TABLE 5. *Possible clinical and pathological risk factors for survival in CCC (univariate analysis)*

Factors	No. of patients	Survival rate (%) 3-year	5-year	Median survival (months)	*P* value
Overall	59	35.8	24.5	22.7	
Age (median, 66 years)					
≤66	33	29.2	25.1	22.1	
>66	26	43.8	24.7	28.4	
Gender					
Male	37	37.6	28.2	21.5	
Female	22	34.1	21.9	24.7	
Biliary drainage					
Not performed	43	36.6	30.5	25.9	
Performed	16	33.3	11.1	17.5	
ICG R15 (normal range, ≤10%)					
≤10	43	36.7	30.1	32.1	
>10	16	34.7	13	25.9	
CA19-9 (median, 306 IU/mL)					.006
≤306	30	50.4	35	37	
>306	29	21.1	14.1	14.8	
CEA (median, 2.9 mg/dL)					.002
≤2.9	30	50.4	38.1	36.7	
>2.9	29	20.6	10.3	14.8	
With VR					
Yes	21	37.1	14.9	23.1	
No	38	34.9	27.9	22.1	
Red blood cell transfusion					<.001
Performed	26	11.5	3.9	17.5	
Not performed	33	58.4	45.9	54.9	
Macroscopic type of the tumor					.004
Mass-forming	44	25.8	12.3	17.5	
Periductal or intraductal	15	6.6	56.5	N.A.	
Serosal invasion					
Positive	7	21.4	0	7	
Negative	52	37.6	27.6	23.1	
Histologic differentiation					
Well or papillary	12	41.7	31.3	25.9	
Others	47	34.4	24.1	22.1	
Invasion of resected major portal vein					.011
Absent	29	49	38.5	31.7	
Present	30	22.7	N.A.	17.5	
T category					.001
1, 2	26	55.3	47.4	54.9	
3, 4	33	20.2	N.A.	17.5	
Lymph node involvement					.016
Negative	23	50.6	37.5	37.4	
Positive	36	26.8	17.9	17.5	
Invasion of the lymphatic system					.014
Absent or slight	29	52.6	33.8	36.7	
Moderate to marked	30	19	19	16.6	
Invasion of the venous system					.017
Absent or slight	25	45.4	40.3	31.1	
Moderate to marked	34	28.9	0	16.6	
Invasion of the nervous system					.036
Absent or slight	18	60.2	40.1	54.9	
Moderate to marked	41	25	16.7	21.5	
Intrahepatic daughter nodule					.003
Absent	42	44.4	31.3	31.1	
Present	17	14.1	7.1	11.1	
Proximal bile ductal margin					.003
Negative	50	40.5	29.8	27.2	
Positive	9	11.1	0	9.3	
R0 resection					
Achieved	42	40.6	29.5	27.2	
Not achieved	17	23.2	N.A.	21.5	

ICGR15 indicates indocyanine green retention value at 15 minutes; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembrionic antigen; VR, vascular (hepatic artery, portal vein or inferior vena cava) resection and reconstruction; N.A., not available.

TABLE 6. *Risk factors associated with postoperative survival in patients with perihilar cholangiocarcinoma (Cox Multivariate Regression Analysis)*

Variable	β	SE	*P* value	Odds ratio	95% CI
BDC patients					
R0 resection	0.622	0.275	.024	1.862	1.085–3.194
Histologic differentiation (well, papillary versus others)	0.759	0.300	.011	2.135	1.186–3.844
Lymph node involvement	1.198	0.282	<.001	3.314	1.906–5.763
CCC patients					
Intrahepatic daughter nodule	0.841	0.239	<.001	2.318	1.450–3.705
Preoperative serum CEA level over 2.9 ng/mL	0.958	0.341	.005	2.606	1.337–5.080
Red blood cell transfusion requirement	0.961	0.399	.016	2.614	1.195–5.719
Invasion of the lymphatic system	1.521	0.423	<.001	4.577	1.997–10.494
Proximal bile duct margin	2.001	0.673	.003	7.398	1.976–27.688

$P < 0.1$ was set as the cut-off for variable elimination.

sometimes difficult. Actually, in 12 of 59 cases (20%) finally diagnosed as CCC, 17 of 99 cases (17%) finally diagnosed as BDC, it was not easy to discriminate between CCC and BDC by the review of pathologist. In our series, in approximately 20% of the cases it was not easy to discriminate between BDC and CCC. Hepatobiliary surgeons and pathologists should be aware of the differences between BDC and CCC. In addition, the examination of a greater number of cases and the use of immunohistological or genetic techniques may provide a better understanding of these two conditions.[22]

With respect to the surgical procedures, HPD[20] to secure the distal bile duct margin was done significantly more frequently in BDC patients. This difference implies that, among the BDC cases, there was extensive longitudinal extension along the extrahepatic bile duct, which may account for the significantly higher cancer-positive rates of the proximal and distal bile duct margins among the BDC patients. The rate of portal vein resection and reconstruction was comparable in the BDC and CCC patients. In our protocol, the decision to perform a right- or left-sided hepatectomy is made based on the predominant location of the tumor. When the tumor involved the right and left or proximal bile duct equally, right-sided hepatectomy was the first choice; this situation commonly occurs in BDC patients. Thus, a right-sided hepatectomy and prior PVE were performed significantly more frequently in BDC patients.

The bile duct margins must be clear of cancer to achieve cure; many previous reports have suggested that the presence of clear margins is an independent prognostic factor,[3–6,10–13,21] which is consistent with the results seen in our CCC patients. In BDC patients, the proportion of positive proximal bile duct margin was 29%, and R0 resection was achieved in 59%. This figure is lower than the 70% or greater cancer-negative surgical margins previously reported in large series.[3,5,7,9,12,13,23] The relatively high rate of cancer-positive surgical margins in the present study may be attributed to the strict diagnostic criteria used for bile duct margins or due to institutional differences in the diagnostic criteria. Nevertheless, the overall 5-year survival rate of 38.4% for BDC patients in our series is at least comparable to previous reports.[3,5,7,9,12,13,23] Further research is needed to clarify the diagnostic accuracy of bile duct margins[24,25] and the impact of positive bile duct margins on survival.[26,27]

Although the rates of red blood cell (RBC) transfusion requirement in both CCC and BDC group were comparable, CCC patients who underwent RBC transfusion showed a significantly shorter survival than those who did not undergo transfusion. On the other hand, there was no significant survival difference in BDC patients in terms of RBC transfusion. In patients with hepatocellular carcinoma (HCC), Yamamoto et al.[28] pointed out that perioperative RBC transfusion promotes tumor recurrence after hepatectomy. This may support that CCC, classified into primary liver tumor as same as HCC, potentially has a different character from BDC classified into bile duct tumor.

In summary, the overall survival of BDC patients was significantly better than that of CCC patients. On univariate analysis, only a few of the statistically significant clinicopathological factors were the same in the two groups; on multivariate analysis, there were no common significant predictive factors. Thus, BDC and CCC appear to show different biological behaviors. Differentiating between these two conditions would have an impact on the ability to predict postoperative survival.

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Minimally Invasive Intraductal Papillary-mucinous Carcinoma of the Pancreas: Clinicopathologic Study of 104 Intraductal Papillary-mucinous Neoplasms

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Abstract: Invasive intraductal papillary-mucinous carcinoma (I-IPMC) is a heterogeneous entity with various postoperative outcomes. The aim of this study is to characterize early-stage I-IPMC with nonaggressive characteristics. One hundred and four patients with intraductal papillary-mucinous neoplasm (IPMN) were clinicopathologically investigated. The lesions were classified into 53 noninvasive IPMNs (adenoma, borderline, and noninvasive IPMC) and 51 I-IPMCs on the basis of the WHO classification. I-IPMCs were divided further into 26 minimally invasive IPMCs (MI-IPMCs) and 25 invasive carcinomas originating in IPMC (IC-IPMCs) by new diagnostic criteria proposed in this study. We examined invasiveness of I-IPMC on 4 patterns, and defined simple and practical diagnostic criteria of minimal invasion for each invasive pattern. The disease-specific survival rates after 3, 5, and 10 years were 100%, 100%, and 100% for both noninvasive IPMN and MI-IPMC, and 51%, 38%, and 0% for IC-IPMC. The overall and disease-specific survival rates for MI-IPMC were both significantly better than those for IC-IPMC ($P < 0.001$), but there was no significant difference between noninvasive IPMN and MI-IPMC. Multivariate analysis showed that the factors indicative of poor prognosis were a diagnosis of I-IPMC classified as IC-IPMC and a high level of serum carbohydrate antigen 19-9. The prognosis of IC-IPMC was not significantly different from that of pancreatic ductal carcinoma in each of the corresponding tumor-node-metastasis stages. These findings suggest that a category of MI-IPMC provides more accurate and useful information of the stage and the aggressiveness of I-IPMC.

Key Words: intraductal papillary-mucinous neoplasms of the pancreas, minimal invasion, prognosis, clinicopathologic analysis, invasive pattern

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Intraductal papillary-mucinous neoplasm (IPMN) of the pancreas is a well-characterized clinical and pathologic entity. IPMNs are characterized by intraductal proliferation of neoplastic mucinous cells, which usually form papillae and lead to cystic dilation of the pancreatic ducts, forming clinically and macroscopically detectable masses.[15] Similarly to the well-defined adenoma-carcinoma sequence in colorectal cancer,[5] IPMNs progress from intraductal papillary-mucinous adenoma (IPMA) to borderline IPMN, then to intraductal papillary-mucinous carcinoma (IPMC), and eventually to invasive adenocarcinoma.[2,9,10] According to the WHO classification,[13,15] IPMC is classified as either "noninvasive" or "invasive." It is reported that noninvasive IPMN shows a favorable postoperative outcome in comparison with invasive IPMC (I-IPMC), with 5-year survival rates ranging from 77% to 100%.[4,6,16,20,22,24] With regard to the prognosis of I-IPMC, there is a substantial variation in the 5-year survival rates from 24% to 60% in previous reports.[4,6,16,20,22–24] This may be due to heterogeneity of I-IPMCs, including an invasive component of various sizes and biologic behavior. Our hypothesis is that the prognosis of I-IPMC can be substantially determined by the degree and type of invasion, and thus I-IPMC can be classified as either aggressive or nonaggressive by categorization according to the extent and pattern of invasion. Such a classification would be clinically relevant for deciding whether surgery is indicated, for selecting the most appropriate surgical procedure, and for prediction of postoperative outcome.

The concept of minimally invasive cancer was originally introduced for uterine cervical cancer showing very early invasion and a favorable prognosis.[26] Minimally invasive IPMC (MI-IPMC) has been categorized within the classification of pancreatic carcinoma used by the Japan Pancreatic Society (JPS) since 1993.[12] In the JPS classification, I-IPMC is classified into 2 categories: MI-IPMC and invasive carcinoma originating in IPMC (IC-IPMC), the latter being more advanced. A few reports have indicated that MI-IPMC has a better surgical outcome than IC-IPMC.[19,25,27] However, the definition of "minimal invasion" has not been clear. In the original JPS text, it is described only as "slight invasion beyond the pancreatic duct wall."[12]

In this retrospective study, we evaluated the invasiveness of I-IPMC by the examination of 4 invasive

patterns, and tried to define simple and practical diagnostic criteria of minimal invasion for each invasive pattern. The clinical relevance of this subdivision was then evaluated in terms of postoperative survival.

MATERIALS AND METHODS

Study Population

This study was approved by the Ethics Committee of the National Cancer Center, Japan. Between January 1984 and December 2005, 111 patients underwent pancreatic resection for IPMNs at the National Cancer Center Hospital, Japan. There were no operation-related deaths, and all patients underwent macroscopically curative resection without any residual tumor. Seven cases also had ductal carcinoma of the pancreas, which was not directly associated with IPMNs. Excluding these patients, 104 cases of IPMN were included in this study. The patients comprised of 56 males and 48 females with a median age of 66 (41 to 84) years. The operative procedures included 12 pancreatoduodenectomies (PDs), 59 pylorus-preserving PDs (PPPDs), 24 distal pancreatectomies, 3 total pancreatectomies, 5 partial pancreatectomies, and 1 PPPD with distal pancreatectomy. These procedures accounted for 18.9% of all pancreatectomies (n = 551) performed at our institution for pancreatic tumors during the same period.

Every patient was followed up in the outpatient clinic every 1 to 3 months during the first postoperative year, and every 6 to 12 months thereafter. No patient dropped out during follow-up. Clinical or radiologic data and follow-up information for every patient were obtained from the medical records. The median follow-up period after surgery was 37.2 (4.2 to 210) months for all patients, 52.9 (4.2 to 171) months for noninvasive IPMN, 43.4 (13.2 to 210) months for MI-IPMC, and 20.4 (7.1 to 87.7) months for IC-IPMC.

Pathologic Examination

All of the IPMNs were pathologically reexamined and the diagnosis of IPMN was confirmed. Surgically resected specimens were fixed in 10% formalin and cut into serial 5-mm-thick slices, horizontally in the pancreas head, and sagittally in the pancreas body and tail. All the sections were stained with hematoxylin and eosin for pathologic examination. If necessary, additional staining for elastic fibers (elastica stain) was performed. After histopathologic examination of all the sections, the lesion was classified as IPMA, borderline IPMN, noninvasive IPMC, or I-IPMC according to the WHO classification.[13,15] The lesion was graded by the highest degree of atypia. I-IPMCs were divided further into MI-IPMC or IC-IPMC according to our proposed criteria (Table 1) described later. We evaluated the invasiveness of I-IPMC, and the 4 invasive patterns were examined: "infiltrative growth," "mucous rupture," "expansive growth," and "intra-abdominal rupture." The criterion of minimal invasion was proposed for each invasive pattern. I-IPMC showing some features of minimal invasion without any features categorized in IC-IPMC was classified as MI-IPMC. I-IPMC showing at least one invasive feature beyond minimal invasion is classified as IC-IPMC. For example, if an I-IPMC shows mucous rupture and infiltrative growth of tubular adenocarcinoma with 6-mm length of invasion, this tumor is diagnosed as IC-IPMC.

An infiltrative growth pattern, which is commonly found in conventional invasive ductal carcinoma of the pancreas, is considerably aggressive (Figs. 1A–D). Among the 6 patients with IC-IPMC, in whom the depth of infiltration of carcinoma cells ranged from 6 to 20 mm, 3 patients (including a patient with 6-mm-length infiltration of carcinoma cells) had recurrence in the liver or peritoneal cavity, and died of the disease. This suggests that infiltrative growth is strongly associated with a high rate of recurrence and mortality, even if the size of invasion is limited. On the other hand, none of the 17 patients with a maximum infiltration of 5 mm or less had recurrence except 2 patients, 1 of them had 2-mm-length infiltration of tubular adenocarcinoma and the other had 2-mm-length infiltration of pure mucinous carcinoma. Therefore, we adopted a threshold of 5 mm as a diagnostic criterion for minimal invasion in infiltrative growth (Table 1). Lymphatic, venous, and neural invasion are treated as a part of infiltration of cancer cells. Invasion of 5 mm or less is sometimes difficult to detect. In such cases, elastica staining was helpful for differentiating infiltrating carcinoma from intraductal spreading of carcinoma (Figs. 1C, D).

IPMN is characterized by its prominent mucus secretion into the lumen, in some cases, into the space between epithelial cells and basement membrane due to inverted cellular polarity, which subsequently causes disruption of the pancreatic duct wall and spilling of mucus into the interstitial space.[1,7] This is referred to mucous rupture (Fig. 2) and is diagnosed as minimal invasion if mucous lakes are not associated with mucinous carcinoma showing infiltrative growth (Table 1). Mucous rupture was observed only in the vicinity of the pancreatic ductal system, although the location was not confined to the pancreas. We considered mucus lakes near noninvasive IPMC as mucous rupture regardless of the presence of viable cancer cells within it, because viable cancer cells may be present floating in the mucus lake. When viable cancer cells floating in mucus lake are apparently present and are scant (there is very small number of cancer cells or their clusters floating in only the limited mucus lakes. The representative feature was shown in Fig. 2D), this situation is called as "mucous rupture with cellular component." This subcategory includes a kind of pure mucinous carcinoma (alternatively colloid carcinoma)[1] showing a very low cellularity, and nonmucinous cancer cells which are simply detached from the duct wall and are floating in mucus lake. Mucous rupture without floating cancer cells represented the suspected lesion of mucous rupture with cellular component. When there are many cancer cells (more than "scant" level) floating in mucus lake, it is judged as infiltrative growth of mucinous carcinoma (Figs. 2E, F).

TABLE 1. Growth Patterns of I-IPMCs and Diagnostic Criteria for Minimal Invasion

Growth Pattern	Pathologic Features	Minimal Invasion	Beyond Minimal Invasion (IC-IPMC)
Infiltrative growth	Carcinoma cells from the pancreatic duct occupied by IPMC invade the surrounding stroma. Disappearance of the basement membrane or desmoplastic change around the ducts implies interstitial invasion. Infiltrative distance is defined as the length from the deepest point of invasion to the stroma surface of the nearest non-invasive IPMC	1. An infiltrative distance of 5 mm or less is regarded as minimal invasion 2. Venous, lymphatic, or neural invasion within the area (≤5 mm of the infiltrative distance) is also counted in this category 3. This category includes the invasion of various histologic types of cancer, such as tubular adenocarcinoma, mucinous carcinoma, etc	1. An infiltrative distance more than 5 mm is regarded as IC-IPMC 2. Venous, lymphatic, or neural invasion within the area (> 5 mm of the infiltrative distance) is also counted in this category 3. This category includes the invasion of various histologic types of cancer, such as tubular adenocarcinoma, mucinous carcinoma, etc
Mucous rupture	"Mucous rupture" and "expansive growth" are unique features of IPMC that grows intraductally and secretes large amounts of mucus. The mucus spills out, forming a mucus lake around the pancreatic duct, due to rupture of the dilatated pancreatic duct occupied by IPMN through high pressure caused by the hypersecreted mucin. This is referred to mucous rupture. Mucous lakes of various sizes are seen, sometimes containing scanty floating cancer cells	1. If mucous lakes are not associated with mucinous carcinoma showing infiltrative growth, this feature is diagnosed as minimal invasion, regardless of the size and location of the mucus lakes 2. If a mucus lake contains scanty floating cancer cells (there is very small number of cancer cells or their clusters floating in only the limited mucus lakes. The representative feature was shown in Fig. 2D), it is additionally described as "mucous rupture with cellular component." This subcategory includes a kind of pure mucinous carcinoma associated with IPMC 3. If many cancer cells (more than "scant" level) are floating in mucus lakes (Fig. 2E), it is treated as "infiltrative growth" of mucinous carcinoma and the infiltrative distance of 5 mm or less is regarded as minimal invasion	1. If many cancer cells are floating in mucus lakes, it is treated as "infiltrative growth" of mucinous carcinoma and the infiltrative distance of more than 5 mm is classified as IC-IPMC
Expansive growth	A pancreatic duct is markedly dilated, becoming ductectatic or cystic in shape. The basement membrane and subepithelial elastic fibers may be elongated and disrupted. Cystic IPMC may grow expansively into peripancreatic connective tissues, and eventually involves the bowel or major vessels [portal vein (PV), SPV, SMV, or splenic artery (SPA)]	1. Loss of the basement membrane of the pancreatic duct with IPMC is regarded as minimal invasion 2. If I-IPMC grows expansively, even if it ruptures into the bowel, or even if it erodes a major vessel wall unless cancer cells enter the lumen of the major vessel, it is still regarded as minimal invasion 3. If I-IPMC has this type of growth as predominance, it is corresponded to a kind of pure mucinous carcinoma associated with IPMC	1. If I-IPMC forms a fistula with a major vessel, it is regarded as IC-IPMC
Intra-abdominal rupture	IPMC ruptures into the abdominal cavity, and mucus-secreting cancer cells are scattered in it	Peritoneal dissemination may occur. Therefore, this finding should be distinguished from ordinary IPMN and classified separately as ruptured IPMN. MI- or IC-IPMC should be noted additionally	
Judgement		An I-IPMC showing some features of minimal invasion without any features categorized in IC-IPMC is classified as MI-IPMC	An I-IPMC showing at least one invasive feature beyond minimal invasion is classified as IC-IPMC

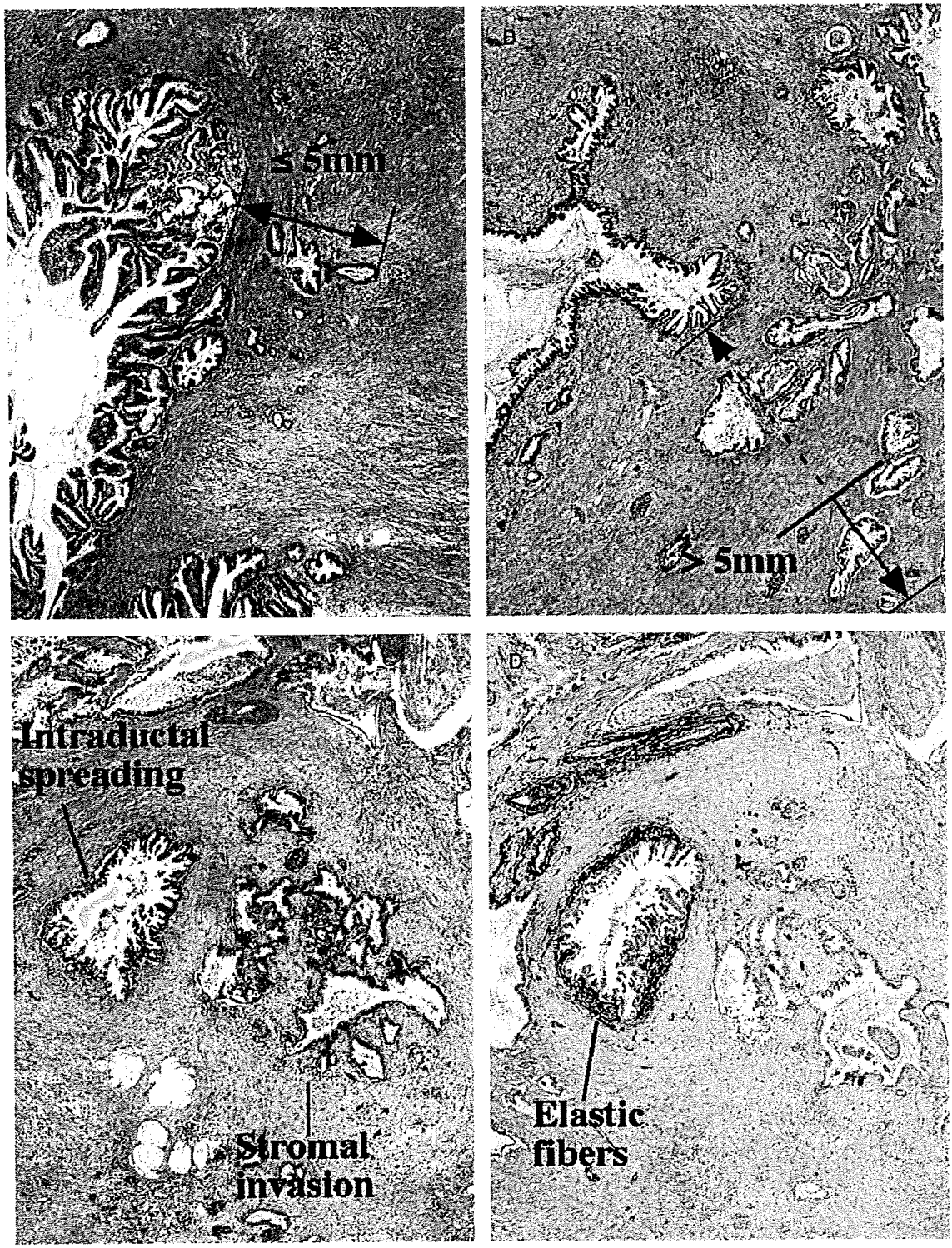

FIGURE 1. Histopathologic features of "infiltrative growth" in I-IPMC. A and B, Histologic features resemble those of conventional invasive ductal carcinoma of the pancreas. The arrows indicate the depth of infiltration of invasive carcinoma. If the depth is less than 5 mm, it is regarded as minimal invasion (A), and if the depth is more than 5 mm it is regarded as IC-IPMC (B). C and D, Elastica stain (D) helps to discriminate infiltrative growth from intraductal spread of carcinoma. The former lacks a positively stained sheath of elastic fibers (black) around the pancreatic duct. Hematoxylin and eosin stain (C).

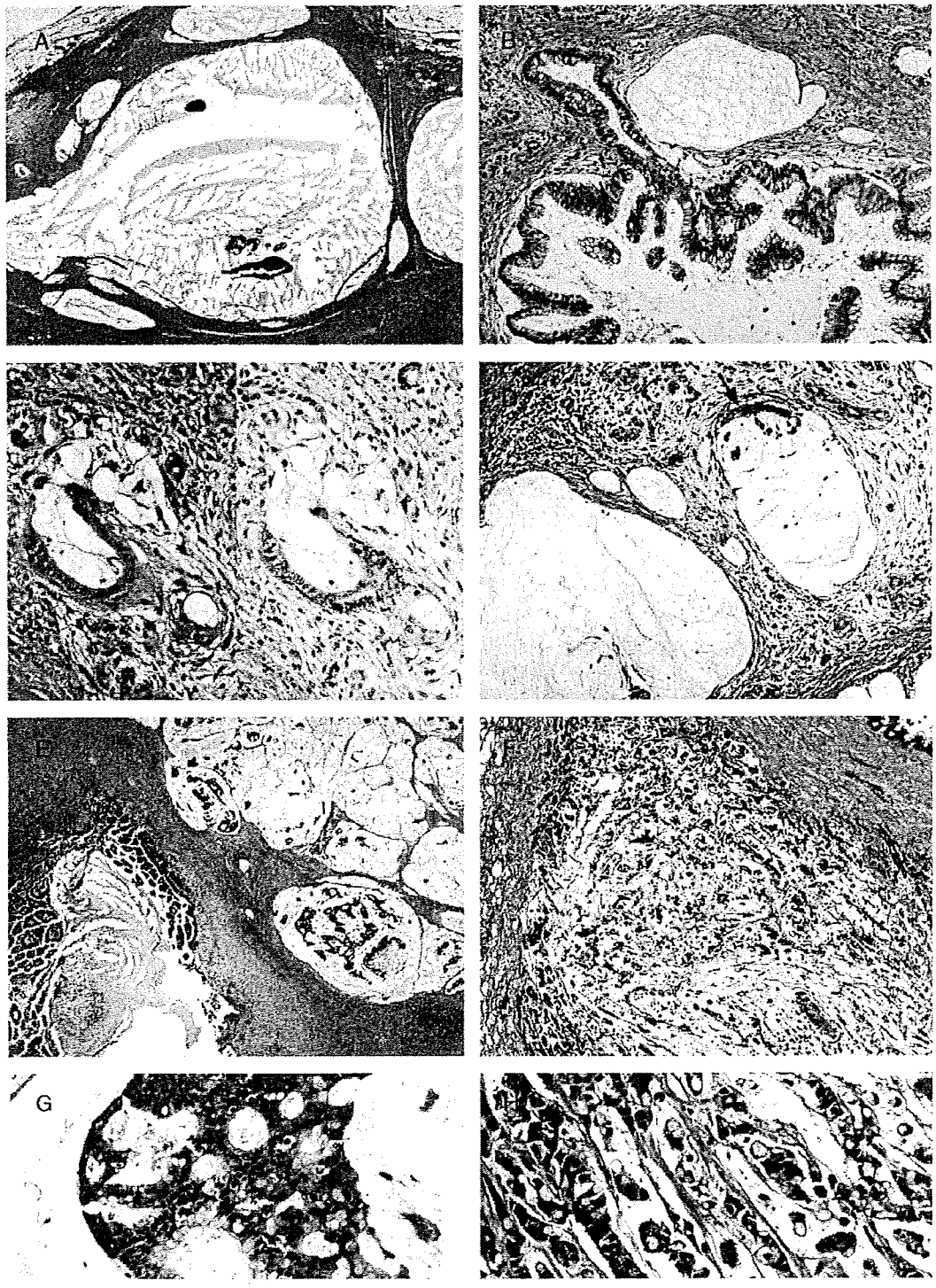

FIGURE 2. Histopathologic features of "mucous rupture" and "infiltrative growth" of mucinous carcinoma in I-IPMC. A to D, "Mucous rupture" pattern. Part of the pancreatic duct is disrupted and mucus leakage is evident. Variable sizes of mucus lakes without viable cancer cells floating are observed (A–C). A small duct covered by elastic fibers (C right column; elastica stain) is broken and the mucus leaks to form mucus lake (C). A small number of cancer cells (arrow) are floating in mucus lakes, which is described as "mucous rupture with cellular component." We could not observe any floating cancer cells in mucus lakes other than this cluster of cancer cells (arrow) in the entire lesion of the I-IPMC (D). E to H, "Infiltrative growth" of mucinous carcinoma. Many cancer cells floating in mucus lakes (E, G) or infiltrating features of mucinous carcinoma (F, H) are categorized as "infiltrative growth" of mucinous carcinoma. G and H, High-power view of (E) and (F), respectively.

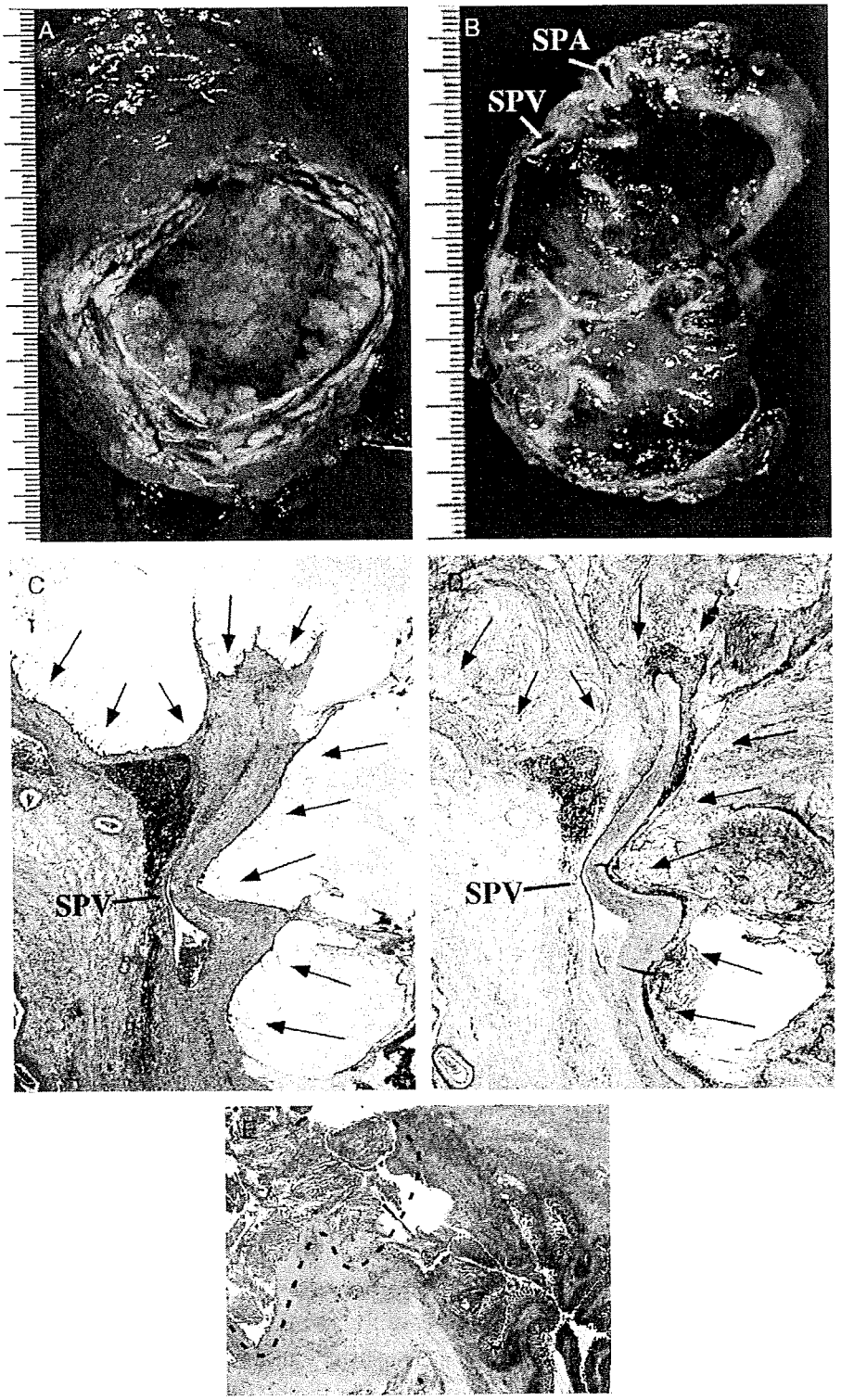

FIGURE 3. Histopathologic features of "expansive growth" in I-IPMC. The pancreatic duct is markedly dilated to a cystlike shape (A, B). Fresh cut view (A) and formalin-fixed cut surface (B) of cystic I-IPMCs. Cystically dilated pancreatic duct is filled with clear mucus and many papillary projections are seen on the inner surface (A). The SPV is compressed (B–D) and its thickened wall is eroded by an enlarged cystic IPMC (arrows) in hematoxylin and eosin stain (C) and elastica stain (D). A fistula has been formed between cystic I-IPMC (dotted line) and duodenum (E).

All of the 24 patients with mucous rupture MI-IPMC survived after surgery.

Expansive growth of ductectatic or cystic IPMN is another characteristic feature of IPMN (Fig. 3). In addition to mucous rupture, an increase of intraductal pressure by hypersecretion of mucus causes marked cystic dilatation of the duct, which continues to grow expansively into extrapancreatic tissue. In some cases, cystic IPMC eventually forms a fistula with surrounding digestive organs (Fig. 3E) or erodes the wall of major blood vessels [portal vein, splenic vein (SPV), superior mesenteric vein (SMV), or splenic artery] (Figs. 3C–E). Such growth and spread are rather passive in contrast to the infiltrative growth that occurs in active invasion and this feature was not associated with poor prognosis, similarly to mucous rupture. IPMC showing expansive growth with loss of the basement membrane of the pancreatic duct in the IPMC is diagnosed as MI-IPMC. If I-IPMC grows expansively, even if it ruptures into the bowel, or even if it erodes a major vessel wall unless cancer cells enter the lumen of the major vessel, it is still regarded as minimal invasion (Table 1). If I-IPMC has this type of growth as predominance, it is corresponded to a kind of pure mucinous carcinoma associated with IPMC.

Although we have not yet experienced intra-abdominal rupture of IPMC, a few cases have been reported.[18] As intra-abdominal rupture was followed by peritoneal dissemination in these reported cases, this type should be distinguished from ordinary IPMN and managed separately as ruptured IPMN.

IC-IPMC was defined as a lesion consisting of IPMN and invasive carcinoma with the predominance of the IPMN component.[12] Such invasive carcinoma exceeds the minimal invasion proposed in Table 1, and shows a continuous transition between invasive carcinoma and intraductal IPMC. In this study, we added new group of cases to the original IC-IPMC category, which had invasive carcinoma apparently originated from IPMN but predominant over the IPMN component. We wanted to compare the prognosis between IC-IPMC and conventional invasive ductal carcinoma of the pancreas in the matched tumor-node-metastasis (TNM) stages.[11]

Statistical Analysis

Comparisons of qualitative variables were performed using the χ^2 test or Fisher exact test. One-way analysis of variance was used to compare the means of 3 or more groups. The postoperative overall and disease-specific survival rates were calculated by the Kaplan-Meier method. Univariate analysis was performed for prognostic factors using the log-rank test. The factors found to be predictive by univariate analysis were subjected to multivariate analysis using the Cox proportional hazards model. Differences at $P < 0.05$ were considered statistically significant. Statistical analyses were performed with SPSS 11.0J software (SPSS Inc, Chicago, IL).

RESULTS

Histopathologic Evaluation of I-IPMC

One hundred and four IPMNs were classified into 27 IPMAs, 11 borderline IPMNs, 15 noninvasive IPMCs, and 51 I-IPMCs according to the WHO classification.[13,15] None of them had an ovarianlike stroma, and all the lesions showed communication with the pancreatic ductal system. I-IPMCs were further divided into 26 MI-IPMCs and 25 IC-IPMCs according to our criteria (Table 1) based on the histopathologic pattern of invasion.

To evaluate the aggressive characteristics of I-IPMC, we examined the invasiveness of I-IPMC. The invasiveness was categorized into 4 patterns: infiltrative growth, mucous rupture, expansive growth, and intra-abdominal rupture (see Materials and Methods). The criterion of minimal invasion was proposed for each corresponding pattern (Table 1), and the representative features are shown in Figures 1 to 3.

Seventeen among 26 patients with MI-IPMC showed infiltrative growth pattern (Fig. 1). Histologic types of the infiltrating cancer cells were tubular adenocarcinoma in 7 patients, mixed tubular adenocarcinoma and mucinous carcinoma in 2 patients, pure mucinous carcinoma in 5 patients, and papillary adenocarcinoma in 3 patients. The average depth of infiltration was 1.5 mm (range from < 1 to 5 mm). None of the 17 patients with a maximum infiltration of 5 mm or less had recurrence with exception of 2 patients, one of them had 2-mm-length infiltration of tubular adenocarcinoma and the other had 2-mm-length infiltration of pure mucinous carcinoma.

The most of the patients with MI-IPMC had mucous rupture and 6 patients had MI-IPMC with mucous rupture as predominant invasive pattern (Fig. 2). Two of them were subcategorized as mucous rupture with cellular component. None of these 6 patients had recurrence.

Expansive growth (Fig. 3) was often observed in cystically growing tumors and 4 patients with MI-IPMC mentioned below showed expansive growth as predominance. In 2 patients with I-IPMC, a fistula was formed between the IPMN and the duodenum. No cancer cells infiltrating the duodenal wall were detected in either case by histologic examination (Fig. 3E). It was suspected that the fistulas were formed by rupture of the expansively growing IPMN into the adjacent duodenum. The lesion was classified as MI-IPMC (expansive growth) in 1 patient, but the other patient had definite invasive cancer in the pancreas tail distant from the fistula, and was therefore diagnosed as having IC-IPMC. Whereas the former patient had no recurrence 107 months after surgery, the latter patient developed local lymph node (LN) metastasis 6 months after surgery and died of the disease. In the other 3 patients with expansive growth of MI-IPMC, the IPMN had grown deeply into the retropancreatic tissue, compressing the wall of the SPV or SMV. In one of them, the tunica media of the SPV was involved without a fistula

TABLE 2. Comparison of Invasive Lesion Between MI-IPMC and IC-IPMC

	MI-IPMC (n = 26)	IC-IPMC (n = 25)	P*
Growth pattern			
Infiltrative growth	17	25	
Mucous rupture or expansive growth as predominance	10†	0	
Vessel or neural invasion	4	25	**< 0.001**
Lymphatic invasion	0	23	**< 0.001**
Venous invasion	2	24	**< 0.001**
Intrapancreatic neural invasion	2	22	**< 0.001**
Extrapancreatic involvement	4	23	**< 0.001**
Serosa	0	4	0.051
Retropancreatic tissue	3‡	21	**< 0.001**
Duodenum	1‡	8	**0.002**
Extrahepatic bile duct	0	3	0.110
Portal venous system	1‡	8	**0.011**
Arterial system	0	1	0.490
Extrapancreatic nerve plexus	0	4	0.051
Invasion to surgical margin	0	4	0.051
Metastasis	0	17	**< 0.001**
Local LN	0	17	**< 0.001**
Distant organs	0	4§	0.051
TNM stage			**< 0.001**
IA	22	1	
IB	0	0	
IIA	4	7	
IIB	0	13	
III	0	0	
IV	0	4§	
Histology of infiltrative growth			
Pap	3	2	
Tub1	7	5	
Tub2	0	8	
Tub + Muc	2	7	
Tub3	0	1	
Muc	5	1	
AS	0	1	

Statistically significant value is in bold characters.

*P value was calculated by χ^2 or Fisher exact test.

†6 patients showed mucous rupture (2 of them showed mucous rupture with cellular component) and 4 patients showed expansive growth (one of them showed infiltrative growth as well).

‡Due to expansive growth.

§One patient with liver metastasis, 3 patients with para-aortic LN metastasis.

AS indicates adenosquamous carcinoma; Muc, mucinous carcinoma; Pap, papillary adenocarcinoma; Tub1, well-differentiated tubular adenocarcinoma; Tub2, moderately differentiated tubular adenocarcinoma; Tub3, poorly differentiated tubular adenocarcinoma.

between tumor and SPV (Figs. 3C–E). These 3 patients did not have postoperative recurrence at 28, 52, and 96 months after surgery, respectively. We thought mucous rupture and expansive growth is dormant invasion, considering its nonaggressive nature, which is characteristic to IPMN.

Comparison of the pathologic characteristics and TNM staging[11] between invasive lesions of MI-IPMCs and IC-IPMCs are summarized in Table 2. Vessel or neural invasion and extrapancreatic involvement were much more common in IC-IPMC than in MI-IPMC. No LN metastasis was observed in patients with MI-IPMC, whereas 17 patients (68%) with IC-IPMC showed LN metastasis. With regard to the histology of the invasive component of the IC-IPMC, most of the patients had tubular adenocarcinoma and only 1 patient had pure mucinous carcinoma. Among 26 patients with MI-IPMC, 9 had tubular adenocarcinoma and 11 had pure mucinous carcinoma.

Prognostic Significance of the Classification of I-IPMC

The median survival period for the 104 patients was 142 months, and the 3, 5, and 10-year overall survival rates were 86%, 78%, and 59%, respectively. There was no statistically significant difference in overall survival among patients with IPMA, borderline IPMN, and noninvasive IPMC ($P = 0.54$). Therefore, they were integrated into noninvasive IPMN for subsequent analysis. The survival rates 3, 5, and 10 years after surgery were 95%, 92%, and 70% for noninvasive IPMN, 95%, 79%, and 79% for MI-IPMC, and 51%, 38%, and 0% for IC-IPMC (Fig. 4A). The disease-specific survival rates after 3, 5, and 10 years were 100%, 100%, and 100% for noninvasive IPMN, 100%, 100%, and 100% for MI-IPMC, and 51%, 38%, and 0% for IC-IPMC (Fig. 4B). Overall and disease-specific survival for MI-IPMC was significantly better than for IC-IPMC ($P < 0.001$), whereas there was no significant difference in overall survival between noninvasive IPMN and MI-IPMC ($P = 0.66$).

Overall survival was compared between I-IPMC and conventional invasive ductal carcinoma of the pancreas during the same period (Figs. 5A–D). The stages of IC-IPMCs were assessed on the basis of size and spread of invasive carcinoma in the lesion, using the International Union against Cancer (UICC) TNM classification,[11] and classified as stage IA, IB, and IIA, stage IIB, and stage III and IV. Between IC-IPMC and conventional invasive ductal carcinoma of the pancreas at each corresponding TNM stage, there was no statistically significant difference in survival rate, though IC-IPMC had a tendency to show a favorable outcome.

Prognostic Factors in I-IPMCs

Clinicopathologic factors possibly affecting the postoperative outcome of I-IPMCs were studied (Table 3). The following variables were significantly related to unfavorable prognosis: presence of jaundice, cancer cells present at the surgical margin except the pancreatic margin, presence of major vascular invasion [portal vein, SMV, SPV, or splenic artery], presence of lymphatic invasion, presence of venous invasion, presence of intrapancreatic neural invasion, presence of LN metastasis, presence of para-aortic LN metastasis, CA19-9 > 300 U/mL, size of invasive cancer > 2 cm, histopathologic diagnosis of IC-IPMC (vs. MI-IPMC), and tubular adenocarcinoma as histologic type of invasive cancer in I-IPMC. Multivariate analysis (backward elimination method) showed that a histopathologic diagnosis of I-IPMC classified as IC-IPMC and

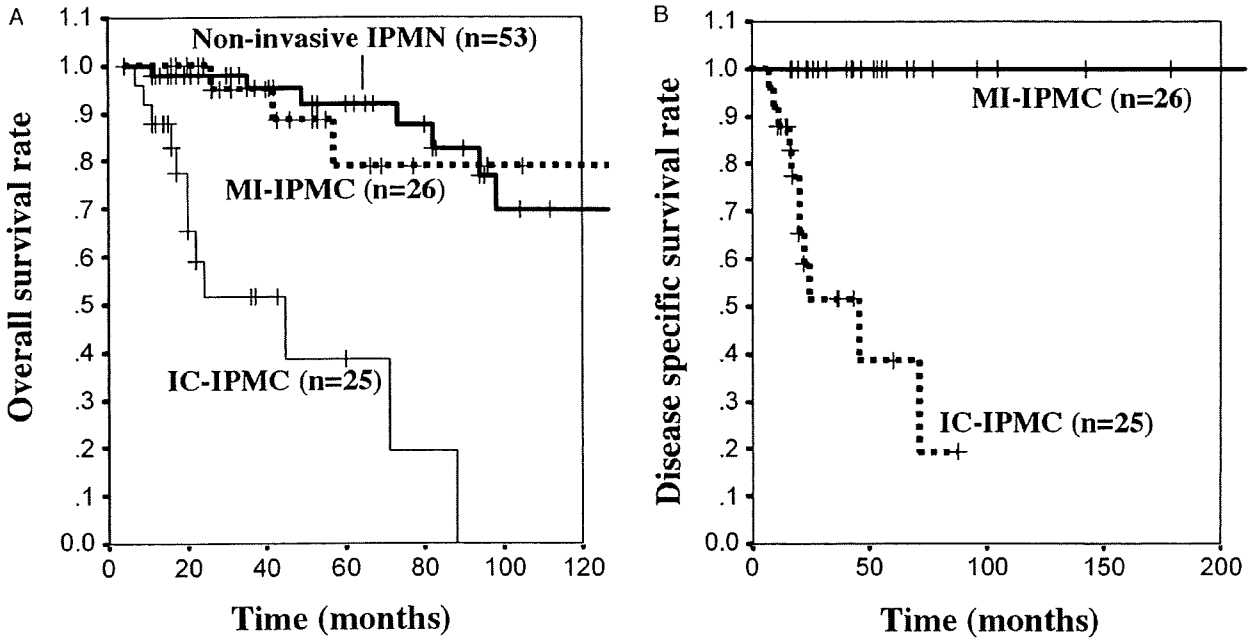

FIGURE 4. Kaplan-Meier survival curves of the 104 patients with IPMNs. A, Overall survival of patients with MI-IPMC was significantly better than that of patients with IC-IPMC ($P<0.001$), whereas no significant difference was found between patients with noninvasive IPMN and those with MI-IPMC ($P=0.66$). B, Disease-specific survival of patients with MI-IPMC was significantly better than that of patients with IC-IPMC ($P<0.001$), with no disease-related death among 26 patients with MI-IPMC during a median follow-up period of 43.4 (13.2 to 210) months.

CA19-9 > 300 U/mL were significant prognostic factors (Table 4).

Postoperative Recurrence of IPMNs

Postoperative recurrence was observed in 15 patients exclusively among those with I-IPMC (Table 5). Two patients with MI-IPMC suffered recurrence of MI-IPMC and invasive cancer in the remnant pancreas 36 and 48 months after surgery, respectively. At initial surgery, both patients had undergone PPPD for IPMNs in the pancreas head with negative surgical margins. The former underwent completion pancreatectomy in a second operation, and pathologic examination revealed another MI-IPMC in the remnant pancreas distant from the site of pancreato-jejunostomy. In the latter patient, recurrence of invasive ductal carcinoma was also found distant from the pancreato-jejunostomy, and additional partial resection of the remnant pancreas was performed. Both patients are currently doing well with no evidence of recurrence 8 and 20 months after the second operation, respectively. The remaining 13 recurrences were observed in patients with IC-IPMC. The site of recurrence was local (remnant pancreas) in 2 patients, LN in 2 patients, the lung in 1 patient, the liver in 4 patients, and peritoneal dissemination in 4 patients (Table 5). The time interval between surgery and recurrence was less than 20 months in all cases, with an especially short duration of 6.15 ± 0.82 months for patients with peritoneal dissemination.

Analysis of the Pancreatic Surgical Margin

Intraoperative frozen section analysis of the pancreas margin was performed in 96 patients, and 17 patients needed additional pancreatic resection owing to the confirmed or suspected presence of cancer cells at the pancreatic surgical margin (Table 5). Additional resection was performed more frequently in patients with MI-IPMC and IC-IPMC than in those with noninvasive IPMN, regardless of IPMN size ($P = 0.007$). The final pancreatic margin status was negative in 75 patients, positive for IPMA in 25, borderline IPMN in 2, noninvasive IPMC in 1, and invasive carcinoma in 1.

DISCUSSION

Many groups have investigated the malignant potential of IPMNs,[4,6,16,20,22–24] and the recent consensus is that its aggressiveness is dependent on the presence of invasive cancer, the extent of cancer invasion, and the biologic characteristics of the cancer cells.[2,3,8,10,14,15] However, no sufficient pathologic and presurgical staging system has yet been established for evaluating the malignant potential of I-IPMC. In this study, we examined 104 IPMNs surgically resected at the same hospital and proposed histopathologic criteria for classification of I-IPMC. I-IPMC shows heterogeneous features, which reflect the presence of heterogeneous cancer types with different biologic behaviors. Therefore, the criteria of MI-IPMC should differ in accordance with each histopathologic pattern of invasion. Our proposed

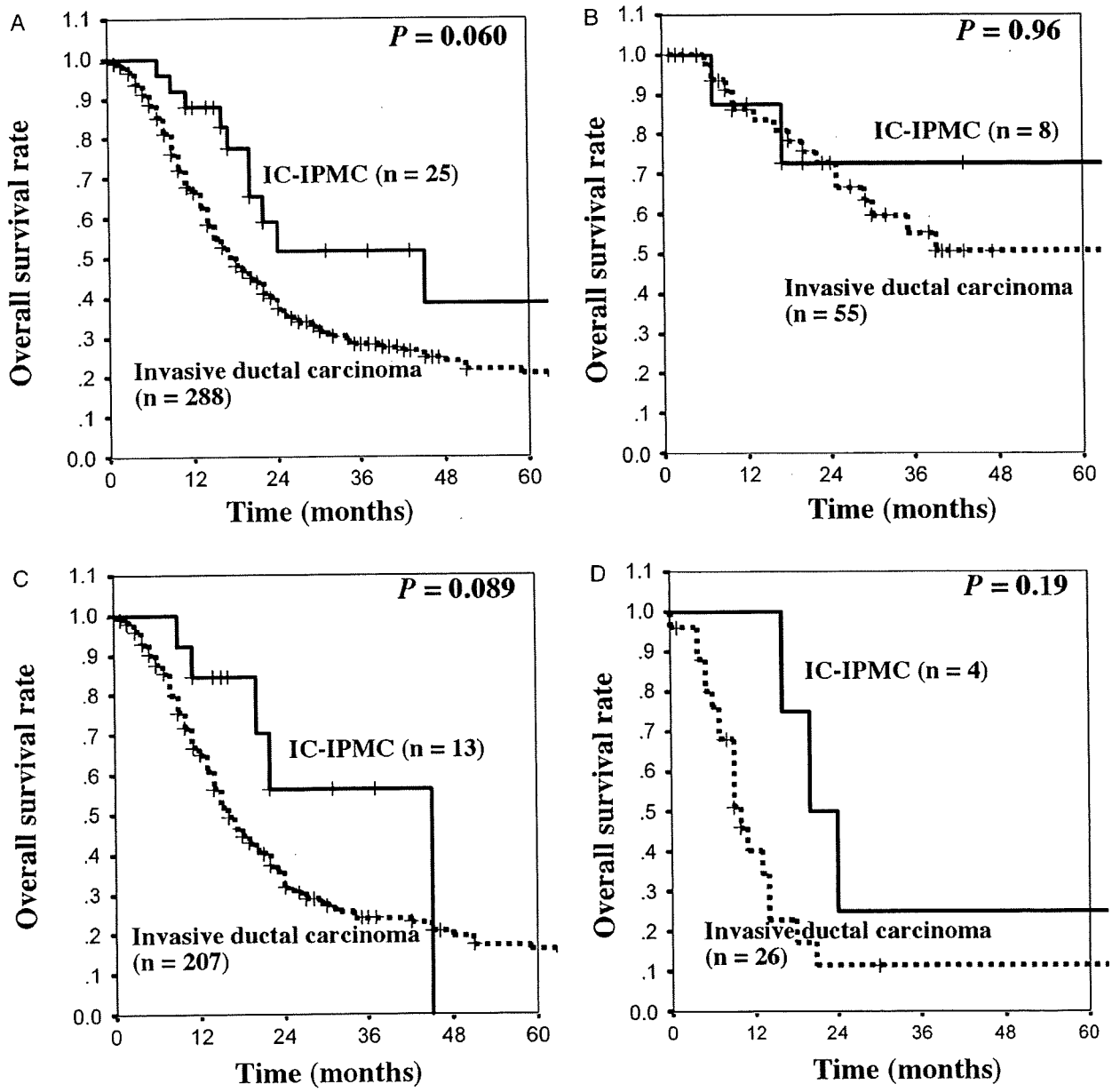

FIGURE 5. Kaplan-Meier survival curves of the 25 patients with IC-IPMC and the 288 patients with invasive ductal carcinoma of the pancreas. Comparison of overall survival of the patients with IC-IPMC and that of patients with conventional invasive ductal carcinoma at all stages (A), and in stage IA, IB, and IIA (B), stage IIB (C), and stage III and IV (D). Although the patients with IC-IPMC tended to have a better outcome than those with conventional invasive ductal carcinoma at each corresponding stage, the difference was not statistically significant.

criteria of invasiveness were successful in categorizing IPMCs in our series into noninvasive IPMC, MI-IPMC, and IC-IPMC. Patients with IC-IPMC had a significantly worse outcome than those with MI-IPMC. However, there was no difference in postoperative outcome between patients with MI-IPMC and those with noninvasive IPMC. This is the first report to propose practical criteria for MI-IPMC that can separate early-stage nonaggressive I-IPMC from total I-IPMC. Discrimination between MI-IPMC and IC-IPMC can provide important information for predicting the postoperative outcome of patients with IPMNs and also for deciding additional clinical management.

When IC-IPMCs were staged according to the size and spread of an invasive carcinoma component, the survival curve showed a similar decline to that of conventional invasive ductal carcinomas of the corresponding TNM stage, suggesting that it is the invasive

TABLE 3. Prognositic Factors of I-IPMCs in Univariate Analysis

Variables	n	Survival Rate 1 y	3 y	5 y	P (Log-rank Test)
Sex					0.262
M	27	96.3	80.4	80.4	
F	24	91.7	70.4	41.5	
Age (y)					0.082
≤70	33	100.0	80.6	72.6	
> 70	18	83.3	67.3	46.2	
Tumor location					0.937
Ph included	33	93.9	72.4	60.8	
Ph excluded	18	94.4	84.0	67.2	
Tumor distribution					0.821
Confined in 1 segment	35	91.4	75.7	63.2	
Diffuse (≥ 2 segments)	16	100.0	76.6	63.8	
PV resection					0.471
+	5	80.0	53.3	53.3	
–	46	95.7	78.0	63.0	
Chief complaint*					0.225
+	28	92.9	65.6	49.2	
–	23	95.6	90.0	81.0	
Jaundice					**0.011**
+	6	83.3	27.8	0.0	
–	45	95.6	80.7	66.2	
IPMN type					0.571
MPD or mixed	40	97.5	76.2	59.0	
BD	11	81.8	72.7	72.7	
MPD diameter					0.422
≤8 mm	31	90.3	75.4	52.2	
> 8 mm	20	100.0	75.6	75.6	
Additional resection of pancreas†					0.864
+	14	92.9	63.7	63.7	
–	37	94.6	78.4	60.3	
Surgical margin (except for pancreas margin)‡					**< 0.001**
+ or ±	4	75.0	0.0	0.0	
–	47	95.7	84.4	69.1	
Major vascular invasion (SMV, SPV, PV, or SPA)					**0.009**
+	10	90.0	48.2	0.0	
–	41	95.1	82.0	71.2	
Lymphatic invasion					**< 0.001**
+	23	87.0	44.7	22.4	
–	28	100.0	95.5	81.7	
Venous invasion					**0.006**
+	26	88.5	53.5	42.8	
–	25	100.0	94.4	78.0	
Intrapancreatic neural invasion					**< 0.001**
+	24	87.5	52.7	39.5	
–	27	100.0	94.4	78.4	
Local LN metastasis					**< 0.001**
+	18	88.9	47.1	23.5	
–	33	97.0	88.9	76.4	
Para-aortic LN metastasis					**< 0.001**
+	3	100.0	0.0	0.0	
–	48	93.8	32.3	67.4	
CEA (ng/mL)					0.455
≤5	35	94.3	83.4	64.3	
> 5	16	93.8	58.4	58.4	
CA19-9 (U/mL)					**< 0.001**
≤300	40	97.5	84.5	79.8	
> 300	11	81.8	40.9	0.0	
IPMN size (mm)					0.552
≤40	15	93.3	70.2	43.9	
> 40	36	94.4	78.2	72.2	
IPMN size (mm)					0.762
≤70	33	90.9	74.8	60.0	
> 70	18	100.0	77.0	66.0	
Size of invasive lesion (mm)					0.001
≤20	32	96.9	88.7	75.1	
> 20	19	89.5	48.7	32.4	
Pathologic diagnosis					**< 0.001**
MI-IPMC	26	100.0	94.7	78.6	
IC-IPMC	25	88.0	50.6	38.0	
Histology of invasive cancer in I-IPMC					
Tubular adenocarcinoma§	29	89.7	61.8	54.1	0.065
Pure mucinous carcinoma	12	100.0	100.0	57.1	
Tubular adenocarcinoma§	29	89.7	61.8	54.1	**0.010**
Nontubular adenocarcinoma	22	100.0	94.4	71.6	
Pure mucinous carcinoma	12	100.0	100.0	57.1	0.162
Nonpure mucinous carcinoma	39	92.3	68.6	62.9	
Past history of another cancer in other organs					0.316
+	10	90.0	72.0	54.0	
–	41	95.1	79.8	63.3	
TNM stage					
MI-IPMC	26	100.0	94.7	78.6	**0.040**
Stages IA, IB, and IIA	8	87.5	70.0	70.0	0.42
Stage IIB	13	84.6	56.4	0.0	0.82
Stages III and IV	4	100.0	25.0	25.0	

Statistically significant value is in bold characters.
*Diabetes mellitus exacerbation and jaundice included.
†Due to existence of neoplastic cells in pancreas margin in frozen section analysis.
‡Presence of invasive carcinoma cells in the stroma.
§Mixed tubular adenocarcinoma and mucinous carcinoma were included.
BD indicates branch duct; MPD, main pancreatic duct; Ph, pancreatic head; PV, portal vein.

carcinoma rather than IPMN itself that determines the prognosis.

In our series, none of the patients with MI-IPMCs showed LN metastasis, whereas the patients with IC-IPMCs had a high rate (68%) of LN metastasis. This finding implies that complete resection of a lesion without LN dissection may be sufficient for the treatment of MI-IPMC, whereas radical pancreatectomy with LN dissection is indicated for IC-IPMC. In this context, preoperative distinction between MI-IPMCs and IC-IPMCs is clinically very important.

TABLE 4. Multivariate Analysis of Effects of Clinicopathologic Factors on Postoperative Survival of I-IPMC

	Hazard Ratio	95% Confidence Interval	P*
IC-IPMC (vs. MI-IPMC)	7.1	1.9-26.5	< 0.001
CA19-9 > 300 (U/mL)	4.4	1.4-13.8	0.010

*P value was calculated by Cox hazards model (backward elimination method).

TABLE 5. Pancreatic Margin Status and the Recurrence of IPMNs After Surgery

	IPMA or Borderline IPMN (n = 38)	Noninvasive IPMC (n = 15)	MI-IPMC (n = 26)	IC-IPMC (n = 25)	Total (n = 104)	P
Additional pancreas resection*	3	0	5	9	17	0.007†
Final margin status						0.071
Negative	26	10	20 (2)	19 (11)	75	
IPMA	12	5	5	3 (1)‡	25	
Borderline IPMN	0	0	1	1	2	
Noninvasive IPMC	0	0	0	1	1	
Invasive cancer	0	0	0	1 (1)§	1	
Recurrence						
MI-IPMC (in the remnant pancreas)	0	0	1	0	1	
Invasive cancer (in the remnant pancreas)	0	0	1	0	1	
Local recurrence of invasive cancer	0	0	0	2	2	
Local LN	0	0	0	2	2	
Distant metastasis (lung or liver)	0	0	0	5	5	
Peritoneal dissemination	0	0	0	4	4	
Total	0	0	2	13	15	

*Due to the presence of neoplastic cells in the pancreatic surgical margin in the frozen section analysis.
†Comparison between noninvasive IPMN and I-IPMC.
‡Liver metastasis.
§Local recurrence, numbers in the parentheses denotes the number of patients who developed recurrence after the operation.

Another significant finding was a predominantly high recurrence rate among patients with IC-IPMC (52%), compared with 2.5% for patients with noninvasive IPMN or MI-IPMC. In the latter group, recurrence was observed in the remnant pancreas distant from the cut end, suggesting that IPMC occurred multifocally. Although this recurrence rate is not as high as that reported previously,[4] careful follow-up seems to be necessary after surgery, especially in patients with IC-IPMCs.

Our criteria are not contradictory to the previous studies, in which the postoperative outcome of I-IPMC with pure mucinous carcinoma (colloid carcinoma) was better than that of patients with I-IPMC with tubular adenocarcinoma in the invasive lesion.[1,24] Tubular adenocarcinoma shows active infiltrative growth similar to conventional pancreatic ductal adenocarcinoma, suggesting that it rapidly grows and progresses into advanced cancer. In fact, tubular adenocarcinoma occurred at a higher rate in IC-IPMC than in MI-IPMC, and was an unfavorable prognostic factor ($P = 0.010$; Table 3). It has been reported that mucinous carcinoma associated with IPMN or mucinous cystic tumor has a better outcome than conventional ductal carcinoma. According to Adsay's criteria (a carcinoma with more than 80% of mucinous carcinoma is defined as pure mucinous carcinoma),[1] 12 I-IPMCs were diagnosed as pure mucinous carcinoma associated with IPMC in our series, which contained 11 MI-IPMC (5 with infiltrative growth of pure mucinous carcinoma, 2 with predominantly mucous rupture with cellular component, and 4 with expansive growth) and 1 IC-IPMC. Among these 12 patients with pure mucinous carcinoma associated with IPMC, 1 patient with MI-IPMC with infiltrative growth and 1 patient with IC-IPMC had recurrence of the carcinoma. Although 12 patients had the recurrent cancers and 10 of them died among 29 patients of I-IPMCs with tubular adenocarcinoma (8 in MI-IPMC and 21 in IC-IPMC). Patients with pure mucinous carcinoma as histologic type of invasive cancer tended to have better prognosis than patients with tubular adenocarcinoma as invasive cancer ($P = 0.065$; Table 3). Our study also suggested that some mucinous carcinoma has aggressive behavior. The prognosis of mucinous carcinoma in the other organs such as colon, has been reported to be worse than the ordinary adenocarcinoma, especially worse for mucinous carcinoma with rich cellular component.[17,21] In ductal carcinoma of the pancreas, mixed mucinous carcinoma with other histologic types of carcinoma (usually tubular adenocarcinoma) shows bad prognosis comparable with the other types of conventional ductal adenocarcinoma.[7,14] In this situation, it is desired that a diagnostic criterion is established to distinguish aggressive and nonaggressive mucinous carcinoma correctly. In this study, addition to the classification of tubular adenocarcinoma of the I-IPMC into aggressive and nonaggressive state, we also classified mucinous carcinoma relevant to clinical behavior based on the invasiveness and cellularity. Compared with mucous rupture, more aggressive mucinous carcinoma shows massive invasion with much more cancer cells floating and proliferating in mucus lakes, and is often accompanied by partial invasion of tubular adenocarcinoma.

Lymphatic, venous, and intrapancreatic neural invasion were frequently observed in IC-IPMC (Table 2) and were significant prognostic factors in I-IPMC (Table 3). In this study, we tried to select early-stage I-IPMC with nonaggressive characters from I-IPMCs with such worse prognostic factors. We successfully selected it by categorizing the infiltrating depth of cancer cells, which included lymphatic, venous, and/or neural invasion. Indeed, all the patients with MI-IPMC having vessel or neural invasion within 5-mm length from IPMC duct

showed good postoperative outcome. In addition, lymphatic, venous, and intrapancreatic neural invasion were not significant variables for the prognosis in multivariate analysis (Table 4).

The present results suggest that IC-IPMC (not MI-IPMC) should be currently paid attention as I-IPMC with aggressive characteristics. In this situation, preoperative detection of IC-IPMC can be beneficial for selecting the most ideal operative procedure, especially on considering additional LN dissection. We are now investigating possible criteria for classifying these cancers preoperatively, and our findings suggest that it may be feasible to use radiologic data for this purpose. Multidetector row computed tomography was found to be useful to distinguish IC-IPMC from MI-IPMC and noninvasive IPMNs with more than 80% sensitivity and 100% specificity in the study using 123 patients with IPMNs (manuscript in preparation).

In future, we would like to test our criteria using another large series of samples or in a prospective study, to obtain more watertight pathologic criteria for classification of I-IPMC.

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Clinicopathological and prognostic significance of EGFR, VEGF, and HER2 expression in cholangiocarcinoma

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Epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), and human epidermal growth factor receptor 2 (HER2) have been considered as potential therapeutic targets in cholangiocarcinoma, but no studies have yet clarified the clinicopathological or prognostic significance of these molecules. Immunohistochemical expression of these molecules was assessed retrospectively in 236 cases of cholangiocarcinoma, as well as associations between the expression of these molecules and clinicopathological factors or clinical outcome. The proportions of positive cases for EGFR, VEGF, and HER2 overexpression were 27.4, 53.8, and 0.9% in intrahepatic cholangiocarcinoma (IHCC), and 19.2, 59.2, and 8.5% in extrahepatic cholangiocarcinoma (EHCC), respectively. Clinicopathologically, EGFR overexpression was associated with macroscopic type ($P=0.0120$), lymph node metastasis ($P=0.0006$), tumour stage ($P=0.0424$), lymphatic vessel invasion ($P=0.0371$), and perineural invasion ($P=0.0459$) in EHCC, and VEGF overexpression with intrahepatic metastasis ($P=0.0224$) in IHCC. Multivariate analysis showed that EGFR expression was a significant prognostic factor (hazard ratio (HR), 2.67; 95% confidence interval (CI), 1.52–4.69; $P=0.0006$) and also a risk factor for tumour recurrence (HR, 1.89; 95% CI, 1.05–3.39, $P=0.0335$) in IHCC. These results suggest that EGFR expression is associated with tumour progression and VEGF expression may be involved in haematogenic metastasis in cholangiocarcinoma.
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Cholangiocarcinoma arises from the ductal epithelium of the bile duct tree and is classified anatomically into intrahepatic cholangiocarcinoma (IHCC) and extrahepatic cholangiocarcinoma (EHCC). The incidence and mortality rates of cholangiocarcinoma, especially those of IHCC, are increasing worldwide (Khan *et al*, 2005). Complete resection is the only way to cure the disease at present. Moreover, because cholangiocarcinoma is difficult to diagnose at an early stage and extends diffusely, most patients have unresectable disease at clinical presentation, and prognosis is very poor (5-year survival is 0–40% even in resected cases) (Khan *et al*, 2005; Sirica, 2005). Therefore, novel effective therapeutic strategies are urgently required to improve the prognosis. Among potential therapeutic targets, several studies have revealed overexpression of epidermal growth factor receptor (EGFR) or human epidermal growth factor receptor 2 (HER2) protein, amplification, and mutation of these genes (Ito *et al*, 2001; Aishima *et al*, 2002; Ukita *et al*, 2002; Altimari *et al*, 2003; Gwak *et al*, 2005; Nakazawa *et al*, 2005; Leone *et al*, 2006) as well as overexpression of vascular endothelial growth factor (VEGF) protein (Hida *et al*, 1999; Tang *et al*, 2006) in cholangiocarcinoma.

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Epidermal growth factor receptor and HER2 are members of the ErbB receptor tyrosine kinase family. Binding of ligands, such as epidermal growth factor and transforming growth factor alpha (TGFα), to their extracellular ligand-binding domain initiates intracellular signalling cascades, leading to progression, proliferation, migration, and survival of cancer cells (Olayioye *et al*, 2000; Yarden and Sliwkowski, 2001). Vascular endothelial growth factor plays a key role in tumour-associated neo-angiogenesis, which contributes to providing a tumour with oxygen, nutrition, and a route for metastasis. It binds to VEGFR (vascular endothelial growth factor receptor), and leads to survival, proliferation, and migration of endothelial cell (Tabernero, 2007). Expression of these molecules has been reported to have prognostic significance in several cancers (Gusterson *et al*, 1992; Han *et al*, 2001; Nicholson *et al*, 2001; Des Guetz *et al*, 2006; Mohammed *et al*, 2007). Recently, agents targeted at these molecules have been used clinically, such as trastuzumab in breast cancer (Gonzalez Angulo *et al*, 2006), gefitinib, and erlotinib in non-small cell lung cancer, and bevacizumab in colorectal cancer (Tabernero, 2007). In cholangiocarcinoma, a phase II study of erlotinib (Philip *et al*, 2006) and some case reports of combined chemotherapy including cetuximab (Sprinzl *et al*, 2006; Huang *et al*, 2007) have been reported.

However, no previous studies have clarified associations between the expression of these molecules and clinicopathological

factors or prognosis in patients with cholangiocarcinoma. To elucidate the biological significance and potential of these molecules as therapeutic targets, we investigated EGFR/VEGF/HER2 expression and attempted to elucidate their associations with various clinical features as well as patient survival in 236 cases of cholangiocarcinomas.

MATERIALS AND METHODS

Patients

A total of 236 patients with cholangiocarcinoma (male 160; female 76) who had undergone tumour resection and been diagnosed histologically as having adenocarcinoma of the bile duct at the National Cancer Center Hospital, Tokyo, between January 1991 and August 2004, were enrolled in the present study. Median patient age and follow-up period were 65 years and 875 days, and median tumour sizes of IHCC and EHCC were 4.8 and 3.0 cm, respectively. Detailed characteristics of patient with IHCC and EHCC are presented in Tables 1 and 2. All patients were followed for more than 100 days. Follow-up examination was performed using computed tomography, abdominal ultrasonography, and measurement of the serum carcinoembryonic antigen and carbohydrate antigen 19-9 (CA19-9) levels every 3-6 months. Recurrence was diagnosed by clinical, radiological, or pathological methods, but mainly by radiological evaluation including computed tomography and ultrasonography. Clinical and pathological profiles were obtained from the database of hepatobiliary tumours based on the medical records of the patients. This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan, and written informed consent was obtained from all patients.

All cases were anatomically classified into two groups: IHCC and EHCC. Tumours arising from the bilateral hepatic duct or distal common bile duct were classified as EHCC. The numbers of IHCC and EHCC cases were 106 and 130, respectively.

Histological assessment

Tumour staging and histological classification were assessed according to *TNM Classification of Malignant Tumours* (Sobin and Wittekind, 2002) defined by the International Union Against Cancer (UICC) and the *World Health Organization Histological Classification of Tumours* (Hamilton and Altonen, 2000). Macroscopic types of IHCC were defined with reference to *General Rules for the Clinical and Pathological Study of Primary Liver Cancer* (Liver Cancer Study Group of Japan, 2003): (1) the mass-forming type (MF), which develops an apparent tumour in the liver; (2) the periductal infiltrating type (PI), which spreads along the bile duct; (3) the intraductal growth type (IG), which is confined within the bile duct, and divided into two groups: the mass-forming group (MF and MF mixed with PI or IG) and the non-mass forming group (PI and/or IG). Macroscopic types of EHCC were divided into polypoid type and non-polypoid type (including nodular, scirrhous constricting, and infiltrating types). Other clinicopathological factors were categorised into groups that are presented in Table 1 (IHCC) and Table 2 (EHCC). Because the classifications and clinicopathological factors used in IHCC and EHCC are different, statistical analyses were performed separately.

Immunohistochemistry

Immunohistochemistry (IHC) for EGFR, VEGF, and HER2 was performed using a polymer-based method (Envision™ + Dual Link System-HRP (Dako, DK-2600 Glostrup, Denmark)). Sources and dilutions of primary antibodies were as follows: anti-EGFR (mouse monoclonal, clone 31G7; Zymed, South San Francisco, CA, USA; 1:100), anti-VEGF (rabbit polyclonal; Zymed; 1:50), and anti-HER2 (rabbit polyclonal; Dako; 1:300).

Formalin-fixed, paraffin-embedded serial tissue sections (4 μm) were placed on silane-coated slides for IHC. Sections cut through the maximum tumour diameter were selected for IHC evaluation. The sections were deparaffinised and rehydrated in xylene and grade-diluted ethanol (50-100%), and submerged for 20 min in 0.3% hydrogen peroxide with absolute methanol to block endogenous peroxidase activity. Antigen retrieval for EGFR, VEGF, and HER2 was carried out by adding Digest-all™3 pepsin solution (Zymed) at 37°C for 10 min for EGFR, near boiling in 0.01 M citrate buffer (pH 6.0) for 15 min for VEGF, and heating in 0.01 M citrate buffer at 121°C for 10 min by pressure cooker for HER2. After protein blocking, the sections were incubated with each primary antibody at room temperature for 1 h, followed by incubation with

Table 1 Characteristics of the IHCC patients

Factors	Categories	Population
Age	<65 years old	54 (50.9%)
	⩾65 years old	52 (49.1%)
Gender	Male	64 (60.4%)
	Female	42 (39.6%)
Tumour size	⩽5.0 cm	55 (55.6%)
	>5.0 cm	44 (44.4%)
Macroscopic type	Non-mass forming	17 (16.0%)
	Mass forming	89 (84.0%)
Invasion of portal vein	Negative	23 (21.9%)
	Positive	82 (78.1%)
Invasion of hepatic vein	Negative	56 (54.9%)
	Positive	46 (45.1%)
Intrahepatic metastasis	Negative	75 (70.8%)
	Positive	31 (29.2%)
Lymph node metastasis	Negative	62 (58.5%)
	Positive	44 (41.5%)
UICC pT	1+2	71 (68.3%)
	3+4	33 (31.7%)
UICC stage	1+2	45 (42.5%)
	3A+3B+3C	61 (57.5%)
Histological classification	Well	22 (20.8%)
	Mod	79 (74.5%)
	Por	5 (4.7%)
Lymphatic vessel invasion	Negative	20 (18.9%)
	Positive	86 (81.1%)
Venous invasion	Negative	19 (17.9%)
	Positive	87 (82.1%)
Perineural invasion	Negative	29 (27.4%)
	Positive	77 (72.6%)
Hepatic surgical margin	Negative	89 (84.0%)
	Positive	17 (16.0%)
Bile duct margin	Negative	91 (85.8%)
	Positive	15 (14.2%)

Well = well differentiated adenocarcinoma; Mod = moderately differentiated adenocarcinoma; Por = poorly differentiated adenocarcinoma. In some factors, data were not available for all cases.

Table 2 Characteristics of the EHCC patients

Factors	Categories	Population
Age	<65 years old	60 (46.2%)
	⩾65 years old	70 (53.8%)
Gender	Male	96 (73.8%)
	Female	34 (26.2%)
Tumour size	⩽3.0 cm	72 (56.3%)
	>3.0 cm	56 (43.7%)
Macroscopic type	Polypoid	21 (16.8%)
	Non-polypoid	104 (83.2%)
Depth of tumour invasion	Within FM	13 (10.0%)
	Beyond FM	117 (90.0%)
Invasion of portal vein	Negative	97 (74.6%)
	Positive	33 (25.4%)
Invasion of hepatic artery	Negative	127 (97.7%)
	Positive	3 (2.3%)
Lymph node metastasis	Negative	71 (54.6%)
	Positive	59 (45.4%)
UICC pT	1+2	49 (37.7%)
	3+4	81 (62.3%)
UICC stage	1A+1B	37 (28.5%)
	2A+2B+C	93 (71.5%)
Histological classification	Pap	20 (15.4%)
	Well	31 (23.8%)
	Mod	62 (47.7%)
	Por	17 (13.1%)
Lymphatic vessel invasion	Negative	16 (12.3%)
	Positive	114 (87.7%)
Venous invasion	Negative	19 (14.6%)
	Positive	111 (85.4%)
Perineural invasion	Negative	23 (17.7%)
	Positive	107 (82.3%)
Dissected periductal structures margin	Negative	109 (83.8%)
	Positive	21 (16.2%)
Bile duct margin	Negative	92 (70.8%)
	Positive	38 (29.2%)
Invasion to other organ	Negative	53 (40.8%)
	Positive	77 (59.2%)

FM = fibromuscular layer; Pap = papillary adenocarcinoma; Well = well differentiated adenocarcinoma; Mod = moderately differentiated adenocarcinoma; Por = poorly differentiated adenocarcinoma. In some factors, data were not available for all cases.

Envision+ Dual Link reagent at room temperature for 30 min, and visualised using 3,3′-diaminobenzidine tetrahydrochloride as a chromogen. Finally, the sections were counterstained with haematoxylin. Sections were gently rinsed in phosphate-buffered saline between the incubation steps.

Evaluation of immunohistochemistry

All sections were evaluated by DY, HO, and TS without the knowledge of any clinical or pathological information, and cases for which consensus could not be reached were discussed to decide the evaluation. Based on the Herceptest™ (Dako) criteria, intensities of both EGFR and HER2 were defined as follows: 0, no membrane staining or membrane staining in ⩽10% cancer cells; 1+, faint and partial membrane staining in >10% cancer cells; 2+, moderate and complete membrane staining in >10% cancer cells; 3+, strong and complete membrane staining in >10% cancer cells. Intensities of VEGF were defined as follows: 0, no cytoplasmic staining or cytoplasmic staining in ⩽30% cancer cells; 1+, faint cytoplasmic staining, equivalent to the intensity of normal bile duct epithelium within the same section, in >30% cancer cells; 2+, moderate cytoplasmic staining in >30% cancer cells; 3+, strong cytoplasmic staining in >30% cancer cells. For cases showing mixed intensity, the predominant intensity was selected as the final IHC score. A final IHC score of 2+ or 3+ was defined as positive for expression of each protein.

Statistical analysis

Associations between results of IHC and clinicopathological factors were assessed by χ^2 test. Cumulative survival rates and survival curves were calculated by the Kaplan–Meier method, and log-rank test was performed for the comparison of survival curves. Cox's proportional hazard model was performed to estimate hazard ratio (HR) and 95% confidence interval (CI) of each outcome (death and recurrence). Multivariate analyses were performed using the factors identified to be risk factors for each outcome by univariate analyses, without UICC pT and UICC Stage, which are composed of other factors. All *P*-values reported are two-sided, and significance level was set at $P<0.05$. All statistical analyses were performed with the Statview 5.0 statistical software package (Abacus Concepts, Berkeley, CA, USA).

RESULTS

Expression of EGFR, VEGF, and HER2 protein in cholangiocarcinoma

Representative cases of positive staining for each protein are shown in Figure 1 (A, EGFR; B, HER2; C, VEGF). Epidermal growth factor receptor, VEGF, and HER2 were expressed in 29 (27.4), 57 (53.8), and 1 (0.9%) of the 106 IHCCs, respectively, and in 25 (19.2), 77 (59.2), and 11 (8.5%) of the 130 EHCCs, respectively. Microscopically, EGFR was mostly overexpressed in the moderately and/or poorly differentiated component, which is characterised by infiltration (52 of 54 EGFR-positive cases, Figure 1D), whereas only two cases showed EGFR overexpression in the well-differentiated component. In contrast, HER2 was preferentially expressed in the well-differentiated component. In 6 of 12 HER2-positive cases, HER2 was expressed only in well-differentiated component (Figure 1E), and 5 progressive cases showed positive HER2 staining in both the well and moderately and/or poorly differentiated components and 1 case only in moderately differentiated component. There was no association between VEGF expression and histological features.

Associations between EGFR, VEGF, and HER2 expression and clinocopathological factors

Statistical analyses of HER2 were performed only in EHCC cases because of the small number of HER2-positive cases in IHCC. In IHCC, VEGF expression was significantly associated with intrahepatic metastasis ($P=0.0224$). There was no significant association between EGFR expression and any clinicopathological factors.

In EHCC, EGFR expression was significantly associated with macroscopic type (0% in the polypoid type, 24.0% in the non-polypoid type; $P=0.0120$), lymph node metastasis ($P=0.0006$), UICC Stage ($P=0.0424$), lymphatic vessels invasion ($P=0.0371$), and perineural invasion ($P=0.0459$). Human epidermal growth factor receptor 2 expression was significantly associated with

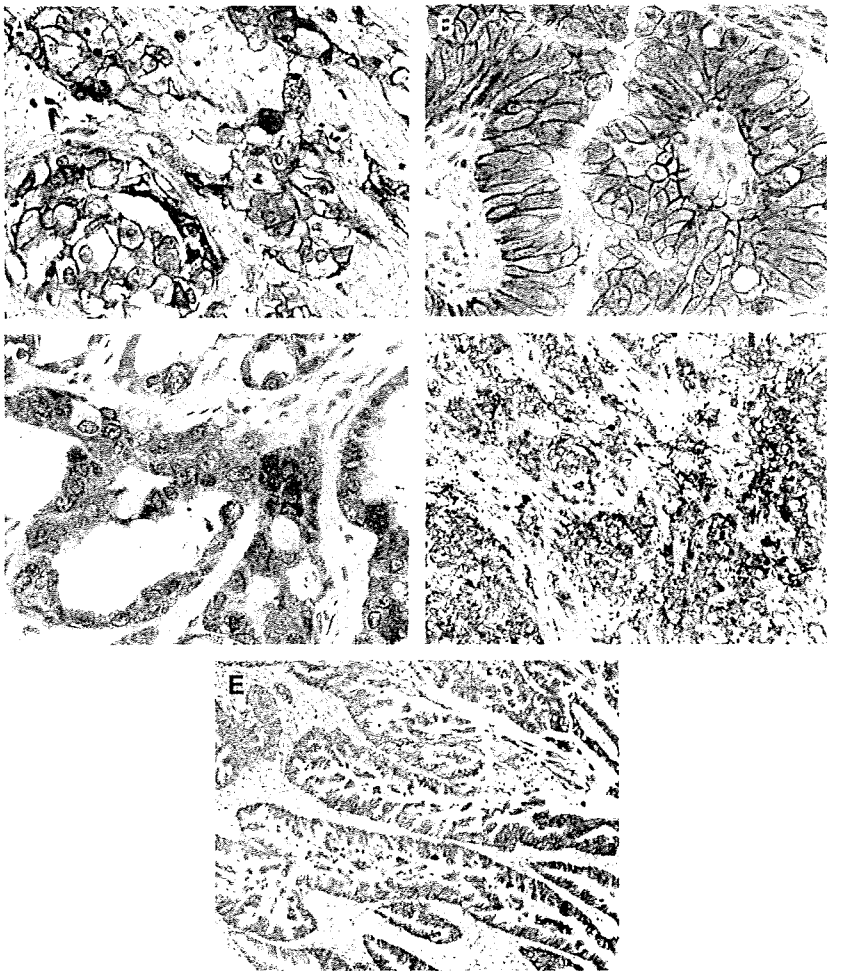

Figure 1 Representative immunohistochemical staining of (**A**) EGFR, (**B**) HER2, and (**C**) VEGF in cholangiocarcinoma (× 400 magnification). (**D**) Epidermal growth factor receptor tends to be expressed in the poorly differentiated component (× 100 magnification). (**E**) Human epidermal growth factor receptor 2 is preferentially expressed in more differentiated areas such as the glandular or papillary component (× 100 magnification).

macroscopic type (23.8% in the polypoid type, 5.8% in the non-polypoid type; $P=0.0078$), histological classification (25% in papillary adenocarcinoma, 9.7% in well differentiated adenocarcinoma, 3.2% in moderately differentiated adenocarcinoma, 5.9% in poorly differentiated adenocarcinoma; $P=0.0237$), and invasion to other organs (3.9% in invasive cases, 15.1% in non-invasive cases; $P=0.0242$). VEGF expression was not significantly associated with any factors in EHCC.

Detailed results of associations between EGFR/VEGF/HER2 expression and clinicopathological factors are shown in Supplementary information 1 (IHCC) and Supplementary information 2 (EHCC).

Univariate and multivariate analyses regarding overall survival and tumour recurrence in cholangiocarcinoma

The number of dead and the median survival time were 70 cases and 724 days in IHCCs, and 76 cases and 1197 days in EHCCs, respectively. The number of recurrence and the median recurrence time were 64 cases and 522 days in IHCCs, and 78 cases and 960 days in EHCCs, respectively.

Overall 5-year cumulative survival for patients with IHCC and EHCC was 33.0 and 41.6%, respectively, and no significant difference was identified between the groups ($P=0.0599$). The survival curves stratified by EGFR expression status are shown as Figure 2. Five-year survival for patients with EGFR-positive and EGFR-negative tumours was 17.7 and 47.1% for IHCC, and 26.4 and 45.6% for EHCC, respectively. There was a significant difference between EGFR-positive and -negative cases for both IHCC ($P=0.0008$) and EHCC ($P=0.0204$).

The results of multivariate analyses following univariate analyses regarding overall survival and tumour recurrence are shown in Table 3 (IHCC) and Table 4 (EHCC).

In IHCC, 13 factors including EGFR expression were identified as significantly prognostic by univariate analysis. Multivariate analysis revealed that EGFR expression was an independent prognostic factor (HR, 2.67; 95% CI, 1.52–4.69; $P=0.0006$), along with mass-forming macroscopic group (HR, 2.96; 95% CI, 1.06–8.31; $P=0.0390$), intrahepatic metastasis (HR, 2.91; 95% CI, 1.60–5.29; $P=0.0005$), and lymph node metastasis (HR, 1.96; 95% CI, 1.04–3.69; $P=0.0375$). In EHCC, 14 factors including EGFR expression were identified as significantly prognostic by univariate analysis. Multivariate analysis revealed that lymph node metastasis (HR, 2.03; 95% CI, 1.16–3.55; $P=0.0133$) and a histological classification of moderately differentiated adenocarcinoma (HR for papillary adenocarcinoma, 4.23; 95% CI, 1.08–16.50; $P=0.0380$) and poorly differentiated adenocarcinoma (HR for papillary adenocarcinoma, 13.22; 95% CI, 3.10–56.45; $P=0.0005$) were significant prognostic factors.

Multivariate analysis following univariate analysis for risk factors of tumour recurrence revealed that EGFR expression in IHCC was a significant risk factor of tumour recurrence (HR, 1.89;