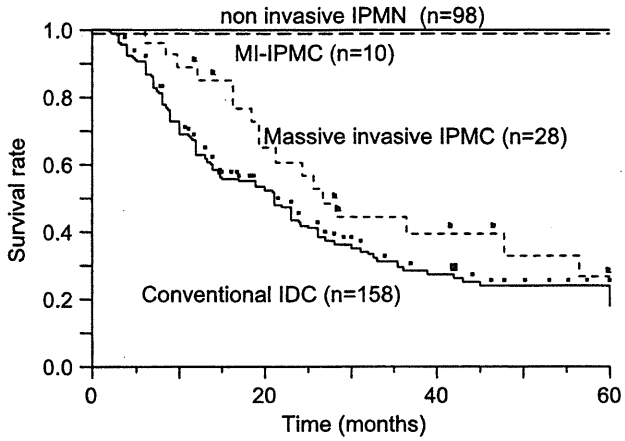

FIGURE 2. Kaplan-Meier survival curves of patients with all types of IPMC and conventional IDC. $P = 0.067$, minimally invasive IPMC versus massively invasive IPMC; $P = 0.115$, massively invasive IPMC versus conventional IDC. Noninvasive IPMN includes IPMA, IPMB, and CIS.

oncocytic-type IPMN (n = 5, 2.8%). Intraductal papillary mucinous neoplasms that could not be specifically categorized into any of the above subtypes were segregated as unclassified (n = 8, 4.5%). Most of the gastric-type IPMNs were adenomas or borderline tumors, and a large number of the intestinal-type IPMNs were borderline tumors or carcinomas. On the other hand, the oncocytic and pancreatobiliary-type IPMNs were only carcinomas (Table 1). The invasive components of the IPMCs were classified as either tubular or colloid carcinoma, and colloid carcinoma was defined as a tumor consisting of colloid differentiation in 80% of the infiltrating carcinoma or greater.[10] Other pathological variables (lymphatic invasion, vascular invasion, and perineural invasion) were recorded based on the Japan Pancreas Society classification.[25] The definition of minimal invasion in this study was stromal invasion of 5 mm or less beyond the pancreatic duct (Fig. 1F), as described previously.[18] We described cases as having massive invasion if the stromal invasion was greater than 5 mm. We sometimes observed cases with a mucus lake without cancer cells in the stroma and considered such lesions to be noninvasive IPMCs. Clinical data were obtained from a combination of medical record reviews, reports of outside medical records, and communication with patients. 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 and the principles embodied in the Declaration of Helsinki.

TABLE 2. Univariate Analyses Using Cox Proportional Hazards Model in Invasive IPMC

Variable	Risk Ratio	95% Confidence Interval	*P*
Age, y			
≧60/<60	0.486	0.172–1.570	0.212
Sex			
Male/female	0.475	0.180–1.280	0.137
Subtype			
Intestinal/nonintestinal	0.174	0.039–0.550	0.002
Invasive pattern			
Tubular/colloid	5.031	1.387–32.32	0.011
Lymph node metastasis			
Positive/negative	3.227	1.181–3.728	0.022
Invasive degree			
Minimal/massive	0.000		0.014
Venous invasion			
Positive/negative	0.933	0.262–2.646	0.903
Lymphatic invasion			
Positive/negative	4.183	1.486–12.062	0.008
Serosal invasion			
Positive/negative	3.281	0.733–10.778	0.109
Retroperitoneal invasion			
Positive/negative	12.726	4.050–48.292	<0.001

Statistical Analysis

All statistical calculations were carried out using JMP 7.0.1 software (SAS Institute, Cary, NC). The disease-specific survival rates were calculated by the Kaplan-Meier method, and differences between curves were analyzed by the log-rank test. All deaths from causes other than IPMN were censored at the time of the last follow-up. The clinicopathologic parameters were compared using the χ^2 test. We also conducted univariate analyses of the prognostic factors with a survival analysis using Cox proportional hazards model. All differences were considered to be statistically significant if $P < 0.05$.

RESULTS

Patient Outcomes and Prognostic Factors in All IPMNs

There were no disease-specific deaths in the IPMA, IPMB, and CIS groups (Fig. 2). We focused only on invasive IPMC in this study. Univariate analyses for disease-specific survival in invasive IPMC identified lymph node metastasis ($P = 0.022$), subtype ($P = 0.002$), massive invasion ($P = 0.014$), tubular carcinoma ($P = 0.011$), retroperitoneal invasion ($P < 0.001$), and lymphatic invasion ($P = 0.008$) as significant prognostic factors for a poor prognosis (Table 2).

Pathological Features of Minimally Invasive IPMC

Among the 42 patients with invasive IPMC, 10 patients (23.8%) showed minimal invasion, and the average depth of tumor invasion was 2.6 mm in this subgroup (Table 3). The

TABLE 3. Characteristics of All Patients With Minimally Invasive Carcinoma

Case	Age, y	Sex	Invasive Type	Invasive Size, mm	Prognosis, mo	Dead or Alive	Subtype
1	75	M	Colloid	4.0	24.7	Alive	I
2	63	M	Tubular	4.5	1.4	Alive	I
3	74	M	Tubular	0.3	13.4	Alive	I
4	77	M	Tubular	0.7	12.5	Alive	I
5	69	M	Tubular	4.8	14.5	Alive	I
6	72	M	Tubular	0.6	7.5	Alive	I
7	62	F	Tubular	3.0	120.5	Alive	I
8	65	F	Tubular	2.0	10.3	Alive	PB
9	59	F	Colloid	4.5	21.5	Alive	I
10	58	M	Tubular	3.0	20.6	Alive	PB

F indicates female; I, intestinal type; M, male; PB, pancreatobiliary type.

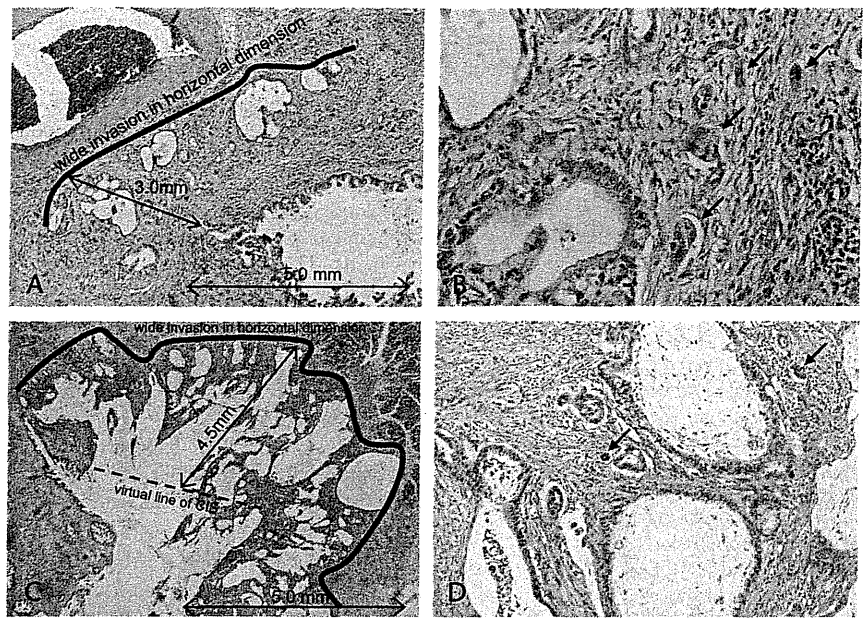

FIGURE 3. Histopathologic features in 2 patients with MI-IPMC with lymph node metastasis. The bold and broken lines indicate the invasive border and depth of invasion, respectively. Both tumors show wide invasion in the horizontal dimension (A, C) and poorly differentiated carcinoma cells (arrow; B, D).

5-year disease-specific survival rate of MI-IPMC was 100% (Fig. 2), and none of these patients showed postoperative recurrence. Venous, lymphatic, serosal, and retroperitoneal invasions were not detected in MI-IPMC.

Lymph node metastasis was detected in 2 (20%) of 10 patients with MI-IPMC and 17 (53.1%) of 32 patients with massively invasive IPMC, compared with none of the patients with CIS. There were significant differences in the frequencies of lymph node metastasis between patients with CIS and patients with MI-IPMC ($P = 0.016$) and between patients with MI-IPMC and patients with massively invasive IPMC ($P < 0.001$), although there was no significant difference in the frequencies of lymph node metastasis between patients with MI-IPMC and patients with massively invasive IPMC ($P = 0.058$). Of the 2 MI-IPMCs with lymph node metastasis, one was pancreatobiliary type and showed 3.0-mm invasion of a tubular carcinoma and neural invasion (Fig. 3A), and the other was intestinal type and showed 4.5-mm-deep invasion of a colloid carcinoma (Fig. 3C). Both patients showed a poorly differentiated carcinoma with sprouting growth and wide invasion in the horizontal dimension (Fig. 3, B and D).

Surgical Outcomes of Patients With Colloid and Tubular Invasive IPMCs

The survival rate was significantly higher for patients with colloid carcinoma derived from IPMN than for patients with tubular carcinoma derived from IPMN ($P = 0.019$; Fig. 4A). Even in the analyses of massively invasive IPMC, the prognosis was significantly better for patients with colloid carcinoma

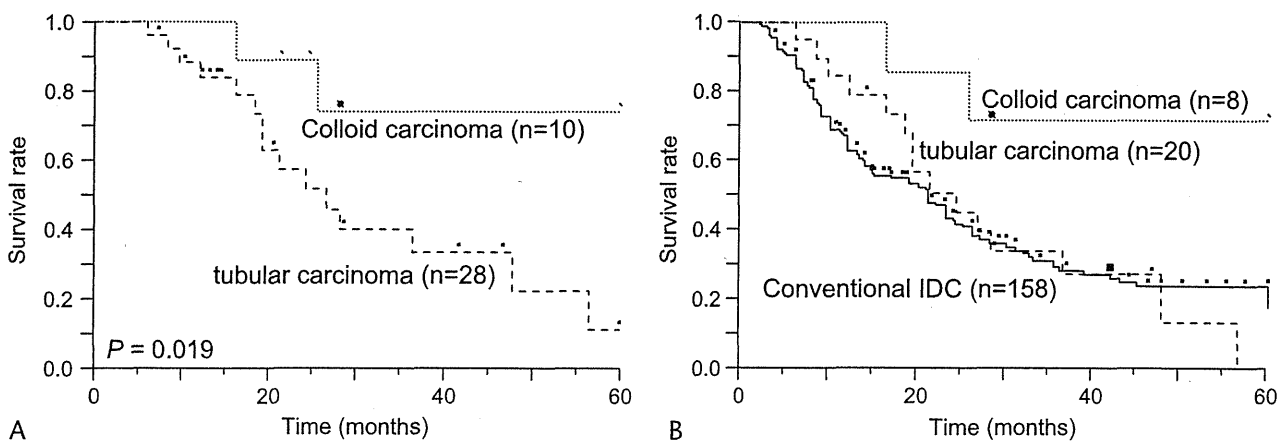

FIGURE 4. Kaplan-Meier survival curves for patients with colloid carcinoma and patients with tubular carcinoma. Survival curves for invasive IPMC (A) and massively invasive IPMC (B) are shown. A, $P = 0.019$, colloid carcinoma versus tubular carcinoma. B, $P = 0.013$, colloid carcinoma versus tubular carcinoma; $P = 0.015$, colloid carcinoma versus conventional IDC; $P = 0.898$, tubular carcinoma versus conventional IDC.

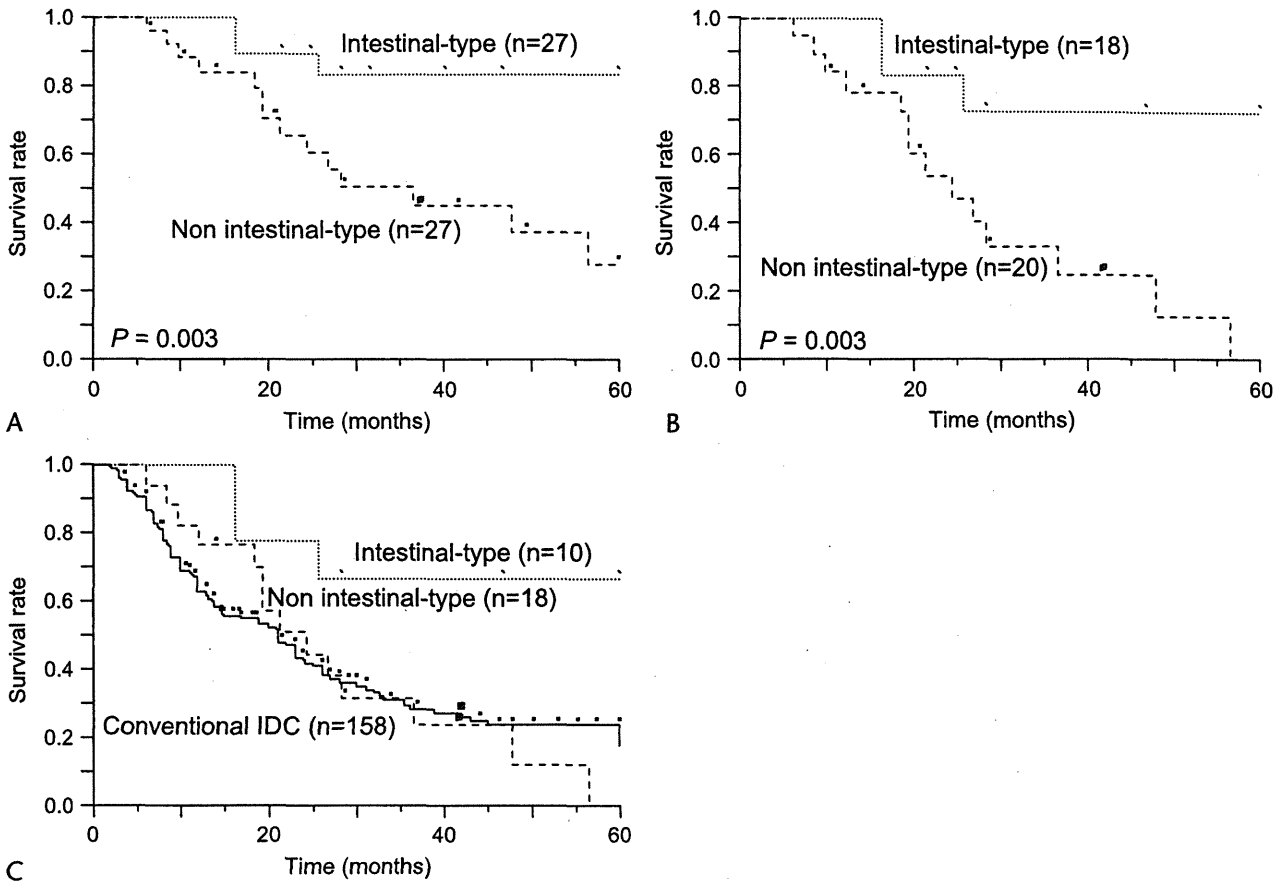

FIGURE 5. Kaplan-Meier survival curves for patients with IPMC divided by subtypes or conventional IDC. Survival curves for all types of IPMC (A), invasive IPMC (B), and massively invasive IPMC (C) are shown. A, $P = 0.003$, intestinal-type IPMC versus non–intestinal-type IPMC. B, $P = 0.003$, intestinal-type IPMC versus non–intestinal-type IPMC. C, $P = 0.013$, intestinal-type IPMC versus non–intestinal-type IPMC; $P = 0.012$, intestinal-type IPMC versus conventional IDC; $P = 0.907$, non–intestinal-type IPMC versus conventional IDC.

derived from IPMN than for patients with tubular carcinoma derived from IPMN ($P = 0.013$; Fig. 4B). For a comparison with conventional IDC (n = 158), we excluded MI-IPMC patients, because there were no cases of conventional IDC with invasion of less than 5 mm in our series. The disease-specific survival rate was significantly higher in patients with colloid carcinoma derived from IPMN than in patients with conventional IDC ($P = 0.015$), but there was no significant difference between patients with tubular carcinoma derived from IPMN and patients with conventional IDC ($P = 0.898$; Fig. 4B).

Surgical Outcomes of IPMC Based on Subtype

In the analyses of invasive and noninvasive IPMCs, patients with intestinal-type IPMC showed a significantly higher survival rate than patients with non–intestinal-type IPMC ($P = 0.003$; Fig. 5A). In the analyses of invasive carcinoma only (including minimally and massively invasive cases), the disease-specific survival rate was significantly higher in patients with invasive carcinoma derived from intestinal-type IPMN than in patients with invasive carcinoma derived from non–intestinal-type IPMN ($P = 0.003$; Fig. 5B). Carcinomas in situ or MI-IPMCs were more frequently observed in intestinal-type IPMC (23 of 33 cases, 69.7%) than in non–intestinal-type IPMC (16 of 38 cases, 42.1%) ($P = 0.019$; Table 4). Therefore, we analyzed only patients with massively invasive IPMC and found that the survival rate was significantly higher in patients with invasive carcinoma derived from intestinal-type IPMN than in patients with invasive carcinoma derived from non–intestinal-type IPMN or conventional IDC ($P = 0.013$ and $P = 0.012$, respectively; Fig. 5C). On the other hand, there was no significant difference between the survival rates of patients with invasive carcinoma derived from non–intestinal-type IPMN and patients with conventional IDC ($P = 0.903$).

TABLE 4. Relationship Between Invasive Degree and Subtype of IPMN

	CIS	MI-IPMC	Massive	
Intestinal type	15	8	10	*$P = 0.019$
Nonintestinal type	14	2	22	
G type	2	0	7	
PB type	3	2	11	
O type	4	0	1	
U type	5	0	3	

*CISs or MI-IPMCs were observed more frequently in intestinal-type IPMC than in non–intestinal-type IPMC.

I type indicates intestinal type; G type, gastric type; PB type, pancreatobiliary type; O type, oncocytic type; U type, unclassified type.

TABLE 5. Relationship Between Subtypes of Massive Invasive IPMC and Pathological Features

	Intestinal (n = 10)	Nonintestinal (n = 22)	
Invasive pattern			
Colloid	8	0	<0.001
Tubular	2	22	
Lymph node metastasis			
Positive	6	9	0.315
Negative	4	13	
Lymphatic invasion			
Positive	1	12	0.001
Negative	9	10	
Venous invasion			
Positive	3	7	0.918
Negative	7	15	
Neural invasion			
Positive	5	11	1.000
Negative	5	11	
Serosal invasion			
Positive	0	6	0.021
Negative	10	15	
Retroperitoneal invasion			
Positive	2	12	0.060
Negative	8	10	
Distant metastasis			
Positive	1	0	0.122
Negative	9	22	

Histological Comparisons of Massively Invasive Carcinomas Derived From Intestinal-Type and Non–Intestinal-Type IPMNs

Among the 32 patients with massively invasive IPMC, colloid carcinoma was more frequently detected in the invasive components derived from intestinal-type IPMN (8 of 10 cases, 80.0%) than in those derived from non–intestinal-type IPMN (0 of 22 cases, 0%) ($P < 0.001$; Table 5). Lymphatic ($P = 0.001$) and serosal ($P = 0.021$) invasions were less frequently observed in the invasive components of intestinal-type IPMC than in those of non–intestinal-type IPMC (Table 5).

DISCUSSION

In this study, we evaluated the propriety of the previous definition of MI-IPMC and investigated the prognosis of colloid carcinoma for a subsequent precise analysis of the prognosis of patients with intestinal and non–intestinal-type IPMCs. The present analyses focusing on different morphological subtypes revealed that the high frequencies of minimal invasion and colloid carcinoma in intestinal-type IPMC contributed to higher survival rates compared with those in non–intestinal-type IPMC. Interestingly, we also found that, even when confined to massively invasive IPMC only, the prognosis of patients with invasive carcinoma derived from intestinal-type IPMN was significantly better than that of patients with invasive carcinoma derived from non–intestinal-type IPMN and patients with conventional IDC. These differences were caused by the less aggressive nature of intestinal-type IPMC.

There is no generally accepted agreement with regard to the definition of minimal invasion in IPMC. Recently, Nara et al[18] defined MI-IPMC using a threshold of 5 mm, even if the invasive carcinoma cells were above the "scant" level, and reported that their patients with minimal invasion had no lymph node metastases and a better prognosis than that of patients with massively invasive IPMC. However, our present series included 2 MI-IPMC patients with lymph node metastasis, and both of these patients had invasion in the horizontal dimension. In uterine cervical cancer, the upper limit of the depth of invasion is 3 to 5 mm, and the definition of "microinvasion" includes the horizontal dimension of the tumor in addition to the depth of invasion.[26] Furthermore, the diagnosis of microinvasion is accurately performed by cervical conization, whereas in pancreatic cancer, preoperative pathological diagnosis of the depth of invasion is impossible and can only be estimated using radiologic images.

The threshold of 5 mm for the definition of MI-IPMC seems to be reasonable, considering its favorable prognosis, and we used this definition in the present study. However, considering the rate of lymph node metastasis in this study and the difficulty in making a preoperative diagnosis, omitting lymph node dissection in patients with suspected MI-IPMC may not be reasonable. The number of patients with minimal invasion in our study was small, and further examinations will be needed to define MI-IPMC based on the volume of invasive carcinoma cells in addition to the depth of invasion.

When we compared the prognoses of patients with invasive IPMC and patients with conventional IDC, we excluded patients with MI-IPMC and colloid carcinoma, neither of which is seen in conventional IDC. We found no significant difference in the prognoses between patients with tubular carcinoma derived from IPMN and patients with conventional IDC. These data suggest that the favorable prognosis of IPMC reported previously may be caused by the existence of colloid carcinoma and MI-IPMC. This is the first report to describe the prognoses of patients with invasive IPMC and patients with conventional IDC, taking both colloid carcinoma and minimal invasion into account.

Finally, we compared the prognoses of patients with different morphological subtypes of invasive IPMC. Adsay et al[14] reported that intestinal-type IPMN may have a distinct indolent pathway of carcinogenesis. We observed a high frequency of minimal invasion in the invasive components of intestinal-type IPMC as well as in the invasive components of CIS. Interestingly, even when confined to massively invasive IPMC, invasive carcinoma derived from intestinal-type IPMN showed a better prognosis and less aggressive behavior than invasive carcinoma derived from non–intestinal-type IPMN. These findings suggest that intestinal-type IPMN may have an indolent nature and that the biological behavior of intestinal-type IPMC may be less aggressive, even when it has proceeded to massive invasion.

Based on the results of the present study, we conclude that the definition of MI-IPMC using the invasion criterion of 5 mm is reasonable, considering its better prognosis, although lymph node dissection cannot be omitted, and further investigations are necessary to elucidate the significance of the volume of invasion. The better prognosis of invasive carcinoma derived from intestinal-type IPMN was associated with the high frequencies of the minimally invasive form of the disease and colloid carcinoma, and the less invasive nature regarding lymphatic and peripancreatic invasions.

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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Claudin-4 Expression Predicts Survival in Pancreatic Ductal Adenocarcinoma

Kosuke Tsutsumi, MD, PhD[1], Norihiro Sato, MD, PhD[1], Reiko Tanabe, MD[1], Kazuhiro Mizumoto, MD, PhD[1], Katsuya Morimatsu, MD[2], Tadashi Kayashima, MD[1], Hayato Fujita, MD, PhD[1], Kenoki Ohuchida, MD, PhD[1], Takao Ohtsuka, MD, PhD[1], Shunichi Takahata, MD, PhD[1], Masafumi Nakamura, MD, PhD[1], and Masao Tanaka, MD, PhD, FACS[1]

[1]Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [2]Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

ABSTRACT

Background. Identification of prognostic markers would be useful in the clinical management of patients with pancreatic ductal adenocarcinoma (PDAC). The clinical relevance of claudin-4 (CLDN4), recently identified as overexpressed in PDAC, is unknown.

Methods. Using quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR), we analyzed *CLDN4* mRNA expression in a panel of 9 pancreatic cancer cell lines and formalin-fixed paraffin-embedded (FFPE) tissues from 100 patients with PDAC. The *CLDN4* expression levels were then correlated with clinicopathological variables and patient outcome. We also performed immunohistochemical analysis in 20 FFPE samples of PDAC to investigate the expression of CLDN4 protein.

Results. Increased expression of *CLDN4* was confirmed in all the pancreatic cancer cell lines tested compared with normal ductal epithelial cells and fibroblasts. We found that low expression of *CLDN4* was significantly associated with shorter survival in patients with PDAC (hazard ratio; 1.362, 95% confidence interval; 1.011–1.873, $P = 0.0419$). Patients with high *CLDN4* expression survived longer for a median of 63.0 months, compared with 14.7 months in patients with low *CLDN4* expression ($P = 0.0067$). In immunohistochemical analysis, the level of *CLDN4* mRNA expression was significantly correlated with the expression of CLDN4 protein ($P = 0.0168$).

Conclusion. Increased expression of *CLDN4* mRNA predicts better prognosis in PDAC.

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive cancers, representing the fourth leading cause of cancer-related death in the industrialized world.[1,2] According to a recent report, the 5-year survival rate of PDAC remains extremely low, at less than 5%.[3] This is partly because the majority of patients with PDAC are diagnosed at an advanced stage, with only 15–20% presenting with resectable tumors. Even after surgical resection, the 5-year survival rate is only 15–25% because of the high incidence of local recurrence and/or disseminated disease.[4–6] Despite recent advances in multimodal therapeutic approaches including surgery, chemoradiotherapy, and immunotherapy, the fatal prognosis of PDAC has remained essentially unchanged over the last few decades. Stratification of the patients according to the prognostic information helps with a therapeutic decision. Therefore, efforts are underway to identify novel markers, such as molecular biological markers, to predict outcome and, ultimately, to identify a subset of patients with a favorable prognosis who benefit from surgery and/or chemoradiotherapy.

The most commonly used tumor marker, CA19-9, has been shown to be associated with prognosis in patients with pancreatic cancer.[7–9] However, in the clinical setting we often experience that CA19-9 level is unreliable to predict the response to therapy and overall survival of patients with pancreatic cancer. In addition to the commonly used tumor markers, previous studies have demonstrated prognostic relevance of a number of molecular genetic markers in pancreatic cancer, including p53, p21, SMAD4, transforming growth factor-β (TGF-β),

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N. Sato, MD, PhD
e-mail: norisato@fukuoka-med.jrc.or.jp

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Bcl-2, matrix-metalloproteinases (MMP), p16, β-catenin/E-cadherin, vascular endothelial derived growth factor, platelet-derived endothelial growth factor, basic fibroblast growth factor (bFGF), human equilibrative nucleoside transporter-1, and S100A4.[10–21] The prognostic relevance of these molecular markers, however, remains largely unknown and should be investigated in prospective studies.

Claudin-4 (CLDN4), encoding a transmembrane protein involved in tight junction formation and function, has recently attracted considerable interest for its widespread involvement in human cancers. CLDN4 has been shown to be overexpressed in a variety of cancers, including ovarian cancer, prostate cancer, and breast cancer.[22–24] CLDN4 has been identified as overexpressed in pancreatic cancers by gene expression profiling.[25–27] We have recently shown that *CLDN4* expression is associated with neoplastic progression of intraductal papillary mucinous neoplasms (IPMNs) of the pancreas, a precursor to pancreatic cancer, and, especially, with a distinct pathway to intestinal differentiation. Functionally, expression of CLDN4 decreases the invasive capacity and metastatic potential of pancreatic cancer cells.[28] Therefore, it is highly probable that CLDN4 may play a role in pancreatic ductal carcinogenesis and aggressive phenotype of this highly lethal neoplasm. However, the prognostic implication of CLDN4 in PDAC remains unknown.

In an attempt to elucidate the clinicopathological implication of CLDN4 in PDAC, we used quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) to determine *CLDN4* mRNA expression in formalin-fixed paraffin-embedded (FFPE) tissues from a large series of PDAC.

MATERIALS AND METHODS

Cultured Cells

A total of 9 pancreatic cancer cell lines, BxPC-3, KP-2, PANC-1, SUIT-2 (provided by Dr. H. Iguchi, National Shikoku Cancer Center, Matsuyama, Japan), CAPAN-1, CFPAC-1, HS766T, SW1990 (American Type Culture Collection, Manassas, VA), MIA PaCa-2 (Japanese Cancer Resource Bank, Tokyo, Japan), 3 primary cultures of pancreatic fibroblasts derived from patients with PDAC (established in our laboratory), and a primary culture of normal human pancreatic ductal epithelial cells (Cell Systems, Applied Cell Biology Research Institute, Kirkland, WA) were used in the present study. Cells were maintained, and primary cultures of pancreatic fibroblasts were produced using the outgrowth method as described previously.[29,30]

Clinical Samples of PDAC; Patient Demographics and Clinicopathological Characteristics

A total of 100 FFPE tissue samples of histology-proven PDAC obtained from patients undergoing grossly curative resection at our department (Kyushu University Hospital, Fukuoka, Japan) from 1992 to 2007 were selected for the present study simply based on the availability of sufficient quantities of cancer cells and capability of PCR amplification. Mixed neoplasms and histological variants of PDAC (including adenosquamous carcinoma, signet ring cell carcinoma, medullary carcinoma, hepatoid carcinoma, and colloid carcinoma) were excluded from this study because of their peculiar biological behaviors. Invasive carcinomas derived from intraductal papillary mucinous neoplasm (IPMN) were also eliminated. These PDAC patients comprised 67 males and 33 females with a median age of 66 years (range, 36–82 years). Of these, 63 patients had PDAC in their pancreatic head, 17 patients in pancreatic body, and 20 patients in pancreatic tail. Pancreaticoduodenectomy (conventional Whipple operation or pylorus-preserving procedure) was performed in 62 patients, distal pancreatectomy in 37 patients, and total pancreatectomy in 1 patient. Regional lymph node dissection was performed in all patients. There were 26 patients who received no postoperative chemotherapy because of their poor performance status, while 71 patients received postoperative chemotherapy that based 5-fluorouracil and/or gemcitabine. Survival was measured from the time of pancreatic resection, and death was the endpoint. The median observation time for overall survival was 15 months, ranging from 1 to 101 months. A total of 65 patients died during the follow-up, and the other patients were alive and censored. In all samples, histological findings were evaluated according to the criteria of the World Health Organization.[31] The stage of cancers was determined according to the UICC classification.[32] The clinicopathological characteristics are noted in Table 1.

Written informed consent was obtained from all patients, and this study was approved by our institution's surveillance committee and conducted according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and the Helsinki Declaration.

Isolation of RNA

Total RNA was extracted from cultured cells using a High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) with DNase I (Roche Diagnostics) treatment according to the manufacturer's instructions. As far as the isolation of total RNA from FFPE samples of PDAC, we firstly performed macrodissection of cancer

TABLE 1 Clinicopathological characteristics of the patients ($n = 100$)

Median age	66 years (range, 36–82 years)
Gender (male/female)	67/33
Tumor location	
Head	63
Body	17
Tail	20
pT category	
pT1	6
pT2	3
pT3	90
pT4	1
pN category	
pN0	32
pN1	68
UICC stage	
I	8
II	88
III	1
IV	3
Histological grade	
G1	22
G2	40
G3	38
Lymphatic invasion	
Positive	78
Negative	22
Vessel invasion	
Positive	69
Negative	31
Neural invasion	
Positive	86
Negative	14
Residual tumor category	
R0	65
R1	35
Postoperative chemotherapy	
Yes	71
No	26
Unknown	3

tissue using a small blade. By cutting into the outer edge of tumor marginally and removing the noncancerous component with a small quantity of the cancer cell, only the cancerous component was selectively isolated to obtain a high cancerous cellularity of ~75%. Meanwhile, the FFPE samples of normal pancreatic tissue were macrodissected to enrich the population of normal ductal cells by removing the surrounding connective tissue, fat tissue, large vessel, lymph node, and acinar and islet cells, as possible. Total RNA was extracted from the isolated cells using the RNeasy FFPE Kit (Qiagen, Tokyo, Japan) according to kit instructions. The extracted RNA was quantified by reading the absorbance at 260 nm, and its purity was evaluated from the 260/280 ratio of absorbance with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE).

Quantitative Assessment of CLDN4 mRNA Level by Real-Time RT-PCR

One-step quantitative real-time RT-PCR was performed using a LightCycler 480 II Real-Time PCR System (Roche Diagnostics, Mannheim, Germany) for 40 cycles of 15 seconds at 95°C and 1 minute at 55°C with the QuantiTect SYBR Green Reverse Transcription-PCR kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.[33] In addition, we designed specific primers—*CLDN4*, 5′-GAG GGC CTA TGG ATG AAC TG-3′ (forward) and 5′-AGC AGC GAG TCG TAC ACC TT-3′ (reverse); *18S* rRNA, 5′-CTT TCG AGG CCC TGT AAT TG-3′ (forward) and 5′-CCT CCA ATG GAT CCT CGT TA-3′ (reverse)—using Primer 3 and performed BLASTN searches to ensure the primer specificities. The PCR product sizes of these primers were small (*CLDN4*; 71 base pairs [bp] and *18S* rRNA; 63 bp, respectively), which allowed accurate and sensitive qRT-PCR despite the fragmented RNA extracted from FFPE tissue specimens.[34,35] The level of *CLDN4* mRNA expression was calculated from a standard curve generated with total RNA from SUIT-2, a human pancreatic cancer cell line. The expression of *CLDN4* mRNA was normalized to that of *18S* rRNA. PCR was repeated at least twice in all samples and three times if the sample showed more than 10% deviation in RT-PCR values. Standard deviation and coefficient of variation were low for both intra-assay and inter-assay variability.

Immunohistochemical Staining

A rabbit anti-CLDN4 antibody (working dilution, 1:100; blocking, normal goat serum; Zymed Laboratories, South San Francisco, CA) was used as the primary antibody. Sections were cut at 4-μm thickness from 20 FFPE materials of PDAC, then deparaffinized in xylene and rehydrated through a graded series of ethanol. After inhibition of endogenous peroxidase and antigen retrieval (microwave irradiation in a citrate buffer), sections were exposed to the primary antibody at 4°C overnight and stained with a streptavidinbiotin-peroxidase kit (Nichirei, Tokyo, Japan). The sections were then finally reacted in 3,3′-diaminobenzidine, counterstained with hematoxylin,

and mounted. Negative controls were prepared by omitting the primary antibody.

Membranous staining was considered positive for CLDN4 protein as described recently.[36] Negative expression was defined as the percentage of stained cells <25%, while positive expression as the percentage of stained cells ≥25%, according to the recent report.[36] All slides were evaluated independently by 2 investigators (K.M. and K.T.) without any knowledge of background of each case.

Statistical Analysis

All statistical analyses were performed with JMP statistical software (version 6.0.3; SAS, Inc., Cary, NC). The expression of *CLDN4* mRNA was classified into either high or low expression group using recursive descent partition analysis, as described by Hoffmann and coworkers.[37] Categorical variables were compared with a chi-square test (Fisher exact probability test). Continuous variable were analyzed by Wilcoxon test (Mann–Whitney *U* test) because normal distributions were not obtained. 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. Statistical differences were considered significant when *P* value was less than 0.05.

RESULTS

Comparison of CLDN4 mRNA Levels Between Pancreatic Cancer Cells and Fibroblasts and Normal Ductal Epithelial Cells

Using quantitative real time RT-PCR, we first confirmed the *CLDN4* mRNA expression level in a panel of 9 pancreatic cancer cell lines, 3 primary cultures of pancreatic fibroblasts derived from PDAC, and 1 primary culture of normal pancreatic ductal epithelial cells. The relative expression levels of *CLDN4* mRNA in all the pancreatic cancer cell lines tested were much higher than those in normal ductal epithelial cells and fibroblasts (Fig. 1).

Quantitative Analysis of CLDN4 mRNA Expression in Clinical Samples of PDAC, Chronic Pancreatitis, and Normal Pancreatic Tissue

Total RNA was isolated from FFPE tissues from a total of 113 patients. PCR amplification was successfully achieved in 113 of the 118 RNA samples (95.8%). We then analyzed *CLDN4* mRNA in these 113 RNA samples (including 100 PDACs, 7 normal pancreatic tissues, and 6 chronic pancreatitis samples). The expression levels of *CLDN4* mRNA were significantly higher in PDAC (relative value of 0.03) than in normal pancreatic tissue (0.003, $P < 0.0001$) and chronic pancreatitis (0.01, $P = 0.0144$) (Fig. 2). Furthermore, *CLDN4* mRNA levels were significantly higher in chronic pancreatitis than in normal pancreatic tissue, although the sample number was relatively small (Fig. 2, $P = 0.0152$).

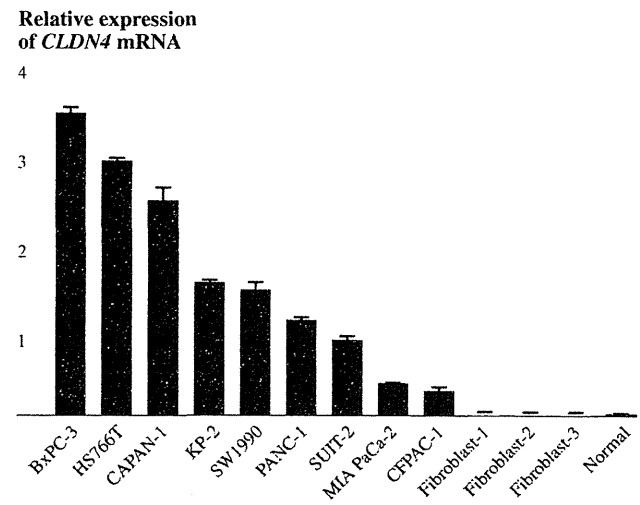

FIG. 1 Comparison of *CLDN4* mRNA expression between 9 pancreatic cancer cell lines and 3 primary cultures of fibroblasts derived from PDAC (fibroblast-1, 2, 3) and 1 primary culture of normal pancreatic ductal epithelial cells (normal). Expression of *CLDN4* mRNA is normalized to that of *18S* rRNA. The relative expression levels of *CLDN4* mRNA in all the pancreatic cancer cell lines tested are much higher than those in normal ductal epithelial cells and fibroblasts

Relationship Between CLDN4 mRNA Expression and Clinicopathological Factors in PDAC

In an attempt to investigate the correlation of *CLDN4* expression with clinicopathological features, we first divided all the PDAC patients into 2 groups, a high *CLDN4* expression group ($n = 43$) and low *CLDN4* expression group ($n = 57$), using recursive descent partition analysis (cutoff value: 0.034). We then compared the clinicopathological variables between the groups (Table 2). The clinicopathological variables analyzed included patient's age (≥65 years vs. <65 years), tumor location (pancreatic head vs. pancreatic body or tail), pT category (pT1/pT2 vs. pT3/pT4), pN category (pN0 vs. pN1), UICC stage (I vs. II vs. III/IV), histological grade (G1 vs. G2 vs. G3), lymphatic invasion, vessel invasion, neural invasion, residual tumor category (R0 vs. R1), and postoperative chemotherapy received. There were no significant difference in age, tumor location, pT status, pN status, UICC stage, histological grade, R factor, and postoperative chemotherapy between

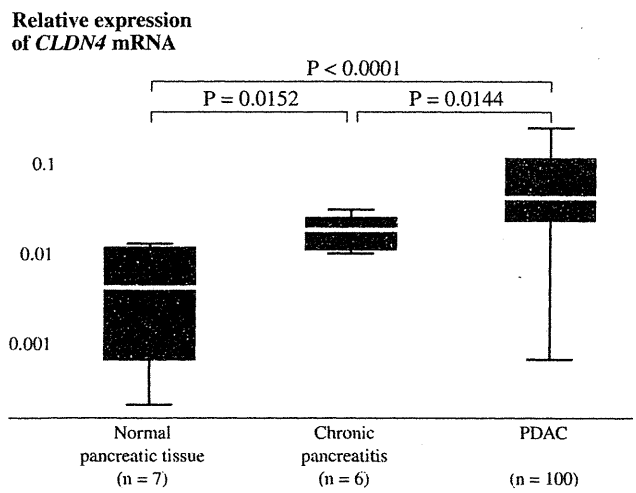

FIG. 2 Expression levels of *CLDN4* mRNA in clinical samples of normal pancreatic tissue ($n = 7$), chronic pancreatitis ($n = 6$), and PDAC ($n = 100$). Results are presented as a *box-and-whisker* plot. Expression of *CLDN4* mRNA is normalized to that of *18S* rRNA. The expression levels of *CLDN4* mRNA in PDAC (median, 0.03) are significantly higher than those in normal pancreatic tissue (median, 0.003) and chronic pancreatitis (median, 0.01) (PDAC vs normal pancreatic tissue; $P < 0.0001$, PDAC vs chronic pancreatitis; $P = 0.0144$, Wilcoxon test). *CLDN4* mRNA levels are also significantly higher in chronic pancreatitis than in normal pancreatic tissue (chronic pancreatitis vs normal pancreatic tissue; $P = 0.0152$, Wilcoxon test)

the high *CLDN4* expression group and low *CLDN4* expression group. However, lymphatic invasion (ly), vessel invasion (v), and neural invasion (ne) by histopathological examinations were observed more frequently in the low *CLDN4* expression group than in the high *CLDN4* expression group (ly; $P = 0.0014$, v; $P = 0.0133$, ne; $P = 0.0205$).

Relationship Between CLDN4 mRNA Expression and Survival in PDAC

We then explored the correlation between *CLDN4* mRNA expression and survival of patients with PDAC. Survival analysis revealed a significantly longer postoperative survival in the high *CLDN4* expression group (median survival time of 63.0 months and 5-year survival time of 53.7%, Table 3) compared with the low *CLDN4* expression group (median survival time of 14.7 months and 5-year survival time of 14.4%, Table 3) (Fig. 3, log-rank test; $P = 0.0067$, Wilcoxon test; $P = 0.0217$).

Univariate and Multivariate Analyses for Factors Influencing Survival in Patients with PDAC

To further determine the prognostic value of *CLDN4* mRNA expression for PDAC, clinicopathological variables as well as *CLDN4* expression status were analyzed using Cox proportional hazard model. Univariate analysis demonstrated significant prognostic factors in addition to *CLDN4* expression ($P = 0.0067$), including significant prognostic factors included pN status ($P = 0.0101$), lymphatic invasion ($P = 0.0047$), vessel invasion ($P = 0.0035$), residual tumor status ($P < 0.0001$), and postoperative chemotherapy ($P < 0.0001$) (Table 3).

TABLE 2 Relationship between *CLDN4* mRNA expression and various clinicopathological factors

	CLDN4 mRNA expression		*P* value
	High expression group ($n = 43$)	Low expression group ($n = 57$)	
Age			0.3058
≥65 years	22	35	
<65 years	21	22	
Tumor location			0.1961
Head	24	39	
Body/Tail	19	18	
pT category			0.9269
pT1/pT2	4	5	
pT3/pT4	39	52	
pN category			0.7421
pN0	13	19	
pN1	30	38	
UICC stage			0.8726
I	4	4	
II	37	51	
III/IV	2	2	
Histological grade			0.7759
G1	8	14	
G2	18	22	
G3	17	21	
Lymphatic invasion			0.0014
Positive	27	51	
Negative	16	6	
Vessel invasion			0.0133
Positive	24	45	
Negative	19	12	
Neural invasion			0.0205
Positive	33	53	
Negative	10	4	
Residual tumor category			0.1965
R0	31	34	
R1	12	23	
Postoperative chemotherapy			0.1318
Yes	34	37	
No	8	18	

TABLE 3 Univariate survival analysis of conventional prognostic factors and *CLDN4* mRNA expression

	Median survival time (months)	5-year survival rate	*P* value
CLDN4 mRNA expression			0.0067
High ($n = 43$)	63.0	53.7%	
Low ($n = 57$)	14.7	14.4%	
Age			0.4741
≥65 years ($n = 57$)	20	18.6%	
<65 years ($n = 43$)	22	32.2%	
Tumor location			0.4300
Head ($n = 63$)	19	23.6%	
Body/Tail ($n = 37$)	26	25.4%	
pT category			0.1797
pT1/pT2 ($n = 9$)	63	62.2%	
pT3/pT4 ($n = 91$)	19	21.0%	
pN category			0.0101
pN0 ($n = 32$)	43	35.3%	
pN1 ($n = 68$)	13.7	20.5%	
UICC stage			0.0589
I ($n = 8$)	63	70.0%	
II ($n = 88$)	19	21.9%	
III/IV ($n = 4$)	12	0.0%	
Histological grade			0.0701
G1/2 ($n = 62$)	26	26.7%	
G3 ($n = 38$)	12	22.4%	
Lymphatic invasion			0.0047
Positive ($n = 78$)	14.7	17.5%	
Negative ($n = 22$)	63	59.1%	
Vessel invasion			0.0035
Positive ($n = 69$)	13.7	16.0%	
Negative ($n = 31$)	45	45.6%	
Neural invasion			0.4154
Positive ($n = 86$)	20	21.6%	
Negative ($n = 14$)	24.2	46.8%	
Residual tumor category			<0.0001
R0 ($n = 65$)	32.7	37.0%	
R1 ($n = 35$)	11	6.4%	
Postoperative chemotherapy			<0.0001
Yes ($n = 71$)	26	34.1%	
No ($n = 26$)	9	0.0%	

Multivariate analysis revealed *CLDN4* mRNA expression (hazard ratio [HR], 1.362; 95% confidence interval [95% CI], 1.011–1.873; $P = 0.0419$), histological grade (HR, 1.526; 95% CI, 1.137–2.037; $P = 0.0052$), residual tumor (HR, 1.804; 95% CI, 1.356–2.412; $P < 0.0001$), and postoperative chemotherapy (HR, 1.660; 95% CI, 1.226–2.238; $P = 0.0012$) to be independent prognostic factors (Table 4).

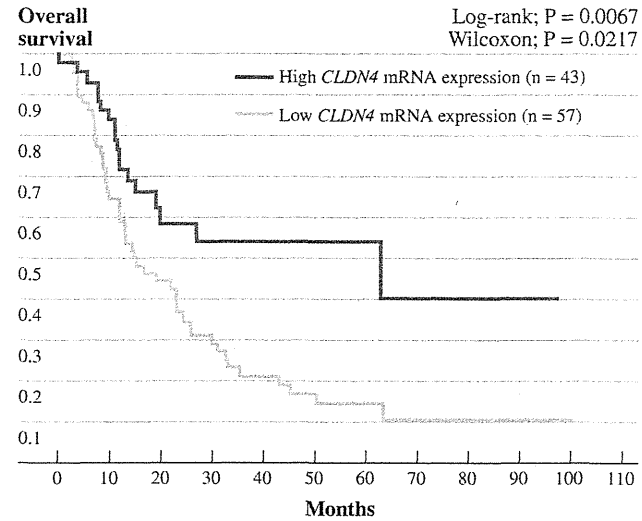

FIG. 3 Overall survival after resection of PDAC with high *CLDN4* mRNA expression versus low *CLDN4* mRNA expression. Results are presented as Kaplan–Meier actuarial survival curves. The patients with PDAC are categorized into 2 groups, a high *CLDN4* mRNA expression group ($n = 43$) and low *CLDN4* mRNA expression group ($n = 57$), using recursive descent partition analysis (cutoff value: 0.034). Low expression of *CLDN4* mRNA is significantly associated with shorter survival than that of high expression of *CLDN4* mRNA (Log-rank test: $P = 0.0067$, Wilcoxon test: $P = 0.0217$)

Correlation Analysis Between CLDN4 mRNA Level and the Protein Expression in PDAC

We performed immunohistochemical analysis in 20 FFPE samples of PDAC to investigate the expression pattern of CLDN4 protein. The immunohistochemical analysis indicated 8 samples with positive expression of CLDN4 and 12 samples with negative expression of CLDN4 (Fig. 4a). When the relationship between protein and mRNA expression levels was investigated in these cases, the positive protein expression group had significantly higher *CLDN4* mRNA levels than those of the negative protein expression group (Fig. 4b, $P = 0.0168$).

DISCUSSION

Molecular biomarkers that could be used as prognostic indicators of outcome would be useful in determining an individualized treatment plan for a patient. In the present study, we have demonstrated, for the first time, that increased *CLDN4* mRNA expression is significantly associated with better survival in patients with PDAC. Therefore, *CLDN4* mRNA expression could be used to select a subset of patient who can benefit from aggressive treatment including surgical resection and adjuvant chemotherapy. Although further studies are warranted to confirm the prognostic relevance of CLDN4 in larger prospective studies, our present results suggest that this

TABLE 4 Multivariate survival analysis (Cox regression model) of conventional prognostic factors and *CLDN4* mRNA expression

	Hazard ratio (HR)	95% confidence interval (95% CI)	*P* value
Low *CLDN4* expression	1.362	1.011–1.873	0.0419
pN category (pN1)	1.136	0.828–1.598	0.4388
UICC stage (III/IV)[a]	–	–	0.6473
Histological grade (G3)	1.526	1.137–2.037	0.0052
Lymphatic invasion (+)	1.036	0.656–1.714	0.8842
Vessel invasion (+)	1.117	0.779–1.659	0.5591
Residual tumor (R1)	1.804	1.356–2.412	<0.0001
Postoperative chemotherapy (−)	1.660	1.226–2.238	0.0012

[a] HR and 95% CI of UICC stage were not shown because of 3 parameters

molecule may serve as a novel prognostic marker in PDAC.

CLDN4 contributes to maintaining epithelial cell polarity and establishing the intercellular barrier as a transmembrane protein involved in tight junction formation and function. Therefore, it has been thought that the reduction of CLDN4 expression in endothelial cells decreases paracellular resistance and facilitates invasion of epithelial cancer cells through endothelial cell layers, similar to the mechanism of E-cadherin.[16,38–41] The functional consequence of CLDN4 expression in PDAC remains poorly understood, but a previous report showed that CLDN4 overexpression decreased invasiveness and metastatic potential of pancreatic cancer cells.[28] This finding is in agreement with our present results showing that increased expression of *CLDN4* mRNA is associated with less invasive pathology and significantly longer survival.

Previous studies have demonstrated prognostic implication of CLDN4 expression in human cancers. In nasopharyngeal cancer, decreased CLDN4 expression independently predicted shorter distant metastasis-free survival by Cox regression analysis.[42] Similarly, a recent immunohistochemical study of a large series of gastric cancer demonstrated that overall survival was significantly shorter in patients with low CLDN4 expression.[43] These findings are consistent with our results in PDAC, further highlighting the prognostic relevance of CLDN4 in a wide range of human cancers.

Frequent overexpression of CLDN4 in cancer has led to an idea that this molecule could be a target for cancer therapy. In fact, *Clostridium perfringens* enterotoxins, for which CLDN4 is the receptor, has been shown to result in an acute dose-dependent cytotoxic effect in pancreatic cancer xenografts.[44] More recently, a monoclonal antibody

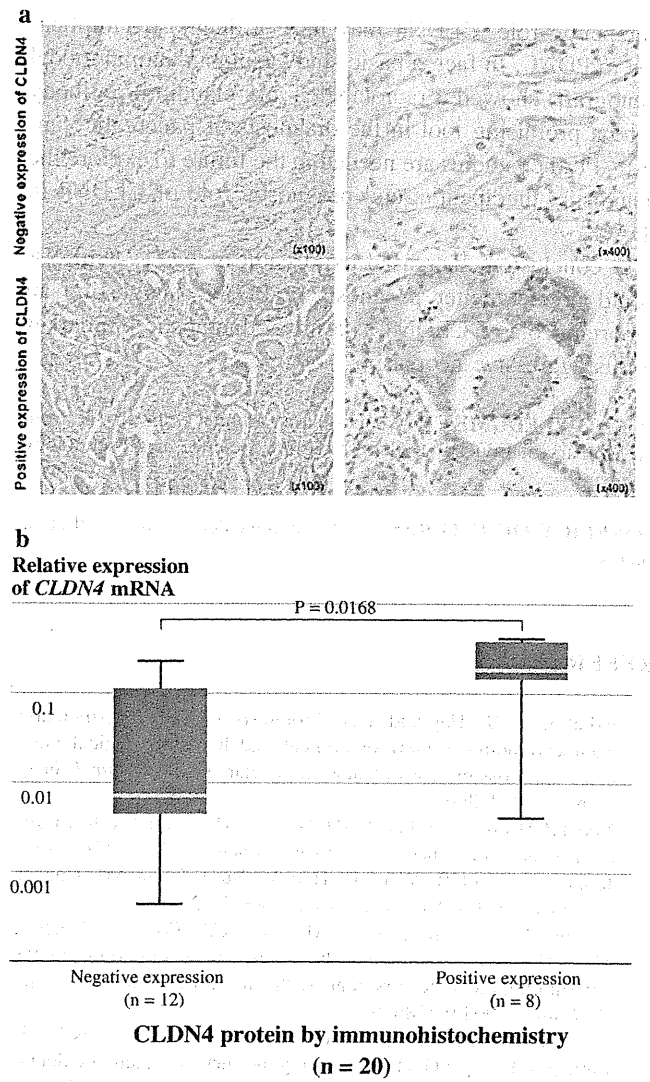

FIG. 4 **a** Representative micrographs of CLDN4 immunostaining in PDAC. The *upper line* shows negative expression of CLDN4. Meanwhile, the *lower line* shows positive expression of CLDN4. The original magnifications are, respectively, shown on the pictures (left side, 100×; right side, 400×). **b** Relationship between the expression of CLDN4 protein and *CLDN4* mRNA level. Results are presented as a *box-and-whisker* plot. Expression of *CLDN4* mRNA is normalized to that of *18S* rRNA. Immunohistochemical study in 20 FFPE samples indicates 12 samples with negative expression of CLDN4 and 8 samples with positive expression of CLDN4. The positive protein expression group has significantly higher *CLDN4* mRNA levels than those of the negative protein expression group ($P = 0.0168$, Wilcoxon test)

against CLDN4 and C-terminal fragment of *Clostridium perfringens* enterotoxin has been demonstrated to have antitumor activity in pancreatic cancer.[45,46] These findings and our present results suggest that CLDN4 represents a promising therapeutic target for PDAC especially in a subset of patients with high CLDN4 expression and a relatively better prognosis. In addition to its prognostic and

therapeutic role, CLDN4 may have a diagnostic implication in PDAC. In fact, a basic study using an animal model (xenograft) showed a radiolabeled anti-claudin 4 antibody to be a promising tool in the diagnosis of pancreatic cancer.[47] Further studies are needed in the future to explore the diagnostic, therapeutic, and prognostic role of CLDN4 in PDAC.

In conclusion, increased expression of *CLDN4* mRNA would predict better prognosis in PDAC, suggesting an important role of CLDN4 as a novel diagnostic biomarker in this aggressive neoplasm.

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CONFLICT OF INTEREST The authors declare no conflict of interest.

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Follow-up study after resection of intraductal papillary mucinous neoplasm of the pancreas; special references to the multifocal lesions and development of ductal carcinoma in the remnant pancreas

Takao Ohtsuka, M.D.[a], Hiroshi Kono, M.D.[a], Reiko Tanabe, M.D.[a], Yosuke Nagayoshi, M.D.[a], Yasuhisa Mori, M.D.[a], Yoshihiko Sadakari, M.D.[a], Shunichi Takahata, M.D.[a], Yasunori Oda, M.D.[b], Shinichi Aishima, M.D.[b], Hisato Igarashi, M.D.[c], Tetsuhide Ito, M.D.[c], Kousei Ishigami, M.D.[d], Masafumi Nakamura, M.D.[a], Kazuhiro Mizumoto, M.D.[a], Masao Tanaka, M.D.[a,*]

[a]Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 8128582, Japan; [b]Department of Anatomic Pathology, [c]Department of Medicine and Bioregulatory Science, and [d]Department of Clinical Radiology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

KEYWORDS:
IPMN;
Multifocal;
Pancreatic ductal adenocarcinoma

Abstract

BACKGROUND: Frequency and characteristics of metachronous occurrence of multifocal intraductal papillary mucinous neoplasms (IPMNs) or distinct pancreatic ductal adenocarcinomas (PDACs) in the remnant pancreas during follow-up evaluation after pancreatectomy for IPMNs have not been well known. The aim of this study was to investigate the outcomes after resection of IPMNs, especially focusing on the metachronous occurrence of multifocal IPMNs and distinct PDACs.

METHODS: Medical records of 172 patients who underwent resection of IPMNs were reviewed retrospectively, and the data regarding the occurrence of metachronous IPMNs or PDACs in the remnant pancreas during a mean postoperative follow-up period of 64 months were collected.

RESULTS: The incidence including synchronous and metachronous multifocal occurrence of IPMNs was 20% (34 of 172), and that of distinct PDACs was 9.9% (17 of 172). Ten metachronous IPMNs developed in the remnant pancreas after a mean time of 23 postoperative months (range, 12–84 mo), and 2 with main duct IPMNs (both were carcinoma in situ) required remnant pancreatectomy. Six distinct PDACs developed in the remnant pancreas after a mean time of 84 postoperative months (range, 12–150 mo). Four of them were found to have a tumor with a size of less than 2 cm, whereas the remaining 2 PDACs were found to be unresectable more than 10 years after resection of IPMNs.

CONCLUSIONS: Intense long-term follow-up evaluation is necessary for the early detection of metachronous occurrence of distinct PDACs as well as malignant IPMNs after resection of IPMNs.

Recent intense investigations regarding intraductal papillary mucinous neoplasms (IPMNs) of the pancreas have provided us with much information about their unique clinical and biological characteristics.[1–16] One of them is that IPMNs have a wide spectrum from adenoma to invasive

* Corresponding author. Tel.: +81-92-642-5441; fax: +81-92-642-5458.
E-mail address: masaotan@med.kyushu-u.ac.jp
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carcinoma, and therefore it is important to determine the malignant potential of IPMNs preoperatively. We recently were able to treat most patients with IPMNs appropriately according to international consensus guidelines,[1] which suggest several predictive factors for malignant IPMNs, and, as a result, outcomes after the treatment of IPMNs seem to be satisfactory.[2–5]

There are 2 types of recurrence after resection of IPMNs: metastatic or local recurrence of invasive carcinoma and metachronous multifocal occurrence of IPMNs in the remnant pancreas. In addition, recent reports have shown that distinct pancreatic ductal adenocarcinomas (PDACs) occur synchronously or metachronously during management of IPMNs, with a frequency of approximately 10%, and IPMNs thus have been recognized recently as one of the predictors for PDAC.[6–11] Several reports have shown the long-term outcome after resection of IPMNs; however, they have not distinguished such various types of recurrent diseases.[3–5] Therefore, the frequency and characteristics of metachronous occurrence of multifocal IPMNs or distinct PDACs in the remnant pancreas after pancreatectomy for IPMNs have been unclear, and we do not know how frequent and how long we should check the patients after resection of IPMNs. The aim of this study was to investigate the outcomes after resection of IPMNs, especially focusing on the metachronous occurrence of multifocal IPMNs and concomitant PDACs.

Patients and Methods

Medical records of 172 patients who underwent pancreatectomy for IPMN at the Department of Surgery and Oncology at Kyushu University Hospital between 1987 and 2010 were reviewed retrospectively. There were 105 men and 67 women, with a mean age of 66 years (range, 33–85 y). Surgical indication was determined based on the suggestions described in the international consensus guidelines[1]; all the cases of main-duct IPMNs, and branch-duct IPMNs with symptoms such as abdominal pain, jaundice, and pancreatitis, the presence of mural nodule, cyst size 30 mm or larger, and dilation of the main pancreatic duct throughout the study period. However, several patients with branch-duct IPMNs without any of the earlier-listed factors underwent pancreatectomy at the initial period of this study. IPMNs were classified into main-duct and branch-duct types based on the preoperative radiologic findings as described in our previous reports.[15,16] The lesions having no predictive factors usually were left in the remnant pancreas in the patient with multifocal IPMNs. Pathologic results were determined by the World Health Organization criteria published in 2010[17]: low-grade, intermediate-grade, and high-grade dysplasia (carcinoma in situ), and invasive carcinoma. The highest degree of pathologic grade was determined in multifocal lesions. Staging of PDAC was defined according to the Japanese General Rules for pancreatic cancer.[18] In all cases, resection margins of the pancreas were checked histologically by frozen sections during surgery as well as permanent sections postoperatively. Pancreatectomy would be completed for negative margin status of main pancreatic duct in frozen section, whereas additional resection would be considered for the presence of tumor cells at the cut margin of the main pancreatic duct.

Follow-up evaluation by physical examination and radiologic studies was performed according to the pathologic results. Routine radiologic examination included computed tomography (CT) and/or magnetic resonance imaging (MRI)/magnetic resonance cholangiopancreatography (MRCP). If there were positive findings in these modalities such as the presence of a new lesion, dilation of the main pancreatic duct, or morphologic changes of residual IPMNs, endoscopic ultrasound sonography (EUS) and endoscopic retrograde pancreatography (ERP) with pancreatic juice cytology were added. EUS-guided fine-needle aspiration has not been performed to date at our institution because of the apprehension of seeding of the tumor cells when malignant. Surveillance was performed every 6 months for noninvasive IPMNs (low- to high-grade dysplasia) using CT and MRI/MRCP during the initial 2 years after surgery, and every 12 months after that. Recently, surveillance for noninvasive IPMNs has been changed to alternating CT and MRI/MRCP every 6 months. For invasive IPMNs and IPMNs with distinct PDACs, surveillance was performed every 3 months using CT during the initial 2 years to focus on the detection of recurrent metastatic/local diseases, and then every 6 months using CT and MRI/MRCP after that for the detection of metachronous IPMNs/PDACs as well as recurrent diseases. Our recent surveillance flow chart is shown in Fig. 1. Recurrent IPMNs were defined as metastatic lesions in the liver, lung, or distant lymph node; dissemination; or local recurrence owing to microscopic residual cancer cells. Metachronous occurrence of IPMNs or PDACs was defined as new occurrence of the tumor in the remnant pancreas after complete resection of the tumor. In such cases, surgical margin of the

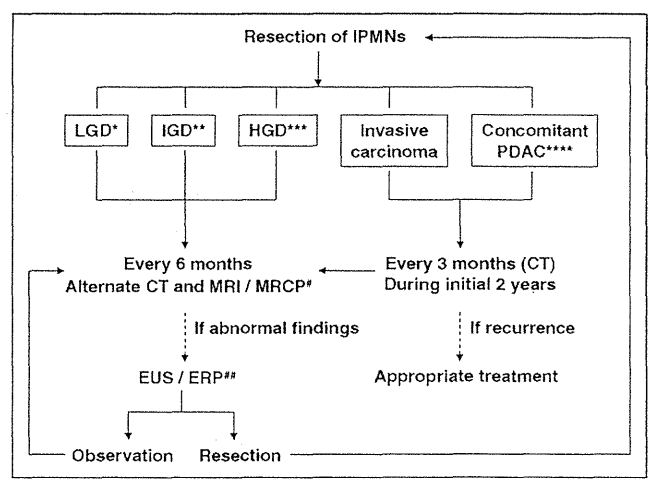

Figure 1 Flow diagram of surveillance after resection of IPMNs of the pancreas according to pathologic grade. HGD, high-grade dysplasia; IGD, intermediate-grade dysplasia; LGD, low-grade dysplasia.

Table 1 Characteristics of 172 patients with intraductal papillary mucinous neoplasms (IPMNs) of the pancreas

	LGD (n = 77)	IMGD (n = 33)	HGD (n = 26)	Invasive carcinoma (n = 36)	Total (n = 172)
At the initial assessment and operation					
Location (pancreas head/pancreas body to tail)	35/42	8/25	16/10	25/11	84/88
Type (Main duct/Branch duct)	8/69	7/26	13/13	16/20	44/128
Synchronous multifocal IPMNs*	13 (10)	4 (0)	2 (1)	5 (5)	24 (16)
Synchronous distinct PDACs	9	2	0	3	14
During follow-up period					
Recurrent IPMNs	0	0	0	16	16
Recurrent PDAC	6	1	0	2	9
Metachronous IPMNs†	1	5 (1)	3 (1)	1	10 (2)
Metachronous distinct PDACs††	4 (3)	1	0	1	6 (3)
Comprehensive assessments					
Multifocal IPMNs	14	9	5	6	34
Distinct PDACs††	13 (3)	3	0	4	17 (3)
Disease-specific survival rate (%) (5-/10-year)	94/91	96/89	100/100	31/24	83/78

LGD = low grade dysplasia; IMGD = intermediate grade dysplasia; HGD = high grade dysplasia; PDAC = pancreatic ductal adenocarcinoma.
*The number in parenthesis indicates the number of patients having residual IPMNs in the remnant pancreas after operation.
†The number in parenthesis indicates the number of patients who underwent remnant total pancreatectomy for metachronous IPMNs.
††The number in parenthesis indicates the number of patients having both synchronous and metachronous PDACs, and thus, a total of 17 patients had distinct PDACs synchronously and/or metachronously.

pancreas at the initial surgery was free from tumor cells, and the new lesion usually developed apart from the surgical margin.

Death related to invasive IPMNs or concomitant PDACs was considered disease specific, and a disease-specific survival rate was calculated using the Kaplan–Meier method.

Results

Table 1 shows the characteristics of the study population. There were 44 main-duct and 128 branch-duct types. Twenty patients (12%) had a history of pancreatitis preoperatively. There were 77 patients with low-grade dysplasia, 33 patients with intermediate-grade dysplasia, 26 patients with high-grade dysplasia, and 36 patients with invasive carcinoma. Eight branch-duct IPMNs did not have any malignant predictors described in consensus guidelines,[1] all of which were low-grade dysplasia, whereas the other 164 had at least one of the predictors. Seventy-seven patients had pathologically mural nodules, and 45 of them were malignant (58%; 20 noninvasive and 25 invasive carcinomas). The mean follow-up period of this study population was 64 months (range, 1–283 mo), and 5-year/10-year disease-specific survival rates of low-grade dysplasia, intermediate-grade dysplasia, high-grade dysplasia, invasive carcinoma, were 94%/91%, 96%/89%, 100%/100%, and 31%/24%, respectively (Table 1).

There were 24 multiple IPMNs at the time of initial diagnosis (Table 1). Complete resection of IPMNs including 5 cases of total pancreatectomy was achieved in 156 patients, whereas IPMNs were left in the remnant pancreas in the remaining 16 patients (Table 1). In the latter group, 14 lesions were branch-duct IPMNs without any predictive factor for malignancy described in the consensus guidelines,[1] and the remaining 2 were low-grade dysplasia left at the cut margin of the main pancreatic duct. IPMNs and synchronous concomitant PDACs were resected in 14 patients. In these 14 patients with PDACs, there was 1 patient in stage 0, 4 patients in stage I, 2 patients in stage II, 5 patients in stage III, and 2 patients in stage IVa.

There were 16 and 9 patients having local or distant metastatic recurrence after resection of invasive IPMNs and distinct PDACs, respectively, and there was no recurrence in low- to high-grade dysplasia of IPMNs (Table 1). Ten metachronous IPMNs occurred in the remnant pancreas after a mean of 23 postoperative months (range, 12–84 mo). They were 6 branch-duct type and 4 main-duct types. In all 10 patients, the pancreatic cut margins were negative for tumor cells at the initial surgery. All 6 branch-duct IPMNs were small size cysts without any sign of mural nodule or clinical symptom, and did not require resection. Two of the 4 main-duct IPMNs were resected by remnant total pancreatectomy, and their pathologic results were both noninvasive carcinomas. The other 2 metachronous main-duct IPMNs were observed without resection because of the absence of informed consent.

Of 16 patients with IPMN left in place in the remnant pancreas, 2 patients had recurrence of invasive IPMNs. The other 14 lesions showed no morphologic change during the mean follow-up period of 47 months (range, 6–156 mo). No patients had a metachronous occurrence of new IPMNs in the remnant pancreas in this group. The prevalence of multifocal IPMNs including both synchronous and metachronous in this study population was 20% (34 of 172) (Table 1).

All 14 IPMNs with concomitant PDACs were branch-duct type. In these patients, 3 metachronous PDACs developed after resection of low-grade dysplasia of IPMNs and concomitant PDACs (Table 1). In addition, 3 PDACs metachronously occurred after resection of IPMNs. Therefore, occurrence of PDACs after resection of IPMNs was observed in 6 patients after a mean time of 84 postoperative months (range, 12–150 mo), and thus the prevalence of distinct PDACs in the resected IPMNs was 9.9% (17 of 172) (Table 1). Three patients with metachronous PDACs had branch-duct IPMNs as well, and thus all 17 patients who had distinct PDACs had branch-duct IPMNs. By contrast, metachronous PDACs developed irrespective of the pathologic grade and tumor location (head or body to tail) of IPMNs and there seemed to be no predisposing time point after the initial surgery. Three of 6 metachronous PDACs were found by CT with a tumor size of less than 2 cm, whereas the remaining 2 PDACs were found as unresectable cancers with liver metastases after more than 10 years after resection of IPMNs. The intervals between a prior normal imaging study and detection of PDACs in 2 patients with unresectable PDACs were 8 and 13 months. The remaining 1 PDAC (noninvasive) was diagnosed by pancreatic juice cytology obtained by ERP, which was detected by neither CT nor MRI/MRCP.

Fifty-three extrapancreatic malignancies were found in 47 patients (27%) before (n = 28), at the time of (n = 18), and after initial surgery (n = 7). There were 16 colorectal, 11 gastric, 6 prostate, 4 gynecologic, 3 lung, 3 bladder, 2 breast, 2 renal, and 6 other malignancies.

Comments

The present study focusing on the metachronous occurrence of IPMNs or distinct PDACs in the remnant pancreas after resection of IPMNs has shown that strict surveillance would provide a chance to detect noninvasive IPMNs and early stage PDACs. Those metachronous lesions developed even long term after initial surgery irrespective of pathologic grade, and thus all the patients should be strictly followed up for a long time after resection of IPMNs. By contrast, there still have been unresolved issues regarding diagnostic modalities and intervals of surveillance.

The incidence of multifocal occurrence of IPMNs in this study is consistent with that in previous reports, ranging from 14.6% to 36%.[3–5] In patients with synchronous multifocal IPMNs, the international consensus guidelines[1] recommend that only those having predictive factors for malignancy should be resected, and those without predictive factors might be left in the remnant pancreas. We basically have followed the recommendation of the guidelines,[1] and we have not experienced any cases showing morphologic changes in IPMNs left in the remnant pancreas during the follow-up period. We also have experienced metachronous occurrence of IPMNs after compete resection of initial IPMNs, and all of them could be managed surgically or nonsurgically. Surgical management for multifocal IPMNs described in the guidelines[1] seem to be appropriate; however, long-term strict follow-up evaluation is necessary to detect metachronous occurrence of IPMNs.

Several recent reports[9–11] have investigated the development of distinct PDACs during follow-up evaluation of branch-duct IPMNs, which are not an indication for surgery, and reported an incidence ranging from 4.5% to 8%. This report focuses on the metachronous occurrence of PDACs in the remnant pancreas after resection of IPMNs. Uehara et al[7] found 5 PDACs during follow-up evaluation of 60 cases of branch-duct IPMNs, and 4 of them were resectable. Thus, branch-duct IPMNs would be a good predictor for early detection of PDACs during observations of small branch-duct IPMNs or after resection of branch-duct IPMNs with malignant predictors.

By contrast, some metachronous PDACs were found as unresectable cancers more than 10 years after the initial surgery for IPMNs. A noteworthy fact is that annual examination was not sufficient to detect all the cases of resectable PDACs. This finding suggests the possibility of the rapid growth of distinct PDACs. Therefore, we are now checking all the patients on follow-up evaluation at least every 6 months by imaging even long term after resection of IPMNs, as shown in Fig. 1. However, prospective surveillance is necessary to confirm whether this algorism would be adequate or not.

One of the metachronous PDACs was found as carcinoma in situ by pancreatic juice cytology obtained during ERP, although CT and MRI showed no morphologic abnormality. ERP is not usually included in the routine follow-up modalities in our department; however, the present case shows the use of pancreatic juice cytology for the detection of early pancreatic cancer during management of IPMNs. EUS fine-needle aspiration cytology recently has taken the place of pancreatic juice cytology by ERP in terms of definitive pathologic diagnosis of pancreatic cancer.[19] By contrast, we recently reported a patient with multiple IPMNs with concomitant noninvasive and invasive PDACs, which were detected by pancreatic juice cytology during preoperative ERP.[20] In this patient, multiple PDACs could not be detected by either CT/MRI or EUS, and we consider that pancreatic juice cytology during ERP has an important role to detect early pancreatic cancers, which cannot be detected by regular imaging modalities. Further investigation is necessary to determine whether ERP should be included in routine follow-up diagnostic modalities, and how frequently ERP should be performed during follow-up evaluation of the remnant pancreas after resection of IPMNs.

Another important issue during management of IPMNs is the occurrence of extrapancreatic malignancies. The prevalence of extrapancreatic malignancies in this study is consistent with those in previous reports, ranging from 24% to 32%,[1,21] and various organs seem to be involved. Therefore, careful attention should be paid to extrapancreatic diseases

as well as metachronous diseases in the remnant pancreas during surveillance after resection of IPMNs.

Recent advances in molecular biology have provided many insights into biological behaviors of IPMNs as well as PDACs,[22–26] however, molecular analyses regarding genetic changes in multiple occurrences of IPMNs and PDACs in the same pancreas have not been used to date. Others recently have shown that patients with IPMNs often have extrapancreatic tumors[1,21] and therefore patients with IPMNs might have some systemic genetic abnormalities causing carcinogenesis. Those types of investigations would provide some hints for the early detection of PDACs during management of IPMNs.

In conclusion, intense long-term, follow-up evaluation is necessary for the early detection of metachronous occurrence of IPMNs as well as distinct PDACs after resection of IPMNs.

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Prognostic Implications of Lymph Node Metastases in Carcinoma of the Body and Tail of the Pancreas

Tevfik T. Sahin, MD, Tsutomu Fujii, MD, PhD, FACS,* Mitsuro Kanda, MD, PhD,* Shunji Nagai, MD, PhD,* Yasuhiro Kodera, MD, PhD, FACS,* Akiyuki Kanzaki, MD,* Kazuo Yamamura, MD,* Hiroyuki Sugimoto, MD, PhD,* Hideki Kasuya, MD, PhD, FACS,* Shuji Nomoto, MD, PhD,* Shin Takeda, MD, PhD,* Satoshi Morita, PhD,† and Akimasa Nakao, MD, PhD, FACS**

Objective: The current classification of pancreatic cancer is based only on anatomic location of metastatic lymph nodes (LNs). On the other hand, the number of metastatic LNs has been used in staging of colorectal, esophageal, and gastric cancers. The aim of this study was to assess the prognostic impact of the number or ratio of the metastatic LNs in pancreatic body and tail carcinoma.

Methods: Eighty-five patients with pancreatic body and tail adenocarcinoma who underwent pancreatectomy were included. Location, number, ratio of metastatic LNs, and the survival of patients were analyzed.

Results: Forty patients with LN metastasis had poor prognosis ($P = 0.007$). The prognoses of patients with 5 or more metastatic LNs were poorer than those with less than 5 metastatic LNs ($P = 0.046$), and patients with a metastatic LN ratio of 0.2 or more had the worst prognosis. Multivariate analysis revealed that 5 or more metastatic LNs and metastatic LN ratio of 0.2 or more were independent prognostic factors for survival ($P = 0.0015$ and $P = 0.014$, respectively).

Conclusion: These results indicate that the number and the ratio of metastatic LNs can be used to predict poor patient survival and as a staging strategy.

Key Words: lymph node metastasis, metastatic lymph node ratio, left-sided pancreatic cancer, distal pancreatectomy

(*Pancreas* 2011;40: 1029–1033)

Pancreatic cancer continues to be the gastrointestinal malignancy with the worst prognosis, with only 3% of the patients surviving 5 years after diagnosis.[1] In particular, carcinoma of the body and tail of the pancreas has dismal prognosis because it often develops without symptoms leading to inability to resect the carcinoma at the time of diagnosis.[2–4] Nevertheless, extended pancreatectomy with lymph node dissection remains the only chance for cure of the disease.

Many prognostic markers such as lymph node metastasis, resection margin, and residual tumor have been identified.[5] Pancreatic cancer frequently spreads to lymph nodes and surrounding tissues such as nerve plexi and vessels.[6] Several factors have been reported to be relevant for survival of patients with pancreatic cancer,[7–9] and many studies identified that lymph node metastasis results in a dismal prognosis in pancreatic cancer.[10–14] In this study, the prognostic influence of lymph node metastasis focusing on left-sided pancreatic cancer is anticipated owing to the fact that previous reports of lymph node metastasis focused primarily on pancreatic head cancer. It is believed that there is a difference in clinical characteristics between pancreatic head cancer and left-sided cancer owing to the anatomic location and lymph stream. Therefore, they must be considered separately.

In staging of pancreatic cancer, the classifications of the Union Internationale Contra le Cancer (UICC) and the Japan Pancreas Society consider only anatomic location of metastatic lymph nodes.[15,16] On the other hand, the number of metastatic lymph nodes has been used in staging of colorectal, esophageal, and gastric cancers.[16–20] Furthermore, current data in various gastrointestinal system cancers have emphasized the importance of the ratio of metastatic to examined lymph nodes on patients' prognosis.[21–23] There are similar studies of pancreatic head cancer that also concluded that metastatic lymph node ratio is an independent risk factor for poor survival[24]; however, there is no study evaluating the detailed influence of lymph node metastasis focused on carcinoma of the body and tail of the pancreas. Therefore, the aim of this study was to assess the impact of lymph node metastasis on patients' survival in left-sided pancreatic cancer.

MATERIALS AND METHODS

Patients and Operative Procedure

A total of 553 patients with pancreatic cancer who underwent surgery between October 1991 and November 2010 were retrieved from the prospective database of the Department of Surgery II, Nagoya University. Among these patients, 132 had carcinoma mainly at the pancreatic body and tail. Forty-seven of 132 cases were excluded because they were not resectable owing to distant metastasis or locally advanced disease, and 85 patients who underwent radical pancreatectomy with lymph node dissection were selected for analyses. Lymph node dissection including lymph nodes along the common hepatic artery (lymph node No. 8), the splenic artery (lymph node No. 11), inferior edge of the pancreas (lymph node No. 18), and lymph nodes at the splenic hilum (lymph node No. 10) during distal pancreatectomy was routinely performed (Fig. 1).[25] Para-aortic lymph node dissection was not routinely performed unless it was needed to obtain a negative retroperitoneal margin. During surgery, resection margins were carefully evaluated pathologically including the remnant pancreas and retroperitoneal region. To obtain a clear margin, resection of the portal vein or the superior mesenteric vein was aggressively performed when invasion was determined.[26] Spleen-preserving surgery, laparoscopic surgery, and neoadjuvant radiotherapy or chemotherapy were not performed in our institution. This study covered almost

From the *Department of Surgery II, Nagoya University Graduate School of Medicine, Nagoya, Japan; and †Department of Biostatistics and Epidemiology, Yokohama City University Graduate School of Medicine and Medical Center, Yokohama, Japan.

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Reprints: Tsutomu Fujii, MD, PhD, FACS, Department of Surgery II, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan (e-mail: fjt@med.nagoya-u.ac.jp).

Drs Tsutomu Fujii, Mitsuro Kanda, and Shunji Nagai contributed equally to this research.

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