ORIGINAL ARTICLE

PHENOTYPIC CHARACTERIZATION OF EARLY BILIARY TRACT CARCINOMAS PROPOSES TWO CARCINOGENESIS PATHWAYS

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Abstract   Early biliary tract carcinomas（BTCs）are divided into three groups: early gallbladder carcinoma（GBC）, early extrahepatic bile duct carcinoma（EBC）, and early duodenal ampullary carcinoma（DAC）. These early carcinomas frequently show metaplastic changes. However, phenotypic characterization has not yet been examined. We examined 76 lesions of surgically resected early biliary tract carcinomas（pTis/pT1 tumors according to TNM classification）. The predominant carcinoma phenotypes were classified with hematoxylin and eosin（HE）stain, and immunohistochemical examinations（MUC1, MUC2, MUC5AC, MUC6, and CD10）were performed. We also analyzed phenotypes of the surrounding non-neoplastic mucosa. Of the 33 early GBCs, 18（54.5%）were biliary type, and 15（45.5%）were metaplastic type（gastric foveolar type/intestinal type）carcinoma. Of the 26 early EBCs, 18（69.2%）were biliary type carcinoma, and eight（30.8%）were metaplastic type carcinoma. Of the 17 early DACs, eight（47.1%）were biliary type carcinoma, nine（52.9%）were metaplastic type carcinoma. Biliary type carcinomas less frequently showed metaplastic changes, while metaplastic type carcinomas were frequently surrounded by the metaplastic mucosa. Early GBC and early DAC more frequently showed metaplastic changes, compared to the early EBC. In conclusion, we speculated that two carcinogenesis pathways of early BTC（GBC, EBC, and DAC）:（1）carcinomas arising from the proper epithelium（mainly EBC）and（2）carcinomas from the metaplastic epithelium（mainly GBC and DAC）.

Introduction

Patient's prognosis of biliary tract carcinomas（BTCs）, including extrahepatic bile duct carcinoma（EBC）, gallbladder carcinoma（GBC）, and duodenal ampullary carcinoma（DAC）, are still poor. The incidence of BTCs has been increasing worldwide over the past several decades[1, 2]. In Japan, the mortality of BTCs is six most common malignancies, and more than 18,000 peoples died of this cancer in every year[3]. Symptoms indicating BTCs are jaundice, pain in the upper right area of the abdomen, general malaise, anorexia, body weight loss, and so on[4-10]. However, these complain are not specific, and patients often do not have any serious symptoms in early stage cancer. Surgical resection is the only hope for curative treatment in biliary tract cancer, but curative resection rate has remained low at around 40%[11]. Therefore, in order to improve prognosis, it is the most important things how to detect and confirm malignant neoplasm at early stage[12].

Previous studies have reported several risk

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factors of BTCs as follows: (i) pancreaticobiliary maljunction, primary sclerosing cholangitis, and hepatolithaiasis, clonorchiasis (risk factor of EBC); and (ii) pancreaticobiliary maljunction, choliolithaiasis, gallbladder polyp, and adenomyomatosis (risk factor of GBC)[13]. These condition result in chronic inflammation and persistent stimulation. Furthermore, atypical epithelium, dysplasia, and metaplasia in the surroundin mucosa of GBC, and are are often observed associated with carcinoma in situ[14, 15]. Several molecular biological studies have mentioned the existence of metaplasia-dysplasia-carcinoma sequence[16-18].

In WHO classification, BTCs are histologically divided into three groups (i.e., biliary type, gastric foveolar type, and intestinal type) based on the carcinoma phenotypes. In this study, we analyzed the relationship between phenotypic characterization in the mucosal neoplastic tissues, as well as in the non-neoplastic surrounding mucosae, and speculated the carcinogenesis pathways.

Materials and methods

Definition of early biliary tract carcinomas (BTCs)

Early BTCs were divided into three groups: early gallbladder carcinoma (GBC), early extrahepatic bile duct carcinoma (EBC), and early duodenal ampullary carcinoma (DAC).

Early GBC was categorized as ''early carcinoma limited to the mucosae or invading as far as muscular layer." Early EBC was categorized as "early carcinoma limited to the mucosae or invading as far as fibromuscular layer." Early DAC was categorized as "early carcinoma limited to the mucosae or invading as far as sphincter of Oddi." All cases we regarded as early BTCs correspond to pTis or pT1 in the American Joint Committee on Cancer (AJCC) and Union for International Cancer Control

(UICC) TMN system[19, 20].

Tissue specimens

A total of 82 surgically resected early BTC specimens were obtained by surgical resection from January 1996 to December 2014, and retrieved from the pathology files of Hirosaki University Hospital, Hirosaki, Japan. The series consisted of 33 GBCs, 26 EBCs and 17 DACs. The 32 cases (33 lesions) of GBC consisted of 15 men and 17 women with a median age of 73 years (range, 41-94 years). The 25 cases (26 lesions) of EBCs consisted of eight men and 17 women with a median age 67 years (range, 53-80 years). DACs consisted of 11 men and six women with a median age of 69 years (range, 47-82 years).

Pathological evaluation

The surgically resected specimens were fixed routinely in 10% neutral buffered formalin, and the whole tumor nodules were processed into paraffin blocks for pathological examination. Tissue sections were cut into four-μm-thick slices, and stained with hematoxylin and eosin (HE) stain.

The 82 BTCs were classified into three histological phenotypes according to the WHO classification as follows: (a) Biliary type: the carcinoma was composed of short or long tubular glands lined by cells that vary in height from cuboidal to tall columnar, superficially resembling biliary epithelium; (b) Gastric foveolar type: the carcinoma was composed of tall columnar cells with basally oriented nuclei and abundant mucin-containing cytoplasm; and (c) Intestinal type: the carcinoma was composed of tubular glands closely resembling those of colonic adenocarcinoma, or consisted of glands lined predominantly of goblet cells usually with a variable number of neuroendocrine and
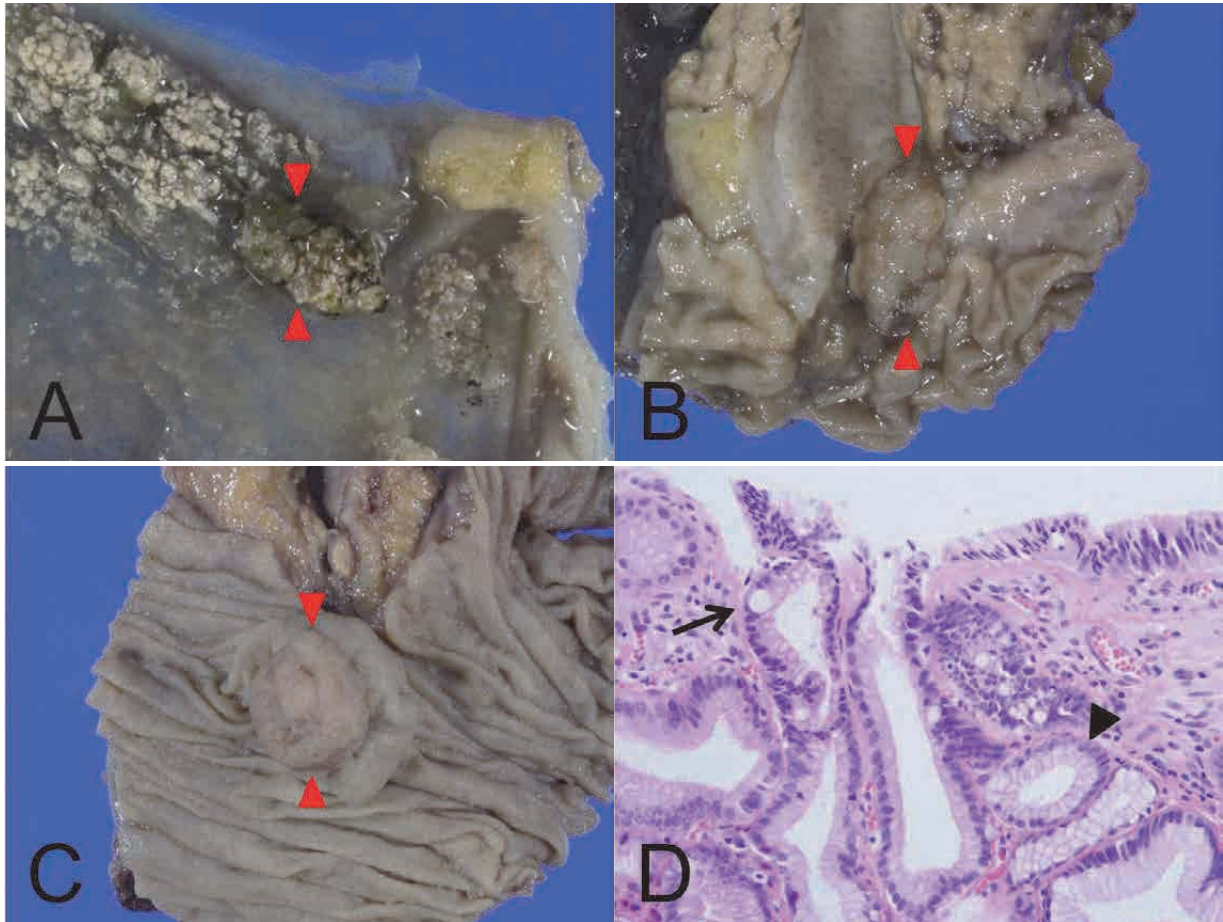
Figure 1 Gross photograph of early biliary tract carcinoma（red arrow head）（A: GBC, B: EBC, C: DAC）and non-neoplastic surrounding mucosa with pseudopyloric glandmetaplasia（arrow head）and goblet cell metaplasia（arrows）.

Paneth cells. We used a term "metaplastic type" as the "gastric foveolar type and/or intestinal type." The existence of any phenotype was acknowledged regardless of the amount, even if it was minimal or underdeveloped. However, each case was assigned a final phenotype on the basis of predominant pattern（>50% of the lesion）.

Non-neoplastic surrounding mucosae around the early BTCs frequently show not only proper epithelium phenotype, but also metaplastic epithelium（Figure 1）. In this study, the metaplastic phenotypes were divided into the gastric type and intestinal type.

Immunohistochemistry

For histological examination, EBTC specimens were routinely fixed in 10% neutral buffered formalin, and embedded in paraffin and thin-sectioned. Tissue sections 4-μm-thick were mounted on saline-coated glass slides. Immunohistochemical examination was performed on deparaffinized sections using the standard avidin-biotin-peroxidase complex method with automated immunostainer（Benchmark XT; Ventana Medical System, Tucson, AZ, USA）. We investigated the phenotype of EBTC using MUC1, MUC2, MUC5AC, MUC6 antibodies. CD10 was used for aid in the evaluation of malignant or benign

Table 1. Histological phenotypes of early biliary tract carcinoma.

	B type	G type	I type	O type
Early BTC（Our study）（n=76）	57.9%（44/76）	32.9%（25/76）	9.2%（ 7/76）	0%
GBC（n=33）	54.5%（18/33）	39.4%（13/33）	6.1%（ 2/33）	0%
EBC（n=26）	69.2%（18/26）	26.9%（ 7/26）	3.8%（ 1/26）	0%
DAC（n=17）	47.1%（ 8/17）	29.4%（ 5/17）	23.5%（ 4/17）	0%
ICPN[※]	50.0%（61/123）	35.8%（44/123）	8.1%（10/123）	6.5%（8/123）

BTC: biliary tract carcinoma, GBC: gallbladder carcinoma, EBC: extrahepatic bile duct carcinoma, DAC: early duodenal ampullary carcinoma, ICPN: intracholecystic papillary-tubular neoplasms, B type: biliary type carcinoma, G type: gastric foveolar type carcinoma, I type: intestinal type carcinoma, O type: oncocytic type carcinoma. ※: Adsay V, et al, references[22].

lesions of the epithelium[21]. The antibodies we used were: MUC1 （1:50, clone Ma696）, MUC2 （1:50, clone Ccp58）, MUC5AC （1:100, clone CLH2）, MUC6 （1:100, clone CLH5; all from Novocastra Laboratories, Newcastle, UK）, CD10 （1:100, clone 56C6; DAKO, Carpiteria, CA, USA）.

The histological indicator of gastric-type metaplasia （GM） is the presence of MUC5AC and/or MUC6, and the indicator of intestinal-type metaplasia （IM） is the presence of MUC2.

When at least one of the three indicators of metaplasia （MUC2, MUC5AC, and MUC6） was present in the carcinoma tissue, and in the non-neoplastic surrounding mucosa within 5 mm from the carcinoma margin, we interpreted them as carcinoma with metaplastic changes and non-neoplastic metaplastic mucosa, respectively.

Evaluation of immunohistochemistry

Luminal membranous immunoreactivities of the tumor cells were recognized as positive reactions for MUC1 and CD10. Cytoplasmic immunoreactivities of the tumor were recognized as positive for MUC2, MUC5AC and MUC6. According to the above immunohistochemical expression of each mucin and CD10, the cases were divided into two groups; a negative group in which less than 10% of tumor cells were stained, and a positive group in which more than 10% were stained.

Statistical analysis

Statistical comparisons between two groups were analyzed using the Pearson's Chi-square test for categorical data. Differences were considered to be statistically significant if the P-value was <0.05.

Results

Histological and immunohistochemical findings of early BTC.

We reviewed 76 cases of early BTCs （33 early GBCs, 26 early EBCs, and 17 early DACs） and divided them into three phenotypes （biliary type, gastric foveolar type and intestinal type）. Of the 33 early GBCs, 18 （54.5%） were biliary type and 15 （45.5%） were metaplastic type （gastric foveolar type or intestinal type）. Of the 26 early EBCs, 18 （69.2%） were biliary type and eight （30.8 %） were metaplastic type. Of the 17 early DACs, eight （47.1%） were biliary type and nine （52.9%） were metaplastic type （Table 1）. Results of immunohistochemical analyses of early BTC are shown in Table 2 and Figure 2.

There was statistically significant difference in the frequency of MUC2 expression between biliary type carcinoma and intestinal type carcinoma （P<0.01; Figure 3A）. In addition, there were statistically significant differences in the frequency of MUC5AC/MUC6 expression between biliary type carcinoma and gastric foveolar type carcinoma （MUC5AC: P<0.05,

**Table 2.** Mucin expression of early biliary tract carcinoma.

		Carcinoma					Surrounding mucosa				
		MUC1	MUC2	MUC5 AC	MUC6	CD10	MUC1	MUC2	MUC5 AC	MUC6	CD10
Early BTC (Our study) (n=76)		46.1% (35/76)	43.4% (33/76)	43.4% (33/76)	51.3% (39/76)	68.4% (52/76)	28.9% (22/26)	36.8% (28/76)	27.6% (21/76)	77.6 (59/76)	64.5% (49/76)
GBC (n=33)		54.5% (18/33)	45.5% (15/33)	45.5% (15/33)	48.5% (16/33)	63.6% (21/33)	45.5% (15/33)	54.5% (18/33)	42.4% (14/33)	84.8% (28/33)	81.8% (27/33)
	B type (n=18)	55.6% (10/18)	44.4% (8/18)	50.0% (9/18)	44.4% (8/18)	83.3% (15/18)	50.0% (9/18)	61.1% (11/18)	44.4% (8/18)	83.3% (15/18)	94.4% (17/18)
	G type (n=13)	53.8% (7/13)	46.2% (6/13)	38.5% (5/13)	61.5% (8/13)	38.5% (5/13)	30.8% (4/13)	38.5% (5/13)	30.8% (4/13)	84.6% (11/13)	61.5% (8/13)
	I type (n=2)	50.0% (1/2)	50.0% (1/2)	50.0% (1/2)	0% (0/2)	50.0% (1/2)	100% (2/2)	100% (2/2)	100% (2/2)	100% (2/2)	100% (2/2)
EBC (n=26)		30.8% (8/26)	23.1% (6/26)	30.8% (8/26)	34.6% (9/26)	73.1% (19/26)	11.5% (3/26)	19.2% (5/26)	15.4% (4/26)	61.5% (16/26)	53.8% (14/26)
	B type (n=18)	33.3% (6/18)	11.1% (2/18)	5.6% (1/18)	11.1% (2/18)	72.2% (13/18)	11.1% (2/18)	11.1% (2/18)	5.6% (1/18)	44.4% (8/18)	50.0% (9/18)
	G type (n=7)	28.6% (2/7)	42.9% (3/7)	85.7% (6/7)	100% (7/7)	71.4% (5/7)	14.3% (1/7)	42.9% (3/7)	42.9% (3/7)	100% (7/7)	57.1% (4/7)
	I type (n=1)	0% (0/1)	100% (1/1)	100% (1/1)	0% (0/1)	100% (1/1)	0% (0/1)	0% (0/1)	0% (0/1)	100% (1/1)	100% (1/1)
DAC (n=17)		52.9% (9/17)	70.6% (12/17)	58.8% (10/17)	82.4% (14/17)	70.6% (12/17)	23.5% (4/17)	29.4% (5/17)	17.6% (3/17)	88.2% (15/17)	47.1% (8/17)
	B type (n=8)	62.5% (5/8)	50.0% (4/8)	50.0% (4/8)	75.0% (6/8)	37.5% (3/8)	37.5% (3/8)	25.0% (2/8)	12.5% (1/8)	87.5% (7/8)	62.5% (5/8)
	G type (n=5)	60.0% (3/5)	80.0% (4/5)	80.0% (4/5)	100% (5/5)	100% (5/5)	20.0% (1/5)	40.0% (2/5)	40.0% (2/5)	80.0% (4/5)	40.0% (2/5)
	I type (n=4)	25.0% (1/4)	100% (4/4)	50.0% (2/4)	75.0% (3/4)	100% (4/4)	0% (0/4)	25.0% (1/4)	0% (0/4)	100% (4/4)	25.0% (1/4)

BTC: biliary tract carcinoma, GBC: gallbladder carcinoma, EBC: extrahepatic bile duct carcinoma, DAC: duodenal ampullary carcinoma, B type: biliary type carcinoma, G type: gastric foveolar type carcinoma, I type: intestinal type carcinoma.

MUC6: $P<0.01$; Figure 3A). The gastric foveolar type carcinoma tended to show frequent MUC6 expression, compared to the intestinal type carcinoma ($P=0.053$). CD10 expression in carcinoma exhibited no significant difference in three phenotypes. The surrounding mucosa tended to show frequent MUC6 expression, compared to biliary type (gastric foveolar type vs biliary type: $P=0.066$, intestinal type vs biliary type: $P=0.080$; Figure 3C). There was statistically significant difference in the frequency of MUC6 expression between biliary type carcinoma and metaplastic type carcinoma ($P<0.05$; Figure 3D).

Metaplastic changes of early biliary tract carcinoma and the surrounding mucosa.

We reviewed the phenotypes of both carcinoma and surrounding mucosa and classified into four groups, **i.e.,** (1) carcinoma without metaplastic change (biliary type carcinoma), and surrounding mucosa without metaplastic change (proper surrounding mucosa); (2) biliary type carcinoma, and surrounding mucosa with metaplastic change (metaplastic surrounding mucosa); (3) carcinoma with metaplastic change (metaplastic type carcinoma), and proper surrounding mucosa; (4) metaplastic type
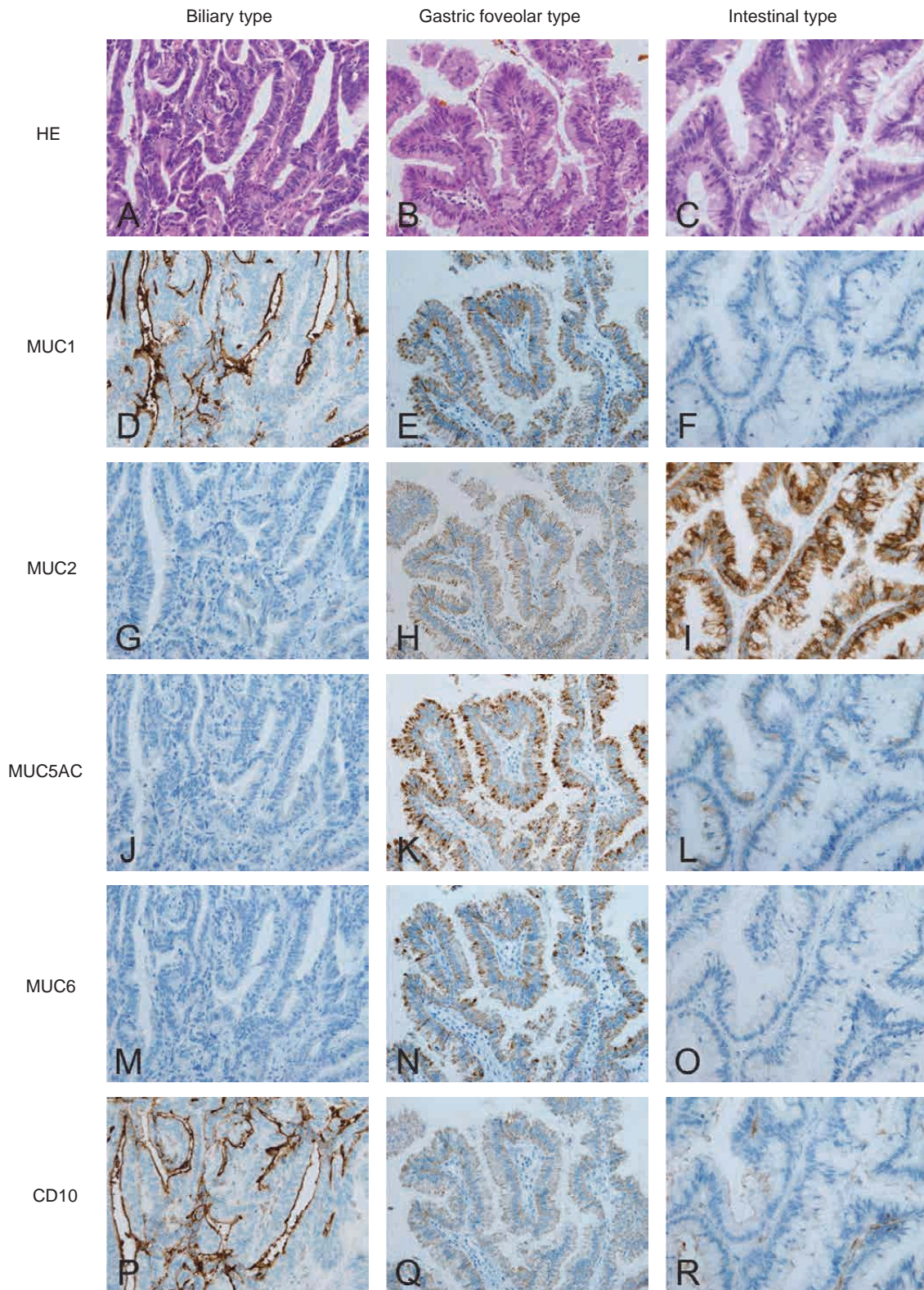
Figure 2 Representative mucin expression images of early BTC. A case of biliary type carcinoma (A, D, G, J, M, P). A case of gastric foveolar type (B, E, H, K, N, Q) and a case of intestinal type (C, F, I, L, O, R). Immunohistochemical staining of MUC1 (D)-(F), MUC2 (G)-(I), MUC5AC (J)-(L), MUC6 (M)-(O), and CD10 (P)-(R). Biliary type carcinoma (A, D, G, J, M, P) shows MUC1 and CD10 positive, MUC2, MUC5AC, MUC6 negative. Gastric foveolar type carcinoma (B, E, H, K, N, Q) shows MUC1, MUC2, MUC5AC, and MUC6 positive, CD10 negative. Intestinal type (C, F, I, L, O, R) shows MUC2 positive, MUC1, MUC5AC, MUC6, CD10 negative.

A.

B.

C.

D.
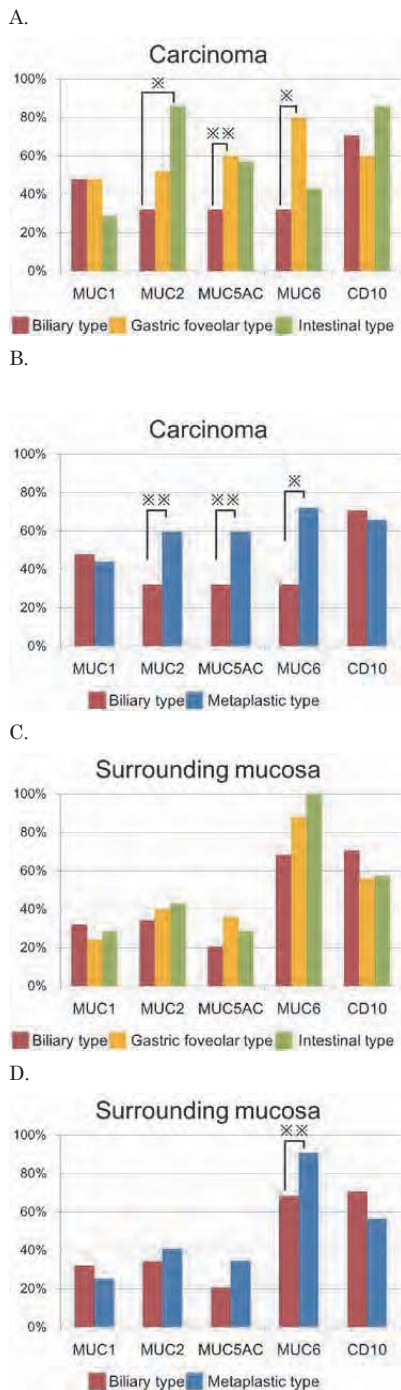
Figure 3 Relationship between expression rate of immunohistochemical staining and histological phenotype. (A) The expression rate of MUC2 was significantly higher in intestinal type carcinoma than in biliary type carcinoma. The expression of MUC5AC and MUC6 was significantly higher in gastric foveolar type than in biliary type carcinoma. (B) No statically significant differences were recognized in surrounding mucosa of the tumor. (C) In surrounding mucosa of the tumor, the expression rate of MUC6 was significantly higher in metaplastic type carcinoma than in biliary type carcinoma. ※P<0.01, ※※P<0.05 (significant difference in the expression).

carcinoma, and metaplastic surrounding mucosa (Table 3 and Figure 4).

Of the total 33 early GBCs, the biliary type carcinoma [(1) + (2), described above] were total seven (7/33; 21.2%), while the metaplastic type carcinoma [(3) + (4), described above] were total 12 (12/33; 36.4%). Of the total 26 early EBCs, the biliary type carcinoma were total 14 (14/26; 53.8%), while the metaplastic type carcinoma were total eight (8/26; 30.8%). Of the total 17 early DACs, the biliary type carcinoma were total two (2/17; 11.8%), while the metaplastic type carcinoma were total nine (9/17; 30.8%).

Discussion

In the present study, we investigated histological phenotype of EBTC and metaplastic changes in the tumor and surrounding non-neoplastic mucosa, using 76 surgically-resected primary biliary tract carcinomas. EBTC were classified into three groups with hematoxylin and eosin stain; biliary type, gastric foveolar type, and intestinal type. In our study the proportion of histological phenotypes were almost the same as intracholecystic papillary-tubular neoplasm (ICPN) of the gallbladder[22]. As well as several studies have reported between histological phenotype and mucin expression in biliary tract cancer[23, 24], in our study MUC2 expression in EBTC was one of the markers to distinguish between biliary type carcinoma and intestinal type carcinoma similarly. MUC5AC and MUC6 expression were one of markers to distinguish gastric foveolar type carcinoma from biliary type carcinoma in EBTC. Furthermore, MUC6 can be used to distinguish between gastric foveolar type carcinoma and intestinal type carcinoma. Some investigators showed the diagnostic utility of CD10 in benign and malignant extrahepatic bile duct lesions[21]. They showed 96% of high

Table 3. Metaplastic changes of early biliary tract carcinoma and the surrounding mucosa.

	eraly BTC (n=76)			B type (n=44)			M type (n=32)		
	GBC (n=33)	EBC (n=26)	DAC (n=17)	GBC (n=18)	EBC (n=18)	DAC (n=8)	GBC (n=15)	EBC (n=8)	DAC (n=9)
C(−)S(−)	9.1% (3/33)	34.6% (9/26)	5.9% (1/17)	16.7% (3/18)	50.0% (9/18)	12.5% (1/8)	0.0% (0/15)	0% (0/8)	0% (0/9)
C(−)S(+)	21.2% (7/33)	19.2% (5/26)	5.9% (1/17)	22.2% (4/18)	27.8% (5/18)	12.5% (1/8)	20.0% (3/15)	0% (0/8)	0% (0/9)
C(+)S(−)	0% (0/33)	3.8% (1/26)	0% (0/17)	0% (0/18)	5.6% (1/18)	0% (0/8)	0.0% (0/15)	0% (0/8)	0% (0/9)
C(+)S(+)	69.7% (23/33)	42.3% (11/26)	88.2% (15/17)	61.1% (11/18)	16.7% (3/18)	75.0% (6/8)	80.0% (12/15)	100% (8/8)	100% (9/9)

BTC: biliary tract carcinoma, GBC: gallbladder carcinoma, EBC: extrahepatic bile duct carcinoma, DAC: duodenal ampullary carcinoma, B type: biliary type carcinoma, M type: metaplastic type (gastric foveolar type plus intestinal type) carcinoma, C(−): carcinoma without metaplastic change, C(+): carcinoma with metaplastic change, S(−): surrounding mucosa without metaplastic change, S(+): surrounding mucosa with metaplastic change.
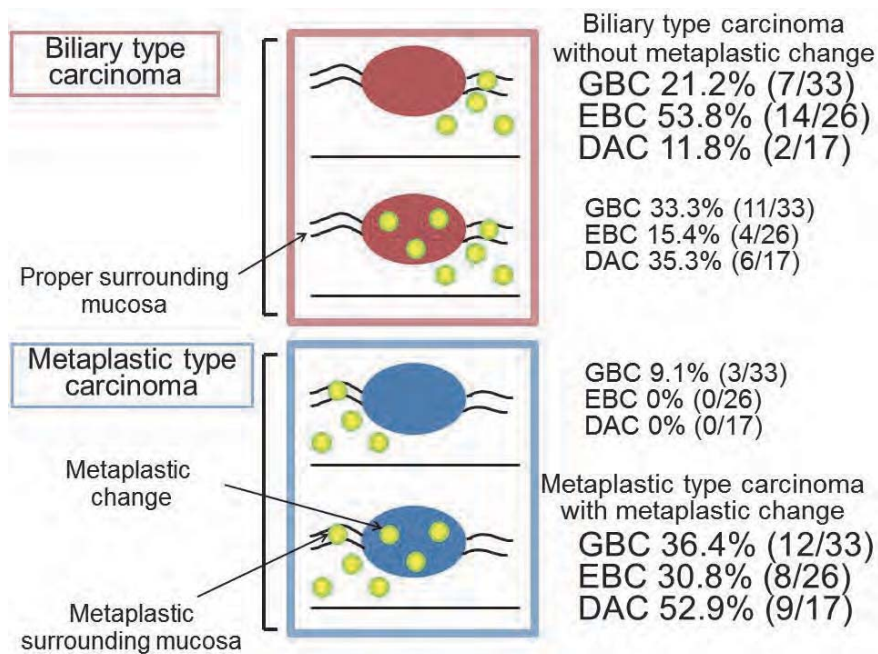

Figure 4 Relationship between phenotype and metaplastic change in the tumor tissue of mucosae and in the non-neoplastic surrounding mucosae.
We reviewed the phenotypes of both carcinoma and surrounding mucosa and classified into four groups, **i.e.,** (1) carcinoma without metaplastic change (biliary type carcinoma), and surrounding mucosa without metaplastic change (proper surrounding mucosa); (2) biliary type carcinoma, and surrounding mucosa with metaplastic change (metaplastic surrounding mucosa); (3) carcinoma with metaplastic change (metaplastic type carcinoma), and proper surrounding mucosa; (4) metaplastic type carcinoma, and metaplastic surrounding mucosa. Biliary type cartcinoma ((1) + (2)) may arise from proper epithelium. Metaplastic type carcinoma ((3) + (4)) may arise from metaplastic epithelium.

grade dysplasia of the bile duct lesions lacked expression of CD10. But in our study over 68 % of EBTC express CD10 and CD10 expression was not useful to distinguish between phenotypes.

EGBC and EDAC more frequently showed metaplastic carcinoma compared to EEBC (Table 1). Besides, by a combination of carcinoma and metaplastic changes, it showed that EGBC
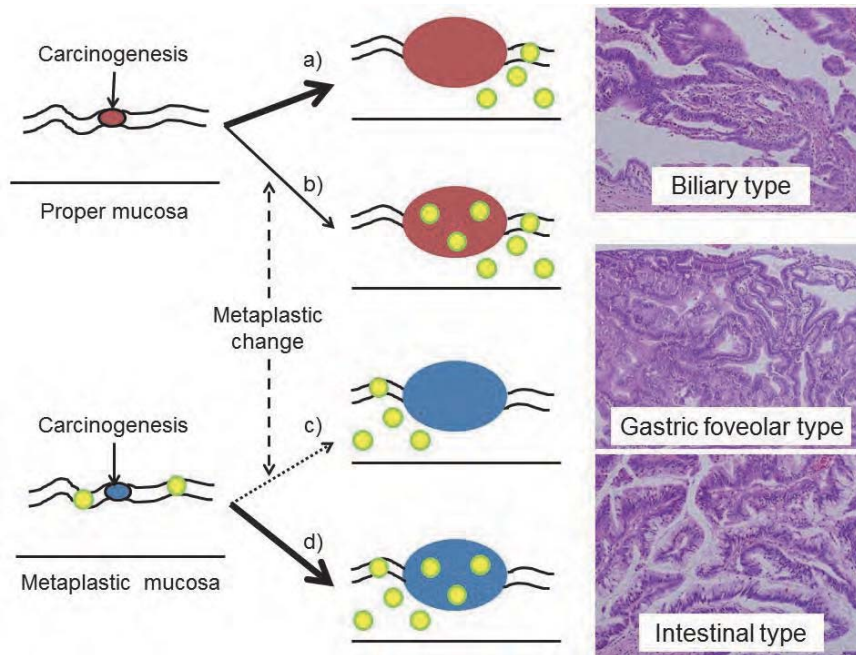
Figure 5 GBC: d)＞b)＞a)
　　　　　　EBC: mainly proper epiyhelium
　　　　　　DAC: d) main

and EDAC have more frequently metaplastic changes, compared to EEBC (Table 3). It may be the reason why membranous epithelium of EEBC is thiner than that of EDAC and EGBC, and therefore metaplastic change occurs less frequently in EEBC than in EGBC and EDAC. And it may be also the reason why ampullary region is near intestine and it makes chronic inflammation and persistent stimulation, and consequently metaplastic changes occur much frequently in EDAC than in EEBC. In all lesions of metaplastic carcinoma, metaplastic changes existed in surrounding mucosa. On the other hand, in some lesions of biliary type carcinoma, metaplastic changes were lacked in surrounding mucosa (Table 3).

From this, we speculated that biliary type carcinoma may arise from the proper epithelium and metaplastic carcinoma may arise from metaplastic epithelium: 21.2% (7/33) of EGBC, 53.8% (14/26) of EEBC, and 11.8% (2/17) of EDAC may arise from the proper epithelium; 36.4% (12/33) of EGBC, 30.8% (8/26) of EEBC, and 52.9% (9/17) of EDAC may arise from metaplastic epithelium. Previous study also indicated that EGBC could have two carcinogenesis pathway (1) carcinomas arising from proper epithelium, and (2) carcinomas from metaplastic epithelium. Our data can be regarded as a result of support.

Conflict t of interest

The authors declare that they have no conflict of interest.

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