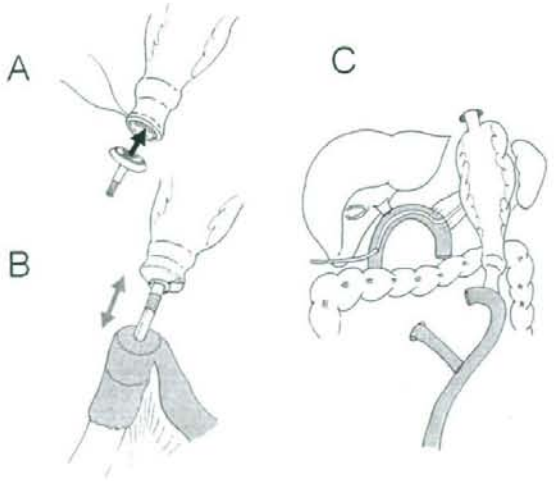

Fig. 1A–C. Schematic illustration of Roux-en-Y duodenojejunostomy using a circular stapler. **A** A purse-string suture was placed around the duodenal stump, and an anvil was placed within. **B** Duodenojejunostomy was accomplished using a circular stapler. **C** Pancreaticojejunostomy, hepaticojejunostomy, and Roux-en-Y duodenojejunostomy with an external pancreatic drainage

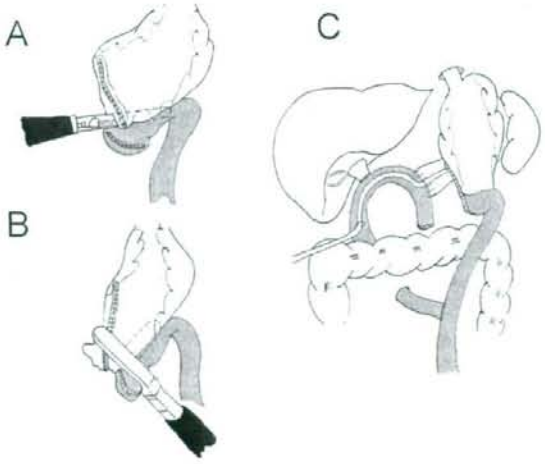

Fig. 2A–C. Schematic illustration of Roux-en-Y gastrojejunostomy using linear staplers. **A** Gastrojejunostomy using a linear stapler. A side-to-side anastomosis was made between the posterior wall of the stomach and the side wall of the jejunum. **B** The edges of the stomach and the jejunal loop were divided using a linear stapler. **C** Pancreaticojejunostomy, hepaticojejunostomy, and Roux-en-Y gastrojejunostomy with an external pancreatic drainage

28 mm, US Surgical, Norwalk, CT [$n = 1$]) (Fig. 1). The duodenum was divided 3–4 cm anal of the pylorus ring. A segmental jejunum was sacrificed and resected, preserving the mesojejunum, and the anal jejunum was used for the duodenojejunostomy. An anvil device was inserted into the duodenal stump, a circular stapler was inserted into the jejunal loop, and the mechanical anastomosis was completed. The stump of the jejunal loop was closed using a linear stapler.

Roux-en-Y Reconstruction Using a Linear Stapler During Standard Whipple Procedure (SW)

In four SWs an antecolic gastrojejunostomy was performed by Roux-en-Y reconstruction using a linear stapler (Endo-GIA Reticulator 60, US Surgical) (Fig. 2). A segmental jejunum was removed, preserving the mesojejunum, and the anal jejunum was used for the gastrojejunostomy. A small incision was made on the posterior wall of the stomach, near the stump on the greater curvature. A linear stapler was inserted into the remnant stomach and the jejunum to adjust the posterior wall of the stomach and the side wall of the jejunum. A mechanical anastomosis was made, and the stumps of the stomach and the jejunum were closed using another linear stapler.

In the remaining two PDs, a circular stapler was used to make a gastrojejunostomy on the posterior wall of

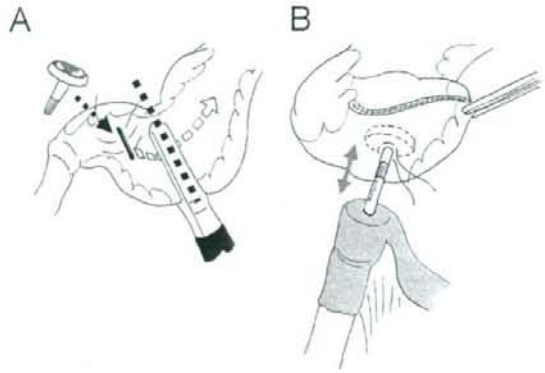

Fig. 3A,B. Schematic illustration of Roux-en-Y gastrojejunostomy using a circular stapler. **A** The anvil was inserted through a small incision in the antrum into the fornix of the stomach. The incision was closed with interrupted sutures. **B** The handle of the anvil was pulled out though the posterior wall of the stomach, and the root of the handle was closed using a purse-string suture. Thereafter, a gastrojejunostomy was accomplished using the circular stapler

the stomach (Fig. 3). Before dividing the stomach during SW the anvil was inserted into the fornix of stomach through a small incision in the antrum, which was then sutured. The handle of the anvil was pulled out of the posterior wall of the remnant stomach, and a mechani-

cal anastomosis was completed. The stump of the jejunum was closed using another linear stapler.

In both the mechanical Roux-en-Y reconstructions during PPPD and SW, the anal jejunojejunostomy was created by serosa-to-serosa continuous suture. Gastrostomy was not done in any of the patients. In the pancreaticojejunostomy, an internal stent (n = 5) or an external drainage tube (n = 19) was placed in 24 patients. In the hepaticojejunostomy, an internal stent (n = 16) or external drainage tube (n = 2) was placed in 18 patients. In 23 patients a jejunal drainage tube (n = 8) to decompress the anastomotic loop or a jejunal feeding tube (n = 15) was inserted through the right-sided jejunal loop. The nasogastric tube was removed on POD 1 in all 26 patients. Two closed drains were inserted beside the pancreaticojejunostomy, and intermittent suction was applied 24 h a day for 4–6 days in the former term. In the latter 2 years, intermittent suction was not used. Patients were discharged home when they were able to resume eating about half the amount of their regular diet and had only minimal output from one abdominal drain.

Definition of Outcome Measures

The results of the upper gastrointestinal (UGI) study were evaluated within 10 PODs according to the degree of passage of the contrast medium. Grade A showed good passage of the medium without any dilatation of the stomach; grade B showed mild dilatation of the remnant stomach or formation of the niveau in the stomach, and passage of the medium could be maintained when the patient leaned forward; and grade C showed severe dilatation of the remnant stomach or no passage of the contrast medium.

Delayed gastric emptying was defined as failure to start a regular diet within 14 PODs according to previous reports.[5,6,9-11,13] Postoperative pancreatic fistula was graded according to the definitions proposed by an international study group on pancreatic fistula,[16] namely, when the amylase concentration of the drain fluid obtained on, or after POD 3 was greater than three times the serum amylase concentration. Pancreatic fistulas were classified into grades A, B, and C according to severity; grade A was a "transient fistula," not associated with a delay in hospital discharge; a grade B fistula led to a delay in discharge, with persistent drainage for more than 3 weeks; and a grade C fistula was usually associated with major complications. Grades B and C fistula were considered to be major complications.

Comparison of the Two Reconstruction Methods

We compared the operative times, blood loss, results of the UGI study, morbidity and mortality, and operative and hospital costs between the patients who underwent conventional reconstruction and those who underwent the stapled Roux-en-Y reconstruction.

Statistical Analysis

Statistical analysis was done using the chi-square test or Fisher's exact test for univariate analysis. We used the Mann–Whitney U-test to compare the variables between the two groups. Data are expressed as medians and ranges. A P value of less than 0.05 was considered significant.

Results

There was no in-hospital mortality (0%) and the overall morbidity rate was 63%. Pancreatic fistula developed in 33 (46%) patients (grade A in 12, grade B in 20, and grade C in 1) and was clinically significant in 29%. Twenty-five (35%) patients suffered DGE. Major morbidity included grade C pancreatic fistula in one patient, who suffered sepsis and required percutaneous drainage of the abdominal fluid under computed tomography (CT) guidance. Another major morbidity was anastomotic bleeding after stapled reconstruction, which resulted in shock status of the patient on POD 16. Three other patients suffered bleeding from the stapled anastomotic site: on POD 1 in one and on POD 2 in two. The bleeding was indicated by decreased hematocrit levels as the patients' general condition was stable. All three underwent endoscopic clipping of the bleeding points, and recovered conservatively. None of the patients suffered arterial bleeding associated with the pancreatic fistula.

There were no remarkable differences in the patients' backgrounds, operative parameters, incidence of pancreatic fistula, or overall morbidity rates between the patients who underwent conventional reconstruction and those who underwent Roux-en-Y stapled reconstruction (Table 1). Gastrostomy was not done in the stapled reconstruction group patients, but none of these patients required reinsertion of a nasogastric tube. The results of the UGI study were significantly better in the 26 patients with stapled reconstruction than in the 43 patients who underwent conventional reconstruction (P = 0.03). The exception was the median duration between surgery and the UGI study, which was significantly longer in the conventional reconstruction group than in the stapled reconstruction group (6 days vs 5 days, $P < 0.001$). The duration between surgery and start of oral intake, the incidence of DGE, and the hospital stay were significantly reduced in the stapled reconstruction group vs. the conventional reconstruction group ($P <$ 0.001, P = 0.04, and P = 0.008, respectively). The opera-

Table 2. Operative outcomes of patients who underwent conventional reconstruction versus Roux-en-Y stapled reconstruction

	Conventional ($n = 46$)	Roux-en-Y, stapled ($n = 26$)	P value
Removal of nasogastric tube or closure of gastrostomy (POD)	6	1	<0.001*
Results of UGI study ≤10 PODs			
Grade A	22	20	0.03*
Grade B or C	21	6	
Day of UGI study	6 (4–10)	5 (3–9)	<0.001*
Resumption of regular diet (POD)	13 (5–62)	6 (4–20)	<0.001*
DGE			
Absent	26	21	0.04*
Present	20	5	
Pancreatic fistula			
None, Grade A	34	17	0.44
Grade B or C	12	9	
Placement of drains	13 (6–72)	11 (2–65)	0.08
Minor complications	27	16	0.81
Major complications	1	1	0.68
Hospital stay (days)	27 (14–89)	21 (10–37)	0.008*
Operative costs ($)	8000	8700	0.009*
Hospital costs ($)	18000	17000	0.049*
Mortality	0	0	ND

UGI, upper gastrointestinal; POD, postoperative days; DGE, delayed gastric emptying; ND, not determined
* $P < 0.05$

tive costs were US$700 higher ($P = 0.009$), but the hospital cost was significantly ($1000) lower ($P = 0.049$) in the stapled reconstruction group than in the conventional group (Table 2).

Discussion

The findings of the present study suggest that the new Roux-en-Y reconstruction, using circular or linear staplers during PD, might improve the early passage of duodenojejunostomy and gastrojejunostomy, and thereby reduce the incidence of DGE. The stapled anastomosis felt very neat to the touch, which might contribute to preventing postoperative anastomotic edema. In addition, Roux-en-Y reconstruction would eradicate bile reflux and position the stomach tube vertically; thereby potentially assisting in advancing the stomach contents.[11] The incidence of anastomotic bleeding in this initial study (15%) was unexpectedly higher than that after gastric or colorectal surgery. Anastomotic bleeding was a major drawback in stapled reconstruction, so we strongly recommend confirming hemostasis of the anastomotic site via the jejunal loop and administering proton pump inhibitors postoperatively. Furthermore, we hope that the quality of staplers will improve in the future.

The passage of contrast medium in the UGI study and the recommencement of oral intake were improved significantly ($P = 0.03$ and < 0.001, respectively) and the incidence of DGE was reduced significantly ($P = 0.04$) by the introduction of stapled reconstruction (Table 2). Delayed gastric emptying has been reported to be affected by several other factors including gastric dysrhythmias due to intra-abdominal complications,[5,6] gastric atony after duodenal resection in response to the reduction of motilin,[7,17] pylorospasm secondary to vagotomy,[8] and angulation of the reconstructed alimentary tract.[18] A prospective randomized trial showed that erythromycin,[7] cyclic enteral feeding,[9] and antecolic reconstruction[10] all reduced DGE, whereas a retrospective study showed that ante-mesenteric reconstruction,[6] vertical reconstruction,[11] and straight-lined antecolic reconstruction[12] improved the oral intake. However, to our knowledge, the present study is the first to show the possible advantages of stapled Roux-en-Y reconstruction during PD.

This study is a retrospective analysis of one surgeon's experience. During the initial 3 years, oral intake was resumed very late, partly because we feared early bleeding, which might evoke pancreatic fistula. Since the introduction of stapled reconstruction in August 2006, the incidence of pancreatic fistula has been gradually decreasing, probably as a result of the improved surgical skill in pancreaticojejunostomy during PD. Fortunately, since the introduction of internal stents in pancreaticojejunostomy and in hepaticojejunostomy, external drainage is no longer needed. This may contribute to early discharge from hospital. Gastrostomy was placed in 97% of the patients who underwent the conventional

method, but it was not required in any of those who underwent stapled reconstruction. This strongly affected the duration of gastric decompression. The difference in hospital stay between the two groups may be reflected not only by the methods of alimentary reconstruction, but also by the placement of drainage or feeding tubes, and management of drainage tubes. A multi-institutional prospective randomized trial will be necessary to evaluate the efficiency of this stapled reconstruction during PD.

The possible advantages of the Roux-en-Y stapled reconstruction we described are as follows: standardization of reconstruction, irrespective of the attending surgeon; easy reconstruction; possible prevention of anastomotic edema and subsequent stricture, brought about by continuous two-layer anastomosis; and a complete diversion of the bile and pancreatic juice from the anastomosis. On the other hand, its disadvantages are as follows: higher cost; a risk of bleeding at the anastomotic site; and mass production of industrial waste. It is noteworthy that the operative costs were higher in the stapled group, but the overall hospital costs were higher in the conventional method group. This is most likely due to the reduced costs for hospital stay and nutritional support required in the stapled group.

In conclusion, our retrospective analysis shows that this new method of Roux-en-Y reconstruction using staplers during PD might reduce the incidence of delayed gastric emptying vs. conventional hand-sewn reconstruction. However, this study is a historical cohort analysis of one surgeon's experience. The improvement in the surgeon's skills, the change in drainage-tube placement, and the early return to a normal diet may have created bias. Thus, further study will be necessary to evaluate the utility of this new method.

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Clinicopathological Characteristics of Intrahepatic Cholangiocellular Carcinoma Presenting Intrahepatic Bile Duct Growth

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Background: Intrahepatic cholangiocellular carcinoma (ICC) presenting intraductal growth (IG) has distinct clinicopathological features with a favorable prognosis. The mass-forming (MF) plus IG type of ICC has not been previously investigated.

Methods: One hundred forty-four patients with ICC underwent surgical resections and were classified according to the macroscopic type. The clinicopathological features of the IG type (n = 7) and MF plus IG type (n = 14) were retrospectively analyzed. The clinicopathological differences between the MF plus IG type and MF plus periductal infiltrating (PI) type (n = 37) were compared.

Results: All of the patients with the IG type had no sign of recurrence and survived. The 5-year overall survival rates was significantly better in the MF plus IG type than in the MF plus PI type (41.3% vs. 13.3%, $P = 0.034$). Significant differences were recognized between the MF plus IG type and MF plus PI type, in terms of vascular resection ($P = 0.030$), mucosal extension ($P = 0.006$), and postoperative recurrence ($P = 0.004$).

Conclusions: The MF plus IG type had significantly better prognosis than the MF plus PI type. The IG type and MF plus IG type should be distinguished from other types even if hepatic hilar confluence is involved, because of the favorable outcome after surgery.

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KEY WORDS: **intrahepatic cholangiocellular carcinoma; intraductal growth type; mass-forming plus intraductal growth type; surgical resection**

INTRODUCTION

Intrahepatic cholangiocellular carcinoma (ICC) is the second most common primary liver cancer after hepatocellular carcinoma accounting for only 5% of all primary liver cancers [1]. ICCs have been classified into several types based on the macroscopic appearance of the tumors, because of their different clinical features and prognosis [2–6]. The intraductal growth (IG) type, which is classified as one of major macroscopic types according to the classification of primary liver cancer of the Liver Cancer Study Group of Japan [7], shows intraductal papillary and/or granular extension sometimes associated with superficially spreading mucosal carcinoma or intraductal tumor thrombi. This type of ICC has been less frequently encountered and it also has a significantly better prognosis [8–11].

The mass-forming (MF) type ICC creates an apparent tumor in the liver, but sometimes spreads to the major Glisson's branches or hepatic hilar confluence. Such tumors have been divided as subtypes of MF type ICC, which are macroscopically recognized as the MF plus periductal infiltrating (PI) type and MF plus IG type ICC [7]. Several authors [5,12,13] reported that the MF plus PI type ICC might be one of the advanced states of the MF type ICC and long term survival is not expected in this type tumor if treated by surgery alone. However, the clinicopathological features of the MF plus IG type ICC have not been previously described.

The present study retrospectively analyzed the clinicopathological features and surgical outcome of ICC presenting intrahepatic bile duct growth with special reference to the distinct macroscopic types. The clinicopathological differences between the MF plus IG type and MF plus PI type ICC were also compared.

PATIENTS AND METHODS

Between January 1993 and April 2006, 144 patients with ICC underwent macroscopic curative resections at the National Cancer Center Central Hospital, Tokyo, Japan. All the resected specimens were reviewed to investigate the macroscopic types of the tumors according to the Classification of Primary Liver Cancer by the Liver Cancer Study Group of Japan by the pathologist (Hidenori Ojima) [7]. The macroscopic types included: the MF type, the PI type (characterized by tumor infiltration along the bile duct, occasionally involving the surrounding blood vessels and/or hepatic parenchyma), or the IG type (characterized by papillary and/or granular growth, or both, into the bile duct lumen, occasionally showing superficial extension); when more than one type of lesion was found, all of the types detected and the predominant type were recorded. The distribution of the macroscopic tumor types in these cases was: MF type (n = 74); MF plus PI type (n = 37); MF plus IG type (n = 14); PI type (n = 9); IG type (n = 7); PI plus MF type (n = 2); non-classifiable (n = 1). The PI plus MF type ICC is mainly characterized by the PI type as the predominant type and has minimum component of MF appearance. In this study, this type ICC was distinguished from the MF plus PI type ICC. Patients with the IG and MF plus IG type ICC were the focus of the present study. Figure 1 shows the representative dynamic CT images, macroscopic appearance and schematic illustration of the MF plus IG type ICC (Case 10).

The extent of the hepatic resection was depended on both the tumor location and the mode of extension. Dissection of the regional lymph nodes was performed in patients with the MF type ICC when the

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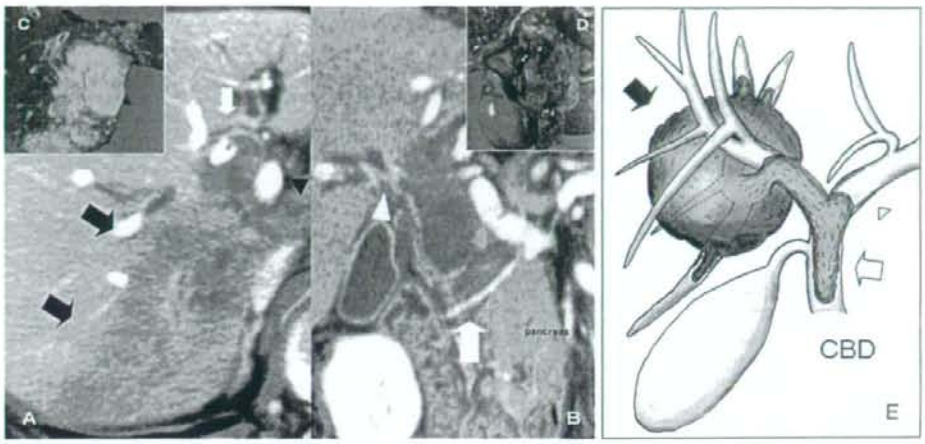

Fig. 1. Representative dynamic CT images of the MF plus IG type ICC (Case 10). **A**: A massive ill-defined tumor (black arrows) occupied the entire posterior segment of the right lobe with intraductal extension to the hilar confluence (a white arrow). An arrow head indicates the invasion to the caudate lobe with bile duct dilation. **B**: A tumor located in the common bile duct extended just above the upper margin of the pancreas (a white arrow). The tumor involved the right hepatic artery (an arrow head). **C**: A gross appearance of a mass-forming plus intraductal growth type of intrahepatic cholangiocellular carcinoma. An irregular whitish tumor was located in the posterior segment of the right lobe. **D**: Intraductal component of the tumor extended to the common hepatic bile duct. **E**: Schematic illustration shows mass-forming type component (a black arrow) located on the mainly posterior segment. Intraductal extension from the posterior branch to the common bile duct was demonstrated as intraductal growth (a white arrow). Mucosal extension (a white arrow head) was seen into the left hepatic duct. CBD: common bile duct.

patients were suspected to have lymph node involvement. When the hepatic hilus was involved with tumors, an extended lobectomy with a combined resection of the caudate lobe, extrahepatic bile duct and regional lymph nodes was performed. Frozen sections of the specimens of the bile duct stumps were histologically examined. When the resected margin was positive, further excision of the bile duct was performed as far as anatomically possible.

The pathology of the resected liver tumors was studied according the general rules for the Clinical and Pathological Study of primary liver cancer [7]. The gross type, differentiation, mucosal extension, lymph node metastasis, portal vein invasion, hepatic vein invasion, intrahepatic metastasis, and surgical margin were noted. The branch of the bile duct tumor extensions and the segment of tumor location were defined using the Couinaud's classification [14]. Mucosal extension was defined as non-invasive carcinoma spread beyond the main tumor mass. A positive surgical margin was defined by the presence of cancer cells at the cut surface of the resected margin of the liver, the dissected periductal structures including the radial resection margin in the hepatoduodenal ligament and the bile duct cut ends including the proximal hepatic side and distal duodenal side.

All patients were followed in the outpatient clinic where abdominal ultrasound, CT scan, and the measurement of carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) levels were done every 3–6 months after surgery. The median follow-up duration of the IG type and MF plus IG type ICC were 60 months (20–130 months) and 37 months (4–142 months), respectively.

A total of 12 clinicopathological factors, were compared between patients with the MF plus IG type and MF plus PI type ICC. Survival estimates were calculated using the Kaplan–Meier method and differences in univariate survival were examined using the log rank test. Comparisons were performed by independent two-sample t-test. Pearson's χ test and Fisher's exact test were used for nominal variables. All statistical analyses were performed using the Software Package for Social Sciences, version 11.5J for Windows® (SPSS, Chicago, IL). $P < 0.05$ was considered to be statistically significant.

RESULTS

Table I shows the clinicopathological features of the IG type ICC. There were three men and four women and their mean age was 63 years (range: 53–73). Epigastric discomfort was manifested in two cases and general fatigue in one case, but no patients developed jaundice. No biliary tract stones were observed in all cases. The tumor size ranged from 14 to 80 mm with a mean of 39 mm. Parenchymal invasion was observed in only one patient (13%) and interstitial invasion in four patients (57%) with the IG type ICC. There were no cases with lymph node metastasis, portal vein invasion, hepatic vein invasion, and intrahepatic metastasis. All patients with the IG type ICC have shown no sign of recurrence and were alive at the time of submission of this manuscript. One patient who had a positive surgical margin is still alive at 20 months with no sign of recurrence. No adjuvant therapy was administrated in this patient. The clinicopathological findings of the MF plus IG type ICC are summarized in Table II. There were nine men and five women and their mean age was 65 years (range: 51–78). One patient died due to postoperative liver failure (Case 3). Jaundice was manifested in four cases and epigastric discomfort in three cases. Biliary tract stones were present in one patient. Hepatitis B surface antigen was positive in two patients and hepatitis C virus antibody was positive in two patients. The tumor size ranged from 20 to 90 mm with a mean of 52 mm. Lymph node metastases, portal vein invasion, hepatic vein invasion, and intrahepatic metastasis were observed in 36% (5/14), 79% (11/14), 29% (4/14), and 21% (3/14), respectively. Six patients (35%) died of tumor recurrence and the remaining seven patients are currently alive with no sign of recurrence. Oral uracil-tegafur (UFT) was administrated in one patient with positive surgical margin (Case 10), and no adjuvant therapy was done in the other two patients with positive surgical margin.

A comparison of the clinicopathological factors in the patients with the MF plus IG type and MF plus PI type ICC is shown in Table III. Significant differences were recognized between the two groups in terms of the presenting mucosal extension ($P = 0.006$) and vascular

TABLE I. Characteristics of the Patients With Intraductal Growth Type of Intrahepatic Cholangiocellular Carcinoma

No	Sex/age	Symptom	Location	Size (mm)	Operation	Bile duct resection	Surgical margin	Histologic differentiation	Parenchymal invasion	Interstitial invasion	Mucosal extension	Survival time (month)
1	F/57	−	B2+3	30	ELL	+	−	Pap	−	+	−	130
2	M/63	−	B2	42	ELL	−	−	Pap	−	−	+	35
3	F/53	+	B6	14	ERL	+	−	Pap	+	+	−	90
4	F/70	+	B2+3	33	ELL	−	−	Well	−	+	+	77
5	M/59	−	B3	15	ELL	+	−	Well	−	−	−	45
6	F/63	+	B8~Bi	80	RL+PD	+	−	Well	−	+	+	22
7	M/73	−	B5,6,7,8+RHD,LHD	60	ERL	+	+	Well	−	−	+	20

B, branch, defined by the Couinaud's nomenclature; Bi, inferior portion of bile duct; RHD, right hepatic duct; LHD, left hepatic duct; ELL, extended left lobectomy; RL, right lobectomy; ERL, extended right lobectomy; PD, pancreatoduodenectomy; Pap, papillary.

resection ($P = 0.030$). Among the three patients with a surgical margin positive in the MF plus IG type, one patient had cancer cells only in the mucosal layer (Case 4) and two patients had tumors exposed on the cut surface of the liver (Cases 6 and 10). On the other hand, among the 17 patients with a positive surgical margin in the MF plus PI type, 2 patients had cancer cells only in the ductal mucosal layer and 7 patients had cancer cells in the connective tissue surround the bile duct and 13 patients had tumors exposed on the cut surface of the liver and the radial resection margin in the hepatoduodenal ligament.

The overall survival rates in the patients with the MF plus IG type and MF plus PI type of ICC are shown in Figure 2. The 5-year overall survival rates and median survival time were significantly better in the MF plus IG type than in the MF plus PI type (41.3% and 55 months vs. 13.3% and 19 months, $P = 0.034$). The 5-year overall survival rates and median survival time of the MF type were 37.8% and 28 months, respectively. There were significant differences in the overall survival between the IG type and MF type, and between the IG type and MF plus IG type, respectively ($P = 0.014$, 0.041). On the other hand, there was no significant difference in the overall survival between the MF plus IG type and MF type ($P = 0.517$).

Recurrence was observed in 5 (36%) of the MF plus IG type and 29 (78%) of the MF plus PI type ICC, respectively. Recurrence was significantly frequent in patients with the MF plus PI type ($P = 0.004$). The most frequent site of recurrence in the MF plus IG type ICC was the liver (n = 3). Local recurrence was more frequent in patients with the MF plus PI type (n = 7, 19%) than the MF plus IG type (n = 1, 7%), but there were no significant differences between the two groups ($P = 0.302$).

DISCUSSION

One of the distinct macroscopic types presenting IG is the IG type ICC, which has been rarely described [5,8,13,15–17]. Yamamoto et al. [5] reported the 5-year survival rate in 10 patients with the IG type was 69%. Suh et al. [8] reported that the 5-year survival rate in 16 patients with the IG type was 80%. Ohtsuka et al. [13] reported that all 9 patients with the IG type have remained alive during follow-up periods ranging from 8 to 72 months, with only one patient developing a recurrence. The present study confirmed that the incidence of this type was only 6% (7/144) with the favorable biological behavior due to the absence of lymph nodes metastases, vascular invasion and intrahepatic metastases with well or papillary differentiation.

Another macroscopic type of ICC presenting IG is the MF plus IG type ICC, which has been scarcely documented, because there are only two case reports describing the clinicopathological features of the MF plus IG type ICC [17,18]. This type of ICCs sometimes mimic the MF plus PI type ICCs, which also invade the large Glisson's sheath or hepatic hilar confluence but demonstrate poor prognosis even after an extended surgical resection [5,12,13]. The current study clarified the clinicopathological characteristics of the MF plus IG type ICC and

TABLE II. Characteristics of the Patients With Mass-Forming Plus Intraductal Growth Type of Intrahepatic Cholangiocellular Carcinoma

No	Sex/age	Symptom/ Jaundice	Location	Size (mm)	Operation	Bile duct resection	Surgical margin	Histologic differentiation	N	VP	VV	IM	Mucosal extension	Survival time (month)	Outcome	Recurrence
1	M/68	−/−	S2,3	58	ELL	+	−	Well	−	−	−	−	+	142	Alive	None
2	F/70	+/+	S2,3,4	80	ELL	+	−	Well	−	−	−	−	+	55	Died	Liver, bone
3[a]	M/72	−/−	S4	31	ELL	+	−	Por	+	+	−	+	−	6	Died	None
4	M/53	−/−	S6,7	60	PR	−	+	Well	−	+	−	−	+	83	Alive	None
5	M/55	+/−	S5,6,7,8,1	60	ERL	+	−	Mod	+	+	−	−	+	53	Alive	None
6	M/65	+/−	S4,5,1,6	80	ERL	+	+	Mod	+	+	+	+	+	4	Died	Liver
7	F/78	−/−	S7	35	RL	−	−	Mod	−	+	−	−	−	44	Alive	None
8	M/56	+/+	S4,2	40	ELL	+	−	Mod	+	+	+	−	+	14	Died	Local recurrence
9	M/74	+/−	S2+3	20	ELL	−	−	Mod	−	+	−	−	+	39	Alive	None
10	F/68	+/+	S7	90	ERL	+	+	Well	−	+	−	−	+	17	Died	Liver
11	F/66	−/−	Post~RHD	62	ERL	+	−	Mod	−	−	−	−	−	5	Alive	None
12	M/65	+/+	S4	60	ELL	+	−	Mod	−	+	+	−	+	12	Died	Bone
13	M/70	−/−	S4	19	ELL	+	−	Mod	+	+	−	−	+	19	Alive	None
14	F/51	−/−	S4	40	ELL	+	−	Mod	−	+	+	+	+	22	Alive	None

[a]Died of liver failure caused by bile peritonitis 169 days after surgery.

S, segment; defined by the Couinaud's nomenclature Post, posterior segment; RHD, right hepatic duct; ELL, extended left lobectomy; PR, partial resection; ERL, extended right lobectomy; RL, right lobectomy; Mod, moderately; Por, poorly; N, lymph node metastasis; VP, portal vein invasion; VV, hepatic vein invasion; IM, intrahepatic metastasis.

TABLE III. Relationship Between the Macroscopic Classification and Other Clinicopathologic Factors

Factors	MF + PI type (n = 37)	MF + IG type (n = 14)	*P*-value
Symptom			
Absent	20 (54%)	7 (50%)	0.796
Present	17 (46%)	7 (50%)	
Jaundice			
Absent	24 (65%)	10 (71%)	0.657
Present	13 (35%)	4 (29%)	
Size			
>5.0 cm	21 (57%)	6 (43%)	0.375
≤5.0 cm	16 (43%)	8 (57%)	
Vascular resection and reconstruction			
Absent	27 (73%)	14 (100%)	0.030
Present	10 (27%)	0 (0%)	
Extrahepatic bile duct resection			
Absent	5 (13%)	5 (36%)	0.075
Present	32 (87%)	9 (64%)	
Histologic differentiation			
Well papillary	5 (14%)	4 (29%)	0.225
Others	31 (86%)	10 (71%)	
Mucosal extension			
Absent	24 (65%)	3 (21%)	0.006
Present	13 (35%)	11 (79%)	
Lymph node metastasis			
Absent	14 (39%)	9 (64%)	0.106
Present	22 (61%)	5 (36%)	
Portal vein invasion			
Absent	7 (19%)	3 (21%)	0.840
Present	30 (81%)	11 (79%)	
Hepatic vein invasion			
Absent	18 (51%)	10 (71%)	0.201
Present	17 (49%)	4 (29%)	
Intrahepatic metastasis			
Absent	26 (70%)	11 (79%)	0.553
Present	11 (30%)	3 (21%)	
Resectional margin			
Positive	17 (46%)	3 (21%)	0.110
Negative	20 (54%)	11 (79%)	

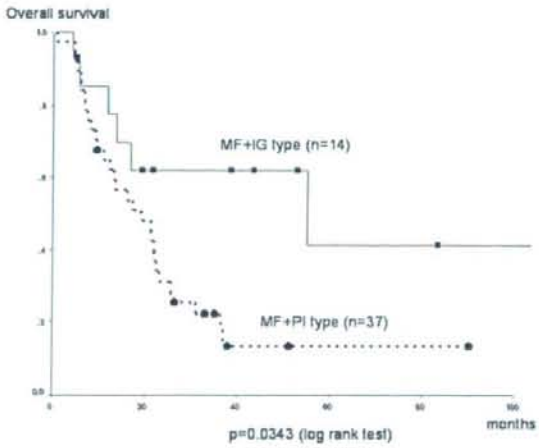

Fig. 2. Overall survival rates after the surgical resection of intrahepatic cholangiocellular carcinoma, according to the gross appearance.

disclosed that this type had a favorable prognosis, in comparison to the MF plus PI type ICC. On the other hand, there was no significant difference in the overall survival between the MF plus IG type and MF type. However, the limitation of the current study was retrospectively evaluated in the relatively small case series. The large number of patient's accumulation might be necessary to deduce the definitive conclusion, and it is important for farther studies to be sponsored by large cooperative groups.

The reason for a favorable prognosis in patient with the MF plus IG type ICC is uncertain, because there are no statistical differences in terms of the prognostic variables such as tumor size, portal vein invasion, hepatic vein invasion, and lymph node metastases in comparison to those of the MF plus PI type. One possibility is the infiltrative characteristics of the MF plus PI type ICC, because the incidence of vein resection was more frequent in patients with the MF plus PI type. Okano et al. [19] reported that macroscopic bile duct invasion of colorectal liver metastases might reflect an indolent biological behavior and Esaki et al. [20] reported that a favorable long-term result might be expected in patients with hepatocellular carcinoma with macroscopic bile duct invasion. With regard to ICC, IG with MF tumors might represent more favorable biological behavior than periductal infiltration with MF.

Mucosal cancer extension is an important clinical characteristic of the IG type and MF plus IG type ICC. It was observed in 4 patients (57%) with the IG type and 11 patients (79%) with the MF plus IG type ICC. Sakamoto et al. [21] reported that mucosal extension was

observed in 88% of papillary or nodular tumor of hilar bile duct carcinomas, but in only 4% of nodular-infiltrating or diffusely infiltrating tumors. This indicates that the IG type of ICC might have similar characteristics to papillary or nodular tumor of bile duct carcinoma with well histological differentiation. Accurate operative assessment of cancer extension in the bile duct margins may be extremely difficult in these diseases, thus, a frozen section diagnosis is necessary in this type of ICC during surgery.

Local recurrence was rare in patients with the MF plus IG type in comparison to those in patients with the MF plus PI type. This might be because a positive resection margin in the connective tissue surrounding the bile duct was frequently observed in patients with the MF plus PI type. A positive resection margin in the connective tissue surrounding the bile duct, which was strongly associated with the positive radial resection margin in the hepatoduodenal ligament, is linked with poorer prognosis in comparison to a positive mucosal margin. If the resection margin is positive only in the ductal mucosal layer in patients with the MF plus IG type ICC, additional resection during surgery should be considered.

Preoperative identification of the IG of tumor has been remarkably improved with recent advancements in imaging [22]. The IG type and MF plus IG type ICC should be distinguished from other macroscopic types of ICC even in involving the hepatic hilar confluence, because they are associated with a favorable outcome after surgery. The current results may therefore be useful for assessing the preoperative diagnosis, planning surgical treatment and considering postoperative chemotherapy of ICC.

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Intraductal carcinoma component as a favorable prognostic factor in biliary tract carcinoma

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The aim of this study is to evaluate the prognostic impact of an intraductal carcinoma component and bile duct resection margin status in patients with biliary tract carcinoma. An intraductal carcinoma component was defined as carcinoma within the bile duct outside the main tumor nodule consisting of a subepithelial invasive component. Surgically resected materials from 214 patients were evaluated by histological observations. Seventy-nine patients (36.9%) with an intraductal carcinoma component infrequently developed large tumors and infrequently showed deep invasion and venous, lymphatic and perineural involvement in the main tumor nodule. An intraductal carcinoma component was inversely correlated with advanced clinical stage, and was shown to be a significantly favorable prognostic factor by both univariate and multivariate analyses. Proximal (hepatic) side bile duct resection margin status was categorized into negative for tumor cells, positive with only an intraductal carcinoma component [R1 (is)], and positive with a subepithelial invasive component (R1). Forty-five patients (21.0%) with an R1 resection margin had a poorer prognosis than 148 patients (69.2%) with a negative resection margin, whereas 21 patients (9.8%) with an R1 (is) resection margin did not. In patients with an R1 resection margin, the risk of anastomotic recurrence was higher, and the period until anastomotic recurrence was shorter, than in patients with an R1 (is) resection margin. Surgeons should not be persistent in trying to achieve a negative surgical margin when the intraoperative frozen section diagnosis is R1 (is), and can choose a safe surgical procedure to avoid postoperative complications. (*Cancer Sci* 2009; 100: 62–70)

Biliary tract carcinoma still has a poor prognosis, and most cases are at an advanced stage when patients present with symptoms. Previous studies of extrahepatic bile duct carcinoma and hilar cholangiocarcinoma have indicated that surgical resection is the only curative treatment for affected patients.[1–10] Biliary tract carcinoma is remarkable because of its tendency for superficial extension by wide intraductal carcinoma.[11–14] However, it is difficult to accurately estimate the extent of the intraductal carcinoma component in the biliary tract on the basis of preoperative imaging studies.[13,15–18] It is feasible that intraoperative histological diagnosis using frozen sections may detect tumor involvement at the bile duct resection margin. Surgeons are required to make an immediate decision about the resection area based on intraoperative frozen section diagnosis. However, to our knowledge, no previous study has examined the clinicopathological significance and prognostic impact of an intraductal carcinoma component with reference to bile duct resection margin status in patients with biliary tract carcinoma.

In this retrospective study, the presence or absence of an intraductal carcinoma component and bile duct resection margin status were evaluated by histological observations of all surgically resected materials from 214 patients with biliary tract carcinoma who underwent radical surgery with curative intent. In order to provide a yardstick for surgeons who depend on the results of frozen section diagnosis during surgery, we examined the correlation between an intraductal carcinoma component and bile duct resection margin status on the one hand, and clinicopathological parameters on the other, and also the prognostic impact of an intraductal carcinoma component and bile duct resection margin status.

Materials and Methods

Patients and specimens. The study included 214 patients with biliary tract carcinoma who underwent radical surgery with curative intent at the National Cancer Center Hospital, Tokyo, Japan, between May 1965 and December 2003. Patients who died in hospital or within 100 days after surgery, and patients who underwent biopsy or palliative surgery, were not included. The included patients comprised 150 men and 64 women, ranging in age from 33 to 83 (mean 63.4) years.

The main tumor nodule was located in the lower, middle and upper thirds of the extrahepatic bile duct, the entire extrahepatic bile duct, the hilar bile duct, and intrahepatic bile duct adjacent to the hilar area in 27, 38, 14, 5, 77, and 53 patients, respectively. Patients with carcinoma of the peripheral intrahepatic bile duct were excluded. Pancreatoduodectomy (PD), extrahepatic bile duct resection (EHBD), hepatic resection with extrahepatic bile duct resection (HR+EHBD), hepatic resection (HR) and combined hepatectomy and pancreatoduodectomy (HPD) were performed in 47, 19, 124, 16 and 8 patients, respectively. The formalin-fixed surgically resected specimens were cut into slices at intervals of 0.5–0.7 cm, and all the sections were embedded in paraffin and routinely processed for microscopical examination. All tumors were classified according to the pathological tumor-node-metastasis (TNM) classification.[19] Intrahepatic bile duct carcinomas adjacent to the hilar area, for which TNM criteria have never been established, were classified according to the TNM classification for extrahepatic bile duct carcinoma. This study was approved by the Ethical Committee of the National Cancer Center, Tokyo.

Evaluation of an intraductal carcinoma component and bile duct resection margin status. The intraductal carcinoma component was defined as carcinoma within the bile duct and its small branch outside the main tumor nodule consisting of a subepithelial invasive component (Fig. 1). For cases in which intraoperative frozen section diagnosis of the ductal stump had been performed, the proximal (hepatic) side bile duct resection margin status was histologically assessed by review of the frozen section and its re-fixed permanent section with reference

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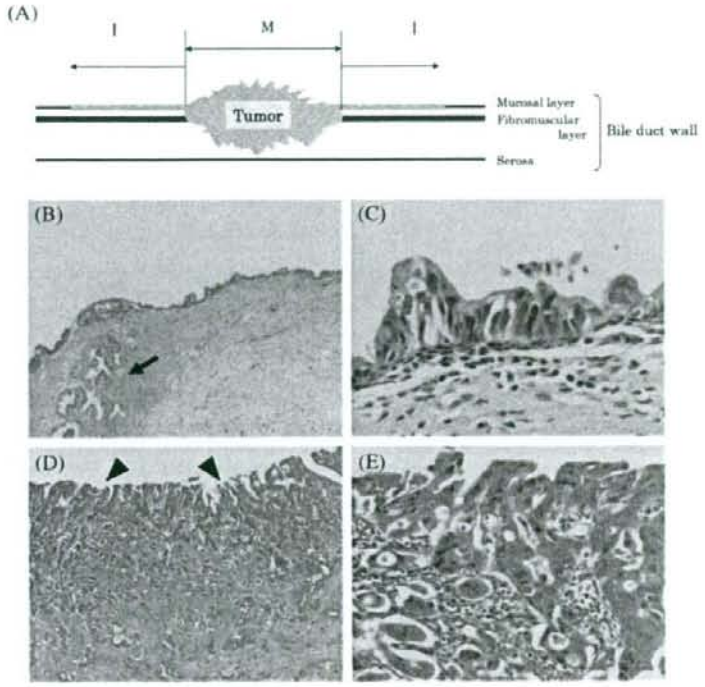

Fig. 1. Definition of an intraductal carcinoma component (I). (A) I is defined as intraductal carcinoma in the bile duct and its small branch outside the main tumor nodule (M) consisting of a submucosal invasive component. (B and C) Microscopic view of an example of I in the bile duct and its small branch (arrow). Hematoxylin and eosin (H&E) stain, original magnification ×40 (B) and ×400 (C). (D and E) Microscopic view of an example of M. Carcinoma *in situ* inside M (arrow heads) is not considered as I in this study. H&E stain, original magnification ×40 (D) and ×200 (E).

to the extent of the tumor in formalin-fixed surgically resected specimens. For cases in which intraoperative frozen section diagnosis of the ductal stump had not been performed, proximal side bile ductal resection margin status was histologically assessed by review of the formalin-fixed surgically resected specimens.

Follow-up and assessment of anastomotic recurrence at the bile duct resection margin. All 214 patients were followed for more than 100 days, and the mean duration of follow-up was 1215 days. Follow-up examination was performed using computed tomography, abdominal ultrasonography, and measurement of the serum carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) levels every 3–6 months by surgeons. Anastomotic recurrence at the proximal side of the bile duct resection margin was diagnosed only in patients with a positive resection margin. In such patients, when a mass lesion was detected after dilatation of the bile duct in the residual liver because of obstruction of the anastomosis site, using radiological evaluation including computed tomography and ultrasonography, surgeons considered that the patient had anastomotic recurrence (not local recurrence in which perineural invasion around the hepatic artery and/or involved regional nodes first formed a mass lesion). Causes of death were determined from the medical records.

Statistical analyses. Correlations between presence or absence of an intraductal carcinoma component and bile duct resection margin status on the one hand and clinicopathological parameters on the other were analyzed using chi-squared test.

Person-days of follow-up were calculated from the date of surgical resection until date of death or end of the study period (March 8, 2005), whichever occurred first. The crude rate of all-cause deaths was calculated by dividing the number of deaths by the number of person-days. Similarly, person-days of follow-up were calculated from the date of surgical resection until date of death, date of diagnosis of anastomotic recurrence, or end of the study period (March 8, 2005), whichever occurred first. The crude rate of recurrence at the proximal side bile duct resection margin was calculated by dividing the number of cases with recurrence by the number of person-days. Survival curves were constructed using the Kaplan–Meier method, and differences in survival were evaluated using the log-rank test. The Cox proportional hazards model was used to estimate hazard ratio (HR) and 95% confidence interval (CI) of death or anastomotic recurrence by clinicopathologic factors using the SAS program (PROC PHREG) (SAS Institute Inc., Cary NC, US). All tests were two-sided and differences at $P < 0.05$ were considered statistically significant.

Results

Univariate analysis of correlation between an intraductal carcinoma component and clinicopathological parameters. An intraductal carcinoma component was positive in 79 (36.9%) of the 214 examined patients. Correlations between an intraductal carcinoma component and clinicopathological parameters were examined using univariate analysis (Table 1). Location of the main tumor nodule ($P = 0.007$), and histologic type ($P < 0.0001$) were significantly correlated with an intraductal carcinoma component (Table 1). Tumor size ($P = 0.01$), depth of invasion ($P < 0.0001$), venous involvement ($P < 0.0001$), lymphatic involvement ($P = 0.0006$), perineural involvement ($P < 0.0001$), the pathological assessment of the primary tumor (pT) ($P < 0.0001$), and pathological TNM stage ($P < 0.0001$) were each inversely correlated with presence of an intraductal carcinoma component: patients with an intraductal carcinoma component infrequently developed large tumors, infrequently showed deep invasion into the bile duct wall and venous, lymphatic and perineural involvement in the main tumor nodule, and were infrequently at an advanced stage when diagnosed (Table 1).

Table 1. Correlation between an intraductal carcinoma component and clinicopathological parameters in patients with biliary tract carcinoma

	No. of cases		*P* for difference*
	Intraductal carcinoma component		
	Negative (n = 135)	Positive (n = 79)	
Age (years)			0.01
<65	73	29	
≥65	62	50	
Sex			0.85
Male	94	56	
Female	41	23	
Location of the main tumor nodule			0.007
Lower third of extrahepatic bile duct	15	12	
Middle third of extrahepatic bile duct	17	21	
Upper third of extrahepatic bile duct	6	8	
Entire extrahepatic bile duct	2	3	
Hilar bile duct	54	23	
Intrahepatic bile duct	41	12	
Histologic type			<0.0001
Adenocarcinoma	129	55	
Papillary adenocarcinoma	1	21	
Others	5	3	
Tumor size (cm)			0.13
<3	54	40	
≥3	81	39	
Differentiation of adenocarcinoma			0.50
Well	34	18	
Moderate	80	29	
Poor	15	8	
Depth of invasion			<0.0001
Carcinoma *in situ* or invasion to fibromuscular layer	1	16	
Invasion into subserosa or beyond bile duct wall	134	63	
Venous involvement			<0.0001
Absent	6	19	
Present	129	60	
Lymphatic involvement			0.0006
Absent	9	18	
Present	126	61	
Perineural involvement			<0.0001
Absent	10	24	
Present	125	55	
pT classification			<0.0001
pT1-2	11	40	
pT3-4	124	39	
pN classification			0.06
pN0	64	48	
pN1	71	31	
TNM stage			<0.0001
0, IA, IB	8	32	
IIA	53	14	
IIB	62	28	
III	12	5	

*Chi-squared test.

Univariate analysis of correlation between an intraductal carcinoma component or clinicopathological parameters on the one hand and prognosis of patients on the other. Overall survival rates after resection were 33.2% at 5 years and 22.9% at 10 years. Hazard ratio (HR) and 95% confidence interval (CI) of all-cause deaths by an intraductal carcinoma component and other clinicopathological parameters were examined using univariate analysis (Table 2). Patients with an intraductal carcinoma component showed a significantly more favorable prognosis than patients without such a component (Table 2).

Multivariate analysis of prognostic impact of an intraductal carcinoma component. When all 214 patients were examined by multivariate analysis adjusted for age, operation day, type of surgical resection, tumor size, histologic type and tumor differentiation, depth of invasion, venous involvement, lymphatic involvement, perineural involvement and TNM stage, patients with an intraductal carcinoma component showed a significantly more favorable prognosis than patients without such a component (Table 3). When only the 117 patients who underwent complete resection (proximal side bile duct resection margin for all

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Table 2. Crude hazard ratio (HR) and 95% confidence interval (CI) of all-cause deaths by an intraductal carcinoma component and clinicopathological parameters

	No. of deaths	Person-days	Crude death rate†	Crude HR	95% CI	*P* for trend
Intraductal carcinoma component						
Negative	96	136 804	70.2	1.00		
Positive	35	123 209	28.4	0.39	0.27, 0.58	
Age (years)						
<65	58	137 562	42.2	1.00		
≥65	73	122 451	59.6	1.33	0.94, 1.87	
Sex						
Male	94	179745	52.3	1.00		
Female	37	80 268	46.1	0.84	0.57, 1.23	
Location of the main tumor nodule						
Lower third of extrahepatic bile duct	17	43 899	38.7	1.00		
Middle third of extrahepatic bile duct	23	43 696	52.6	1.04	0.56, 1.96	
Upper third of extrahepatic bile duct	10	18 228	54.9	1.17	0.54, 2.56	
Entire of extrahepatic bile duct	3	4837	62.0	1.07	0.31, 3.67	
Hilar bile duct	46	87 502	52.6	1.09	0.63, 1.91	
Intrahepatic bile duct	32	61 851	51.7	1.14	0.63, 2.06	
Histologic type						
Adenocarcinoma	123	211 330	58.2	1.00		
Papillary adenocarcinoma	5	37 000	13.5	0.25	0.10, 0.62	
Others	3	11 683	25.7	0.51	0.16, 1.61	
Tumor size (cm)						
<3	46	134 392	34.2	1.00		
≥3	85	125 621	67.7	1.82	1.27, 2.61	
Differentiation of adenocarcinoma						
Well	36	70 278	51.2	1.00		
Moderately	67	126 210	53.1	1.13	0.75, 1.69	
Poorly	20	14 842	134.8	2.56	1.47, 4.44	
Depth of invasion						
Carcinoma *in situ* or invasion to fibromuscular layer	3	37 435	8.0	1.00		
Invasion into subserosa or beyond bile duct wall	128	222 578	57.5	6.44	2.04, 20.3	
Venous involvement						
Absent	6	63 305	9.5	1.00		
Present	125	196 708	63.5	5.80	2.54, 13.3	
Lymphatic involvement						
Absent	9	76 294	11.8	1.00		
Present	122	183 719	66.4	4.67	2.25, 9.67	
Perineural involvement						
Absent	11	72 565	15.2	1.00		
Present	120	187 448	64.0	3.67	1.95, 6.89	
pT classification						
pT1-2	23	89 367	25.7	1.00		
pT3-4	108	170 646	63.3	2.32	1.47, 3.66	
pN classification						
pN0	57	176 738	32.3	1.00		
pN1	74	83 275	88.9	2.56	1.80, 3.65	
TNM stage						
0,IA,IB	15	77 359	19.4	1.00		<0.01
IIA	39	91 428	42.7	2.26	1.24, 4.12	
IIB	65	75 858	85.7	4.21	2.37, 7.46	
III	12	15 368	78.1	3.80	1.77, 8.15	

†per 100 000 person-days.

patients, distal [duodenal] side bile duct resection margin for patients who underwent HR + EHBR, resected margin of the pancreas for patients who underwent PD were all negative) were examined in order to eliminate the effect of surgical curability, an intraductal carcinoma component was still a favorable prognostic factor (Table 3).

Correlation between an intraductal carcinoma component and bile duct resection margin status. Although an intraductal carcinoma component has been proven to be a favorable prognostic factor, it is feasible that patients with such components frequently have tumor involvement at the bile duct resection margin. Therefore, the correlation between an intraductal carcinoma component and proximal side bile duct resection margin status (negative or positive) was examined statistically (Table 4). An intraductal carcinoma component was found to be correlated with bile duct resection margin status: patients with an intraductal carcinoma component more frequently had a positive resection margin than patients without such a component ($P = 0.0192$, Table 4). In

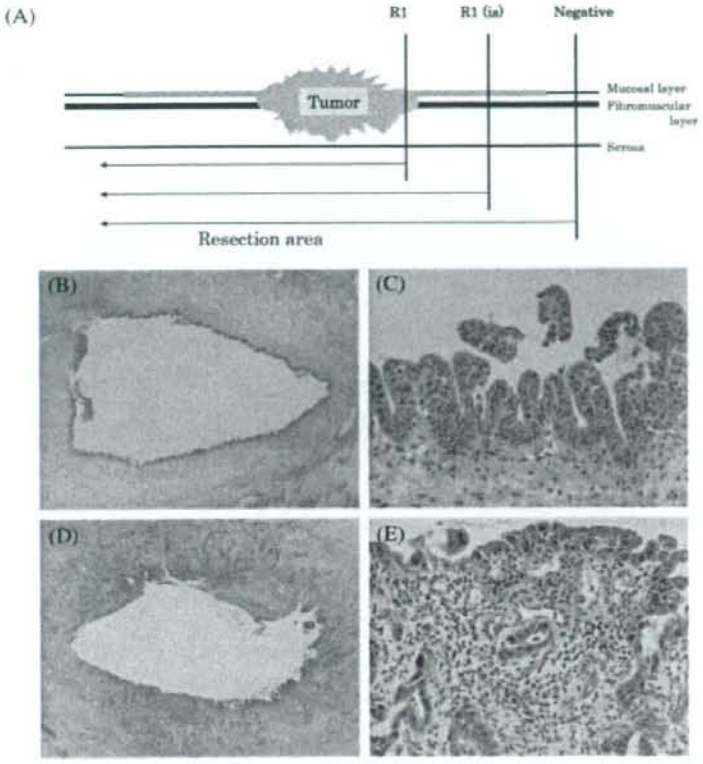

Fig. 2. Definition of bile duct resection margin status. (A) Bile duct resection margin status was categorized as negative for tumor cells (negative), positive with only an intraductal carcinoma component [R1 (is)], and positive with a subepithelial invasive component (R1). (B and C) Microscopic view of an example of an R1 (is) bile duct resection margin. Hematoxylin and eosin (H&E) stain, original magnification ×12.5 (B) and ×200 (C). (D and E) Microscopic view of an example of an R1 bile duct resection margin. H&E stain, original magnification ×12.5 (D) and × 200 (E).

Table 3. Adjusted hazard ratio (HR) and 95% confidence interval (CI) of all-cause death by an intraductal carcinoma component

	Adjusted HR	95% CI
Total (214 cases)		
Intraductal carcinoma component†		
Negative	1.00	
Positive	0.50	0.31, 0.79
Complete resection cases (117 cases)		
Intraductal carcinoma component†		
Negative	1.00	
Positive	0.31	0.13, 0.73

†Adjusted for age, operation day, type of surgical resection, tumor size, histologic type and tumor differentiation, depth of invasion, venous involvement, lymphatic involvement, perineural involvement, and TNM stage.

Table 4. Correlation between an intraductal carcinoma component and proximal side bile duct resection margin status

	No. of cases		*P* for difference*
	Bile duct resection margin status		
	Negative (n = 148)	Positive (n = 66)	
Intraductal carcinoma component			0.0192
Negative	101	34	
Positive	47	32	

*Chi-squared test.

order to further examine the clinicopathological significance and prognostic impact of bile duct resection margin status with reference to an intraductal carcinoma component, proximal side bile duct resection margin status was categorized as negative for tumor cells (negative), positive with only an intraductal carcinoma component [R1 (is)], or positive with a subepithelial invasive component (R1) (Fig. 2). According to the International Union Against Cancer, when invasive carcinoma is completely resected but histology shows an *in situ* component at the resection margin, the residual tumor is defined as R1 (is).[20] When the surgeon considers that resection has been complete but histology shows invasive carcinoma at the resection margin, the residual tumor is defined as R1.[20]

Univariate analysis of correlations between bile duct resection margin status and clinicopathological parameters. Bile duct resection margin status was negative, R1 (is) and R1 in 148 (69.2%), 21 (9.8%) and 45 (21.0%) of the 214 examined patients, respectively. Correlations between bile duct resection margin status and clinicopathological parameters were examined by univariate analysis (Table 5). Location of the main tumor nodule ($P = 0.0004$), histological type ($P = 0.008$) and venous involvement ($P = 0.009$) were each significantly correlated with bile duct resection margin status (Table 5).

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Table 5. Correlation between bile duct resection margin status and clinicopathological parameters in patients with biliary tract carcinoma

	No. of cases			*P* for difference*
	Proximal side ductal resection margin			
	Negative ($n = 148$)	R1 (is) ($n = 21$)	R1 ($n = 45$)	
Age (years)				0.09
< 65	77	6	19	
≥ 65	71	15	26	
Sex				0.81
Male	103	16	31	
Female	45	5	14	
Location of the main tumor nodule				0.004
Lower third of extrahepatic bile duct	23	2	2	
Middle third of extrahepatic bile duct	25	8	5	
Upper third of extrahepatic bile duct	8	1	5	
Entire of extrahepatic bile duct	0	1	4	
Hilar bile duct	53	7	17	
Intrahepatic bile duct	39	2	12	
Histologic type				0.008
Adenocarcinoma	129	13	42	
Papillary adenocarcinoma	15	6	1	
Others	4	2	2	
Tumor size (cm)				0.34
< 3	65	12	17	
≥ 3	83	9	28	
Differentiation of adenocarcinoma				0.16
Well	33	7	12	
Moderately	78	4	27	
Poorly	18	2	3	
Depth of invasion				0.06
Carcinoma *in situ* or invasion to fibromuscular layer	14	3	0	
Invasion into subserosa or beyond bile duct wall	134	18	45	
Venous involvement				0.009
Absent	20	5	0	
Present	128	16	45	
Lymphatic involvement				0.18
Absent	22	3	2	
Present	126	18	43	
Perineural involvement				0.57
Absent	26	3	5	
Present	122	18	40	
pT classification				0.08
pT1-2	37	8	6	
pT3-4	111	13	39	
pN classification				0.48
pN0	80	12	20	
pN1	68	9	25	
TNM stage				0.35
0, IA, IB	28	7	5	
IIA	48	5	14	
IIB	59	9	22	
III	13	0	4	

*Chi-squared test.

Univariate and multivariate analysis of prognostic impact of bile duct resection margin status. Univariate analysis revealed that although an R1 (is) bile duct resection margin had no prognostic impact in comparison with a negative bile duct resection margin, patients with an R1 bile duct resection margin showed a poorer prognosis than patients with a negative bile duct resection margin (Table 6). Surgical resection procedure, which was not examined in Table 3, is addressed in Table 6. None of the 66 patients with a positive resection margin [both R1 (is) and R1] had received any adjuvant therapy until recurrence was diagnosed.

When adjusted for age, operation day, surgical resection procedure, tumor size, histologic type, tumor differentiation, depth of invasion and venous involvement, although an R1 (is) bile duct resection margin had no prognostic impact in comparison with a negative bile duct resection margin, patients with an R1 bile duct resection margin showed a poorer prognosis than patients with a negative bile duct resection margin (Table 7).

Table 6. Crude hazard ratio (HR) and 95% confidence interval (CI) of all-cause deaths by bile duct resection margin status and clinicopathological parameters

	No. of deaths	Person-days	Crude death rate*	Crude HR	95% CI	*P* for trend*
Bile duct resection margin status						
Negative	82	209 492	39.1	1.00		<0.01
R1 (is)	11	22 476	48.9	1.00	0.53, 1.88	
R1	38	28 045	135.5	2.80	1.88, 4.18	
Surgical resection procedure						
PD	29	62 430	46.5	1.00		
EHBR	14	26 842	52.2	1.03	0.54, 1.94	
HR+EHBR	75	129 281	58.0	1.08	0.70, 1.67	
HR	11	32 569	33.8	0.86	0.43, 1.73	
HPD	2	8891	22.5	0.45	0.11, 1.87	

*per 100 000 person-days.
PD: pancreatoduodectomy, EHBD: extrahepatic bile duct resection, HR + EHBD: hepatic resection with extrahepatic bile duct resection, HR: hepatic resection, HPD: combined hepatectomy and pancreatoduodectomy. R1 (is): resection margin with intraductal carcinoma component, R1: resection margin with subepithelial invasive component.

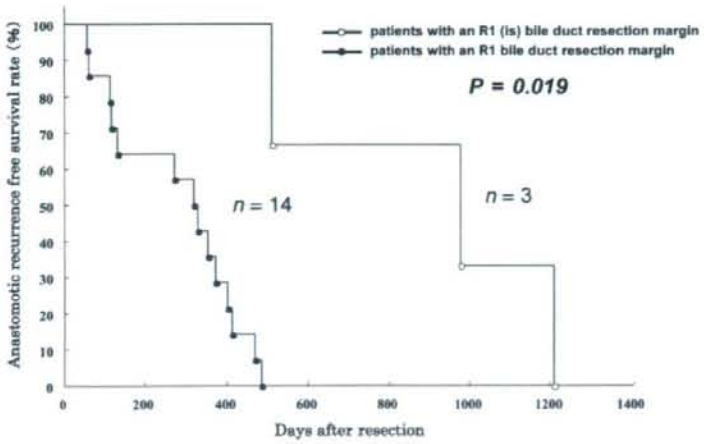

Fig. 3. Anastomotic recurrence-free survival rate of patients with a positive bile duct resection margin. Kaplan-Meier analysis revealed that the period until anastomotic recurrence at the bile duct resection margin after surgical resection in patients with a subepithelial invasive component bile duct resection margin (solid circles) was significantly shorter than that in patients with an intraductal carcinoma component bile duct resection margin (clear circles) ($P = 0.019$).

Table 7. Adjusted hazard ratio (HR) and 95% confidence interval (CI) of all-cause death by bile duct resection margin status

	Adjusted HR†	95% CI
Total (214 cases)		
Bile duct resection margin status		
Negative	1.00	
R1 (is)	1.06	0.53, 2.10
R1	1.95	1.27, 3.00

†Adjusted for age, operation day, type of surgical resection, tumor size, histologic type and tumor differentiation, depth of invasion, and venous involvement. R1 (is): resection margin with intraductal carcinoma component, R1: resection margin with subepithelial invasive component.

Correlation between bile duct resection margin status and anastomotic recurrence at the bile duct resection margin. In order to understand the background factors responsible for the difference in prognostic impact between R1 (is) and R1 bile duct resection margins, the correlation between bile duct resection margin status [R1 (is) *vs* R1] and anastomotic recurrence at the bile duct resection margin was examined by multivariate analysis. The risk of anastomotic recurrence in patients with an R1 bile duct resection margin was 4.5 times higher than that in patients with an R1 (is) bile duct resection margin (Table 8). In addition, the Kaplan-Meier method revealed that the period until anastomotic recurrence after surgical resection in patients with an R1 bile duct resection margin was significantly shorter than that in patients with an R1 (is) bile duct resection margin (Fig. 3, $P = 0.019$).

Discussion

Unlike several previously published studies analyzing the prognostic parameters in small series of patients with biliary tract carcinoma,[21–28] the present study examined in detail the clinicopathological parameters of 214 patients who underwent radical surgery with curative intent and had been strictly followed up at a single institution.

It has recently been reported that biliary tract carcinoma has a marked tendency for superficial extension by wide intraductal carcinoma[11–14] In this study, we defined the intraductal carcinoma component as carcinoma within the bile duct and its small branch outside the main tumor nodule consisting of a subepithelial invasive component. Surprisingly, an intraductal carcinoma component outside the main tumor nodule was significantly correlated with lower aggressiveness in the main tumor nodule:

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Table 8. Adjusted hazard ratio (HR) and 95% confidence interval (CI) of anastomotic recurrence by bile duct resection margin status in patients with a positive bile duct resection margin

	No. of recurrent cases	Person-days	Adjusted HR†	95% CI
Bile duct resection margin status				
R1 (is)	3	22 029	1.00	
R1	14	25 237	4.48	1.09, 18.5

†Adjusted for operation day and type of surgical resection. R1 (is): resection margin with intraductal carcinoma component, R1: resection margin with subepithelial invasive component.

patients with an intraductal carcinoma component infrequently developed large tumors and infrequently showed deep invasion into the bile duct wall and venous, lymphatic and perineural involvement in the main tumor nodule (Table 1). Furthermore, patients with an intraductal carcinoma component were infrequently at the advanced stage when diagnosed (Table 1). During the preparation of this paper, Nakanishi *et al.*[29] reported that cases of extrahepatic bile duct carcinoma with intraepithelial spread showed a more differentiated histological grade, less deep invasion, infrequent portal vein or hepatic invasion and a better prognosis than cases without such spread. In addition, we demonstrated statistically significant inverse correlations between the intraductal carcinoma component on the one hand and lymphatic and perineural involvement and TNM stage on the other, whereas Nakanishi *et al.* failed to show such correlations. Moreover, we demonstrated an inverse correlation between the intraductal carcinoma component and tumor aggressiveness by both univariate and multivariate analyses in a larger cohort than that reported by Nakanishi *et al.*[29], whereas that report performed only univariate analysis.

We have revealed that human cancer cell lines showing wide intraepithelial spreading in mouse inoculation models show strong adhesiveness to extracellular matrix proteins, which are components of the basement membrane of epithelial tissues, *in vitro*.[30] The expression patterns of cell-matrix adhesion molecules, such as integrins, in human cancer cell lines showing wide intraepithelial spreading in mouse inoculation models differ from those in human cancer cell lines that do not show such spreading.[30] In addition, it has been confirmed that there is a similar difference in the expression pattern of cell-matrix adhesion molecules between cancer cells showing, and not showing, such spreading in surgically resected clinical samples.[30] Cell-matrix adhesion molecules generally participate in cancer-stromal interactions and determine the invasiveness of human cancers.[31] Therefore, it is feasible that cancer cells showing wide intraepithelial spreading also show strong adhesiveness to the basement membrane of cancer nests and a less invasive tendency. This may be the reason why an intraductal carcinoma component was inversely correlated with aggressiveness in the main tumor nodule in this study. Although the molecular mechanism responsible for such an inverse correlation in biliary tract carcinoma needs to be further clarified, the presence of an intraductal carcinoma component may become an indicator of lower tumor aggressiveness. In fact, patients with an intraductal carcinoma component showed a significantly better prognosis than patients without such a component, irrespective of whether all patients from the present cohort or only patients who underwent complete resection were examined. The correlation between an intraductal carcinoma component and a favorable prognosis is consistent with the similar correlation observed in ductal carcinoma of the pancreas: the presence of an intraductal carcinoma component is reportedly a significantly good prognostic parameter for patients with invasive ductal carcinoma of the pancreas after surgical resection.[32–34]

On the other hand, the presence of an intraductal carcinoma component was significantly correlated with a positive bile duct resection margin (Table 4). Recently, Wakai *et al.* have reported that invasive carcinoma at the ductal resection margin appears to have a strong impact on patient survival, whereas residual carcinoma *in situ* does not, after surgical resection for extrahepatic bile duct carcinoma,[14] although they did not mention any background factors for the difference in prognostic impact between invasive carcinoma and carcinoma *in situ* at the ductal resection margin. We also defined two types of positive bile duct resection margin: R1 (is) (positive with only an intraductal carcinoma component) and R1 (positive with a subepithelial invasive component). An R1 bile duct resection margin was more frequently associated with poorer prognosis than a negative bile duct resection margin, whereas an R1 (is) bile duct resection margin was not more frequently associated with poorer prognosis than a negative bile duct resection margin (Tables 6 and 7). As in other malignancies, tumor recurrence after radical surgery for biliary tract carcinoma leads to eventual death.[26] Although Wakai *et al.* did not perform statistical analysis about anastomotic recurrence, the risk of anastomotic recurrence at the bile duct resection margin was significantly higher (Table 8), and the period until such recurrence was significantly shorter (Fig. 3), in patients with an R1 bile duct resection margin than in patients with an R1 (is) bile duct resection margin. Therefore, at least part of the difference in prognostic impact between R1 (is) and R1 bile duct resection margins is attributable to the difference in the risk of anastomotic recurrence.

However, the incidence of anastomotic recurrence in patients whose bile duct resection margin was positive [R1 (is) and R1] was only 26% (17 of 66 cases); some patients may die because of 'local recurrence' or distant metastasis before anastomotic recurrence becomes clinically obvious. Sakamoto *et al.* have proposed that anastomotic recurrence should be distinguished from 'local recurrence' derived from perineural invasion around the hepatic artery and/or involved regional nodes.[35] It is self-evident that all patients with an R1 (is) bile duct resection margin possess an intraductal carcinoma component that is inversely correlated with tumor aggressiveness, including perineural and lymphatic involvement and clinical stage. The intraductal carcinoma component may be partly responsible for the difference in prognostic impact between R1 (is) and R1 with reference not only to 'anastomotic recurrence' but also to 'local recurrence' derived from perineural invasion and/or involved regional nodes.

We analyzed both the intraductal carcinoma component and resection margin status in the same cohort and examined in detail the inconsistency of less tumor aggressiveness and a positive surgical margin in patients with intraductal carcinoma components. Surgeons are frequently required to decide the resection area based on the results of intraoperative histological diagnosis of bile duct resection margin status using frozen sections, and generally intend to achieve a negative bile duct resection margin. If the frozen section diagnosis is R1, the risk of death will be actually reduced if surgeons make efforts to achieve a negative bile duct resection margin. However, if the frozen section diagnosis is R1 (is), an intraductal carcinoma component is present and the prognosis for such patients will be

favorable. Moreover, if surgeons perform additional resection to achieve a negative bile duct resection margin, then the prognosis for patients whose initial bile duct resection margin is R1 (is) may not be improved significantly. Therefore, surgeons should not be persistent in trying to achieve a negative surgical margin when the intraoperative frozen section diagnosis is R1 (is), and can choose a safe surgical procedure to avoid postoperative complications.

Acknowledgments

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Periostin, secreted from stromal cells, has biphasic effect on cell migration and correlates with the epithelial to mesenchymal transition of human pancreatic cancer cells

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Periostin is a secretory protein that has been suggested to function as a cell adhesion molecule and promote the invasiveness or growth rate of tumors. However, little is known about the association of its expression and epithelial to mesenchymal transition (EMT), which is considered to play a crucial role in cancer cell metastasis. Thus, the authors investigated whether periostin could be involved in the process of EMT and the role of this gene in pancreatic cancer development. The expression of periostin was observed mainly in stromal cells but very little in cancer cells by immunohistochemistry and real-time RT-PCR. *In vitro*, pancreatic stellate cells (PSCs) exhibited a much higher basal expression of periostin compared with cancer cells. Periostin secreted in the supernatant from 293T cells that expressed periostin (approximately 150 ng/ml) inhibited the migration of pancreatic cancer cells. Coculture assay revealed that periostin expression in PSC was induced by pancreatic cancer cells. To assess the direct role of periostin in pancreatic cancer cells, the authors generated pancreatic cancer cell lines that stably express periostin. The induced expression of periostin (to 150 ng/ml) altered the morphology of cancer cells, changing them from mesenchymal to epithelial phenotypes with the induction of epithelial markers and a reduction of mesenchymal markers, and showed reduced cell migration *in vitro* and formed smaller tumors as well as suppressed metastasis *in vivo*. On the other hand, high concentration of recombinant periostin (1 μg/ml) promoted cell migration with AKT activation. The findings suggest that periostin has biphasic effect on the development of pancreatic cancer.

Key words: pancreatic cancer; periostin; EMT; pancreatic stellate cell

Pancreatic adenocarcinoma is one of the most malignant gastrointestinal tumors. Once pancreatic cancer is clinically evident, it progresses rapidly to develop metastatic lesions, frequently by the time of diagnosis. The pathogenic mechanisms that regulate the aggressive behavior of this cancer still remain to be clarified.

Tumor metastasis is a multistep pathological process involved in the final phase of tumor development. During this process, epithelium-derived tumor cells undergo fibroblast-like transformation, referred to as epithelial-mesenchymal transition (EMT).[1,2] EMT is the process by which an epithelial cell shows transitory changes in cell structure and becomes a more motile mesenchymal cell with migratory properties during embryogenesis. This transition is considered to be an important event during malignant tumor progression and metastasis.[3–5]

Periostin is a secretory protein that has been suggested to function as a cell adhesion molecule for preosteoblasts and to participate in osteoblast recruitment, attachment and spreading.[6,7] Recently, the expression of periostin has been implicated in the development of variety of carcinomas such as neuroblastoma,[8] epithelial ovarian cancer[9] and non-small cell lung carcinoma.[10] Periostin has been also reported to promote the metastatic growth of colon cancer by augmenting cell survival via the Akt/PKB pathway.[11] In addition, periostin has been suggested to promote the invasiveness or growth rate and confer resistance to hypoxia in pancreatic cancer cells, although the source of this protein was stromal cells rather than cancer cells.[12] It has been suggested that an interaction between cancer cells and stromal cells plays a pivotal role in cancer development since a pancreatic cancer cell supernatant stimulated the secretion of periostin from pancreatic satellite cells (PSC).[13] These findings raise the question of whether or not periostin can lead pancreatic cancer cells to the state of EMT through epithelial-mesenchymal interaction.

Here, we investigated the functional role of periostin during tumor progression *in vitro* and in *vivo*. We demonstrated that stromal cells were important sources of periostin and that pancreatic cancer cells increased the expression of periostin in PSC, which suppressed tumor metastasis through the blockade of EMT.

Material and methods

Tissues

Pancreatic cancer tissues were obtained from patients who underwent surgical operations for the tumors. The tissues collected at the time of surgery were fixed in 10% paraformaldehyde overnight and embedded in paraffin wax. Thirty-two pancreatic cancer tissues were used for the immunohistochemistry. Informed consent was obtained from all patients before surgery.

Immunohistochemistry

The localization of periostin in human pancreatic tissues was investigated by immunohistochemistry. The tissue sections were deparaffinized and the antigens were retrieved by boiling the sections in Target Retrival Solution (Dako, Carpenteria, CA) in a microwave oven. Then, the sections were incubated in methanol with 0.3% hydrogen peroxide for 30 min to block the endogenous peroxidase activity. After incubation with the periostin antibody (US Biological, Massachusetts, MA) overnight at 4°C, a histofine kit (Nichirei, Tokyo, Japan) was used. Visualization of the immunoreaction was carried out in 0.06 mM 3,3′-diaminobenzidine tetrahydrochloride (Dojin, Kumamoto, Japan) containing 2 mM hydrogen peroxide in PBS for several minutes at room temperature. For the negative control, the immunostaining process was performed by replacing the primary antibody with PBS. The negative control sections showed no specific immunoreactivity.

The degree of immunostaining was evaluated as follows: negative, no positive cells were found; weak, small clusters of positive cells were observed; moderate, clusters of positive cells were

Abbreviations: 293T-peri, periostin expressing vector transfected cells; BrdU, 5-bromo-2-deoxyuridine; EMT, epithelial to mesenchymal transition; EV cells, empty vector transfected cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC Human umbilical vein endothelial cells; Panc1.EV, empty vector transfected Panc1 cells; PP1, Panc1 cells stably overexpressing periostin; PSC, pancreatic stellate cells; RT-PCR, reverse transcription-polymerase chain reaction.

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observed in some areas; and intense, immunoreactive cells were observed in most areas of tissue sections. The evaluation of immunostaining was done independently by 2 observers (K.S. and A.K.) who had not been informed of the histological diagnosis.

RNA extraction and RT-PCR for pancreatic cancer cell lines and tissues

Total RNA was prepared using the RNeasy kit (QIAGEN, Hilden, Germany) with DNase I treatment to eliminate any DNA contamination according to the protocol provided by the manufacturer. First strand cDNA was generated from 1 μg total RNA using RETROscript kit (Ambion, Austin, TX) according to the manufacturer's protocol. PCR was performed on 2 μl of RT products in a 25-μl reaction mixture using Ex Taq polymerase (Takara, Ohtsu, Japan) with 10 μmol/l of 3′ and 5′ primers. The gene expression was normalized to the respective glyceraldehydes-3-phosphate dehydrogenase (GAPDH) expression level. The PCR condition for our cDNA templates was set to ensure that replication was in the linear phase for each primer set used. To quantify the gene expression level, we also employed quantitative real-time RT-PCR by using LightCycler and LightCycler-FastStart DNA Master SYBR Green I (Roche diagnostics, Basel, Switzerland). Each experiment was repeated at least 3 times, and representative data are shown. The primer pairs used were as follows: human periostin, forward 5′ TGTTGCCCTGGTTATATGAG3′ and reverse 5′ACTCGGTG CAAAGTAAGTGA3′; rat periostin, forward 5′TGCAAAAAGA GGTCTCCAAGGT3′ and reverse 5′AGGTGTGTCTCCCTGAA GCAGT3′; human GAPDH, forward 5′GGCGTCTTCACCACCA TGGAG3′ and reverse 5′AAGTTGTCATGGATGACCTTGGC3′; rat GAPDH, forward 5′ACATCATCCCTGCATCCACT3′ and reverse 5′GGGAGTTGCTGTTGAAGTCA3′. The annealing temperature for these primer sets was 58°C. All reactions were performed according to the manufacturer's protocol. The specificity of each PCR reaction was confirmed by melting curve analyses. The primers for rat periostin and GAPDH did not detect human periostin or GAPDH (data not shown). The level of target gene expression in each sample was normalized to the respective GAPDH expression level.

Pancreatic tissue samples and microdissection

The pancreatic tissues collected at the time of surgery were embedded immediately in Tissue-Tek OCT compound medium (Sakura, Tokyo, Japan), frozen in liquid nitrogen and stored at −80°C. The frozen tissues were cut into 8-μm-thick sections using a crsyostat (Jung CM3000; Leica, Nussloch, Germany) and then fixed with cold methanol and stained with toluidine blue. Histologically cancerous ductal cells and stromal cells from the sections were dissected using LaserScissors Pro300 (Cell Robotics, Albuquerque, NM) according to the manufacturer's protocols. These microdissected samples were subjected to RNA extraction. RNA was prepared using the RNeasy Micro kit (QIAGEN, Hilden, Germany) with DNase I treatment to eliminate DNA contamination according to the protocol provided by the manufacturer.

Cell culture and isolation

The human pancreatic cancer cell lines (AsPC-1, BxPC3, Panc-1 and MIAPaca2) were purchased from American Type Culture Collection (Manassas, VA), routinely grown in Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal bovine serum (Miles, Kankakee, IL) at 37°C, 5% CO_2 in a humidified environment. Four colonic cancer cell lines (WiDr-Tc, SW480, CoLo205, DLD1), human fibroblast (KMST6) and human embryonic kidneyfibroblast cell line (293T), which were provided by Cancer Cell Repository (Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum, penicillin sodium and streptomycin sulfate. Human pancreatic stellate cells (PSCs) were isolated from the surgically resected normal pancreas tissues of patients with pancreatic cancer,[14] under the approval of the Ethics Committee of Tohoku University School of Medicine. The cells were maintained in Ham's F-12/DMEM containing 10% heat-inactivated fetal bovine serum (ICN Biomedicals, Aurora, OH), penicillin sodium and streptomycin sulfate. Human umbilical vein endothelial cells (HUVEC) and their optimized culture medium were purchased from Clonetics (San Diego, CA). HUVEC were grown on 0.2% gelatin-coated tissue culture dishes (Corning, Corning, NY). Immortalized rat PSC line (SIPS) isolation and culture were performed as previously described.[15]

Coculture of rat pancreatic stellate cell line (SIPS) and Panc1

Panc1 cells were added to monolayer of immortalized rat PSCs (1×10^6 cells/10 mm dish) at a ratio of 1:1 and cultured for 24 hr at 37°C. Total RNA was extracted, and the levels of rat periostin mRNA in the immortalized rat PSCs were investigated by real-time RT-PCR.

Generation of periostin-overexpressing Panc1, KMST6, 293T cells

The expression plasmid of HA-tagged rat periostin was kindly provided by Dr. Inoue (Siga University, Japan). Transfection of the cells with expression vectors (rat periostin cDNA or vector alone) was performed using FuGENE 6(Roche, Indianapolis, IN) as recommended by the supplier. Transfected Panc1 and KMST6 were selected with 800 mg/ml of G418 (Invitrogen) to generate stably expressing cell lines. After G418 selection, the clones were subjected to immunoprecipitation and Western blot analysis with a specific antibody against HA to confirm the periostin expression. After establishment of the empty vector (EV) or periostin transfected clonal cell lines, the same passages were used for each experiment.

Reagent

Recombinant human periostin was purchased from Biovendor (Heidelberg, Germany). Periostin treatment was performed in 0.1 M acetate buffer (pH4) at the concentration of 100 ng/ml or 1 μg/ml.

Immunoprecipitation

Immunoprecipitation was performed using a protein G immunoprecipitation kit (Roche Applied Science) according to the manufacturer's instructions. Briefly, 500 μg of protein from cell lysates or supernatant were incubated with 1 μg of anti-HA antibody at 4°C for 1 hr. Fifty microliters of protein G (provided with the kit) were used per sample. Western blot analysis was performed as described later.

Western blot analysis

For whole cell protein extraction, cells were lysed by the addition of lysis buffer (150 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate). The protein concentration in each sample was determined using a Bradford assay kit (Dojin, Kumamoto, Japan). After the addition of 5 × sample buffer (pH 6.8, 125 mM Tris-HCL, pH 6.8, 4% sodium dodesylsulphate, 20% glycerol and 0.04% bromphenolblue, 10% mercaptoethanol), aliquots were boiled at 100°C for 5 min and subjected to Western blotting. The nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) was blocked for 1 hr at room temperature in a buffer containing 10 mM Tris-HCL (pH 7.5), 100 mM NaCl, 0.1% Tween 20 and 5% dry milk, and then incubated with primary antibody overnight at 4°C. The primary antibodies used in this study were as follows: HA antibody (Sigma, St Louis, MO), monoclonal mouse E-cadherin antibody (BD Transduction Laboratories, Lexington, KY), monoclonal mouse β-catenin antibody (BD Transduction Laboratories), monoclonal mouse phosphospecific Akt antibody (BD Transduction Laboratories) and monoclonal mouse total Akt antibody (BD Transduction Laboratories). The membrane was washed with a buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl,