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ORIGINAL ARTICLE – PANCREATIC TUMORS

MicroRNA-203 Expression as a New Prognostic Marker of Pancreatic Adenocarcinoma

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ABSTRACT

Background. Detection of aberrant microRNA (miR) expression may contribute to diagnosis and prognosis of various cancers. The aim of this study is to evaluate the correlation between miR-203 expression and prognosis of patients with pancreatic adenocarcinoma after curative resection.

Methods. A total of 113 formalin-fixed paraffin-embedded tissue samples of pancreatic adenocarcinoma, 20 samples of chronic pancreatitis, and 8 samples of normal pancreas were obtained. We investigated the association of miR-203 expression measured by quantitative reverse-transcription polymerase chain reaction assays with clinicopathological parameters and survival times.

Results. miR-203 was overexpressed in pancreatic adenocarcinoma samples compared with chronic pancreatitis ($P < 0.001$) and normal pancreas ($P = 0.001$) samples. An association between miR-203 expression and clinicopathological factors of pancreatic adenocarcinoma was not observed. On univariate analysis, the high-miR-203 group and the subgroup (20%) of cases with the highest miR-203 overexpression had significantly shorter survival time ($P = 0.048$ and $P = 0.024$, respectively). Multivariate analysis revealed that miR-203 expression was an independent predictor of poor prognosis in cases with no residual tumor (relative risk 2.298, $P = 0.027$).

Conclusions. miR-203 expression is a new prognostic marker in pancreatic adenocarcinoma patients.

MicroRNAs (miRs) are 18–25-nucleotide (nt) noncoding RNAs that negatively regulate target genes by translational repression or degradation of RNAs.[1,2] Biologically, miRs serve critical roles by targeting a number of functional genes, and have been shown to regulate cell proliferation, development, differentiation, and apoptosis.[3–5] Aberrant expression of miRs, which has been demonstrated in various hematological and solid malignancies, contributes to carcinogenesis and cancer development by promoting expression of oncogenes or by inhibiting tumor suppressor genes.[4,6–8] Unique miR expression signatures have been demonstrated in various solid tumors compared with normal tissues, and detection of aberrant expression of specific miRs may contribute to diagnosis of malignancies and prediction of prognosis.[9,10]

Pancreatic adenocarcinoma is one of the most lethal tumors among all cancer types, and is the fourth leading cause of tumor-related deaths in the industrialized world.[11] Only 10–20% of patients with pancreatic adenocarcinoma have a chance of curative resection, and, even in resected cases, the 5-year survival rate is only 15–25% due to a high recurrence rate.[12–14] To date, p53, transforming growth factor-β, basic fibroblast growth factor, Bcl-2, matrix metalloproteinases, β-catenin/E-cadherin, vascular endothelial-derived growth factor, and human equilibrative nucleoside-1 have been suggested as biomarkers to predict prognosis of pancreatic adenocarcinoma patients.[15–23] However, there are conflicting findings with regard to their validity as prognostic markers, and none of the markers

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described above are used in clinical practice.[15] To further classify patients into different risk categories and to aid clinicians in choosing suitable treatments for individual patients, new and better prognostic markers are needed. miR profiles of pancreatic adenocarcinomas have been established by previous studies, and specific miRs may provide novel diagnostic or prognostic markers in pancreatic adenocarcinoma.[24–26] Bloomston et al. performed miR microarray analysis on 65 ductal adenocarcinomas of the pancreas and matched normal pancreatic tissues. Their results showed that 30 miRs, including miR-21 and miR-155, were upregulated in pancreatic adenocarcinoma compared with normal pancreatic tissues, and high miR-196a-2 expression was correlated with poor survival.[24]

miR-203 is a keratinocyte-derived miR that promotes epithelial differentiation from proliferative basal progenitors in the dermis by suppressing p63, a member of the p53 family.[27,28] miR-203 is involved in the apoptotic program observed in head and neck squamous cell carcinoma, and in hematopoietic malignancies, miR-203 functions as a tumor suppressor by targeting ABL1.[29,30] However, miR-203 is overexpressed in pancreatic adenocarcinoma compared with levels in normal pancreatic tissues and chronic pancreatitis, suggesting that miR-203 may be linked to specific characteristics of tumors and their progression patterns.[25,26] In our preliminary study of a miR array, miR-203 was also upregulated in the pancreatic cancer cell lines CAPAN-1 and CFPAC-1, compared with normal human pancreatic duct epithelial (HPDE) cells.

The aim of this study is to evaluate the correlation between miR-203 expression and clinicopathological features of pancreatic adenocarcinoma, especially patient prognosis in pancreatic adenocarcinoma patients who underwent curative pancreatectomy. We extracted miR from 113 formalin-fixed paraffin-embedded (FFPE) tissue samples of resected pancreatic adenocarcinomas by macrodissection and measured miR-203 expression levels by quantitative reverse-transcriptional polymerase chain reaction (qRT-PCR). We found that miR-203 was more highly expressed in pancreatic adenocarcinoma than in normal pancreas tissue and in chronic pancreatitis, and that miR-203 overexpression indicated poor prognosis in pancreatic adenocarcinoma patients who underwent pancreatectomy.

METHODS

Pancreatic Cell Lines

Six pancreatic cancer cell lines [AsPC-1, BxPC-3 (Dr. Iguchi, National Shikoku Cancer Center, Matsuyama, Japan), CAPAN-1, CAPAN-2, CFPAC-1, SW1990 (American Type Culture Collection (ATCC), Rockville, MD)], three colon cancer cell lines [RCM1, DLD-1 (Japanese Collection of Research Bioresources (JCRB), Osaka), HCT116 (ATCC)], two gastric cancer cell lines [AGS (Dr. Katano, Kyushu University, Fukuoka, Japan), MKN45 (JCRB)], two breast cancer cell lines [MCF7, MDA-MB231 (ATCC)], two esophageal squamous cell carcinoma cell lines [KYSE220, KYSE270 (JCRB)], a uterine cervical cancer cell line [HeLa (Dr. Kamiyoshi, Kyushu University)], a human primary normal pancreatic epithelial cell line (Cell Systems, Kirkland, WA), an immortalized pancreatic ductal epithelial cell line [HPDE6-E6E7 clone 6 (Dr. Ming-Sound Tsao, University of Toronto, Toronto, Canada)], human umbilical vein endothelial cells [HUVEC-2 (BD Biosciences, San Jose, CA)], and two primary cultures of pancreatic fibroblasts derived from patients with invasive pancreatic adenocarcinoma (established in our laboratory) were used in the present study. Cells were maintained as described in Supplementary Method 1. Primary cultures of pancreatic fibroblasts were produced using the outgrowth method as described previously.[31]

Patients and Pancreatic Tissues

Our study series consisted of 113 patients who underwent pancreatic resection for pancreatic adenocarcinoma at our institution from 1992 through 2007. Prognoses were examined in October 2008, and follow-up data of 107 cases were available. The median observation time for overall survival was 14.7 months, ranging from 0.5 to 98 months. Seventy-four patients died during follow-up, and the other patients were alive and censored. Tissues from normal pancreas ($n = 8$) and chronic pancreatitis ($n = 20$) cases were also included in our study. Normal pancreatic tissues and pancreatitis-affected tissues were taken from peripheral tissues away from nonneoplastic pancreas resected due to bile duct disease or chronic pancreatitis. All resected specimens were fixed in formalin and embedded in paraffin for pathological diagnosis. All tissues adjacent to the specimens were evaluated histologically according to the criteria of the World Health Organization.[32] The stage of tumors was assessed according to the International Union against Cancer (UICC) classification.[33] The clinicopathological characteristics of the tumor collection are presented in Table 1. The study was approved by the Ethics Committee of Kyushu University and conducted in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and the Helsinki Declaration.

Macrodissection

After a review of representative hematoxylin and eosin-stained slides, four to seven sections of 5-μm thickness

TABLE 1 Clinicopathological characteristics of the patients ($N = 113$)

Median age	66 years (range 36–86 years)
Sex (male/female)	71 (62.8%)/42 (37.2%)
Histological diagnosis	
Ductal adenocarcinoma	111 (98.2%)
Intraductal papillary–mucinous carcinoma (invasive type)	2 (1.8%)
pT category	
pT1	6 (5.3%)
pT2	4 (3.5%)
pT3	102 (90.3%)
pT4	1 (0.9%)
pN category	
pN0	37 (32.7%)
pN1	76 (67.3%)
UICC stage	
I	8 (7.1%)
II	101 (89.4%)
III	1 (0.9%)
IV	3 (2.7%)
Histological grade	
G1	22 (20.2%)
G2	44 (40.4%)
G3	43 (39.5%)
Residual tumor category	
R0	73 (64.6%)
R1	40 (35.4%)
Vessel invasion	
Positive	73 (64.6%)
Negative	40 (35.4%)
Neural invasion	
Positive	94 (83.2%)
Negative	19 (16.8%)

were obtained from FFPE blocks of pancreatic adenocarcinoma, chronic pancreatitis, and normal pancreas for macrodissection. Adjacent normal tissues including normal acinar tissues and adipose tissues in the sections of pancreatic adenocarcinoma and chronic pancreatitis were macroscopically removed with a scalpel. Only cancerous parts and pancreatitis-affected areas of sections were used for isolation of miR.

Isolation of miR

miR was extracted from FFPE samples of pancreatic tissues using the RNeasy FFPE kit (Qiagen, Tokyo, Japan) by modifying the manufacturer's instructions. Detailed procedures are described in Supplementary Method 2. Total RNA samples were also extracted from cultured cells at 90% confluence using a High Pure RNA isolation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Extracted RNA was quantified by absorbance at 260 nm, and its purity was evaluated from the 260/280 ratio of absorbance using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA).

Quantitative Reverse-Transcriptional Polymerase Chain Reaction

Quantitative RT-PCR was performed using a Chromo4 Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) using a TaqMan® MicroRNA reverse transcription kit and TaqMan® Universal PCR Master Mix (Applied Biosystems, Tokyo, Japan). For the measurement of miR-203 and RNU6B expression (U6 snRNA, a reference gene), we performed two-step qRT-PCR with specific primers for miR-203 and RNU6B (designed by Applied Biosystems) following the manufacturer's protocol. Briefly, the reaction mixture was initially incubated at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min, and then held at 4°C for the reverse-transcription step until the preparation for the PCR reaction had been completed. PCR amplification was initiated with one cycle of 95°C for 10 min to activate the AmpliTaq Gold® enzyme (Applied Biosystems), followed by 40 cycles of 94°C for 15 s and 60°C for 60 s. Each sample was run in triplicate. The level of miR-203 expression was calculated from a standard curve constructed with small RNAs from CAPAN-1. The expression levels of miR-203 were normalized to those of RNU6B.

Statistical Analysis

Data of miR-203 expression in FFPE samples were analyzed using the Mann–Whitney *U*-test. miR-203 expression was divided into high- and low-level groups using recursive descent partition analysis, as described by Hoffmann et al.[34] This analysis finds a set of cutoff points for gene expression values that best predict the survival time by searching all possible cutoffs, and it can determine the most significant split by the largest likelihood-ratio chi-square statistic.[34] Categorical variables were compared with a chi-square test (Fisher's exact probability test). Survival curves were constructed with the Kaplan–Meier product-limit method and compared by log-rank test. To evaluate independent prognostic factors associated with survival, multivariate Cox proportional-hazards regression analysis was used, with miR-203 expression, pN status, UICC stage, residual tumor status (R factor), histological grade (G), and vessel invasion as covariates. Statistical

significance was defined as a P value <0.05. All statistical analyses were performed using JMP 7.01 software (SAS Institute, Cary, NC, USA).

RESULTS

Pancreatic Cancer Cell Lines Showed Upregulation of miR-203

We conducted a miR array to investigate the difference in miR expression between HPDE cells and pancreatic cancer cells. The results from the miR array were validated by miR RT-PCR analysis. We found that the expression level of miR-203 in HPDE cells was lower than that in CAPAN-1 or CFPAC-1 cells (Fig. 1a). Next, we measured miR-203 expression in six pancreatic cancer cell lines, a normal pancreatic epithelial cell line, cancer-associated fibroblasts, and HUVEC-2 cells. Five pancreatic cancer cell lines (i.e., all except AsPC-1 cells) expressed miR-203 at higher levels than did nonmalignant cells, which expressed little or no miR-203 (Fig. 1b). Additionally, we examined miR-203 expression in colon cancer, gastric

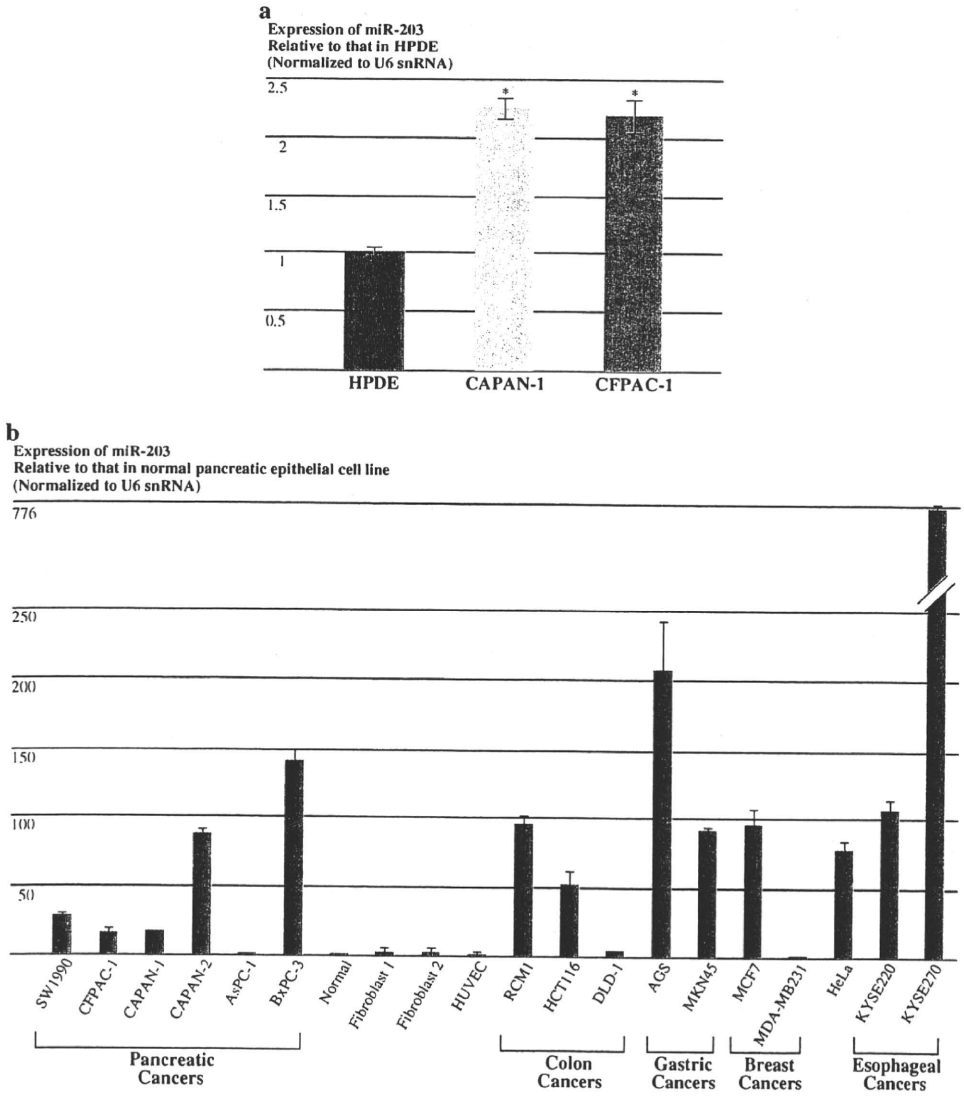

FIG. 1 **a** miR-203 expression in two pancreatic cancer cell lines and HPDE cells. Expression levels of miR-203 were normalized to the corresponding levels of U6 snRNA. Each sample was analyzed in triplicate, and values are expressed as levels (mean ± standard deviation) relative to those in HPDE cells. miR-203 expression in CAPAN-1 and CFPAC-1 cells was higher than in HPDE cells. * $P < 0.05$. **b** Relative miR-203 expression in six pancreatic cancer cell lines (SW1990, CFPAC-1, CAPAN-1, CAPAN-2, AsPC-1, and BxPC3), as well as colon cancer (RCM1, HCT116, and DLD-1), gastric cancer (AGS and MKN45), breast cancer (MCF7 and MDA-MB231), esophageal cancer (KYSE220 and KYSE270), and uterine cervical cancer (HeLa) cell lines, and nonmalignant cells (normal pancreatic epithelial cell line, two cultures of cancer-associated fibroblasts, and HUVEC-2 cells). Expression levels of miR-203 were normalized to the corresponding levels of U6 snRNA. Each sample was analyzed in triplicate, and values are expressed as levels (mean ± standard deviation) relative to those in the normal pancreatic epithelial cell line. Normal, normal pancreatic epithelial cell line

cancer, breast cancer, esophageal cancer, and uterine cervical cancer cell lines. Most cancer cell lines expressed higher levels of miR-203 compared with nonmalignant cells (Fig. 1b), suggesting that upregulation of miR-203 was induced in a broad array of malignant solid tumors.

Quantitative Analysis of miR-203 Expression in Normal Pancreas, Chronic Pancreatitis, and Pancreatic Adenocarcinoma

We measured miR-203 expression levels in normal pancreas, chronic pancreatitis, and pancreatic adenocarcinoma by qRT-PCR to determine whether miR-203 is differentially expressed between the tissue types. miR-203 expression was detected in all FFPE samples of pancreatic tissues, and the expression levels of miR-203 were normalized to RNU6B for analyses. In the cancer tissues, the expression levels of miR-203 ranged widely, with the highest observed expression level being 546-fold that of the lowest expression level. miR-203 expression was significantly higher in pancreatic adenocarcinoma than in chronic pancreatitis ($P < 0.001$) and normal pancreas ($P = 0.001$, Fig. 2). The median expression levels of miR-203 in normal pancreas, chronic pancreatitis, and pancreatic adenocarcinoma were 0.024, 0.013, and 0.064, respectively.

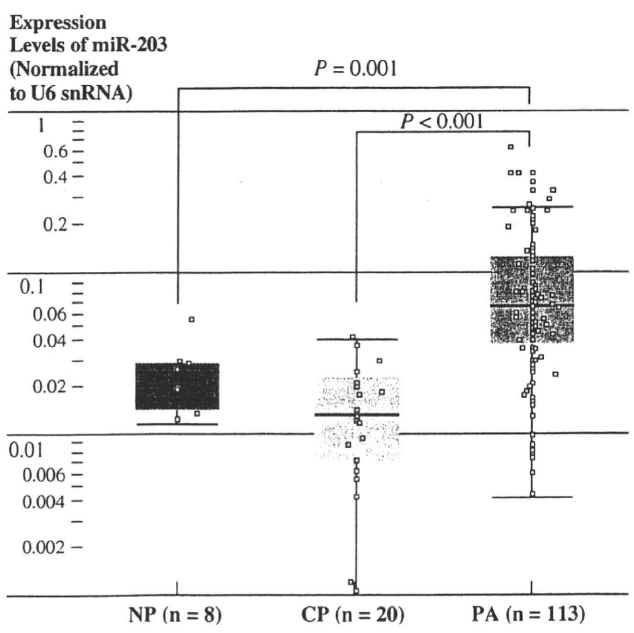

FIG. 2 Quantitative analysis of miR-203 expression in normal pancreas, chronic pancreatitis, and pancreatic adenocarcinoma. Expression levels of miR-203 were normalized to the corresponding levels of U6 snRNA. miR-203 expression was significantly higher in pancreatic adenocarcinoma (PA) than in chronic pancreatitis (CP; $P < 0.001$) and normal pancreas (NP; $P = 0.001$)

Relationship between miR-203 Expression in Resected Pancreatic Adenocarcinoma and Various Clinicopathological Factors

We constructed two groups with high versus low miR-203 expression (cutoff value of 0.054) based on recursive descent partition analysis. The high-expression and low-expression miR-203 groups were composed of 63 and 50 cases, respectively. We did not find any significant association between miR-203 expression and clinicopathological factors (Supplementary Table 1).

Univariate Analysis of miR-203 Expression for Survival Time of Patients with Pancreatic Adenocarcinoma after Curative Resection

On univariate survival analysis, the conventional prognostic markers, pN status, UICC stage, histological grade, R factor, and positive vessel invasion reached significance for overall survival (Table 2). The high-miR-203 group had significantly shorter survival than the low-miR-203 group (Fig. 3a, $P = 0.048$). The median survival time and the 5-year survival rate were 15 months and 14.6% in the high-miR-203 group, and 26 months and 27.3% in the low-miR-203 group. Moreover, we compared the top 20% (22 cases) of these cases in terms of miR-203 expression with the bottom 20% (22 cases) by constructing other Kaplan–Meier survival curves in order to confirm the findings that miR-203 overexpression was a predictor of poor prognosis in pancreatic adenocarcinoma patients. The 20% of cases with the highest miR-203 expression showed a more significantly shorter survival (Fig. 3b, $P = 0.024$).

Multivariate Analysis of miR-203 Expression for Survival Time of Patients with Pancreatic Adenocarcinoma after Curative Resection

Multivariate survival analysis was performed on all parameters that were found to be significant on univariate analysis. Overall survival time was significantly dependent on histological grade ($P < 0.001$) and R factor ($P < 0.001$) but not miR-203 (Table 3). Additionally, we performed multivariate analysis for R0 cases. When limited to cases with R0, miR-203 was found to be an independent predictor of poor prognosis (Table 4). These results suggest that miR-203 expression may be an independent prognostic marker in pancreatic adenocarcinoma patients when the tumors are excised completely without microscopic residue of cancer cells.

TABLE 2 Univariate survival analysis of conventional prognostic factors and miR-203 expression ($N = 107$)

Characteristics	No. of cases	Median survival time (months)	5-Year survival rate	P value
miR-203 expression				0.048
High	58	15	14.6%	
Low	49	26	27.3%	
Age				0.863
≥65 years	60	20.9	24.5%	
<64 years	47	19.0	32.8%	
pT category				0.210
pT1/pT2	10	63	56.0%	
pT3/pT4	97	19	16.5%	
pN category				<0.001
pN0	36	43	32.8%	
pN1	71	13.3	13.1%	
UICC stage				0.049
I	8	63	70.0%	
II	95	19	17.1%	
III/IV	4	12	0%	
Histological grade				<0.001
G1/2	62	27	25.6%	
G3	42	12	12.7%	
Residual tumor				<0.001
R0	68	26	29.6%	
R1	39	12	4.6%	
Vessel invasion				0.004
Positive	69	13.7	12.3%	
Negative	38	31	33.3%	
Neural invasion				0.941
Positive	90	20.9	18.1%	
Negative	17	19	27.9%	

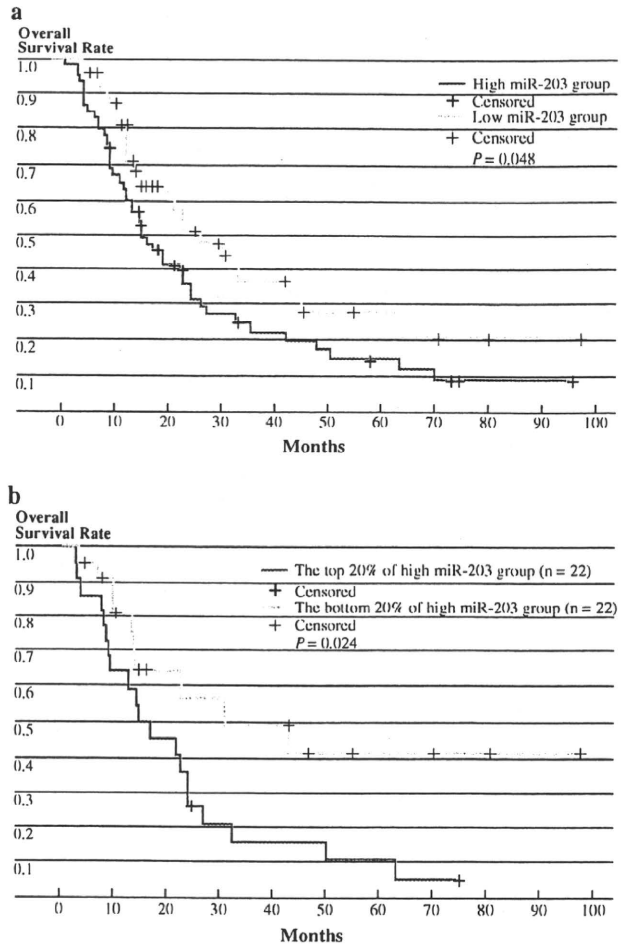

FIG. 3 **a** Overall survival after resection of pancreatic adenocarcinomas with high miR-203 expression versus low miR-203 expression. High miR-203 expression was significantly associated with shorter survival ($P = 0.048$). miR-203 expression levels were normalized to RNU6B levels. **b** Overall survival after resection of pancreatic adenocarcinomas in the top 20% (22 cases) of cases in terms of high miR-203 expression versus the bottom 20% (22 cases) having the lowest miR-203 expression. The top 20% of cases in terms of miR-203 overexpression showed significantly shorter survival ($P = 0.024$). miR-203 expression levels were normalized to RNU6B levels

DISCUSSION

Only a few among 600 miRs have been found to be associated with prognosis in solid tumors; however, miRs were discovered only recently. High miR-21 expression has been correlated with poor prognosis in colon cancer and pancreatic adenocarcinoma without nodal metastasis, and high miR-155 expression is a significant indicator of poor survival in lung carcinoma.[10,35,36] In the present study, we showed that high miR-203 expression was significantly correlated with poor survival in pancreatic adenocarcinoma and may represent an independent prognostic marker in pancreatic adenocarcinoma patients who have undergone curative surgery. This finding is inconsistent with the report of Bueno et al. that miR-203 functions as a tumor suppressor by targeting ABL1, which is specifically activated in chronic myelogenous leukemia and acute lymphoblastic leukemia.[30] Their study showed that miR-203 re-expression reduced ABL1 levels and inhibited tumor cell proliferation, suggesting that miR-203 may be of therapeutic benefit in hematopoietic malignancies. However, malignant tumors have a specific signature of miR expression according to the type of cancer.[8] Moreover, miRs target various genes and intricately regulate biological function. We showed that upregulation of miR-203 was observed in not only pancreatic adenocarcinomas but also in other solid tumors including colon, gastric, breast, and uterine cervical cancers. miR-203 overexpression in pancreatic adenocarcinoma may regulate some genes involved in cancer progression that are unique to solid malignancy, resulting in poor prognosis. On the other hand,

TABLE 3 Multivariate survival analysis (Cox regression model) of conventional prognostic factors and miR-203

	Relative risk	95% Confidence interval	*P* value
miR-203	1.212	0.720–2.068	0.472
pN status	1.488	0.810–2.857	0.205
UICC stage	–	–	0.759
Histological grade (G3)	3.001	1.756–5.120	<0.001
R factor	2.633	1.572–4.423	<0.001
Vessel invasion	1.644	0.483–1.390	0.090

The relative risk of UICC stage is not shown because of the two parameters involved

TABLE 4 Multivariate survival analysis (Cox regression model) of conventional prognostic factors and miR-203 in pancreatic adenocarcinoma with R0

	Relative risk	95% Confidence interval	*P* value
miR-203	2.298	1.097–4.986	0.027
pN status	1.524	0.683–3.687	0.311
UICC stage	–	–	0.645
Histological grade (G3)	4.373	1.942–10.045	<0.001
Vessel invasion	1.496	0.707–3.380	0.300

The relative risk of UICC stage is not shown because of the two parameters involved

the results of our study did not clarify whether miR-203 expression plays a role in cancer progression, because miR-203 overexpression was not correlated with any clinicopathological factors of pancreatic adenocarcinoma. miR-203 may be involved in other malignant behaviors including stress resistance, rather than conventional progressive pathways including tumor invasion of adjacent tissues or metastasis to lymph nodes. The function of miR-203 in carcinogenesis and the development of tumors should be clarified.

Recently, sensitive quantitative analysis of gene expression from FFPE tissues was made possible with qRT-PCR following improvements in extraction and analysis of RNA from FFPE tissues.[37,38] Large-scale analysis of RNA expression based on a huge resource of FFPE samples with clinical follow-up data provides invaluable information about prognostic factors.[39] Hoffmann et al. performed qRT-PCR using total RNA isolated from 65 FFPE samples of pancreatic adenocarcinoma, reporting that hypoxia-inducible factor 1α expression had a strong association with prognosis.[34] In the present study, using qRT-PCR we measured miR-203 expression levels for 113 FFPE samples of pancreatic adenocarcinoma, 20 samples of chronic pancreatitis, and 8 samples of normal pancreas. Nonneoplastic cells, including normal epithelial cells, cancer-associated fibroblasts, and endothelial cells expressed little miR-203, and the expression that was recorded in FFPE tissues was considered to be derived from malignant epithelial cells. Our results showed that miR-203 was expressed in pancreatic adenocarcinoma to a greater extent than in chronic pancreatitis or in normal pancreas, and was associated with poor prognosis in pancreatic adenocarcinoma patients. Methodologically, qRT-PCR analysis of miRs extracted from FFPE samples holds a great advantage compared with conventional messenger RNA (mRNA) expression analysis. miRs are stable, short-sized RNAs, while mRNAs extracted from FFPE tissue can be highly degraded.[2] Analysis of miRs in clinical samples using qRT-PCR is very effective, especially for small amounts of tissue or fragmented RNA obtained by endoscopic retrograde pancreatography or endoscopic ultrasound fine-needle aspiration. The reproducibility of qRT-PCR using clinical samples is influenced by a variety of factors, including tissue preparation, miR extraction, and the qRT-PCR protocol. In our study, the difference in expression levels of miR-203 between nonmalignant and malignant tumors was less than threefold, which may be too small to analyze independent samples for individual patient prognosis. However, the median relative expression level of miR-203 (0.23) in the 20% of pancreatic adenocarcinoma cases with the highest miR-203 expression was 14.4-fold that (0.016) in the 20% of cases with the lowest miR-203 expression, and 9.6-fold that in normal pancreas. By setting a high cutoff value, we could obtain reproducible results and yield useful information. In addition, to date, no single biomarker has been found to have sufficient predictive accuracy for prognosis in pancreatic adenocarcinoma. Measurement of miR-203 expression in combination with that of several other miRs may possibly provide improved diagnostic accuracy.

We showed that miR-203 expression was significant for prognosis in all pancreatic adenocarcinoma patients on univariate analysis; however, on multivariate analysis, miR-203 expression was significant for prognosis only in R0 cases. This result appears somewhat inconsequential in biological terms, because miR-203 expression is not associated with R factor or R1-predicting factors including T stage and perineural/vascular invasion. It cannot be denied that there is the possibility of statistical error due to subset analyses. However, we consider that this is because the effect of miR-203 expression on survival outcome was too weak in R1 patients. The median survival time of patients with R1 was 12 months, which may be too short to evaluate the correlation between miR-203 and prognosis in R1 patients. At any rate, we can state that miR-203 is an independent predictor of poor prognosis in pancreatic

adenocarcinoma patients when the tumors are excised completely without microscopic residue of cancer cells.

In conclusion, our results suggest that microRNA-203 expression is a new prognostic marker for pancreatic adenocarcinoma. Measurement of miR levels is a novel approach to determine malignancy of pancreatic tumors and predict prognosis from clinical samples. The clinical significance of preoperative analysis of miR-203 expression needs to be explored further.

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Predictors of the Presence of Concomitant Invasive Ductal Carcinoma in Intraductal Papillary Mucinous Neoplasm of the Pancreas

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Objective: Invasive ductal carcinoma (DC) of the pancreas arising as an independent lesion in association with intraductal papillary mucinous neoplasm (IPMN) has occasionally been reported. However, clinicopathological features related to the presence of DC in patients with IPMN remain largely unknown. The purpose of the present study was to determine the factors predicting the presence of concomitant DC in those with IPMN.

Materials and Methods: We retrospectively reviewed the clinicopathological data of a consecutive series of 236 patients with IPMN treated by surgical resection or followed up at our institution between January 1987 and June 2008. In an attempt to identify predictors for the presence of DC, clinicopathological variables were compared between IPMN patients with concomitant DC and those without concomitant DC.

Results: Of 236 patients with IPMN, concomitant DC was detected synchronously or metachronously in 22 patients (9.3%). All the 22 IPMNs were of branch duct type and histological grades of 12 resected IPMNs were adenoma (n = 8) and borderline (n = 4). Multivariate analysis revealed 2 significant predictive factors for the presence of DC in IPMN, including worsening diabetes mellitus ($P < 0.001$) and an abnormal serum CA 19–9 level ($P = 0.024$).

Conclusion: In view of the high prevalence of DC careful inspection of the entire pancreatic gland is necessary for early detection of DC in patients with branch duct IPMNs, especially when worsening diabetes mellitus and an abnormal serum CA 19–9 level are manifested.

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Intraductal papillary mucinous neoplasm (IPMN) of the pancreas is a distinct clinicopathological entity that was initially described in 1982 by Ohashi et al as "mucin-producing tumor of the pancreas" with better prognosis as compared with ductal carcinoma (DC) of the pancreas.[1] IPMN is, by definition, a grossly visible noninvasive neoplasm that arises in the main pancreatic duct (MPD) or its major branches. Currently, IPMNs are being increasingly recognized and now account for 8% to 20% of all resected pancreatic neoplasms, probably ascribable to the widespread use of screening ultrasound, the development of cross-sectional imaging and the increased awareness of this entity among clinicians.[2,3]

Clinically, most IPMNs are less aggressive and survival after surgical resection is better than that of conventional DC. Importantly, however, a concomitant DC is sometimes found in a distinct location of the pancreatic gland where no IPMN is identifiable, suggesting that IPMNs serve not only as a precursor to invasive carcinoma but also as a predictor of an independent DC.[4,5]

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In previous studies, DC of the pancreas was found synchronously or metachronously in 8% to 9.2% of patients with IPMN.[5,6] These findings suggest that careful imaging investigation is needed in patients with IPMN, especially in those at an increased risk of associated invasive carcinoma. However, clinicopathological features associated with the occurrence of concomitant DC in patients with IPMN have not been fully investigated. In the present study, we retrospectively reviewed the clinicopathological data from a consecutive series of 236 patients with IPMN in an attempt to identify predictors for the presence of DC of the pancreas.

PATIENTS AND METHODS

Patients and Clinicopathological Variables

Clinicopathological data were extracted from a consecutive series of 236 patients with IPMN treated by surgical resection or watched without resection at our institution between January 1987 and June 2008. IPMNs were diagnosed macroscopically by imaging studies (CT, MRI/MRCP, EUS, and/or ERCP) and microscopically by pathologic examination of the resected specimens.[7,8] This is a cohort study to evaluate possible predictors of the presence of concomitant but separate DC at the initial diagnosis. We used the criteria for resection proposed in the international consensus guidelines for the management of IPMN and mucinous cystic neoplasm published in 2006 (Sendai criteria) for selecting patients for surgical resection. Before the proposal of the Sendai criteria, we used to resect all main duct IPMNs and branch duct IPMNs which were greater than 3 cm in size and/or symptomatic according to a Japanese consensus.

CT and MRI were used for evaluation of the size of the cyst and the MPD in the follow-up. In the nonoperative patients, the mean follow-up period was 31.8 ± 30.4 months. During the follow-up, operation was recommended to the patients when there was an increase in the cyst size to greater than 30 mm, appearance of mural nodules, or enlargement of the MPD, or the patient became symptomatic.

Factors analyzed included patient's age, gender, and clinical symptoms/manifestations including abdominal pain or back pain, abdominal fullness or discomfort, jaundice, worsening diabetes mellitus (DM), and general fatigue. The patients were defined as asymptomatic if IPMN or DC was discovered incidentally or during follow-up of the underlying diseases by imaging. Serum levels of carcinoembryonic antigen (CEA, normal range, 0–3.2 ng/mL) and CA 19–9 (normal range, 0–37 U/mL) as well as pancreatic juice cytology obtained endoscopically during ERCP were also analyzed. Pathologically, several parameters were reviewed, including the type of IPMN, of the WHO classification diameter of the MPD, maximal diameter of the cystic lesion, and appearance of mural nodule in the MPD or branch ducts. The histologic subtypes of IPMN including intestinal, gastric foveolar, oncocytic, and pancreatobiliary types were evaluated by using the different expressions of MUC1, MUC2, and MUC5AC.[9–11]

Statistical Analysis

All statistical analyses were performed with SPSS software (version 13.0; SPSS Inc, Chicago). Descriptive statistics were re-

ported as percentage or mean (standard deviation; SD) unless specified otherwise. Univariate analyses between factors and the occurrence of pancreatic carcinoma in IPMN patients were compared by using independent t test for continuous data and χ^2 test or Fisher exact test for categorical data. Stepwise backward logistic regression analysis was used as the method for variable selection, variables with P value >0.05 were eliminated from the model. Survival analysis was performed by the Kaplan-Meier analysis and P value was calculated by the log-rank test. The P value <0.05 was considered to be statistically significant.

RESULTS

Clinicopathological Features of Patients With IPMN Associated With Concomitant Ductal Carcinoma of the Pancreas

Of 236 patients with IPMN, 200 had branch duct IPMN and 36 had main duct IPMN. Only 3 of the 36 patients with main duct IPMN did not undergo resection due to their advanced age or refusal. Of 200 branch duct IPMN patients, 22 (9.3% of all IPMNs, or 11% of branch duct IPMNs) had concomitant DC and the other 178 had IPMNs alone. They included 15 men and 7 women with a mean age of 70 years. The detailed management in 22 patients with concomitant DC is given in Table 1 (resection in 17, no resection in 5). In the 178 IPMN alone group, surgical resection was performed in 93 patients and 85 patients were observed.

Twenty of the 22 patients with DC and branch duct IPMN developed their DC synchronously. Only 2 patients developed DC metachronously in the remnant pancreas. One of them was found to have DC in the pancreatic remnant at 4-year follow-up after pylorus-preserving pancreatoduodenectomy for a branch duct IPMN with a 10-mm mural nodule in the pancreatic head. Since this patient already had severe diabetes, partial pancreatectomy was added, leaving a small portion of the pancreatic tail in place. Another patient developed DC in the pancreatic head 7 years after distal pancreatectomy for a branch duct IPMN and concomitant but separate carcinoma in situ in the pancreatic tail. The patient underwent completion total pancreatectomy.

The most common sign/symptom seen in the 22 IPMN patients with concomitant DC was worsening DM seen in 10 patients (45%), followed by abdominal pain or discomfort in 6 patients (27%) and weight loss in 4 patients (18%). Back pain was

TABLE 1. Clinicopathological Findings of 22 Patients with Intraductal Papillary Mucinous Neoplasm and Concomitant Ductal Carcinoma of the Pancreas

No.	Sex	Age	Chief Complaint	Location of IPMN/DC	Operation	Increased Serum CA 19-9/CEA	MPD (mm)	WHO Classification	Staging of DC	DC Size (mm)
1	M	63	Worsening DM, weight loss	Ph/Ph	PPPD	+/−	3	IPMB	IVa	25
2	M	67	Abdominal pain	Pt/Ph	PPPD	+/+	5	None	IVa	27
3	M	80	Back pain	Pb and Pt/Ph	PPPD	+/+	2	None	IVa	45
4	M	75	Abdominal pain	Ph/Ph	PPPD	−/−	10	IPMB	III	22
5	M	62	Abdominal pain	Ph/Pb	PPPD and SR*	−/−	5	IPMB	III	25
6	F	70	Worsening DM, weight loss	Ph/Ph	PPPD	+/−	3	IPMA	II	30
7	M	72	Worsening DM, weight loss	Ph/Ph	PPPD	+/−	10	IPMA	III	14
8	M	69	Worsening DM, weight loss	Pt/Ph	PPPD	−/−	4	IPMA	I	11
9	M	63	None (F/U rectal cancer)	Ph/Pb	TP	−/−	3	IPMA	I	15
10	M	74	Worsening DM	Ph/Pb	DP	−/−	4	None	IVa	18
11	M	73	None (F/U malignant lymphoma)	Ph/Pb	DP	+/−	10	None	IVa	40
12	M	69	Abdominal pain	Pt/Pt	DP	−/−	8	IPMA	III	25
13	M	59	None (F/U gastric cancer)	Pb/Pb	DP	−/−	3	IPMA	0	CIS
14	M	55	None (F/U colon cancer and HCC)	Pt/Pb	DP and PD†	−/−	2	IPMA	0	CIS
15	M	60	Worsening DM, abdominal pain	Pb/Pb	DP	−/+	6	IPMB	III	25
16	F	82	Worsening DM	Ph/Pb	DP	+/−	3	None	III	75
17	F	54	Worsening DM	Diffuse/Pb	DP	−/−	4	IPMA	I	15
18	F	82	None (check up)	Ph/Pb	None	−/−	3	None	IVa	15
19	F	79	Worsening DM, back pain	Ph/Pb	None	+/+	3	None	IVb	25
20	M	67	Abdominal fullness	Ph/Pb	None	−/+	3	None	IVb	38
21	F	83	Worsening DM	Ph/Pb	None	+/−	9	None	IVa	30
22	F	80	Jaundice	Diffuse/Ph	None	+/−	3.8	None	III	30

*PPPD with segmental resection 4 yr later.

†DP with completion total pancreatectomy (PPPD) 7 yr later.

PPPD indicates pylorus-preserving pancreaticoduodenectomy; SR, segmental resection of the pancreas; DP, distal pancreatectomy; TP, total pancreatectomy; CIS, carcinoma in situ; Ph, pancreatic head; Pb, pancreatic body; Pt, pancreatic tail; +, abnormal serum tumor marker; −, normal level tumor marker.

found in 2 patients and jaundice in only 1. Among the 5 patients who had no sign/symptom, 4 patients were found to have DC during the follow-up of a previously identified cancer, including gastric cancer, rectal cancer, malignant lymphoma, and coincidental hepatocellular carcinoma and colon cancer (synchronously with pancreatic cancer). The remaining patient was found to have DC incidentally during annual health check-up. Elevated serum levels of CA 19–9 and CEA were seen in 10 (46%) and 5 patients (23%), respectively. We used CT scan to measure the size of branch duct IPMN and MPD accurately. The size of the cyst at the initial diagnosis was 24.6 ± 10.6 mm (min 10 mm, max 40 mm) in 22 patients with IPMN and concomitant DC and not different from 29.3 ± 14.9 mm (min 5 mm, max 84 mm) in the remaining 178 patients with IPMN alone (P = 0.14). Dilatation of MPD (6 mm or larger in diameter) was noted in only 6 of the 22 patients (27%). EUS was performed in 160 of a total of 236 patients (67.8%) in the present series. The other patients did not undergo EUS because of limitation of preoperative time or their reluctance. Pancreatic juice cytology was performed in 18 patients who underwent ERCP and 6 of them (33%) were identified as positive (class V) (Table 1).

IPMNs were located in the head of the pancreas in 12 patients (55%), body and tail in 8 patients (36%), and in multiple places in the entire gland in 2 patients (9%). A mural nodule was preoperatively detected in only 1 patient (case 5). DC was located in the pancreatic body and tail in 14 patients (64%) and in the head in 8 patients (36%). Eight patients (36%) underwent pancreatoduodenectomy (case 1–8, Fig. 1) and 8 patients (36%) underwent distal pancreatectomy (case 10–17, Fig. 2). Total pancreatectomy was performed in 1 patient (case 9, Fig. 1) with DC in the pancreatic body and a 3-cm IPMN in the pancreatic head. Additionally, the pathologist eventually identified several areas of carcinoma in situ and invasive DC in the pancreatic body and tail of this patient. One patient underwent segmental resection of the pancreatic body 4 years after PPPD (case 5) and another patient had additional pancreatic head resection 7 years after distal pancreatectomy for a small IPMN and a separate DC in situ (case 14). Four patients did not undergo surgery because of advanced age (2 patients) or metastatic disease (2 patients), and 1 patient with DC spreading to the major vessels underwent bypass surgery (Fig. 3). Concomitant extrapancreatic malignancies were also found in 4 of 22 patients (23%), including gastric cancer, rectal cancer, colon and hepatocellular carcinoma, and malignant lymphoma.

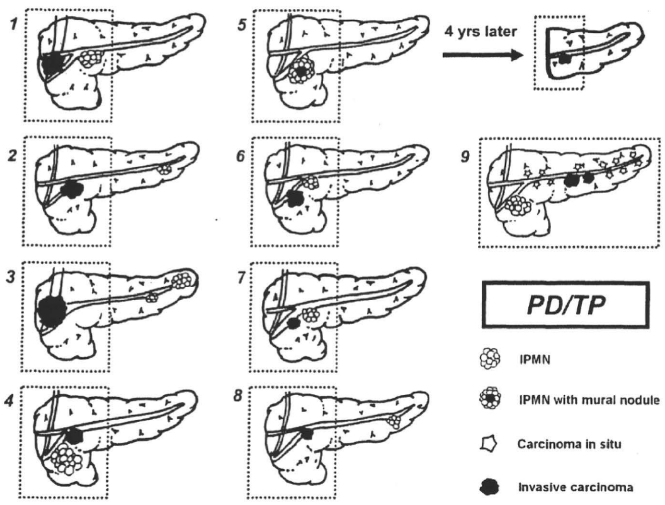

FIGURE 1. Schematic representation of 9 patients with IPMN and concomitant DC who underwent pancreatoduodenectomy (PD) (8 patients) or total duodenectomy (1 patient).

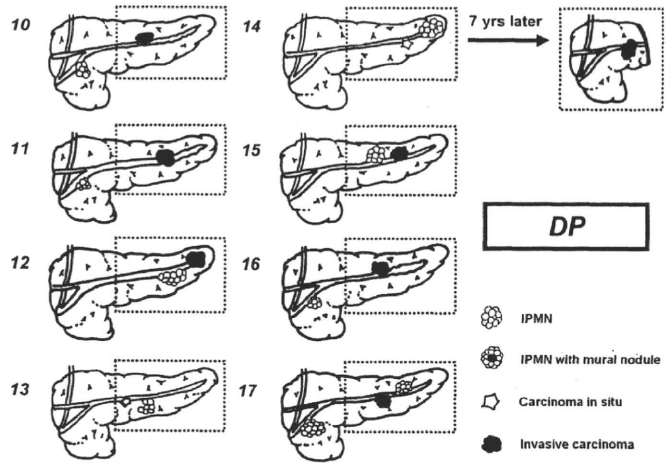

FIGURE 2. Schematic representation of 8 patients with IPMN and concomitant DC who underwent distal pancreatectomy (DP).

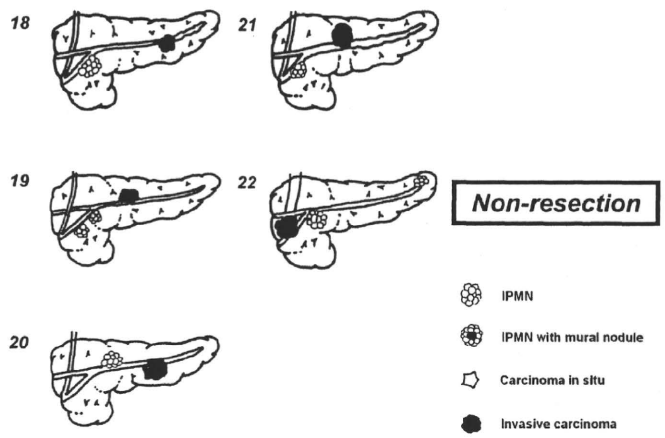

FIGURE 3. Schematic representation of 5 patients with IPMN and concomitant DC who did not undergo pancreatic resection.

Intraoperative frozen section histology of the pancreatic excisional stump reported no positive margins; however, final pathologic diagnosis identified 3 patients with positive margins, including 2 peripancreatic tissue dissection margin and 1 peripheral exposure wedge margin. Histopathological examination of 12 resected IPMNs revealed 8 IPMA and 4 IPMB according to the WHO classification. The most common histologic subtype in this group was gastric-foveolar as seen in 9 patients (data not shown).

The survival time of patients with IPMN associated with separate DC was significantly shorter than that of patients with IPMN alone ($P < 0.001$, Fig. 4). The median survival time of IPMN with DC was 49.0 months (95% CI: 5.17–92.83), the 25th percentile of survival time of IPMN with DC and IPMN alone were 12 and 98 months, respectively.

Clinicopathological Factors Predicting the Presence of Concomitant DC

The clinicopathological features were compared between 22 patients with branch duct IPMN associated with DC and 178 patients with branch duct IPMN without DC, excluding those with main duct IPMN. Data on some features were not necessarily obtained in all patients. Whereas univariate analysis showed no

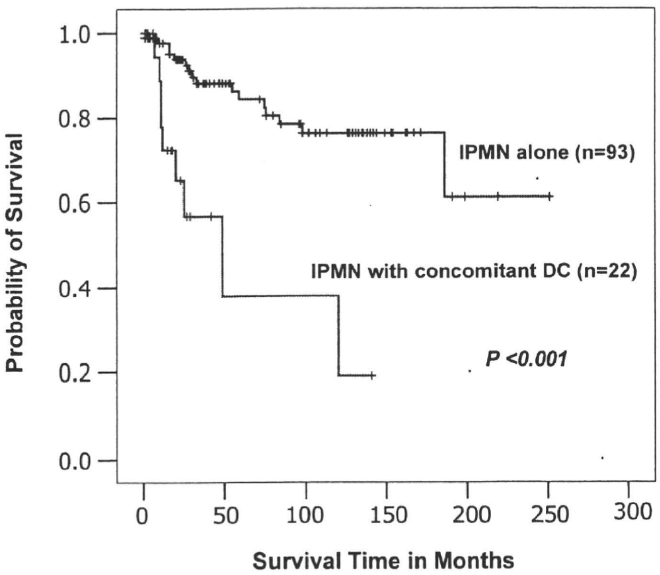

FIGURE 4. Kaplan-Meier acturial survival curves comparing patients with IPMN alone and patients with IPMN with concomitant DC.

significant differences between the 2 groups in age, sex, clinical manifestations such as abdominal or back pain and jaundice, the presence of mural nodules in IPMN, location of IPMN or DC, the size of the MPD, serum CEA level, histologic grade of adenoma (IPMA of WHO Classification), and immunocytochemistry of MUC proteins, 4 potentially predictive factors for the presence of DC were found, including worsening DM ($P < 0.001$), weight loss ($P < 0.001$), abnormal serum CA 19–9 ($P = 0.010$), and pancreatic juice cytology (Table 2). Furthermore, multivariate analysis revealed worsening DM (odds ratio 15.73 [95% CI: 4.40–56.25]; $P < 0.001$), and abnormal serum CA 19–9 (odds ratio 3.70 [95% CI: 1.19–11.48]; $P = 0.024$) to be independent predictive factors related to the occurrence of synchronous or metachronous separate DC in patients with IPMN (Table 3).

DISCUSSION

In the present study, we retrospectively reviewed the clinicopathological data from a consecutive series of 236 patients with IPMN in an attempt to elucidate the clinicopathological features of invasive DC arising in a different part of the pancreas in association with IPMN. Our results demonstrate that 22 of the 236 patients (9.3%) were found to have concomitant DC as an independent lesion synchronously or metachronously. Interestingly, all of these 22 IPMNs were of branch duct type with nondilated MPD. Furthermore, multivariate analysis revealed worsening DM and abnormal serum CA 19–9 levels to be independent factors predicting the presence of DC in patients with IPMN. These findings further support our previous notion that IPMN may be a potential diagnostic clue to DC of the pancreas.[4,5,8,12,13] We conclude that careful inspection of the entire pancreatic gland and postoperative surveillance of the remnant pancreas even after pancreatic resection is necessary in patients with branch duct IPMN, especially when worsening DM and abnormal serum CA 19–9 levels are manifested.

Recently, IPMN has attracted increasing attention because this neoplasm is associated with extrapancreatic malignancies.[14–18] We and others have also experienced synchronous or metachronous occurrence of DC of the pancreas in patients with IPMN. A previous study from our group identified independent DC in 7 (9.2%) of 76

TABLE 2. Univariate Analysis of Potential Predictive Factors of the Occurrence of Ductal Carcinoma in the Pancreas with Intraductal Papillary Mucinous Neoplasm

	IPMN With Cancer Concomitant DC		IPMN Alone		
Variables	**Number**	**%**	**Number**	**%**	***P***
Age					
Mean ± SD	70.00 ± 8.97		66.40 ± 10.16		0.114
Gender					0.157
Male	15	68.18	93	52.25	
Female	7	31.82	85	47.75	
Symptoms					
Worsening DM	10	45.45	6	3.37	<0.001*
Weight loss	4	18.18	0	0.00	<0.001*
Back pain	2	9.09	40	22.47	0.176*
Abdominal pain or discomfort	6	27.27	48	26.97	0.976
Jaundice	1	4.55	2	1.12	0.296*
General fatigue	0	0.00	5	2.81	1.000*
Nodule in main duct IPMN (M)	0	0.00	2	1.12	1.000*
Nodule in branch duct IPMN (B)	1	4.55	33	18.54	0.134*
Location of IPMN					
Pancreatic head	15	68.18	125	71.84	0.721
Pancreatic body and tail	9	40.91	69	39.66	0.910
MPD size (mm)					0.892*
<6.00	16	72.73	127	71.35	
≥6.00	6	27.27	51	28.65	
Serum CEA level					0.333*
Normal	17	77.27	105	86.07	
Abnormal	5	22.73	17	13.93	
Serum CA 19-9 level					0.001*
Normal (<37 U/mL)	12	54.55	107	86.29	
Abnormal (>37 U/mL)	10	45.45	17	13.71	
WHO					0.137*
IPMA	8	66.67	46	51.69	
IPMB	4	33.33	20	22.47	
IPMC	0	0.00	23	25.84	
Pancreatic juice cytology					0.001*
Class I	1	5.56	40	28.37	
Class II	6	33.33	68	48.23	
Class III	5	27.79	24	17.02	
Class IV	0	0.00	3	2.13	
Class V	6	33.33	6	4.26	

*Fisher exact test.

TABLE 3. Multivariate Analysis of Predictive Factors of the Occurrence of Ductal Carcinoma in the Pancreas with Intraductal Papillary Mucinous Neoplasm

	Adjusted		
Variables	**Odds Ratio**	**95% CI**	***P***
Worsening DM	15.73	4.40–56.25	<0.001
Abnormal serum CA 19-9 level	3.70	1.19–11.48	0.024

patients with IPMN.[5] Sohn et al[19,20] reported 3 patients with IPMN who had subsequently developed DC of the pancreas in the pancreatic remnant after margin-negative pancreatoduodenectomy. In another recent study reported by Uehara et al,[6] 60 patients with branch duct IPMN were followed up for an average period of 87 months and DC of the pancreas was found in 5 patients (8%). These studies suggest a relatively high incidence (8%–9% in most reports) of concomitant occurrence of DC in the pancreas harboring IPMNs, although the exact prevalence should be carefully evaluated in a case-control study of larger series.

Importantly, in our present series, DC of the pancreas was identified at a resectable stage in 17 (77%) of 22 patients and survival after resection (median survival time of 49 months) was better as compared with patients with conventional DC without IPMNs. Similarly, in a previous report by Uehara et al,[6] 4 of 5 ductal carcinomas identified during follow-up of branch duct IPMN were resectable. These findings highlight the importance of IPMN as a clue to the early detection of DC of the pancreas. Interestingly, in a prospective controlled study of screening for early pancreatic neoplasia in individuals with a strong family history of pancreatic cancer, Canto et al[21] identified 8 patients with pancreatic neoplasia including 7 patients with IPMN, suggesting that IPMN can be considered as a sentinel lesion detectable in advance of developing DC of the pancreas.

There is only limited information on clinicopathological factors that predict the presence of DC in patients with IPMN. A previous study showed that patients with branch duct IPMN >70 years old developed DC significantly more frequently than those under 69.[6] In the present study, we found 2 independent factors predicting the presence of DC in patients with IPMN, including worsening DM, and elevated serum CA 19–9. Patients with IPMN are known to present with a variety of symptoms, including abdominal pain, jaundice, weight loss, and nausea and vomiting.[6,20,22,23] In previous studies, only 12% to 15% of worsening DM was reported as one of the clinical signs of IPMN, but in our present analysis this was the most common manifestation detected in 10 of 22 patients (45.5%) with DC associated with IPMN. Nowadays, the most extensively used tumor-associated marker for the diagnosis of pancreatic cancer is CA 19–9, with reported sensitivity of 83% and specificity of 82%.[24] In the present study, the sensitivity of CA 19–9 to predict concomitant DC was only 45% with specificity of 82%, while the positive predictive value and negative predictive value were 32% and 89%, respectively. The incidence of abnormal serum CA 19–9 in IPMN with DC >2 cm in size was significantly higher than in IPMN with DC of smaller size (64.3% vs. 12.5%; $P = 0.031$). Therefore, CA 19–9 is one of the predictors of the presence of DC in patients with IPMN, but not sensitive enough to detect a small DC.

The molecular mechanisms underlying the coexistence of DC and IPMN of the pancreas remain unknown. The field defect theory was considered to explain multifocal disease as a result of a widespread neoplastic field defect in the ductal epithelium of the pancreas.[19,20,22,23,25] In addition, there were several studies comparing the molecular mechanisms between IPMN and pancreatic cancer.[6,19,26–28] Recently, a global genomic analysis using microarray-based comparative genomic hybridization revealed significant differences in chromosomal aberrations between IPMN and conventional invasive DC of the pancreas, suggesting that these 2 neoplasms have different genetic background.[29] Further studies are requisite to determine the genetic and epigenetic markers for identifying the relationship between IPMN and DC.

In conclusion, a significant percentage (>9%) of patients with branch duct IPMN may have concomitant DC as an independent lesion synchronously or metachronously. Worsening DM and abnormal serum CA 19–9 levels are independent factors predicting the presence of DC in patients with IPMN. Careful inspection of the entire pancreatic gland is necessary for early detection of invasive DC in patients with branch duct IPMNs, especially when worsening DM and abnormal serum CA 19–9 levels are manifested.

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Original Article

Enhanced Cell Migration and Invasion of CD133$^+$ Pancreatic Cancer Cells Cocultured With Pancreatic Stromal Cells

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BACKGROUND: Recently, cancer stem cells have been reported as a new therapeutic target in pancreatic cancer as well as other cancers, but the specific role of these cells is unknown. **METHODS:** The authors investigated the functional roles of CD133$^+$ cells, 1 of the putative cancer stem cell candidates in pancreatic cancer. CD133 expression was assessed in human pancreatic cancer and cancer cell lines by quantitative real-time reverse transcriptase polymerase chain reaction and flow cytometry. Next, they compared the ability of CD133$^+$ and CD133$^-$ cells to proliferate, migrate, and invade using 2 pancreatic cancer cell lines. In particular, they evaluated the relationship between CD133$^+$ cells and primary pancreatic stromal cells. **RESULTS:** CD133 was expressed in primary human pancreatic cancer tissues and some cancer cell lines, whereas there was little expression in primary normal pancreatic epithelial cells and primary pancreatic stromal cells. CD133$^+$ cells, isolated by flow cytometry, showed increased cell proliferation under anchorage-independent conditions ($P < .01$), and enhanced migration and invasion, particularly when cocultured with primary pancreatic stromal cells ($P < .001$). Chemokine-related receptor-4 (CXCR4), markedly overexpressed in CD133$^+$ cells, may be responsible for the increased invasive ability of the cells cocultured with pancreatic stromal cells, which express stromal derived factor-1, the ligand to CXCR4. **CONCLUSIONS:** These data suggest that CD133$^+$ cells exhibit more aggressive behavior, such as increased cell proliferation, migration, and invasion, especially in the presence of pancreatic stromal cells. The targeting therapy for the interaction between CD133$^+$ cancer cells and stromal cells may be a new approach for the treatment of pancreatic cancer. ***Cancer* 2010;116:3357–68.** *© 2010 American Cancer Society.*

KEYWORDS: CD133, CXCR4, cell invasion, cancer-stromal interaction, pancreatic cancer.

Pancreatic cancer is the fifth leading cause of cancer-related death in Japan[1] and the fourth leading cause in the United States,[2] with annual deaths reaching >17,000 in Japan[1] and almost 30,000 in the United States.[2] The prognosis for pancreatic cancer is the worst of all cancers, because of the lack of improvement in early detection, diagnosis, and treatment strategies. Therefore, novel diagnostic modalities for early diagnosis and new therapeutic strategies are urgently needed.

Recently, cancer stem cells have been defined as a very small subset of cells within the tumor population that have the capacity to initiate and sustain tumor growth. It is most notable that cancer stem cells possess the ability to self-renew and undergo multilineage differentiation.[3] The study of cancer stem cells has developed through 2 important techniques, fluorescence-activated cell sorting (FACS) and tumor xenograft models in immunocompromised mice. Bonnet and Dick[4] isolated cancer stem cells in human acute myeloid leukemia for the first time by using cell surface markers (CD34$^+$ CD38$^-$) and a xenograft model in nonobese diabetic/severe combined immunodeficiency mice.

CD133 was reported as a marker of cancer stem cells in the brain,[5-7] colon,[8,9] liver,[10,11] and prostate.[12] In pancreatic cancer, Li and colleagues[13] have determined that pancreatic cancer is hierarchically organized and originates from a primitive stem/progenitor group of cells for which CD44$^+$ CD24$^+$ ESA$^+$ precursors constitute 1 of the most immature stages. However, Hermann and colleagues[14] have reported that a distinct subpopulation of CD133$^+$ cancer stem cells

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determined the metastatic phenotype of individual tumors. Therefore, there are 2 possible sources for cancer stem cells in pancreatic cancer; 1 is $CD44^+$ $CD24^+$ ESA^+ cells, and the other is $CD133^+$ cells. Hermann et al.[14] reported that these 2 populations overlap but are not identical. In this study, we analyzed the 2 kinds of cancer stem cell markers and found that CD44 was indeed expressed in almost 100% of pancreatic cancer cell lines. Therefore, CD44 seemed to be an inappropriate marker to isolate CD44-positive and -negative cells. Therefore, we used CD133 as a candidate marker of cancer stem cells in pancreatic cancer, and investigated the biological function of $CD133^+$ cells.

Previous studies have attributed the high expression levels of specific adenosine triphosphate-binding cassette drug transporters to the increased resistance of $CD133^+$ cancer stem cells to chemotherapeutic agents in hepatocellular carcinomas[15] and brain tumors.[16] Others have also demonstrated that cancer stem cells promoted radioresistance in glioblastoma[17] and breast cancer.[18] In pancreatic cancer, however, there are few reports regarding the biological functions of $CD133^+$ cancer cells. In the present study, we investigated the biological function of $CD133^+$ cells isolated by FACS in pancreatic cancer cell lines. Our data showed that $CD133^+$ cells might play important roles in cell proliferation, migration, and invasion in pancreatic cancer.

Invasive ductal adenocarcinoma of the pancreas is often characterized by abundant desmoplastic stroma. Recently, the stromal reaction has been a focus of attention as a characteristic feature of the majority of pancreatic cancers.[19] Therefore, we also investigated the involvement of $CD133^+$ cells in tumor-stromal interactions and found that $CD133^+$ cells significantly increased cell migration and invasion when cocultured with primary pancreatic stromal cells compared with $CD133^-$ cells. These data suggest that $CD133^+$ cells contribute to cancer-stromal interaction more than $CD133^-$ cells.

MATERIALS AND METHODS

Human Pancreatic Tissue Samples

The tissue samples analyzed in this study were obtained from patients who underwent a surgical procedure to resect a portion of the pancreas in Kyushu University Hospital, Fukuoka, Japan. Normal pancreatic tissues were also taken from areas of peripheral tissue away from the tumor or from the non-neoplastic pancreas resected because of biliary disease. Tissues were removed as soon as possible after resection and used for the experiments in the present study. The samples were suspended in Hank solution with collagenase, and mechanically dissociated using scissors and then minced with a sterile scalpel blade over ice to yield 1 × 1-mm pieces. The pieces were washed with Hank solution 3× before analysis by flow cytometry. The primary cultures of pancreatic stromal cells were established and maintained as described previously.[20] Written informed consent was obtained from all patients, and the study was approved by the ethics committee of Kyushu University and conducted according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

Cell Lines and Cultures

The following 14 pancreatic cancer cell lines were used: AsPC-1, KP-1N, KP-2, KP-3, PANC-1, and SUIT-2 (Dr. H. Iguchi, National Shikoku Cancer Center, Matsuyama, Japan); MIA PaCa-2 (Japanese Cancer Resource Bank, Tokyo, Japan); CAPAN-1, CAPAN-2, CFPAC-1, H48N, HS766T, and SW1990 (American Type Culture Collection, Manassas, Va); and NOR-P1 (established by Dr. N. Sato in our laboratory). A human pancreatic ductal epithelial cell line (HPDE6-E6E7 clone 6) immortalized by transduction with the E6/E7 genes of human papillomavirus 16 was kindly provided by Dr. Ming-Sound Tsao (University of Toronto, Toronto, Ontario, Canada), and maintained as described previously.[20] Primary cultures of human normal pancreatic epithelial cells were obtained from Cell Systems (Kirkland, Wash), and maintained in Cell Systems Corporation (CS-C) medium containing 10% fetal bovine serum (FBS), according to the supplier's instructions. Total cell numbers were quantified using a particle distribution counter (CDA500; Sysmex, Kobe, Japan).

Flow Cytometry and Cell Sorting

Cells were incubated in phosphate-buffered saline containing 1% FBS with phycoerythrin (PE)-conjugated antihuman CD133/2 antibody (Miltenyi Biotec, Aubum, Calif). Isotype-matched mouse immunoglobulins served as controls. For flow cytometry, samples were analyzed using an EPICS ALTRA flow cytometer (Beckman Coulter, Fullerton, Calif) and a FACS Calibur (Becton Dickinson, Bedford, Mass). For cell sorting by flow cytometry, samples were analyzed and sorted on the EPICS ALTRA. For the positive and negative population, only the top 10% most brightly stained cells or the bottom 10% most dimly stained cells were selected, respectively. Aliquots of $CD133^+$ and $CD133^-$ sorted cells were evaluated for

purity with the same machine, using the CD133/2 antibody. We also analyzed expression of cell surface markers using the antibodies of chemokine-related receptor-4 (CXCR4)-PE (R & D systems, Minneapolis, Minn).

Transfections

Transfections were performed by electroporation using a Nucleofector system (Amaxa Biosystems, Cologne, Germany). All studies were performed in triplicate. Cells (1-2 × 10^6) were centrifuged at 1200 rpm for 5 minutes, and the medium was removed. The cells were resuspended in 98 μL of Nucleofector solution (Amaxa Biosystems) at room temperature, followed by the addition of 2 μL of 50 μmol CXCR4-siRNA or a control-siRNA (both obtained from B-Bridge International, Mountain View, Calif). The transfected cells were resuspended and cultured in regular culture medium containing 10% serum for 24 hours before analysis.

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction Assay for CD133 mRNA Expression

Total RNA was isolated from cell lines using the High Pure RNA isolation kit (Roche Diagnostics, Indianapolis, Ind), and RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Del) at 260 and 280 nm (A260/280). RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, Calif). Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assays were performed using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Tokyo, Japan) with a Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, Calif) according to the manufacturer's instructions. PCR was performed for 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, followed by a thermal denaturation protocol. The expression levels of CD133 mRNA in all cell lines were normalized to the expression level of 18S rRNA. The following primer pairs were used for PCR amplification: CD133-forward, 5′-GCCACCGCTCTAGATACTGC-3′; CD133-reverse, 5′-GCTTTTTCCTATGCCAAACCA-3′; CXCR4-forward, 5′-GAAGCTGTTGGCTGAAAGG-3′; CXCR4-reverse, 5′-CTCACTGACGTTGGCAAAGA-3′; stromal cell-derived factor-1 (SDF-1) (CXCL12)-forward, 5′-GAT TGTAGCCCGGCTGAAGA-3′; SDF-1 (CXCL12)-reverse, 5′-TTCGGGTCAATGCACACTTGT-3′; 18S rRNA-forward, 5′-GATATGCTCATGTGGTGTTG-3′; and 18S rRNA-reverse, 5′-AATCTTCTTCAGTCGC TCCA-3′.

Cell Proliferation Assay

Cell proliferation was evaluated by measuring the fluorescence intensity of propidium iodide (PI) as described previously.[21,22] $CD133^+$ and $CD133^-$ cells in pancreatic cancer were seeded at 2 × 10^4 cells/well in 24-well tissue culture plates (Becton Dickinson). In anchorage-independent proliferation experiments, cells were plated at 1 × 10^5 cells/well in 24-well plates coated with Ultra Low Attachment Surface (Corning Inc., Corning, NY). Cell proliferation was evaluated after culture for a further 72 or 120 hours after the initial cell number determination by the PI assay. PI (30 μM) and digitonin (600 μM) were added to each well to label all nuclei with PI. The fluorescence intensity, corresponding to the total cell number, was measured using a CytoFluor II multiwell plate reader (PerSeptive Biosystems, Framingham, Mass) with 530-nm excitation and 645-nm emission filters. A separate well, which possessed the same medium but no cells, was used for a baseline PI signal as a control. We evaluated the difference between each sample well and the control well. Cell proliferation was defined relative to the cell number measured at the beginning of the experiment. All experiments were performed in triplicate wells and repeated at least 3×.

Migration Assay and Invasion Assay

Migration of pancreatic. cancer cells was measured by counting the number of cells that migrated from transwell chambers 24 hours after seeding cells into the upper chamber. Invasive ability of pancreatic cancer cells was measured by the number of cells invading Matrigel-coated transwell chambers. We also assessed cancer-stromal interaction by coculture with pancreatic cancer-related stromal cells.

In the migration assay, a total of 5 × 10^4 isolated cells were resuspended in 250 μL of Dulbecco modified Eagle medium (DMEM) containing 10% FBS and placed in the upper transwell chamber (8 μm pore size, Becton Dickinson, Franklin Lakes, NJ). The upper chamber was placed in a 24-well culture dish containing 750 μL of the medium described above supplemented with 5 × 10^4 primary pancreatic stromal cells. After 24 hours of incubation at 37°C, the number of migrated cells was counted. In the invasion assay, 5 × 10^4 isolated cells were plated in the upper Matrigel-coated transwell chambers (filled with 20 μg of Matrigel/well) and reconstituted with 10% FBS-containing medium for 2 hours before the experiment.

Cells were seeded into the upper chambers in 250 μL of DMEM supplemented with 10% FBS. The same medium (750 μL) was placed in the lower wells. After 72 hours of incubation at 37°C, the number of invading cells was counted.

Cells that had degraded the Matrigel and invaded the lower surface of the Matrigel-coated membranes were fixed with 70% ethanol, stained with H & E, and counted in 5 random fields at ×200 magnification under a light microscope. The results were expressed as the average number of invading cells per field.

Statistical Analysis

All data were expressed as the mean ± standard deviation and evaluated using an analysis of variance on Microsoft Office Excel software (Redmond, Wash). Values of $P < .05$ were accepted as statistically significant in any analysis.

RESULTS

CD133 Is Markedly Overexpressed in Pancreatic Cancer Tissues Compared With Normal Pancreatic Tissues

We measured the expression of CD133 in human pancreatic cancer tissues and normal pancreatic tissues by flow cytometry, and found that the expression of CD133 in cancer tissues was much higher than in nonmalignant tissues (Fig. 1A, $P < .001$).

We performed quantitative RT-PCR and flow cytometry to measure the expression of CD133 in 14 pancreatic cancer cell lines, HPDE cells, and primary cultures of normal pancreatic epithelial cells and pancreatic fibroblasts (Fig. 1B and C). There was a wide range of relative CD133 expression levels among the pancreatic cancer cell lines. The primary normal pancreatic epithelial cells and fibroblasts did not express detectable levels of CD133. We selected SUIT-2 and KP-2 cells in which >10% of cells were determined to be isolated $CD133^+$ cells and $CD133^-$ cells by FACS for the following experiments.

More $CD133^+$ Cells Survive Than $CD133^-$ Cells Under Anchorage-Independent Conditions

To investigate cell proliferation and cell survival, we performed PI assays using SUIT-2 and KP-2 cells. In anchorage-dependent conditions, there was no significant difference in cell proliferation between $CD133^+$ and $CD133^-$ cells (Fig. 2A). However, in anchorage-independent conditions, cell survival of $CD133^+$ cells was significantly increased compared with $CD133^-$ cells in both SUIT-2 and KP-2 cells (Fig. 2B; $P = .004$).

$CD133^+$ Cells Show High Migration and Invasion Ability, Especially When Cocultured With Primary Pancreatic Stromal Cells

Next, we compared the migration and invasion abilities of $CD133^+$ and $CD133^-$ cells using SUIT-2 and KP-2 cells. The $CD133^+$ SUIT-2 and KP-2 cells exhibited slightly increased cell migration compared with $CD133^-$ cells 24 hours after seeding in monoculture conditions (Fig. 3A, $P = .0028$; Fig. 3B, $P = .035$, respectively). When cocultured with pancreatic stromal cells, $CD133^+$ cells exhibited markedly increased invasive potential compared with $CD133^-$ cells (Fig. 3A, $P = .0002$; Fig. 3B, $P < .0001$, respectively). Although we found no significant difference in cell invasion between $CD133^+$ and $CD133^-$ cells at 72 hours after seeding under monoculture conditions (Fig. 3C; $P = .12$), $CD133^+$ cells exhibited markedly increased invasive potential compared with $CD133^-$ cells when cocultured with pancreatic stromal cells (Fig. 3C; $P < .0001$). Similar results were again found in KP-2 cells (Fig. 3D, $P = .023$ and $P < .0001$, respectively).

$CD133^+$ Cells Expressed CXCR4 at Much Higher Level Than $CD133^-$ Cells

To assess the mechanism underlying the increase in the migration and invasion ability of $CD133^+$ cells in cocultures with pancreatic stromal cells, we focused on 2 major tumor-stromal cell interactions in pancreatic cancer, CXCR4/SDF-1[14] and c-Met/hepatocyte growth factor (HGF).[20] SUIT-2 and KP-2 $CD133^+$ cells expressed a significantly higher level of CXCR4 mRNA than $CD133^-$ cells (Fig. 4A; $P = .0003$ and .001, respectively). However, there was no significant difference in c-Met mRNA expression (Fig. 4A). These results suggest that CXCR4 may be responsible for the increased migration and invasion ability of $CD133^+$ cells when cocultured with pancreatic stromal cells secreting SDF-1 (Fig. 4B, CAF-3).

Down-Regulation of CXCR4 Decreases Cell Migration and Invasion Only in $CD133^+$ Cells

To assess whether the CXCR4 /SDF-1 axis plays an important role in the migration and invasion of $CD133^+$ cells in pancreatic cancer, we down-regulated CXCR4 using RNA interference. At 24 hours (Day 1) after transfection with 100 pmol of CXCR4-siRNA or control-siRNA, $CD133^+$ and $CD133^-$ cells transfected with CXCR4-siRNA showed 0.3-fold lower levels of CXCR4 mRNA than the cells transfected with control-siRNA (Fig. 5A). At 120 hours (Day 5) after transfection, CXCR4-siRNA was

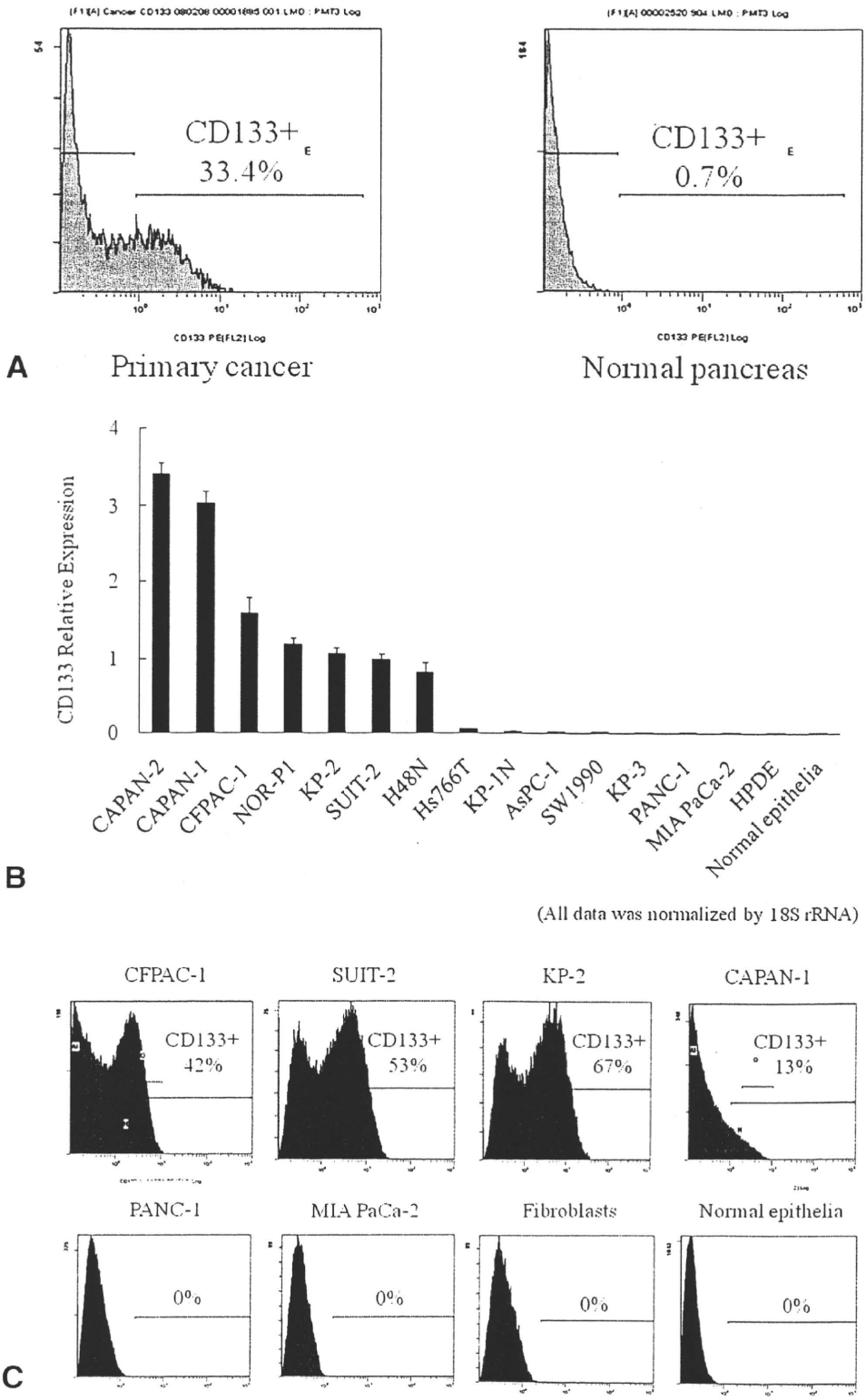

Figure 1. Levels of CD133 expression in human pancreatic tissues and cell lines are shown. (A) Levels of CD133 expression in pancreatic cancer tissues and normal pancreatic tissues as determined by flow cytometry are shown. (B) Levels of CD133 mRNA expression were assessed by quantitative reverse transcriptase polymerase chain reaction and normalized to the level of 18S rRNA in each sample. Data represent the mean ± standard deviation of triplicate measurements. (C) Levels of CD133 expression in pancreatic cancer cell lines were assessed by flow cytometry. Data represent triplicate measurements.