Increased Expression of *ADAM 9* and *ADAM 15* mRNA in Pancreatic Cancer

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Abstract. *Background: A disintegrin and metalloproteases (ADAMs) comprise a multifunctional family of membrane-anchored proteins. ADAM 9 and ADAM 15 are involved in cell migration and invasion. Expression of ADAM 9 and ADAM 15 was reported to be altered in several types of cancer. Materials and Methods: Quantitative real-time reverse transcription-polymerase chain reaction was performed to measure the expression of ADAM 9 mRNA in bulk pancreatic tissues. Results showed no significant difference in the expression of ADAM 9 mRNA between pancreatic cancer and non-neoplastic pancreas. Primary cultured pancreatic fibroblasts also expressed ADAM 9 mRNA. Therefore, a laser microdissection and pressure catapulting technique was employed to isolate cancer cells from tumor tissues. The expression of ADAM 9 and ADAM 15 mRNA was measured in microdissected samples (cancer cells, n=11; normal epithelial cells, n=13 for ADAM 9; cancer cells, n=9; normal epithelial cells, n=9 for ADAM 15). Results: Pancreatic cancer cells expressed significantly higher levels of ADAM 9 and ADAM 15 mRNA than did normal pancreatic epithelial cells (p=0.016 for ADAM 9; p=0.004 for ADAM 15). Conclusion: ADAM 9 and ADAM 15 are involved in pancreatic cancer. Microdissection-based analysis appears to be indispensable for the accurate analysis of the expression of certain ADAM family members in pancreatic cancer.*

Pancreatic cancer is one of the most lethal cancers with an overall 5-year survival rate after resection of approximately 10-20% (1). Despite improvements in chemotherapy and radiotherapy, the prognosis for pancreatic cancer has remained poor for decades because of its extremely aggressive nature. In order to improve the prognosis for pancreatic cancer, the identification of novel target genes for therapy is necessary.

Approximately 40 members have been identified of the family of multifunctional membrane-anchored proteins known as ADAMs (a disintegrin and metalloproteases). ADAMs are composed of a metalloprotease, a disintegrin, a cysteine-rich, an epidermal growth factor (EGF)-like, a transmembrane and a cytoplasmic domain (2). Each domain exerts its own unique functions. The metalloprotease domain cleaves the ectodomains of cytokines, growth factors, receptors and other molecules. The disintegrin domain binds integrins and is involved in cell-cell and cell-matrix interactions (3). Each ADAM exerts diverse functions according to the targets of the metalloprotease domain and the binding sites of the disintegrin domain.

ADAM 9 has been reported to be overexpressed in several cancers (4-9). Targets of the ADAM 9 metalloprotease domain are epidermal growth factor receptor (EGFR) ligands (10), and the disintegrin domain of ADAM 9 binds alfa(v)beta(5) (11), alfa(9)beta(1) (12) and alfa(6)beta(1) (13) integrins and degrades extracellular matrix (ECM) components including fibronectin and gelatin (12). On the basis of these functions, ADAM 9 is believed to be involved in malignancy. Many recent studies have suggested a relation between ADAM 9 and various cancers. ADAM 9 has been reported to be up-regulated in breast (4, 5) and prostate cancer (6) and has been associated with tumor aggressiveness and progression in liver (7) and non-small cell lung cancer (9). Gene expression profiling by microarray has suggested that *ADAM 9* is overexpressed in pancreatic cancer and its cell lines (14, 15). Immunohistochemistry has also suggested that ADAM 9 expression is associated with poor tumor differentiation and poor patient prognosis in pancreatic cancer (16).

ADAM15 has been reported to be dysregulated in several cancers (17-19). Targets of the ADAM 15 metalloprotease domain include amphiregulin, epiregulin (20) and CD23 (21). Amphiregulin and epiregulin constitute EGFR ligands. The disintegrin domain of ADAM 15 binds alfa(5)beta(1)

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(22), alfa(v)beta(3) (23) and alfa(9)beta(1) (24) integrins and degrades ECM components including Type IV collagen and gelatin (12). On the basis of these functions, *ADAM 15* is also thought to be involved in malignancy. ADAM 15 and ADAM 9 have been shown to be up-regulated in gastric cancer (17). ADAM 15 expression has also been reported to be associated with aggressive prostate and breast cancers (18). Schutz *et al*. (19) reported that ADAM 15 was frequently detected in lung carcinoma cell lines and tissues, and that lung cancer cells located at the invasion front expressed significantly higher levels of ADAM 15 than those located within the tumor center. To date, however, there have been no reports regarding the expression of ADAM 15 in pancreatic cancer.

In the present study, to clarify the involvement of ADAM 9 and ADAM 15 in pancreatic cancer, we investigated mRNA expression using one-step quantitative real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR).

Materials and Methods

Cultured cells and pancreatic tissues. The following 13 human pancreatic cancer cell lines were used: ASPC-1, BxPC-3, KP-1N, KP-2, Panc-1 and SUIT-2 (Dr. H. Iguchi, National Kyushu Cancer Center, Fukuoka, Japan); MIA PaCa-2 (Japanese Cancer Resource Bank, Tokyo, Japan); Capan-1, Capan-2, CFPAC-1, SW1990 and HS766T (American Type Culture Collection, Manassas, VA, USA), and NOR-P1 (established from a metastatic subcutaneous tumor of a patient with pancreatic cancer; 25). Six primary cultures of pancreatic fibroblasts derived from patients with invasive pancreatic adenocarcinoma were used in the present study. Cells were maintained as described elsewhere (26). Tissue samples were obtained during surgery at Kyushu University Hospital (Fukuoka, Japan) during the period February 15, 2001 to July 15, 2005, as described elsewhere (27). A total of 23 pancreatic cancer tissue samples were obtained from the primary tumor of each resected pancreas. The diagnosis of pancreatic cancer was confirmed by histological examination of resected specimens. Twelve non-neoplastic pancreata were obtained away from the pancreatic tumor or were normal pancreata resected due to cholangiocarcinoma, as described elsewhere (27). Experienced pathologists performed histological examination of all tissues adjacent to the specimens. Written informed consent was obtained from all patients and the study was approved by our institution's surveillance committee and conducted according to the Helsinki Declaration.

Isolation of total RNA. Total RNA was extracted according to the standard acid guanidinium thiocyanate phenol chloroform protocol (28), with glycogen (Funakoshi, Tokyo, Japan). Pancreatic cancer cells and normal pancreatic epithelial cells were isolated from frozen sections using a laser microdissection and pressure catapulting system (LMPC) and then extracted total RNA from these isolated cells. RNA extracts were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Rockland, DE, USA), at 260 nm and 280 nm. RNA integrity was assessed with an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA).

Quantitative analysis of ADAM 9 and ADAM 15 mRNA levels via one-step real-time RT-PCR with gene-specific primers. One-step quantitative real-time RT-PCR was used with gene-specific primers to examine mRNA levels of *ADAM 9* and *ADAM 15*. We designed specific primers (ADAM 9 forward: 5'-gttcctgtggagcaaagagc-3', reverse: 5'-ccagcgtccaccaacttatt-3'; ADAM 15 forward: 5'-agcctcaaaaaggtgcttca-3', reverse: 5'-ccctggtagcagcagttctc-3'; 185 rRNA forward: 5'-ccatccaatcggtagtagcg-3', reverse: 5'-gtaacccgttgaaccccatt-3') BLAST searches to ensure the specificity of these primers. One-step quantitative real-time RT-PCR with gene-specific primers was performed with a QuantiTect SYBR Green RT-PCR Kit (Qiagen K.K., Tokyo, Japan) and a LightCycler Quick System 350S (Roche Diagnostics K.K., Tokyo, Japan). The reaction mixture was incubated at 50˚C for 20 min to allow for reverse transcription, during which first-strand cDNA was synthesized by priming with a gene-specific primer. PCR was initiated with one cycle of 95˚C for 15 min to activate modified Taq polymerase followed by 40 cycles of 94˚C for 15 sec, 55˚C for 25 sec and 72˚C for 10 sec, followed by one cycle of 95˚C for 0 sec, 65˚C for 15 sec and +0.1˚C/s to 95˚C for melting analysis to visualize nonspecific PCR products, different fragments showed separate distinct melting peaks. Each primer set used in the present study produced a single melting peak and a single prominent band of expected size on microchip electrophoresis. Each sample was run twice and any sample showing greater than 10% deviation from the RT-PCR value was tested a third time. Levels of mRNA in each sample were calculated from a standard curve generated with total RNA from Capan-1 human pancreatic cancer cells. Levels of *ADAM 9* and *ADAM 15* mRNA were normalized to that of *18S rRNA*. Samples were used that showed ADAM levels greater than the levels of *18S rRNA* corresponding to 0.05 ng of total RNA derived from Capan-1 cells. When levels of *ADAM 9* or *ADAM 15* mRNA were not detected due to the limitation of our real-time RT-PCR-based assay (the level corresponding to 0.01 ng of total RNA derived from Capan-1 cells), expression values were defined as this level.

Microdissection-based quantitative analysis of ADAM 9 and ADAM 15 mRNA. Frozen tissue samples were cut into 8-μm-thick sections. One section was stained with hematoxylin and eosin (H&E) for histological examination. We used a laser microdissection and pressure catapulting system (LMPC; PALM MicroLaser Technologies AG, Bernried, Germany), according to the manufacturer's instructions to selectively isolate pancreatic cancer cells from 11 sections and normal pancreatic epithelial cells from 13 sections for quantification of *ADAM 9* mRNA, and pancreatic cancer cells from 9 sections and normal pancreatic epithelial cells from 9 sections for quantification of *ADAM 15* mRNA. After microdissection, total RNA was extracted from the isolated cells and was subjected to one-step real-time RT-PCR for quantitative measurement of *ADAM 9* or *ADAM 15* mRNA.

Statistical analysis. Data were analyzed using the Mann-Whitney *U*-test because the data did not follow a normal distribution. Statistical significance was set at $p<0.05$.

Results

Quantitative analysis of ADAM 9 mRNA expression in bulk tissues of pancreatic cancer and non-neoplastic pancreas. ADAM 9 mRNA expression did not differ significantly between bulk tissues of pancreatic cancer and non-

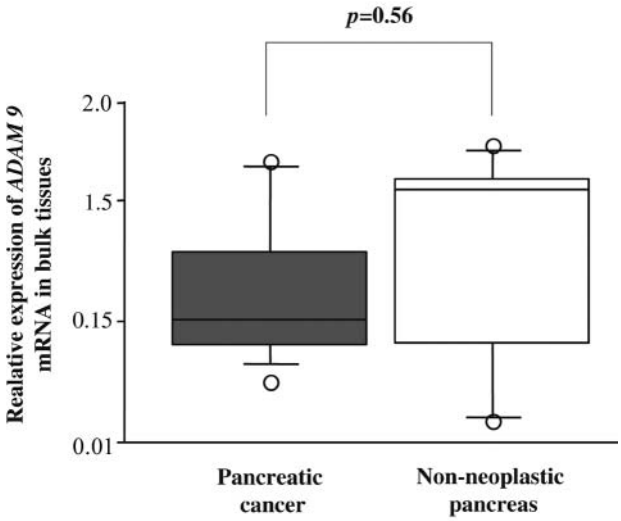

Figure 1. *Quantitative analysis of ADAM 9 mRNA expression in bulk tissues of pancreatic cancer and non-neoplastic pancreas. Expression of ADAM 9 mRNA was measured using one-step quantitative real-time RT-PCR with gene-specific primers and was normalized to that of 18S rRNA. Expression of ADAM 9 mRNA did not differ significantly between bulk tissues of pancreatic cancer and non-neoplastic pancreas (p=0.56).*

neoplastic pancreas (Figure 1, $p=0.56$). Pancreatic cancer tissues typically contain a few carcinoma cells amid a large amount of other structures such as stromal components composed of fibroblasts, endothelial cells and inflammatory cells. Non-neoplastic pancreata included in the present study were typically taken from the distal normal portion of resected pancreata with pancreatic tumors. The histological appearance of these non-neoplastic pancreata showed chronic pancreatitis, possibly secondary pancreatitis due to the occlusion of pancreatic ducts by tumor. These pancreata were also typically composed of various components including inflammatory lesions, stromal lesions and remnant normal structures. These findings suggest that analysis of bulk tissues does not accurately reflect expression levels of *ADAM 9* mRNA in pancreatic cancer cells and normal pancreatic epithelial cells.

Quantitative analysis of ADAM 9 and ADAM 15 mRNA expression in human pancreatic cancer cell lines and primary cultures of pancreatic fibroblasts. The present bulk tissue analysis showed no significant difference in the level of *ADAM 9* mRNA between bulk tissues of pancreatic cancer and non-neoplastic pancreas. We performed histological examination of these tissues and found that most of the pancreatic cancer samples and a subset of the non-neoplastic pancreas samples consisted predominantly of stromal components. In order to evaluate the effect of stromal cells in these tissues on *ADAM 9* and *ADAM 15* mRNA expression, we measured *ADAM 9* and *ADAM 15* mRNA in primary cultures of pancreatic fibroblasts and compared these levels with those in cultured pancreatic cancer cell lines.

Primary cultures of pancreatic fibroblasts showed levels of *ADAM 9* mRNA similar to those of pancreatic cancer cell lines (Figure 2A). These results suggest that the presence of fibroblasts in bulk tissues may affect the expression profiles of *ADAM 9* mRNA in cancer. Pancreatic cancer cell lines also showed significant levels of *ADAM 9* mRNA, but we found no correlation between the histological grade of differentiation and the level of *ADAM 9* mRNA.

The levels of *ADAM 15* mRNA in primary cultures of pancreatic fibroblasts were much lower than those in pancreatic cancer cell lines (Figure 2B). All of the pancreatic cancer cell lines examined in the present study expressed significant levels of *ADAM 15* mRNA, but we found no correlation between the histological grade of differentiation and the level of *ADAM 15* mRNA.

Quantitative analysis of ADAM 9 and ADAM 15 mRNA expression in microdissected pancreatic cancer cells and normal pancreatic epithelial cells. On the basis of the results obtained with bulk tissues and cultured cells, we performed LMPC to isolate pancreatic cancer cells and normal pancreatic epithelial cells so as to obtain accurate *ADAM 9* and *ADAM 15* mRNA expression profiles. We then measured *ADAM 9* and *ADAM 15* mRNA expression in these cells with one-step quantitative real-time RT-PCR with gene-specific primers. We typically isolated pancreatic cancer cells from invasive components of pancreatic cancer tissues and normal epithelial cells from normal pancreata or chronic pancreatitis-affected pancreata. Microdissected pancreatic cancer cells showed significantly higher levels of *ADAM 9* mRNA (median, 1.79) than did normal pancreatic epithelial cells (median, 0.85; $p=0.016$) (Figure 3A). Similarly, microdissected pancreatic cancer cells showed significantly higher levels of *ADAM 15* mRNA (median, 0.12) than did normal pancreatic epithelial cells (median, 0.02; $p=0.004$) (Figure 3B). We found no correlation between the histological grade of differentiation and the level of *ADAM 9* or *ADAM 15* mRNA in microdissected pancreatic cancer cells, similar to the results obtained with pancreatic cancer cell lines (Figure 2A).

We measured both *ADAM 9* and *ADAM 15* mRNA in seven microdissected samples of pancreatic cancer cells. We found no significant correlation between *ADAM 9* and *ADAM 15* mRNA levels. None of the samples showed high levels of both *ADAM 9* and *ADAM 15* mRNA. Two of the seven samples of pancreatic cancer cells (28.5%) showed low levels of both *ADAM 9* and *ADAM 15* mRNA. Five of the seven samples (71.5%) showed significant levels of *ADAM 9* or *ADAM 15* mRNA. These results suggest that *ADAM 9* and *ADAM 15* may play independent roles in pancreatic cancer.

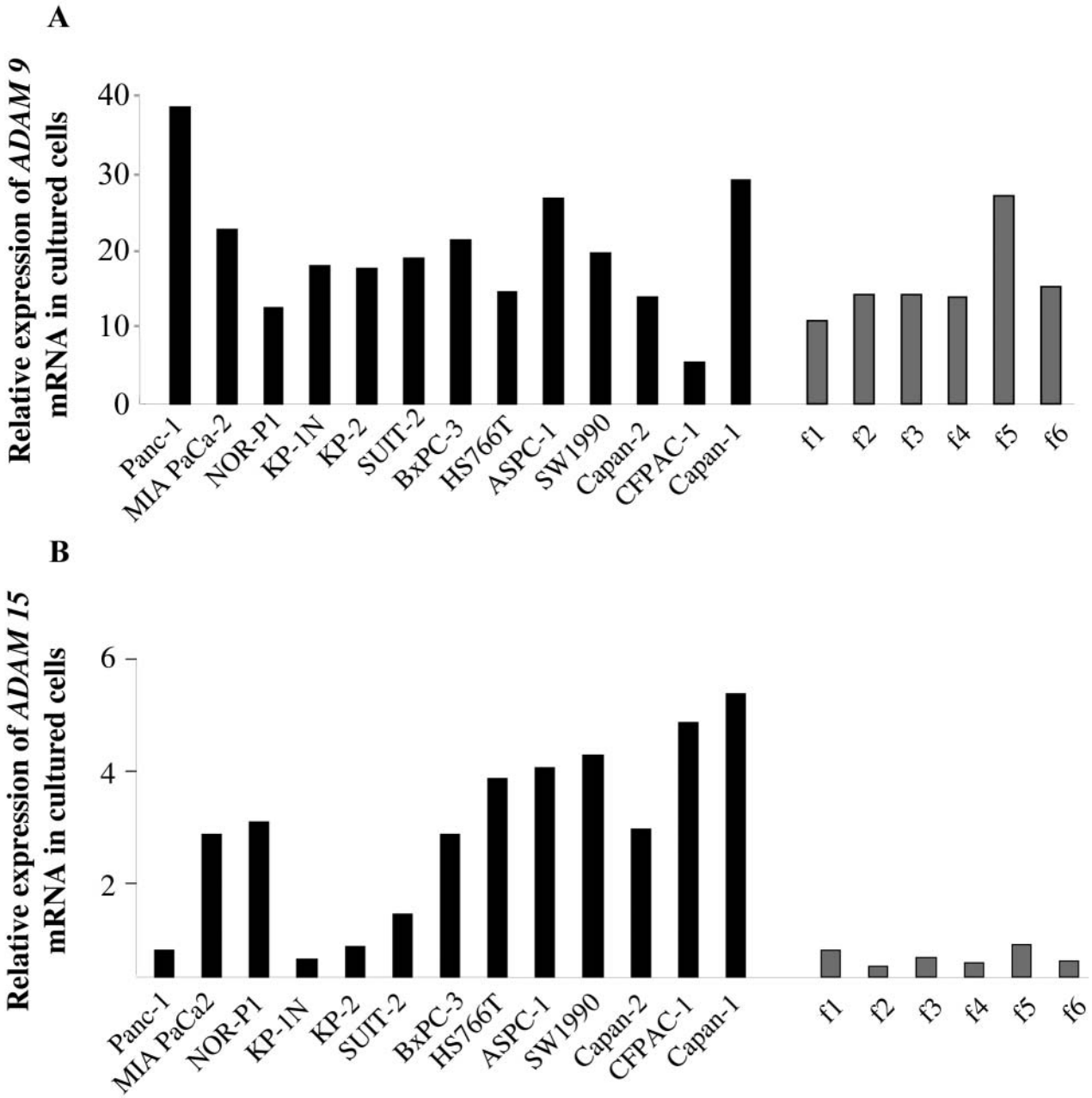

Figure 2. *Quantitative analysis of ADAM 9 and ADAM 15 mRNA expression in human pancreatic cancer cell lines (Panc-1 to Capan-1) and primary cultured pancreatic fibroblast (f1-f6). We measured ADAM 9 and ADAM15 mRNA expression in 13 pancreatic cancer cell lines and six cultures of primary cultured pancreatic fibroblast. All of the pancreatic cancer cell lines and the primary cultured pancreatic fibroblasts expressed ADAM 9 and ADAM 15 mRNA. We found no correlation between the histological grade of differentiation and levels of ADAM 9 and ADAM 15 mRNA in cultured pancreatic cancer cells. Primary cultured pancreatic fibroblasts showed levels of ADAM 9 mRNA similar to those of the pancreatic cancer cell lines, but the levels of ADAM 15 mRNA were much lower in primary cultured pancreatic fibroblasts than in pancreatic cancer cell lines.*

Five out of nine samples of normal pancreatic epithelial cells (55.6%) showed *ADAM 15* mRNA levels below the level corresponding to 0.01 ng of total RNA derived from Capan-1 cells, although these cells showed higher levels of *18S rRNA* than the levels corresponding to 0.08 ng of total RNA derived from Capan-1 cells, suggesting that the relative levels of *ADAM 15* mRNA normalized to the levels of *18S rRNA* in these samples were lower than 0.125, which was used as the

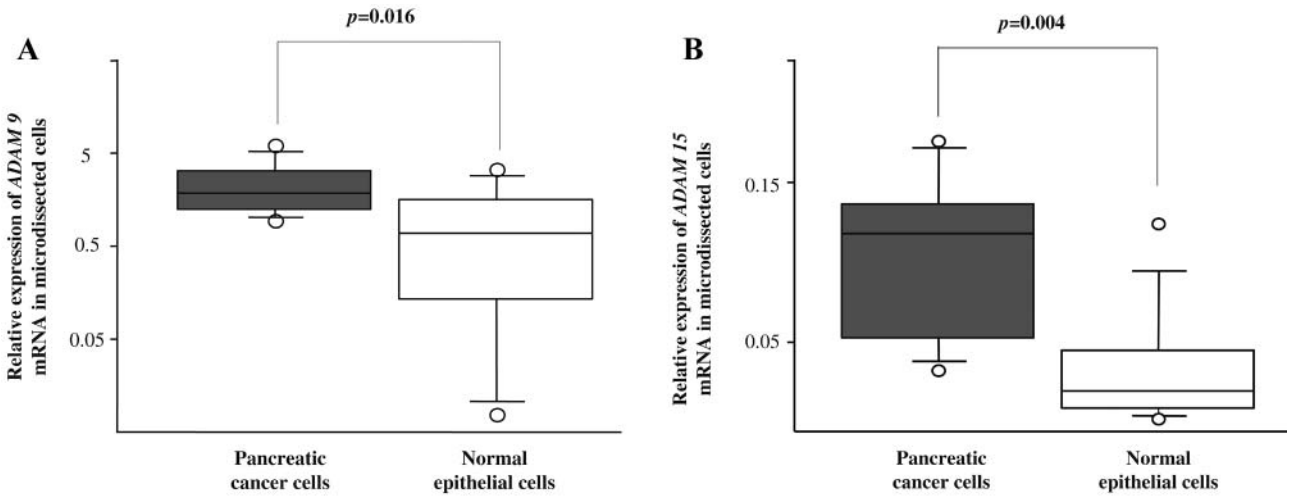

Figure 3. *Quantitative analysis of ADAM 9 and ADAM 15 mRNA in pancreatic cancer cells and normal pancreatic epithelial cells. We isolated pancreatic cancer cells and normal pancreatic epithelial cells from frozen sections by microdissection and performed one-step quantitative real-time RT-PCR with gene-specific primers to analyze the levels of ADAM 9 and ADAM 15 mRNA in these cells. Expression of ADAM 9 and ADAM 15 mRNA was normalized to that of 18S rRNA. We found that pancreatic cancer cells showed significantly higher levels of ADAM 9 and ADAM 15 mRNA than did normal pancreatic epithelial cells (p=0.016 for ADAM 9; p=0.004 for ADAM 15).*

value for statistical analysis. In contrast, 9 out of 13 samples of normal pancreatic epithelial cells (79.2%) showed significant levels of *ADAM 9* mRNA. In the present study, cultured pancreatic fibroblasts showed significant levels of *ADAM 9* mRNA but did not show any *ADAM 15* mRNA. These results suggest that *ADAM 15* may not play a major role under normal conditions and may not be involved in tumor-stromal cell interactions; expression of *ADAM 15* may be more specific than that *ADAM 9* to pancreatic cancer cells.

In the present microdissection-based experiments, 8 out of 11 samples (72.7%) were derived from resected tumors with lymph node metastasis. Levels of *ADAM 9* mRNA in these 8 samples (median, 2.17) were higher than those in the samples without lymph node metastasis (median, 1.07), although the difference was not statistically significant ($p=0.07$). Seven out of 11 patients included in the present study (63.6%) survived longer than 15 months, and the levels of *ADAM 9* mRNA in the samples from these patients (median, 1.31) were lower than those in the other samples (median, 2.38), although the difference was not statistically significant ($p=0.06$). These results are consistent with previous reports suggesting that ADAM 9 may play roles in tumor aggressiveness, progression and prognosis (8, 16).

Discussion

In this study, we performed quantitative analysis of *ADAM 9* and *ADAM 15* mRNA levels in human pancreatic cancer cell lines, primary cultured pancreatic fibroblasts, microdissected pancreatic cancer cells and normal pancreatic epithelial cells with quantitative real-time RT-PCR. Analysis of bulk tissues showed no significant difference in the levels of *ADAM 9* mRNA between pancreatic cancer and non-neoplastic pancreas. However, using microdissection we found that pancreatic cancer cells showed significantly higher levels of *ADAM 9* mRNA than did normal epithelial cells. Pancreatic cancer tissues typically contain a few ductal carcinoma cells amid a large amount of stromal components. We also confirmed that primary cultured pancreatic fibroblasts express significant levels of *ADAM 9* mRNA. Grutzmann *et al*. (14) performed microdissection and microarray based gene expression analysis and found differential expression of *ADAM 9* between pancreatic cancer cells and normal epithelial cells. However, Tan *et al*. (29) reported no overexpression of *ADAM 9* using microarray analysis of bulk pancreatic cancer tissues. Thus, microdissection is necessary for the accurate analysis of *ADAM 9* in pancreatic cancer.

All of the primary cultured pancreatic fibroblasts analyzed expressed *ADAM 9* and *ADAM 15* mRNA. Levels of *ADAM 9* mRNA were similar to those of cultured pancreatic cancer cell lines. Mazzocca *et al*. (30) reported that ADAM 9 protein was expressed by stromal liver myofibroblasts in metastatic liver tumors and suggested that ADAM 9 secreted by stromal liver myofibroblasts may promote carcinoma invasion. Primary cultured pancreatic fibroblasts examined in the present study were obtained from tumors with the histological appearance of invasive ductal carcinoma, indicating that tumor-associated

pancreatic fibroblasts may play an important role in the progression of pancreatic cancer. However, *ADAM 15* may not be involved in tumor-stromal cell interactions because the expression levels of *ADAM 15* mRNA in primary cultures of pancreatic fibroblasts were much lower than those in cultured pancreatic cancer cell lines.

In the present study, we isolated pancreatic cancer cells from tumors for microdissection-based quantitative analysis. Results showed that the levels of *ADAM 9* mRNA were significantly higher in pancreatic cancer cells than in normal epithelial cells. In addition, the levels of *ADAM 9* mRNA appeared to be related to lymph node metastasis and prognosis. ADAM 9 sheds and activates several growth factors, such as heparin-binding EGF and transforming growth factor-alpfa (10), and also binds several integrins (11-13) and degrades ECM components such as fibronectin and gelatin (12). Mazzocca *et al*. (30) showed that ADAM 9 promotes carcinoma invasion, suggesting that ADAM 9 plays a role in pancreatic cancer, contributing to its aggressiveness and poor prognosis.

Microdissection-based quantitative analysis showed that the levels of *ADAM 15* mRNA were significantly higher in pancreatic cancer cells than in normal epithelial cells. The targets of ADAM 15 ectodomain shedding are EGF family ligands (20). The disintegrin domain of ADAM 15 binds several integrins (22-24) and degrades ECM components such as Type IV collagen and gelatin (12). ADAM 15 has been reported to be up-regulated in malignant tumors and related to tumor aggressiveness (17-19). However, Herren *et al*. (31) reported that ADAM 15 overexpression enhanced cell-cell interactions and reduced cell migration. The role of ADAM 15 in cancer progression remains controversial. Our results showed that *ADAM 15* mRNA was up-regulated in pancreatic cancer, suggesting that ADAM 15 may play a role in the progression of pancreatic cancer. However, we found no relation between the level of *ADAM 15* mRNA and the status of lymph node metastasis, prognosis or histological differentiation, possibly due to the small number of samples analyzed. In the present study, levels of *ADAM 15* mRNA were much lower in primary cultured pancreatic fibroblasts than in pancreatic cancer cell lines. Microdissection-based analysis showed that normal pancreatic epithelial cells expressed extremely low levels of *ADAM 15* mRNA but significant levels of *ADAM 9* mRNA, suggesting that the expression of ADAM 15 may be more specific than the expression of ADAM 9 to pancreatic cancer cells.

Conclusion

We confirmed upregulation of *ADAM 9* and *ADAM 15* mRNA expression in microdissected pancreatic cancer cells and found that LMPC is indispensable for the accurate analysis of these genes in pancreatic cancer. *ADAM 9* and *ADAM 15* may play roles in the progression of pancreatic cancer and may present promising target genes for the diagnosis and treatment of pancreatic cancer.

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