Impact of the histological phenotype of extrahepatic bile duct carcinoma.
（　肝外胆管癌における組織学的表現型の影響　）

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Impact of the histological phenotype of extrahepatic bile duct carcinoma

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Abstract. The classification of histological phenotypes was originally conceived for pancreatic intraductal papillary mucinous neoplasms. Recently, it has been introduced for extrahepatic cholangiocarcinoma. The aim of the present study was to clarify the associations between histological phenotype and clinicopathological features of extrahepatic cholangiocarcinoma, using 99 cases of surgically- resected extrahepatic cholangiocarcinoma. All cases were divided into one of two histological phenotypes: Biliary-type (BT; 56 cases, 56.6%) or metaplastic-type (MT; 43 cases, 43.4%). The clinicopathological features were compared between these two phenotypes. BT tumors exhibited significantly poorer differentiation, more frequent lymph node metastasis (BT vs. MT, 42.9 vs. 30.2%; P=0.042), more severe venous invasion (v2/3: BT vs. MT, 64.3 vs. 23.3%; P<0.001), and more severe perineural invasion (ne2/3: BT vs. MT, 78.6 vs. 48.8%, P=0.002). Furthermore, the overall (P=0.015) and disease-free (P=0.003) survival times were significantly decreased in patients with BT vs. MT tumors. In conclusion, extrahepatic cholangiocarcinoma with a BT phenotype has greater malignant potential, and may be an important predictive factor for poor prognosis.

Introduction

Extrahepatic bile duct carcinoma (cholangiocarcinoma) is an epithelial cancer that originates from the bile ducts and exhibits features of cholangiocytic differentiation. Its incidence rate has no significant geographical variation. It accounts for 0.16 and 0.15% of all invasive cancers in males and females, respectively, in the USA (1). Despite recent advances in diagnostic and therapeutic techniques, complete surgical resection of the tumor remains the best way to cure extrahepatic bile duct carcinoma; however, even in patients who have undergone curative resection, poor prognosis is extremely common due to the high recurrence rate of this tumor (2-4). In a recent study, the biliary- type histological phenotype was reported to be a factor for poor prognosis in

diseases such as intraductal papillary mucinous neoplasm (IPMN) (5) and gallbladder cancer (6). The classification of histological phenotypic subtype of IPMN is performed based on pancreatic IPMN; tumors are classified into four types according to histological cell morphology: Pancreaticobiliary type, intestinal type, gastric type, and oncocytic type (7). Different histological subtypes have a tendency to occur at different primary sites, such as branch- duct type and main- duct type, and have varying incidence rates of malignant transformation (8). On the other hand, intraductal papillary neoplasm of the bile duct has also been accepted as a counterpart of pancreatic IPMN, and the concept of the phenotypic classification has now been introduced for bile duct tumors (3, 9). However, the clinicopathological features and prognosis associated with the phenotype of extrahepatic cholangiocarcinoma have not been clarified. Therefore, in the present study, the phenotypes of patients with extrahepatic cholangiocarcinoma who underwent macroscopic curative resection were classified, and the clinicopathological features and prognosis were examined accordingly in order to clarify the significance of phenotypic classification.

Patients and methods

Ethics statement. The ethics committee of the Hirosaki University Graduate School of Medicine approved the current study (approval number. 2017- 1006).

Patients and samples. A total of 99 consecutive bile duct carcinoma surgical cases treated between January 2005 and December 2011 were investigated, after obtaining each patient's informed consent for use of their clinical records and pathological specimens at Hirosaki University Hospital. The series consisted of 72 men and 27 women with a median age of 68 years (range, 31- 83 years). The carcinomas were located in the perihilar (32 cases) and distal bile duct (67 cases). The clinicopathological features of the patients are summarized in Table I. Curative resection and regional lymph node dissection were dependent on the location of the primary tumor: Pancreaticoduodenectomy or pylorus- preserving pancreaticoduodenectomy was performed in 61 patients, bile duct resection in 1 patient, combined hepatectomy with bile duct resection in 30 patients, and combined hepatectomy and pancreaticoduodenectomy in 7 patients. Survival data were obtained from hospital medical charts, and the median observation period was 31 months.

Pathological analysis. All surgically resected specimens were routinely fixed with 10% formalin, then embedded in paraffin and stained with hematoxylin and eosin for

pathological evaluation. The following histological features were assessed: Depth of invasion (T stage), histological differentiation, lymphovascular invasion (ly), venous vessel invasion (v), perineural invasion (ne), lymph node metastasis (N) and histological phenotype. Histological phenotype was defined as biliary type (BT) or metaplastic type (MT), as follows: BT is composed of short or long tubular glands lined by cells that vary in height from cuboidal to tall columnar, superficially resembling biliary epithelium (Fig. 1A); and MT comprises gastric type [GT; composed of tall columnar cells with basally oriented nuclei and abundant mucin-containing cytoplasm (Fig. 1B)] and intestinal type [IT; composed of tubular glands closely resembling those of colonic adenocarcinomas (Fig. 1C); the glands are lined predominantly by columnar cells with pseudostratified ovoid or elongated nuclei]. These data were evaluated according to the General Rules for Surgical and Pathological Studies on Cancer of the Biliary Tract (10) with reference to the World Health Organization classification (11), and were staged according to the Tumor - Node - Metastasis classification of the International Union Against Cancer (12).

Immunohistochemistry. For histological examination, extrahepatic bile duct carcinoma specimens were routinely fixed with formalin, embedded in paraffin, sectioned to a thickness of 4 μm, and mounted on saline coated glass slides. Immunohistochemical examination was performed on deparaffinized sections using the standard avidin - biotin - peroxidase complex method with a BenchMark XT automated immunostainer (Ventana Medical Systems, Inc., Tucson, AZ, USA). The different phenotypes were investigated for mucin (MUC) expression using primary antibodies against MUC1 (#NCL-MUC-1, dilution, 1:50; clone Ma696), MUC2 (#NCL - MUC - 2, dilution, 1:50; clone Ccp), MUC5AC (#NCL - MUC - 5AC, dilution, 1:100; clone CLH2) and MUC6 (#NCL - MUC - 6, dilution, 1:100; clone CLH5), all purchased from Novocastra (Leica Biosystems, Newcastle, UK). After washing in PBS three times, secondary immunostaining was performed with an i- VIEW DAB Universal Kit (Roche Diagnostics, Tokyo, Japan) for 28 min at 42˚C.

Evaluation of immunohistochemistry. Three evaluators, who were blinded to the clinical characteristics of the patients, assessed all 99 specimens. MUC1 was determined to be positive in the presence of luminal membranous immunoreactivity of the tumor, whereas the cytoplasmic immunoreactivities were considered when determining MUC2, MUC5AC and MUC6 positivity. The results were classified into groups based on the percentage of positively stained cells, as follows: Negative group, <5% of cancer cells

stained; and positive group, ≥5% of cells stained.

Statistical analysis. Statistical comparisons between two groups were analyzed using the Pearson's $\chi 2$ test for categorical data and the Student's t- test for continuous data. Survival curves were constructed using the Kaplan- Meier method. The Cox proportional hazards model was used for multivariate analysis. Differences were considered to be statistically significant when $P<0.05$. All statistical evaluations were performed using SPSS software (version 22.0; IBM Corp., Armonk, NY, USA).

Results

Clinicopathological features according to cholangiocarcinoma phenotype. The clinicopathological findings pertaining to patients with BT and MT tumors are summarized in Table II. In total, 56 patients had BT cholangiocarcinoma and 43 patients had MT cholangiocarcinoma (42 patients with GT and 1 patient with IT). The mean tumor diameter was 37.8 mm (range, 10- 75 mm) in BT, and 34.4 mm (range, 13- 85 mm) in MT, with no significant difference observed (P=0.307). Carcinoma in situ developed in 26 patients with BT (46.4%), and 19 patients with MT (44.2%; P=0.826). No significant differences were observed in the levels of carcinoembryonic antigen (cut-off value, 5 ng/ml; P=0.950), and carbohydrate antigen 19- 9 (cut- off value, 100 U/ml; P=0.673) between the BT and MT groups. With regard to T- stage, pT3- 4 cancer was observed in 32 patients with BT (57.1% of group), and 17 patients with MT (39.5% of group), with no significant difference observed (P=0.084). Regarding lymphatic invasion, ly2- 3 was observed in 29 patients with BT (51.8% of group), and 15 patients with MT (34.9% of group), with no significant difference observed (P=0.095). However, significant differences between the two groups were observed for four factors: Histological differentiation [papillary adenocarcinoma or well/moderately differentiated adenocarcinoma observed in 45 patients with BT (80.4%) and 38 patients with MT (88.4%); P=0.018]; N stage [pN1 observed in 24 patients with BT (42.9%) and 13 patents with MT (30.2%); P=0.042]; venous invasion [v2/3 observed in 36 patients with BT (64.3%) and 10 patients with MT (23.3%); P<0.001]; and perineural invasion [ne2/3 observed in 44 patients with BT (78.6%) and 21 patients with MT (48.8%); P=0.002].

MUC immunostaining according to cholangiocarcinoma phenotype. Immunostaining for MUC1, MUC2, MUC5AC and MUC6 was performed in three groups divided according to phenotype (BT, GT and IT; summarized in Table III). MUC1- positivity was observed in 45 patients (80.3%) with BT, 23 patients (54.3%) with GT, and 0

patients (0%) with IT. MUC2- positivity was observed in 7 patients (12.5%) with BT, 7 patients (16.6%) with GT, and 1 patient (100%) with IT. MUC5AC- positivity was observed in 18 patients (32.1%) with BT, 33 patients (78.6%) with GT, and 1 patient (100%) with IT. MUC6- positivity was observed in 20 patients (35.7%) with BT, 27 patients (64.3%) with GT, and 0 patients (0%) with IT. Significant differences in the ratios of tumors positively expressing MUC1, MUC5AC and MUC6 were observed between the BT and MT groups (P=0.004, P<0.001 and P=0.008, respectively).

Survival according to cholangiocarcinoma phenotype. Overall survival (OS) and Disease free survival (DFS) were evaluated in the BT and MT groups using the Kaplan-Meier method. The 1 year DFS rates were 32.2% in the BT group and 81.0% in the MT group; the 3 year DFS rates were 36.4% in the BT group and 59.2% in the MT group; and the 5 year DFS rates were 22.8% in the BT group and 54.3% in the MT group. The mean DFS times were 38.6 months [95% confidence interval (CI), 27.06- 50.32 months] in the BT group and 58.9 months (95% CI, 47.24- 70.62 months) in the MT group; the BT group exhibited a significantly shorter DFS than the MT group (P=0.003; Fig. 2). In the BT and MT groups, respectively, the 1 year OS rates were 87.3 and 90.5%, the 3year OS rates were 46.1 and 66.3%, and the 5 year OS rates were 31.4 and 55.5%. The mean OS times were 51.2 months (95% CI, 39.43- 62.91 months) in the BT group, and 64.0 months (95% CI, 53.55- 74.51 months) in the MT group. Similarly, OS was significantly shorter in the BT group compared with the MT group (P=0.015; Fig. 3).

Univariate and multivariate analyses of survival. Univariate analysis of overall survival time following surgery using the log-rank test was performed for the 99 patients with extrahepatic cholangiocarcinoma. In addition to the phenotype of the tumor (BT; P=0.012), the histological grade (G3- 4; P=0.018), N classification (N1; P<0.001), extent of venous invasion (v2/3; P<0.001) and perineural invasion (ne2/3; P=0.030) were identified as variables that were significantly associated with poor prognosis. On multivariate analysis, N classification [N1; P=0.020; hazard ratio (HR)= 2.02 (95% CI, 1.13- 3.62)] was identified as an independent prognostic factor. Multivariate analysis of survival showed that the BT phenotype had a HR of 0.82 (95% CI, 0.45-1.52; P=0.532, and therefore it was not considered to be an independent prognostic factor in patients with extrahepatic cholangiocarcinoma (Table IV).

Discussion

In the present study, we classified cholangiocarcinoma into two phenotypes, i.e. the biliary type (BT) and the metaplastic type (MT), then examined the clinicopathological features, and prognosis of each group. Compared to MT, BT exhibited higher staging than MT, which we found was associated with lymph node metastasis, severe venous invasion, and severe perineural invasion. Furthermore, with regards to OS and DFS, we found that survival was significantly shorter in BT than MT. Furukawa et al. reported pancreatobiliary type of IPMN are significantly poor prognosis than gastric type and intestinal type (5). Yamamoto et al. classified gallbladder carcinoma into non-metaplastic type and metaplastic type. They reported that non-metaplastic type exhibited a higher incidence of direct invasion to the liver, and significantly shorter survival (13). Our colleagues previously reported that carcinogenesis of cholangiocarcinoma is reported to have two pathways. One is an origin from biliary epithelium, in which a biliary phenotype is expressed. The other is an origin from metaplastic epithelium, in which gastric and intestinal phenotypes are expressed (14). In this study, 99 specimens were classified into three phenotypes: 56 cases of BT, 42 cases of GT, and a case of IT. Focusing on the malignant potential of BT, GT and IT were combined as MT in accordance with Yamamoto's report (13), and then survival differences between BT and MT were investigated. As a result, it was revealed that extrahepatic cholangiocarcinoma with BT expression had severely malignant potential, compared to that with MT. However, multivariate analysis using Cox proportional hazards model showed that BT phenotype expression was not an independent prognostic factor for overall survival. Lymph node metastasis and venous infiltration had a greater influence on prognosis. In BT phenotype group, locally-advanced cases (N1, v2/3, pn2/3) dominated. It was considered that BT

phenotype expression might be strongly associated with multiple prognostic factors, and therefore it could not be independent as a prognostic factor.

In order to examine the immunohistological difference between BT and MT, we examined MUC protein expression in tumor tissue. As a result, BT showed a significantly higher rate of MUC1 positivity at 82.0 % compared to MT (P = 0.004). It was suggested that there was a strong correlation between BT and MUC1 expression. The MUC1 protein is a mucin core protein responsible for the mucous lining inner cavities such as the gastrointestinal tract and the airways. Mucin is divided into secretory mucin and membrane-bound mucin according to the type of core protein. The former is a major component of mucous secreted from epithelial cells, and primarily includes the core proteins MUC2, MUC5AC, and MUC6. On the other hand, mucin molecules of the latter have an extracellular domain, transmembrane domain, and intracellular domain. They can pass through the cell membrane, and the main core proteins include MUC1, MUC3, and MUC4. Of particular note, the membrane-bound mucin MUC1, acts as an adhesion molecule for cancer cells (15-17), and is thought to contribute to extravascular migration of cancer cells and metastasis such as in lung cancer, breast cancer, gastric cancer, pancreatic cancer, and colorectal cancer (18). Furthermore, research has progressed for the application of MUC1 not only as a tumor marker in several malignant neoplasms (19,20), but also as target in immunotherapy (21-23). Park et al. examined the expression of MUC1, MUC2, MUC5AC, and MUC6 in cholangiocarcinoma, and they reported that MUC1-positive patients exhibited severely advanced histological differentiation, T factor, perineural invasion, and venous invasion, and thus MUC1 expression within tumor tissue was a potential factor for poor prognosis (24).

In the present study, it was clarified that BT strongly correlated with the rate of patients positive for MUC1 expression. Therefore, it was suggested that the function of MUC1 as a cancer cell adhesion molecule, and its properties as a metastasis inducer caused high- grade malignancy in extrahepatic BT cholangiocarcinoma. Furthermore, compared to MT, it was revealed that BT had significantly shorter DFS and OS, and thus BT could be a predictive factor for prognosis of extrahepatic cholangiocarcinoma. There has been no reported study to date of the difference in clinicopathological features and prognosis according to extrahepatic cholangiocarcinoma phenotype. On the basis of these results, it was concluded that this was the first report for describing the correlation between the extrahepatic cholangiocarcinoma phenotype and its prognosis.

There were some limitations to this study. First, it was a retrospective study involving a limited number of cases. Second, BT phenotype expression was not an independent prognostic factor for extrahepatic cholangiocarcinoma. Also, multivariate analysis indicated that lymph node metastasis and venous infiltration had a greater influence on prognosis. Therefore, the correlation between the malignant potential of BT and those prognostic factors should be clarified in future.

In conclusion, the extrahepatic cholangiocarcinoma could be classified into BT and MT phenotypes. It was revealed that extrahepatic cholangiocarcinoma with BT had severely malignant potential, and could be a predictive for shorter DFS and OS.

Acknowledgements

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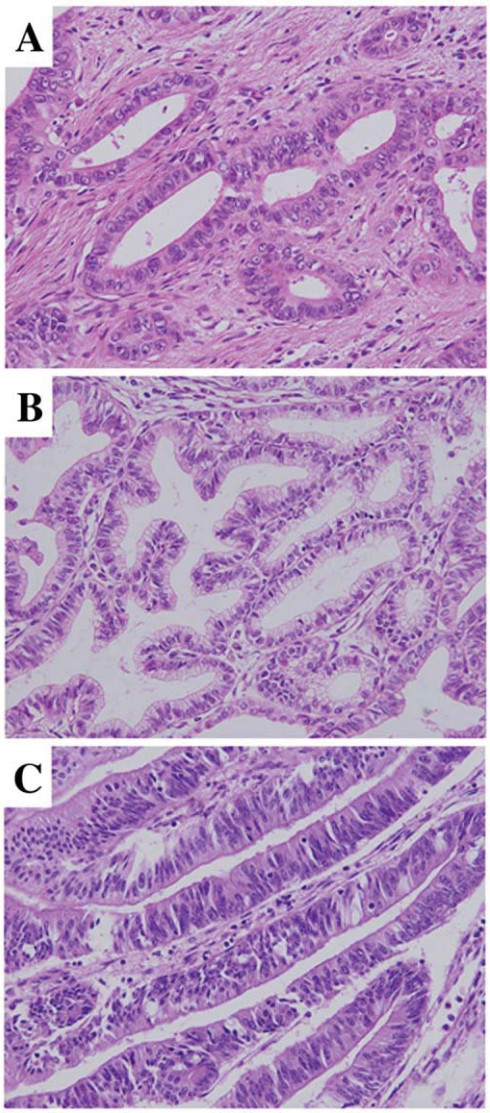
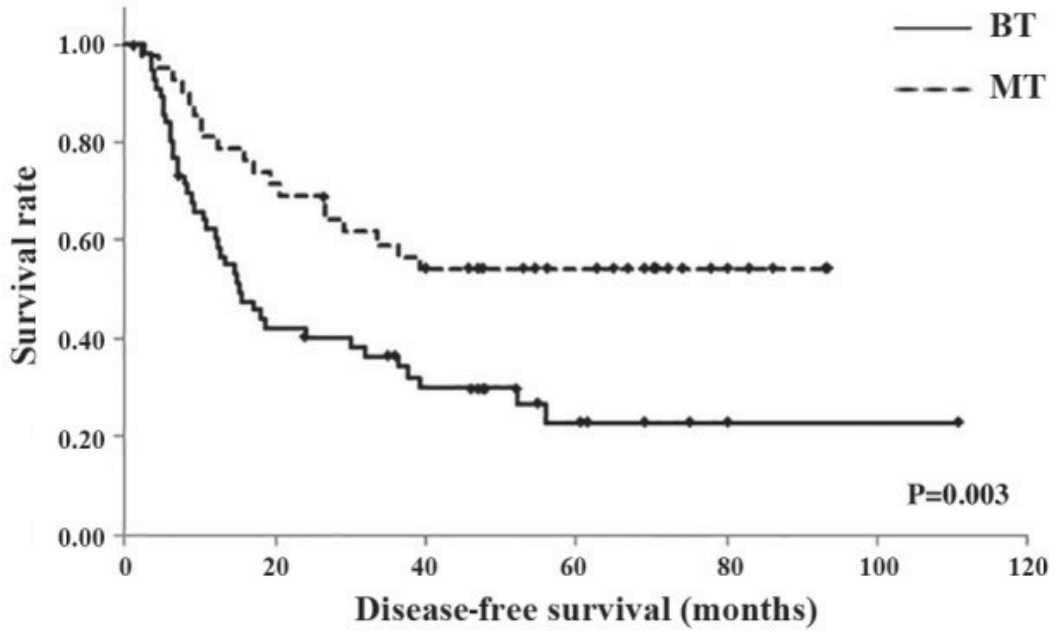
Figure 1.



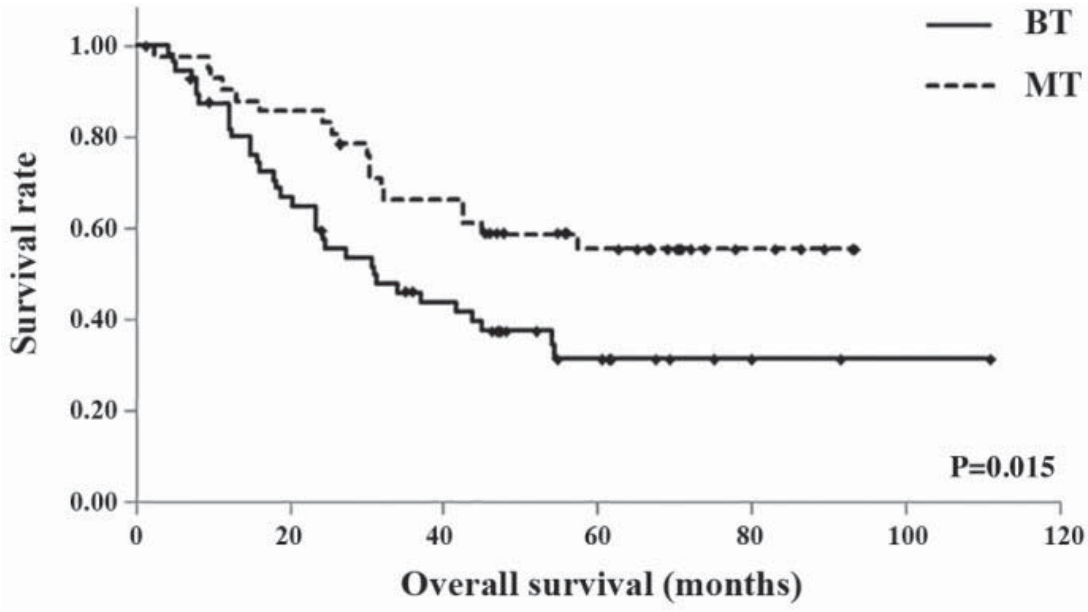
Three morphological subtypes of cholangiocarcinoma (hematoxylin and eosin staining). (A) Biliary-type tumors were composed of short or long tubular glands lined by cells varying in height, from cuboidal to tall columnar, superficially resembling biliary epithelium (magnification, ×100). (B) Gastric foveolar-type tumors were composed of tall columnar cells with basally oriented nuclei and abundant mucin-containing cytoplasm (magnification, ×100). (C) Intestinal-type tumors were composed of tubular glands closely resembling those of colonic adenocarcinomas, and consisted of glands lined predominantly with goblet cells (magnification, ×100).

Figure 2.



Kaplan-Meier estimates of disease-free survival in patients with cholangiocarcinoma. Patients with BT exhibited reduced DFS times (log-rank P=0.003). BT, biliary-type cholangiocarcinoma; MT, metaplastic-type cholangiocarcinoma.

Figure 3.



Kaplan-Meier estimates of overall survival in patients with cholangiocarcinoma. Patients with BT exhibited reduced OS times (log-rank P=0.015). BT, biliary-type cholangiocarcinoma; MT, metaplastic-type cholangiocarcinoma

Table 1

		number (n=99)
Gender		
	Male	72
	Female	27
Age (years)		
	≥70	44
	<70	55
Location		
	Hilar	32
	Distal	67
Size (mm)		33 (10-85)
CEA		
	<5	81
	≥5	18
CA19-9		
	<100	69
	≥100	30
Superficial spreading		
	Positive	45
	Negative	54
*Histological differentiation		
	pap, well, mod	83
	por, others	16
Phenotype		
	Biliary type	56
	Gastric type	42
	Intestinal type	1
T classification		
	pT1, 2	49
	pT3, 4	50
N classification		
	pN0	64

	pN1	35
M classification		
	pM0	94
	pM1	5
Lymphatic invasion		
	ly0, 1	55
	ly1, 3	44
Venous vessel invasion		
	v0, 1	53
	v2, 3	46
Neural invasion		
	ne0, 1	34
	ne2, 3	65

* pap, papillary adenocarcinoma; well, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma

Table 2

		Biliary type (n=56)	Metaplastic type (n=43)	P-value
Gender				0.206
	Male	43	29	
	Female	13	14	
Age (years)				0.230
	≥70	29	17	
	<70	27	26	
Location				0.770
	Hilar	17	15	
	Distal	39	28	
Mean Size (mm)		37.8 (10-75)	34.4 (13-85)	0.307
CEA				0.950
	<5	51	39	
	≥5	5	4	
CA19-9				0.673
	<100	40	29	
	≧100	16	14	
CIS				0.826
	Positive	26	19	
	Negative	30	24	
*Histological differentiation				
	pap, well, mod	45	38	0.018
	por, others	11	5	
T classification				0.084
	pT1, 2	24	26	
	pT3, 4	32	17	
N classification				0.042
	pN0	32	33	
	pN1	24	13	
Lymphatic invasion				0.095

	ly0, 1	27	28	
	ly2, 3	29	15	
Venous vessel invasion				p <0.001
	v1, 2	20	33	
	v2, 3	36	10	
Prineural invasion				0.002
	ne0, 1	12	22	
	ne2, 3	44	21	

* pap, papillary adenocarcinoma; well, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma

Table 3

	Metaplastic type, n=43 (Gastric foveolar type + Intestinal type)	Nonmetaplastic type, n=56 (Biliary type)	P value
Mucin expression			
MUC1	23 (53.5%)	45 (80.3%)	0.004
MUC2	8 (18.6%)	7 (12.5%)	0.406
MUC5AC	34 (79.1%)	18 (32.1%)	<0.001
MUC6	27 (62.8%)	20 (35.7%)	0.008

Table 4

Table 4: Cox proportional analysis of the survival

Variables	Univariate analysis			Cox proportional analysis		
	Values (%)	MST (months)	p-value	HR	95%CI	p-value
Phenotype of the tumor			0.012	0.53	0.45-1.52	0.82
Metaplastic type	43 (43.4%)	n.r.			-	
Biliary type	56 (56.6%)	32.0				
Histological grade			0.018	1.86	0.82-4.30	0.14
G1/G2	28 (28.3%)	n.r.				
G3/G4	71 (71.7%)	31.0				
N classification			<0.001	2.02	1.13-3.62	0.02
N0	65 (65.6%)	n.r.				
N1	34 (34.4%)	23.0				
Venous infiltration			<0.001	1.73	0.90-3.36	0.11
v0/1	53 (53.5%)	n.r.				
v2/3	46 (46.5%)	30.0				
Perineural invasion			0.03	1.01	0.52-2.18	0.86
pn0/1	34 (34.3%)	n.r.				
pn2/3	65 (65.5%)	32.0				

MST, median survival time; n.r., not reached