

Impact of the histological phenotype of extrahepatic bile duct carcinoma

KENSUKE OKANO^{1,2}, TADASHI YOSHIZAWA², TAKUYA MIURA¹,
KEINOSUKE ISHIDO¹, DAISUKE KUDO¹, NORIHISA KIMURA¹, TAI-ICHI WAKIYA¹,
YUNYAN WU², SATOKO MOROHASHI², KENICHI HAKAMADA¹ and HIROSHI KIJIMA²

Departments of ¹Gastroenterological Surgery, and ²Pathology and Bioscience, Hirosaki
University Graduate School of Medicine, Hirosaki, Aomori 036-8562, Japan

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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

Correspondence to: Dr Hiroshi Kijima, Department of Pathology and Bioscience, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan
E-mail: hkijima@hirosaki-u.ac.jp

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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- μ m, 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, \geq 5% of cells stained.

Statistical analysis. Statistical comparisons between two groups were analyzed using the Pearson's χ^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 patients 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

Table I. Patient characteristics (n=99).

Characteristic	Value
Sex, n	
Male	72
Female	27
Age (years), n	
≥70	44
<70	55
Location, n	
Hilar	32
Distal	67
Size (mm)	
Mean	33
Range	10-85
Carcinoembryonic antigen (ng/ml), n	
<5	81
≥5	18
Carbohydrate antigen 19-9 (U/ml), n	
<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
pT classification, n	
pT1-2	49
pT3-4	50
pN classification, n	
pN0	64
pN1	35
pM classification, n	
pM0	94
pM1	5
Lymphatic invasion, n	
ly0-1	55
ly2-3	44
Venous vessel invasion, n	
v0-1	53
v2-3	46
Neural invasion, n	
ne0-1	34
ne2-3	65

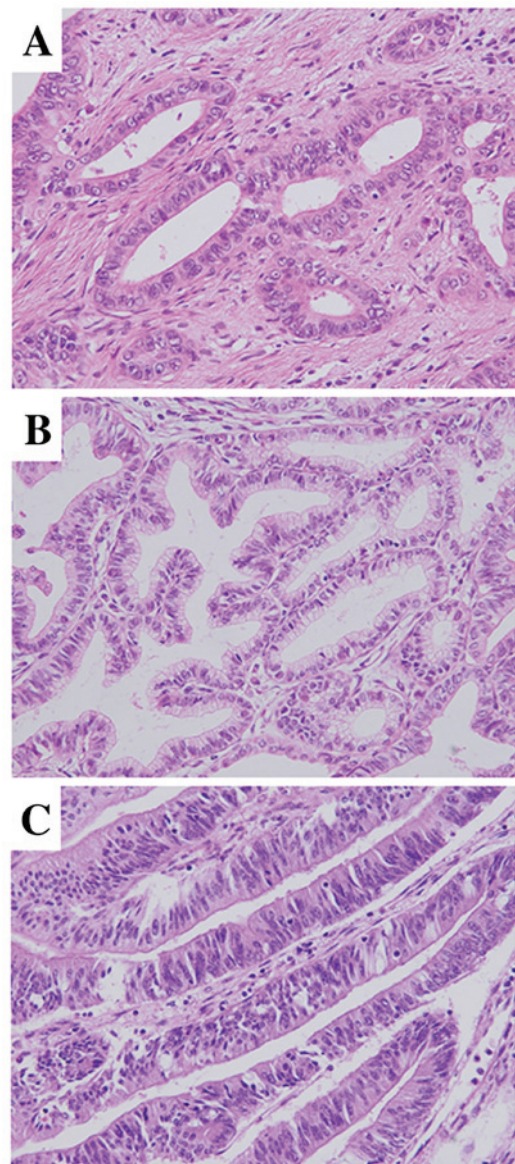


Figure 1. Three morphological subtypes of cholangiocarcinoma (hematoxylin and eosin staining). (A) Biliary-type tumors were composed of short or long tubular glands lined by cells varying in height, from cuboidal to tall columnar, superficially resembling biliary epithelium (magnification, x100). (B) Gastric foveolar-type tumors were composed of tall columnar cells with basally oriented nuclei and abundant mucin-containing cytoplasm (magnification, x100). (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, x100).

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),

Table II. Clinicopathological features according to histological phenotype in cholangiocarcinoma.

Feature	Biliary type	Metaplastic type	P-value
Total patients, n	56	43	-
Sex, n (%)			0.206
Male	43 (76.8)	29 (67.4)	
Female	13 (23.2)	14 (32.6)	
Age (years), n (%)			0.230
≥70	29 (51.8)	17 (39.5)	
<70	27 (48.2)	26 (60.5)	
Location, n (%)			0.770
Hilar	17 (30.4)	15 (34.9)	
Distal	39 (69.6)	28 (65.1)	
Size (mm)			0.307
Mean	37.8	34.4	
Range	10-75	13-85	
Carcinoembryonic antigen (ng/ml), n (%)			0.950
<5	51 (91.1)	39 (90.7)	
≥5	5 (8.9)	4 (9.3)	
Carbohydrate antigen 19-9 (U/ml), n (%)			0.673
<100	40 (71.4)	29 (67.4)	
≥100	16 (28.6)	14 (32.6)	
Carcinoma <i>in situ</i> , n (%)			0.826
Positive	26 (46.4)	19 (44.2)	
Negative	30 (53.6)	24 (55.8)	
Histological differentiation, n (%)			0.018
Pap, well, mod	45 (80.4)	38 (88.4)	
Poor, other	11 (19.6)	5 (11.6)	
T classification, n (%)			0.084
pT1-2	24 (42.9)	26 (60.5)	
pT3-4	32 (57.1)	17 (39.5)	
N classification, n (%)			0.042
pN0	32 (57.1)	33 (76.7)	
pN1	24 (42.9)	10 (23.3)	
Lymphatic invasion, n (%)			0.095
ly0-1	27 (48.2)	28 (65.1)	
ly2-3	29 (51.8)	15 (34.9)	
Venous vessel invasion, n (%)			<0.001
v1-2	20 (35.7)	33 (76.7)	
v2-3	36 (64.3)	10 (23.3)	
Perineural invasion, n (%)			0.002
ne0-1	12 (21.4)	22 (51.2)	
ne2-3	44 (78.6)	21 (48.8)	

Pap, papillary adenocarcinoma; well, well-differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma.

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 3-year 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

Table III. MUC expression in cases of cholangiocarcinoma.

MUC type	Cases exhibiting positive expression, n (%)		P-value
	Metaplastic type ^a (n=43)	Biliary type (n=56)	
MUC1	23 (53.5)	45 (80.4)	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

^aGastric foveolar + intestinal types. MUC, mucin.

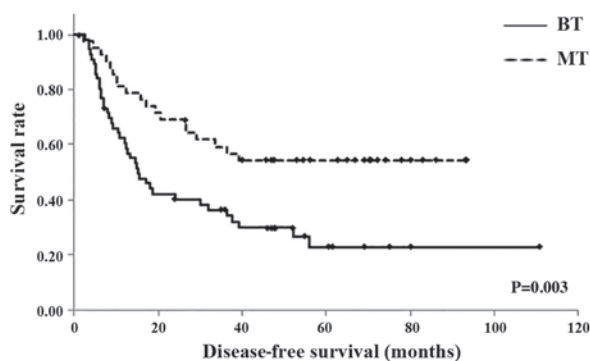


Figure 2. Kaplan-Meier estimates of disease-free survival in patients with cholangiocarcinoma. Patients with BT exhibited reduced DFS times (log-rank $P=0.003$). BT, biliary-type cholangiocarcinoma; MT, metaplastic-type cholangiocarcinoma.

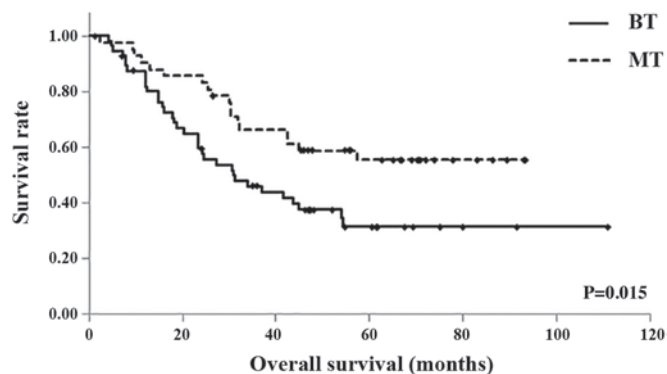


Figure 3. Kaplan-Meier estimates of overall survival in patients with cholangiocarcinoma. Patients with BT exhibited reduced OS times (log-rank $P=0.015$). BT, biliary-type cholangiocarcinoma; MT, metaplastic-type cholangiocarcinoma.

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, cholangiocarcinoma specimens were classified into two phenotypes (BT and MT), and examined according to the clinicopathological features and prognosis of the patients. Compared with MT, BT tumors tended to have higher T stages, and this was also found to be associated with increased rates of lymph node metastasis, severe venous invasion, and severe perineural invasion. Furthermore, with regard to OS and DFS, survival was significantly decreased in patients with BT compared with those with MT tumors.

In a previous study, Furukawa *et al* (5) reported that patients with pancreatobiliary-type IPMN have a significantly poorer prognosis than those with GT or IT tumors. Yamamoto *et al* (13)

classified gallbladder carcinoma into non-metaplastic and metaplastic types, and reported that patients with non-metaplastic-type tumors exhibited a higher incidence of direct invasion to the liver and significantly shorter survival times (13). Previously, our colleagues reported that carcinogenesis of cholangiocarcinoma can occur via two pathways: One originating from the biliary epithelium, in which a biliary phenotype is expressed; and one originating from the metaplastic epithelium, in which gastric and intestinal phenotypes are expressed (14).

In the present study, 99 specimens were classified into three phenotypes: 56 cases of BT, 42 cases of GT, and 1 case of IT. Focusing on the malignant potential of BT, the GT and IT groups were combined as an MT group, as reported by Yamamoto *et al* (13), and the survival differences between the BT and MT groups were then investigated. As a result, it was revealed that extrahepatic cholangiocarcinomas with a BT phenotype had greater malignant potential compared with those with the MT phenotype. However, multivariate analysis using a Cox proportional hazards model showed that BT phenotype expression was not an independent prognostic factor for OS, and lymph node metastasis and venous infiltration were found to have a greater influence on prognosis. In the BT phenotype group, features of locally advanced disease (pN1, v2-3 and ne2-3) were more commonly observed. Thus, it was hypothesized that the BT phenotype may be strongly associated with multiple prognostic factors, and could not be considered an independent prognostic factor.

Table IV. Univariate and multivariate analyses of overall survival.

Variable	Univariate analysis			Cox proportional hazards analysis		
	Cases, n (%)	MST (months)	P-value ^a	HR	95% CI	P-value
Phenotype of tumor			0.015			
Metaplastic type	43 (43.4)	n.a.		-	-	-
Biliary type	56 (56.6)	32.0		0.82	0.45-1.52	0.532
Histological grade			0.018			
G1-2	28 (28.3)	n.a.		-	-	-
G3-4	71 (71.7)	31.0		1.86	0.82-4.30	0.137
N classification			<0.001			
N0	65 (65.7)	n.a.		-	-	-
N1	34 (34.3)	23.0		2.02	1.13-3.62	0.018
Venous infiltration			<0.001			
v0-1	53 (53.5)	n.a.		-	-	-
v2-3	46 (46.5)	30.0		1.73	0.90-3.36	0.105
Perineural invasion			0.030			
ne0-1	34 (34.3)	n.a.		-	-	-
ne2-3	65 (65.7)	32.0		1.01	0.52-2.18	0.862

^aP-value calculated by log-rank test based on the Kaplan-Meier survival analyses. MST, median survival time; n.a., not applicable (median could not be calculated as >50% of patients remained alive at the end of follow-up); HR, hazard ratio; CI, confidence interval.

In order to examine the immunohistological differences between BT and MT, MUC protein expression was investigated in the tumor tissues. BT tumors exhibited a significantly higher rate of MUC1 positivity (80.4%) compared with MT (P=0.004). Thus, there appears to be a strong association between BT and MUC1 expression. The MUC1 protein is a MUC core protein responsible for the mucous lining of inner cavities, such as the gastrointestinal and respiratory tracts. MUCs are divided into secretory MUCs and membrane-bound MUCs 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, MUC molecules of the latter have an extracellular domain, transmembrane domain, and intracellular domain. Membrane-bound mucins can pass through the cell membrane, and the main core proteins include MUC1, MUC3 and MUC4. Of particular note, membrane-bound MUC1 acts as an adhesion molecule for cancer cells (15-17), and is considered to contribute to extravascular migration of cancer cells and metastasis, such as in lung, breast, gastric, pancreatic and colorectal cancers (18). Furthermore, research has suggested the application of MUC1 as a tumor marker in several malignant neoplasms (19,20), as well as a target in immunotherapy (21-23). Park *et al* (24) examined the expression of MUC1, MUC2, MUC5AC and MUC6 in cholangiocarcinoma, and reported that MUC1-positive patients exhibited considerably advanced histological differentiation, T stage, perineural invasion, and venous invasion; thus, MUC1 expression within tumor tissue was reported to be a potential factor for poor prognosis.

In the present study, it was demonstrated that BT was strongly associated with an increased ratio of tumors with positive MUC1 expression. Therefore, it was suggested that

the function of MUC1 as a cancer cell adhesion molecule and its properties as a metastasis inducer contributed to high-grade extrahepatic BT cholangiocarcinoma. Furthermore, compared with MT tumors, it was revealed that patients with BT tumors had significantly shorter DFS and OS times, and thus it was hypothesized that BT could be a predictive factor for prognosis in cases of extrahepatic cholangiocarcinoma. To the best of our knowledge, no studies reported to date have investigated the difference in clinicopathological features and prognosis according to extrahepatic cholangiocarcinoma phenotype, and this is the first study to do so.

The present study had certain limitations. First, it was a retrospective study involving a limited number of cases. Second, BT phenotype expression was not determined to be an independent prognostic factor for extrahepatic cholangiocarcinoma on multivariate analysis, and 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, extrahepatic cholangiocarcinoma may be classified into BT and MT phenotypes, and tumors with the BT phenotype appear to have a higher malignant potential. Thus, the BT phenotype could potentially be an important factor associated with poor prognosis.

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