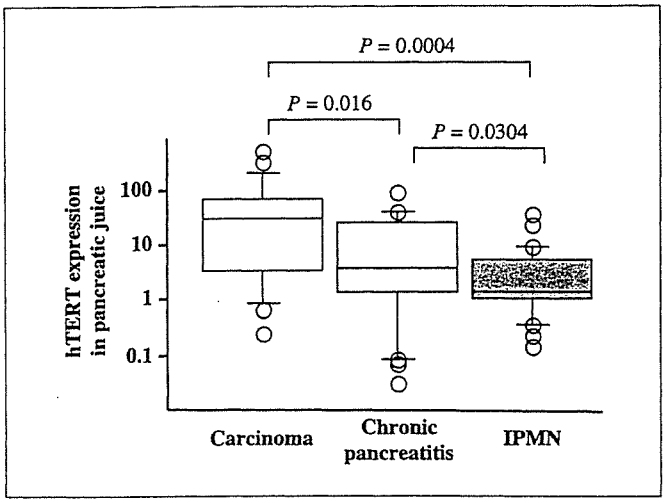

Fig. 1. Relative expression of hTERT mRNA in pancreatic juice was normalized to β-actin expression. There were significant differences in the expression of hTERT between carcinoma juice and chronic pancreatitis or IPMN juice after Bonferroni correction ($P < 0.0125$). However, there was no significant difference between juice samples derived from patients with chronic pancreatitis and IPMN.

revealed that the sensitivity and specificity of differentiation between carcinoma and IPMN were 43.5% (95% CI, 23.2-65.5) and 100% (95% CI, 87.9-100), respectively, when the cutoff point was set at 36.4. Moreover, the sensitivity and specificity of differentiation between carcinoma and chronic pancreatitis were 21.7% (95% CI, 7.5-43.7) and 100% (95% CI, 90.2-100), respectively, when the cutoff point was set at 87.9. These data suggest that hTERT analysis may offer some advantage over cytologic analysis, especially for differentiation between carcinoma and IPMN.

Microdissection-based quantitative analysis of mRNA shows differential expression of hTERT mRNA in IDCs, nonmalignant IPMNs, lymphocytes, and normal ducts. To support the results of pancreatic juice analyses, the expression levels of hTERT mRNA in IPMN cells isolated from 8 resected IPMN tissues, which did not include malignant IPMN, and hyperplastic epithelial cells isolated from 10 tissues with the histologic appearance of pancreatitis were compared with those in normal ductal cells ($n = 7$) or IDC cells ($n = 13$). It has been reported that activated lymphocytes exhibit hTERT expression possibly leading to false-positive results in chronic pancreatitis samples. Therefore, we also isolated lymphocytes from 7 tissues with the histologic appearance of pancreatitis and investigated the expression levels of hTERT mRNA.

IDC cells showed a significantly higher level of hTERT mRNA expression than levels shown by normal duct cells, IPMN cells, hyperplastic cells, and lymphocytes (Fig. 3). Although IPMN cells isolated from several tissues with the histologic appearance of severe atypia exhibited a level similar to that of carcinoma cells, other IPMN cells with low-grade atypia had low hTERT expression. Hyperplastic epithelial cells isolated from tissue with the histologic appearance of pancreatitis had a level of hTERT mRNA expression similar to that of IPMN cells, although a few hyperplastic epithelial cells had a high level of hTERT mRNA expression, similar to that of IPMN cells with severe atypia (Fig. 3). Lymphocytes isolated from tissues with the histologic appearance of pancreatitis showed significantly lower levels of hTERT expression than IDC cells. However, lymphocytes expressed significantly higher levels of hTERT mRNA than normal duct cells. In addition, the median level of hTERT mRNA in lymphocytes was greater than the levels in IPMN cells and hyperplastic cells. Notably, lymphocytes from several tissues exhibited a high hTERT expression level approaching that of carcinoma cells.

Discussion

In the present study, pancreatic juice analyses showed that the power to discriminate between carcinoma and IPMN was significantly greater than that between carcinoma and chronic pancreatitis. In addition, we showed differential hTERT mRNA expression levels between normal ductal cells and carcinoma cells and relatively high hTERT mRNA expression levels in lymphocytes isolated from several resected tissues with the histologic appearance of pancreatitis.

Seki et al. (18) reported that detection of hTERT mRNA in pancreatic juice samples by nested PCR is useful for the diagnosis of pancreatic cancer. However, they also reported that several pancreatic juice samples from patients with chronic pancreatitis exhibited positive expression of hTERT mRNA. Our present results showed that the quantitative analysis of hTERT expression does not reduce the false-positive diagnosis rate in pancreatitis-derived juice. Several studies (25-27) have shown that activated lymphocytes exhibit telomerase activity. To investigate the cause of false-positive samples derived from patients with pancreatitis, we did microdissection and measured hTERT expression in lymphocytes and hyperplastic epithelium isolated from selected tissues with the histologic appearance of pancreatitis. These lymphocytes had relatively high levels of hTERT expression. In addition, hyperplastic epithelial cells isolated from 2 of 10 tissues with the histologic appearance of pancreatitis also exhibited relatively high levels of hTERT

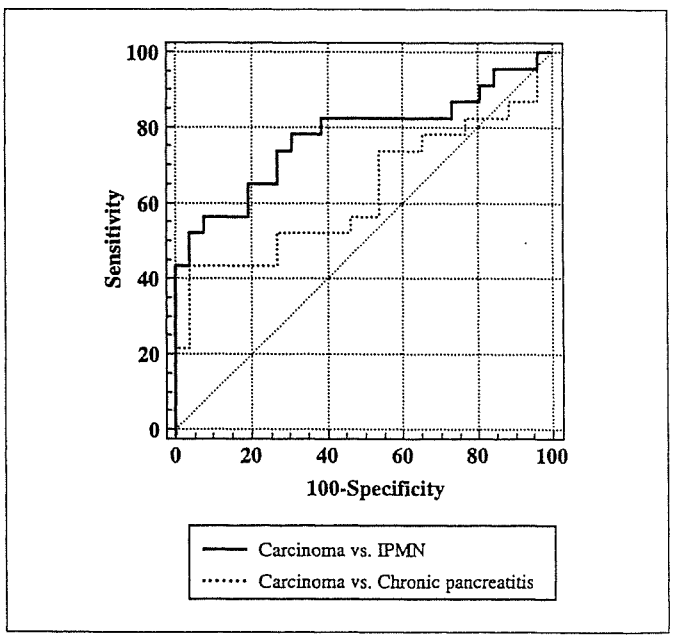

Fig. 2. The area under the ROC was 0.778 for carcinoma versus IPMN (95% CI, 0.636-0.884) and 0.629 for carcinoma versus chronic pancreatitis (95% CI, 0.479-0.762). Significant difference between areas for carcinoma versus IPMN and carcinoma versus chronic pancreatitis was observed (difference between areas, 0.149; 95% CI, 0.005-0.293; $P = 0.042$).

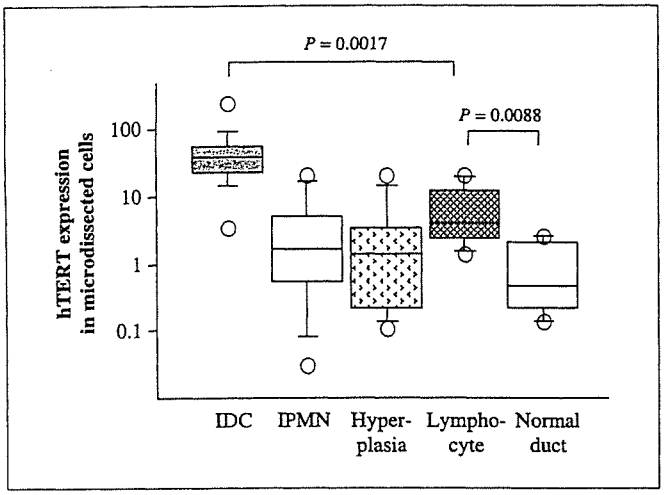

Fig. 3. Microdissection-based quantitative analysis of hTERT mRNA. IDC cells exhibited a significantly high level of hTERT mRNA expression compared with that of normal duct cells, IPMN cells, hyperplastic cells, and lymphocytes. Most IPMN cells and hyperplastic epithelial cells had low levels of hTERT expression. Lymphocytes expressed a significantly higher level of hTERT mRNA than normal duct cells. Lymphocytes from several tissues exhibited a high hTERT expression level approaching that of carcinoma cells.

mRNA. These observations suggest that a subset of lymphocytes or hyperplastic epithelial cells expressing high levels of hTERT mRNA may cause false-positive diagnoses in patients with chronic pancreatitis. To avoid the effect of lymphocytes in pancreatic juice, it may be useful to isolate target epithelial cells from cytologic specimens of pancreatic juice by microdissection. A trial is now in progress in our laboratory.

Inoue et al. (28) reported that detection of telomerase activity in pancreatic juice samples is useful for differentiation between nonmalignant and malignant IPMN. Most nonmalignant IPMN-derived juice samples in our study had low levels of hTERT mRNA, consistent with a previous report (28). However, several IPMN-derived juice samples exhibited high hTERT mRNA expression levels. One IPMN patient with pancreatic juice expressing a high level of hTERT mRNA underwent surgery, and histologic examination of the resected tissue showed severely atypical IPMN. Moreover, cells isolated from IPMN with histologically severe atypia had high hTERT mRNA expression. Our data indicate that precancerous IPMN cells expressing high levels of hTERT mRNA may have strong potential to progress to cancer.

It is possible that some of our pancreatic carcinoma samples were misclassified as IPMN or chronic pancreatitis samples. All patients who did not undergo resection were followed up for at least 6 months, but a longer observation period may be necessary to rule out the possibility of pancreatic carcinoma completely because a subset of pancreatic cancers, such as *in situ* malignant IPMN, show very slow growth.

In conclusion, the quantitative analysis of hTERT mRNA in pancreatic juice offers some advantage over cytologic analysis for differentiation between carcinoma and IPMN but probably not for differentiation between carcinoma and chronic pancreatitis.

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Quantitative analysis of MUC1 and MUC5AC mRNA in pancreatic juice for preoperative diagnosis of pancreatic cancer

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Pancreatic juice is a promising type of diagnostic sample for pancreatic cancer, and members of the mucin (MUC) family are diagnostic candidates. To evaluate the utility of MUC family members as diagnostic markers, we measured MUC mRNA expression in pancreatic tissues and pancreatic juice obtained from patients with different pancreatic diseases as well as in pancreatic cancer cell lines by real-time PCR. Furthermore, to support the possibility of early diagnosis by quantification of MUC1 and MUC5AC, immunohistochemistry and microdissection-based quantitative analysis of mRNA were carried out. There was no significant correlation between MUC1 and MUC5AC expression in cell lines. When β-actin was used as a reference gene, median MUC1 and MUC5AC mRNA expression levels were remarkably greater in tumoral tissues than in non-tumoral tissues, but median MUC4 and MUC6 mRNA expression levels were not. Receiver operating characteristic curve analysis showed that quantitative analysis of MUC1 and MUC5AC mRNA in pancreatic juice is better diagnostic modality than that of MUC4 and MUC6 mRNA. Immunohistochemistry showed that MUC1 and MUC5AC were highly expressed in invasive ductal carcinomas (IDC) and moderately expressed in high-grade pancreatic intraepithelial neoplasia (PanIN); no staining was observed in normal ducts. Analysis of cells isolated by microdissection showed stepwise upregulation of MUC1 and MUC5AC in the development of high-grade PanIN to IDC. Our results suggest that MUC1 and MUC5AC are upregulated stepwise in pancreatic carcinogenesis and that quantitative assessment of MUC1 and MUC5AC mRNA in pancreatic juice has high potential for preoperative diagnosis of pancreatic cancer. © *2005 Wiley-Liss, Inc.*

Key words: MUC1; MUC5AC; pancreatic cancer; pancreatic juice

Pancreatic cancer is the fifth leading cause of cancer death and has the lowest survival rate of any solid cancer.[1,2] Curative resection is the only way to improve prognosis; other conventional therapeutic approaches, including chemotherapy and radiotherapy, show limited efficacy.[3,4] Despite improvements in diagnostic imaging, diagnosis before surgery remains difficult due to the inaccessibility of the organ and the highly malignant nature of the disease. The vast majority of patients with pancreatic cancer suffer a poor clinical course. There are 2 difficulties in preoperative diagnosis: distinguishing pancreatic carcinoma from other disorders such as pancreatitis and detecting it early enough to perform curative resection.

Endoscopic retrograde cholangiopancreatography (ERCP) combined with cytology of pancreatic juice is currently used as a minimally invasive diagnostic tool to distinguish pancreatic cancer from other disorders.[5,6] The sensitivity of cytology is not satisfactory, however, varying from 30–80%.[7,8] It has also been reported that detection of mutations in K-ras, p53 and other oncogenes in pancreatic juice is useful for preoperative diagnosis.[9–12] K-ras mutation occurs at a specific codon (codon 12 or 13), whereas mutations in other oncogenes, such as p53, occur as numerous hot spots. Detection of these mutations may be too complex and time-consuming to introduce into clinical practice. Detection of K-ras mutations has been used in a large clinical study.[13] The sensitivity of detection of ras mutation in pancreatic juice was 38.1%,[13] however, which is inconsistent with previous reports describing a sensitivity of 79–90%.[9–12] These conflicting data indicate the difficulty of introducing assays for the detection of DNA mutations into clinical practice. Novel biomarkers or diagnostic strategies are needed urgently to distinguish pancreatic cancer from other disorders preoperatively and, if possible, to detect early pancreatic cancer.

Mucins (MUC) are high molecular weight glycoproteins. Fourteen mucin genes have been described currently, and 8 are now well characterized, including MUC 1–4, MUC5B, MUC5AC, MUC6 and MUC7.[14–16] Alterations in the expression of MUC have been reported in both pre-neoplastic and neoplastic lesions.[17–19] In addition, recent studies have reported that MUC are expressed differently between non-neoplastic and neoplastic pancreatic lesions. Overexpression of MUC1 and MUC6 and *de novo* expression of MUC4 and MUC5AC in pancreatic cancer have been observed at RNA and protein levels.[18,20–23] DNA microarray technology has been applied to pancreatic cancer and has shown that MUC4 and MUC5AC are upregulated in pancreatic cancer compared to levels in normal pancreatic tissues.[24,25] Thus, MUC are promising diagnostic markers for pancreatic cancer.

In our study, we measured the expression of MUC family mRNA in pancreatic cell lines, tissues and juice quantitatively by real-time PCR. MUC1 and MUC5AC mRNA levels were significantly increased in tumoral tissues of pancreas and in pancreatic juice from patients with pancreatic cancer. The preoperative diagnostic abilities of quantitative analysis of MUC1, MUC4, MUC5AC and MUC6 mRNA in pancreatic juice were compared by evaluation of receiver operating characteristic (ROC) curves.[26] In addition, to investigate whether quantification of MUC1 and MUC5AC in pancreatic juice is useful for detection of early pancreatic cancer, immunohistochemistry (IHC) studies and microdissection-based quantitative analysis of mRNA were carried out on samples of normal ducts, high-grade pancreatic intraepithelial neoplasia (PanIN) and invasive ductal carcinomas (IDC).

Material and methods

Cell lines, pancreatic tissues and pancreatic juice

The following 15 pancreatic cancer cell lines were used: ASPC-1, BxPC-3, KP-1N, KP-2, KP-3, Panc-1, Suit-2 (Dr. H. Iguchi, National Kyushu Cancer Center, Fukuoka, Japan), MIA PaCa-2 (Japanese Cancer Resource Bank, Tokyo, Japan), NOR-P1 (established at our laboratory), Capan-1, Capan-2, CFPAC-1, H48N, HS766T and SW1990 (American Type Culture Collection, Manassas, VA). A human pancreatic duct epithelial cell line (HPDE)

Abbreviations: ERCP, endoscopic retrograde cholangiopancreatography; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPDE, human pancreatic duct epithelial cell line; IDC, invasive ductal carcinoma; IHC, immunohistochemistry; MUC, mucin; PanIN, pancreatic intraductal neoplasm; ROC, receiver operating characteristic.

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immortalized by transduction with the E6/E7 genes of human papilloma virus 16 was kindly provided by Dr. M.-S. Tsao (University of Toronto, Canada). Cells were maintained as described previously.[27,28] Tissue samples were obtained at the time of surgery at Kyushu University Hospital, Fukuoka, Japan. Thirty-two tumoral tissue samples were obtained from cancerous lesions of resected pancreases with primary pancreatic ductal carcinoma, and 42 nontumoral tissue samples were taken from the peripheral soft tissue away from the tumors. The tissue samples were removed as soon as possible after resection and divided into more than 3 large tissue samples. The first samples were stored at −80°C for analysis of whole tissue samples. The second samples were embedded in OCT compound (Sakura, Tokyo, Japan) and snap-frozen for analysis by microdissection. The third samples were fixed in formalin, embedded in paraffin, and cut into 4-μm thick sections for IHC or hematoxylin and eosin (H&E) staining. All tissues adjacent to the specimens were examined histologically, and the diagnosis was confirmed. Pancreatic juice samples were collected from 63 patients who underwent ERCP for suspected malignancy of the pancreas at Kyushu University Hospital from January 1, 2002, to October 31, 2004, as described previously.[29,30] We used pellets of cellular material from pancreatic juice for preparation of RNA.

The diagnosis of pancreatic ductal adenocarcinoma was confirmed by histologic examination of resected specimens where they were available. In inoperable cases, clinical diagnosis was made on the basis of imaging findings. The diagnosis of pancreatitis or cholelithiasis was made on the basis of either histologic examination of resected specimens or clinical observation with conventional diagnostic imaging for at least 6 months. Written informed consent was obtained from all patients, and the study was conducted according to the Helsinki Declaration.

Quantitative assessment of MUC levels by real-time PCR

We designed a real-time PCR protocol for the quantitative analysis of MUC family members (MUC1, MUC4, MUC5AC and MUC6) and housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin). We designed specific primers (MUC1 forward primer, 5′-agacgtcagcgtgagtgatg-3′; reverse primer, 5′-gacagccaaggcaatgagat-3′; MUC4 forward primer, 5′-gggaagaaaggcccaactac-3′; reverse primer, 5′-ctatgctgacgggttggaat-3′; MUC5AC forward primer, 5′-ccttcgacggacagagctac-3′; reverse primer, 5′-tctcggtgacaacacgaaag-3′; MUC6 forward primer, 5′-ccaatccccaagctcattta-3′; reverse primer, 5′-tggtgcctgtactggtgtgt-3′; GAPDH forward primer, 5′-caatgacccccttcattgacc-3′; reverse primer, 5′-gatctcgctcctggaagatg-3′; and β-actin forward primer, 5′-aaatctggcaccacaccttc-3′; reverse primer, 5′-ggggtgttgaaggtctcaaa-3′), carried out BLAST searches to ensure the gene specificity of these primers. Quantitative RT-PCR was carried out with a Quantitect SYBR Green RT-PCR kit (QIAGEN, Tokyo, Japan) and a LightCycler Quick System 350S (Roche Diagnostics, Mannheim, Germany), as described previously.[31] In brief, the total volume of the reaction mixture was 20 μl, and it contained 10 μl of 2× SYBR Green Buffer, 0.2 μl of RT Mix, 1 μl of each primer (10 μM) and 1 μl of total RNA (0.01 μg/μl). The reaction mixture was first incubated at 50°C for 15 min to allow for reverse transcription. PCR was then initiated at 95°C for 10 min to activate modified Taq polymerase, followed by a 45-cycle amplification (95°C for 15 sec, 55°C for 20 sec and 72°C for 10 sec) and 1 cycle (95°C for 0 sec, 65°C for 15 sec and 0.1°C/sec to 95°C) for melting analysis. Each sample was run twice. In addition, all samples showing over 10% deviation in their values were tested in a third run. Deviation in concentration was calculated after use of the calibration curve. The mRNA expression of each gene was calculated on a standard curve constructed with the use of total RNA from the Capan-1 pancreatic cancer cell line. The threshold for observation of product ranged from 20–33 cycles for MUC1 and MUC4, 20–35 cycles for MUC5AC, 20–31 cycles for MUC6 and 15–30 cycles for β-actin and GAPDH primers. For relative quantification, the expression of each gene was normalized against that of the indicated housekeeping gene and expressed as the ratio of relative expression of each target gene mRNA to that of each housekeeping gene mRNA.

IHC studies

Tissue sections of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene and rehydrated in graded alcohols. Sections to be analyzed for MUC1 and MUC5AC staining were microwaved in 1 mM EDTA, pH 8.0, for 10 min, endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS, nonspecific binding was blocked by a 20 min incubation in protein blocking solution consisting of PBS plus 1.5% normal goat serum (DAKO, Glostrup, Denmark), and then the sections were incubated with the appropriate dilution of mouse monoclonal MUC1 or MUC5AC antibody (Novocastra Laboratories, Ltd., Newcastle upon Tyne, UK) overnight at 4°C. The sections were then incubated in the appropriate dilution of biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) for 30 min with a Vectastain Elite ABC kit (Vector Laboratories). Positive reactions were visualized by incubation of the slides with stable 3,3′-diaminobenzidine tetrahydrochloride (Dojindo laboratories, Kumamoto, Japan). The sections were rinsed with distilled water and counterstained with hematoxylin for 10 sec.

Evaluation of antibody reactivity

The degree of monoclonal anti-MUC1 or anti-MUC5AC reactivity in each tissue section was scored by the percentage of stained normal or neoplastic epithelial cells in the section (0 points for no staining, 1 point for <20% of cells staining, 2 points for 20–75% of cells staining and 3 points for >75% of cells staining), and the intensity of immunoreactivity was graded on a scale from 0–3. The total score was obtained as the product of intensity and extent of staining. Negative sections had a score of 0, weakly positive sections had a score of 1–3, moderately positive sections had a score of 4–6 and strongly positive sections had a score >6. Experienced pathologists carried out this evaluation.

Microdissection-based quantitative analysis of mRNA

Frozen tissue samples were cut into 8-μm thick sections. One section was stained with H&E for histologic examination. IDC cells, PanIN cells and normal pancreatic ductal epithelial cells were isolated selectively using laser microdissection and a pressure catapulting system (LMPC; Palm Microlaser Technologies AG, Bernried Germany) in accordance with the manufacturer's protocols. After microdissection, total RNA was extracted from the selected cells as described previously[32] and was subjected to real-time PCR for quantitative measurement of MUC1 and MUC5AC.

Statistical analysis

Data were analyzed by Mann-Whitney *U*-test and Spearman rank correlation test because normal distribution was not obtained. Statistical significance was defined as <0.05, but because we carried out multiple comparisons on our real-time PCR data of 4 genes in whole tissue and pancreatic juice, Bonferroni's correction was used; thus, the adjusted significance level was <0.0125 in the analyses of pancreatic whole tissue and juice. The optimal cut-off points for each marker for discriminating between pancreatic carcinoma and other benign diseases were sought by constructing ROC curves, which were generated by calculating the sensitivities and specificities of each marker at several predetermined cut-off points.[33]

Results

Comparison of expression of MUC mRNA in pancreatic cancer cell lines and in HPDE

We measured MUC1, MUC4, MUC5AC and MUC6 mRNA levels in 15 pancreatic cancer cell lines and in HPDE cells by

TABLE I – EXPRESSION PROFILES OF MUC MRNAS IN PANCREATIC CANCER CELL LINES AND HPDE[1]

	MUC1	MUC4	MUC5AC	MUC6
HPDE	0.86[2]	ND	ND	ND
Panc-1	5.62	ND	0.090	0.517
MIA PaCa-2	4.25	0.394	0.007	0.181
ASPC-1	33.78	0.395	0.003	0.930
Suit-2	0.18	0.083	0.012	0.269
SW1990	13.10	ND	0.037	0.658
KP-1N	1.38	0.550	0.016	5.360
BxPC3	3.80	1.436	0.022	1.217
Capan-2	2.42	0.743	1.024	0.991
CFPAC	48.00	0.623	0.711	0.140
KP-3	130.38	0.939	ND	3.149
NOR-P1	9.16	ND	ND	ND
H48N	8.10	20.125	0.213	2.785
HS766T	1.00	ND	0.771	0.301
KP-2	8.25	0.502	0.026	1.276
Capan-1	1.00	1.000	1.000	1.000

[1]Expression of each gene was normalized to that of beta-actin.–[2] Values are expressed relative to 1.000 for expression in Capan-1 cells. HPDE, human pancreatic ductal epithelial cell line; ND, not detected.

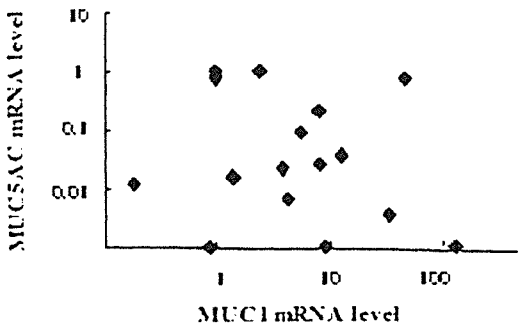

FIGURE 1 – No correlation between MUC1 and MUC5AC mRNA levels in 16 cell lines. The relative expression of MUC1 and MUC5AC in 15 pancreatic cell lines and HPDE was normalized to β-actin. There was no significant correlation between the expression of MUC1 and MUC5AC.

real-time PCR. Expression profiles of MUC mRNA in pancreatic cancer cell lines and HPDE are shown in Table I. In HPDE, only MUC1 mRNA was detectable. In all pancreatic cancer cell lines except Suit-2, the expression of MUC1 mRNA was greater than that in HPDE. The expression of MUC4 mRNA was detectable and was measured quantitatively in 11 pancreatic cancer cell lines. The expression of MUC5AC mRNA was detectable and was measured quantitatively in 13 pancreatic cancer cell lines. The expression of MUC6 mRNA was detectable and was measured quantitatively in all pancreatic cancer cell lines except NOR-P1. There was no significant correlation between MUC1 and MUC5AC mRNA levels in these pancreatic cancer cell lines (Fig. 1; $p = 0.4749$). We found no significant correlation between any other genes examined. (data not shown). These data suggest that the genes examined in our study may be independent markers for diagnosis of pancreatic cancer.

Notably, relative MUC mRNA expression levels were generally considerably lower in cultured cells than in tissues or juice samples (Table I, Fig. 2*b*, Fig. 3*a*). This may be due to differences in β-actin mRNA expression between cultured cells and cells derived from tissue or juice. Several studies have suggested that β-actin expression is affected by cell cycle and experimental conditions.[34–36] Cultured cells are highly proliferative compared to primary tumors. Therefore, when normalized to β-actin, relative expression of target mRNA may decrease in cultured cells compared to that in tissues.

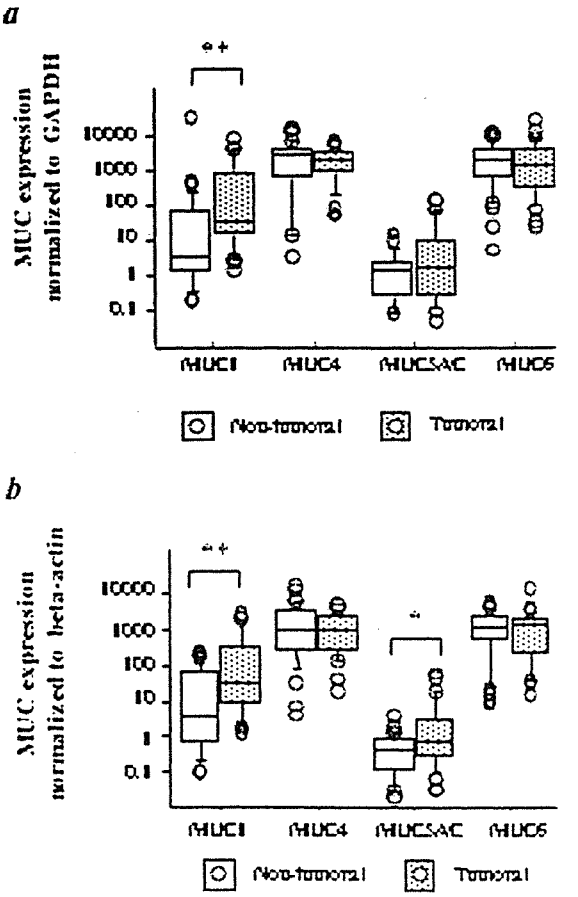

FIGURE 2 – Relative expression of MUC in tumoral and non-tumoral whole tissues. (*a*) There was a significant difference in the relative expression of MUC1 after Bonferroni's correction but not of MUC4, MUC5AC or MUC6 between tumoral and non-tumoral tissues when GAPDH was used as the reference gene. (*b*) When β-actin was used as the reference gene, the median relative expression of MUC1 and MUC5AC was 7.2- and 2.8-fold greater in tumoral tissues, respectively, than in non-tumoral tissues, whereas those of MUC4 and MUC6 in tumoral tissues was similar to that in non-tumoral tissues. $^{*}p < 0.05$; $^{**}p < 0.0125$.

Quantitative analysis of MUC mRNA in tumoral and non-tumoral pancreatic tissues

We measured MUC1, MUC4, MUC5AC and MUC6 mRNA levels in tumoral and non-tumoral pancreatic whole tissues. To quantify target gene expression, 2 major housekeeping genes (GAPDH and β-actin) were used for relative quantification. The results of relative quantification normalized to the indicated housekeeping genes are shown in Figure 2*a* (GAPDH) and Figure 2*b* (β-actin). When normalized to GAPDH, a significant difference between tumoral and non-tumoral tissues was found only in MUC1 mRNA levels (Fig. 2*a*; $p = 0.0017$). When normalized to β-actin, median expression levels of MUC1 and MUC5AC mRNA were 7.2- and 2.8-fold greater in tumoral tissues, respectively, than in non-tumoral tissues, although the difference in MUC5AC mRNA expression levels was not significant after Bonferroni's correction (level of significance adjusted to $p < 0.0125$) (Fig. 2*b*; $p = 0.0033$ for MUC1, $p = 0.0223$ for MUC5AC). Schek *et al.*[37] and Crnogorac-Jurcevic *et al.*[38] also reported that the expression of GAPDH increases in pancreatic cancer. These data suggest that β-actin may be a better reference gene than GAPDH for diagnosis by quantification of MUC mRNA in pancreatic tissues. Therefore, in subsequent analyses of pancreatic juice, β-actin was used as the reference gene.

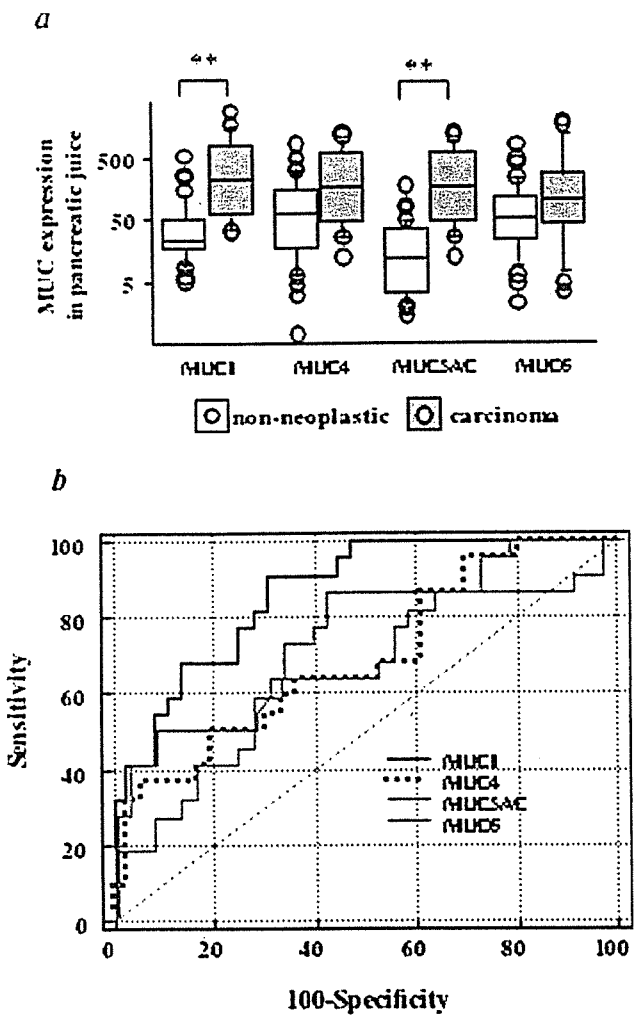

FIGURE 3 – Relative expression of MUC in pancreatic juice normalized to β-actin and ROC curve analysis. (*a*) The relative expression of MUC1, MUC4, MUC5AC and MUC6 in pancreatic juice was normalized to β-actin expression. There were significant differences in the expression of MUC1 and MUC5AC between carcinoma juice and non-neoplastic samples after Bonferroni's correction (**$p < 0.0125$). (*b*) The sensitivity of each marker was determined at several specificity levels. ROC curve analysis showed that the discriminative abilities of MUC1 and MUC5AC were greater than that of MUC4 or MUC6.

There were no significant differences in MUC4 and MUC6 mRNA levels between tumoral and non-tumoral tissues ($p = 0.5385$ for MUC4, $p = 0.5858$ for MUC6, even when normalized to β-actin). This was inconsistent with previously published findings.[18,20] Swartz *et al.*[39] and other investigators[18,20,40] reported detection of MUC4 or MUC6 expression in PanIN, reactive epithelium, squamous metaplasia or mononuclear cells. In our present study, we investigated H&E staining of adjacent tissue of non-tumoral tissues and found reactive epithelial change or appearance of mild pancreatitis with mononuclear cells in some samples, possibly due to sampling from site distal to the tumor. It is also possible that these large samples included PanIN lesions. In addition, the tumoral samples used in our present study showed remarkable variation in the amount of stromal tissue present in the tumors. As described previously,[20] this variation affects relative mRNA expression levels in tumoral samples. The use of whole tissues or bulky tumors for analysis may contribute to conflicting results between our present study and previous studies.

Quantitative analysis of MUC1, MUC4, MUC5AC and MUC6 mRNA expression in pancreatic juice

We measured MUC1, MUC4, MUC5AC and MUC6 in 63 pancreatic juice samples, including samples from 24 pancreatic carcinomas and 39 pancreases with non-neoplastic disease (pancreatitis, $n = 33$; cholelithiasis, $n = 6$). The amount of total RNA extracted from pancreatic juice samples was too small to measure quantitatively because few cells are present in pancreatic juice. Therefore, an adequate reference gene was necessary for relative quantification of target gene expression. Based on our present analysis of tissue samples, the β-actin gene was used as the reference gene for relative quantification. Relative expression levels of MUC1 and MUC5AC were significantly greater in carcinoma samples than in pancreatitis-affected or cholelithiasis-affected samples after Bonferroni's correction (Fig, 3*a*; $p < 0.0001$ for MUC1, $p < 0.0001$ for MUC5AC). There were, however, no significant differences in relative MUC4 and MUC6 expression between carcinoma juice and pancreatitis-affected or cholelithiasis-affected samples after Bonferroni's correction ($p = 0.0185$ for MUC4, $p = 0.0304$ for MUC6).

The ROC curves for MUC 1, MUC4, MUC5AC and MUC6 mRNA expression are presented in Figure 3*b*. The sensitivity of each marker was determined at several specificity levels. The area under the curve (AUC) was 0.864 for MUC1 (95% confidence interval [CI] = 0.748–0.939) and 0.762 for MUC5AC (95% CI = 0.632–0.864). For MUC4, the AUC was 0.686 (95% CI = 0.550–0.801), and for MUC6, the AUC was 0.646 (95% CI = 0.510–0.767). A significant difference between AUC for MUC1 and MU6 was observed (difference between areas, 0.217; 95% CI = 0.048–0.386; $p = 0.012$). These data showed that the discriminative abilities of MUC1 and MUC5AC were greater than that of MUC4 or MUC6.

In our study, cytologic Class IV or V[41] was considered positive for a diagnosis of malignancy. The cytologic sensitivity for diagnosis of pancreatic cancer was only 22.0% (95% CI = 14.7–29.3), which was similar to that cited in previous reports,[29] although the specificity was 100%. ROC curve analyses showed the sensitivity and specificity of MUC1 analysis to be 41.7% (95% CI = 22.1–63.3) and 97.4% (95% CI = 86.5–99.6), respectively, when the cut-off point was set at 264.18. MUC5AC analysis showed similar sensitivity and specificity when the cut-off point was set at 82.33. These data suggest that analysis of MUC1 and MUC5AC may offer some advantage over cytology alone.

IHC studies and microdissection-based quantitative analysis of mRNA shows differential expression of MUC1 and MUC5 AC in IDC, high-grade PanIN and normal ducts

To clarify whether quantification of MUC1 and MUC5AC in pancreatic juice may lead to early detection of pancreatic cancer, the expression of protein and mRNA levels of these genes in high-grade PanIN was compared to those in normal ducts or IDC. Representative IHC data obtained with the anti-MUC1 and anti-MUC5AC antibodies are shown in Figure 4. Strong MUC1 and MUC5AC staining was identified in 52.2% and 48.3% of IDC, respectively, whereas in >70% of normal ducts, no expression of either gene was identified (Tables II, III). Importantly, most high-grade PanIN showed weak or modest expression of both genes.

By microdissection, normal duct cells, PanIN cells and IDC cells were isolated from frozen sections and were subjected to quantification of MUC1 or MUC5AC mRNA. As with the protein levels observed in the IHC study, MUC1 and MUC5AC mRNA were expressed differentially in normal duct cells, PanIN cells and IDC cells (Fig. 5*a*,*b*). In particular, there were significant differences in MUC5AC mRNA levels among normal duct cells, PanIN cells and IDC cells ($p = 0.0248$ between PanIN cells and IDC cells; $p = 0.006$ between PanIN cells and normal duct cells).

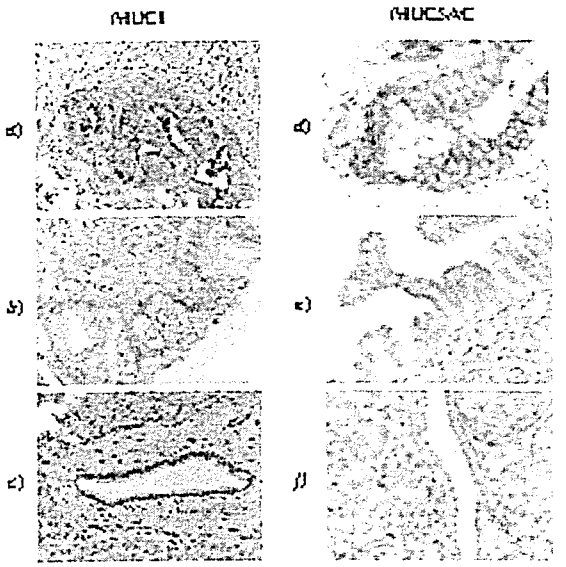

FIGURE 4 – IHC studies. Immunoreactivity for MUC1 (*a–c*) and MUC5AC (*d–f*). Representative images of IDC (*a,d*), high-grade PanIN (*b,e*) and normal ducts (*c,f*) (original magnification = 400×). Differential expression of MUC1 and MUC5AC protein levels was found among IDC, high-grade PanIN and normal ducts.

TABLE II – MUC1 IMMUNOREACTIVITY IN PANCREATIC TISSUE

	Normal (n = 27)	PanINs (n = 12)	IDC (n = 23)
Negative	20 (74.1%)	1 (8.3%)	0 (0%)
Weak	5 (18.5%)	9 (75%)	1 (4.3%)
Moderate	2 (7.4%)	2 (16.7%)	10 (43.5%)
Strong	0 (0%)	0 (0%)	12 (52.2%)

TABLE III – MUC5AC IMMUNOREACTIVITY IN PANCREATIC TISSUE

	Normal (n = 29)	PanIN (n = 11)	IDC (n = 29)
Negative	24 (82.8%)	0 (0%)	0 (0%)
Weak	4 (13.8%)	4 (36.4%)	5 (17.2%)
Moderate	1 (3.4%)	7 (63.6%)	10 (34.5%)
Strong	0 (0%)	0 (0%)	14 (48.3%)

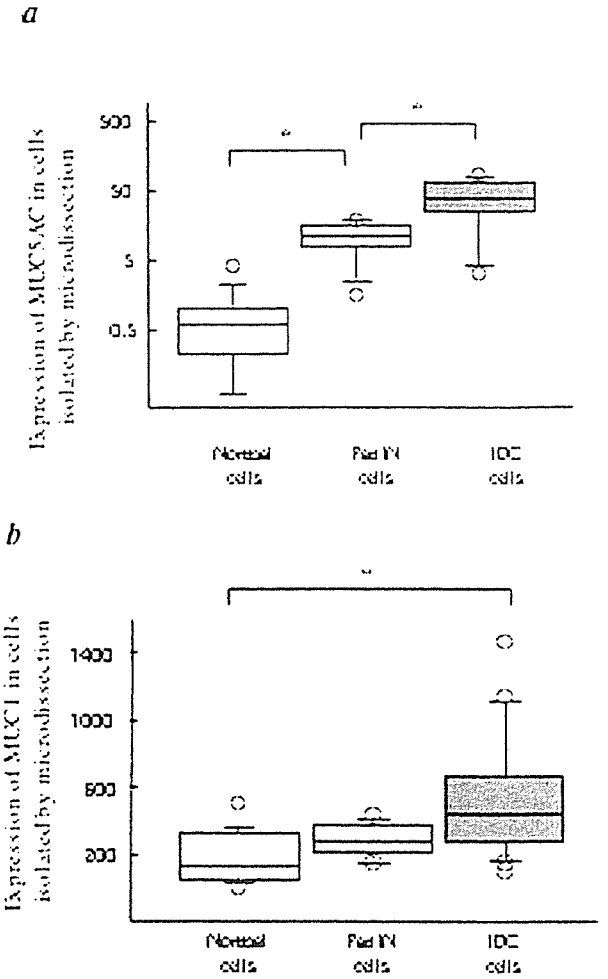

FIGURE 5 – Microdissection-based quantitative analysis of MUC5AC (*a*) and MUC1 (*b*) mRNA. By microdissection, IDC cells, high-grade PanIN cells and normal ductal cells were isolated from frozen sections, and total RNA extracted from these cells was subjected to quantitative analysis of MUC1 and MUC5AC mRNA by real-time PCR. Differential expression of MUC1 and MUC5AC mRNA levels was found among IDC, high-grade PanIN and normal ducts.

Discussion

This is the first report of quantitative analysis of MUC1 and MUC5AC mRNA levels in pancreatic juice for preoperative diagnosis of pancreatic cancer. In our present study, ROC curve analyses of MUC1 and MUC5AC mRNA showed the usefulness of quantifying these 2 markers for diagnosis of pancreatic cancer and showed some advantage over cytologic examination alone. In addition, in support of the possibility that quantification of MUC1 and MUC5AC may lead to the detection of early pancreatic cancer, IHC and microdissection-based quantitative analysis of mRNA showed that MUC1 and MUC5AC are differentially expressed in normal ducts, high-grade PanIN and IDC.

A cut-off point with high values can be selected if high specificity is needed, although sensitivity decreases. If pancreatic cancer is diagnosed, the patient may require quite invasive surgery compared to other disease. Therefore, the diagnosis of pancreatic cancer should be of high specificity. In this respect, quantitative analysis of MUC genes may be superior to qualitative analysis of DNA markers.

In addition, in comparison to DNA mutation assays, quantitative analysis of mRNA with real-time PCR is much more suitable to clinical practice due to its simplicity and rapidity. We can also quantify multiple target genes at a time with real-time PCR, suggesting that this analysis is also suitable for the evaluation of aberrant mRNA expression during pancreatic carcinogenesis, possibly leading to improved diagnostic accuracy. In comparison to analysis of cell pellets, microdissection to isolate target cells in pancreatic juice will make it much easier to detect differential expression between non-malignant and malignant cells as in our present study, leading to decreased overlap between cancer and control samples. Microdissection studies are currently in progress in our laboratory.

Early diagnosis is necessary to improve the prognosis of patients with pancreatic cancer. Maitra *et al.*[42] and Kim *et al.*[18] carried out IHC and reported that overexpression of MUC1 and *de novo* expression of MUC5AC were observed at all stages of PanIN and invasive ductal adenocarcinoma, which was confirmed by our present IHC and microdissection-based quantitative mRNA analysis. Notably, however, our present data showed differential expression of MUC1 and MUC5AC between normal ducts, high-grade PanIN and IDC, suggesting that alteration of MUC1 and MUC5AC expression may be an early event in pancreatic carcinogenesis and that expression of these genes may increase stepwise with disease progression. Therefore, accurate quantitative, not qualitative, analysis of MUC1 and MUC5AC mRNA levels in

pancreatic juice may provide for the discrimination of early pancreatic cancer from premalignant lesions.

In our study, most of the samples were from patients with advanced cancer (Stages III or IV). Only one tumoral tissue sample and one pancreatic juice sample were obtained from 2 separate patients with Stage II pancreatic cancer. Therefore, we could not show the clear evidence of early diagnosis in the present study. From a clinical standpoint, however, we need not only early detection of pancreatic cancer but also differential diagnosis from other diseases. Even advanced pancreatic cancer is occasionally difficult to distinguish from chronic pancreatitis. The results of analysis of pancreatic juice in our study suggest the usefulness of quantitative analyses of MUC1 and MUC5AC for discrimination of advanced pancreatic cancer from non-neoplastic lesions including chronic pancreatitis.

In conclusion, our data suggest that quantitative analysis of MUC1 and MUC5AC mRNA levels in pancreatic juice is a promising approach for preoperative diagnosis of pancreatic cancer and possibly for diagnosis of early pancreatic cancer.

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RAPID COMMUNICATION

Overexpression of *c-met* in the early stage of pancreatic carcinogenesis; altered expression is not sufficient for progression from chronic pancreatitis to pancreatic cancer

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Abstract

AIM: To investigate *c-met* expression during early pancreatic carcinogenesis.

METHODS: We used 46 bulk tissues and 36 microdissected samples, including normal pancreas, chronic pancreatitis, and pancreatic cancer, for quantitative real-time reverse transcription-polymerase chain reaction.

RESULTS: In bulk tissue analyses, pancreatic cancer tissues expressed significantly higher levels of *c-met* than did chronic pancreatitis and normal pancreas tissues. *c-met* levels did not differ between chronic pancreatitis and normal pancreas tissues. In microdissection-based analyses, *c-met* was expressed at higher levels in microdissected pancreatic cancer cells and pancreatitis-affected epithelial cells than in normal ductal epithelial cells (both, $P < 0.01$). Interestingly, pancreatitis-affected epithelial cells expressed levels of *c-met* similar to those of pancreatic cancer cells.

CONCLUSION: Overexpression of *c-met* occurs during the early stage of pancreatic carcinogenesis, and a single alteration of *c-met* expression is not sufficient for progression of chronic pancreatitis-affected epithelial cells to pancreatic cancer cells.

Key words: *c-met*; Pancreatic cancer; Chronic pancreatitis; Pancreatic carcinogenesis

Yu J, Ohuchida K, Mizumoto K, Ishikawa N, Ogura Y, Yamada D, Egami T, Fujita H, Ohashi S, Nagai E, Tanaka M. Overexpression of *c-met* in the early stage of pancreatic carcinogenesis; altered expression is not sufficient for progression from chronic pancreatitis to pancreatic cancer. *World J Gastroenterol* 2006; 12(24): 3878-3882

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INTRODUCTION

Pancreatic cancer is the fourth and fifth leading cause of cancer death in the United States and Japan, respectively, and has the lowest survival rate of any solid cancer[1-3]. Because the lack of specific symptoms in patients with pancreatic cancer makes early diagnosis difficult, initial diagnosis typically occurs when the tumor has reached an advanced stage[4]. Therefore, we need a better understanding of the early carcinogenesis of pancreatic cancer to facilitate early detection.

The protooncogene *c-met* encodes a member of the family of receptor tyrosine kinases that is a 190-ku glycoprotein comprised of a transmembrane 145-ku β subunit and an extracellular 50-ku α subunit[5,6]. The met receptor binds to and is activated by hepatocyte growth factor/scatter factor (HGF/SF)[7], leading to increased proliferation, altered motility, and enhanced invasion[8]. The wild-type *c-met* gene is amplified or overexpressed in many types of human cancer, including cancers of the breast, stomach, liver, endometrium, nasopharynx, and pancreas[9-14].

Pancreatic ductal cells, which give rise to the most common type of human pancreatic carcinoma, are particularly sensitive to inflammatory and carcinogenic processes[15]. There are reports that pancreatic inflammation may play a key role in early pancreatic carcinogenesis[16]. Rivera *et al* found that *k-ras* mutations that lead to uncontrolled cell growth and may be the principal molecular event in the pathogenesis of pancreatic cancer are present in chronic pancreatitis, providing a genetic basis for the potential progression of chronic pancreatitis to pancreatic cancer[15]. Taken together, the data indicate that the molecular changes associated with chronic pancreatitis

should be studied as a part of a comprehensive strategy to understand pancreatic carcinogenesis. It was reported that *c-met* mRNA expression was increased during development of chronic pancreatitis in dibutyltin-treated mice[17]. Furukawa *et al* also reported that strong immunostaining of c-Met was present in 58% of specimens demonstrating pancreatic hyperplastic epithelia and in 78% of specimens demonstrating ductal adenocarcinoma, respectively[18]. To understand the clinical significance of *c-met* expression in pancreatic carcinogenesis, we need to examine *c-met* expression during early pancreatic carcinogenesis. Therefore, we need accurate measurements of the levels of *c-met* expression in specific cells, such as normal epithelial cells, pancreatitis-affected epithelial cells, and invasive ductal carcinoma (IDC) cells.

The microdissection method, which can isolate specific cells from a frozen section, is used for genetic analysis of specific lesions[19]. Because tumor cells typically represent only 60%-70% of the cells in pancreatic cancer bulk tissues and because the percentage of chronic pancreatitis-affected epithelial cells is very low in bulk chronic pancreatitis tissues[19], cell microdissection is necessary and useful for reliable molecular analyses related to pancreatic carcinogenesis.

In the current study, we examined *c-met* mRNA levels in 46 human pancreatic bulk tissue samples and cells microdissected from 36 samples of pancreas, including normal pancreas, chronic pancreatitis, and pancreatic cancer by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). Our goals were to quantify *c-met* expression in chronic pancreatitis, which is an early stage of pancreatic carcinogenesis, and to clarify when overexpression of c-met occurs during pancreatic carcinogenesis.

MATERIALS AND METHODS

Pancreatic tissues

Tissue samples were obtained at the time of surgery at Kyushu University Hospital (Fukuoka, Japan) as described previously[20]. In brief, tissue samples were removed as soon as possible after resection and divided into at least three bulk tissue specimens. The first sample was embedded in OCT compound (Sakura Findek, Tokyo, Japan), snap-frozen for microdissection, and stored at -80℃ until use. The second sample was fixed in formalin, embedded in paraffin, and cut into 4-μm-thick sections for hematoxylin and eosin (H&E) staining. The third sample was snap-frozen for bulk tissue analysis and stored at -80℃ until use. Tissues adjacent to the specimens were examined histologically, and the diagnosis was confirmed by pathologists. Thirteen pancreatic cancer tissue specimens were obtained from tumoral lesions of resected pancreas with primary pancreatic cancer. Twelve normal pancreatic and 11 pancreatitis-affected pancreatic tissue specimens were taken from peripheral tissues away from the tumor or pancreas resected due to mass-forming pancreatitis. 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.

Table 1 *c-met* and *β-actin* primer sequences and product size

Primer	Forward Sequence 5′-3′	Reverse Sequence 5′-3′	Product size
c-met	tgatgatgaggtggacaca	ctatggcaaggagcaaaga	149
β-actin	aaatctggcaccacaccttc	ggggtgttgaaggtctcaaa	139

Pancreatic cancer cell lines

Fourteen pancreatic cancer cell lines, ASPC-1, BxPC-3, KP-1N, KP-2, Panc-1, Suit-2 (provided by Dr. H. Iguchi, National Shikoku Cancer Center, Matsuyama, Japan), MIA PaCa-2, NOR-P1 (established in our laboratory), Capan-1, Capan-2, CFPAC-1, H48N, HS766T, and SW1990 (American Type Culture Collection, Manassas, Virginia), and four primary cultured pancreatic fibroblasts derived from resected pancreatic tumors were used. Cells were maintained as described previously[21].

RNA Isolation

Total RNA was extracted from bulk tissues with an RNeasy Mini Kit (Qiagen, Tokyo, Japan) per the manufacturer's protocol. Total RNA was extracted from cells isolated by microdissection with the standard acid guanidinium thiocyanate-phenol-chloroform protocol[22] with or without glycogen (Funakoshi, Tokyo, Japan).

Quantitative analysis of c-met mRNA expression by real-time RT-PCR

Quantitative real-time RT-PCR was performed with a QuantiTect SYBR Green RT-PCR Kit (Qiagen) with a LightCycler Quick System 350S (Roche Applied Science, Mannheim, Germany) as described previously[23]. In brief, the reaction mixture was first incubated at 50℃ for 15 min to allow for reverse transcription. PCR was initiated with one cycle of 95℃ for 10 min to activate modified Taq polymerase followed by 45 cycles of 94℃ for 15 s, 55℃ for 20 s, and 72℃ for 10 s, and one cycle of 95℃ for 0 s, 65℃ for 15 s, and + 0.1℃/s to 95℃ for melting analysis. Each sample was run twice. In addition, any sample showing more than 10% deviation in the values was tested a third time. The 10% deviation was calculated from the concentrations determined from the calibration curve. The level of *c-met* mRNA expression was calculated from a standard curve constructed with total RNA from the Capan-1 pancreatic cancer cell line. The range of threshold cycles was from 20-35 cycles for *c-met* primers[24] and from 5-30 cycles for *β-actin* primers[25] (Table 1). Expression of *c-met* mRNA was normalized to that of *β-actin* mRNA.

Microdissection-based quantitative analysis of c-met mRNA

Frozen tissues were cut into 8-μm-thick sections. One section was stained with H&E for histologic examination. IDC cells from 13 sections, pancreatitis-affected epithelial cells from 12 sections, and normal ductal epithelial cells from 11 sections were selectively isolated with a laser microdissection and pressure catapulting system (P.A.L.M. Microlaser Technologies, Bernried, Germany) in accordance with the manufacturer's protocols. After microdis-

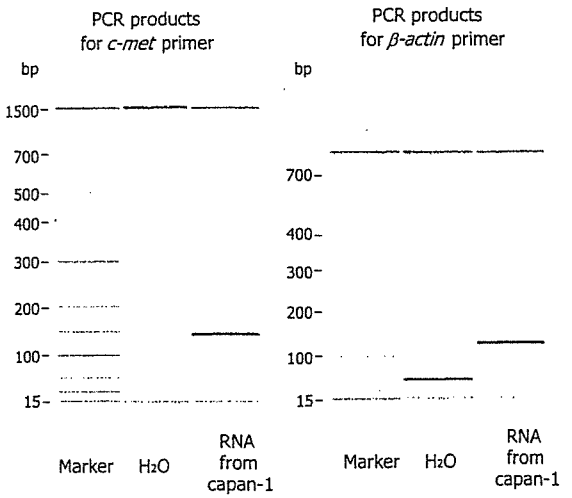

Figure 1 PCR products were analyzed with an Agilent Bioanalyzer 2001. A single 149-bp band was observed for *c-met* primer pairs (left panel). A single 138-bp band was observed for *β-actin* primer pairs (right panel). Each primer pair used in the present study produced a single melting peak on real-time RT-PCR and a single prominent band of the expected size on microchip electrophoresis.

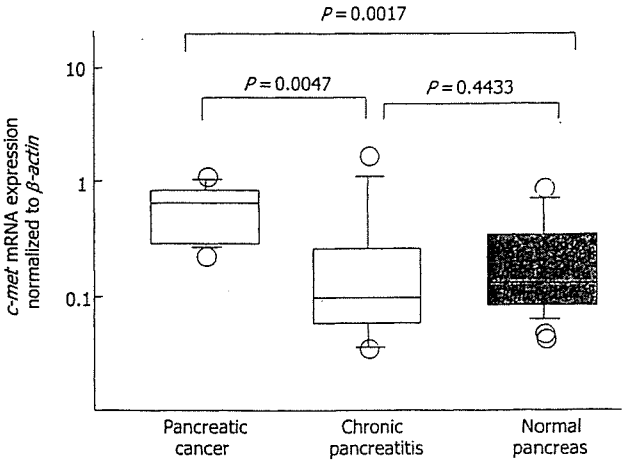

Figure 2 We performed quantitative real-time RT-PCR to quantify *c-met* expression in pancreatic cancer tissues (n = 11), normal pancreatic tissues (n = 20), and chronic pancreatitis tissues (n = 15). *c-met* was overexpressed in pancreatic cancer tissues in comparison to expression in normal pancreatic (P = 0.0017) and chronic pancreatitis tissues (P = 0.0047). Levels of *c-met* did not differ between normal pancreatic and chronic pancreatitis tissues (P = 0.4433).

section, total RNA was extracted from the selected cells and subjected to real-time RT-PCR for quantitative measurement of *c-met* as described previously[19].

PCR products sized by the Agilent 2100 Bioanalyzer

Microchip electrophoresis was performed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). A DNA 1000 Assay Kit was purchased from Agilent Technologies. As shown in Figure 1, each PCR product was analyzed with DNA 1000 Lab Chips (Agilent Technologies) per the manufacturer's protocol[26].

Statistical analysis

Data were analyzed with the Kruskal-Wallis test for comparison of three groups and Mann-Whitney U test for comparison of two groups because normal distribution was not obtained after logarithmic transformation. Statistical significance was defined as $P < 0.05$. Because we performed multiple comparisons of our real-time RT-PCR data, we conservatively used the Bonferroni correction, and therefore, the adjusted significance level was $P < 0.017$.

RESULTS

Quantitative analyses of c-met expression in bulk pancreatic tissues

In the bulk tissue analyses, we measured *c-met* expression in pancreatic cancer tissues (n = 11), normal pancreatic tissues (n = 20), and chronic pancreatitis tissues (n = 15). As shown in Figure 2, the level of *c-met* expression in normal pancreatic tissues was similar to that in chronic pancreatitis tissues. The median values were 0.130 for normal pancreatic tissues and 0.107 for chronic pancreatitis tissues (P = 0.44). The median value of *c-met* expression in pancreatic cancer tissues was 0.678, which was approximately 5-fold greater than that in normal pancreatic tissues (P = 0.0017) and 6-fold greater than that in chronic pancreatitis tissues (P = 0.0047). All data from bulk tissue analyses indicated that *c-met* was overexpressed in pancreatic cancer, but not chronic pancreatitis tissues.

Quantitative Analysis of c-met expression in 14 pancreatic cancer cell lines and 4 primary pancreatic fibroblast cultures

To confirm expression of *c-met* mRNA in pancreatic cancer cell lines, total RNA was isolated from 14 pancreatic cancer cell lines. As shown in Figure 3, *c-met* was expressed in all 14 pancreatic cancer cell lines with median value of 0.742. The 4 primary cultures of normal pancreatic fibroblast1, 2, 3 and 4 (Panc-f1, Panc-f2, Panc-f3, and Panc-f4) expressed low levels of *c-met* mRNA with a median value of 0.023. Pancreatic cancer and chronic pancreatitis tissues usually contain abundant stromal cells, such as fibroblasts. Most of the bulk pancreatic cancer tissues in the present study also contained many desmoplastic changes and the chronic pancreatitis tissues contained stromal components. Therefore, the level of *c-met* expression detected in bulk tissue analyses may not represent the true levels of expression by specific cells due to dilution of these specific cells by contaminating cells such as stromal fibroblasts.

Quantitative analysis of c-met in microdissected pancreatic cancer cells, pancreatitis-related epithelial cells, and normal pancreatic epithelial cells

In general, bulk pancreatic tissue is complex, containing ductal epithelial cells, acinar cells, fibroblasts, islet cells, and mesenchymal cells. Tumor cells comprise only 60%-70% of the cells in bulk tissue specimens of pancreatic cancer[19]. Therefore, data from bulk tissue analyses may not accurately reflect *c-met* levels in specific cells, such as pancreatic cancer cells, pancreatitis-affected epithelial cells, and normal ductal epithelial cells. It has been reported that *c-met* is expressed in acini and pancreatic islets in normal human pancreas[27]. To avoid the influence of contami-

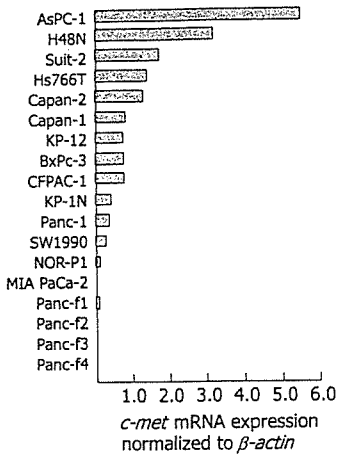

Figure 3 *c-met* expression in pancreatic cancer cell lines and primary pancreatic fibroblasts. The median value of *c-met* expression from pancreatic cancer cell lines was 0.742. In contrast, the median value of *c-met* expression in 4 primary cultures of pancreatic fibroblasts (Panc-f1, Panc-f2, Panc-f3, and Panc-f4) was 0.023.

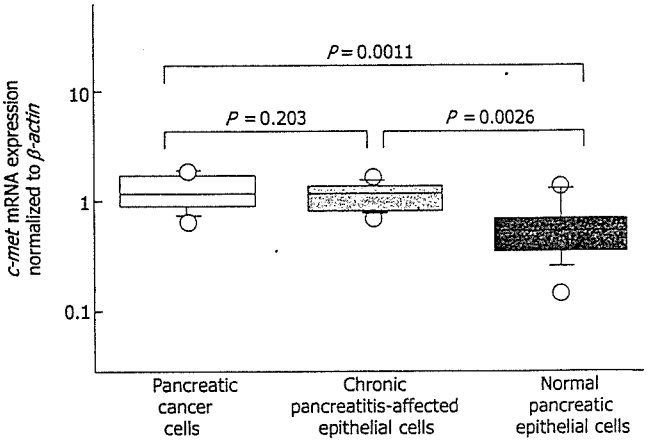

Figure 4 Quantitative analysis of *c-met* mRNA levels in microdissected pancreatic cancer cells (n = 13), pancreatitis-affected epithelial cells (n = 11), and normal pancreatic ductal epithelial cells (n = 12). *c-met* levels in pancreatic cancer cells (median, 1.208) were 2.21-fold higher than those in normal ductal epithelial cells (median, 0.546; P = 0.0011). *c-met* levels in pancreatitis-affected epithelial cells (median, 1.211) were 2.22-fold higher than those in normal ductal epithelial cells (median, 0.546; P = 0.005). *c-met* levels in microdissected normal ductal epithelial cells were the lowest with a median value of 0.546. Interestingly, pancreatitis-affected epithelial cells expressed levels of *c-met* that approached those in pancreatic cancer cells.

nating non-ductal cells, we used a laser-microdissection method to select specific cells for analysis. For microdissection analyses, we isolated pancreatic cancer cells from 13 sections, normal pancreatic ductal epithelial cells from 12 sections, and pancreatitis-affected epithelial cells from 11 sections. As shown in Figure 4, *c-met* expression in pancreatic cancer cells (median, 1.208) was 2.21-fold higher than that in normal pancreatic duct epithelial cells (median, 0.546; P = 0.0011). *c-met* level in normal pancreatic ductal epithelial cells was the lowest. Interestingly, the *c-met* level in pancreatitis-affected epithelial cells (median, 1.211) was significantly higher than that in normal pancreatic duct epithelial cells (median, 0.546; P = 0.005). These data suggested that *c-met* was overexpressed in chronic pancreatitis-affected epithelial cells at levels close to those in pancreatic cancer cells.

DISCUSSION

We performed quantitative real-time RT-PCR to measure *c-met* expression in pancreatitis-affected epithelial cells, which may progress to pancreatic cancer[28-30], to clarify the significance of *c-met* expression in an early stage of pancreatic carcinogenesis. Bulk tissue analyses revealed that *c-met* was overexpressed in pancreatic cancer, but *c-met* expression did not differ between chronic pancreatitis and normal pancreatic tissues. In microdissection-based analyses, we found that pancreatitis-affected epithelial cells expressed high levels of *c-met* that approached those of pancreatic cancer cells. Although it may be difficult to make comparison because microdissection data are based on c-met mRNA levels in single cell, our findings appear to be inconsistent with those of previous immunohistochemical studies in which the positive rates of c-Met were 58%, 80%, and 78% of specimens demonstrating hyperplastic epithelia, severely dysplastic epithelia, and pancreatic ductal adenocarcinoma, respectively[18]. However, Furukawa *et al*[18] also reported that pancreatic cancer patients with diffuse c-Met immunostaining survived longer than c-Met-negative patients. Welm *et al* reported that overexpression of c-*Met* alone did not result in development of tumors, although c-Met acted cooperatively with other genetic alterations, such as overexpression of *MYC*, to induce mammary tumorigenesis[31]. In addition, there have been several reports that the cumulative risk of pancreatic cancer among patients with chronic pancreatitis is only 1% to 4%[28-30]. Taken together, these data suggest that overexpression of *c-met* occurs in the early stage of pancreatic carcinogenesis but is not sufficient for progression of chronic pancreatitis-affected epithelial cells to pancreatic cancer cells. However, these data also suggest that pancreatitis-affected epithelial cells expressing high levels of *c-met* in conjunction with other genetic or epigenetic changes may have the potential to progress to pancreatic cancer. Therefore, *c-met* may be a useful marker for identifying persons with high-risk lesions that may progress to pancreatic cancer.

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Identification of Prognostic Factors Associated with Early Mortality after Surgical Resection for Pancreatic Cancer—Under-analysis of Cumulative Survival Curve

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Abstract

Background: The cumulative survival curve after surgery for advanced pancreatic cancer is characterized by a steep downward slope in the early postoperative period. The aim of this investigation was to identify the characteristics associated with early mortality in patients undergoing pancreatic resection for pancreatic cancer.

Methods: Thirty-seven patients with extended radical pancreatectomy combined with intraoperative radiation therapy were studied. The cumulative survival curve in this series was depicted using the Kaplan-Meier method. Assuming that there were two distinct survival curves, below and above the breakpoint, each part of the curve was modeled as an exponential distribution. Three parameters, the breakpoint, the high hazard rate below the breakpoint, and the low hazard rate above the breakpoint were estimated by the maximum likelihood method. Prognostic factors associated with early mortality after surgery were evaluated using univariate and multivariate Cox proportional hazards regression analyses.

Results: The breakpoint of the survival curve was estimated at 41 months. The short-survival group (SSG) was defined as deceased earlier than 41 months after surgery, and included 31 patients (83.8 %). The long-survival patient group (LSG) consisted of 6 patients who were alive more than 41 months after surgery. Eighteen SSG patients (58.1 %) died of hepatic metastases, whereas no LSG patients died of hepatic metastases. Abdominal pain and/or back pain during clinical course was identified by multivariate analysis as a prognostic factor for patients undergoing pancreatic resection.

Conclusions: The high hazard rate in the early postoperative period was closely linked with death due to liver metastases. The preoperative presence of local pain was a prognostic factor associated with early mortality.

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Pancreatic adenocarcinoma remains a lethal disease. Despite improvements in imaging technology regarding diagnosis, fewer than 20% of cases have been potentially resectable at the time of initial diagnosis.[1]

Complete pancreatic resection has been able to yield actuarial 5-year survival rates of 15%–25% following pancreaticoduodenoctomy[2–4] and 8%–14% following distal pancreatectomy.[5,6] The common failure patterns in treating pancreatic cancer have been recurrence in the locoregional tumor bed and/or the development of hepatic metastases. We introduced extended radical pancreatectomy combined with intraoperative radiation therapy (IORT) in 1984. Compared with other treatment modalities, this approach has provided the best control of local recurrence,[7] although it has not contributed to survival benefit. The cumulative survival curve after surgery for advanced pancreatic cancer is characterized by a steep decline in the early postoperative period followed by a gentle downward slope. This indicates that most patients die not long after resection. Our aim is to identify the characteristics associated with early mortality in patients undergoing pancreatic resection for pancreatic cancer.

PATIENTS AND METHODS

Patients and Surgical Technique

Between December 1984 and December 1999 at Kumamoto University, we performed pancreatic surgery on 88 patients with pancreatic cancer. Of the 88 patients, 41 underwent extended radical pancreatectomy combined with IORT, because they had potentially curable disease as assessed by physical examination and the following objective imaging criteria: (1) no evidence of remote metastases; (2) no evidence of tumor extension to the celiac axis or the superior mesenteric artery; (3) possible resection and reconstruction of the superior mesenteric vein (SMV) or the portal vein (PV) in cases of tumor extension to the SMV or PV. This combined therapy, which had provided the best control of local recurrence, involves the dissection of the juxtaparaaortic and regional lymph nodes, together with the connective tissue and nervous plexus around the aorta, extending from the diaphragm above to the inferior mesenteric artery below. After dissection, a dose of 30 Gy with 9–12 MeV electron beam radiation was administered to the operative field, as described previously.[7] Four cases of hospital death were excluded from this study. Twelve patients received preoperative and/or postoperative 5-fluorouracil (5-FU)-based chemotherapy. No patients received extra radiation therapy.

Survival Curve Analyzed

The cumulative survival curve in this series was depicted using the Kaplan-Meier method. Assuming that there were two distinct arms of the survival curve below and above the breakpoint, each part of the curve was modeled as an exponential distribution. The exponential model corresponded to an assumption of a uniform hazard within that time period. Three parameters, the breakpoint, the high hazard rate below the breakpoint, and the low hazard rate above the breakpoint were estimated by the maximum likelihood method. The model fitness, the magnitude of −2 log likelihood was also evaluated, changing the breakpoint and the case having no breakpoint. The better fit model had a lower value of −2 log likelihood. The short survival group (SSG) was defined based on analyses of the survival curve using these statistical methods.

Characteristics Analyzed

Prognostic factors associated with early mortality after surgery were evaluated for the following variables: clinical and histological features, laboratory examination data, and intraoperative factors. The analyzed factors were as follows: clinical features (abdominal pain and/or back pain, body weight loss, preoperative obstructive jaundice and/or biliary infection); histological features (tumor location, tumor size, tumor stage, residual tumor); laboratory examination data (white blood cell, lymphocytes, hemoglobin, total protein, albumin, serum amylase level, elastase-1, the 75 g glucose tolerance test, the N-benzoyl-L-tyrosyl-P-aminobenzoic acid (NBT-PABA) test, tumor-associated carbohydrate antigens, CA 19-9 and/or DUPAN-2 and/or Span-1); intraoperative factors (operation time, bleeding volume, volume of blood transfusion). We conducted an interview with each patient during clinical course to inquire about the experience of abdominal pain and/or back pain and body weight loss. Preoperative biliary drainage (endoscopic stents or percutaneous drains) was performed in the event of biliary infection and/or jaundice (serum total bilirubin level more than 5 mg/dl). Blood samples for the complete blood count, chemistries, amylase, and tumor-associated carbohydrate antigens were taken on admission. All but one patient were measured for serum tumor-associated carbohydrate antigens. Within the normal limits of serum CA 19-9, DUPAN-1, and Span-1 levels are 37 U/ml, 150 U/ml, and 30 U/ml, respectively. The 75 g glucose tolerance test and the NBT-PABA test were performed before operation. Diagnosis of glucose intolerance was made on the basis of a

fasting venous blood glucose level of more than 110 mg/dl, and/or glucose level more than 140 mg/dl 2 hours after uptake of 75 g glucose. The exocrine function of the pancreas was assessed by the NBT-PABA test. Insufficiency of exocrine function was diagnosed by lower than 70% excretion of total PABA in the urine. Histopathological examination of each resected specimen was conducted. The resected specimens were fixed in a 20% formalin solution. The paraffin sections for histological examination were prepared and stained with hematoxylin and eosin. According to the classification of pancreatic cancer defined by the Japanese Pancreas Society,[8] we evaluated histological characteristics including tumor location, tumor size, tumor stage, and residual tumor.

Statistical Analysis of Prognostic Factors

Prognostic factors associated with early mortality after surgery were first investigated using univariate Cox proportional hazards regression analyses, and significant independent variables were subsequently tested in a multivariate Cox proportional hazards regression analysis. Statistical difference was considered to be significant at $P < 0.05$.

RESULTS

Patient Characteristics

The clinical characteristics of the 37 patients are outlined in Table 1. Study subjects included 15 women and 22 men. The average age of the patients was 59.1 years, ranging from 37 to 75 years. The primary pancreatic lesion was located in the head in 30 patients and in the body in 7 patients. Of these 37 patients, 28 were affected by stage IVa disease. Twenty-three patients underwent R0 resection.

Table 1.
Patients characteristics

No. of patients	37
Age (years)	
Average	59.1
Range	37–75
Male/female	22/15
Site of primary lesion	
Head	30
Body	7
Tail	0
Tumor stage	
I	0
II	1
III	5
IVa	28
IVb	3
Pancreatectomy	
PpPD	11
PD	17
DP	6
TP	3

PpPD, pylorus preserving pancreaticoduodenectomy; PD, pancreaticoduodenectomy; DP, distal pancreatectomy; TP, total pancreatectomy.

Analyses of Survival Curve

The cumulative survival curve in this series was depicted using the Kaplan-Meier method. Five-year actual survival was 14.0%. This Kaplan-Meier survival curve could not be approximately represented by an exponential distribution with the constant hazard rate of 0.028 (−2 log likelihood was 311.851) if there was no breakpoint. Assuming that there are two distinct arms of the survival curve, below and above the breakpoint, each part of the curve was modeled as the exponential distribution. Three parameters, the breakpoint, the high hazard rate below the breakpoint, and the low hazard rate above the breakpoint were estimated by the maximum likelihood method. The magnitude of −2 log likelihood was also evaluated, changing the breakpoint and the cases having no breakpoint. Using the maximum likelihood estimate, if the breakpoint was 41, the value of the −2 log likelihood was minimal (300.072) (Table 2). Therefore, we estimated the breakpoint at 41 months. This Kaplan-Meier survival curve could be approximately represented by two distinct exponential curves with the different hazard rates below and above the breakpoint (Fig. 1). The hazard rate of the survival curves below and above the breakpoint was 0.038 and 0.0072, respectively. The difference of −2 log likelihood between the constant hazard rate model and the two distinct hazard rate model was 11.779, and it was highly significant ($P = 0.003$).

According to these analyses, SSG was defined as death earlier than 41 months after surgery, including 31 patients (73.0%). The long surviving patient group (LSG) consisted of 6 patients who were still alive more than 41 months postoperatively.

Outcomes

All 37 patients were followed until death, or from 56 to 234 months for surviving patients. The median follow-up for surviving patients is 82.7 months. The outcomes of

Table 2.
Maximum likelihood estimate

Break point (BP)	20	30	41*	50	60	none
−2 log likelihood	311.381	307.246	300.072	302.753	305.391	311.351
Hazard rate Before BP	0.0312	0.0359	0.0382	0.0381	0.0341	0.0278
Hazard rate After BP	0.0246	0.0159	0.0072	0.0081	0.0094	0.0278

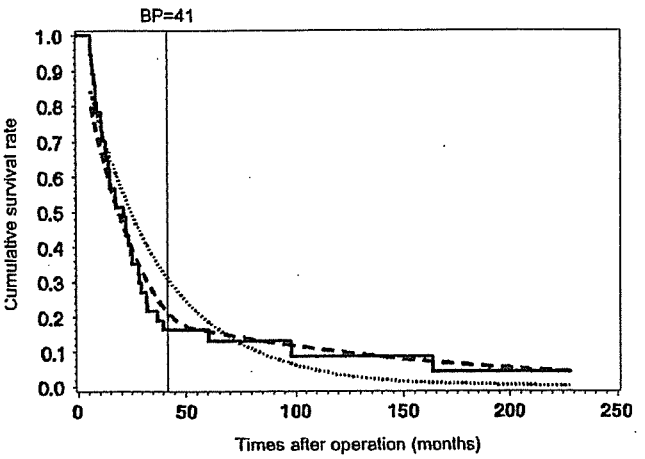

Figure 1. The cumulative survival curve in this series was depicted using the Kaplan-Meier method (a solid line). This curve could not be approximately represented by an exponential curve with a hazard rate of 0.028, if there was no breakpoint (a light dotted line), but it could be approximately represented by two distinct arms of the exponential curves that composed the survival curves below and above the breakpoint (bold dotted line). BP: breakpoint.

these patients are described in Table 3. In SSG, there were 29 (95.8%) cases of cancer-related death, compared with 5 such cases (33.3%) in LSG. The most striking outcome was that 18 patients (58.1%) died of hepatic metastases in SSG, whereas no patients died of hepatic metastases in LSG.

Table 3.
Mode of recurrence in patients with extended pancreatic resection with IORT

	SSG (31 patients)	LSG (6 patients)
Cancer-related Death		
Liver metastases	18	0
Lung metastases	2	1
Peritoneal dissemination	6	0
Pleural dissemination	0	1
Recurrence of remnant pancreas	2	0
Local recurrence	1	0
Other cause of death	2	1
Alive	0	3

SSG, short survival group; LSG, long surviving patients group; IORT, intraoperative radiation therapy.

Table 4.
Univariate analysis of prognostic factors associated with early mortality; clinical and pathological characteristics

	Hazard ratio	95% CI	*P* value
Abdominal pain and/or back pain	3.216	1.517–6.817	0.0023
Body weight loss	1.330	0.660–2.681	0.4252
Obstructive jaundice and/or cholangitis	1.534	0.748–3.147	0.2434
Tumor location (head)	1.118	0.481–2.596	0.7957
Tumor size (cm)	1.073	0.886–1.299	0.4691
Tumor stage IV	2.107	0.735–6.045	0.1655
Residual tumor (R1-2)	0.803	0.395–1.632	0.5438

Prognostic Factors Associated with Early Mortality

First, using a Cox proportional hazards model, a univariate analysis was performed to determine whether any of the characteristics were predictors of survival. Among clinical and pathological variables, abdominal pain and/or back pain during the preoperative clinical course was the only significantly prognostic factor (Table 4). Among laboratory data, preoperative elevation of serum amylase, abnormality in NBA-PABA test, and elevation of tumor-associated carbohydrate antigens levels were statistically significant prognostic factors (Table 5). The intraoperative factors, including operation time, bleeding volume, and blood transfusion were also analyzed. No significant predictive factor for early death was identified among the intraoperative variables (Table 6).

A Multivariate Analysis of Prognostic Factors

A multivariate Cox proportional hazards regression analysis of the factors that significantly predicted survival in the univariate analysis revealed that abdominal pain and/or back pain during the preoperative clinical course was the only prognostic factor (Table 7).

Table 5.
Univariate analysis of prognostic factors associated with early mortality; laboratory data

	Hazard ratio	95% CI	*P* value
White blood cell	1.000	1.000–1.000	0.7187
Lymphocytes	1.000	1.000–1.001	0.4768
Hemoglobin	1.293	0.984–1.699	0.0649
Total protein	1.051	0.563–1.961	0.8766
Albumin	1.339	0.566–3.169	0.5067
Elevation of serum Amylase level	3.213	1.488–6.937	0.0030
Elevation of Elastase-1	1.674	0.718–3.904	0.2332
Abnormal glucose metabolism	0.940	0.459–1.925	0.8652
NBT-PABA test	2.613	1.165–5.861	0.0198
tumor-associated carbohydrate antigens	2.974	1.179–7.501	0.0209

Table 6.
Univariate analysis of prognostic factors associated with early mortality; intraoperative factors

	Hazard ratio	95% CI	*P* value
Operation time (hr)	1.089	0.935–1.269	0.2743
Bleeding volume (L)	1.204	0.886–1.636	0.2343
Blood transfusion (unit)	1.025	0.998–1.052	0.0671

DISCUSSION

Pancreatic adenocarcinoma remains a lethal disease. A small coterie of patients can be salvaged, at least temporarily, by surgical resection. Compared with other treatment modalities, extended radical pancreatectomy combined with IORT has provided the best control of local recurrence,[7] although it does not appear to have made a contribution to survival benefit. The most crucial problem, apparent in more than half the patients in this series, is occurrence of hepatic metastases after operation, despite the absence of any evidence of liver metastases by preoperative radiological imaging and intraoperative examination. Even after curative resection of 21 patients with pancreatic cancer, liver metastasis occurred in 11 patients (52.4 %).[9] Liver metastasis occurred in a total of 18 cases (48.6 %) in this study, and these patients died of hepatic metastases within 41 months after operation. It is suggested that the steep downward slope of the survival curve in the early postoperative phase reflects the poor prognosis of patients who died of hepatic metastases.

Generally, survival curves of malignant diseases with poor prognosis do reveal a steep downward slope in the early postoperative phase. This implies that improvement of treatment causes the steep downward slope to change to a gentle downward slope in the survival curve. Improvement of short-term survival for pancreatic cancer can be made by establishing means of preventing liver metastases, but two crucial factors must be considered. First, is the need for more precise tools for preoperative diagnosis of liver metastases, because the high recurrence rate of liver metastases in the early period after surgery indicates that liver micro-metastases might be present at the time of operation. One advantageous diagnostic tool to detect small liver metastases is computed tomography during arterial portography combined with computed tomography–assisted hepatic arteriography (CTAP + CTHA). CTAP + CTHA has a higher sensitivity than conventional CT for detection of liver metastases.[10] The second factor is treatment for free cancer cells in the blood and latent liver micro-metastases that may not be detected by preoperative radiological imaging and intraoperative examination. Analyses of slope of survival curve in this study can be useful to evaluate the efficacy of current and future treatment for liver metastases after surgery.

Table 7.
Multivariate analysis of prognostic factors associated with early mortality

	Hazard ratio	95% CI	*P* value
Abdominal pain and/or back pain	3.780	1.431–9.988	0.0073
Elevation of serum Amylase level	1.630	0.619–4.289	0.3226
NBT-PABA test	1.781	0.662–4.793	0.2528
Tumor-associated carbohydrate antigens	1.870	0.597–5.858	0.2825

Prognostic factors associated with early mortality after surgery were evaluated using univariate and multivariate Cox proportional hazards regression analyses. If three patients who had other causes of death were considered censored, there was no difference in the statistical results. From the multivariate Cox proportional hazards regression analysis, abdominal and/or back pain was found to be the only statistically significant prognostic factor in this study. The causes of abdominal and/or back pain might be the occurrence of pancreatitis or invasion of tumor into the retroperitoneal nerve. From the univariate analysis, the preoperative serum amylase level was found to be one of the prognostic factors. These findings suggest that inflammation caused by obstruction of the main pancreatic duct and its branches may promote rapid tumor progression. The causative link between

inflammation and cancer has been described, with evidence presented for several cancers of the gastrointestinal tract.[11] We have reported that a human pancreatic cancer cell line, Capan-1, expresses the chemokine receptor 2, which is an interleukin 8 (IL-8) receptor.[12] Interleukin-8 might contribute to tumor progression via nuclear factor kappa B (NFκB) activation, because IL-8 activates NFκB. Overexpression of downstream genes of NFκB, such as urokinase plasminogen activator, are important in cancer metastasis.[13] Moreover, pancreatitis also promotes elevation of serine protease level in the pancreas and blood. It was reported that serine protease enhanced blood-borne metastases of tumor cells due to tumor cell aggregation.[14] This evidence suggests that there might be a strong correlation between inflammation and tumor progression of pancreatic cancer.

Elevation of tumor-associated carbohydrate antigen levels was also a predictive factor after surgery in this study under a univariate analysis. Sialyl Lewis^a antigen (CA 19-9) was reported to play an important role in the endothelial leukocyte adhesion molecule-1 mediated binding between human cancer cells and activated endothelial cells.[15] It has been suggested that tumor cells expressing Sialyl Lewis^a antigen have a high degree of adhesion to endothelial cells in the process of metastases. There are also reports of a positive correlation between expression of the tumor-associated carbohydrate antigens and the hepatic metastatic potential of pancreatic cancer.[9,16] These carbohydrate antigens might facilitate metastasis of cancer cells to the liver.

In conclusion, the Kaplan-Meier survival curve after surgery for advanced pancreatic cancer could be approximately represented by two distinct exponential curves, with the different hazards rates below and above the breakpoint. The high hazard rate in the early postoperative period was closely linked with death from liver metastases. Therefore, prevention and treatment of liver metastases should be a goal in improving survival of pancreatic cancer patients. The effects of prevention and treatment can be evaluated by analysis of the survival curve in this study. The preoperative presence of local pain, which may be caused by inflammation, was a prognostic factor associated with early mortality. Thus the relation between this factor and tumor progression should be precisely analyzed.

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