The Significance of p53 and *K-ras* Immunocytochemical Staining in the Diagnosis of Malignant Biliary Obstruction by Brush Cytology during ERCP

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Background/Aims: Brush cytology during ERCP can provide a pathologic diagnosis in malignant biliary obstruction. *K-ras* and p53 mutations are commonly found in biliary and pancreatic cancers. We evaluated the diagnostic yield of brush cytology and the changes obtained by adding p53 and *K-ras* staining.

Methods: One hundred and forty patients with biliary obstruction who underwent ERCP with brush cytology during a 7-year period were included. The sensitivity and specificity of brush cytology only and with the addition of p53 and *K-ras* staining were obtained.

Results: Malignant biliary obstruction was confirmed in 119 patients. The sensitivity and specificity of brush cytology were 78.2% and 90.5%, respectively. The sensitivity of cytology was 77.3% at the ampulla-distal common bile duct (CBD), 92.6% at the mid common hepatic duct (CHD), and 94.7% at the proximal CBD-CHD ($p < 0.05$); these values did not differ with the degree or the length of the obstruction. In the 97 patients who received additional p53 and *K-ras* staining, the sensitivity of cytology plus p53 was 88.2%, cytology plus *K-ras* was 84.0%, and cytology plus p53 and *K-ras* was 88.2%. The sensitivity of cytology plus p53 was higher than that of brush cytology only (95% confidence interval: 83.69-92.78 vs 72.65-83.65) but not that of cytology plus *K-ras*. **Conclusions:** Brush cytology for malignant biliary obstruction has a high diagnostic accuracy. Adding p53 staining can further improve the diagnostic yield, whereas *K-ras* staining does not. (*Gut Liver* 2010;4:219-225)

Key Words: Malignant biliary obstruction; Brush cytology; ERCP; Immunocytochemical stain

INTRODUCTION

Brush cytology during ERCP for tissue sampling at biliary stricture is a simple and safe method that can be performed during the same session. Although this technique has a specificity of nearly 100%, it has only a modest sensitivity that ranges from 30-69%.¹⁻⁶ Improvement of instrument for brush cytology has facilitated the acquisition of tissue at biliary stricture, but the sensitivity remains below 85%⁷ and cancer detection rate is still low.⁸ Sometimes cytologic specimen with adequate cellularity can not be interpreted either malignant or benign due to borderline characteristics. Therefore, additional cytologic criteria or special staining methods are required for accurate interpretation of cytologic specimen obtained by ERCP brush cytology. An analysis of tumor-specific genetic alterations in the cytologic specimens may increase the diagnostic value of brush cytology.

Mutations of *K-ras* oncogene are one of the most common genetic alterations in human cancers and are frequently found in both bile duct carcinoma and pancreatic cancer that cause biliary stricture.⁹⁻¹³ Also, the prevalence of p53 mutations in cancers of pancreas, bile duct, and ampulla of Vater is between 50 and 70%.^{14,15} Mutant *K-ras* protein and mutant p53 protein product can accu-

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mutate in the nucleus, where they can be detected with simple, quick, and cheap immunocytochemical techniques available in routine laboratories. However, there has been no study evaluating the advantage of *K-ras* immunocytochemical staining in brush cytology for diagnosis of malignant biliary stricture. Recently published two studies have shown improved diagnostic sensitivity of p53 immunocytochemical staining compared to conventional cytological diagnosis using pancreatic ductal brush cytology specimens in pancreatic cancers.^{16,17} But, the results of subsequent studies did not support this finding.¹⁸⁻²⁰

In this study, we prospectively assessed the diagnostic values of *K-ras* and/or p53 immunocytochemical staining in addition to conventional cytology using samples obtained by ERCP brush cytology for the diagnosis of malignant extrahepatic bile duct stricture.

MATERIALS AND METHODS

1. Patients

All consecutive patients who underwent ERCP with brush cytology for the evaluation of extrahepatic bile duct stenosis and had the diagnosis confirmed either by surgery or clinical outcome between 1998 and 2005 at Daegu Catholic University Hospital were included. Patients who did not have available follow up data for discrimination of benign or malignant biliary strictures were excluded. All the patients had given a written informed consent prior to ERCP with brush cytology. Total 140 patients with biliary obstruction were included.

2. Brush cytology for cytopathologic sampling

Olympus EVIS system, JF or TJF 200 series (Olympus, Tokyo, Japan) was used for ERCP.

During ERCP after obtaining cholangiogram, specimens for brush cytology was obtained from obstructed segment of bile duct under fluoroscopic guidance using 6 Fr standard cytology brush, Combo-cath sheath cytology brush[®] (Boston Scientific Co., Natick, MA, USA) or Geenen brush[®] (Wilson-Cook Medical Inc., Winston-Salem, NC, USA). The brush was inserted over a standard 0.035-inch guidewire. The entire procedure was performed during a single session by one dedicated endoscopist (H.G.K.). After brushing the stricture by 6-10 to-and-pro movements, the brush was withdrawn. After removing from the channel of endoscope, brush was immediately smeared on four slides in the following order: glass slide for routine Papanicolaou stain, polylysine coated ProbeOn slide[®] (Cole-Parmer, Vernon Hills, IL, USA) for immunocytochemical stain of p53 protein and *K-ras* protein, and two glass slides for Papanicolaou stain. After smear-

ing, slides were immersed immediately in a 95% alcohol jar and transported to the cytology laboratory. The results of conventional brush cytology were compared between malignant tumors of the biliary tree (bile duct cancer, ampullary cancer, or gallbladder cancer) and pancreatic cancer.

3. Final clinical diagnosis

The diagnosis of malignancy was confirmed either by surgery, ultrasound- or computed tomogram (CT)-guided biopsy or by clinical follow-up for at least 12 months.

The diagnosis of benign disease was confirmed when all tests including CT and serum CA 19-9 level failed to show malignant disease and the patient showed no evidence of disease progression for at least 12 months follow-up.

4. Cytopathologic and immunocytochemical diagnosis

Single dedicated pathologist (C.H.C.) processed and interpreted the specimens. The results of routine Papanicolaou stain were classified by using standard cytologic criteria as follows: positive for malignancy, atypical cell, negative for malignancy. The pathologic diagnosis of malignancy was confined only to positive for malignancy. Three-dimensional cluster was defined as a group of cells with high nucleocytoplasmic ratio with ovoid or angulated irregular nuclei and variable amount of cytoplasm that are arranged large thick clusters in three-dimensional structure with marked cellular overlapping or crowding (Fig. 1).

Among 140 patients with biliary stricture, 119 patients had been confirmed of malignant stricture. The ProbeOn

Fig. 1. Photomicrograph of brush cytology showing a three-dimensional cluster in malignant stricture (Papanicolaou stain, $\times 100$). Cells are arranged into large thick clusters in the three-dimensional structure with marked cellular overlapping or crowding.

Fig. 2. Immunocytochemical staining with p53 and *K-ras*. Cell nuclei are stained with p53 stain (A, $\times 400$) and *K-ras* stain (B, $\times 200$), respectively.

slide[®] glasses for immunocytochemical staining of 118 malignant strictures were divided in the midline. The half was stained for *K-ras* mutation, and the other half for p53 mutation.

For immunocytochemical stain, every slide was divided in the mid-portion of smeared area, the half was stained by *K-ras* antibody (1:10; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and the other half was stained by p53 antibody (1:100; DAKO, Glostrup, Denmark) respectively. Positive immunocytochemical stain for p53 mutation was defined as detection of more than five clusters of tumor cells with strong intranuclear staining for p53 in medium magnification ($\times 200$) (Fig. 2). Positive immunocytochemical stain for *K-ras* was defined as detection of more than five clusters of tumor cells with cytoplasmic staining for *k-ras* in medium magnification ($\times 200$) (Fig. 2).

5. Cholangiographic analysis of biliary stricture

Cholangiographic findings were classified according to level, length, and tightness of biliary stricture. And sensitivities of brush cytology in diagnosis of biliary stricture were analyzed according to level, length, and tightness of biliary stricture. The anatomic levels of stricture were divided into ampulla and distal common bile duct (CBD) (ampulla-distal CBD), mid CBD, common hepatic duct (CHD) or hilum (CHD-hilum). The length of stricture was divided into short stricture less than or equal to 1 cm and long stricture longer than 1 cm. The tightness of stricture was divided into tight, intermediate, and loose. Tight stricture was defined as stricture through which the insertion of 0.035-inch guidewire was difficult, but possible and injected contrast could hardly reach proximal side of the stricture. Intermediate stricture was defined as threadlike stricture through which the guidewire was able to traverse without difficulty. Loose stricture was defined as stricture with wide lumen through which injected con-

trast filled easily.

6. Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 12.0 (SPSS Inc., Chicago, IL, USA). Factors influencing outcome of brush cytology was analyzed with χ^2 test. Sensitivity at adding p53 and *K-ras* immunocytochemical staining in malignant obstruction was compared with that of routine cytology in 95% confidence interval (CI). In all statistical analysis, p value less than 0.05 was considered to be statistically significant.

RESULTS

1. Characteristics of patients and final diagnosis

One hundred forty patients with biliary stricture were included in the study. Mean age was 67.5 years old (range, 37 to 90 year old), and 79 patients were male (Table 1). Final diagnoses were confirmed by surgery in 34 patients, by endoscopic or percutaneous biopsy in 10 patients, and by long-term clinical follow-up in 96 patients (mean follow-up, 19.1 months), respectively.

Of 140 patients with biliary strictures, 119 (82.9%) strictures were malignant and 21 (17.1%) were benign (Table 1). Final diagnoses of 119 malignant strictures were as follows: 61 (43.6%) extrahepatic bile duct cancers, 19 (13.6%) pancreatic cancers, 16 (11.4%) hilar bile duct cancers, 13 (9.3%) ampullary cancers, 8 (5.7%) gallbladder cancers, and 2 (1.4%) metastatic cancers (one from recurrent gastric cancer and the other from unknown primary cancer). Of 21 patients with benign strictures, 12 patients (8.6%) had benign CBD stricture, 3 patients (2.1%) strictures by chronic pancreatitis, 2 patients (1.4%) papillitis, and 4 patients (2.9%) strictures by other causes (Table 1).

Table 1. Patient Characteristics

Characteristic	
Total no. of patients	140
Median age (range), yr	67.5 (37-90)
Male:Female, no.	79:61
Final diagnosis	
Malignant, no. (%)	119 (82.9)
Ampullary cancer	13 (9.3)
Extrahepatic bile duct cancer	61 (43.6)
Hilar bile duct cancer	16 (11.4)
Gallbladder cancer	8 (5.7)
Pancreas cancer	19 (13.6)
Metastatic cancer	2 (1.4)
Benign, no. (%)	21 (17.1)
Benign CBD stricture	12 (8.6)
Chronic pancreatitis	3 (2.1)
Papillitis	2 (1.4)
Others	4 (2.9)

CBD, common bile duct.

2. Outcomes of conventional brush cytology in malignant stricture

Of the 119 patients with malignant bile duct strictures, 93 were accurately diagnosed with conventional cytology (positive for malignancy). Of the 21 patients with benign bile duct strictures, 19 were diagnosed as benign (negative for malignancy) with conventional cytology.

For the malignant biliary stricture, the sensitivity of conventional brush cytology was 78.2%, and specificity was 90.5% (Table 2). In brush cytology for malignant biliary stricture, positive predictive value, negative predictive value, and diagnostic accuracy were 97.9%, 42.4%, and 80.0%, respectively (Table 2). The sensitivity of brush cytology was 82.7% (81 were positive among 98 malignancies) for CBD or GB cancers and 63.2% (12 were positive among 19 malignancies) for pancreatic cancers. The sensitivity of brush cytology was higher for bile tree cancer than for pancreatic cancer ($p=0.05$) (Table 3).

3. Factors influencing outcome of conventional brush cytology

Sensitivities of brush cytology for malignant stricture at ampulla-distal CBD, mid CBD, and CHD-hilum were 79.31% (95% CI, 73.92-84.70), 72.0% (95% CI, 66.03-77.97), and 80.6% (95% CI, 74.97-86.14), respectively. Sensitivities of brush cytology did not differ significantly according to the level of bile duct obstruction.

Sensitivities of brush cytology for loose or intermediate, and tight malignant stricture were 76.47% (95% CI, 70.83-82.11) and 76.36% (95% CI, 70.71-82.02), respectively. Sensitivities of brush cytology did not differ sig-

Table 2. Results of Conventional Brush Cytology in the Diagnosis of Biliary Stricture

		Brush cytology		
		Malignant	Benign	Total
Final diagnosis	Malignant	93	26	119
	Benign	2	19	21
	Total	95	45	140
Brush cytology in diagnosing the malignant biliary stricture				
Sensitivity: $93/119=78.2\%$				
Specificity: $19/21=90.5\%$				
Positive predictive value: $93/95=97.9\%$				
Negative predictive value: $19/45=42.4\%$				
Accuracy: $112/140=80.0\%$				

Table 3. Comparison of Sensitivities of Conventional Brush Cytology between Biliary-Tree Cancer and Pancreatic Cancer

	Positive in brush cytology	Sensitivity	p-value
Biliary tree cancer* (n=98)	81	82.7%	0.050
Pancreatic cancer (n=19)	12	63.2%	

*Includes bile duct cancer, ampullary cancer, and GB cancer.

nificantly according to the tightness of obstruction.

Sensitivities of brush cytology for short and long malignant stricture were 82.69% (95% CI, 77.35-88.03) and 71.43% (95% CI, 65.42-77.44), respectively. Sensitivities of conventional brush cytology did not differ significantly according to the length of obstruction.

4. Presence of three-dimensional cluster in brush cytology

Three-dimensional cluster was observed in 88 cases out of 119 malignant strictures (73.9%), and in 11 cases out of 21 benign strictures (52.4%). This was more frequently found in malignant stricture than benign stricture ($p=0.045$).

5. Addition of p53 and K-ras immunocytochemical stain in the diagnosis of malignant biliary obstruction

In 119 malignant strictures, p53 and *K-ras* immunocytochemical stains were done in addition to Papanicolaou smear using ProbeOn slide[®]. The sensitivity of p53 immunocytochemical stain plus Papanicolaou smear in malignant stricture was 88.20% (95% CI, 83.69-92.78), which was significantly higher than that of Papanicolaou smear alone of 78.15% (95% CI, 72.65-83.65). The sensitivity of *K-ras* immunocytochemical stain plus Papanicolaou smear in malignant stricture was 84.03% (95% CI,

79.16-88.91), which was not significantly higher than that of Papanicolaou smear alone. The sensitivity of both p53 and *K-ras* immunocytochemical stains plus Papanicolaou smear in malignant stricture was 88.20% (95% CI, 83.69-92.78), which was similar to that of p53 immunocytochemical stain plus Papanicolaou smear.

DISCUSSION

Among various techniques for tissue sampling from biliary stricture, brushing during ERCP is the most frequently used technique that is technically easy and safe, and requires little time.^{1,21} Cancer detection rate of brush cytology was not high when brush cytology was first described by Osnes *et al.*²² in 1975 because the brush could not pass through the stricture and brushing was done at distal end of stricture. In 1989, Foutch *et al.*²³ introduced new method of brushing using the catheter with brush that can be passed in monorail fashion over the guide-wire. They reported 60% of sensitivity and 72% of accuracy for the diagnosis of bile duct cancer.

Studies evaluating the variables influencing positive result of brush cytology in malignant biliary obstruction are scarce. Theoretically, multiple factors can influence the yield of brush cytology: length of stricture, tightness of stricture, location of stricture, and origin of malignancy. Because brushing scrapes tissue from superficial mucosal surface, low cellular yield can occur in the setting of extrinsic compression of biliary tree by pancreatic cancer.^{4,24} In our study, the sensitivity of conventional brush cytology was higher in biliary stricture by bile duct cancer than biliary stricture by pancreatic cancer (82.7% vs 63.2%, respectively, $p=0.05$). According to a recent study, mass size more than 1 cm and stricture longer than 1 cm were significant variables for positive yields of biliary brush cytology.²⁵ In our study, there were no differences in the sensitivities of brush cytology according to tightness of stricture and length of stricture.

There have been various attempts at increasing sensitivity of brush cytology, including brushing after dilatation of stricture,⁷ using new brush,⁸ and the addition of immunocytochemical staining such as *K-ras* and p53 mutation.²⁶ Mutation of p53 tumor suppressor gene and mutation of *K-ras* oncogene were relatively common in resected samples of pancreas cancer and cholangiocarcinoma.^{12,27-29} But the value of adding immunocytochemical staining such as *K-ras* and p53 mutation to conventional Papanicolaou stain in brushing cytology at bile duct or pancreatic duct are controversial.

In the study of preoperative cytology compared with surgical specimen, p53 immunoreactivity in brush cytol-

ogy was helpful in the diagnosis of malignant bile duct cancer, but only 12 cases (24.5%) out of 49 patients with pancreato-biliary malignancy were immunoreactive.¹⁸ However, p53 immunocytochemical staining was positive in 18/20 cases (90%) of pancreatic cancer, while none of eight cases of chronic pancreatitis were positive in the other study.¹⁶ The authors concluded that combining p53 immunocytochemical staining with conventional cytology provided differential diagnosis of pancreatic cancer from chronic pancreatitis. Immunocytochemical detection of p53 protein as an adjunct in cytologic diagnosis from pancreatic duct brushings was also valuable for differentiating adenoma from carcinoma in mucin-producing tumors of pancreas.²⁰ In a relatively large series of 143 cases of pancreato-biliary brush cytology, p53 immunocytochemical staining was positive in 45 cases (50.6%) of 89 malignant strictures while 63 cases (70.8%) were identified in conventional cytology.¹⁹ They concluded that routine diagnostic use of p53 immunocytochemical staining, as an adjunct to conventional cytology was not supported. In our study, the sensitivity of p53 immunocytochemical stain plus Papanicolaou smear in malignant stricture was significantly higher than that of Papanicolaou smear alone (88.20% vs 78.15%, respectively).

According to a study using PCR-based test with biopsy or resected tissue, *K-ras* codon 12 mutation is very common in pancreas cancer (76%) and common in bile duct cancer (39%).¹⁰ In their study, combination of PCR study for *K-ras* codon 12 mutation using brush sample with conventional light microscopic cytology from 312 malignant biliary stenosis showed higher sensitivity (62%) than conventional cytology alone (36%). The other study with PCR-based studies in supernatant of bile showed *K-ras* mutation was higher (56.7%) and simultaneous analysis of p53 and *K-ras* in bile could enhance genetic diagnosis of bile tract carcinoma (80.0%).³⁰ But *K-ras* mutation was not useful for differentiating pancreatic cancer from chronic pancreatitis in pancreatic ductal brushing during ERCP in recent PCR study.³¹ There is lack of study for *K-ras* immunocytochemical staining using ERCP brush cytology in the patient with biliary stricture. In our study, the sensitivity of *K-ras* immunocytochemical stain plus Papanicolaou smear in malignant stricture was not significantly higher than that of Papanicolaou smear alone. The sensitivity of both p53 and *K-ras* immunocytochemical stain plus Papanicolaou smear in malignant stricture was similar to that of p53 immunocytochemical stain plus Papanicolaou smear.

In the slide of conventional cytology, the cells sometimes arrange as three-dimensional cluster, which is a

large thick cluster in three-dimensional structure with marked cellular overlapping or crowding. One study about biliary tract cytology described that the presence of three-dimensional epithelial cluster with marked atypia signified malignancy and warranted diagnosis of malignant neoplasm even when only single cluster was seen.³² In our study, the presence of three-dimensional cluster was more frequent in malignant stricture than benign stricture.

Limitations of this study are single center study involving small number of patients and small percentage of malignant biliary obstruction from pancreatic cancer. Further study involving multiple centers could overcome this shortcoming. However, biliary obstruction resulting from pancreatic cancer is not as common as in Western countries.

In conclusion, the detection of three-dimensional cluster can improve the sensitivity of conventional brush cytology during ERCP for the diagnosis of malignant stricture. p53 immunocytochemical staining as an adjunct in conventional cytology may improve the sensitivity of brush cytology in malignant stricture, but *K-ras* may not.

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