ORIGINAL

Relationship between mucin expression and preoperative bile juice cytology in biliary tract carcinoma

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Abstract : The present study evaluated correlations between preoperative bile juice cytology and mucin expression of surgical specimens in biliary tract carcinoma. Twenty-five patients with biliary tract carcinoma surgically treated at our hospital, whose bile juice cytology had been evaluated before operation, were allocated to this study. Biliary cytology was classified into three categories based on the Papanicolaou classification. Immunohistochemical staining of tissues was performed using MUC1 and MUC2 monoclonal antibodies. Lesions showing MUC1 expression of ++ or higher and MUC2 expression of - were classified as Group A, and the remaining lesions as Group B. According to the epithelial site, preoperative cytology was highly correlated in Group A, while it was negative in Group B ($p<0.05$). In the advanced site of carcinomas, preoperative cytology tended to highly be positive in Group A, while it tended to be negative in Group B ($p<0.05$). These results suggest that the bile juice cytology results are affected by characteristics of mucin expression in the tissue. Based on the possibility that mucin expression correlates with the prognosis of each carcinoma, a positive cytological result suggests a poor prognosis for the carcinoma, which may be informative for predicting the post-operative courses and choosing treatments. J. Med. Invest. 54 : 41-47, February, 2007

Keywords **:** *biliary tract cancer, mucin, bile juice cytology*

INTRODUCTION

Currently, a variety of diagnostic methods and treatment strategies for biliary tract carcinoma are available. The prognosis for patients with this carcinoma, however, is still not as good as expected and thus the development of novel early diagnosis and treatment methods is desired. Although that bile juice cytology is useful for a definite diagnosis, this technique may give a false-negative result in some patients with advanced cancer, and various methods including different sampling methods, have been tried using to improve true positive rates (1, 2-6).

MUC1 is a mucin highly expressed in tumor cells of the digestive tract. MUC1 is sialylated in association with cancerization of cells and is considered to play a role as an anti-adhesion factor that inhibits cell-to-cell adhesion including tumor cell to tumor cell and tumor cell to extracellular matrix (7, 8). MUC2 is considered to control cell proliferation (9, 10).

There have been many studies on the relationship between mucin expression and various cancer

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prognoses (7, 8, 11-13), and we reported that mucin expression in surgically obtained biliary tract carcinoma specimens was closely related to survival prognosis (14). To our knowledge, however, there are no studies evaluating the relationship between mucin expression and the results of cytological examination for any type of cancer. Therefore, we conducted this study to examine whether mucin expression is a factor contributing to the results of cytological examination of aspirated bile juice in patients with biliary tract carcinoma.

METHODOLOGY

Materials

Twenty-five patients with biliary tract carcinoma (14 males, 11 females ; gallbladder carcinoma : 13 cases, cancer of the ampulla of Vater : one case, cancer of the common bile duct : seven cases and hepatic hilar cholangiocarcinoma : four cases) surgically treated at our hospital and whose bile juice cytology had been evaluated before operation were allocated to this study. Gallbladder cancer stages were evaluated according to the TNM Classification of Malignant Tumors (ICD-O C23, 24.0 and 24.1) of the UICC. All patients approved the study with informed consent.

Cytological examination

Cytological examination of bile juice was performed three times or until a specimen showed a positive reaction according to the Papanicolaou classification system. Two experienced cytologists examined all samples to determine whether they contained malignant cells. Specimens of bile juice aspiratate or effluent were obtained by percutaneous transhepatic cholangiodrainage (PTCD), percutaneous transhepatic gallbladder drainage (PTGBD), or endoscopic nasobiliary drainage (ENBD) and kept on ice in an ice box and promptly submitted to the clinical laboratory for examination. Biliary cytology was classified into three categories based on the Papanicolaou classification : negative, suspicious and positive.

Mucin immunohistochemistry

Serial sections were immunostained with monoclonal antibodies against MUC1 and MUC2. For immunostaining of mucin, antibodies MY.1E12 (kindly provided by Prof. Tatsuro Irimura, The University of Tokyo) and CCP58 (Pharmingen Co. Ltd., San Diego, CA) were diluted 100 and 50 times, respectively, before application. To enhance the immunoreactivity of CCP58, sections were pretreated in a microwave oven ; deparaffinized sections were transferred to 0.01M citrate buffer at pH 6, and were heated in a 500W microwave oven for 12 min. To inactivate endogenous peroxidase, all sections were treated in methanol containing 3% hydrogen peroxidase for 10 min. After washing with PBS, the sections were incubated overnight at 4℃ with each monoclonal antibody. They were then washed with PBS, and incubated with biotinylated goat anti-rabbit IgG antibody (Vector Laboratories, Inc., Burlingame, CA, USA) and avidin-biotinylated complex for 40 min at room temperature. The reactions were visualized by staining with 3-3` diaminobenzidine, and the sections were counter stained with hematoxylin.

The immunoreactivity of each antibody was graded on the extent of staining of cancer cells : 0% (-), 1-10% (+), 11-50% (++), 50%< (+++). Lesions showing MUC1 expression of ++ or higher and MUC2 expression of - were classified as belonging to Group A, and the remaining lesions as belonging to Group B. Because mucin expression of the infiltrative lesion of the cancer differed from mucin expression in the intra-epithelial lesion, the mucin expression was assessed separately in each part.

Statistical Analysis

Categorical data were analyzed using chi-square test and p values of <0.05 were considered significant.

RESULTS

Bile juice cytology

A total of 40 specimens for bile juice cytology were collected from 25 patients (a mean number of cytological examinations per patient, 1.6). Ten (40.0%) of the 25 patients studied showed positive cytology (Table 1). As mentioned above, patients underwent preoperative bile cytology up to three times. No significant relationship was demonstrated between the number of cytological examinations and their results ; six patients showed a positive result in a single test but three remained suspicious in all three tests (Table 2). Evaluation of the relationship between clinical stage and the results of bile juice cytology yielded no significant relationship ; three patients at clinical stage I or early-stage biliary tract carcinoma showed positive cytology, but four

at clinical stage IV, or an advanced stage, revealed suspicious or negative cytology (Table 3).

Mucin-expression group and its relationship to the cytological results

There was no significant relationship between the extent of each mucin expression in the intra-epithelial lesion and the cytological results of bile juice. No significant relationship was also found between the expression of MUC1 alone or MUC2 alone in the infiltrative lesion and the cytological results. Then, the evaluation was performed after categorizing each portion into Group A and B based on the extent of mucin expression. In the intra-

Table 1. Mucin staining results of 25 patients with biliary tract carcinoma

Case	Age/Sex	Diag.	Stage	HPG	Cytology Class/Exam.	Intra-epithelial lesion	Infiltrative lesion
						MUC1/MUC2	MUC1/MUC2
1	65/M	CBD	IIA	G1	5/2	+++/−	+++/−
2	65/F	GB	IIB	G2	4/1	+++/−	+++/−
3	48/F	GB	III	G1	2/2	+++/+	+++/+
4	64/M	GB	IIB	G2	4/2	+++/−	+++/−
5	73/M	GB	0	G1	2/1	++/+	++/−
6	78/F	GB	IB	G3	4/1	+/+	+++/+
7	72/M	GB	IA	G1	5/1	+++/−	++/−
8	61/F	GB	IIA	G1	2/1	+/+++	+/++
9	71/M	GB	IV	G4	3/1	+/−	+/−
10	79/F	GB	IIB	G1	2/1	++/+++	+++/+++
11	62/F	GB	IB	G1	3/2	++/++	+/−
12	74/F	GB	IB	G1	2/1	+++/−	++/−
13	61/M	HHC	IIB	G2	5/3	+++/−	+++/−
14	66/M	CBD	IV	G1	3/2	+++/+++	+++/+
15	52/M	AV	III	G2	3/2	−/+	−/−
16	75/F	CBD	IA	G2	5/1	+++/++	+++/−
17	61/F	HHC	IV	G2	2/1	++/−	++/−
18	46/M	CBD	IIA	G1	3/3	++/+	+++/+
19	64/M	CBD	IIB	G2	5/1	++/−	++/−
20	57/M	HHC	IB	G1	3/3	+/−	+/−
21	40/M	GB	0	G1	1/1	+++/−	+++/−
22	67/M	CBD	III	G1	5/2	++/−	++/−
23	76/F	CBD	IA	G1	3/3	+++/−	+++/−
24	71/F	GB	IV	G1	3/1	+/−	−/−
25	62/M	HHC	IIB	G2	5/1	++/−	+/−

GB : gallbladder carcinoma, AV : cancer of the ampulla of Vater, CBD : cancer of the common bile duct
HHC : hepatic hilar cholangiocarcinoma, HPG : Histopathological Grading
G1 : Well differentiated, G2 : Moderately differentiated, G3 : Poorly differentiated, G4 : Undifferentiated

Table 2. Correlation between cytological results and the number of examinations

Cytological results	Number of examinations		
	1	2	3
Positive (n=10)	6	3	1
Suspicious (n=8)	2	3	3
Negative (n=7)	6	1	0
Total (n=25)	14	7	4

Table 3. Correlation between clinical stage and cytological results

Cytology	Clinical stage				
	0	IA, IB	IIA, IIB	III	IV
Positive (n=10)	0	3	6	1	0
Suspicious (n=8)	0	3	1	1	3
Negative (n=7)	2	1	2	1	1
Total (n=25)	2	7	9	3	4

epithelial lesion of the cancer, 12 patients were in Group A, including 8 patients who were positive for cytology test and 1 who was suspicious and 3 who were negative ; in contrast, of 13 patients in Group B, 2 patients were positive for cytology test, 7 suspicious and 4 negative. In the infiltrative lesion, 13 patients were in Group A, including 8 patients being positive for cytology test, 1 suspicious and 4 negative ; in contrast, of 12 patients in Group B, 2 patients were positive for cytology test, 7 suspicious and 3 negative. These results demonstrated that mucin expression in both the intra-epithelial and infiltrative lesions of cancer was significantly related to the cytological results of bile juice when each portion was classified into Group A or B based on the extent of mucin expression (intra-epithelial portion *vs.* infiltrative portion, both p=0.0164) (Table 4, Figs. 1(A, B), 2(A, B)).

Table 4. Correlation between mucin expression group and cytological results

		Positive	Suspicious	Negative	
Intra-epithelial lesion	Group A (n=12)	8	1	3	*
	Group B (n=13)	2	7	4	
Infiltrative lesion	Group A (n=13)	8	1	4	*
	Group B (n=12)	2	7	3	

* : p<0.05

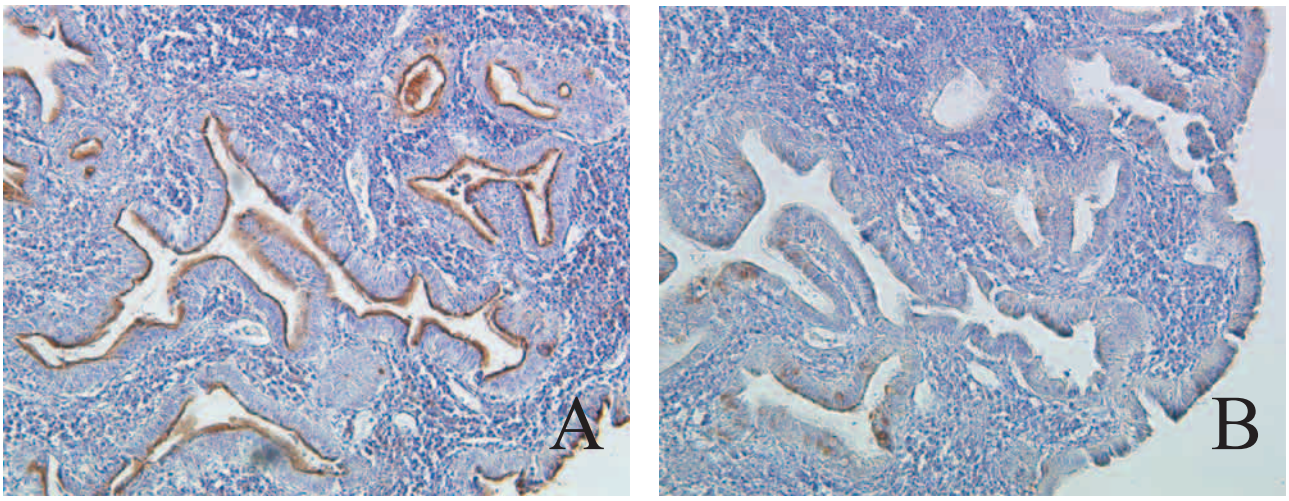

Fig. 1 : This case is a 65-year-old male with common bile duct cancer, tissue type G1, stage IIA, and class V bile juice cytology, strongly positive for MUC1 (A) in the infiltrative lesion of cancer, and negative for MUC2 (B), and was Group A for the extent of mucin expression.

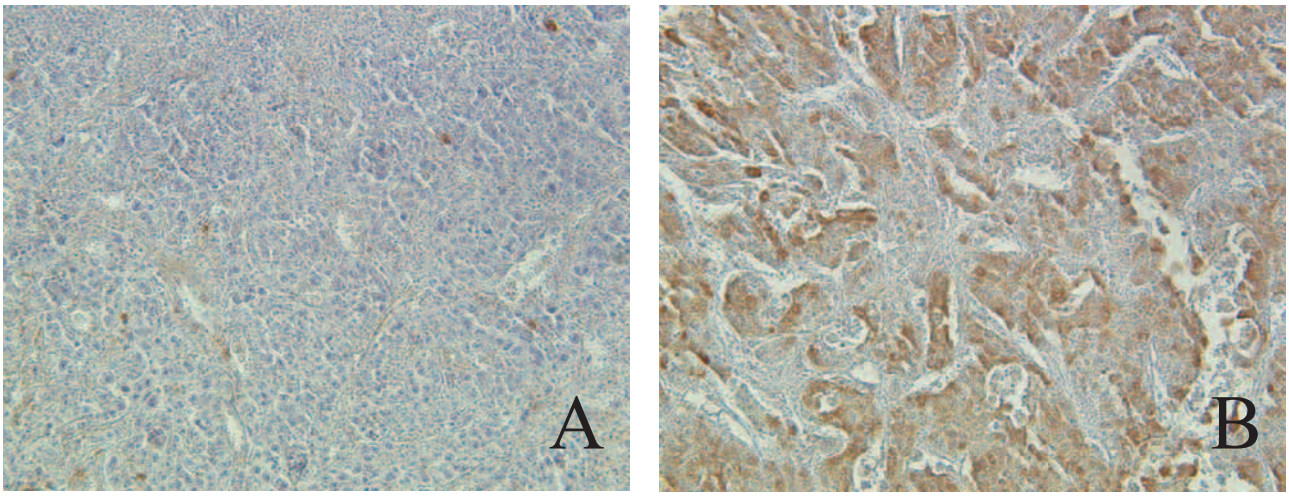

Fig. 2 : This case is a 61-year-old female with gallbladder carcinoma, tissue type G1, stage IIA, and class II bile juice cytology, mildly positive for MUC1 (A) in the intra-epithelial lesion of the cancer, and strongly positive for MUC2 (B), and was Group B for the extent of mucin expression.

DISCUSSION

Today, imaging diagnosis has progressed considerably. However, in many cases it is still difficult to determine whether the cause of a biliary tract disorder, especially stenosis, is malignant or benign. In such cases, the results of bile juice cytology, specimens of which are obtained via endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic biliary drainage (PTBD), will provide information. Although bile exfoliative cytology is popular for the diagnosis of biliary tract carcinoma, its sensitivity varies from 60% to 82% (1, 2-6), and still remains inadequate (between 68% and 76%) even when specimens are obtained by the biliary tract brushing technique (1, 4, 5). For the diagnosis of malignant biliary stenosis, only the technology for collecting cells has been stressed and no attention has been paid to individual patient's factors.

At the same time, there have been many studies on the relationship between mucin expression and cancer prognoses (7, 8, 11-13). Mucin is a major secretory component of mucus produced by epithelial cells and is considered to play a role in protecting cells from the effects of extracellular substances or in lubricating cell surfaces. Abundant recent data derived from studies on mucin produced by epithelial tumor cells have demonstrated that one type of mucin (MUC1) also adheres to cellular membranes. Using this feature, the existence of a relationship between the extents of expression of MUC1 and MUC2 and the prognosis of various digestive tract cancers has been demonstrated ; the cancers studied included gastric carcinoma (10, 11), colon cancer (15), gall bladder cancer (7, 8, 14, 16), extrahepatic bile duct cancer (12, 17), intrahepatic bile duct cancer (18), papillary carcinoma (13), pancreatic cancer (9), and intraductal papillary mucinous tumor of the pancreas (19) ; among the presumable features, the anti-adhesion effect of MUC1 and cell growth-control function of MUC2 are assumed to contribute to this association (10). Meanwhile, the relationship between mucin expression and the results of cytology has not been explored in any type of cancer. We hypothesized that cancer cell exfoliation results from mucin expression, and therefore we examined the relationship between mucin expression and the results of preoperative bile juice cytology in patients with biliary tract carcinoma.

In this study, no relationship between the number of cytological examinations of bile and the results of the cytology was observed, the increased number of cytological examinations was not always associated with the detection of cancer cells in bile, and there were patients in whom cancer cells were detected by a single examination. In further study, we need a quantitative cytological examination of bile juice to reassess our hypothesis more precisely because we simply implemented qualitative bile exfoliative cytology in this study. In addition, no significant correlation was found in this study between the results of bile cytology and the histopathological type or clinical stage ; positive bile cytology was observed even in patients with early stage cancer, and contrarily, negative bile cytology was noted in patients with advanced stage cancer, probably due to little exfoliation of cancer cells.

Because mucin expression in the epithelial portion of a cancer was reported to differ from that in the infiltrative portion (8, 20), we also evaluated mucin expression in both the epithelial portion near the bile duct and the infiltrative portion. When cytological specimens were classified according to the extents of expressions of MUC1 and MUC2, the intra-epithelial portion and the infiltrative portion showed almost the same results ; only one patient presented a different mucin expression group ratio, although MUC1 and MUC2 were expressed to different extents in different areas. Yonezawa *et al.* reported that diagnosis using both MUC1 and MUC2 would be useful to assume the prognoses of various cancers (9, 12, 18). Our data demonstrated a close relationship between mucin expression groups and the results of bile cytology. MUC 1 is sialylated in association with cancerization of cells and considered to play a role as an anti-adhesion factor that inhibits cell to cell adhesion, including tumor cell to tumor cell and tumor cell to extracellular matrix. Thus, MUC1 probably has a function related to anti-adhesion (7, 8). MUC2 is considered to control cell proliferation (9, 10). These features probably function in the epithelium of the bile duct. The close relationship between prognosis of patients with biliary tract carcinoma and mucin expression suggests that the results of bile cytology can be a valuable marker for assessing the prognosis of this cancer. Moreover, the results of bile cytology classified according to the extent of mucin expression may provide information useful for the choice of additional treatment after surgery. Studies using preoperative bile cytology with a long-term follow-up are expected to support the relationship that we observed in this study.

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REFERENCES

1. Tomasz RK, Alastair D, James SD, Robert D, Kenneth EFH, Brian RD : A prospective study of biliary cytology in 100 patients with bile duct strictures. Hepatology 18 : 1399-1403, 1993
2. Cohan RH, Illescas FF, Braun SD, Newman GE, Dunnick NR : Fine needle aspiration biopsy in malignant obstructive jaundice. Gastrointest Radiol 11 : 145-150, 1986
3. Iitsuka Y, Hiraoka H, Kimura A, Kudoh H, Koga S : Diagnostic significance of bile cytology in obstructive jaundice. Jpn J Surg 14 : 207-211, 1984
4. Govil H, Reddy V, Kluskens L, Treaba D, Massarani-Wafai R, Selvaggi S, Gattuso P : Brush cytology of the biliary tract : retrospective study of 278 cases with histopathologic correlation. Diagn Cytopathol 26 : 273-277, 2002
5. Siddiqui MT, Gokaslan ST, Saboorian MH, Carrick K, Ashfaq R : Comparison of thinprep and conventional smears in detecting carcinoma in bile duct brushings. Cancer (Cancer Cytophathol) 99 : 205-210, 2003
6. Rupp M, Hawthorne CM, Ehya H : Brushing cytology in biliary tract obstruction. Acta Cytol 34 : 221-226, 1990
7. Kashiwagi H, Kijima H, Dowaki S, Ohtani Y, Tobita K, Yamazaki H, Nakamura M, Ueyama Y, Tanaka M, Inokuchi S, Makuuchi H : MUC 1 and MUC2 expression in human gallbladder carcinoma : a clinicopathological study and relationship with prognosis. Oncol Rep 8 : 485-489, 2001
8. Kashiwagi H, Kijima H, Dowaki S, Ohtani Y, Tobita K, Tsukui M, Tanaka Y, Matsubayashi H, Tsuchda T, Yamazaki H, Nakamura M, Ueyama Y, Tanaka M, Tajima T, Makuuchi H : DF3 expression in human gallbladder carcinoma : Significance for lymphatic invasion. Int J Oncol 16 : 455-459, 2000
9. Yonezawa S, Sato E : Expression of mucin antigens in human cancers and its relationship with malignancy potential. Pathol Int 47 : 813-830, 1997
10. 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 4 : 2605-2614, 1998.
11. 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 198 : 665-674, 2002
12. Tamada S, Goto M, Nomoto M, Nagata K, Shimizu T, Takeda S, Sakoda K, Imai K, Yonezawa Y : Expression of MUC1 and MUC2 mucins in extrahepatic bile duct carcinoma : its relationship with tumor progression and prognosis. Pathol Int 52 : 713-723, 2002
13. Kitamura H, Yonezawa S, Tanaka S, Kim YS, Sato E : Expression of mucin carbohydrates and core proteins in carcinomas of the ampulla of vater : their relationship to prognosis : Jpn J Cancer Res 87 : 631-640, 1996
14. Takagawa M, Muguruma N, Oguri K, Imoto Y, Okamoto K, Ii K, Ito S : Prediction of the prognosis in the gallbladder carcinoma by mucin and p53 immunohistochemistry. Dig Dis Sci 50 : 1410-1413, 2005
15. Ajioka Y, Allison LJ, Jass JR : Significance of MUC1 and MUC2 mucin expression in colorectal cancer. J Clin Pathol 49 : 560-564, 1996
16. 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 7 : 1333-1342, 2001
17. 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 15 : 1966-1975, 1999
18. 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 30 : 1347-1355, 1999
19. Nakamura A, Horinouchi M, Goto M, Nagata K, Sakoda K, Takao S, Imai K, Young SK,

Sato E, Yonezawa S : New classification of pancreatic intraductal papillary-mucinous tumour by mucin expression : its relationship with potential for malignancy. J Pathol 197 : 201-210, 2002

20. Hiraga Y, Tanaka S, Haruma K, Yoshihara M, Sumii K, Kajiyama G, Shimamoto F, Kohno N : Immunoreactive MUC1 expression at the deepest invasive portion correlates with prognosis of colorectal cancer. Oncology 55 : 307-319, 1998