The Evaluation of *MSX2* mRNA Expression Level in Biliary Brush Cytological Specimens

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Abstract. *Background: To distinguish cholangiocarcinoma from inflammatory disease remains difficult when stricture is present in the bile duct. Endoscopic brushing cytology is a convenient method for stricture in the bile duct, however, the diagnostic sensitivity of this method for malignancy is reported to be low (<60%). Msh homeobox 2 is frequently expressed in carcinoma cells of epithelial origin but not in normal tissues. Aim: To assess whether MSX2 expression level in brushing samples allows differentiation of malignant from benign bile duct stricture. Patients and Methods: Cytological brushing specimens were obtained from strictures of the bile duct during endoscopic retrograde cholangiopancreaticography (ERCP) in 71 patients. The brushing fluid was subjected to cytological diagnosis and RNA extraction. The expression level of MSX2 was evaluated by one-step real-time RT-PCR. Results: MSX2 expression levels were significantly higher in malignant than in benign bile duct stricture (p=0.004). The sensitivity and specificity for cholangiocarcinoma of cytology and MSX2 expression in strictures of the bile duct were: 55.3% and 100%, and 72.3% and 58.3%, respectively. Conclusion: The sensitivity of MSX2 expression level for cholangiocarcinoma was much higher than that of cytology. This suggests that the evaluation of MSX2 level in ERCP brushing samples would be a useful tool to distinguish malignant from benign bile duct stricture.*

Cholangiocarcinoma is a neoplasm that arises anywhere in the biliary tract and is considered to be originated from cholangiocytes (1). The incidence of cholangiocarcinoma, especially intrahepatic cholangiocarcinoma, has been shown to be increasing (2). Despite the improvement of diagnostic methods, the mortality of cholangiocarcinoma is growing and the 5-year survival of patients with this neoplasm ranges from 20 to 43%, indicating the poor prognosis of this malignancy (1, 3). Surgical resection is the only curative therapy, but most patients present at advanced clinical stage. Magnetic resonance imaging (MRI)/magnetic resonance cholangiopancreatography (MRCP), positron-emission tomography (PET), endoscopic ultrasonography (EUS) and computed tomography (CT) are the most frequently used modalities for diagnosis and tumor staging (4). However, it is still difficult to obtain histological evidence of malignancy. Although EUS-guided fine-needle aspiration (FNA) can be performed on tumors in patients with negative cytology or to sample enlarged lymph nodes for preoperative staging (5-7), this approach has potential for tumor seeding (8). On the other hand, brushing cytology for bile duct stricture during endoscopic retrograde cholangiopancreatography (ERCP) is convenient and safe but the sensitivity of the diagnosis for cholangiocarcinoma is not high (about 35-60%) (1, 4, 9-11).

Msx2, a member of the homeobox gene (*Hox* genes) family, is expressed in a variety of sites, including premigratory cranial neural crest, tooth, retina and lens, apical ectodermal ridge and mammary gland in a variety of vertebrates (12-15). In the development of these organs, the expression patterns of this gene suggest its active involvement in epithelial–mesenchymal interactions (16, 17). Recently, we have shown that *MSX2* (HOX-8), the human homolog of *Msx2,* was frequently expressed in pancreatic carcinoma cell lines and tissues but not in benign cultured cells or normal human pancreatic tissues (18, 19). In addition, enhanced levels of transcripts for *MSX2* have been shown in a variety of carcinoma cell lines of epithelial origin compared to their corresponding normal tissues (20). However, little is known about the involvement of *MSX2* in the development of

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cholangiocarcinoma. Therefore, we investigated the expression of *MSX2* in cholangiocarcinoma to assess whether this expression would differentiate malignant from benign stricture of the bile duct during ERCP.

Patients and Methods

Microdissection of cholangiocarcinoma tissue and RNA extraction. Cholangiocarcinoma tissues were obtained from patients who underwent surgical operations for the tumors. The tissues collected at the time of surgery were immediately embedded in Tissue-Tek O.C.T. compound medium (Sakura, Tokyo, Japan), frozen in liquid nitrogen, and stored at –80˚C. The frozen tissues were cut into 8 μm-thick sections using a cryostat (Jung CM3000; Leica, Nussloch, Germany), and 6 cholangiocarcinoma tissues and 6 normal tissues were subjected to laser-captured microdissection using a Leica CIR MIC system (Leica microsystems, Wetzkar, Germany). Sample collection and usage were performed under the written informed consent obtained from each patient before surgery. Total cellular RNA was extracted from each sample by using the mirVana™ miRNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol.

Brushing cytological samples and RNA extraction. Cytological brushing specimens were obtained from bile duct strictures during ERCP from 71 patients at the Tohoku University Hospital between April 2007 and March 2010. Informed consent was obtained from all patients, and the study was approved by the Ethics Committee at the Tohoku University (article no. 2008-506). Characteristics of all patients are summarized in Table I. Final diagnosis was cholangiocarcinoma (n=47) and benign biliary stricture (n=24, Table II). All of the diagnoses of cholangiocarcinoma were confirmed by brushing cytology or histological examination after surgical resection. Sample collection and usage were carried out under written informed consent prior to the ERCP. The brushing cytology of biliary stricture was performed in every patient by using Rapid Exchange™ Cytology Brushes (Boston Scientific Corporation, Tokyo, Japan), and part of the brushing cytological samples were subjected to RNA extraction for *MSX2* mRNA quantification. Total cellular RNA was extracted from each sample by using the mirVana™ miRNA Isolation Kit (Applied Biosystems) according to the manufacturer's protocol.

Real-time RT-PCR. One-step quantitative real-time RT-PCR was performed on each sample using 4 ng total RNA with a QuantiTect SYBR Green RT-PCR Kit (Qiagen) using LightCycler (Roche Diagnostics, Basel, Switzerland). RNA concentration was determined with an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). The primer pairs used were: *MSX2*, forward 5 CCGCCTCGGTCAAGTCGGAAAAT3 and reverse 5 TGGAGAGGTACTGTTTCTGACGG3'; and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), forward 5' GGCGTCTTCA CCACCATGGAG3' and reverse 5'AAGTTGTCA TGGATGACCTT GGC3'. All reactions were performed according to the manufacturer's protocol. Plasmids including coding region of *MSX2* or *GAPDH* were used as standards and the copy number of *MSX2* in each sample was normalized to the respective *GAPDH* copy number. The specificity of each PCR reaction was confirmed by melting curve analyses.

Table I. *Clinicopathological features of cholangiocarcinoma and benign bile duct stricture*.

Clinicopathological feature	Cholangiocarcinoma	Benign	*P*-value*
Age <60 years	10	10	0.071
Age ≥60 years	37	14	
Male	34	16	0.825
Female	13	8	
Extrahepatic stricture	16	17	0.007
Intrahepatic stricture	31	7	

*Analyzed by χ^2 test, $p<0.05$ was considered to be statistically significant.

Statistical analysis. The correlation between the clinicopathological features and final diagnoses were assessed by the chi-square test. The difference between two groups was analyzed by Mann-Whitney *U*-test. A *p*-value of <0.05 was regarded as statistically significant. The average relative expression level of MSX2 is displayed as the mean±standard error (SE).

Results

We first examined the expression level of *MSX2* mRNA in the microdissected cholangiocarcinoma or non-cancerous bile duct epithelium. As shown in Figure 1A and B, cholangiocarcinoma cells or non-cancerous bile duct cells were collected using the laser-captured microdissection. The relative expression level of *MSX2* in each sample is depicted in Figure 1C, and cholangiocarcinoma was revealed to have a significantly higher expression level of *MSX2* than non-cancerous bile duct epithelium ($p=0.035$), indicating that the expression level of this gene would be useful in distinguishing cholangiocarcinoma cells from non-cancer cells.

In 71 patients, endoscopic brushing was successfully performed and satisfactory specimens were obtained. No major complications, including acute cholangitis, occurred after brushing cytology in the current study. Clinicopathological characteristics of patients who underwent brushing cytology during the ERCP are summarized in Table I. Patients with cholangiocarcinoma were generally elder, and tended to have intrahepatic lesions. This result might be explained by the fact that benign biliary stricture patients included patients with chronic pancreatitis and autoimmune pancreatitis. The diagnoses of patients with benign biliary stricture are summarized in Table II. The sensitivity of routine brush cytology for cholangiocarcinoma was 55.3% (26/47) with 100% specificity. No significant association of tumor location with positive rate of cytology in the tumor location was found (Table III).

MSX2 mRNA in brushing samples was successfully detected and quantified by normalization to the respective *GAPDH* copy number (Figure 2A). The mean expression level of *MSX2* mRNA was 1.558±0.574 (*MSX2*/*GAPDH*

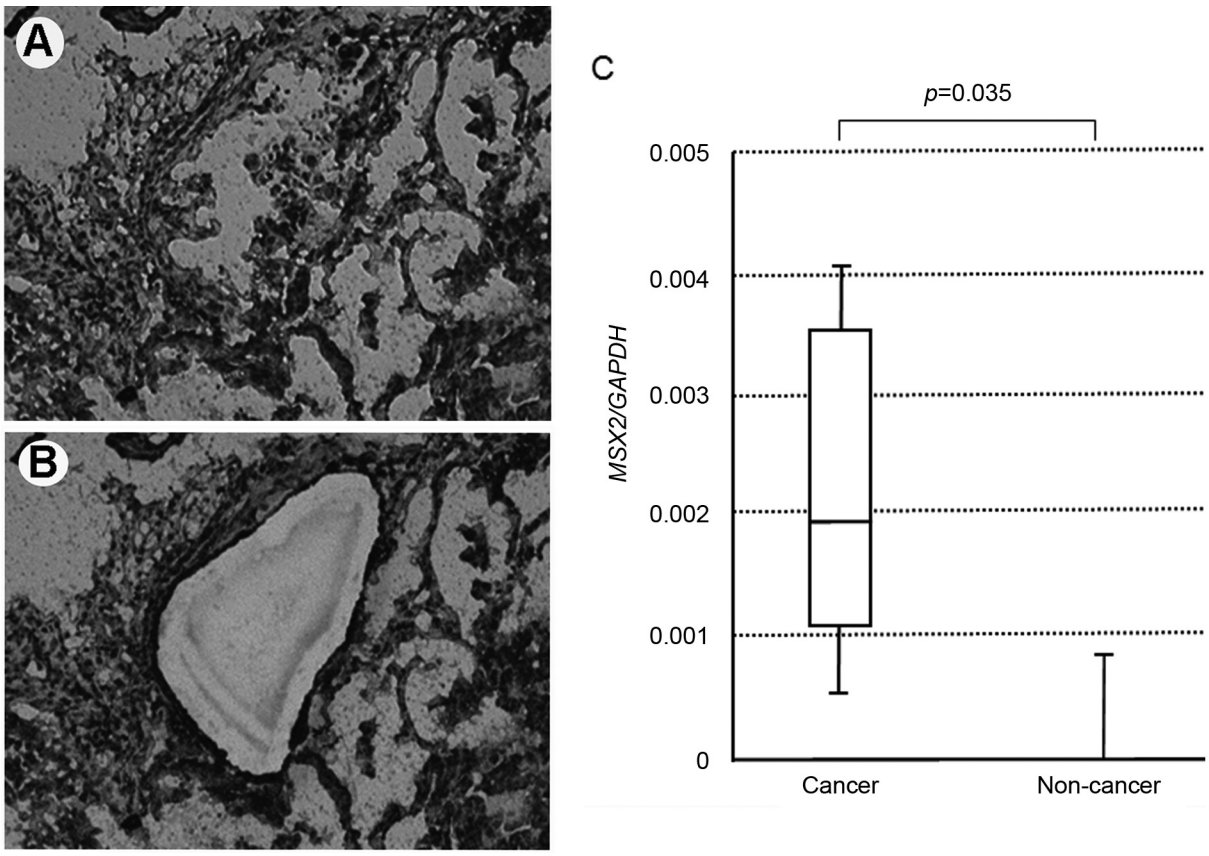

Figure 1. *Expression of MSX2 mRNA in microdissected lesions was detected by quantitative real-time RT-PCR. RNA extraction was performed from the microdissected lesions. Hematoxylin-stained cancer lesions (A) were selectively dissected (B) using a Leica CIR MIC system. Twelve lesions were microdissected and RNAs were extracted. RNAs were then subjected to one-step quantitative real-time RT-PCR and the expression of target gene was normalized to that of GAPDH expression. MSX2 mRNA expression was significantly higher in cancer lesions than non-cancerous lesions (p=0.035, Mann-Whitney U-test)(1C). MSX2/GAPDH represents the MSX2 copy number/GAPDH copy number/μl.*

Table II. *The diagnoses of 24 benign biliary strictures.*

Chronic pancreatitis	7
Autoimmune pancreatitis	6
Choledocholithiasis	6
Primary sclerosing cholangitis	2
Congenital biliary dilatation	2
Unknown	1

Table III. *Association of tumor site with cytological examination.*

	Location		
Cytology	Intrahepatic	Extrahepatic	*P*-value*
Negative	14	7	0.828
Positive	17	9	

*χ^2 test.

copy number/μl, mean±SE) and 0.226±0.046 in cholangiocarcinoma and non-cancerous stenosis, respectively (Figure 2B). The expression level of *MSX2* mRNA was significantly higher in cholangiocarcinoma samples compared to those of non-cancerous stenosis (p=0.004).

Based on these findings, we tried to apply the measurement of *MSX2* mRNA expression in brushing cytological samples to the diagnosis of cholangiocarcinoma. By receiver operating characteristic curve (ROC) analysis, we determined the cut-off value of *MSX2* mRNA expression for the cancer diagnosis to be 0.176. *MSX2* expression level was judged positive when the expression level was equal or higher than the cut-off value. Using this cut-off value, diagnosis of cholangiocarcinoma by *MSX2* mRNA

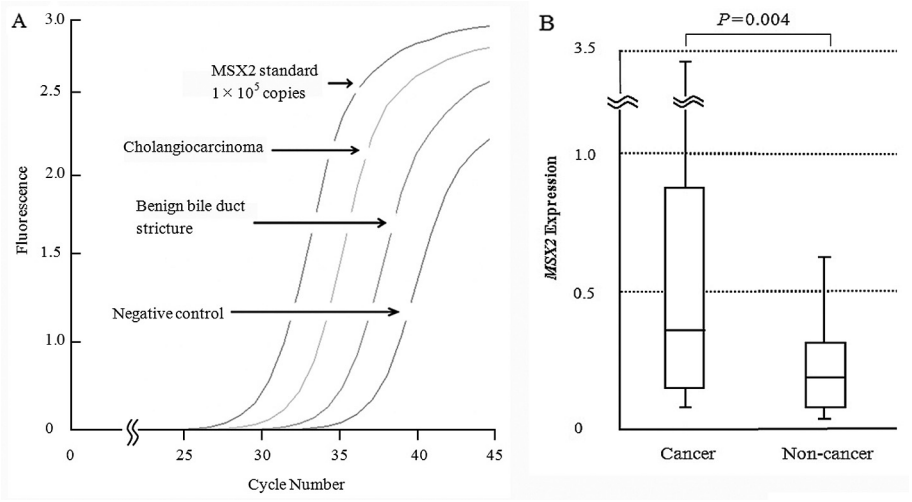

Figure 2. *Expression of MSX2 mRNA in brush samples from cholangiocarcinoma and benign bile stricture. A: Representative real-time RT-PCR curve showing significant difference in MSX2 expression level between cholangiocarcinoma and bile duct stricture caused by chronic pancreatitis. B: Expression of MSX2 was significantly higher in specimens from cholangiocarcinoma than in those from benign bile duct stricture (p=0.004, Mann-Whitney U-test).*

Table IV. *The sensitivity and specificity of each diagnostic strategy.*

	Sensitivity (%)	Specificity (%)
MSX2 expression	72.3	58.3
Cytology	55.3	100
MSX2+cytology	80.9	58.3

Table V. *Association of tumor location with MSX2 expression level.*

	Location		
MSX2[a]	Intrahepatic	Extrahepatic	*P*-value*
Negative	8	5	0.959
Positive	23	11	

[a]Negative, expression <0.176; positive, expression ≥0.176. *χ^2 test.

Table VI. *Association of MSX2 expression level with cytology.*

	MSX2[a]		
Cytology	Negative	Positive	P-value*
Negative	8	13	0.267
Positive	5	21	

[a]Negative, expression <0.176; positive, expression ≥0.176. *χ^2 test.

measurement in brushing cytology reached a sensitivity of 72.3%, with specificity of 58.3% (Table IV). When evaluation of the *MSX2* expression level was combined with cytology, sensitivity for malignancy reached 80.9%.

No significant association of tumor location with positive rate of *MSX2* expression in the tumor was found (Table V). In 26 cases with positive cytology, 21 cases (80.7%) demonstrated an *MSX2* expression level higher than the cut-off value (Table VI). Figure 3 shows a representative case for which both cytology and *MSX2* expression were positive. The 47-year-old female was referred to our Department for further examination of the dilatation of the intrahepatic bile duct. She was found to have bile duct stenosis at the upper extrahepatic bile duct as shown in Figure 3A. Brushing cytology was performed and the sample contained enough cells representing adenocarcinoma (Figure 3B). The expression level of *MSX2* was 1.154, above the cut-off value. The final diagnosis by histology for surgical tissue showed adenocarcinoma cells in the bile duct (Figure 3C).

On the other hand, there were 13 cases showing positive *MSX2* expression among 21 cases of negative cytology. Figure 4 demonstrates a representative case with negative cytology but having positive *MSX2* expression. This case was of a 67-year-old female who was admitted to our hospital due to jaundice. She had lower extrahepatic bile duct stenosis and brushing cytology was conducted (Figure 4A). Since the brushing cytological sample contained only few atypical cells (Figure 4B), this case was diagnosed as class II. This case underwent surgical operations, and the final diagnosis was confirmed by histological diagnosis as cholangiocarcinoma

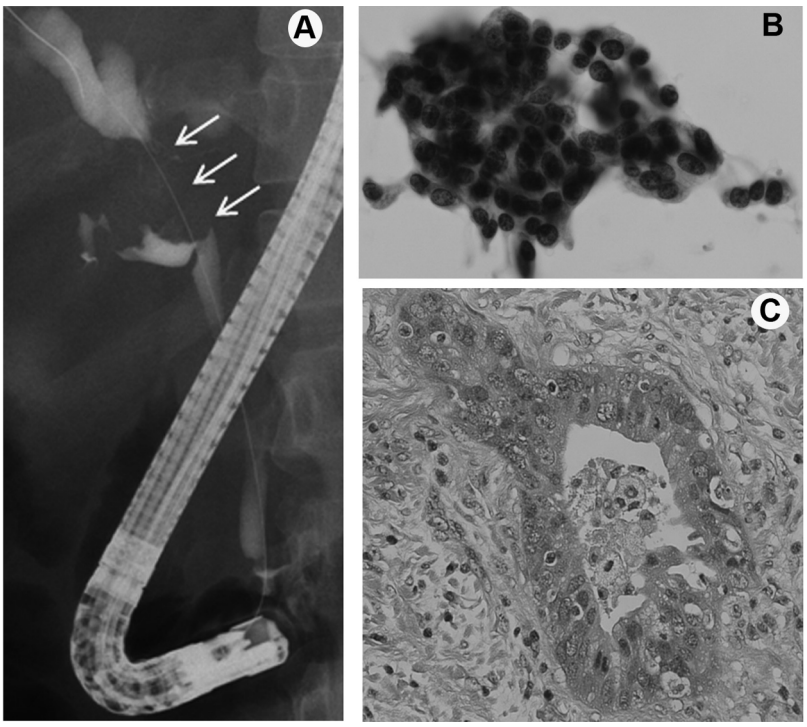

Figure 3. *Representative case of positive cytology with positive MSX2 expression level. A: ERCP showed the stricture of the upper extrahepatic bile duct and the brushing cytology was performed. B: The brushing specimen contained cells representing adenocarcinoma (original magnification, ×400). The expression level of MSX2 in the specimen (1.154) was higher than the cut-off value (0.176). C: Histological examination revealed adenocarcinoma of the bile duct (original magnification, ×400).*

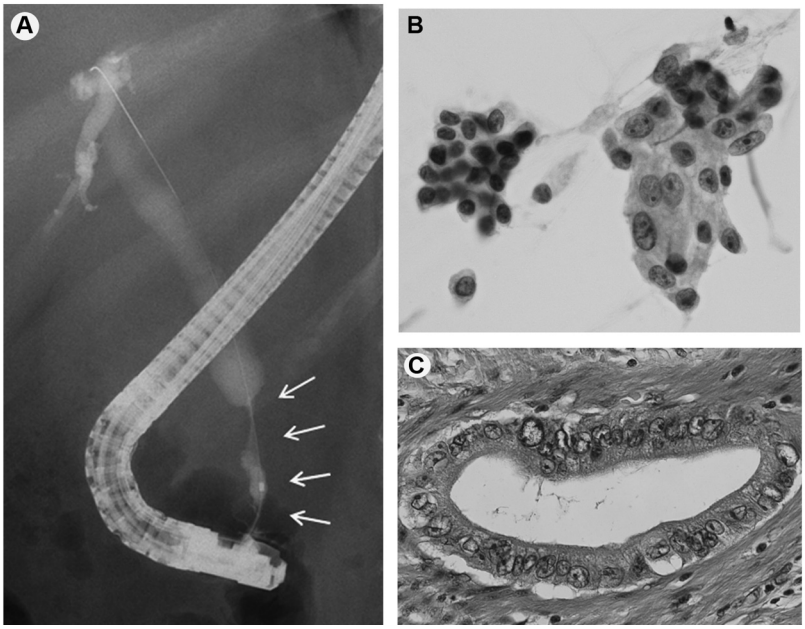

Figure 4. *Representative case with negative cytology but having positive MSX2 expression. A: Bile duct stenosis was found in the lower extrahepatic bile duct (arrow). B: Brushing cytological sample contained only few atypical cells and was diagnosed as class II (original magnification, ×600). The relative expression level of MSX2 was 1.467, above the cut-off value. C: The final diagnosis was confirmed by histological diagnosis of surgical tissues as cholangiocarcinoma (original magnification, ×400).*

(Figure 4C). In this case, the relative expression level of *MSX2* was 1.467, above the cut-off value, suggesting that the *MSX2* level was able to detect malignant cells in which definitive cytological diagnosis could not be obtained.

Discussion

The differential diagnosis for bile duct strictures caused by cholangiocarcinoma or inflammatory disease is generally difficult since it is hard to obtain accurate histological evidence of malignancy from the bile duct before surgery. Brushing cytology for bile duct strictures during ERCP is convenient and safe. However, the sensitivity of this method for cholangiocarcinoma is not satisfactory (<60%) (1, 4, 9-11). To improve the sensitivity of brushing cytology for cholangiocarcinoma, we investigated *MSX2* expression in brushing samples. We have previously shown that *MSX2* was frequently expressed in pancreatic carcinoma cells but not in benign pancreatic duct cells (19, 20) and evaluation of *MSX2* level in ERCP brushing samples was able to differentiate pancreatic cancer from chronic pancreatitis (21). To our knowledge, no study has reported data regarding *MSX2* in cholangiocarcinoma. Therefore, we investigated *MSX2* expression in brushing samples of the bile duct to determine whether this expression could help in the diagnosis of cholangiocarcinoma. In this study, we clearly revealed that: i) *MSX2* mRNA expression was significantly higher in microdissected cholangiocarcinoma cells than non-cancerous cells; ii) significantly high expression of *MSX2* mRNA was detected in brushing specimens from cholangiocarcinomas compared to those from benign lesions; iii) diagnostic sensitivity of the analysis of the *MSX2* expression level was much higher than that of cytological examination.

Previously, immunostaining of *p53* or Ki-67 in forceps biopsy specimens was reported to improve the sensitivity for diagnosis of cholangiocarcinoma, combined with histology (22). The sensitivity was improved from 53% to 75%, but the specificity was reduced. Similarly, detection of telomerase activity in bile duct biopsy specimens improved the sensitivity of bile duct cancer diagnosis combined with *p53* overexpression, but in a relatively small sample size (16 cases) (23). Another approach by detecting aspartyl beta-hydroxylase and homeobox B7 mRNA in brushing cytological samples also improved the sensitivity of brushing cytology up to 82% (24). Compared to these methods, our method yielded a similar sensitivity, suggesting that validation of the *MSX2* expression level in the brushing samples would be good tool to distinguish malignant from benign bile duct stricture in parallel to other molecular analyses as mentioned above.

The measurement of *MSX2* expression had advantages in the cases whose brushing cytological specimen contained only few atypical cells, which led to inconclusive cytological results as shown in Figure 4. Since the sensitivity of RT-PCR is high, the existence of low numbers of atypical cells might be detected by this method. In addition, *MSX2* expression level can be evaluated within one day, and this also benefits quicker clinical decision making. Furthermore, obstructive jaundice is a typical clinical manifestation of cholangiocarcinoma (25), and immediate biliary drainage is required in case of cholangitis (26). In these situations, placement of a plastic biliary stent or endoscopic naso-biliary drainage tube is widely performed, but chronic inflammation due to the plastic stent will cause artificial thickening of bile duct epithelium (27). Even in these cases, brushing cytology can be performed, and the measurement of *MSX2* expression can also be carried out.

In conclusion, *MSX2* was significantly expressed in brush samples of cholangiocarcinoma compared to those of non-cancerous bile duct. The sensitivity for malignancy was much higher than cytology, suggesting that the evaluation of the *MSX2* expression level, in addition to cytological examination, would help to differentiate malignant from benign stricture of the bile duct.

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The Authors have no conflicts of interest to declare.

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