Expression of MUC1, MUC2, MUC5AC and MUC6 in cholangiocarcinoma: Prognostic impact

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Received March 13, 2009; Accepted June 3, 2009

DOI: 10.3892/or_00000485

Abstract. Mucin is a high molecular weight glycoprotein that plays an important role to protect the gastrointestinal tract epithelium. However, in cancer cells and during cancer progression, the expression profile of mucins is altered and expression of some mucins is correlated with prognosis for certain malignancies. The aim of this study was to determine the relationship between the expression of MUC1, MUC2, MUC5AC and MUC6 in cholangiocarcinoma and clinicopathological parameters as well as patient survival. In addition, this study was performed to identify whether immunohistochemical staining for mucins is useful to differentiate cholangiocarcinoma from adenocarcinoma of the pancreas and gallbladder. Immunohistochemical staining for MUC1, MUC2, MUC5AC and MUC6 was performed for 85 cases of cholangiocarcinoma, including 34 cases of intrahepatic cholangiocarcinoma (ICC), 51 cases of extrahepatic cholangiocarcinoma (ECC), 11 cases of gallbladder adenocarcinoma and 14 cases of pancreas adenocarcinoma. For cholangiocarcinomas, positivity of immunohistochemical staining for MUC1, MUC2, MUC5AC and MUC6 was 65.8, 23.5, 61.1 and 14.1%, respectively. For cholangiocarcinomas, MUC1 positivity was determined to be statistically significant for poor differentiation (p=0.002), T category (p=0.003), gross type (ICC, p=0.005; ECC, p=0.006) and poor patient survival (p=0.015). MUC5AC was more frequently expressed in advanced tumors (p=0.013). MUC6 expression was significantly detected in well-differentiated cholangiocarcinomas (p=0.006). There was no significant difference for the mucin staining patterns of cholangiocarcinomas, pancreatic adenocarcinomas and gallbladder adenocarcinomas. These results indicate that MUC1 expression in cholangiocarcinomas is closely related to dedifferentiation, infiltrative growth pattern and patient survival. Our results suggest that the expression of MUC1 might be associated with the progression of cholangiocarcinoma.

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Key words: mucin, cholangiocarcinoma, immunohistochemistry

Introduction

Cholangiocarcinoma (CC) accounts for about 3% of all gastrointestinal cancers with a 5-year survival of 20-30% after surgery (1,2). A CC arises from the ductal epithelium of the biliary tree. Depending on location, a CC can be classified as an intrahepatic cholangiocaricnoma (ICC) and an extrahepatic cholangiocarcinoma (ECC). Due to a poor prognosis, investigation of prognostic factors is very valuable for the treatment of patients with a CC.

Mucins are high molecular weight glycoproteins that are heavily glycosylated with many oligosaccharide side chains linked to a protein backbone called apomucin. During the past several years, a number of human mucins (MUC1-MUC20) have been identified (3,4). Mucins can be broadly subdivided into two groups: proteins that are secreted and form extracellular gels (MUC2, MUC5AC, MUC5B and MUC6) and membrane bound mucins (MUC1, MUC3 and MUC4). Mucins are present at the surface of most epithelial cells and play a role in protection and lubrication. It has been suggested that expression of mucins is associated with clinicopathological findings and patient survival in tumors that arise in various organs (5-8). MUC1 overexpression is most evident, and these rigid mucin glycoproteins located on cancer cells play a role in metastasis by inhibiting tumor cell adhesion and allowing escape from immune surveillance (9-11). Therefore, altered expression of mucins appears to be involved in tumor biology. Despite several studies, the role of expression of mucins in CC is still controversial and no definite conclusions have been reached.

Identification of the tissue of origin for tumors that arise in and around the biliary tract is particularly difficult, especially in advanced tumor stages, as the regions are contiguous. Moreover, the cytological differentiation of a CC and a pancreatic or gallbladder adenocarcinoma can be difficult. Nevertheless, a correct diagnosis is essential to select the proper therapy and to determine patient prognosis.

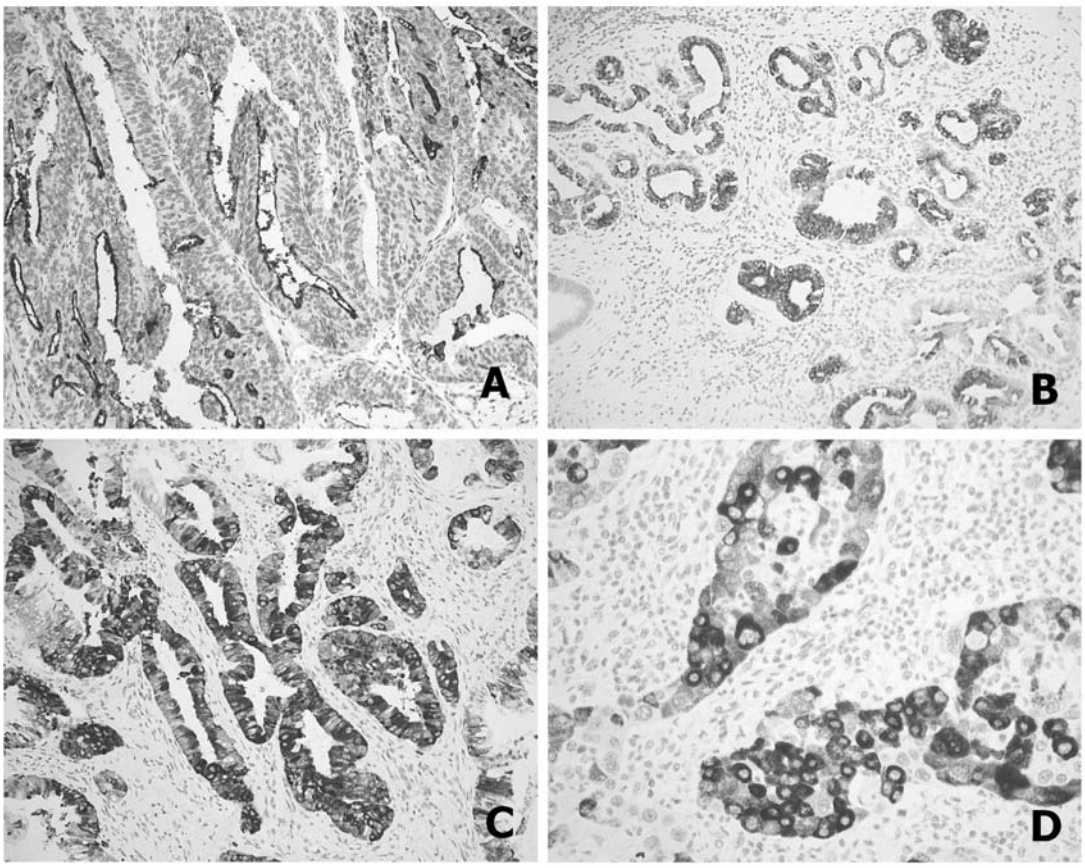

Figure 1. Representative immunohistochemical staining is shown. (A) MUC1 expression in a cholangiocarcinoma is shown. (B) MUC6 expression in a cholangiocarcinoma is shown. (C) MUC5AC expression in a cholangiocarcinoma is shown. (D) MUC5AC expression in a gallbladder adenocarcinoma is shown.

In the current study, we have analyzed the association of MUC1, MUC2, MUC5AC and MUC6 expression with the clinical and pathological findings and with survival in patients with CC. In addition, we have evaluated whether differential patterns of expression of mucins can be used to distinguish CC from gallbladder and pancreatic adenocarcinoma.

Materials and methods

Tissue specimens. A total of 85 CC (34 cases of ICC and 51 cases of ECC), 14 pancreatic adenocarcinoma and 11 gallbladder adenocarcinoma samples were obtained from patients who had undergone surgery between 1998 and 2007 at Chonbuk National University Hospital. Of the 85 patients with CC, 58 patients were male and 27 patients were female. The mean age of the patients at the time of surgery was 63.8 years (age range, 44-82 years). Seven patients had hepatolithiasis and five patients had clonorchiasis. Patients were chosen for analysis based on the availability of paraffin-embedded tissue. Hematoxylin and eosin stained slides were reviewed and were graded according to the WHO classification (12). The following histological features were also examined: vessel invasion, nerve invasion and the presence of a lymph node metastasis at the time of surgery. The pathological stage was reclassified according to the American Joint Committee on Cancer Staging (AJCC) 6th edition (13). Based on gross appearance, ICCs were classified as mass-

Table I. Difference in the expression of mucins for cholangiocarcinoma, gallbladder adenocarcinoma and pancreatic adenocarcinoma.

	Mucin expression (%)			
Tumor type	MUC1	MUC2	MUC5AC	MUC6
Cholangiocarcinoma				
Total, 85				
Positive	56 (65.8)	20 (23.5)	52 (61.1)	12 (14.1)
Negative	29 (34.2)	65 (76.5)	33 (38.9)	73 (85.9)
Gallbladder adenocarcinoma				
Total, 11				
Positive	10 (90.9)	3 (27.3)	9 (81.8)	4 (36.4)
Negative	1 (9.1)	8 (72.7)	2 (18.2)	7 (63.6)
Pancreatic adenocarcinoma				
Total, 14				
Positive	11 (78.6)	1 (7.1)	11 (78.6)	4 (28.6)
Negative	3 (21.4)	13 (92.9)	3 (21.4)	10 (71.4)

Table II. Difference in the phenotype expression of mucins for intrahepatic and extrahepatic cholangiocarcinoma.

	Mucin expression		
	ICC (%)	ECC (%)	P-value
MUC1			
Positive	19 (55.8)	37 (72.5)	0.209
Negative	15 (44.2)	14 (27.5)	
MUC2			
Positive	10 (29.4)	10 (19.6)	0.314
Negative	24 (70.6)	41 (80.4)	
MUC5AC			
Positive	16 (47.1)	36 (70.6)	0.026
Negative	18 (52.9)	15 (29.4)	
MUC6			
Positive	5 (14.7)	7 (13.7)	0.569
Negative	29 (85.3)	44 (86.3)	

ICC, intrahepatic cholangiocarcinoma; ECC, extrahepatic cholangiocarcinoma.

forming, periductal-infiltrative and intraductal growth types. ECCs were classified as periductal-infiltrative and intraductal growth types. Clinical data, including age, sex, presence of a distant metastasis and patient overall survival were obtained from the medical records. This study received local ethics committee approval from the institutional reviewed board of Chonbuk National University Hospital.

Immunohistochemistry. Immunohistochemical analysis was performed by the streptavidin-biotin-peroxidase (SAB) method. Paraffin blocks with representative areas of the tumors were cut into 4-μm thick tissue sections, and endogenous activity was quenched by incubation with 3% hydrogen peroxidase for 30 min after deparaffinization and hydration. Antigen retrieval was subsequently carried out. The primary antibodies used in this investigation were MUC1 (Clone Ma695, Novocastra, Leica Microsystems, Wetzlar, Germany), MUC2 (Clone Ccp58, Novocastra), MUC5AC (Clone CLH2, Novocastra) and MUC6 (CLH5, Novocastra); these antibodies were used at 1:100 dilution. Diaminobenzidine was used as a chromogen and the tissues were counterstained with hematoxylin. Two pathologists (W.S.M. and S.Y.P.) with no previous knowledge of the clinicopathological details evaluated all of the tissue slides. Distinct staining in >10% of the tumor cells was recorded as positive.

Table III. Correlation between the expression of mucins and clinicopathological factors for cholangiocarcinoma.

		MUC1		MUC2		MUC5AC		MUC6	
Category	Total	+ (%)	P-value	+ (%)	P-value	+ (%)	P-value	+ (%)	P-value
Differentiation			0.002		0.234		0.174		0.010
Well	37	16 (43)		12 (32)		23 (62)		10 (27)	
Moderately	37	31 (84)		6 (16)		25 (68)		2 (5)	
Poorly	11	9 (91)		2 (18)		4 (36)		0 (0)	
T category			0.003		0.057		0.013		0.465
T1	40	20 (50)		13 (33)		21 (53)		4 (10)	
≥T2	45	36 (80)		7 (16)		33 (73)		8 (18)	
LN metastasis			0.586		0.578		0.400		0.221
Positive	18	12 (67)		4 (22)		12 (67)		1 (6)	
Negative	67	44 (66)		16 (24)		40 (60)		11 (16)	
Distant metastasis			0.443		0.394		0.331		0.279
Positive	8	6 (75)		1 (13)		6 (75)		0 (0)	
Negative	77	50 (65)		19 (25)		46 (60)		12 (16)	
Nerve invasion			0.015		0.103		0.205		0.386
Positive	29	24 (83)		4 (14)		24 (83)		5 (17)	
Negative	56	32 (57)		16 (29)		28 (50)		7 (13)	
Vessel invasion			0.056		0.520		0.323		0.193
Positive	19	16 (84)		4 (21)		13 (68)		2 (11)	
Negative	66	40 (61)		16 (24)		39 (60)		10 (15)	

Table IV. Correlation between the expression of mucins and clinicopathologic factors for intrahepatic cholangiocarcinoma.

		MUC1		MUC2		MUC5AC		MUC6	
Category	Total	+ (%)	P-value	+ (%)	P-value	+ (%)	P-value	+ (%)	P-value
Differentiation			0.003		0.293		0.098		0.215
Well	14	3 (21)		6 (43)		8 (57)		3 (21)	
Moderately	13	11 (85)		2 (15)		7 (54)		2 (15)	
Poorly	7	5 (71)		2 (29)		1 (14)		0 (0)	
T category			0.072		0.367		0.56		0.465
T1	24	11 (46)		8 (33)		11 (46)		3 (13)	
≥T2	10	8 (80)		2 (20)		5 (50)		2 (20)	
LN metastasis			0.452		0.584		0.389		0.353
Positive	6	4 (67)		2 (33		2 (33)		0 (0)	
Negative	28	15 (54)		8 (29)		14 (50)		5 (18)	
Distant metastasis			0.162		0.338		0.455		0.611
Positive	3	3(100)		0 (0)		2 (67)		0 (0)	
Negative	31	16 (52)		10 (32)		14 (45)		5 (16)	
Nerve invasion			0.305		0.492		0.695		0.724
Positive	2	2(100)		0 (0)		2(100)		0 (0)	
Negative	32	17 (54)		10 (31)		14 (44)		5 (16)	
Vessel invasion			0.162		0.338		0.136		0.611
Positive	3	3(100)		0 (0)		0 (0)		0 (0)	
Negative	31	16 (52)		10 (32)		16 (52)		5 (16)	
Gross type			0.005		0.431		0.126		0.064
IG	12	3 (25)		5 (38)		6 (50)		3 (25)	
PI	7	4 (57)		1 (14)		5 (72)		2 (28)	
MF	15	12 (80)		4 (23)		4 (27)	11 (73.3)	0 (0)	

IG, intraductal growth; PI, periductal infiltrative; MF, mass forming.

Statistical analysis. SPSS 15.0 software (SPSS, Chicago, IL USA) used for statistical analysis. The clinicopathological characteristics were compared with expression of mucins using the χ^2 test. Overall survival was considered as the period of survival between the time of surgery and the date of death by disease or at the last follow-up. Survival curves were calculated by the Kaplan-Meier method and the differences among the curves were analyzed by use of the log rank test. Cox's proportional hazard model was used for multivariate survival analysis. For all tests, a p-value <0.05 was considered to be statistically significant.

Results

Expression of mucins in cholangiocarcinoma, gallbladder and pancreatic adenocarcinoma. The expression pattern of each mucin is shown Fig. 1. MUC1 was expressed in the luminal border and cytoplasm of cancer cells. MUC2, MUC5AC and MUC6 were mainly expressed in the cytoplasm of cancer cells. The expression of MUC1, MUC2, MUC5AC and MUC6 was identified in 56 (65.8%), 20 (23.5%), 52 (61.1%) and 12 (14.1%) of the 85 CCs, respectively. There was no significant difference for expression of mucins among CCs, pancreatic adenocarcinoma and gallbladder adnocarcinoma (Table I).

A higher degree of MUC5AC expression was observed in ECCs than in ICCs (p=0.026) (Table II). There was no significant difference of MUC1, MUC2 and MUC6 expression between ICCs and ECCs. MUC1 expression showed a significant correlation with poor differentiation (p=0.002), higher T category (p=0.003) and the presence of nerve invasion (p=0.015). There was a significant correlation between MUC5AC expression and a higher T category (p=0.013). In contrast, MUC6 was more frequently expressed in well-differentiated tumors (p=0.010). MUC expression showed no correlation with the presence of a lymph node metastasis, distant metastasis and vessel invasion (Table III). When CCs were classified as ICCs and ECCs, MUC1 expression

Table V. Correlation between the expression of mucins and clinicopathological factors for extrahepatic cholangiocarcinoma.

		MUC1		MUC2		MUC5AC		MUC6	
Category	Total	+ (%)	P-value	+ (%)	P-value	+ (%)	P-value	+ (%)	P-value
Differentiation			0.017		0.204		0.486		0.007
Well	23	13 (57)		6 (26)		15 (65)		7 (30)	
Moderately	24	20 (83)		4 (17)		18 (75)		0 (0)	
Poorly	4	4 (100)		0 (0)		3 (75)		0 (0)	
T category			0.079		0.15		0.034		0.282
T1	16	9 (56)		5 (31)		8 (50)		1 (6)	
≥T2	35	28 (80)		5 (14)		28 (77)		6 (17)	
LN metastasis			0.428		0.567		0.233		0.471
Positive	12	8 (67)		2 (17)		10 (83)		1 (8)	
Negative	39	29 (74)		8 (21)		26 (64)		6 (15)	
Distant metastasis			0.421		0.681		0.537		0.462
Positive	5	3 (60)		1 (20)		4 (80)		0 (0)	
Negative	46	34 (74)		9 (20)		32 (69)		7 (15)	
Nerve invasion			0.115		0.287		0.066		0.261
Positive	27	22 (82)		4 (15)		22 (82)		5 (19)	
Negative	24	15 (63)		6 (25)		14 (59)		2 (8)	
Vessel invasion			0.278		0.381		0.215		0.619
Positive	16	13 (81)		4 (25)		13 (81)		2 (13)	
Negative	35	24 (69)		6 (17)		23 (66)		5 (14)	
Gross type			0.006		0.269		0.068		0.429
IG	17	8 (47)		5 (29)		8 (47)		3 (18)	
PI	34	29 (85)		5 (15)		7 (21)		4 (12)	

IG, intraductal growth; PI, periductal infiltrative.

correlated with tumor differentiation (p=0.003) and macroscopic classification (p=0.005) for ICCs (Table IV). For ECCs, MUC1 expression showed a significant correlation with differentiation (p=0.017) and the periductal-infiltrative type (p=0.006). MUC5AC was more frequently expressed for a higher T category in ECCs (p=0.034). There was significant inverse correlation between MUC6 expression and tumor differentiation for ECCs (p=0.007) (Table V).

Survival analysis. The median survival time for patients with a CC was 20.6 months. Based on the use of univariate analysis, patients with MUC1-positive cancers had significantly poorer survival as compared to patients with MUC1-negative cancers (p=0.015). Other factors that correlated with survival were tumor differentiation, T category, the presence of a lymph node metastasis and a distant metastasis (Table VI). The expression of MUC2, MUC5AC and MUC6 showed no correlation with overall survival (Fig. 2). The use of multivariate analysis determined that T category and the presence of a lymph node metastasis were independent prognostic factors (Table VII).

Discussion

This study demonstrated the following findings. i) MUC1 expression was correlated with tumor progression and patient survival for cholangiocarcinoma (CC). ii) MUC5AC was more frequently expressed in an advanced CC. iii) MUC6 expression was more observed in a well-differentiated CC. iv) The expression patterns of mucins in CC, pancreatic adenocarcinoma and gallbladder adenocarcinoma were not different. MUC1 is a membrane bound type mucin detected in most epithelial cells (3,5). MUC1 is frequently expressed both in developing intrahepatic bile ducts in the fetal liver and in CCs, but is absent in the normal adult intrahepatic biliary tree (14,15). In cancer cells, abnormal mucins are synthesized and can potentially be used as markers for the development and progression of tumors. In the present study, we found that MUC1 expression was significantly associated with tumor progression factors such as poor differentiation, an advanced tumor stage and nerve invasion. Moreover, increased expression of MUC1 in CC tissues was correlated with poor survival of CC patients. These results are in

Table VI. Univariate analysis for overall survival for cholangiocarcinoma.

Category	No.	Overall survival, mean, 95% CI			P-value
		Lower bound	Median	Upper bound	
MUC1 expression					0.015
Positive	58	21.635	29.213	36.791	
Negative	27	41.012	56.479	71.945	
Differentiation					0.027
Well	37	37.111	50.350	63.590	
Moderately	37	22.079	33.005	43.932	
Poorly	11	12.706	19.184	25.661	
T category					< 0.001
T1	40	41.895	54.333	66.770	
≥T2	45	15.551	20.061	24.571	
LN metastasis					< 0.001
Positive	18	9.120	12.861	16.603	
Negative	67	35.140	45.172	55.205	
Distant metastasis					0.012
Positive	8	10.352	14.535	18.718	
Negative	77	32.685	42.228	51.770	
Nerve invasion					0.082
Positive	29	21.332	32.784	44.236	
Negative	56	30.189	41.043	51.896	
Vessel invasion					0.412
Positive	19	19.840	28.509	37.177	
Negative	66	32.074	43.003	53.932	

95% CI, 95% confidence interval.

agreement with findings of previous reports (16,17). Several experimental studies may explain the finding that MUC1 expression is correlated with tumor progression (9-11,18,19). MUC1 expressed on cancer cells may inhibit interactions between cytotoxic lymphocytes and tumor cells (11) or may function as an anti-adhesion molecule that inhibits cell-cell adhesion (9,10,18,19).

In the present study, we subdivided the ICCs into three gross types, the mass-forming, periductal-infiltrative and intraductal growth type, according to the recommendations of the Liver Cancer Study Group in Japan. Our study demonstrated that MUC1 expression was significantly higher in the mass-forming type as compared to the other types of ICCs. For ECCs, MUC1 expression was higher in the periductal infiltrative type as compared to the intraductal growth type. However, other types of mucin expression did not correlate with the gross types of ICCs in this study. Similarly, a previous study has reported that the mass-forming type showed significantly higher MUC1 expression as compared to the periductal infiltrative and intraductal growth type (16). In contrast to our results, Suh *et al* have reported that MUC1 expression was not significantly different based on gross type, and MUC2 was highly expressed only in the intraductal growth type and was never expressed in the mass-forming and periductal infiltrative types of ICCs (20).

MUC5AC is a gel-forming mucin that is expressed in gastric foveolar cells and is expressed in tracheobronchial epithelial cells. Aberrant expression of MUC5AC has been reported in preneoplastic lesions and in carcinomas arising from intrahepatic and extrahepatic bile ducts (21). A recent study has shown that the MUC5AC-expressing gastric foveolar type of ICC was more often associated with aggressive tumor development, whereas the pyloric gland type exhibited less aggressive behavior. Furthermore, the determination of serum MUC5AC expression may be predictive of poor patient outcome and the presence of serum MUC5AC has shown high sensitivity and specificity for the presence of a CC (22,23). Boonla *et al* (22) have suggested a possible mechanism for the relationship between MUC5AC expression and malignant progression. First, MUC5AC is negatively-charged and cells that express high levels may repel each other and enhance cell migration. Second, the highly viscous

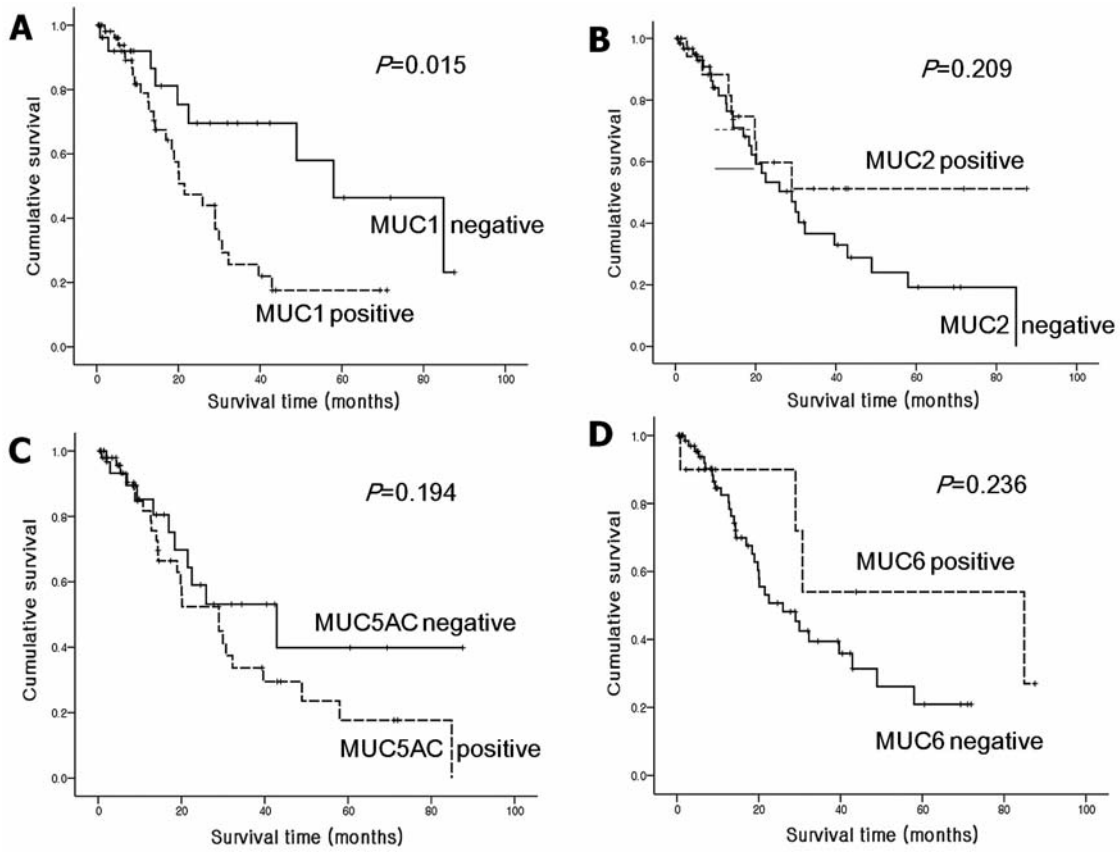

Figure 2. Survival curves of a cholangiocarcinoma patient according to mucin expression of mucins were determined by use of the Kaplan-Meier method. (A) MUC1, (B) MUC2, (C) MUC5AC and (D) MUC6.

Table VII. Multivariate Cox regression analysis for overall survival in cholangiocarcinoma.

Factors	Hazard ratio	95% CI	P-value
MUC1 (positive vs negative)	1.211	0.403-3.640	0.733
Differentiation			
Well	Reference	-	0.613
Moderately	1.420	0.564-3.572	0.456
Poorly	1.743	0.560-5.421	0.337
T category (T1 vs ≥T2)	2.548	1.029-6.310	0.043
LN metastasis (presence vs absence)	3.458	1.250-9.569	0.017
Distant metastasis (presence vs absence)	0.784	0.222-2.770	0.706

LN, lymph node; 95% CI, 95% confidence interval.

gel formed by MUC5AC surrounds the tumor emboli and may protect the tumor from proteolysis and limit the escape of immunogenic cells. In our study, MUC5AC expression was significantly associated with a higher T category. However, the association between high expression of MUC5AC and poor survival of patients with CC was not statistically significant. MUC5AC is frequently over-expressed in ICCs that arise in the large bile duct. According to the level of the involved bile duct, ICCs can be separated as hilar and peripheral types. MUC5AC is frequently expressed in ICCs from the hilar portion of the liver (21,24). We found that the frequency of MUC5AC expression in ECCs (70.6%) was significantly higher as compared to expression in ICCs (47.1%). Similarly, Lee *et al* have shown that MUC5AC is more frequently expressed in ECCs (44.0%) than in ICCs (60.0%) (25). However, only a limited number of studies have compared the expression of MUC5AC in ICCs and ECCs. A further study with a large number of cases may clarify this point.

MUC6 is expressed in a wide variety of epithelial tissues including the gastric pyloric gland, duodenal Brunner's gland, gallbladder and seminal vesicle (26). MUC6 expression has been reported in malignant epithelial tissues of the lung, breast, prostate, pancreas and stomach (25-30). Previous studies have reported that MUC6 expression is related with tumor progression and metastatic potential in various cancers (25-31). A few reports on the clinical impact of the expression of MUC6 in CC patients are available (24,32). MUC6 was expressed predominantly in well-differentiated ICC tumors and MUC6 expression in a pyloric gland type CC exhibited less aggressive behavior (24). Thuwajit *et al* have demonstrated that the expression of MUC6 showed a good correlation with the survival of CC patients (32). Our study also

showed that MUC6 expression was correlated with tumor histological grade (well differentiation). A high production of MUC6 may act as a barrier to cancerous extension, resulting in less aggressive biological behavior in mucinous carcinoma of the breast (29). MUC6 expression in ICCs may be related to histological differentiation and lower levels of invasiveness; however, further investigations are needed to clarify the mechanisms of MUC6 expression that are associated with favorable outcome in patients with CC.

Depending on the primary site of origin, CC, especially ECC, may extend to the gallbladder and pancreas. Since all of these organs may give rise to carcinomas with similar morphological features, identification of the exact origin relies on precise macroscopy but may be impossible in a larger tumor (33). Many studies have made efforts to distinguish CC from pancreas and gallbladder adenocarcinoma. Duval *et al* have tried to identify the site of origin using cytokeratin expression and they have suggested that it is not possible to differentiate these tumors based solely on cytokeratin expression (34). Lee *et al* have reported that pancreas cancers and ECCs were characterized as MUC5AC positive, whereas gallbladder and ampulla of Vater cancers were negative for MUC5AC expression (25). This finding is different from our findings, which revealed that most of the tumors in these sites were highly positive for expression of MUC1 and MUC5AC. Moreover, there was no significant difference for the expression patterns of mucins for CC, gallbladder and pancreas cancer. Our results suggest that the expression profile of mucins cannot identify the site of origin for tumors of the pancreas, gallbladder and bile ducts.

In conclusion, high expression of MUC1 significantly correlates with an infiltrative growth pattern, dedifferentiation, nerve invasion and patient poor survival. These results suggest that MUC1 may play a role in CC progression and MUC1 can be considered as a useful prognostic indicator.

Acknowledgements

This study was supported by a grant of the Korean Ministry of Education, Science and Technology (The Regional Core Research Program/The Center for Healthcare Technology Development).

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