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Original contribution

The expression of gastrointestinal differentiation markers in extrahepatic cholangiocarcinoma: clinicopathological significance based on tumor location☆

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Summary The expression of gastrointestinal differentiation markers is associated with the tumorigenesis and prognosis of digestive cancers. However, little is known about the significance of gastrointestinal differentiation marker profiles in patients with extrahepatic cholangiocarcinoma (CCA), which is classified as perihilar and distal CCA. The purpose of this study was to clarify the role of gastrointestinal differentiation marker expression in extrahepatic CCA based on tumor location. We examined the expression of gastrointestinal differentiation markers in resected perihilar (n = 30) and distal (n = 54) CCAs based on the immunohistochemical expression of the following markers: MUC2, MUC5AC, MUC6, CD10, CDX-2, and cytokeratin 20. Expression scores were determined semiquantitatively based on the rate of positively stained cells. Furthermore, we performed hierarchical clustering of the CCAs based on the immunohistochemical expression scores to evaluate differences in the expression patterns of the 6 gastrointestinal differentiation markers. Consequently, perihilar and distal CCAs were stratified into 2 subgroups each. Among the perihilar CCAs, subgroup 1 was characterized by lower expression of MUC5AC and MUC6, a larger median tumor size, and a significantly worse prognosis compared with subgroup 2. Furthermore, the immunohistological subgroup (subgroup 1 versus 2) and TNM stage (stage III versus II) were independent predictors of patient survival. Among the distal CCAs, subgroup 1 was characterized by lower expression of MUC5AC compared with subgroup 2. We suggest that gastrointestinal differentiation marker profiles are useful for stratifying perihilar and distal CCAs. In addition, gastrointestinal differentiation markers play a crucial role in tumor development, particularly in perihilar CCA.

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1. Introduction

Cholangiocarcinoma (CCA) is defined as a malignant neoplasm arising from the biliary tract epithelium. The incidence of CCA is gradually increasing worldwide, with a particularly high incidence in East Asia [1-3]. The symptoms of CCA usually develop during the later stages of the disease and can be nonspecific, such as obstructive jaundice, weight loss, and cholangitis [3]. Therefore, CCA is still difficult to diagnose and treat. In general, the prognosis is poor, and the 5-year survival rate after diagnosis has not increased during recent years [4,5].

CCAs are classified into intrahepatic and extrahepatic CCAs, and extrahepatic CCAs are divided into perihilar and distal CCAs based on tumor location, according to the seventh edition of the TNM classification [6]. Perihilar CCAs are typically located in the extrahepatic biliary tree proximal to the origin of the cystic duct, whereas distal CCAs are extrahepatic biliary tract lesions found between the origin of the cystic duct and ampulla of Vater [7]. Perihilar and distal CCAs are regarded as separate entities because of differences in their epidemiology, clinical management, and prognosis [5,7,8]. However, more than 90% of CCAs are classified histologically as adenocarcinomas regardless of tumor location [5,9,10]. The histopathological differences between perihilar and distal CCAs have not been clarified.

The expression of gastrointestinal differentiation markers determines the tumor characteristics and is associated with the tumorigenesis and prognosis of gastrointestinal tract cancers [11-14]. Gastrointestinal differentiation of gastrointestinal tract tumors is based on the expression of immunohistochemical markers including MUC2, MUC5AC, MUC6, and CD10 [15]. vMoreover, CDX-2 and cytokeratin (CK) 20 are useful markers of intestinal differentiation [16,17]. Previous studies have shown that the expression of gastrointestinal differentiation markers is closely associated with tumor location. For example, it

Table 2 The antibodies used for immunohistochemical analyses

Antibody	Clone	Source	Dilution	Pretreatment
MUC2	Ccp58	Novocastra Laboratories, (Newcastle, UK)	1:100	PT Link (Dako, Carpinteria, CA)
MUC5AC	CLH2	Novocastra Laboratories	1:100	PT Link
MUC6	CLH5	Novocastra Laboratories	1:100	PT Link
CD10	56C6	Dako	Ready to use	PT Link
CDX-2	DAK-CDX2	Dako	Ready to use	PT Link
CK20	Ks20.8	Dako	Ready to use	PT Link

NOTE. PT Link, boiling bath for 20 minutes at 97°C in ethylenediaminetetraacetic acid buffer, pH 9.

Table 1 Clinicopathological characteristics of patients with perihilar or distal CCA

Parameter	Total (%)	Perihilar CCA (%)	Distal CCA (%)	*P*
No. of cases	84	30	54	
Age (y), median (range)	70.5 (33-85)	68.5 (33-79)	72.0 (52-85)	.116
Sex				
Male	61 (72.6)	25 (83.3)	36 (66.7)	.129
Female	23 (27.4)	5 (16.7)	18 (33.3)	
Largest tumor size (mm), median (range)	25 (10-93)	25 (10-40)	24.5 (10-93)	.568
Macroscopic type				.002 *
Papillary	12 (14.3)	2 (6.7)	10 (18.5)	.593
Nodular	45 (53.6)	11 (36.7)	34 (63.0)	.073
Flat	27 (32.1)	17 (56.6)	10 (18.5)	.002 *
Histological variant				.110
pap	7 (8.3)	0 (0)	7 (13.0)	
tub1	40 (47.6)	18 (60.0)	22 (40.7)	
tub2	33 (39.3)	11 (36.7)	22 (40.7)	
por	4 (4.8)	1 (3.3)	3 (5.6)	
Lymphatic invasion: positive	16 (19.0)	6 (20.0)	10 (18.5)	.869
Venous invasion: positive	9 (10.7)	4 (13.3)	5 (9.3)	.715
Perineural invasion: positive	41 (48.8)	19 (63.3)	22 (40.7)	.047 *
Stage				
I	18 (21.4)	0 (0)	18 (33.3)	
II	51 (60.7)	15 (50.0)	36 (66.7)	
III	15 (17.9)	15 (50.0)	0 (0)	

Abbreviations: pap, papillary adenocarcinoma; por, poorly differentiated adenocarcinoma.; tub1, well-differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma

* $P < .05$.

was postulated that pathological and/or molecular differences in the gastrointestinal differentiation marker profiles exist between left- and right-sided colorectal carcinomas [12,13,18]. In addition, in gastric cancers, the gastric phenotype was characterized by a poor prognosis, high proliferative activity, and specific genetic alterations including microsatellite instability (MSI), whereas the intestinal phenotype was characterized by very well-differentiated tumors with low proliferative activity and a lack of MSI [11,14]. Gastrointestinal differentiation marker expression might play a major role in tumorigenesis and tumor development. However, although several studies have focused on the expression of gastrointestinal differentiation markers in extrahepatic CCA [19,20], the role of gastrointestinal differentiation marker profiles in patients with CCA is not well defined.

Here, we hypothesized that the gastrointestinal differentiation marker profile is associated with tumorigenesis and tumor progression in perihilar and distal CCAs, as well as gastrointestinal tract cancers. In addition, there may be differences in the gastrointestinal differentiation markers expressed between perihilar and distal CCAs. The aim of this study was to identify any associations between gastrointestinal differentiation marker profiles and clinicopathological findings including patient outcomes in patients with perihilar or distal CCA.

2. Materials and methods

2.1. Patients

We examined the data of 84 patients with extrahepatic CCA retrieved from surgical pathology files at Iwate Medical University from 2007 to 2012. Each extrahepatic CCA was classified as perihilar (n = 30) or distal (n = 54) based on the surgical and pathological findings in accordance with the TNM classification [6]. The clinicopathological features of these patients were obtained from hospital records according to the classification of biliary tract cancers established by the Japanese Society of Hepato-Biliary-Pancreatic Surgery [10]. The clinicopathological findings of the patients are summarized in Table 1. Stage could not be compared statistically according to tumor location because of differences in the TNM classification criteria for staging of perihilar and distal CCA. No patient received preoperative chemotherapy or

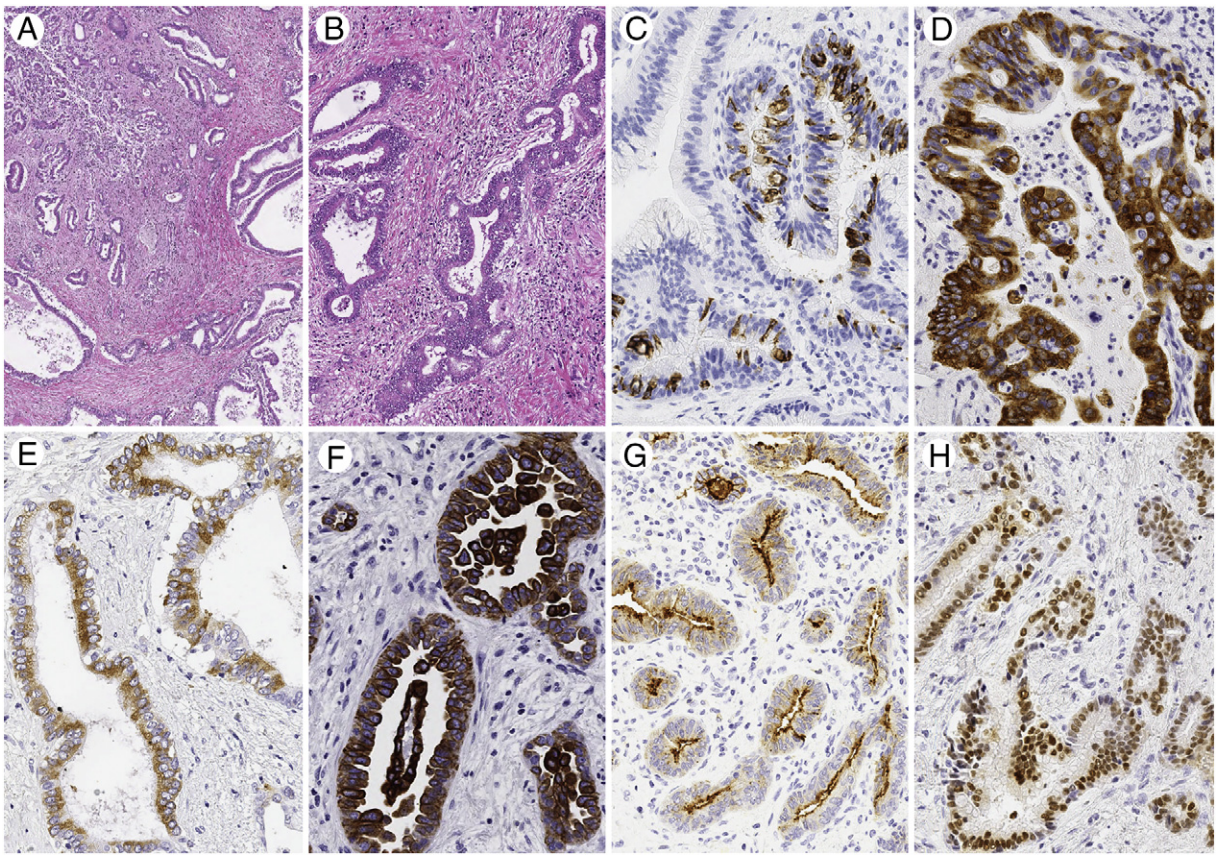


Figure 1 Representative histologic findings and immunohistochemical expression of MUC2, MUC5AC, MUC6, CK20, CD10, and CDX-2 in perihilar CCAs. A and B, Conventional CCA showing tubular adenocarcinoma (hematoxylin and eosin, original magnification ×40 [A] and ×100 [B]). C-F, Positive cytoplasmic expression of MUC2 (C), MUC5AC (D), MUC6 (E), and CK20 (F) (×200). G, Positive brush border expression of CD10 (×200). H, Positive nuclear expression of CDX-2 (×200).

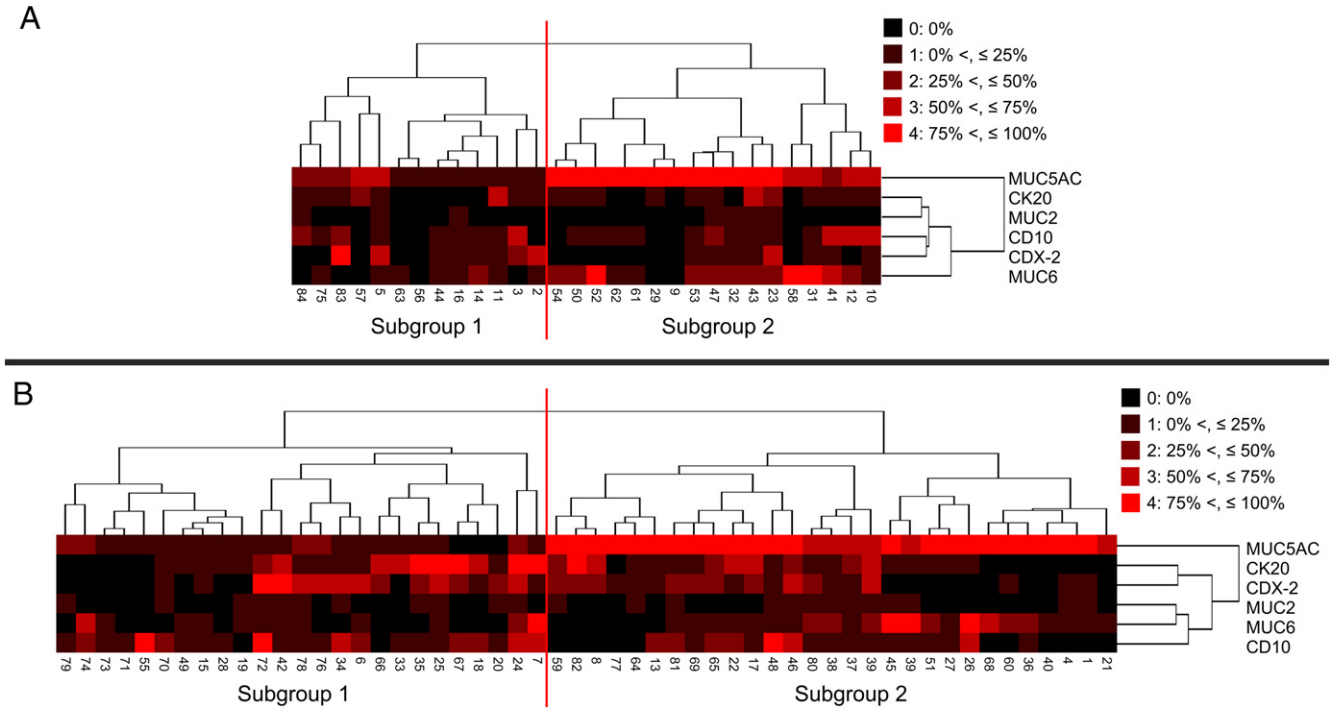


Figure 2 Hierarchical clustering of perihilar and distal CCAs according to immunohistochemical expression of MUC2, MUC5AC, MUC6, CD10, CDX-2, and CK20. A, Perihilar CCA. B, Distal CCA. Numbers indicate the patient numbers.

radiotherapy. The median follow-up period of the patients examined was 36.0 months.

The study was approved by the Ethical Research Committee of Iwate Medical University. All procedures conducted in human participants were performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments, or comparable ethical standards.

Table 3A Clinicopathological characteristics of each subgroup of patients with perihilar CCA

Parameter	Subgroup 1 (%)	Subgroup 2 (%)	*P*
No. of cases	13	17	
Age (y), median (range)	69 (66-76)	67 (33-79)	.135
Sex			
Male	11 (84.6)	14 (82.4)	.869
Female	2 (15.4)	3 (17.6)	
Largest tumor size (mm), median (range)	30 (20-40)	22 (10-30)	.001 *
Macroscopic type			
Papillary	0 (0)	2 (11.8)	.147
Nodular	7 (53.8)	4 (23.5)	
Flat	6 (46.2)	11 (64.7)	
Histological variants			
pap	0 (0)	0 (0)	.478
tub1	7 (53.8)	11 (64.7)	
tub2	6 (46.2)	5 (29.4)	
por	0 (0)	1 (5.9)	
Lymphatic invasion: positive	4 (30.8)	2 (11.8)	.197
Venous invasion: positive	3 (23.1)	1 (5.9)	.169
Perineural invasion: positive	10 (76.9)	9 (52.9)	.176
Stage			
I	0 (0)	0 (0)	.269
II	5 (38.5)	10 (58.8)	
III	8 (61.5)	7 (41.2)	

* $P < .05$.

2.2. Samples used for the histological evaluations

All specimens used in this study had been fixed in 10% neutral-buffered formalin and embedded in paraffin wax. All archival slides containing extrahepatic CCAs were stained with hematoxylin and eosin according to our routine hospital procedure. The archival slides of all cases were reviewed, and the specimens containing features that are morphologically typical of extrahepatic CCAs, including the invasive front, were selected as the representative tissue specimens.

2.3. Antibodies and immunohistochemistry

We examined the following proteins: MUC5AC (foveolar marker), MUC6 (pyloric gland marker), MUC2 (intestinal marker), and CD10 (small intestinal marker) [15]. In addition, CDX-2 is an intestine-specific transcription factor involved in intestinal development [16], and CK20 is expressed in the majority of intestinal adenocarcinomas [16]. CK20, MUC2, and CDX-2 are potentially useful markers for defining intestinal-type ampullary carcinoma [17]. The details of the antibodies used in this study are shown in Table 2.

Paraffin sections (3 μm) were mounted on polylysine-coated glass slides (Matsunami, Tokyo, Japan). Sections were routinely dewaxed, dehydrated, and then subjected to heat-induced epitope retrieval in Target Retrieval Solution, High pH (Dako, Carpinteria, CA). The slides were placed in Peroxidase-Blocking Solution (Dako) to inhibit nonspecific binding. After incubation with the primary antibody, the sections were examined using the EnVision HRP detection system (Dako), as described previously [21]. The antigen-antibody complex was visualized using Liquid DAB+ Chromogen (Dako) and counterstained with hematoxylin before mounting. The representative histologic findings and immunoreactivities of MUC2, MUC5AC, MUC6, CD10, CDX-2, and CK20 in perihilar CCA are shown in Figure 1.

2.4. Immunohistochemical evaluation

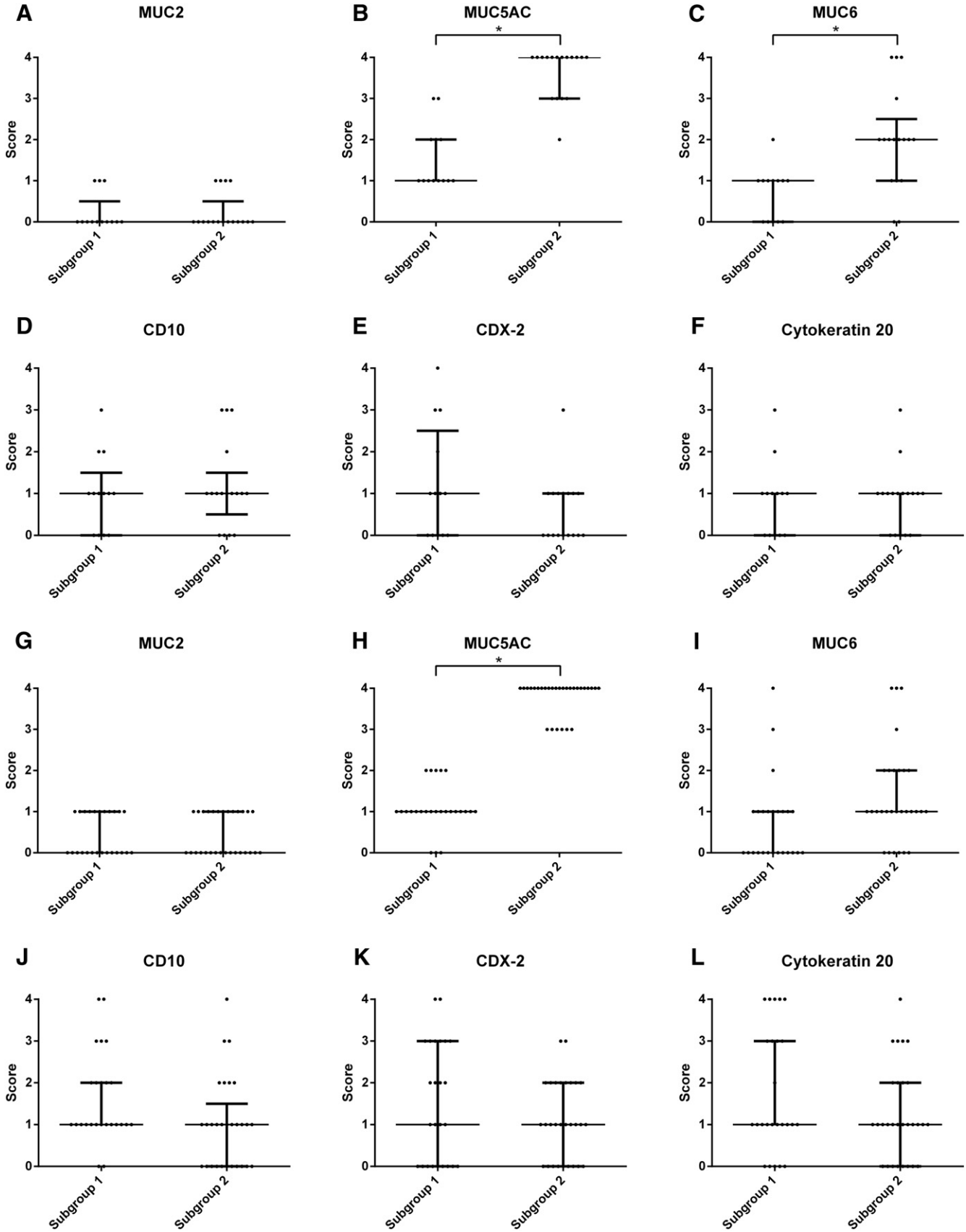
Immunohistochemical evaluation was performed independently by 3 investigators (K. I., M. O., and S. T.), who had no knowledge of the clinicopathological findings or patient outcomes. Discrepancies among the investigators were resolved by consensus using a multiheaded microscope. MUC2, MUC5AC, MUC6, and CK20 are expressed in the cytoplasm; CD10, in the cytoplasm and/or brush border; and CDX-2, in the nucleus. The immunohistochemical expression scores for MUC2, MUC5AC, MUC6, CD10, CDX-2, and CK20 were determined semiquantitatively based on the number of positive cells within each tissue section examined. The percentage of cells stained for each marker was scored as follows, where X represents the percentage of stained cells: 0 (0% cells), 1 ($0\% < X \leq 25\%$ cells), 2 ($25\% < X \leq 50\%$ cells), 3 ($50\% < X \leq 75\%$ cells), and 4 ($75\% < X \leq 100\%$ cells).

2.5. Hierarchical clustering according to marker expression

We conducted hierarchical clustering based on the immunoreactivity scores of each marker to evaluate differences in the expression patterns of MUC2, MUC5AC, MUC6, CD10, CDX-2, and CK20 in patients with perihilar and distal CCAs,

Table 3B Clinicopathological characteristic of each subgroup of patients with distal CCA

Parameter	Subgroup 1 (%)	Subgroup 2 (%)	*P*
No. of cases	25	29	
Age (y), median (range)	71 (52-85)	72 (61-83)	.124
Sex			
Male	19 (76.0)	17 (58.6)	.176
Female	6 (24.0)	12 (41.4)	
Largest tumor size (mm), median (range)	25 (12-93)	24 (10-90)	.495
Macroscopic type			
Papillary	6 (24.0)	4 (13.8)	.612
Nodular	15 (60.0)	19 (65.5)	
Flat	4 (16.0)	6 (20.7)	
Histological variants			
pap	3 (12.0)	4 (13.8)	.211
tub1	8 (32.0)	14 (48.3)	
tub2	11 (44.0)	11 (37.9)	
por	3 (12.0)	0 (0)	
Lymphatic invasion: positive	3 (12.0)	7 (24.1)	.252
Venous invasion: positive	2 (8.0)	3 (10.3)	.766
Perineural invasion: positive	9 (36.0)	13 (44.8)	.510
Stage			
I	11 (44.0)	7 (24.1)	.122
II	14 (56.0)	22 (75.9)	
III	0 (0)	0 (0)	


A
MUC2
B
MUC5AC
*
C
MUC6
*
D
CD10
E
CDX-2
F
Cytokeratin 20
G
MUC2
H
MUC5AC
*
I
MUC6
J
CD10
K
CDX-2
L
Cytokeratin 20
Score
0
1
2
3
4
Subgroup 1
Subgroup 2

respectively. Hierarchical clustering was performed to cluster the CCA samples according to the above scoring system (score 0-4) to achieve maximal homogeneity within each subgroup and the greatest differences between subgroups; this analysis was performed using open-access clustering software (Cluster 3.0; bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). We performed cluster analysis using the city-block distance method, which is a distance measure related to the Euclidean distance; this method proved to be most reliable for assessing the homogeneity of our immunohistochemical expression data. To determine the point at which 2 clusters being combined are considered too different to form a homogenous group, we used the first large increase in the coefficient value. The clustering algorithm was set to centroid linkage clustering, which is the standard hierarchical clustering method used in biological studies.

2.6. Statistical analysis

The statistical analyses were performed using JMP 13 (SAS Institute Inc, Cary, NC) and GraphPad Prism ver. 6.00 (GraphPad Software, San Diego, CA). Statistical significance was evaluated using the χ^2 test (or Fisher exact test, when indicated) and Mann-Whitney *U* test. Overall survival was calculated from the date of surgery until death. Time-to-event data were described using Kaplan-Meier curves, time-to-event intervals were compared between groups using the log-rank test, and hazard ratios (HRs) were calculated using a Cox proportional hazards model. Multivariate analyses using Cox proportional hazards models were used to determine any significant associations between various independent factors and prognosis. The following factors were included in the model: size, lymphatic invasion, venous invasion, perineural invasion, TNM stage, and the subgroup determined by hierarchical clustering. $P < .05$ was considered significant.

3. Results

3.1. Hierarchical clustering based on the expression scores of gastrointestinal differentiation markers and associations of the subgroups with clinicopathological variables

We performed hierarchical clustering based on immunohistochemical expression scores to evaluate differences in the expression patterns of the gastrointestinal differentiation markers MUC2, MUC5AC, MUC6, CD10, CDX-2, and CK20. The patients with perihilar CCA were classified into 2 subgroups based on the results of hierarchical clustering (Figure 2A), and the clinicopathological characteristics were compared between the 2 subgroups (Table 3A). The median tumor size was significantly larger in subgroup 1 than subgroup 2 ($P = .001$). No significant differences were seen in median age, sex, macroscopic type, histological variant type, lymphatic invasion, venous invasion, or perineural invasion between subgroups 1 and 2. The patients with distal CCA were also classified into 2 distinct subgroups (Fig. 2B). There were no differences in median age, sex, median tumor size, macroscopic type, histological variant type, lymphatic invasion, venous invasion, or perineural invasion between the 2 subgroups (Table 3B).

Next, we performed hierarchical clustering based on the immunohistochemical expression scores to clarify the differences in gastrointestinal differentiation marker expression between perihilar and distal CCA. The patients with extrahepatic CCA were classified into 2 subgroups (Supplementary Fig. 1). The expression scores for MUC5AC and MUC6 were significantly higher in subgroup 1 than in subgroup 2 ($P < .001$ and $P = .001$, respectively), and the expression of CDX-2 was higher in subgroup 2 than in subgroup 1 ($P = .048$) (Supplementary Fig. 2). However, tumor location was not different between the 2 subgroups ($P = .819$) (Supplementary Table 1), and no gastrointestinal differentiation markers distinguishing perihilar and distal CCA were identified.

3.2. Associations of each subgroup with gastrointestinal differentiation markers in perihilar and distal CCA

We performed immunohistochemical analyses of the 6 different gastrointestinal differentiation markers for each subgroup and examined the expression patterns (Figure 3). The 2 subgroups of perihilar CCA were characterized by distinct patterns of MUC5AC and MUC6 expression (Fig. 3B and C), with significantly lower expression scores for MUC5AC and MUC6 in subgroup 1 than in subgroup 2 ($P < .001$ and $P = .017$, respectively). There were no differences in the expression scores of MUC2, CD10, CDX-2, or CK20 between the subgroups (Fig. 3A, D, E, and F). In distal CCA, the expression of MUC5AC distinguished the 2 subgroups, with a lower score in subgroup 1 than in subgroup 2 ($P < .001$) (Fig. 3H). There were no differences in the expression scores for MUC2, MUC6, CD10, CDX-2, or CK20 between the 2 subgroups (Fig. 3G, I, J, K, and L).

3.3. Associations of each subgroup with patient outcomes in perihilar and distal CCA

Finally, we examined the correlations between the immunohistological perihilar or distal CCA subgroups and patient

Figure 3 Comparison of the immunohistochemical expression scores of gastrointestinal differentiation markers between the subgroups of perihilar or distal CCA. A-F, Perihilar CCA. G-L. Distal CCA. A and G, MUC2. B and H, MUC5AC. C and I, MUC6. D and J, CD10. E and K, CDX-2. F and L, CK20. Numbers indicate the immunohistochemical expression score. *$P < .05$.

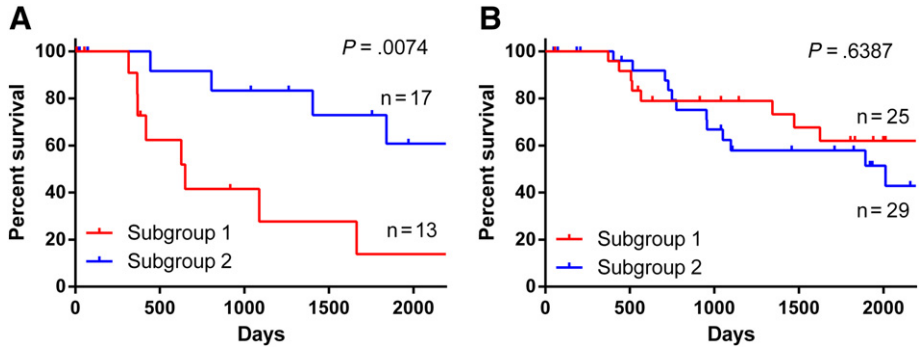


Figure 4 Kaplan-Meier survival curves for patients with CCA according to the perihilar or distal CCA subgroup. A, Perihilar CCA. B, Distal CCA.

outcomes. The 5-year overall survival rate of all patients with perihilar CCA was 40.3%; those of the patients in subgroups 1 and 2 were 13.9% and 60.8%, respectively, and were significantly different (HR, 4.29; 95% confidence interval [CI], 1.60-18.28; P = .007) (Figure 4A).

Next, we performed univariate and multivariate analyses of prognostic factors after curative resection for patients with perihilar CCA using a Cox proportional hazards model (Table 4). Subgroup 1 (versus subgroup 2) and TNM stage III (versus stage II) were associated with a shorter overall survival in the univariate analyses (HR, 4.67; 95% CI, 1.42-17.98; P = .010 and HR, 5.73; 95% CI, 1.56-27.79; P = .007, respectively). In the multivariate analysis, the immunohistological subgroup (subgroup 1 versus 2) and TNM stage (stage III versus II) were confirmed to be independent prognostic factors for perihilar CCA (HR, 7.82; 95% CI, 1.29-66.97; P = .024 and HR, 11.45; 95% CI, 2.13-97.21; P = .003, respectively).

Table 4 Risk factors for overall survival of patients with perihilar CCA

Risk factor	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
Largest tumor size	1.06	0.99-1.14	.057	1.04	0.93-1.19	.423
Lymphatic invasion						
Negative	Reference	–	–	Reference	–	–
Positive	1.24	0.19-4.78	.780	0.34	0.02-2.96	.360
Venous invasion						
Negative	Reference	–	–	Reference	–	–
Positive	2.85	0.41-12.47	.245	0.53	0.05-3.82	.535
Perineural invasion						
Negative	Reference	–	–	Reference	–	–
Positive	2.07	0.64-7.81	.222	0.38	0.04-2.62	.331
Stage						
II	Reference	–	–	Reference	–	–
III	5.73	1.56-27.79	.007 *	11.45	2.13-97.21	.003 *
Hierarchical clustering						
Subgroup 2	Reference	–	–	Reference	–	–
Subgroup 1	4.67	1.42-17.98	.010 *	7.82	1.29-66.97	.024 *

* P < .05.

Among the patients with distal CCA, the 5-year overall survival rate of all patients was 59.4%. Those of the patients in subgroups 1 and 2 were 62.0% and 57.9%, respectively, and were not significantly different (HR, 0.69; 95% CI, 0.29-1.68; P = .424) (Fig. 4B).

4. Discussion

Perihilar and distal CCAs should be considered separate clinical entities because of their differences in terms of patient outcomes, risk factors, and type of surgery performed [7,8]. However, the pathological features of perihilar and distal CCAs were not distinguished in the World Health Organization classification of digestive system tumors or in the classification of biliary tract cancers established by the Japanese Society of Hepato-Biliary-Pancreatic Surgery [9,10]. In this study, we subdivided extrahepatic CCAs into perihilar and distal CCAs and evaluated the value of using gastrointestinal differentiation marker profiles for distinguishing perihilar CCA from distal CCA. Two distinct subgroups of both perihilar and distal CCAs were defined according to the expression patterns of MUC2, MUC5AC, MUC6, CD10, CDX-2, and CK20 by hierarchical clustering. Several studies have shown correlations between the immunohistochemical expression of individual tumor phenotype markers and the clinicopathological findings of overall CCA [19,20,22]. However, no reports have examined such correlations in CCA according to perihilar and distal locations. Our results demonstrated that extrahepatic CCAs can be classified into perihilar and distal CCAs based on their gastrointestinal differentiation marker profiles.

To clarify the significance of gastrointestinal differentiation marker profiles, we analyzed the overall survival of patients

with perihilar or distal CCA. Among patients with perihilar CCA, subgroup 1 and TNM stage were both correlated with poor overall survival in univariate analyses, and the significance of these associations was retained in the multivariate analysis. Recently, a systematic review and meta-analysis showed that a positive surgical resection margin, lymph node involvement, a poor/moderate histological grade, operative transfusion, and T3/T4 stage were associated with poor survival of patients with resectable perihilar CCA [23]. To our knowledge, however, no study has evaluated the association of gastrointestinal differentiation marker expression with patient outcomes in perihilar CCA. Our findings suggest that a specific gastrointestinal differentiation marker profile characterized by low expression of MUC5AC and MUC6 is predictive of a poor prognosis of patients with perihilar CCA. Accordingly, classification of gastrointestinal differentiation marker profiles is expected to predict patient prognosis and aid selection of the appropriate adjuvant therapy for patients with resected perihilar CCA. These results should be validated by a future well-designed prospective study.

Several studies have shown that gastrointestinal differentiation marker expression in gastrointestinal cancer is associated with independent prognostic factors [12,18,24]. Kim et al [25] reported that gastrointestinal differentiation markers were correlated with a longer survival in patients with early gastric cancer. In addition, MUC5AC expression was significantly associated with a favorable prognosis in gastric cancers [25,26]. Similarly, our study demonstrated that perihilar CCA subgroup 2, which was characterized by high expression of MUC5AC, was correlated significantly with a good prognosis compared with subgroup 1. Although the expression of MUC5AC distinguished the 2 subgroups of distal CCA, no correlation between gastrointestinal differentiation marker expression and overall survival was identified. To our knowledge, there has been no report of an association between MUC5AC immunohistochemical expression and patient prognosis in distal CCA. The role of MUC5AC expression may differ between perihilar and distal CCAs.

It is interesting that the role of gastrointestinal differentiation marker expression was found to differ according to the CCA tumor location. Although the reason for this discrepancy remains unknown, some explanations are suggested. First, the difference in the expression patterns of gastrointestinal differentiation markers between perihilar and distal CCAs might be based on the molecular mechanisms associated with each tumor type. Concordant with our results, previous studies showed that expression of gastrointestinal differentiation markers was associated with tumor location in colorectal cancer, with differences in the gastrointestinal differentiation marker profile detected between left- and right-sided tumors [12,13,18]. In addition, several studies showed that molecular alterations in colorectal cancer might depend on the tumor location [12,18,27]. For example, MSI was associated with right-sided colorectal cancers and high expression of gastric markers, whereas microsatellite stability was associated with left-sided colorectal cancers and low expression of gastric markers [18]. Based on this, it is possible that expression of gastrointestinal differentiation markers is associated with bile duct carcinogenesis. Second, the differences in gastrointestinal differentiation marker expression between perihilar and distal CCAs might be attributed to the influence of MUC6 expression. Several studies have shown that MUC6 expression in CCAs is associated with tumor development and patient outcomes [28,29]. We showed that perihilar CCAs, but not distal CCAs, could be separated into 2 subgroups based on MUC6 expression.

The limitations of this study include its retrospective and single-institution design, in which selection bias is inherent. An additional second cohort for validation might be needed to determine patient outcomes in extrahepatic CCA; however, we were limited to a single cohort. Studies involving larger patient cohorts are needed to influence the current treatment strategies for extrahepatic CCA.

In conclusion, 2 distinct subgroups of both perihilar and distal CCAs were defined by the expression patterns of MUC2, MUC5AC, MUC6, CD10, CDX-2, and CK20. Among the perihilar CCAs, subgroups 1 and 2 were characterized by low and high expression, respectively, of MUC5AC and MUC6. Furthermore, perihilar CCA subgroup 1 was correlated with poor overall survival in a multivariate analysis. Finally, our findings are not sufficient to elucidate the reasons underlying the relationship between gastrointestinal differentiation marker profiles and prognosis of patients with CCA. However, we suggest that the role of gastrointestinal differentiation markers differs between perihilar and distal CCAs and that the expression pattern of gastrointestinal differentiation markers plays a crucial role in tumor development, particularly in perihilar CCA.

Acknowledgments

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Appendix A. Supplementary data

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