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The Expression of S100A4 in Human Pancreatic Cancer Is Associated With Invasion

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Objectives: Pancreatic cancer is one of the most lethal malignancies; its poor prognosis is strongly associated with invasion and metastasis. Expression of S100A4 has been reported to correlate with poor prognosis in various cancers. We have investigated the role of S100A4 in pancreatic cancer tumorigenesis and its clinicopathologic significance.

Methods: Protein expression of S100A4 was examined by Western blot in pancreatic cancer cell lines and a human pancreatic ductal epithelium cell line, HPDE-6. Then the expressions of S100A4, TP53, and CD133 were examined immunohistochemically in resected specimens from 83 patients with pancreatic cancer to clarify their clinicopathologic significance. Survival analyses were performed using the Kaplan-Meier method and the Mantel-Cox method.

Results: Forty-eight (58%) of 83 patients with pancreatic cancer positively expressed S100A4, and 50 (60%) and 29 (36%) patients positively expressed TP53 and CD133, respectively. S100A4 expression was significantly correlated with perineural invasion ($P = 0.029$) and invasion pattern ($P = 0.001$). Neither TP53 nor CD133 expression showed significant correlations with any other parameters.

Conclusions: Our present results suggest that S100A4 plays an important role in the invasiveness, particularly with perineural invasion and invasion pattern, of pancreatic cancer. Development of new strategies targeting S100A4 or its downstream effectors is warranted.

Key Words: pancreatic cancer, tissue microarray, S100A4, TP53, clinicopathologic significance, immunohistochemistry

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Pancreatic cancer has an extremely poor prognosis worldwide, because of its aggressive invasion, metastasis to other organs, resistance to chemotherapeutic agents and radiation therapy, and difficulty of diagnosis at an early stage.[1] To improve the survival of patients with pancreatic cancer, especially invasive ductal adenocarcinoma, investigations of the mechanisms of biological aggressiveness and development of novel and effective therapeutic strategies are necessary.

Recently, overexpression of the S100 protein family has been reported in human cancers of various organs, including the pancreas.[2] The S100 protein family is composed of 21 Ca^{2+}-binding proteins characterized by the EF-hand motif and is thought to be implicated in diverse cellular functions, including cell proliferation, differentiation, metabolism, motility, and signal transduction,[3–5] and most of them are located on chromosome 1q21. S100A4 (also called mts1, p9Ka, calvasculin, CAPL, and pEL98) is a member of the S100 protein family; this molecule is emerging as a multifunctional player in proliferation, cellular adhesion, reconstruction of extracellular matrix, angiogenesis, and cellular motility, and its overexpression in pancreatic cancer together with various oncogenic proteins, including prostate stem cell antigen, carcinoembryonic antigen-related cell adhesion molecule 6 and mesothelin, was confirmed in SAGE (serial analysis of gene expression) method.[6] Furthermore, Rosty et al[7] have reported that overexpression of S100A4 correlates with undifferentiated phenotype and DNA hypomethylation of the first intron. We previously demonstrated that S100A4 is frequently up-regulated in pancreatic cancer cell lines and that siRNA-mediated knockdown of *S100A4* causes apoptosis and inhibition of cell motility only in cells with up-regulated S100A4.[8] Hence, it is of great interest to investigate the biological role(s) of S100A4 in the tumorigenesis and progression of pancreatic cancer.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Twelve human pancreatic cancer cell lines, AsPC-1, MiaPaCa-2, PCI-19, PCI-24, PCI-43, PAN-07-JCK, PAN-09-JCK, PK-1, PK-9, PK-45P, PK-45H, and PK-59, as well as HPDE-6, a cell line from normal pancreatic ductal epithelium, were used. These cell lines were described in our previous work and were cultured as described.[8]

Antibodies

Rabbit polyclonal anti-S100A4 antibody (Dako, Glotrup, Denmark), mouse monoclonal anti-TP53 antibody (clone DO-7) (Dako), mouse anti-CD133 monoclonal antibody (AC133) (Miltenyi Biotec, Gladbach, Germany), mouse anti-β actin monoclonal antibody (AC15) (Sigma, St Louis, Mo), and horseradish peroxidase–conjugated anti–mouse or anti–rabbit immunoglobulin antibodies (Amersham Biosciences Corp, Piscataway, NJ) were used for immunohistochemical and Western blot analyses. Anti-S100A4 antibody was used at dilutions of 1:1000 for Western

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blot and 1:25 for immunohistochemistry. Anti-TP53 antibody was used at a dilution of 1:50 for immunohistochemistry.

Western Blot Analysis

Western blot analyses were done following methods described previously.[9] In brief, cells were lysed in a lysis buffer consisting of 65 mM Tris-HCl (pH 6.8) and 3% sodium dodecyl sulfate. After sonication, the lysates were boiled for 5 minutes and centrifuged for 10 minutes. Protein densitometry was performed using DC Protein Assay kit (Bio-Rad, Hercules, Calif) and Ultrospec 3000 (Pharmacia Biotech, Uppsala, Sweden). The cell lysate was diluted with a lysis buffer consisting of 65 mM Tris-HCl (pH 6.8), 3% sodium dodecyl sulfate, 10% β-mercapthoethanol, and 20% glycerol. Equal amounts of protein sample were applied on polyacrylamide gels (Bio-Rad), separated by electrophoresis, and electroblotted onto nitrocellulose membranes. After blocking with 5% skim milk in phosphate-buffered saline containing Tween 20, the membranes were incubated overnight with primary antibodies at 4°C and then incubated with secondary antibodies for 30 minutes at room temperature. Signals were visualized by Enhanced Chemiluminescence Detection Reagent (Amersham Biosciences, Little Chalfont, UK) and digitally processed using LAS 1000 Plus with a Science Lab 99 Image Gauge (Fuji Photo Film, Minamiashigara, Japan).

Tissue Microarray Analysis

Paired surgically resected tissue specimens of primary invasive ductal pancreatic adenocarcinoma and corresponding noncancerous tissues from 83 patients with pancreatic cancer (median age, 63.2 years; range, 43–83 years) were obtained at Tohoku University Hospital, Sendai, Japan, between December 1997 and July 2005. After the tissues were formalin-fixed and paraffin-embedded, corresponding tissue sections were evaluated by hematoxylin-eosin staining. Each block was then cored out with a diameter of 2.3 mm within 1 or more of the representative lesions and the corresponding normal pancreatic tissue(s) using Tissue Microprocessor (Azumaya, Tokyo, Japan). The cored columns were re-embedded in paraffin, and a series of tissue microarray blocks containing 24 columns in 1 block (at most) was prepared. Then 4-μm slide sections were prepared for further analyses; these slides were deparaffinized in xylene and dehydrated in ethanol. The endogenous peroxidase activity was blocked with a 0.3% H_2O_2 solution diluted with methanol for 30 minutes. Antigen retrieval was performed in 0.01 M citrate buffer (pH 6.0) using a microwave oven for 15 minutes, followed by cooling down to room temperature. The slides were first reacted with normal goat/rabbit serum at room temperature and then overnight with the primary antibodies at 4°C. After washing with phosphate-buffered saline buffer, biotinylated secondary antibodies were applied for 30 minutes at room temperature. Then the slides were reacted with peroxidase-labeled streptavidin using HISTOFINE SAB-PO Kit (Nichirei, Tokyo, Japan). DAB (3,3'-diaminobenzidine tetrahydrochloride) (Sigma) diluted with 50 mM Tris-HCl (pH 7.6) and H_2O_2 (final concentration 0.005%) was used to detect the immunoreactivity, and then the nuclei were counterstained with hematoxylin. Immunoreactivity was evaluated by 2 investigators (N.T. and K.A.) without any information about the patients. In this study, tumors with more than 5% stained cells in their cytoplasm or nuclei were defined as positive; lower percentages were defined as negative.[10] The study was approved by the Ethics Committee of Tohoku University School of Medicine.

Statistical Analysis

Statistical analyses were carried out using the SPSS statistics 16.1 software (SPSS Japan, Tokyo, Japan). Associations between the expression of S100A4 and clinicopathologic characteristics were performed using χ^2 test or Fisher exact test. Survival time was calculated from the day of the last follow-up. The Kaplan-Meier method was used to analyze patient survival, and the Mantel-Cox method was used to evaluate the statistical significance of the results.

RESULTS

Expression of S100A4 in Human Pancreatic Cancer Cell Lines

In our previous report, we utilized quantitative reverse transcriptase–polymerase chain reaction to demonstrate that *S100A4* mRNA is frequently overexpressed in pancreatic cancer cell lines; 19 of 21 cell lines showed more than 2-fold higher expression levels than that of HPDE, a cell line established from normal pancreatic ductal epithelium; among these 19 cell lines, 6 expressed levels more than 100-fold higher.[8] In this study, we first analyzed protein expression levels using representative cell lines, as shown in Figure 1; the protein expression levels were in good agreement with previously analyzed results of the quantitative reverse transcriptase–polymerase chain reaction experiments.[8]

Expression of S100A4, TP53, and CD133 in Human Pancreatic Cancer and the Clinicopathologic Significance

We next analyzed primary resected pancreatic ductal adenocarcinomas immunohistochemically. Specimens from 83 patients (median age, 63.2 years; range, 43–83 years) surgically resected at

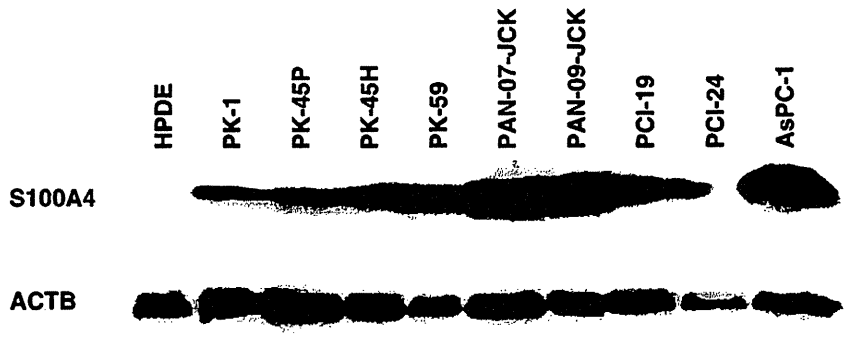

FIGURE 1. Expressions of S100A4 in a human pancreatic ductal epithelium cell line (HPDE) and 9 pancreatic ductal adenocarcinoma cell lines analyzed by Western blot. Anti-S100A4 rabbit polyclonal antibody was used, and the expression of the β-actin (ACTB) was monitored as the internal control.

TABLE 1. Association Between Clinicopathologic Features and Expression of S100A4, TP53, and CD133

		S100A4 Expression				TP53 Expression				CD133 Expression		
Clinical Feature	Cases (n)	Positive	Negative	*P*	Cases (n)	Positive	Negative	*P*	Cases (n)	Positive	Negative	*P*
JPS stage												
I+II	2	0	2	0.278	2	0	2	0.155	1	0	1	0.637
III + IVA + IVB	81	48	33		81	50	31		79	29	50	
Differentiation												
Well + moderate	70	38	32	0.129	70	42	28	0.917	70	28	42	0.085
Poor	13	10	3		13	8	5		10	1	9	
Nodal involvement												
Positive	59	36	23	0.357	59	36	23	0.821	54	17	37	0.201
Negative	24	12	12		24	14	10		26	12	14	
Vascular invasion												
Positive	62	37	25	0.558	62	39	23	0.394	59	19	40	0.207
Negative	21	11	10		21	11	10		21	10	11	
Lymphatic invasion												
Positive	39	26	13	0.125	39	27	12	0.115	35	10	25	0.208
Negative	44	22	22		44	23	21		45	19	26	
Intrapancreatic neural invasion												
Positive	56	37	19	0.029	56	16	11	0.899	52	22	30	0.125
Negative	27	11	16		27	34	22		28	7	21	
Neuroplexus invasion												
Positive	34	21	13	0.546	34	22	12	0.489	31	10	21	0.555
Negative	49	27	22		49	28	21		49	19	30	
Retroperitoneal invasion												
Positive	64	38	26	0.601	64	41	23	0.192	58	20	18	0.593
Negative	19	10	9		19	9	10		22	9	13	
Infiltration												
β	56	31	25	0.888	56	36	20	0.278	55	19	36	0.638
γ	27	17	10		27	14	13		25	10	15	
Invasion pattern												
Scirrhous	48	35	13	0.001	48	28	20	0.678	45	15	30	0.538
Intermediate	35	13	22		35	22	13		35	14	21	

Tohoku University Hospital were analyzed; their clinicopathologic characteristics are summarized in Table 1. According to the Japan Pancreas Society (JPS) staging system,[11] 2 of these patients (2.4%) presented with JPS stage I/II disease, and 81 patients (97.6%) with JPS stage III/IV disease. Representative results are shown in Figure 2. In 48 (58%) of 83 cases, S100A4 staining was positive (typical example in Figs. 2B, C). In contrast, no S100A4 immunoreactivity was detected in noncancerous pancreatic ductal epithelia in any of the 83 patients (data not shown). Positive associations between S100A4 expression and intrapancreatic neural invasion ($P = 0.029$) or invasion pattern ($P = 0.001$) were observed, as summarized in Table 1, and typical example of the immunostaining is shown in Supplemental Figure 1, Supplemental Digital Content 1, http://links.lww.com/MPA/A217. However, other features such as the JPS stage, differentiation, nodal involvement, vascular invasion, lymphatic invasion, infiltration type, extrapancreatic neuroplexus invasion, and retroperitoneal invasion were not significantly associated.

Because our previous in vitro studies indicated that knockdown of S100A4 in highly expressing cells suppressed invasion,[8] it is of interest to analyze expression levels in invasive front and metastatic lesions. Typical examples of the results are shown in Supplemental Figure 2, Supplemental Digital Content 2, http://links.lww.com/MPA/A218; S100A4-positivity is high at the invasive front. Furthermore, positivity is higher in metastatic lymph nodes than the primary lesions (Supplemental Figure 2, Supplemental Digital Content 2, http://links.lww.com/MPA/A218). There is a possibility that S100A4-expressing cancer cells have some advantage to metastasis.

We next analyzed the expressions of TP53 and CD133 immunohistochemically. Representative results are shown in Figure 2. TP53-positive staining was observed in 50 (60%) of 83 cases, and CD133 staining in 29 (36%) of 80 cases, but no significant correlations between any of the clinicopathologic features and TP53 or CD133 were detected. Typical example of the area of perineural invasion is shown in supplementary Figure 1, and these results are summarized in Table 1.

Relationship Between S100A4, TP53, and CD133 With Survival

To see if S100A4, TP53, CD133, or some combinations of them are useful for prediction of survival, we analyzed the cumulative survival rate of 81 informative patients with pancreatic cancer using the Kaplan-Meier method. Expression levels of the S100A4 protein did not correlate with a significant difference in survival time (Fig. 3A). Neither TP53 nor CD133 alone was found to be an independent prognostic factor (data not shown). The expression levels of S100A4 and TP53 did not correlate, as shown in Table 2; however, the survival rates of patients with S100A4 and TP53 doubly positive tumors tended to be lower than those with doubly negative tumors (Fig. 3B). The expression profiles of S100A4 and CD133 did not show any correlation with survival (data not shown).

DISCUSSION

The mortality rate of pancreatic cancer increases after 40 years of age and exceeds 120 deaths in 100,000 individuals at 80 years of age.[12] In developed countries, it is the fourth leading

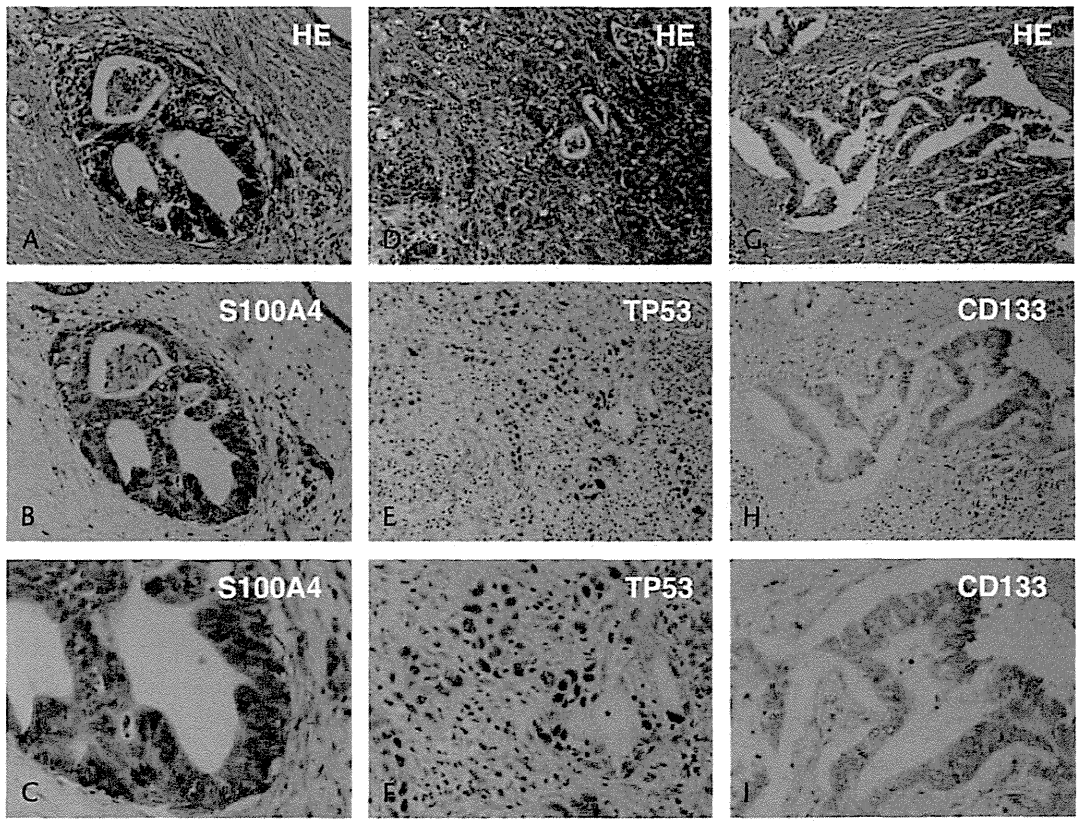

FIGURE 2. Immunohistochemical staining for S100A4, TP53, and CD133 in pancreatic ductal adenocarcinoma specimens. Hematoxylin-eosin staining of ductal adenocarcinoma specimens (A, D, G) and serial sections stained with S100A4, original magnification ×200 [B], ×400 [C]; TP53, original magnification ×200 [E], ×400 [F]; CD133, original magnification ×200 [H] and ×400 [I]).

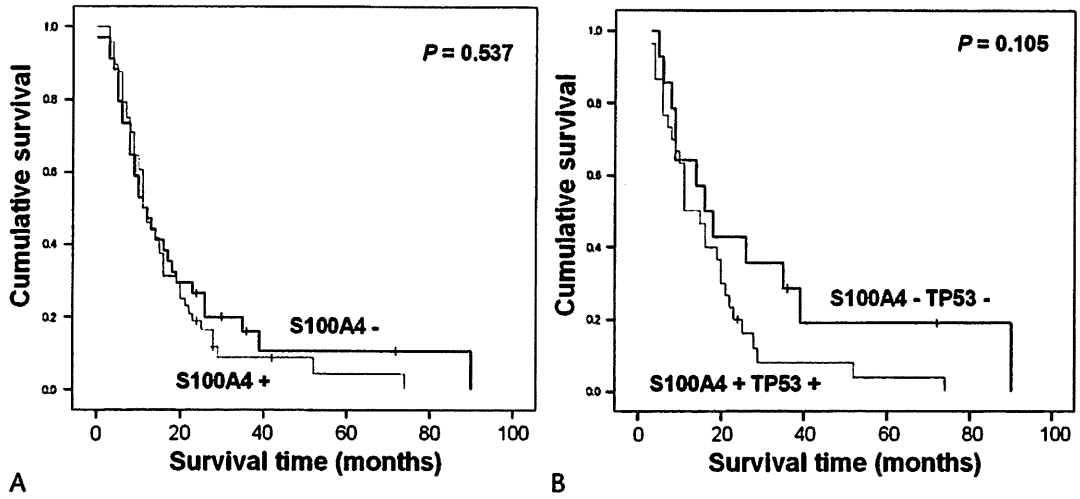

FIGURE 3. Kaplan-Meier survival curves for 81 pancreatic ductal adenocarcinomas. A, Patients with S100A4-positive and S100A4-negative did not show any significant difference. B, Patients harboring both S100A4- and TP53-positive tumors tended to have poorer prognoses than those who were both S100A4 and TP53, but the difference was not significant.

cause of cancer death in men and fifth in women.[13] The poor prognosis for pancreatic cancer results from the difficulty of early diagnosis as well as the disease's aggressive invasion and frequent metastases to other organs. We need to discover effective biomarkers and/or novel therapeutic targets. Among various candidates, the S100 protein family has emerged as promising. Significant correlations between poor differentiation, vascular invasion, and nodal involvement have been reported in several types of cancer, including lung, breast, and thyroid as well as pancreas.[7,14–17,18–21] Based on these findings, S100A4 was highlighted as a candidate molecule, which might have a role, particularly in the late phase of cancer, in invasion and in metastasis.[14–22] In good agreement with a previous report,[7] we observed that S100A4 was widely expressed in adenocarcinomas (48/83, 58%) but was completely negative in normal pancreatic tissue. Ikenaga et al[23] reported a significant relationship between S100A4 expression and survival in patients with pancreatic cancer; an association between S100A4 overexpression and poor prognosis has also been reported in breast cancer,[19,20] gastric cancer,[24] colorectal cancer,[25] and esophageal squamous cell carcinomas.[26] However, we did not find any significant correlation with poor prognosis in pancreatic cancer. Although it has been reported that expression of S100A4 is strongly associated with poor prognosis in early-stage breast cancer patients,[27] in advanced stages, only a few cases have been reported. Ai et al[28] demonstrated that expression of S100A4 in pancreatic cancer correlates with prognosis when half of the cases are in an early stage. In our study, 81 of 83 pancreatic cancer patients were at JPS stage III or higher; this may possibly explain the lack of a significant difference in prognosis.

It is notable that the expression of S100A4 protein is associated with intrapancreatic neural invasion and invasion pattern. One of the most common forms of pancreatic cancer progression is invasion, particularly to nerves. S100 protein is widely used as an immunohistochemical neural marker, so there may be some connection between S100A4 expression and neural invasion. In our previous study, siRNA-mediated *S100A4* knockdown suppressed cell motility,[8] and our recent results of forced expression of S100A4 in low-expressing cells activated cell motility.[29] In our present results that positivity of S100A4 was higher in metastatic lymph nodes than the primary lesions, there is a possibility that S100A4-expressing cancer cells have some advantage to metastasis. If this is the case, then there is a possibility that the invasive character of pancreatic cancer can be controlled by down-regulation of S100A4. Schmidt-Hansen et al[30] investigated the functional significance of S100A4 in tumor-stromal interaction and found that S100A4 was expressed by a variety of cell types in the tumor microenvironment of human breast cancer.[31] Our results showing that significant overexpression of S100A4 is evident in cancer with intrapancreatic neural invasion and that such cancer contains abundant stroma (scirrhous type) suggest that S100A4 may play an important role in the invasion and metastasis of cancer by orchestrating the tumor microenvironment.

We simultaneously analyzed one of the representative tumor suppressor genes, *TP53*, along with CD133, a candidate for cancer stem cell markers of pancreatic cancer.[32] *TP53* is frequently inactivated in pancreatic cancer,[33] and a positive association between its inactivation and poor prognosis has been reported in this disease.[34,35] Furthermore, inactivation of *TP53* gradually increases as the tumor progresses from the high-grade PanIN.[1,36] A genetic and functional relationship between S100A4 and TP53 has been indicated[37–39] and a strong inverse relationship between S100A4 and TP53 expression has also been reported in human lung cancer.[18] S100A4 binds to the extreme end of the C-terminal regulatory domain of TP53 and inhibits phosphorylation of the

TABLE 2. Expressional Association Between S100A4 and TP53 or CD133

		TP53		CD133	
		Positive	Negative	Positive	Negative
S100A4	Positive	30	18	17	28
	Negative	20	15	11	24
	P	0.622		0.555	

TP53 C-terminal peptide by protein kinase C.[39] However, as shown in Table 2, S100A4 seemed to be expressed independently of TP53 expression in our study, and no significant correlations with any clinicopathologic parameters were found. We note here that patient survival with doubly positive tumors for S100A4 and TP53 tended to be shorter than those with neither molecule (Fig. 3B), although the difference was not significant. To clarify this point, we need further studies including more patients at earlier stages. We observed no correlation between S100A4 and CD133 in pancreatic cancer; expression of these 2 proteins seemed to be independent, and CD133 expression did not show any correlation with clinicopathologic feature, including an invasive phenotype. The number of cancer stem cells may not significantly differ among individual tumors.

In conclusion, our results demonstrate that S100A4 is overexpressed in pancreatic adenocarcinomas and is closely correlated with both perineural invasion and invasion pattern. When we take our recent results of knockdown studies of *S100A4* into consideration, S100A4 appears to play an important role in the late-phase tumorigenesis and the invasive phenotype of pancreatic cancer. S100A4 may be a good candidate as a molecular target for diagnosis and treatment of pancreatic cancer.

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Suppressed expression of *NDRG2* correlates with poor prognosis in pancreatic cancer

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ABSTRACT

Pancreatic cancer is a highly lethal disease with a poor prognosis; the molecular mechanisms of the development of this disease have not yet been fully elucidated. N-myc downstream regulated gene 2 (*NDRG2*), one of the candidate tumor suppressor genes, is frequently downregulated in pancreatic cancer, but there has been little information regarding its expression in surgically resected pancreatic cancer specimens. We investigated an association between NDRG2 expression and prognosis in 69 primary resected pancreatic cancer specimens by immunohistochemistry and observed a significant association between poor prognosis and NDRG2-negative staining ($P = 0.038$). Treatment with trichostatin A, a histone deacetylase inhibitor, predominantly up-regulated *NDRG2* expression in the *NDRG2* low-expressing cell lines (PANC-1, PCI-35, PK-45P, and AsPC-1). In contrast, no increased *NDRG2* expression was observed after treatment with 5-aza-2′ deoxycytidine, a DNA demethylating agent, and no hypermethylation was detected in either pancreatic cancer cell lines or surgically resected specimens by methylation specific PCR. Our present results suggest that (1) *NDRG2* is functioning as one of the candidate tumor-suppressor genes in pancreatic carcinogenesis, (2) epigenetic mechanisms such as histone modifications play an essential role in *NDRG2* silencing, and (3) the expression of NDRG2 is an independent prognostic factor in pancreatic cancer.

1. Introduction

Pancreatic cancer is a highly lethal disease; few patients are diagnosed at a state early enough for curative treatments. It is the fourth most common cause of cancer death worldwide [1], and the long-term prognosis remain poor with a 5-year survival rate of less than 5% after the initial diagnosis [2]. One of the major hallmarks of pancreatic cancer is its extensive local tumor invasion and early systemic dissemination. The molecular basis for these characteristics of pancreatic cancer is incompletely understood.

N-Myc downstream regulated gene 2 (*NDRG2*) is a member of *NDRG* gene family that is highly expressed in many normal tissue types, including brain, spinal cord, skeletal muscle, heart, and salivary gland [3–5]. *NDRG* gene family members share 53–65% homologous amino acid sequences with each other. Each member has a distinct tissue specificity of expression and may be intimately involved in cell proliferation, differentiation, development, and stress responses [6].

NDRG2 has been reported to be a candidate tumor suppressor gene, and its expression is downregulated in a number of primary tumors developed in organs of brain and meninges [4,7,8], liver [9,10], pancreas [10], esophagus [11], stomach [12], colorectum [6,13,14], kidney [6], thyroid [15], oral cavity [16], prostate [17], gallbladder [18], blood [19], and lung [20]. *NDRG2* is reported to

Abbreviations: *NDRG2*, N-myc downstream regulated gene 2; 5-aza-dC, 5-aza-2′ deoxycytidine; TSA, trichostatin A; qRT-PCR, quantitative reverse transcription polymerase chain reaction; cDNA, complementary DNA; AU, arbitrary unit; *B2M*, β2-microglobulin; MSP, methylation-specific PCR; HDAC, histone deacetylase.

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suppress proliferation and metastasis, and expressional inactivation of *NDRG2* may play an important role in carcinogenesis [4,9,11,12,21,22]. Several possible mechanisms, including promoter hypermethylation [7–9,13,16,23] and/or repression by MYC [4,11,14,15,24], are responsible for such expressional suppression. However, the precise mechanisms that lead to inactivation of *NDRG2* remain largely unknown, and role of *NDRG2* in human carcinogenesis is not yet well understood.

In the present study using immunohistochemistry, we found a significant association between poor prognosis and suppressed expression of NDRG2 in primary pancreatic cancer. Furthermore, *NDRG2* gene expression was up-regulated by histone deacetylase inhibitor in pancreatic cancer. It is notable that this histone modification has never previously been demonstrated in suppression of *NDRG2* expression in human cancer. These findings suggest that *NDRG2* is likely to be a novel prognostic marker and important indicator for a possible role of *NDRG2* in pancreatic cancer.

2. Materials and methods

2.1. Tissue specimens

A total of 69 pancreatic cancer tissues obtained from surgically resected specimens at Tohoku University Hospital (Sendai, Miyagi, Japan) during the period from 1997 to 2006 were analyzed. The clinical and histopathological characteristics of the pancreatic cancer patients are summarized in Table 1. Staging followed the TNM Classification of Malignant Tumor (6th edition) [25]. None of the patients had received any preoperative adjuvant therapy. The resected tissue specimens from these patients were fixed in 10% formalin and embedded in paraffin. Written informed consent was obtained from all patients. The study was approved by the Ethics Committee of Tohoku University School of Medicine.

Table 1
Relationship between NDRG2 expression and clinicopathological features of pancreatic cancer patients.

	NDRG2 expression		*P*-value
	Positive $n = 18$	Negative $n = 51$	
Gender			
Male	14	32	0.24
Female	4	19	
Age (mean, years)	61.2	64.0	0.25
Tumor size[a] (mm)	38.3	42.3	0.45
UICC Stage			
I	2	2	0.70
II	6	21	
III	6	16	
IV	4	12	
Differentiation			
Wel	2	1	0.14
Mod	15	41	
Por	1	9	
Lymph node metastasis			
N0	6	18	0.88
N1	12	33	
Lymphatic invasion			
Absent	3	5	0.61
Present	15	46	
Venous invasion			
Absent	0	4	0.22
Present	18	47	
Intrapancreatic neural invasion			
Absent	1	2	0.77
Present	17	49	

[a] Average longitudinal diameter.

2.2. Tissue array analysis and immunohistochemistry

A tissue array consisting of 69 paired pancreatic cancer and their corresponding normal tissues was constructed using TISSUE MICROPROCESSOR (AZUMAYA, Tokyo, Japan). Each paraffin-embedded block was cored out at a diameter of 3 mm, and the cored columns were re-embedded in paraffin. For further analyses, 4 μm slide sections were prepared. The immunohistochemical assay was done by the avidin–biotin–peroxidase method described previously [26]. Rabbit polyclonal anti-NDRG2 (1:3000, Atlas Antibodies AB, Stockholm, Sweden) and anti-rabbit (1:1000, Amersham Biosciences, Little Chalfont, UK) secondary antibodies were used. Immunoreactivity was evaluated by two pathologists. NDRG2 immmunoreactivity was detected in both the cytoplasm and the plasma membrane. Normal epithelial cells showed expression of NDRG2 in all specimens. NDRG2 immunoreactivity was defined by comparison the signal intensities of normal and cancerous tissues; strong, moderate, and weak designations denote signals with cancerous tissue that were stronger, similar, or weaker than the normal tissues, respectively. When no NDRG2 signal was detected, we defined the tumor as negative.

2.3. Cell lines analyzed in this study

Nine human pancreatic cancer cell lines (PANC-1, PCI-35, PK-45P, AsPC-1, BxPC-3, PK-1, MIAPaCa-2, PK8 and PK9) and two colorectal cancer cell lines (Clone A and LS174T) were used. These cell lines were also used in our previous studies and were maintained as described [27,28].

2.4. RNA and DNA extraction

Total RNAs from cultured cells were extracted using RNeasy Mini Kit (Qiagen, Valencia, CA), and their concentrations were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Genomic DNAs from cultured cells were extracted using DNeasy Blood & Tissue Kits (Qiagen) according to the manufacturer's instructions, and their concentrations were measured with a NanoDrop ND-1000 Spectrophotometer. All the processes were carried out according to the manufacturers' instructions.

2.5. Quantitative reverse transcription PCR (qRT-PCR)

Each aliquot of 2 μg total RNA was reverse transcribed to synthesize cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. qRT-PCR analyses were performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) following the manufacturer's instructions. Expression of β2-microglobulin (*B2M*) was used as the internal control [29]. The nucleotide sequences for primers, probes, and PCR conditions are listed in Table 2. Amplifications were carried out in the 15 μl reaction mixtures according to methods described previously [30]. The expression ratios of *NDRG2/B2M* were calculated and used for characterization. Each experiment was performed in triplicate.

2.6. 5-Aza-2′-deoxycytidine (5-aza-dC) and trichostatin A (TSA) treatment

Cells were seeded at a density of 2×10^6 cells per 100 mm dish and were maintained for 72 h while replacing the culture medium containing 1 μM 5-aza-dC (Sigma, St. Louis, MO) every 24 h. Subse-

Table 2
Nucleotide sequences of the primers and probes.

	Forward primer (5′–3′)	Reverse primer (5′–3′)	Probe (5′–3′)	Annealing temperature (°C)	PCR cycles	Product size (bp)
qRT-PCR						
NDRG2	GAAGATGCAGTGGTGGAATG	TCAGCTTGCCTGGCTGAGT	TTCCTCAAGATGGCTGACTCCGG	60	30	109
B2M[a]	TTTCAGCAAGGACTGGTCTTT	CCAAATGCGGCATCTTCAAAC	CTGAAAAAGATGAGTATGCCTGCCGTGTG	60	30	171
MSP						
Methylation specific primer	GTTTGCGGGAAGTTCGAGTC	CCGCCGACCCGACTAACG		70	35	134
Unmethylation specific primer	GTGGGTTTGTGGGAAGTTTGAGTTG	CCACCCACCAACCCAACTAACA		70	30	142

[a] Nucleotide sequences for *B2M* primers and probe were previously reported by Ogawa et al. [29].

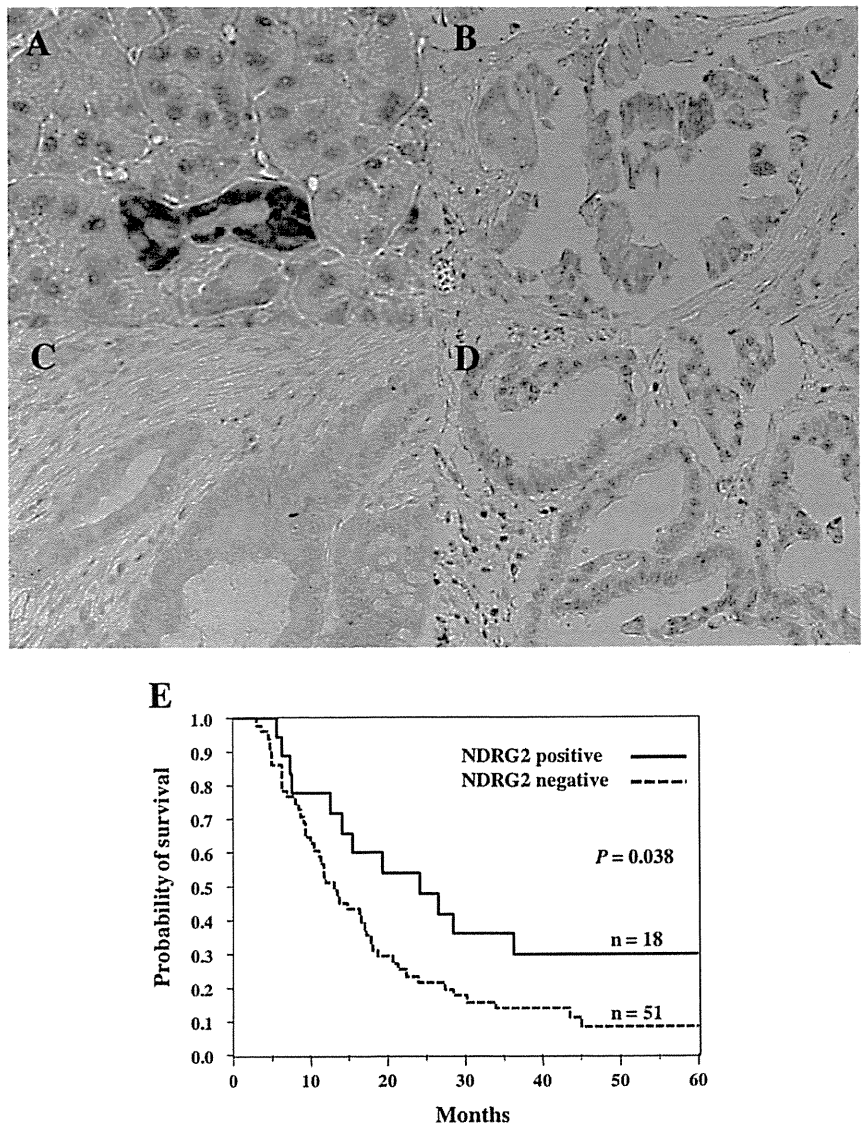

Fig. 1. (A)–(D) Results of representative immmunohistochemical analyses of NDRG2. (A) Strong immunoreactivity was observed in the cytoplasm and plasma membranes of non-neoplastic pancreatic duct (×400 magnification), and pancreatic ductal adenocarcinoma with moderate (B), weak (C), and negative (D) staining (×200 magnification). (**E**) Results of the Kaplan–Meier method indicate a poor overall survival rate of pancreatic cancer patients with negative NDRG2 expression ($P = 0.038$). Solid and dotted lines denote prognoses of NDRG2-positive and -negative patients, respectively.

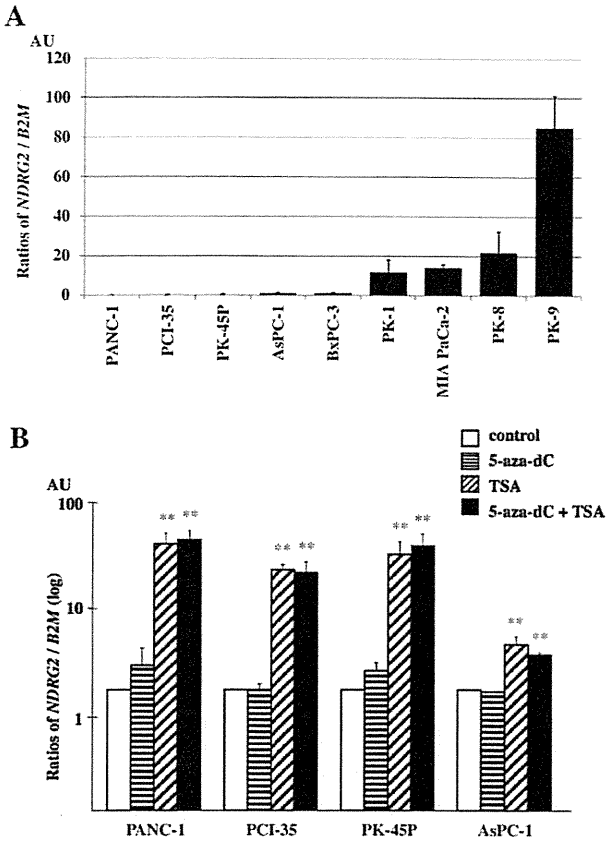

Fig. 2. Expression of *NDRG2* in human pancreatic cancer cell lines by qRT-PCR. Triplicate experiments were done, and the relative expression levels were normalized by the control *B2M* expression (arbitrary units, AU). (A) Results of 9 pancreatic cancer cells are shown. (B) Results of the four low-expressing cells are shown after 5-aza-dC and/or TSA treatments. TSA treatment up-regulated *NDRG2* expression. $^{**}P < 0.01$.

quently, cells were treated with 1 μM 5-aza-dC or 1 μM TSA (Wako, Osaka, Japan) for another 24 h. In TSA only treatment, 2×10^6 cells were plated in a 100 mm dish, and 1 μM TSA was added and cultured for 24 h. All these cells were harvested for qRT-PCR analysis.

2.7. Methylation specific PCR (MSP)

Each aliquot of 2 μg genomic DNA was modified with sodium bisulfite using an Epitect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. The nucleotide sequences of primers for MSP are shown in Table 2. MSP analyses were done by methods described previously [31], and the PCR products were analyzed on 3% agarose gels. Clone A (low *NDRG2* expressing cell line) and LS174T (high *NDRG2* expressing cell line) were used as methylated and unmethylated control cells, respectively.

2.8. Statistical analysis

The Chi-square test was used to examine the correlation between *NDRG2* expression and clinicopathological factors. Survival curves were plotted using the Kaplan–Meier product-limit method, and differences between survival curves were tested using the log-rank test. The gene expression levels before and after 5-aza-dC and/or TSA treatments were analyzed by *t*-test. These statistical analyses were calculated using JMP v9.0 software (SAS Institute Inc., Cary, NC), and results were considered statistically significant when $P < 0.05$.

3. Results

3.1. NDRG2 negative staining correlated with poor prognosis in primary pancreatic cancer

We investigated the expression level of NDRG2 in surgically resected paired cancerous and corresponding normal tissues by immunohistochemical examination. Typical examples are shown in Fig. 1A–D. The spatial distribution of NDRG2 was mainly confined to the cytoplasm and plasma membrane with moderate to strong staining in noncancerous pancreatic ductal cells (Fig. 1A). According to the immunohistochemical results, of the 69 pancreatic cancer specimens examined, one exhibited a moderate NDRG2 expression in tumor cells (Fig. 1B), 17 specimens were weak (Fig. 1C), but no tumor showed stronger NDRG2 expression than normal tissue. The remaining 51 tumors were negative, as shown in Fig. 1D. One moderate and 17 weakly staining tumors were categorized as positive NDRG2 staining (18/69, 26.1%), and 51 tumors (73.9%) were negative. No significant associations were observed in clinicopathological features between positive and negative staining groups (see Table 1). However, the Kaplan–Meier analysis indicated a significant association ($P = 0.038$) between poor prognosis and negative NDRG2 expression in pancreatic cancer patients (Fig. 1E).

3.2. Restoration of NDRG2 expression after 5-aza-dC and/or TSA treatment

To determine whether epigenetic silencing contributes to suppression of the *NDRG2* transcription, we analyzed *in vitro* studies using pancreatic cancer cell lines. The mRNA expressions of *NDRG2* in 9 pancreatic cancer cell lines were determined by

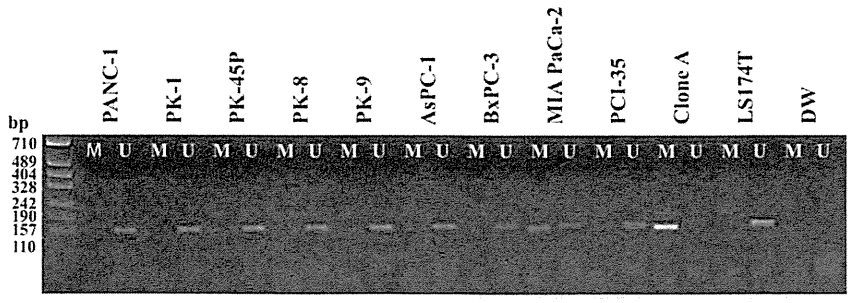

Fig. 3. MSP analyses in pancreatic cancer cell lines. Results of methylation- and unmethylation-specific PCR are indicated by M and U, respectively. All of the cell lines showed unmethylation with one exception: MIA PaCa-2 was partially methylated.

qRT-PCR analyses and found that different cell lines showed different levels of *NDRG2* expression (Fig. 2A). PANC-1, PCI-35, PK45P, AsPC-1 and BxPC-3 showed strong repression, and relatively high expression was observed in PK-9. Results of Western blot analyses correlated well with those of qRT-PCR (data not shown). We selected four pancreatic cancer cell lines (PANC-1, PCI-35, PK45P and AsPC-1) with strong repression of NDRG2 for further analyses. These cells were treated with a demethylating agent, 5-aza-dC, and/or a histone deacetylase (HDAC) inhibitor, TSA; results are shown in Fig 2B. Although no increased *NDRG2* expression was observed after the 5-aza-dC treatment alone, TSA treatment significantly up-regulated *NDRG2* expression. These results suggest that histone modification is one of the main causes for the decreased *NDRG2* expression.

3.3. Promoter hypermethylation was not involved in the decreased NDRG2 expression

It has been reported that hypermethylation is one of the main cause of suppressed expression of *NDRG2* in glioblastoma [7], meningioma [8], hepatocellular carcinoma [9], colorectal cancer [13] and oral squamous-cell carcinoma [16]. We also analyzed other types of cancer cell lines using bisulfite modified sequencing analyses and found that colon cancer cell lines Clone A and LS174T showed methylated and unmethylated CpG islands, respectively (data not shown). Using these cell lines as controls, we studied the methylation status in pancreatic cancer. All the pancreatic cancer cell lines were unmethylated except for MIA PaCa-2, which was partially methylated (Fig. 3). We further analyzed MSP using paired resected normal and cancerous pancreatic tissues from 22 pancreatic cancer patients. As expected, all of the specimens showed the unmethylated pattern, although one was partially methylated (data not shown). These results suggest that the transcriptional repression of *NDRG2* does not mainly depend on hypermethylation.

4. Discussion

Pancreatic cancer is a highly malignant gastrointestinal tumor. Only surgery with adjuvant chemotherapy can achieve a long-term perspective in patients with localized tumors. However, even under optimal treatment conditions, the 5-year survival rate do not exceed 25% [32]. To improve that situation, investigation of new therapeutic agents for pancreatic cancer treatment is essential.

Recently, an accumulation of evidence has indicated that the *NDRG2* gene downregulated in various cancers. In pancreatic cancer, however, little evidence has been reported [10], and no study on an association with prognosis has been reported to date. We demonstrated that NDRG2 expression was significantly reduced and found a significant association between poor prognosis and suppressed expression in pancreatic cancer. As there were no differences, including chemotherapeutic status, between the NDRG2 positive and negative groups, the expression of NDRG2 is likely to be an independent prognostic factor in pancreatic cancer.

We found that histone modification is one of the main mechanisms for downregulating the *NDRG2* expression in pancreatic cancer, and no such mechanisms have previously been reported to control *NDRG2* expression. Histone modification has emerged as a critical component of an epigenetic indexing system demarcating transcriptionally active chromatin domains. In general, while increased histone acetylation is associated with open and active chromatin and increased transcription, deacetylated histones are associated with condensed chromatin and transcriptional repression [33]. Histone deacethylases (HDACs) remove acethyl groups from histones, thereby inducing chromatin condensation and transcriptional repression [34]. Eighteen HDACs have been identified in humans, and they are subdivided into four classes based on their homology to yeast HDACs, their subcellular localization and their enzymatic activities [35]. In pancreatic cancer, high HDAC I expression together with HIF1α were associated with poor prognosis in a series of 39 pancreatic carcinomas [36]. Class I- and class II-selective HDAC inhibitors both synergize in inducing growth arrest and death of pancreatic cells [37]. Other research has also increased our understanding of HDAC function in pancreatic cancer [38]. At least 12 different HDAC inhibitors are undergoing clinical trials as monotherapies or in combination with other adjuvant therapies such as retinoic acid, paclitaxel, gemcitabine, or radiation in patients with various hematologic and solid tumors of the lung, breast, kidney, or bladder as well as with melanoma, glioblastoma, leukemia, lymphomas, and multiple myeloma [39,40]. In pancreatic cancer, promising results have been shown using suberoylanilindehydroxamic acid (SAHA), butyrate, and some other HDAC inhibitors in experimental studies [38,41]. TSA induced G2 arrest and apoptosis in human pancreatic cancer cell lines with mutated TP53 by induction of CDKN1A [42]. Synergistic enhancement of the cytotoxicity of TSA with proteasome inhibitor has also been reported [43]. Our present results that *NDRG2* expression is suppressed mainly by histone-mediated mechanisms and that suppression of *NDRG2* correlates with poor prognosis may provide some valuable clues for the clinical management of patients with pancreatic cancer utilizing a HDAC inhibitor.

The present study indicates that *NDRG2* is likely to be a tumor suppressor gene, reinforcing the data previously reported. In addition, we have demonstrated that inactivation of *NDRG2* associates with poor prognosis in pancreatic cancer. Furthermore, we conclude that epigenetic silencing, such as histone modification, might be the major cause of the frequent loss of NDRG2 expression. Further studies elucidating NDRG2 function will provide a unique and powerful tool for developing novel and useful applications for diagnosis and treatment of patients with pancreatic cancer.

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ORIGINAL ARTICLE

Clinical features and treatment outcome of borderline resectable pancreatic head/body cancer: a multi-institutional survey by the Japanese Society of Pancreatic Surgery

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Abstract
Background Optimal treatment types and prognosis for patients with borderline resectable pancreatic cancer (BRPC) remain unclear because of the lack of studies involving large series of patients.
Methods We retrospectively analyzed various prognostic factors for 624 BRPC (pancreatic head/body) patients treated from June 2002 to May 2007, by distributing questionnaires to member institutions of the Japanese Society of Pancreatic Surgery in 2010. BRPC was defined according to the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines (2009).
Results Among 624 patients, 539 (86.4 %) underwent curative-intent resection, showing an R0 resection rate of 65.9 %. The 3- and 5-year survival rates were 16.1 and 9.9 % in all patients, 22.8 and 12.5 % in the resected patients, and 4.4 and 0 % ($P < 0.0001$) in the unresected patients, respectively. The following factors influencing survival in all patients were selected as independent prognostic factors using multivariate analysis: major arterial involvement on imaging study; preoperative treatment; surgical resection; and postoperative chemotherapy. Among the resected cases, multivariate analysis revealed that major arterial involvement and remnant tumor status were independent prognostic factors.
Conclusion BRPC included two distinct categories of tumors influencing survival: those with portal vein/superior mesenteric vein invasion alone and those with major arterial invasion, which was the most exacerbating factor in the analysis.

Keywords Borderline resectable pancreatic cancer · Aggressive surgical resection · Japanese Society of Pancreatic Surgery

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Introduction

Pancreatic carcinoma is the fifth leading cause of cancer death in Japan, and its prognosis is extremely poor; the disease caused more than 23,000 deaths in 2006 [1]. Although surgical resection is the only curative therapeutic modality, the 5-year postoperative survival rate of patients is <10–15 %. Surgery alone is clearly inadequate for the long-term control of pancreatic carcinoma, and additional therapy is required to improve the outcomes.

Borderline resectable pancreatic cancer (BRPC) has been described as a distinct subset of disease that clarifies the uncertain status between resectable and unresectable tumors. The National Comprehensive Cancer Network (NCCN) has acknowledged the existence of borderline resectable pancreatic adenocarcinoma of the head/body for several years. Moreover, the concept of this distinct subset of tumors has been modified, and the NCCN published a much more precise description of BRPC in 2009. A group at the MD Anderson Cancer Center recently published the first large report regarding BRPC, in which a neoadjuvant approach allowed for identification of the subset of patients who were most likely to benefit from surgery, based on their favorable survival [2]. Takahashi et al. [3] revealed that the prognosis of BRPC was significantly worse than that of resectable PC because of more frequent nerve plexus invasion, portal vein invasion and local recurrence. However, a thorough characterization of patients with BRPC has still not been achieved because of inconsistencies in both the definitions and treatment philosophies that have been adopted in different centers. For example, even an unresectable T4 tumor invading the celiac trunk might sometimes be regarded as potentially resectable at Japanese high-volume centers [4]. In addition, to date there has been no large collective study regarding the treatment of this distinct subset of tumors.

The aim of our study was to determine the precise incidence, prognosis and prognostic predictors of BRPC treated by the 78 member institutions of the Japanese Society of Pancreatic Surgery, paying special attention to significant prognostic factors examined using multivariate analysis.

Methods

Patient selection

The Japanese Society of Pancreatic Surgery (JSPS) holds an annual meeting to discuss developments in pancreatic surgery. One major theme is selected each year and is the subject of questionnaires distributed prior to the meeting. At the 37th Annual Meeting in 2010, BRPC was the main theme, and questionnaires regarding outcomes of advanced pancreatic cancer focusing on BRPC were distributed to member institutions before the commencement of the congress. The detailed composition of the questionnaires is shown in the "Appendix". From June 2002 to May 2007, 3,554 patients with International Union Against Cancer (UICC) T3 or T4 pancreatic cancer underwent surgery with curative intent at the 78 member institutions. There were a total of 714 cases of BRPC, with a mean of 9.0 (range 1–52) cases per institution over the 5-year period covered by the survey. Of the total cases, 41 were omitted owing to incomplete data or unknown outcome. With regard to the tumor location, 624 and 49 cases were located in the pancreatic head/body and pancreatic tail, respectively (Fig. 1). In this study, 624 patients with BRPC of the head/body according to the 2009 NCCN guideline were included.

Definition of borderline resectable pancreatic head/body cancer

In the current study, we defined BRPC according to the NCCN Clinical Practice Guidelines (2009) for pancreatic adenocarcinoma [5] as involving the following four factors: (1) severe unilateral or bilateral superior mesenteric vein (SMV)/portal impingement; (2) less than 180° tumor abutment on the superior mesenteric artery (SMA); (3) abutment or encasement of the hepatic artery (HA), if reconstructible; and (4) SMV occlusion, if it involved a short segment and was reconstructible. SMV/portal impingement is precisely defined as tumor abutment, with or without impingement and narrowing of the lumen, and encasement of the SMV/portal vein (PV) but without encasement of the nearby arteries. Tumor abutment of the SMA should not be >180° of the circumference of the vessel wall. Abutment or encasement of the HA demonstrates gastroduodenal artery encasement up to the HA, with either short segment encasement or direct abutment of the HA. SMV occlusion results from either tumor thrombus or encasement but with suitable vessel proximal and distal to the area of vessel involvement, allowing for safe resection and reconstruction. The above-mentioned four factors regarding the criteria for BRPC were evaluated using triphasic contrast-enhanced multi-detector computed tomography (MDCT) in each institution.

In contrast, the NCCN has defined BRPC that is located in the pancreatic tail as a tumor that shows SMA or celiac

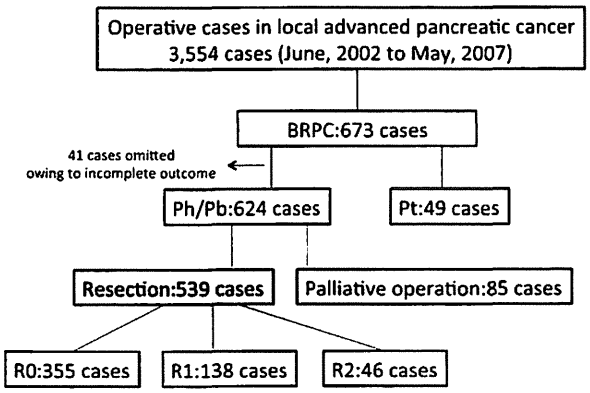

Fig. 1 Results obtained from questionnaires. *BRPC* borderline resectable pancreatic cancer, *Ph/Pb* pancreatic head/pancreatic body, *Pt* pancreatic tail

encasement of <180°. However, we did not use this definition in the preoperative diagnosis of BRPC, since we focused on only pancreatic head/body BRPC in our study.

Evaluation of BRPC prognosis and prognostic predictors

Since there has been no large collective study regarding the characteristics of BRPC, prognostic predictors of BRPC are not well documented. Moreover, even the relevance of surgical resection for BRPC remains unclear. To address these issues, we analyzed the cumulative survival rates of 624 cases, including 85 cases involving palliative surgery (unresected cases), which were considered to be operable preoperatively, but unfortunately proved to be unresectable intraoperatively. Based on the results of questionnaires, as shown in the "Appendix", we identified the most influential prognostic factor using univariate and multivariate analyses.

According to the four factors comprising the NCCN criteria for BRPC, we divided the 624 patients into the following three categories: SMV/PV involvement alone (factors 1 and/or 4); arterial involvement alone (factors 2 and/or 3); and both SMV/PV and arterial involvement. In addition, we defined the cases with para-aortic lymph node involvement as distant metastasis, that is, M1 in the UICC classification. Nevertheless, provided it is not massive, this metastasis has been regarded as potentially curative in Japanese high-volume centers. Therefore, the present study included 24 resected cases with M1. Details of the 63 patients who received preoperative adjuvant treatment are as follows: radiotherapy + gemcitabine (Gem) (29 cases); radiotherapy + Gem + 5-fluorouracil (5-FU) (one case); radiotherapy + the new oral fluorinated pyrimidine derivative S-1 (one case); Gem alone (15 cases); Gem + S1 (two cases); Gem + 5-FU (11 cases); and radiotherapy alone (four cases). On the other hand, 367 of the 424 patients who received postoperative chemotherapy underwent gemcitabine-based therapies such as Gem alone, Gem + 5FU, Gem + S1 and cisplatin + Gem.

Evaluation of surgical procedures and pathological features in 539 resected cases

Even if we can show the relevance of surgical resection in the treatment of BRPC, the adequacy of the surgical approach and how the histological characteristics of the tumor influence the clinical outcome have yet to be addressed. To answer these questions, we compared the cumulative survival curve of 539 resected cases (pancreaticoduodenectomy: 450 cases; distal pancreatectomy: 66 cases; total pancreatectomy: 23 cases) according to the various surgical and pathological variables which are described in the "Appendix". Moreover, we identified the most independent prognostic factors for the resected cases using uni- and multivariate analyses.

Statistical analysis

The data regarding the continuous variables were expressed as mean values ± SD, and the statistical significance was determined using Student's *t* test. Dichotomous variables were evaluated by means of Chi squared analysis. Kaplan–Meier curves for the estimated overall survival were generated, and comparisons between groups were performed using a two-sided log-rank test. To clarify the most influential prognostic factors for BRPC, multivariate analysis using the Cox proportional hazard model was performed, and potential interactions among the entered covariates were examined. Only variables that were statistically significant as determined using univariate analysis were included in the multivariate analysis.

Results

Clinical and demographic variables of patients with BRPC and overall survival

The characteristics of patients with BRPC are summarized in Table 1. Among the 624 patients, 539 (86.4 %) underwent resection with curative intent. Of these patients, combined resection of the portal vein was performed in 430. Unfortunately, however, the treatment resulted in the hospital death

Table 1 Background of overall BRPC cases

	BRPC (n = 624)
Age (years)	63.8 ± 7.9
Gender (male/female)	360/264
Performance status (0–1/2/3/4)	610/14/0/0
Elevation of CA19-9 (yes/no)	470/154
Elevation of CEA (yes/no)	220/404
Maximum tumor size (mm)	35.3 ± 9.4
T factor (T3/T4)	489/135
N factor (N0/N1)	317/307
M factor (M0/M1)	600/24
Preoperative stage (2a/2b/3/4)	247/227/126/24
Surgical procedure (PD/DP/TP/bypass/lap/others)	450/66/23/60/24/1
Resection rate	86.4 % (539/624)
PV/SMV resection	69 % (430/624)
Hospital death (yes/no)	4.6 % (29/624)

BRPC borderline resectable pancreatic cancer, *CEA* carcinoembryonic antigen, *PD* pancreaticoduodenectomy, *DP* distal pancreatectomy, *TP* total pancreatectomy, *lap* laparotomy, *PV/SMV* portal vain/superior mesenteric vein

of 4.6 % of the patients. Among the 539 resected patients, 355 (65.9 %) achieved R0 resection (Fig. 1). As shown in Fig. 2a, the 3- and 5-year survival rates of all 624 patients with BRPC were 16.1 and 9.9 %, respectively.

Prognosis of the 624 BRPC patients and independent prognostic factors

The actuarial overall 3- and 5-year survival rates for the 539 resected patients were significantly better than those for the 85 unresected patients: 17.9 and 11.3 % versus 2.8 and 0 % ($P < 0.0001$) (Fig. 2b). However, especially when we compared the pre- and intraoperative clinical factors between the resected and unresected patients, unresected cases showed significantly more advanced stage disease, a higher incidence of preoperative arterial involvement and intraoperative incidental metastasis (Table 2). Among the 560 patients from whom we excluded the 64 patients with intraoperative findings of distant metastases [33 resected patients (para-aortic lymph node metastasis: 27; peritoneal dissemination: 6) and 31 unresected patients (para-aortic lymph node metastasis: 10; peritoneal dissemination: 9; liver metastasis: 13)], the 3- and 5-year survival rates for the resected patients ($n = 506$) were still significantly better than those for the unresected patients ($n = 54$); they were 22.8 and 12.5 % versus 4.4 and 0 % ($P < 0.0001$) (Fig. 2c). In the 64 patients with intraoperative findings of metastasis, there was no significant difference in 3- and 5-year overall survival rates between the resected ($n = 33$) and unresected cases ($n = 31$); these were 3.6 and 0 % versus 0 and 0 % (not significant, $P = 0.241$).

Next, when we focused on how the NCCN resectability factors affected the prognosis of BRPC, the survival rates were found to be significantly lower in patients with arterial involvement than in those with PV/SMV invasion alone (Fig. 3).

The survival rates and median survival times (MSTs) for patients with BRPC according to the other prognostic factors which were statistically significant, are summarized in Table 3. The patients who underwent intraoperative radiotherapy (IORT) had a significantly worse prognosis than those that did not receive IORT. Using multivariate analysis, the following independent prognostic factors influencing survival were selected: arterial involvement (NCCN resectability factor 2 and/or 3) (yes/no); preoperative treatment (yes/no); surgical resection (yes/no); and postoperative chemotherapy (yes/no) (Table 4).

Prognosis of the 539 resected patients with BRPC and the most influential prognostic predictive factor

Table 5 shows the backgrounds of the 539 resected patients with BRPC, and Table 6 shows the survival rates and MSTs according to each prognostic factor that was statistically significant. In the same way as the overall rate for all patients, the survival rates in the patients who had preoperative major artery involvement were significantly worse than those for the patients who did not have this condition (Fig. 4a). Moreover, the R0 resection rate was significantly lower in patients with arterial factors than in those without them (Fig. 4b). As shown in Table 6, the prognosis was significantly worse for patients who had undergone the

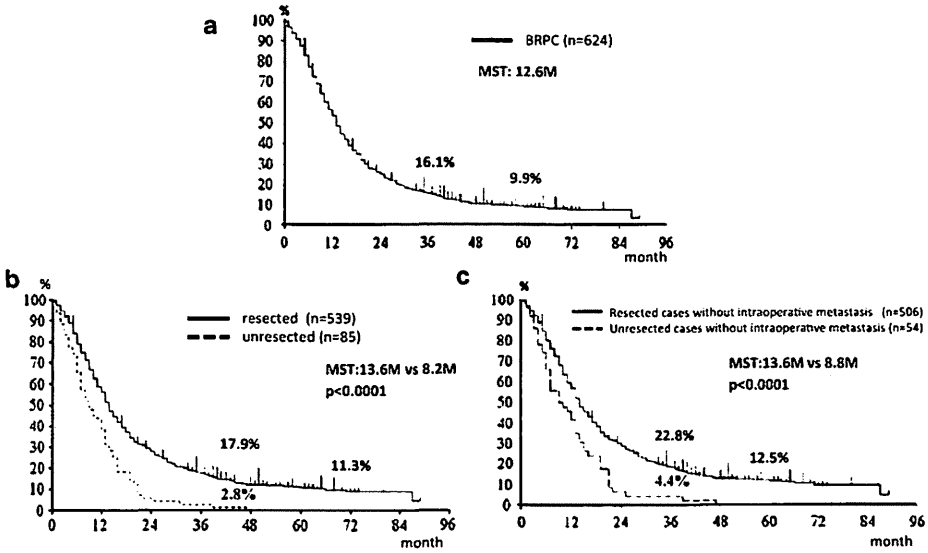

Fig. 2 **a** Kaplan–Meier actuarial overall survival curves for patients with borderline resectable pancreatic cancer (*BRPC*). **b** Kaplan–Meier actuarial overall survival curves for resected and unresected BRPC patients. **c** Kaplan–Meier actuarial overall survival curves for resected and unresected patients without intraoperative metastasis. *MST* median survival time

Table 2 Comparison of background between resected and unresected cases

	Resected (n = 539)	Unresected (n = 85)	P value
Maximum tumor size (mm)	34.7 ± 12.9	39.5 ± 14.6	0.023
Preoperative T factor (T3/T4)	439/100	50/35	<0.001
Preoperative N factor (N0/N1)	274/265	43/42	0.970
Preoperative M factor (M0/M1)	518/21	82/3	0.876
Preoperative stage (2a/2b/3/4)	222/204/92/21	25/23/34/2	<0.001
Major arterial involvement (yes/no)	213/326	56/29	<0.001
Intraoperative metastasis (yes/no)	33/506	31/54	<0.001

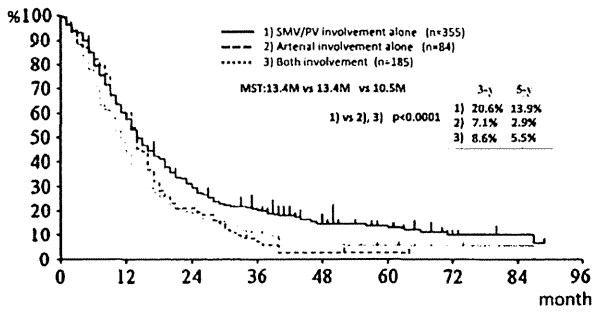

Fig. 3 Kaplan–Meier actuarial overall survival curves plotted for patient groups with the following NCCN resectability factors: SMV/PV involvement alone; arterial involvement alone; and both SMC/PV and arterial involvement. *BRPC* borderline resectable pancreatic cancer, *MST* median survival time

following procedures: total pancreatectomy; total dissection of the SMA plexus; celiac trunk resection; R2 resection; and pathological invasion of the PV/SMV, SMA/HA and peri-SMA plexus. In particular, the 46 patients who underwent R2 resection showed poor 3- and 5-year survival rates that were similar to those of the 54 unresected patients without distant metastasis: 2.6 and 0 % versus 4.4 and 0 %. Based on multivariate analysis, major arterial involvement and status of the tumor remnant (R0 or R1/R2) were selected as independent prognostic factors for the resected patients (Table 7).

Discussion

Recent advances in pancreatic imaging and surgical techniques for pancreatic cancer have revealed a distinct subset of tumors that blurs the distinction between resectable and unresectable disease. Such tumors are classified as BRPC. However, the treatment and prognosis for patients with BRPC is still unclear because of the lack of studies involving large series of patients. Our present study analyzed 624 patients with the objective of determining prognostic factors and establishing a standard treatment schema. This cohort represents the largest collective series of BRPC patients to be evaluated to date.

Table 3 The 3- and 5-year survival rates in the 624 patients with BRPC according to prognostic factors

Variable	n	3-year (%)	5-year (%)	MST (months)	P value
Performance status					
0–1	610	16.7	10.3	12.7	0.00012
2	14	0	0	6.1	
Elevator of CEA					
Yes	404	14.5	7.1	11.2	0.00010
No	220	18.3	11.3	12.1	
Preoperative stage					
IIa	247	22.5	14.5	12.9	<0.0001
IIb	227	11.9	7.4	12.9	IIa versus IIb
III	126	11.9	5.6	11.0	IIa versus III
IV	24	0	0	9.0	
NCCN resectability factors					
PV alone (1)	355	20.6	13.9	13.4	<0.0001
A involvement alone (2)	84	7.1	2.9	13.4	(1) versus (2)
Both (3)	185	8.6	5.5	10.5	(1) versus (3)
Preoperative treatment					
Yes	63	30.2	14.4	12.8	0.0216
No	561	14.0	8.9	9.0	
Resection					
Yes	539	17.9	11.3	13.6	<0.0001
No	85	2.8	0	8.2	
IORT					
Yes	90	11.0	6.1	12.4	0.0037
No	534	16.5	10.1	13.1	
Postoperative treatment					
Yes	424	17.9	10.5	14.5	<0.0001
No	200	10.0	7.1	8.9	

PV alone PV/SMV involvement alone, *A involvement alone* arterial involvement alone, *IORT* intraoperative radiation therapy

Multivariate analysis of the factors influencing survival revealed the following independent prognostic factors: (1) surgical resection; (2) major artery involvement; (3) preoperative treatment; and (4) postoperative chemotherapy in all patients. For patients who had undergone resection, these factors were: (1) major artery involvement; and (2)

Table 4 Result of multivariate analysis for the prognostic factors in overall BRPC cases using Cox proportional hazard model

Variable	Relative risk	Confidence limit	*P* value
Major arterial involvement			
Yes	1.31	1.04–1.57	0.00012
No	1		
Preoperative treatment			
Yes	0.623	0.457–0.828	0.0216
No	1		
Resection			
Yes	1	1.21–2.03	<0.0001
No	1.582		
Postoperative chemotherapy			
Yes	0.554	0.462–0.676	<0.0001
No	1		

Major arterial involvement: cases with NCCN factor (2) and/or (3) preoperatively

BRPC borderline resectable pancreatic cancer

Table 5 Background of the 539 resected BRPC cases

Variables	BRPC with resection ($n = 539$)
Age (years)	63.8±7.9
Gender (male/female)	307/232
Preoperative adjuvant treatment (yes/no)	57/482
NCCN resectability factors (PV alone/A involvement/both)	326/71/142
Surgical procedure (PD/DP/TP)	450/66/23
Dissection of plexus around SMA (half or none/total)	466/73
PV/SMV resection (yes/no)	430/109
SMA resection (yes/no)	24/515
Celiac trunk resection (yes/no)	37/502
HA resection (yes/no)	51/488
Residual tumor (R0/R1/R2)	355/138/46
Histological type of tumor (tubular/papillary/originated from IPMN/adenosquamous/others)	497/12/9/8/13
Tumor differentiation (G1/G2/G3–4/unknown)	100/344/60/35
Pathological invasion of PV/SMV (yes/no/unknown)	353/181/5
Pathological invasion of artery (yes/no/unknown)	467/63/9
Pathological invasion of plexus around SMA (yes/no/unknown)	240/288/11
Postoperative adjuvant chemotherapy (yes/no)	377/162

BRPC borderline resectable pancreatic cancer, *PD* pancreaticoduodenctomy, *DO* distal pancreatectomy, *TP* total pancreatectomy, *SMA* superior mesenteric artery, *PV* portal vein, *SMV* superior mesenteric artery, *HA* hepatic artery, *IPMN* intraductal papillary mucinous neoplasm

Table 6 The 3- and 5-year survival rates in the 539 resected BRPC cases according to surgical and pathological prognostic factor

Variable	*n*	3-year (%)	5-year (%)	MST (months)	*P* value
Surgical procedures					
PD	450	18.7	11.7	13.2	PD versus TP
DP	66	14.7	8.4	16.1	0.0049
TP	23	0	0	8.3	DP versus TP
					0.0032
Dissection of SMA plexus					
Half/none	466	18.0	11.2	13.5	0.00029
Total	73	13.8	10.3	11.1	
Celiac trunk resection					
Yes	37	12.6	6.3	13.3	0.0084
No	502	17.8	11.2	13.3	
Residual tumor					
R0	355	20.6	13.9	14.3	R0 versus R1, R2
R1	138	12.4	5.9	11.7	R1 versus R2
R2	46	20.6	0	9.0	<0.0001
Tumor differentiation					
G1	100	20.2	16.3	18.0	G1 versus G2
G2	344	16.4	11.0	12.7	0.0134
G3–4	60	12.8	9.9	9.7	G1 versus G3
					0.0052
Pathological invasion into PV/SMV					
Yes	353	16.5	9.0	12.2	0.0357
No	181	19.8	14.7	14.5	
Pathological invasion into the artery					
Yes	63	11.1	3.2	9.1	<0.0001
No	467	18.7	12.3	13.7	
Pathological invasion into SMA plexus					
Yes	288	12.8	7.0	11.7	0.00021
No	240	22.4	14.7	11.6	
Preoperative treatment					
Yes	57	33.4	16.0	23.8	0.023
No	482	16.0	10.8	12.1	
Postoperative chemotherapy					
Yes	377	20.5	12.5	16.1	<0.0001
No	162	11.8	8.5	8.1	

BRPC borderline resectable pancreatic cancer, *PV* portal vein, *SMV* superior mesenteric vein, *SMA* superior mesenteric artery

status of the tumor remnant. Furthermore, the R0 resection rate of patients with major arterial involvement was significantly lower than that for patients without major arterial involvement. These results indicate that major artery involvement, not PV/SMV involvement, is the most exacerbating prognostic factor in BRPC, and thus BRPC defined by the NCCN guidelines includes two distinct categories of tumors influencing survival: those with PV/SMV invasion alone and those with major arterial invasion.