• BASIC RESEARCH •

MUC1 and MUC5AC mucin expression in liver fluke-associated intrahepatic cholangiocarcinoma

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Abstract

AIM: To investigate the expressions of MUC1 and MUC5AC in intrahepatic cholangiocarcinoma (ICC). Association of expressions of mucins MUC1 and MUC5AC with clinical findings, metastasis, and survival of the liver fluke-associated ICC patients was determined.

METHODS: The expressions of MUC1 and MUC5AC mucins were examined by immunohistochemical staining in 87 cases of histologically-proven ICC. The expressions of mucins in relationship between clinicopathological significance and prognosis of the patients were evaluated.

RESULTS: Fifty-two patients (60%) exhibited both MUC1 and MUC5AC expressions, whereas 31% expressed either MUC1 or MUC5AC, and 9% expressed neither. High MUC1 immunoreactivity displayed a significant correlation with tumor progression as reflected by vascular invasion ($P<0.001$), whereas high expression of MUC5AC significantly correlated with neural invasion ($P = 0.022$) and advanced ICC stage ($P = 0.008$). Patients with high expression of MUC1 had a significantly shorter survival ($P = 0.0002$). According to multivariate analyses, MUC1 reactivity ($P = 0.026$), histological grading and stage of tumor represented the least probability of survival.

CONCLUSION: MUC1 is overexpressed in liver fluke-associated cholangiocarcinoma and relates to vascular invasion and poor prognosis, whereas MUC5AC mucin is neoexpressed and relates to neural invasion and advanced ICC stage. High MUC1 expression in tumor may be useful for predicting the poor outcome of ICC patients.

INTRODUCTION

Cholangiocarcinoma (CC), malignancy of bile duct epithelia, is a relatively rare cancer in Western countries, but it is found frequently in Southeast Asia, especially in Northeast Thailand. The cancer is still a challenging public health problem in the region.

Risk factors for CC in Asia are obviously different from those in the Western countries, according to epidemiological and experimental studies. In Western countries, primary sclerosing cholangitis is the most strong predisposing factor, with a relative risk of 10-30% to develop CC as compared to the general population[1,2]. In Asia, the animal studies[3-5] and epidemiological evidence[6] support the association of liver fluke infection with the development of cancer in this region. The different risk factors and etiology of CC among different geographic regions may lead to different carcinogenesis and pathogenesis of CC in these regions.

Liver fluke (*Opisthorchis viverrini*) infection is a major risk in Thai, Laos, and Malaysia, while *Clonorchis sinensis* infection is prominent in Japanese, Korean and Vietnamese[7-9]. Liver flukes chronically habitat in the biliary tree, leading to chronic inflammation, bile duct proliferation, dysplasia, and eventually, development of CC. Liver fluke *per se*, however, is not a sufficient cause to develop cancer[3]. Potent carcinogens such as nitrosamine compounds enhance the carcinogenic effect of flukes[10]. These compounds can be obtained from both exogenous and endogenous sources[11,12]. The former is mostly from diet, while the latter is the byproduct of endogenous nitrosation of nitrogenous compounds, such as nitric oxides and derivatives.

CC is a slowly-growing tumor and frequently diagnosed, when the tumor is big enough to obstruct the biliary tract and produce signs and symptoms. Most of the patients are thus diagnosed at the late stage of tumor and their survival is poor, due to the original as well as disseminated tumors. At present, there is no effective tool or specific biomarkers that can indicate the early stage and status of

CC. A specific marker for either early detection or monitoring of the tumor may significantly improve the prognosis and therapeutic management of such patients.

Mucins are heavily O-glycosylated proteins, mainly expressed by ductal and glandular epithelial tissues. To date, 19 human mucin genes have been identified and designated, according to their distinct structures and functions as transmembrane mucins or secreted gel-forming mucins. Mucin genes are expressed in a cell- and tissue-specific manner, for instances, MUC2 and MUC3 in bowel[13], MUC5AC and MUC6 in gastric tissue[14].

Vast production of mucus is frequently found in various carcinomas. The alterations in quantity and quality of mucins have been demonstrated in cancer tissues, including CC[15-18]. Neoexpressed and overexpressed mucins are of clinical values as a maker for supportive diagnosis, prognosis or monitoring therapy[19-21]. In this study, we investigated the expressions of two aberrant apomucins, namely MUC1 and MUC5AC in CC tissues obtained from 87 intrahepatic cholangiocarcinoma (ICC) patients who had an active or past history of *O. viverrini* infection. The correlation between mucin expressions and their malignant potential, clinicopathological findings, and patient survival was analyzed.

MATERIALS AND METHODS

Tissue samples
Between January 1998 and December 2000, 87 surgically resected specimens of ICC were selected from the files of the Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Thailand. Informed consent was obtained from each subject before surgery and the Human Research Ethics Committee, Khon Kaen University approved the research protocol (#HE43210).

Of the 87 ICC patients, 67% had a high titer of antibody against *O. viverrini*, of which 30% had active infection of *O. viverrini* as determined by the presence of parasite eggs in the bile, the others had a past history of liver fluke infection. The age, gender, tumor location, histological grading, and pTNM stage[22] were evaluated by reviewing the medical charts and pathologic records. Tumor size was evaluated using the greatest perpendicular diameter of each liver lesion. Survival of each CC patient was recorded from the date of surgery to the date of death or to December 31, 2000. Twenty-three patients were (26.4%) lost to follow-up.

Immunohistochemistry
All specimens were fixed in 10% neutral formalin buffer, embedded in paraffin, and cut into 4-μm-thick sections. Immunohistochemical staining was performed by an immunoperoxidase method using a mAb MUC-1-Core (Clone Ma695, Novocastra Laboratories, Newcastle-Upon-Tyne, UK) or a polyclonal anti-serum MAN-5ACI[23].

Each section was deparaffinized and the endogenous peroxidase was blocked with 0.3% hydrogen peroxide. The sections were incubated with 1:100 MUC-1-core protein antibody, followed by 1:300 biotin-conjugated goat anti-mouse immunoglobulin (Zymed, San Francisco, CA, USA), or with 1:1 000 MUC-5ACI antisera followed by 1:300 biotin-conjugated goat anti-rabbit immunoglobulin (Zymed, San Francisco, CA, USA). The sections were then incubated with 1:300 peroxidase-conjugated streptavidin. After being washed, the sections were reacted with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co., St. Louis, MO, USA) and 0.1% H_2O_2 in 50 mol/L Tris-HCl pH 7.8. The positive staining was counteracted, when PBS was applied instead of the primary antibody.

The intensity of mucin expression was semi-quantitatively classified into four groups, on the basis of the percentage of positive tumor cells: 0, negative; +1, 1–25%; +2, 26-50%; and +3 >50%. For statistical analysis, the 0 and +1 were categorized as low expression, +2 and +3 as high expression.

Tumor invasion of lymphatic, vascular, or nerve tissues was identified in both tumorous and non-tumorous liver tissues. Lymphatic and vascular invasion was so accounted by the presence of infiltrating cancer cells within the lymphatic or blood vessels, respectively. Neural invasion was presented as positive cancer cells in the perineurium and/or neural fascicle.

Real-time PCR
ICC tissues with either none, low or high expressions of MUC1 or MUC5AC mucin were analyzed by real-time PCR. Total RNA was extracted from the CC tissues, using the standard acid guanidinium thiocyanate-phenol-chloroform protocol. RT-PCR was done using TaqMan® Gold RT-PCR Kit in a two-step reaction. The reverse transcription step was performed in 50 μL reaction for 1 μg total RNA. The reaction mixture was composed of 5.5 mmol/L $MgCl_2$, 500 umol/L of each dNTP, 2.5 μmol/L random hexamers, 20 U RNase inhibitor and 62.5 U of MultiScribe® reverse transcriptase in regular strength TaqMan RT Buffer®. Incubation was at 25 ℃ for 10 min, at 48 ℃ for 30 min and at 95 ℃ for 5 min. The cDNA was stored in solution at -20 ℃ until use.

Primers and probes were originally designed by Primer Express® software. The DNA and cDNA sequences were obtained from the GenBank database. The sequence of the primers and TaqMan® probes for MUC1 and MUC5AC are presented in Table 1. The real-time PCR step was done using an ABI7700 machine (Applied Biosystems) in a 25 μL reaction using the TaqMan® Gold RT-PCR Kit (GAPDH was used as the internal control). Each reaction mixture was composed of 25 ng cDNA, 5.5 mmol/L $MgCl_2$, 200 umol/L of dGTP, dATP, dCTP, and 400 umol/L dUTP, 100 nmol/L of the TaqMan® probe, 200 nmol/L of each primer, 0.25 U of AmpErase® enzyme and 0.625 U of AmpliTaq Gold DNA polymerase® enzyme in regular strength TaqMan Buffer A®. The reaction mixture was incubated at 50 ℃ for 2 min and at 95 ℃ for 10 min before the PCR step. The PCR cycle was performed at 95 ℃ for 15 s at 60 ℃ for 15 s and at 72 ℃ for 30 s. The total number of cycles for GAPDH and MUC1 mucin was 40 and 50 for MUC5AC. The comparative temperature was determined by the PCR machine and Sequence Detector 1.7® program.

Statistical analysis
Statistical analyses for comparisons between clinicopathologic findings, survival and MUC1 or MUC5AC expression were performed using SPSS software (Chicago, IL, USA). Association among a variety of variables, including age,

Table 1 Sequences of primers and TaqMan® probe for MUC1 and MUC5AC apomucins

Mucin	Primer or probe	Sequence	Product size
MUC1	Forward	GCTATGTGCCCCCTAGCAGTAC	73
	Reverse	AGGCTGCTGCCACCGTTA	
	Probe	TCGTAGCCCCTATGAGAAGGTTTCTGCAG	
MUC5AC	Forward	CCCCAACATCAGGAACAGCTT	147
	Reverse	GAGTAGTAGGTTCCCGGCTTCA	
	Probe	AGCGTGGAGAATGAGAAGTATGCTCAGCAC	

Table 2 Expression of MUC1 and MUC5AC apomucins, demographic data, cancer histopathology, and clinical staging of ICC patients

	Variables	n (87)	%
Age (yr)	≤56	42	48.3
	>56	45	51.7
Sex	Male	59	67.8
	Female	28	32.2
Tumor staging	I–III	14	16.1
	IVA	16	18.4
	IVB	57	65.5
Histological grading	Papillary	20	22.9
	Well-differentiated	26	29.8
	Moderately differentiated	11	12.6
	Poorly differentiated	20	22.9
	Unclassified	10	11.5
Tumor size	≤5 cm	24	27.6
	>5 cm	63	72.4
Vascular invasion	No	30	34.5
	Yes	57	65.5
Neural invasion	No	46	52.9
	Yes	41	47.1
Lymphatic invasion	No	23	26.4
	Yes	64	73.6
Mucin expression	MUC1 and MUC5AC	52	59.7
	MUC1 only	15	17.3
	MUC5AC only	12	13.8
	None	8	9.2
MUC1 expression	Low (0, 1+)	53	60.9
	High (2+, 3+)	34	39.1
MUC5AC expression	Low (0, 1+)	41	47.1
	High (2+, 3+)	46	52.8

gender, tumor histology, tumor stage, tumor size, and invasion, was evaluated using the χ^2 test for heterogeneity or the Fisher's exact test. Multivariate analyses for variables influenced by the presence of MUC1 and MUC5AC mucins in the bile duct epithelium were carried out using a logistic regression model.

Patient survival was calculated from the time of resection to either death or the last follow-up. The Kaplan-Meier analysis was used to assess the relation of disease-free survival to the expression of mucins using the log-rank test. Several prognostic factors were evaluated for their association with the overall and disease-free survival in a multivariate analysis using Cox's regression model. $P<0.05$ was considered statistically significant.

RESULTS

Patient characteristics
Of the 87 ICC patients explored, 59 were male and 28 were female with male to female ratio = 2:1. The mean age was 56.7±8.6 years (range, 36-73 years). Most of the patients were at advance ICC stage, with 73% lymphatic, 65% vascular, and 47% neural invasion. Thirty percent of the tumors had well-differentiated histopathological grading and 10 specimens (11%) could not be classified. The majority of patients (70%) possessed a tumor size >5 cm (Table 2).

Real-time PCR and immunohistochemistry of MUC1 and MUC5AC expressions
Alteration in glycosylation of mucin epitopes is frequent in carcinomas and this alters the assessment of mucins by a particular antibody[24,25]. Therefore, immunohistochemical studies using different antibodies against a particular mucin have produced conflicting and inconclusive results[26,27]. To exclude ambiguity, a semi-quantitative analysis of MUC1 and MUC5AC mucins using antibodies in the immunohistochemical staining study was checked first for their reliabilities with the mRNA transcripts obtained by real-time PCR.

The semi-quantitative immunohistochemistry of ICC tissues with none, low or high expressions of MUC1 or MUC5AC mucin using the antibodies selected in this study corresponded to the quantitative analyses of MUC1 and

MUC5AC mucin transcripts by real-time PCR. MUC1 and MUC5AC transcripts were not detectable in the tumor tissues with negative immunohistochemistry for either MUC1 or MUC5AC mucin. The levels of MUC1 and MUC5AC transcripts matched well with those of immunohistochemistry.

The immunohistochemistry demonstrated that MUC1 and MUC5AC mucins were frequently expressed in ICC (Table 2). Fifty-two patients (60%) exhibited both MUC1 and MUC5AC expressions, whereas 31% expressed either MUC1 or MUC5AC, and only 9% expressed neither. The frequency of MUC1 and MUC5AC expressions in ICC was not significantly different.

Expression of MUC1 and clinicopathological findings

MUC1 was expressed on the luminal surface, cell membrane and cytoplasm of cholangiocarcinoma cells (Figure 1A). Expression of MUC1 was observed in 77% (67/87) of ICC patients, 39.1% (34/87) of the patients had high expression. Increased expression of MUC1 did not show a preference to any histological subtypes of tumor, tumor size, or tumor stage (Table 3). However, statistical analysis showed that high expression of MUC1 significantly correlated with poor prognosis. There was a positive correlation between MUC1 expression and vascular invasion ($P<0.001$, Figure 1B).

Expression of MUC5AC and clinicopathological findings

The site of MUC5AC expression was mainly in the cytoplasm and luminal mucin (Figure 1C). MUC5AC mucin was frequently detected in 73% (64/87) ICC tissues, 52.8% (46/87) of which were highly expressed.

Increased expression of MUC5AC showed a preference to advanced stage of the tumor ($P = 0.008$) but not to histological grading, tumor size, or tumor stage (Table 3). Tumor invasion of neighboring vascular, lymphatic and neural tissues was common in ICC patients with MUC5AC expression. However, only neural invasion significantly correlated with high MUC5AC expression ($P = 0.022$, Figure 1D).

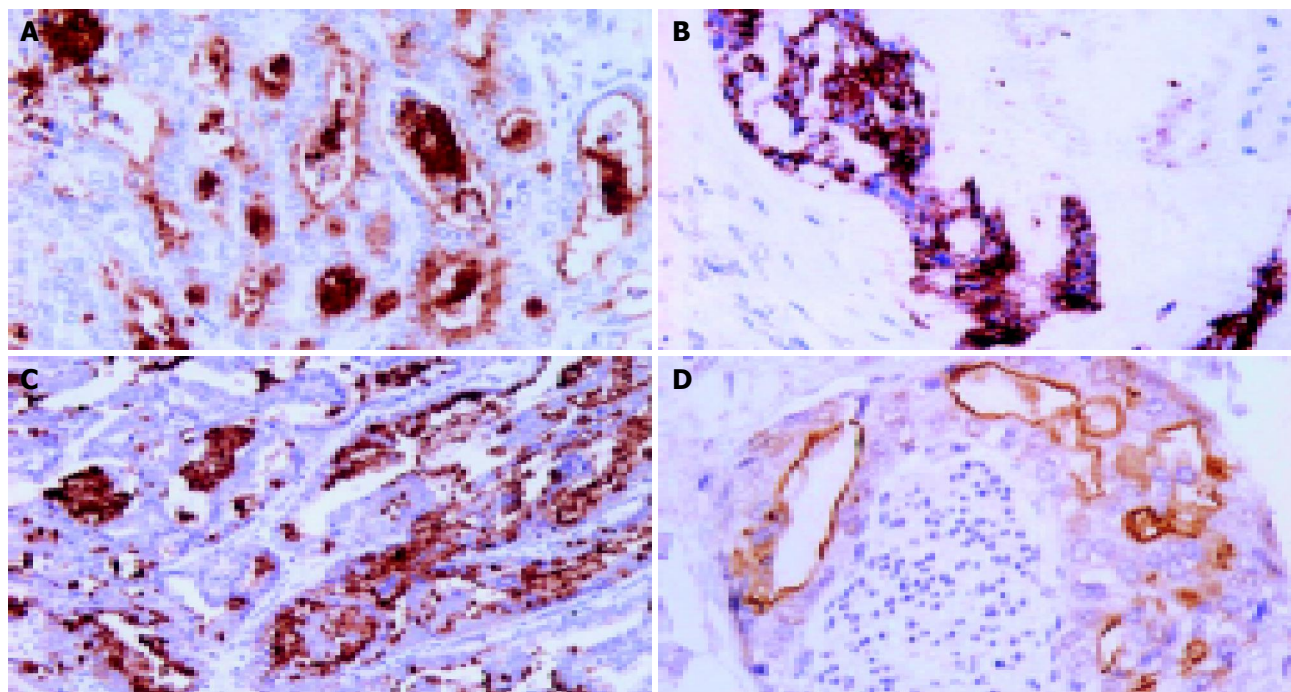### Expressions of MUC1 and MUC5AC mucins and cumulative survival

The median length of survival for patients with low MUC1 expression was 294 d (95%CI, 214-507 d) whereas it was 151 d (95%CI, 90-202 d) for patients with high MUC1 expression. The survival rate of patients with high MUC1 expression was significantly lower than that of patients with low MUC1 expression ($P = 0.0002$, log-rank test, Figure 2A).

The median length of survival for patients with low MUC5AC expression was 256 d (95%CI, 169-430 d) whereas it was 195 d (95%CI, 138-285 d) for patients with high MUC5AC expression. The ICC patients with high MUC5AC expression had a shorter survival than those with low MUC5AC expression, but without statistical significance (Figure 2B). There was no significant difference in survival between ICC patients with either MUC1 expression or MUC5AC expression and those with expressions of both MUC1 and MUC5AC.

The multivariate Cox's regression model showed that MUC1 (but not MUC5AC) expression ($P = 0.026$), histological grading ($P<0.05$), and tumor stage ($P<0.05$) statistically correlated with patient survival independent of age and gender (Table 4).

DISCUSSION

Alterations of epithelial mucin expression have been described in different malignant localizations. Varying degrees of glycosylation of MUC1 mucin between normal

Figure 1 Staining patterns of MUC1 and MUC5AC core proteins. **A** and **C:** Immunohistochemical staining of MUC1 and MUC5AC apomucins expressed in tumor tissues, respectively. **B** and **D:** MUC1 and MUC5AC staining of vascular and neural-invaded tumor cells.

Table 3 Expressions of MUC1 and MUC5AC apomucins in ICC in relation to patients' cancer histopathology and clinical staging

| Variables	MUC1		*P*	MUC5AC		*P*
	Low	High		Low	High	
Age (yr)	53	34	0.894	41	46	0.065
≤56	26	17		15	27	
>56	27	17		26	19	
Gender	53	34	0.698	41	46	0.058
Male	34	24		32	26	
Female	19	10		9	20	
Histological grading	43	24	0.575	34	33	0.095
Papillary	13	7		11	9	
Well-diff.	18	8		9	17	
Moderately diff.	5	6		6	5	
Poorly diff.	7	3		8	2	
Tumor size	51	31	0.964	38	44	0.502
≤5 cm	15	9		13	11	
>5 cm	36	22		25	33	
Staging	50	32	0.940	39	43	0.008
I-III	8	5		6	7	
IVA	6	5		10	1	
IVB	36	22		23	35	
Vascular invasion	53	34	<0.001	41	46	0.284
No	27	2		17	14	
Yes	26	30		24	32	
Lymphatic invasion	53	34	0.995	41	46	0.124
No	14	9		14	9	
Yes	39	25		27	37	
Neural invasion	53	34	0.384	41	46	0.022
No	30	16		27	19	
Yes	23	18		14	24	

diff. = differentiation.

Table 4 Significant prognostic factors for disease-free survival by multivariate analysis

Variables	Crude HR (95%CI)	Adjusted HR (95%CI)	*P*
MUC1 expression			0.026
Low	1	1	
High	2.59 (1.54-4.35)	2.19 (1.11-4.32)	
MUC5AC expression			0.059
Low	1	1	
High	1.36 (0.83-2.24)	2.06 (0.96-4.41)	
Age (yr)			0.571
≤56	1	1	
>56	0.86 (0.53-1.42)	1.19 (0.65-2.17)	
Gender			0.128
Male	1	1	
Female	0.63 (0.37-1.09)	0.60 (0.31-1.18)	
Histological grading			<0.050
Papillary	1	1	
Well-differentiated	1.86 (0.91-3.83)	1.92 (0.82-4.49)	
Moderately differentiated	3.65 (1.59-8.41)	3.00 (1.10-8.20)	
Poorly differentiated	1.78 (0.70-4.53)	3.02 (0.97-9.41)	
Staging			<0.050
I–III	1	1	
IVA	2.49 (0.81-7.62)	5.10 (1.27-20.43)	
IVB	3.57 (1.41-9.00)	3.93 (1.47-10.51)	

and tumor cells are documented in various cancers. The carbohydrate side chains of MUC1 from breast adenocarcinomas are shorter and less densely distributed than those produced by normal breast cells[28-30], whereas those of advanced stage or metastatic colorectal carcinomas had a high level of fully glycosylated mucin[31]. Since different antibodies may require different carbohydrates for their actions, the discrepancies in the immunoreactivity may be obtained, when different mucin antibodies are used[28,29]. In our study, we have shown that the immunoreactivity obtained using MUC1 (Ma695) and MUC5AC (MAN-5ACI) antibodies corresponds to the levels of the mucin transcripts obtained
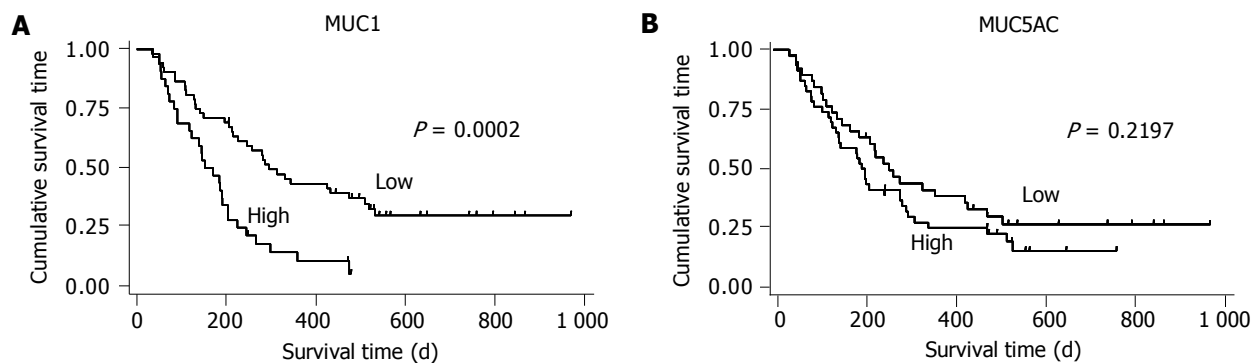
Figure 2 Survival curves using Kaplan-Meier method. **A:** Significant unfavorable prognosis of tumors with high expression of MUC1 compared to low-expression carcinoma (P = 0.0002); **B:** Poor survival of patients with tumors having a high expression of MUC5AC as opposed to patients having a low-expression carcinoma.

by real-time PCR, suggesting that these antibodies are appropriate for semi-quantitative analysis of MUC1 and MUC5AC expressions in the study of immunohistochemistry.

MUC1 is a transmembrane glycoprotein found frequently in the developing intrahepatic bile ducts in fetal liver[32] but not in the normal adult intrahepatic biliary tree[33,34]. Overexpression of MUC1 has been reported continuously in various cancers including CC[35,36]. Therefore, MUC1 apomucin is proposed as an oncofetal antigen in the intrahepatic biliary tree[32]. In contrast, MUC5AC, a gel-forming phenotype of secretory mucin, is rarely expressed in the intrahepatic biliary tree[32,35,37], and only aberrantly in CC tissues[38,39].

In the current study, MUC1 and MUC5AC were significantly expressed in ICC patients. These ectopically expressing mucins showed a significant correlation with poor prognosis. High MUC1 immunoreactivity was associated with vascular invasion, whereas high MUC5AC expression was related to neural invasion. The results suggest that the expression of these two mucins may be influenced by different modulators and may acquire different biological pathways and different clinical courses of ICC.

Association between mucin expression and prognosis of patient outcome has been repeatedly reported in several cancers[17,40,41] including CC. Expressions of mucin MUC4 is a statistically significant risk factor, affecting the survival of patients with ICC[42]. Extensive expression of MUC1 apomucin is an independent risk factor for poor outcome of patients with ICC[35,36,42] and extrahepatic CC[43]. Our study on MUC1 expression in ICC agrees well with the previous reports. However, the association between high expression of MUC5AC mucin in tumor tissue and unfavorable survival observed here is not statistically significant. The result disagrees with our previous finding that the detection of MUC5AC mucin in the serum of CC patients[44] correlates with shorter survival outcomes[39]. This discrepancy may be due to the small number of samples used in the present study.

The link between MUC1 expression and malignant progression is significant in three ways. First, MUC1 is negatively charged and cells expressing high levels may repel each other[45,46]. Cell repulsion is a pivotal event in metastasis. Decreased carbohydrate epitope on MUC1 mucin and other surface molecules markedly increases tumor cells to tumor cell adhesion and hence reduces the ability of these tumor cells to adhere to the surrounding endothelium.

Vascular invasion involves the binding to a ligand (E-selectin) of endothelial cells and epitopes (sialyl Lewis[x/a]) on tumor cells[47,48]. The association between high MUC1 expression and vascular invasion found in our study may indicate the role of MUC1 in tumor adhesion to endothelial cells. This suggestion is supported by the fact that sialyl Lewis[a], a carbohydrate epitope which is present at high level in tumor and serum of CC patients, has been identified as an epitope on MUC1[49]. In addition, our previous study indicates that CC patients with positive sialyl Lewis[a] expression in tumor tissue have a significantly poorer prognosis and the expression of sialyl Lewis[a] is associated with vascular invasion[50].

Overexpression of MUC1 on the membrane of tumor cells can suppress the immunity of patients. Tumor cells with high expression of MUC1 could inhibit the interaction between cytotoxic lymphocytes and tumor cells[51], and high levels of MUC1 correlate with immunosuppression in adenocarcinoma patients[52]. At present, MUC1 mucin is known to serve as a target molecule for killing particular cancer cells by cytotoxic T-lymphocytes and as an antigen for cancer vaccine[53,54]. Attempts to eliminate or control metastasis of tumor cells via anti-MUC1 and phase I/II trials of cancer vaccines using antigen MUC1 are now underway[53].

Perineural invasion is one of the most crucial factors, which tend to yield the poor outcome after resection for biliary tract cancer, because cancer cells in the perineural space often remain on the surgical margin of the tumor after an assumed curative resection[55,56]. In our study, MUC5AC was aberrantly expressed in ICC patients, correlated with neural invasion and advanced stage of tumor, suggesting that MUC5AC mucin plays a role in the late stage carcinogenesis and the poor prognosis of ICC patients is due to neural metastasis.

Even the presently available evidence for the association between MUC5AC mucin expression and neural invasion is limited; however, we can postulate the possible role of MUC5AC mucous layer surrounding the cancer cells in the invasion of cancer cells in several ways. First, the MUC5AC mucin layer may shield the tumor cells from immune recognition by the cellular arm of the immune system, thus favoring metastasis. Second, a number of tumor cells may

cluster as tumor emboli within the mucin layer, which may result in tumor dissemination to distant organ sites. Finally, specific epitopes of carbohydrate ligand on MUC5AC may manipulate specific adherence of tumor cells via some adhesion molecules, e.g., NCAM[57]. Further investigation, including expression of NCAM in ICC patients with positive MUC5AC mucin expression and neural invasion is needed.

In summary, high expressions of MUC1 mucin significantly correlate with poor survival of ICC patients according to the Kaplan-Meier method. Expression of MUC1 and MUC5AC mucins is associated with metastasis, and is thus considered to be a useful prognostic marker for a poor outcome in ICC patients. We suggest that MUC1 and MUC5AC mucin immunohistochemistry may augment the classic histochemistry for diagnosis and prognosis of ICC as well as prediction of the patient outcome.

ACKNOWLEDGMENT

REFERENCES

1   **de Groen PC.** Cholangiocarcinoma in primary sclerosing cholangitis: who is at risk and how do we screen? *Hepatology* 2000; **31**: 247-248

2   **Rosen CB,** Nagorney DM, Wiesner RH, Coffey RJ, LaRusso NF. Cholangiocarcinoma complicating primary sclerosing cholangitis. *Ann Surg* 1991; **213**: 21-25

3   **Thamavit W,** Bhamarapravati N, Sahaphong S, Vajrasthira S, Angsubhakorn S. Effects of dimethylnitrosamine on induction of cholangiocarcinoma in Opisthorchis viverrini-infected Syrian golden hamsters. *Cancer Res* 1978; **38**: 4634-4639

4   **Ohshima H,** Bandaletova TY, Brouet I, Bartsch H, Kirby G, Ogunbiyi F, Vatanasapt V, Pipitgool V. Increased nitrosamine and nitrate biosynthesis mediated by nitric oxide synthase induced in hamsters infected with liver fluke (Opisthorchis viverrini). *Carcinogenesis* 1994; **15**: 271-275

5   **Pinlaor S,** Ma N, Hiraku Y, Yongvanit P, Semba R, Oikawa S, Murata M, Sripa B, Sithithaworn P, Kawanishi S. Repeated infection with Opisthorchis viverrini induces accumulation of 8-nitroguanine and 8-oxo-7, 8-dihydro-2′-deoxyguanine in the bile duct of hamsters via inducible nitric oxide synthase. *Carcinogenesis* 2004; **25**: 1535-1542

6   **Haswell-Elkins MR,** Mairiang E, Mairiang P, Chaiyakum J, Chamadol N, Loapaiboon V, Sithithaworn P, Elkins DB. Cross-sectional study of Opisthorchis viverrini infection and cholangiocarcinoma in communities within a high-risk area in northeast Thailand. *Int J Cancer* 1994; **59**: 505-509

7   **de Groen PC,** Gores GJ, LaRusso NF, Gunderson LL, Nagorney DM. Biliary tract cancers. *N Engl J Med* 1999; **341**: 1368-1378

8   **Kullavanijaya P,** Tangkijvanich P, Poovorawan Y. Current status of infection-related gastrointestinal and hepatobiliary diseases in Thailand. *Southeast Asian J Trop Med Public Health* 1999; **30**: 96-105

9   **Sithithaworn P,** Haswell-Elkins MR, Mairiang P, Satarug S, Mairiang E, Vatanasapt V, Elkins DB. Parasite-associated morbidity: liver fluke infection and bile duct cancer in northeast Thailand. *Int J Parasitol* 1994; **24**: 833-843

10   **Thamavit W,** Pairojkul C, Tiwawech D, Itoh M, Shirai T, Ito N. Promotion of cholangiocarcinogenesis in the hamster liver by bile duct ligation after dimethylnitrosamine initiation. *Carcinogenesis* 1993; **14**: 2415-2417

11   **Satarug S,** Haswell-Elkins MR, Sithithaworn P, Bartsch H, Ohshima H, Tsuda M, Mairiang P, Mairiang E, Yongvanit P, Esumi H, Elkins DB. Relationships between the synthesis of N-nitrosodimethylamine and immune responses to chronic infection with the carcinogenic parasite, Opisthorchis viverrini, in men. *Carcinogenesis* 1998; **19**: 485-491

12   **Satarug S,** Haswell-Elkins MR, Tsuda M, Mairiang P, Sithithaworn P, Mairiang E, Esumi H, Sukprasert S, Yongvanit P, Elkins DB. Thiocyanate-independent nitrosation in humans with carcinogenic parasite infection. *Carcinogenesis* 1996; **17**: 1075-1081

13   **Chang SK,** Dohrman AF, Basbaum CB, Ho SB, Tsuda T, Toribara NW, Gum JR, Kim YS. Localization of mucin (MUC2 and MUC3) messenger RNA and peptide expression in human normal intestine and colon cancer. *Gastroenterology* 1994; **107**: 28-36

14   **Buisine MP,** Devisme L, Degand P, Dieu MC, Gosselin B, Copin MC, Aubert JP, Porchet N. Developmental mucin gene expression in the gastroduodenal tract and accessory digestive glands. II. Duodenum and liver, gallbladder, and pancreas. *J Histochem Cytochem* 2000; **48**: 1667-1676

15   **Jass JR,** Walsh MD. Altered mucin expression in the gastrointestinal tract: a review. *J Cell Mol Med* 2001; **5**: 327-351

16   **Kim GE,** Bae HI, Park HU, Kuan SF, Crawley SC, Ho JJ, Kim YS. Aberrant expression of MUC5AC and MUC6 gastric mucins and sialyl Tn antigen in intraepithelial neoplasms of the pancreas. *Gastroenterology* 2002; **123**: 1052-1060

17   **Lee HS,** Lee HK, Kim HS, Yang HK, Kim YI, Kim WH. MUC1, MUC2, MUC5AC, and MUC6 expressions in gastric carcinomas: their roles as prognostic indicators. *Cancer* 2001; **92**: 1427-1434

18   **Pereira MB,** Dias AJ, Reis CA, Schmitt FC. Immunohistochemical study of the expression of MUC5AC and MUC6 in breast carcinomas and adjacent breast tissues. *J Clin Pathol* 2001; **54**: 210-213

19   **Higashi M,** Yonezawa S, Ho JJ, Tanaka S, Irimura T, Kim YS, Sato E. Expression of MUC1 and MUC2 mucin antigens in intrahepatic bile duct tumors: its relationship with a new morphological classification of cholangiocarcinoma. *Hepatology* 1999; **30**: 1347-1355

20   **Kawamoto T,** Shoda J, Irimura T, Miyahara N, Furukawa M, Ueda T, Asano T, Kano M, Koike N, Fukao K, Tanaka N, Todoroki T. Expression of MUC1 mucins in the subserosal layer correlates with postsurgical prognosis of pathological tumor stage 2 carcinoma of the gallbladder. *Clin Cancer Res* 2001; **7**: 1333-1342

21   **Lee KT,** Liu TS. Altered mucin gene expression in stone-containing intrahepatic bile ducts and cholangiocarcinomas. *Dig Dis Sci* 2001; **46**: 2166-2172

22   **Sobin LH,** Wittekind CH, *TNM classification of malignant tumours.* 5th ed. *New York: John Wiley Sons* 1997: 136-143

23   **Thornton DJ,** Carlstedt I, Howard M, Devine PL, Price MR, Sheehan JK. Respiratory mucins: identification of core proteins and glycoforms. *Biochem J* 1996; **316** (Pt 3): 967-975

24   **Reis CA,** David L, Seixas M, Burchell J, Sobrinho-Simoes M. Expression of fully and under-glycosylated forms of MUC1 mucin in gastric carcinoma. *Int J Cancer* 1998; **79**: 402-410

25   **Baldus SE,** Zirbes TK, Engel S, Hanisch FG, Monig SP, Lorenzen J, Glossmann J, Fromm S, Thiele J, Pichlmaier H, Dienes HP. Correlation of the immunohistochemical reactivity of mucin peptide cores MUC1 and MUC2 with the histopathological subtype and prognosis of gastric carcinomas. *Int J Cancer* 1998; **79**: 133-138

26   **Sakamoto H,** Yonezawa S, Utsunomiya T, Tanaka S, Kim YS, Sato E. Mucin antigen expression in gastric carcinomas of young and old adults. *Hum Pathol* 1997; **28**: 1056-1065

27   **Utsunomiya T,** Yonezawa S, Sakamoto H, Kitamura H, Hokita S, Aiko T, Tanaka S, Irimura T, Kim YS, Sato E. Expression of MUC1 and MUC2 mucins in gastric carcinomas: its relationship with the prognosis of the patients. *Clin Cancer Res* 1998; **4**: 2605-2614

28   **Burchell J,** Taylor-Papadimitriou J. Effect of modification of carbohydrate side chains on the reactivity of antibodies with core-protein epitopes of the MUC1 gene product. *Epithelial Cell Biol* 1993; **2**: 155-162

29   **Girling A,** Bartkova J, Burchell J, Gendler S, Gillett C, Taylor-Papadimitriou J. A core protein epitope of the polymorphic epithelial mucin detected by the monoclonal antibody SM-3 is selectively exposed in a range of primary carcinomas. *Int J Cancer* 1989; **43**: 1072-1076

30   **Hull SR,** Bright A, Carraway KL, Abe M, Hayes DF, Kufe DW. Oligosaccharide differences in the DF3 sialomucin antigen from normal human milk and the BT-20 human breast carcinoma cell line. *Cancer Commun* 1989; **1**: 261-267

31   **Nakamori S,** Ota DM, Cleary KR, Shirotani K, Irimura T. MUC1 mucin expression as a marker of progression and metastasis of human colorectal carcinoma. *Gastroenterology* 1994; **106**: 353-361

32   **Sasaki M,** Nakanuma Y, Terada T, Kim YS. Biliary epithelial expression of MUC1, MUC2, MUC3 and MUC5/6 apomucins during intrahepatic bile duct development and maturation. An immunohistochemical study. *Am J Pathol* 1995; **147**: 574-579

33   **Sasaki M,** Nakanuma Y. Expression of mucin core protein of mammary type in primary liver cancer. *Hepatology* 1994; **20**: 1192-1197

34   **Yamashita K,** Yonezawa S, Tanaka S, Shirahama H, Sakoda K, Imai K, Xing PX, McKenzie IF, Hilkens J, Kim YS. Immunohistochemical study of mucin carbohydrates and core proteins in hepatolithiasis and cholangiocarcinoma. *Int J Cancer* 1993; **55**: 82-91

35   **Sasaki M,** Nakanuma Y, Kim YS. Characterization of apomucin expression in intrahepatic cholangiocarcinomas and their precursor lesions: an immunohistochemical study. *Hepatology* 1996; **24**: 1074-1078

36   **Matsumura N,** Yamamoto M, Aruga A, Takasaki K, Nakano M. Correlation between expression of MUC1 core protein and outcome after surgery in mass-forming intrahepatic cholangiocarcinoma. *Cancer* 2002; **94**: 1770-1776

37   **Vandenhaute B,** Buisine MP, Debailleul V, Clement B, Moniaux N, Dieu MC, Degand P, Porchet N, Aubert JP. Mucin gene expression in biliary epithelial cells. *J Hepatol* 1997; **27**: 1057-1066

38   **Sasaki M,** Nakanuma Y, Kim YS. Expression of apomucins in the intrahepatic biliary tree in hepatolithiasis differs from that in normal liver and extrahepatic biliary obstruction. *Hepatology* 1998; **27**: 54-61

39   **Boonla C,** Wongkham S, Sheehan JK, Wongkham C, Bhudhisawasdi V, Tepsiri N, Pairojkul C. Prognostic value of serum MUC5AC mucin in patients with cholangiocarcinoma. *Cancer* 2003; **98**: 1438-1443

40   **Kashiwagi H,** Kijima H, Dowaki S, Ohtani Y, Tobita K, Yamazaki H, Nakamura M, Ueyama Y, Tanaka M, Inokuchi S, Makuuchi H. MUC1 and MUC2 expression in human gallbladder carcinoma: a clinicopathological study and relationship with prognosis. *Oncol Rep* 2001; **8**: 485-489

41   **Akyurek N,** Akyol G, Dursun A, Yamac D, Gunel N. Expression of MUC1 and MUC2 mucins in gastric carcinomas: their relationship with clinicopathologic parameters and prognosis. *Pathol Res Pract* 2002; **198**: 665-674

42   **Shibahara H,** Tamada S, Higashi M, Goto M, Batra SK, Hollingsworth MA, Imai K, Yonezawa S. MUC4 is a novel prognostic factor of intrahepatic cholangiocarcinoma-mass forming type. *Hepatology* 2004; **39**: 220-229

43   **Takao S,** Uchikura K, Yonezawa S, Shinchi H, Aikou T. Mucin core protein expression in extrahepatic bile duct carcinoma is associated with metastases to the liver and poor prognosis. *Cancer* 1999; **86**: 1966-1975

44   **Wongkham S,** Sheehan JK, Boonla C, Patrakitkomjorn S, Howard M, Kirkham S, Sripa B, Wongkham C, Bhudhisawasdi V. Serum MUC5AC mucin as a potential marker for cholangiocarcinoma. *Cancer Lett* 2003; **195**: 93-99

45   **Wesseling J,** van der Valk SW, Vos HL, Sonnenberg A, Hilkens J. Episialin (MUC1) overexpression inhibits integrin-mediated cell adhesion to extracellular matrix components. *J Cell Biol* 1995; **129**: 255-265

46   **Wesseling J,** van der Valk SW, Hilkens J. A mechanism for inhibition of E-cadherin-mediated cell-cell adhesion by the membrane-associated mucin episialin/MUC1. *Mol Biol Cell* 1996; **7**: 565-577

47   **Regimbald LH,** Pilarski LM, Longenecker BM, Reddish MA, Zimmermann G, Hugh JC. The breast mucin MUCI as a novel adhesion ligand for endothelial intercellular adhesion molecule 1 in breast cancer. *Cancer Res* 1996; **56**: 4244-4249

48   **Nath D,** Hartnell A, Happerfield L, Miles DW, Burchell J, Taylor-Papadimitriou J, Crocker PR. Macrophage-tumour cell interactions: identification of MUC1 on breast cancer cells as a potential counter-receptor for the macrophage-restricted receptor, sialoadhesin. *Immunology* 1999; **98**: 213-219

49   **Yamashita Y,** Ho JJ, Farrelly ER, Hirakawa K, Sowa M, Kim YS. Forskolin and phorbol ester have opposite effects on the expression of mucin-associated sialyl-Lewis(a) in pancreatic cancer cells. *Eur J Cancer* 2000; **36**: 113-120

50   **Juntavee A,** Sripa B, Pugkhem A, Khuntikeo N, Wongkham S. Expression of sialyl Lewis[a] relates to poor prognosis in cholangiocarcinoma. *World J Gastroenterol* 2005; **11**: 249-254

51   **van de Wiel-van Kemenade E,** Ligtenberg MJ, de Boer AJ, Buijs F, Vos HL, Melief CJ, Hilkens J, Figdor CG. Episialin (MUC1) inhibits cytotoxic lymphocyte-target cell interaction. *J Immunol* 1993; **151**: 767-776

52   **Hamanaka Y,** Suehiro Y, Fukui M, Shikichi K, Imai K, Hinoda Y. Circulating anti-MUC1 IgG antibodies as a favorable prognostic factor for pancreatic cancer. *Int J Cancer* 2003; **103**: 97-100

53   **Musselli C,** Livingston PO, Ragupathi G. Keyhole limpet hemocyanin conjugate vaccines against cancer: the Memorial Sloan Kettering experience. *J Cancer Res Clin Oncol* 2001; **127** (Suppl 2): R20-26

54   **Kontani K,** Taguchi O, Ozaki Y, Hanaoka J, Tezuka N, Sawai S, Inoue S, Fujino S, Maeda T, Itoh Y, Ogasawara K, Sato H, Ohkubo I, Kudo T. Novel vaccination protocol consisting of injecting MUC1 DNA and nonprimed dendritic cells at the same region greatly enhanced MUC1-specific antitumor immunity in a murine model. *Cancer Gene Ther* 2002; **9**: 330-337

55   **Koyama K,** Tanaka J, Kato S, Asanuma Y. New strategy for treatment of carcinoma of the hilar bile duct. *Surg Gynecol Obstet* 1989; **168**: 523-530

56   **Koyama K,** Tanaka J, Sato Y, Seki H, Kato Y, Umezawa A. Experience in twenty patients with carcinoma of hilar bile duct treated by resection, targeting chemotherapy and intracavitary irradiation. *Surg Gynecol Obstet* 1993; **176**: 239-245

57   **De Bolos C,** Garrido M, Real FX. MUC6 apomucin shows a distinct normal tissue distribution that correlates with Lewis antigen expression in the human stomach. *Gastroenterology* 1995; **109**: 723-734