancreas adenocarcinomas were analyzed. Sections were prepared from formalin-fixed, paraffin-embedded tissues of samples resected surgically between 1991 and 2004. This study was conducted under the approval of the Ethics Committee of Keio University, School of Medicine. Histologic diagnoses were made according to WHO criteria or the Japan pancreas society classification (20). Perineural invasion, which was frequently observed in surgical specimens, was defined as cancer cell invasion to the perineural space between the perineurium and endoneurium of the peripheral nerve with direct contact to the endoneurium (Fig. 1A), whereas epineural invasion, where cancer cells invade along the perineurium without contact to the endoneurium (Fig. 1B), and nerve involvement, where a cancer nest included nerves without direct contact between them (Fig. 1C), were excluded from perineural invasion (21). Intraneural invasion was also included in perineural invasion.

The degree of perineural invasion was defined microscopically as follows: 0, no perineural invasion; 1, perineural invasion was difficult to find with only one to three occurrences in the lesions; 2,

Table 1. Incidence of perineural invasion in mouse nerve perineural invasion models

	CaPan1	CaPan2	HPAFII	AsPC1	Panc1	*P**
No. mice examined	11	13	11	11	5	
Perineural invasion	6†	9	0	0	0	0.000004
Epineural invasion	3	6	3	4	0	0.216
Nerve involvement	6	8	5	7	3	0.615

*CaPan1 and CaPan2 vs HPAFII, AsPC1, and Panc1.
†Each value indicates the number of mice where perineural invasion, epineural invasion, and nerve involvement was observed, respectively.

perineural invasion was easy to find, in between 1 and 3; and 3, perineural invasion was even easier to find, with more massive occurrences in the lesions and extension beyond the border of the main tumor mass.

Immunohistochemistry. Each section was deparaffinized, rehydrated, and incubated with fresh 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature and then heated in 10 mmol/L citrate buffer (pH 6) for 10 minutes after washing in PBS. Normal goat serum (Invitrogen, Carlsbad, CA) was applied for 30 minutes and removed. The section was then incubated with anti-CD74 antibody at a dilution of 1:100 overnight at 4°C, washed thrice in PBS, and incubated with a secondary antibody for 30 minutes at room temperature. A simple stain MAX-PO (Nichirei, Tokyo, Japan) was used to detect the antibody signals as the secondary antibody. Staining was evaluated by three independent observers (N.K., R.S., and M.S.). An equal staining to lymphocytes was considered positive. The positivity index was expressed as the percentage of positive cancer cells in each lesion. The cases with ≥10% positive cells were defined as CD74 positive.

Statistical analysis. Data are expressed as mean ± SD. The level of CD74 mRNA in the high and low perineural invasion groups was compared using the Mann-Whitney *U* test. The other correlations were analyzed by the χ^2 test. All statistical analyses were done using Statcel2 (OMS Publisher, Saitama, Japan). The results were judged significant at $P < 0.05$.

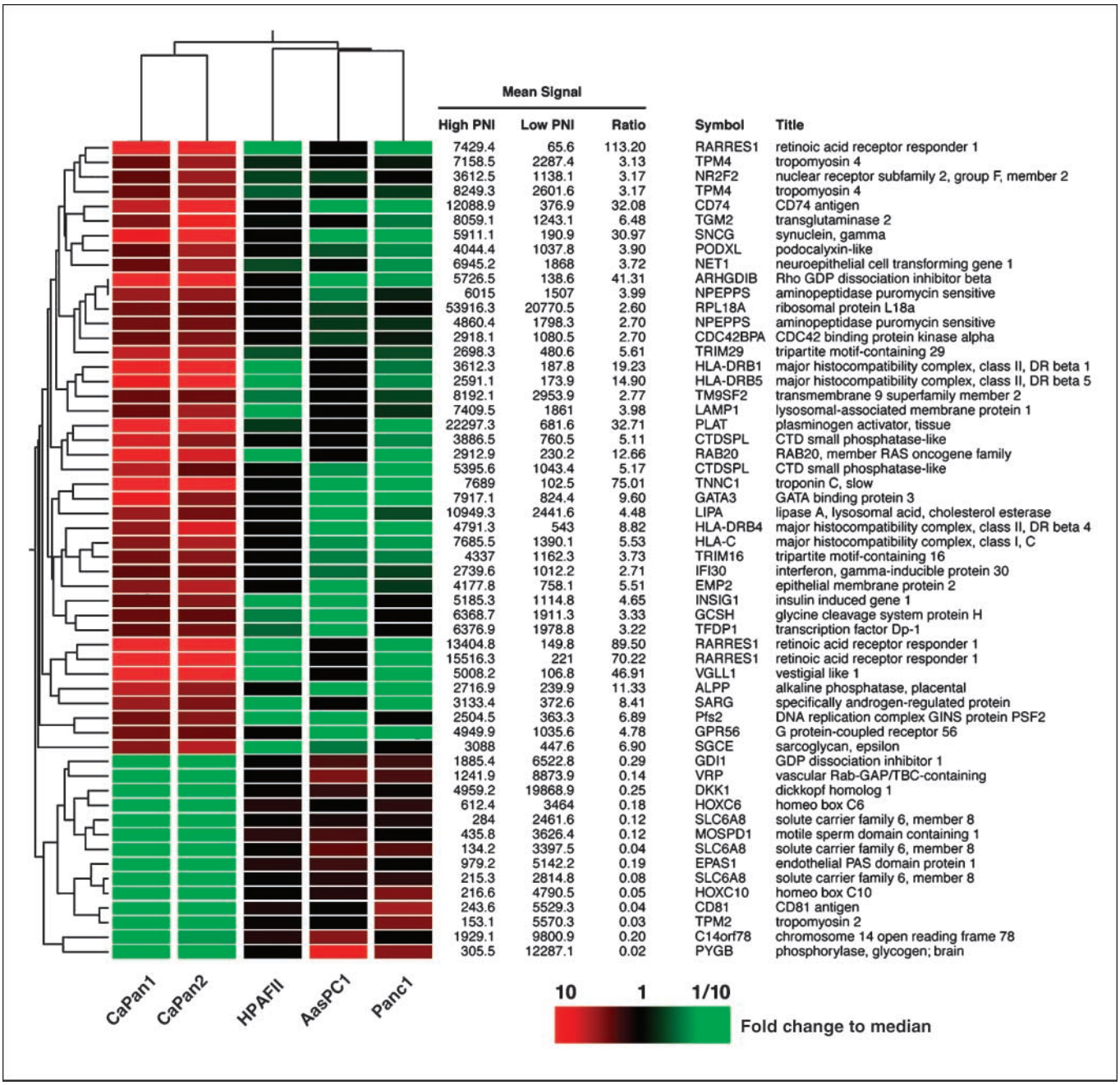

Fig. 3. Two-way hierarchical clustering algolithm. Forty-nine genes displayed a ≥2-fold increase or decrease in expression level. Each color patch in the resulting visual map represents the expression level of the associated gene in the cell line sample, with a continuum of expression levels from *green* (lowest) to *bright red* (highest). A two-way hierarchical clustering algorithm successfully distinguished between the high and low perineural invasion groups. The scale bar reflects the fold increase (*red*) or decrease (*green*) for any given gene relative to the median level of expression across all samples.

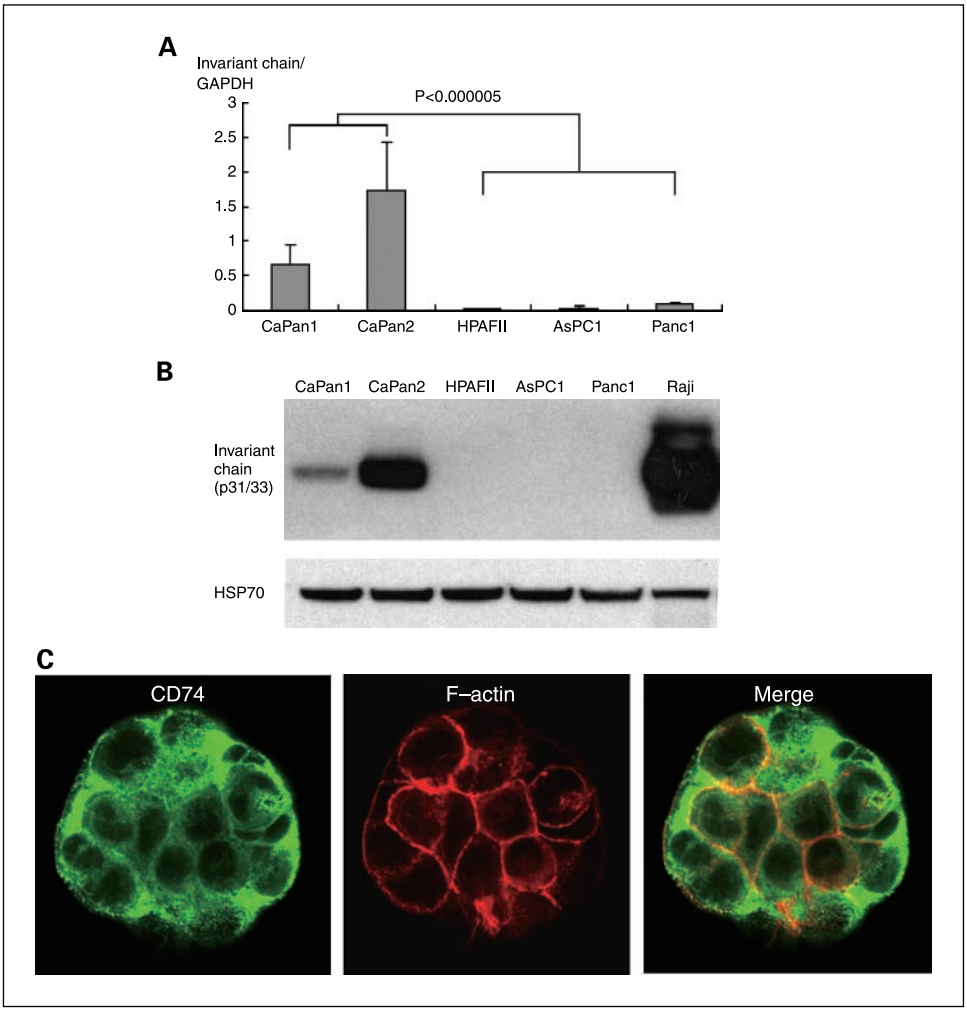

Fig. 4. CD74 expression in pancreatic cancer cell lines. *A,* real-time quantitative RT-PCR analysis of CD74. The expression levels were normalized with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA in each sample. *B,* Western blotting analysis of the CD74 protein. Each cell lysate (20 μg) was analyzed using an anti-CD74 antibody. HSP70 was used as an internal control. *C,* immunofluorescence analysis of CD74 and filamentous actin (*F-actin*) in CaPan2. CaPan2 cells were stained for CD74 (*green*) and filamentous actin (*red*).

Results

Subcutaneous implantation of pancreatic cancer cell lines. To analyze the potential of perineural invasion, seven pancreatic cancer cell lines were s.c. implanted in NOD/SCID mice with human nerve tissue. CaPan1, CaPan2, CFPAC, and MPanc96 showed perineural invasion to human nerves (Fig. 2A and B). Therefore, we confirmed that even cell lines preserved their ability to show perineural invasion to human nerve–like clinical cases. However, pancreatic cancer cell lines frequently did not invade to the transplanted human nerve tissue, probably due to the adverse effect of accompanying inflammatory cells in nerve tissue. Thus, it was difficult to evaluate these models quantitatively. Because we observed these cells also presented perineural invasion to mouse s.c. peripheral nerves (Fig. 2C and D), we employed mouse nerve perineural invasion models for further analyses of perineural invasion.

To analyze quantitatively the frequency of perineural invasion of pancreatic cancer cell lines in mouse s.c. nerves, we selected five cell lines (CaPan1, CaPan2, HPAFII, AsPC1, and Panc1) and implanted these cell lines in the s.c. tissue of 5 to 13 NOD/SCID mice and analyzed the frequency of neural invasion to the mouse s.c. nerves. Perineural invasion of CaPan1 and CaPan2 cells was seen in 6 of 11 and 9 of 13 implanted mice, respectively. In contrast, the other three cell lines did not show perineural invasion, whereas each cell line showed epineural invasion and nerve involvement to the mouse s.c. nerves. These findings indicated that CaPan1 and CaPan2 (high perineural invasion group) had greater potential for perineural invasion *in vivo*, whereas the other three cell lines (low perineural invasion group) had little potential for perineural invasion ($P < 0.000004$; Table 1).

Two-way hierarchical clustering algorithm. To identify genes generally involved in perineural invasion, we compared the expression profiles of the high and low perineural invasion groups. We filtered all genes, with the following limits: (*a*) presence with signal at >2,000; (*b*) >2-fold increase or decrease in average difference between each high perineural invasion cell line and the low perineural invasion group and the high perineural invasion group and each low perineural invasion cell line. In the 49 genes selected under the above criteria, 37 were up-regulated, and 12 were down-regulated in the high perineural invasion group compared with the low perineural invasion group. A two-way hierarchical clustering algorithm successfully distinguished between the high and low perineural invasion groups (Fig. 3).

CD74 expression in pancreatic cancer cell lines. From the genes listed in Fig. 3, we further investigated *CD74*, because it was one of the most highly expressed genes in the high perineural invasion group and is thought to be a membrane

protein that might mediate cancer cell-nerve interaction. To confirm overexpression of CD74 in the high perineural invasion group, we analyzed the level of CD74 mRNA by real-time quantitative RT-PCR. The average relative expression level of CD74 (CD74/glyceraldehyde-3-phosphate dehydrogenase) was significantly higher in the high perineural invasion group than in the low perineural invasion group ($P < 0.000005$), although CaPan1 expressed less CD74 than CaPan2 (Fig. 4A). Next, we used an antibody against CD74 to investigate protein expression of CD74 in five pancreatic cancer cell lines. As in the RT-PCR analysis, CaPan2 and CaPan1 showed significantly higher expression of CD74 than the low perineural invasion group (Fig. 4B). Immunocytochemically, CD74 expression in CaPan2 was found on the cell surface and also more intensely in the cytoplasm (Fig. 4C). Immunohistochemical expression of CD74 in mouse s.c. tumors of these cell lines also showed a similar expression pattern and confirmed the usefulness of the CD74 antibody in immunohistochemistry (Fig. 5A).

Immunohistochemical expression of CD74 in pancreatic cancer tissues. To determine whether CD74 is also overexpressed at the protein level in human pancreatic cancer tissues and involved in perineural invasion, we examined CD74 expression in the surgical cases with an immunohistochemical study. Pancreatic ducts in noncancerous tissues of the normal pancreas or chronic pancreatitis were almost negative or focal immunostaining. However, the lymphocytes always stained strongly and thus served as an internal control of positive staining (Fig. 5B). Neural tissue and acinar cells also showed moderate staining focally. Some pancreatic cancer without perineural invasion showed little or moderate CD74 immunoreactivity, whereas pancreatic cancer with perineural invasion tended to show strong CD74 immunoreactivity (Fig. 5C and D). Strong immunoreactivity was generally observed in the cytoplasm (granular or diffuse) and sometime along the cell membranes of cancer cells. We evaluated the relationship between CD74 and clinicopathologic features. Table 2 shows that degree of perineural invasion was significantly associated with CD74 expression ($P < 0.008$). Other clinicopathologic factors were not associated with CD74 expression, although it seemed to decrease with age ($P < 0.025$).

Discussion

Perineural invasion, together with liver metastasis, is one of the poorest prognostic factors after curative resection (22, 23). Many studies on the perineural invasion of pancreatic cancer and other cancers were clinocopathologic studies using surgical specimens or molecular studies associated with neurotrophins (21, 24–28). Recently, *in vitro* models of perineural invasion using prostate cancer cell lines and mouse dorsal nerves were reported and suggested to be useful to investigate the mechanism of perineural invasion *in vitro* (29, 30). In the present study, we successfully established a perineural invasion model *in vivo* for the first time. We transplanted normal human peripheral nerves into NOD/SCID mice because these mice are an excellent tool to establish human tissue–specific metastasis models (31, 32). As expected, nerve tissues were intact even about 100 days after transplantation. In addition, we showed that pancreatic cancer cell lines preserved their ability of perineural invasion to human nerves. Moreover, they had the ability of perineural invasion to mouse s.c. nerve, too. Finally, using a mouse nerve perineural invasion model, we could clearly separate the high and low perineural invasion groups.

To elucidate the characteristic changes associated with perineural invasion, we globally analyzed the gene expression in the high and low perineural invasion groups. Among up-genes, we further investigated the CD74 molecule and

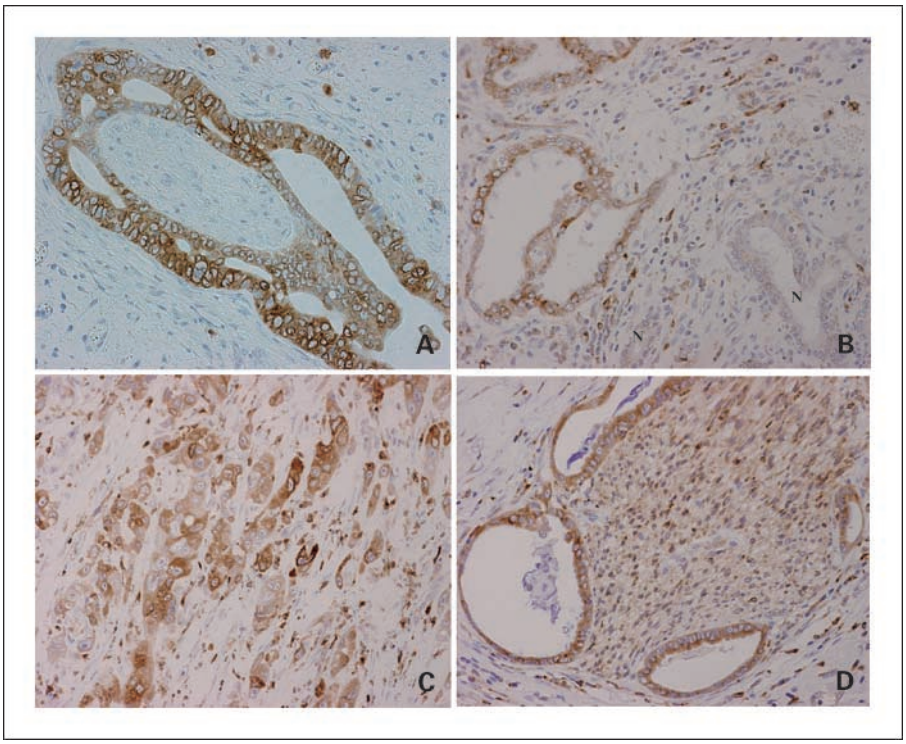

Fig. 5. Immunohistochmical staining of CD74. *A,* CD74 was diffusely expressed in the cytoplasm and cell membrane of CaPan2 demonstrating perineural invasion. *B,* CD74 was negative in the normal pancreatic duct (*N*) but strongly positive in cancer cells and lymphocytes. CD74 was diffusely expressed in poorly differentiated (*C,* perineural invasion 3) and well differentiated (*D,* perineural invasion 3) pancreatic cancer with perineural invasion. Magnification × 200.

Table 2. Immunohistochemical examination of CD74 in human pancreatic cancer tissues

	CD74 expression		*P*
	–	+	
No. cases	15	52	
Mean age (y)	70.5 ± 9.3	63.9 ± 9.6	0.025
Sex			
Male	10	34	0.9267
Female	5	18	
Differentiation			
Well	7	17	0.499
Intermediate	7	33	
Poor	1	2	
Stroma			
Medullary	0	0	0.622
Intermediate	5	21	
Scirrhous	10	31	
INF			
α	0	0	0.718
β	5	20	
γ	10	32	
Lymphatic invasion			
0-1	11	25	0.073
2-3	4	28	
Lymph node metastasis			
Absent	8	14	0.055
Present	7	38	
Vascular invasion			
0-1	10	24	0.161
2-3	5	28	
Perineural invasion			
0-1	10	15	0.008
2-3	5	37	

confirmed its overexpression in pancreatic cancer and association with perineural invasion. Because two cell lines in high perineural invasion group are better differentiated than three cell lines in low perineural invasion group (33), some of the selected genes might represent tumor differentiation but not perineural invasion. However, it was not likely in case of CD74, because there was no correlation between CD74 expression and tumor differentiation in tumor specimens.

CD74 was originally identified as a γ chain that is associated with the α and β chains of HLA-DR (MHC class II). Many studies have shown that CD74 plays an important role in the assembly, transport, and loading of peptides by HLA-DR molecules. HLA-DR and CD74 interacted in the endoplasmic reticulum immediately after their synthesis. This interaction of CD74 prevented binding by HLA-DR molecules to endogenous peptides (34). This was initially observed on antigen-presenting cells, but recently, it was reported that some malignant cells also expressed CD74, suggesting that CD74 expression might prevent presentation of tumor antigens (35–37). Moreover, preclinical studies in B-cell lymphoma and multiple myeloma showed CD74 was a novel and promising therapeutic target because binding of anti-CD74 monoclonal antibodies became rapidly cointernalized (38–40). Recently, serial analysis of gene expression showed CD74 overexpression in pancreatic cancer (16). In the present study, we confirmed that CD74 was positive in 52 cases (78%) and was overexpressed in cancer cells compared with the pancreatic duct in clinical cases. We suggest that CD74 might also be a novel therapeutic target in pancreatic cancer, especially with perineural invasion.

Although the intracellular portion of CD74 seems to lack a signal-transducing domain, cell proliferation and inhibition of apoptosis through extracellular signal-regulated kinase 1/2 are induced by binding of macrophage migration inhibitory factor (41). Secretion of macrophage migration inhibitory factor is seen in both pancreatitis and pancreatic cancer and induces the production or expression of inflammatory molecules, including IFN-γ (42). IFN-γ also induces further expression of cell surface CD74 (43). Hence, CD74 and these associated molecules might play an important role in the perineural invasion of pancreatic cancer in an autocrine/paracrine manner. Moreover, CD74 has a chondroitin sulfate binding site at the extracellular portion, and this can bind cell surface CD44 (44). CD44 is also expressed in pancreatic cancer and human peripheral nerves. Regarding cancer cell-nerve interaction, interaction of CD74 with CD44 may result in cellular activation and migration and promote perineural invasion.

In addition to CD74, some of the up-regulated genes identified in the present analysis have been recently correlated with tumor growth and invasion in pancreatic cancer. For example, tissue plasminogen activator is a serine protease that catalyses the activation of plasminogen. It is required for cell proliferation and angiogenesis in the early stage of pancreatic cancer (45). γ-Synuclein was initially found in infiltrating breast cancer and was referred to as breast carcinoma-specific gene 1. Overexpression of γ-synuclein protein in pancreatic cancer was correlated with perineural invasion and lymph node metastasis in clinical cases (46).

In conclusion, although the precise mechanism remains to be elucidated, overexpression of CD74 was closely associated with perineural invasion in human pancreatic cancer. We think that this perineural invasion model is the first such report and is an appropriate model for analyzing the mechanism of perineural invasion of human pancreatic cancer.

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