Clinical Value of K-*ras* Codon 12 Analysis and Endobiliary Brush Cytology for the Diagnosis of Malignant Extrahepatic Bile Duct Stenosis[1]

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ABSTRACT

Extrahepatic biliary stenosis can be caused by benign and malignant disorders. In most cases, a tissue diagnosis is needed for optimal management of patients, but the sensitivity of biliary cytology for the diagnosis of a malignancy is relatively low. The additional diagnostic value of K-*ras* mutational analysis of endobiliary brush cytology was assessed. Endobiliary brush cytology specimens obtained during endoscopic retrograde cholangiopancreaticography were prospectively collected from 312 consecutive patients with extrahepatic biliary stenosis. The results of conventional light microscopic cytology and K-*ras* codon 12 mutational analysis were compared and evaluated in view of the final diagnosis made by histological examination of the stenotic lesion and/or patient follow-up. The sensitivities of cytology and mutational analysis to detect malignancy were 36 and 42%, respectively. When both tests were combined, the sensitivity increased to 62%. The specificity of cytology was 98%, and the specificity of the mutational analysis and of both tests combined was 89%. Positive predictive values for cytology, mutational analysis, and both tests combined were 98, 92, and 94%, whereas the corresponding negative predictive values were 34, 34, and 44%, respectively. The sensitivity of K-*ras* mutational analysis was 63% for pancreatic carcinomas compared to 27% for bile duct, gallbladder, and ampullary carcinomas. K-*ras* mutational analysis can be considered supplementary to conventional light microscopy of endobiliary brush cytology to diagnose patients with malignant extrahepatic biliary stenosis, particularly in the case of pancreatic cancer. The presence of a K-*ras* codon 12 mutation in endobiliary brush cytology *per se* supports a clinical suspicion of malignancy, even when the conventional cytology is negative or equivocal.

INTRODUCTION

Stenosis of the extrahepatic bile ducts is caused by a variety of malignant and benign disorders. To optimally manage such patients, it is often important to determine the etiology of the stenosis. However, it can be difficult to differentiate malignant from benign causes of biliary stenosis, based on clinical presentation and radiological findings alone, and a definitive diagnosis of malignancy can only be established histo(cyto)-pathologically. Endobiliary brush cytology can be performed during ERCP[3] to collect material for cytopathology. Despite the high specificity of brush cytology, the sensitivity is low (1, 2). An analysis of tumor-specific genetic alterations in these cytology specimens may add to the diagnostic value of brush cytology.

Mutations in the K-*ras* oncogene are attractive for such analyses for a number of reasons. (*a*) K-*ras* mutations are one of the most common genetic alterations in human cancers and are frequent in the two main malignant neoplasms that cause biliary stenosis, pancreatic carcinoma, and bile duct carcinoma (3–9). (*b*) More than 90% of the K-*ras* mutations in these neoplasms occur in codon 12, which makes their detection relatively easy. (*c*) The PCR-based method used for the detection of the K-*ras* mutations is very sensitive and can identify rare mutant DNA copies among an abundance of wild-type DNA (3). (*d*) Results from K-*ras* mutational analyses, as were performed here, can be obtained within 48 h, making the test suitable for routine clinical purposes.

A number of studies have emphasized the diagnostic utility of K-*ras* mutations in material obtained from the head of the pancreas for the diagnosis of pancreatico-biliary malignancies, but most studies were performed on small groups of selected patients (10–22). Furthermore, the specificity of K-*ras* mutational analysis in the clinical diagnosis of neoplastic disease is unclear, because these mutations are also present in intraductal pancreatic proliferations (called "duct hyperplasia"; Refs. 23–25). This study has prospectively assessed the value of K-*ras* mutational analysis of endobiliary brush cytology as compared to conventional cytopathology for the diagnosis of a malignancy

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[3] The abbreviation used is: ERCP, endoscopic retrograde cholangiopancreaticography.

Table 1 Established final diagnosis in 312 patients who were evaluated for bile duct stenosis

Final diagnosis	No. of patients
Malignant stenosis	220
Tissue diagnosis	
Resection stenotic lesion	42
Autopsy	7
Biopsy stenotic lesion	22
Biopsy distant metastasis	45
Clinical diagnosis	
Progressive disease, consistent with malignancy	104
Benign stenosis	74
Tissue diagnosis	
Resection stenotic lesion	10
Clinical diagnosis	
Stable disease or regression	64
Unknown	18
Insufficient clinical information	18

Table 2 Spectrum of the different causes of bile duct stenosis in 294 patients with a final diagnosis

Etiology	No. of patients
Malignant stenosis	220
Pancreatic carcinoma	96
Bile duct carcinoma	73
Gall bladder carcinoma	7
Ampullary carcinoma	8
Lymph node metastasis	10
Lymphoma	1
Unspecified	25
Benign stenosis	74
Inflammatory	
Chronic pancreatitis	26
Cholelithiasis	3
Mirizzi syndrome[a]	1
Primary sclerosing cholangitis	26
Postsurgical[b]	13
Unspecified	5

[a] Gallstone in the gallbladder causing extrahepatic bile duct obstruction by external compression.
[b] Postcholecystectomy or postpapillotomy.

in patients with bile duct stenosis in a large series of consecutive patients with a complete follow-up.

MATERIALS AND METHODS

Patients

The study population consisted of a series of consecutive patients who underwent ERCP with endobiliary brush cytology for the evaluation of an extrahepatic biliary stenosis at the Academic Medical Center in Amsterdam in the period from January 1, 1993, to February 1, 1996. The Medical Ethical Review Committee of the Academic Medical Center approved the study. If a patient underwent ERCP with brush cytology repeatedly during this period, only the first examination was included. This resulted in 312 patients with a mean age of 63 years; 172 patients were male.

A final diagnosis of the nature of biliary stenosis was based on histological and/or clinical findings (Table 1). In the absence of a tissue diagnosis, a clinical diagnosis was established based on clinical symptomatology, the results of imaging studies prior to the ERCP procedure, and, particularly, the course of the disease. Information concerning the clinical follow-up was obtained from the patient's physician. All patients were followed for at least 12 months. The 104 patients with a clinical diagnosis of malignant extrahepatic bile duct stenosis had rapidly progressive disease with symptoms such as jaundice, pain, cachexia, and metastases. Importantly, all these patients died of disease within a mean survival of 5.7 months (range, 0–42 months) after the ERCP procedure, which corresponds to survival rates of patients with cancer of the pancreas and extrahepatic biliary tract in general. The mean survival for all of the 220 patients with a malignant etiology of their stenosis was 9 months (range, 0–50 months). Eight of these 220 patients were still alive at the end of follow-up, and their survival ranged from 22 to 50 months: of these, 6 had a surgical resection of the carcinoma and 2 were biopsied only; thus, they were all tissue proven. In contrast, 71 of the 74 patients with benign disease (including the 10 patients with a tissue diagnosis of benign disease) were all alive after a mean follow-up period of 32 months (range, 15–54 months) and had stable disease or regression of their symptoms. Three patients with benign disease died due to unrelated causes: 2 died from heart disease 10 months and 27 months after ERCP, and 1 died following a hip fracture 18 months after ERCP, all without symptoms of obstructive biliary disease.

In summary, 220 patients (70%) had a malignant etiology for their stenosis, and 74 (24%) had a benign stenosis. In 18 patients (6%), the cause of the stenosis remained unclear because of insufficient information during follow-up, and these patients were excluded from further analysis. The spectrum of the different etiologies of the stenoses in the remaining 294 patients is given in Table 2.

Materials

Brushings of the bile duct stenoses were performed with the GRBH-230-3-3.5 (size of brush device) (Wilson-Cook Medical Inc., Winston-Salem, NC). Four cytology smears from each patient were stained with Giemsa and Papanicolaou for routine diagnostic cytology. The remainder of the brush cytology specimen was suspended in 10 ml of DNA buffer and fixed with 10 ml 100% ethanol. The suspensions were stored at 4°C for subsequent K-*ras* mutational analysis.

Tissue from the area of the bile duct stenosis was available from 71 patients with a malignant cause for their stenosis and from 10 patients with a benign stenosis. These tissues were obtained at resection of the stenotic lesion, from biopsies of the stenotic lesion with malignant findings, and at autopsy (Table 1). In these cases, the available archival tissue blocks were analyzed for K-*ras* mutations, allowing us to compare directly the mutational status of the patient's primary pathology with the analysis of the corresponding brush cytology specimens.

Methods

DNA Isolation. One ml of each brush cytology suspension was used for DNA isolation. In case of the tissue blocks, careful microdissection from 5-μm H&E-stained sections was performed to ascertain a sample of which at least 50% of the

cells comprised the tissue of interest. DNA was extracted as described previously (26).

K-*ras* Mutational Analysis. The protocol of the K-*ras* codon 12 mutational analysis has been described previously (26). With this assay, DNA is subjected to PCR amplification using primers around codon 12. One of the primers generates a restriction enzyme recognition site with the wild-type codon 12 sequence but not with the mutant codon 12 sequence. Digestion of the PCR products with the restriction enzyme is followed by a second round of amplification, which then yields a PCR product enriched for K-*ras* codon 12 mutations. The resulting DNA fragments are denatured and dot-blotted onto nylon membranes and subjected to allele-specific oligonucleotide hybridization with radioactive labeled probes, specific for each possible K-*ras* codon 12 mutation, followed by autoradiography. Cell suspensions with mutant:wild-type ratios of 1:100 and 1:1000 were used as positive controls in every PCR procedure. The suspensions were made of the human colon cancer cell line SW 480 with a homozygous GGT to GTT mutation at codon 12 of K-*ras* and the human colon cancer cell line HT 29 with wild-type K-*ras*. Water was used as a control for contamination, placental DNA was used as a control for nonspecific hybridization, and cloned DNA fragments with the six different K-*ras* codon 12 mutations and the wild-type codon 12 were used as controls for specific hybridization. All PCR products were hybridized with oligonucleotides specific for the wild-type sequence to control for amplification of the patient samples. Both enriched and nonenriched PCR products were dot-blotted next to each other to check the digestion and mutant enrichment. Fig. 1 is an example of an autoradiogram of the K-*ras* analysis. The above mutational analysis has been validated through comparison with sequence analysis in a previous study (27).

The K-*ras* mutational analysis results were evaluated without any information regarding the patient. All mutational analyses were performed in duplicate in separate experiments. If there were discrepancies, a third analysis was performed to resolve the discrepancy. A result was called K-*ras* mutant positive if identical mutations were found in the duplicate analysis and when enrichment for the mutation had occurred.

Light Microscopy. All of the cytology smears were independently evaluated by an experienced cytopathologist (L. A. N.). The following diagnostic categories were used: positive for carcinoma, negative for carcinoma, suspect for carcinoma, and material insufficient or not suitable for diagnosis.

Sensitivity, Specificity, and Positive and Negative Predictive Values. The following definitions were used for evaluation. Sensitivity was defined as the percentage of patients with disease who had positive test results. Specificity was defined as the percentage of patients without disease who had negative test results. Positive predictive value was defined as the percentage of patients with positive test results who had disease. Negative predictive value was defined as the percentage of patients with negative test results who had no disease.

RESULTS

Of the 220 patients with a malignant etiology for their bile duct stenosis, 79 (36%) were diagnosed cytologically, and 92 (42%) had K-*ras* mutations detected in their cytology specimens

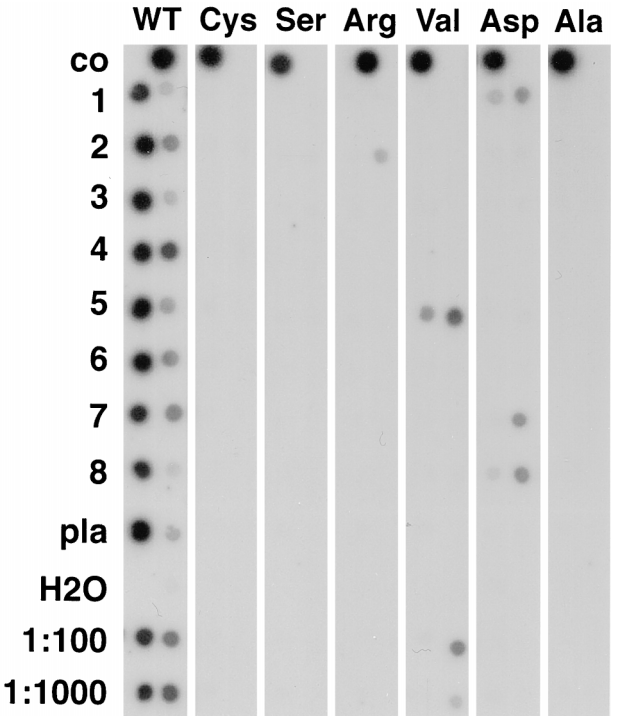

Fig. 1 Example of an autoradiogram of the K-*ras* mutational analysis. Seven nylon membranes each hybridized with a different radioactive labeled oligonucleotide specific for the sequence of the wild-type codon 12 (*left*) and the six possible mutations. For each membrane, the *left lane* contains the nonenriched PCR products, and the *right lane* contains the mutant-enriched PCR products. *Lanes WT*, wild-type (= glycine); *Lane Cys*, cysteine: *Lane Ser*, serine; *Lane Arg*, arginine; *Lane Val*, valine; *Lane Asp*, aspartic acid; *Lane Ala*, alanine. *co*, hybridization controls, on each membrane cloned DNA fragments with a known codon 12 sequence complementary to the labeled oligonucleotides used for the hybridization of that membrane; *1–8*, brush cytology specimens with K-*ras* codon 12 sequences coding for the following amino acids: aspartic acid, arginine, glycine, glycine, valine, glycine, aspartic acid, and aspartic acid, respectively; *pla*, placental DNA; *H2O*, water; *1:100*, one cell with mutant codon 12, coding for the amino acid valine, mixed in 100 cells with wild-type codon 120; *1:1000*, one cell with mutant codon 12, coding for the amino acid valine, mixed in 1000 cells with wild-type codon 12.

(Table 3). Of the 92 patients with mutant K-*ras* in their cytology specimens, 57 patients were not diagnosed with cytology, and thus the two tests combined were able to identify 136 (79+57) patients with malignant disease (62%). Of these 57 patients with mutant K-*ras* and nondiagnostic cytology, 39 had negative cytology results, 14 had suspect for carcinoma cytology, and 4 had material that was insufficient for diagnosis. Positive predictive values and negative predictive values for the cytology, K-*ras* mutational analysis, and both tests combined were 98 and 34%, 92 and 34%, and 94 and 44%, respectively.

Eight of the 74 patients with benign disease on follow-up had K-*ras* mutations identified in their brush cytology (Table 3). All eight patients were alive after a mean follow-up of 30 months (range, 18–50 months), and none had signs of malignant disease at the end of follow-up. Two of these eight patients had a diagnosis of chronic pancreatitis, three had a postsurgical

Table 3 Results of the cytology and K-*ras* mutational analysis in reference to the final diagnosis

	Cytology				
Final diagnosis	Positive	Negative	Suspect	Insufficient	Total
Malignant stenosis	79	104	30	7[a]	220
K-*ras* positive	35	39	14	4	92
K-*ras* negative	44	65	16	3	128
Benign stenosis	2	66	5	1	74
K-*ras* positive	2	6	0	0	8
K-*ras* negative	0	60	5	1	66

[a] One specimen could not be amplified and was called K-*ras* negative.

stenosis, and three patients had primary sclerosing cholangitis. Tissue from the stenotic lesion of one of the patients with primary sclerosing cholangitis was available for K-*ras* mutational analysis. The patient had undergone a hilar resection because of the suspicion of a cholangiocarcinoma. Histopathological findings were cholecystitis with inflammation and fibrosis of the common hepatic duct. The K-*ras* mutation found in the brush cytology specimen was not confirmed in the reactive bile duct epithelium in this case.

Two of the eight patients with "false-positive K-*ras* results," both with a postsurgical stenosis, also had positive cytology (Fig. 2).

In the 71 patients with a definitive tissue diagnosis of a malignancy, cytology was slightly more sensitive for the diagnosis of carcinomas primary to the bile duct compared to the other causes of malignant biliary stenosis, 33% (6 of 18) *versus* 23% (12 of 53; Table 4). The sensitivity of K-*ras* mutational analysis was highest for pancreatic carcinoma, 63% (24 of 38) compared to 27% (9 of 33) for other causes.

Tissue was available for K-*ras* mutational analysis from 60 of the 71 patients who had a definitive tissue diagnosis of a malignancy (Table 5). Twenty-two of 29 (76%) pancreatic carcinomas had a K-*ras* mutation compared to 12 of the 31 (39%) nonpancreatic cancers. In 53 of 60 (88%) patients, the K-*ras* analyses of brush cytology and tissue specimens were concordant: in 27 patients, identical mutations were found, and in 26 patients, both specimens were negative for mutations. In seven patients the results were discrepant. All these patients had wild-type K-*ras* detected in their cytology specimens, and mutant K-*ras* was detected in their primary carcinomas; the cytology of these patients was also negative for carcinoma.

No mutations were found in the tissue specimens of the 10 patients with a benign stenosis.

DISCUSSION

The clinical value of analyzing endobiliary brush cytology specimens for K-*ras* codon 12 mutations in establishing the diagnosis of a malignancy in patients with extrahepatic bile duct stenosis was examined. The study materials were prospectively collected from a large series of consecutive patients who underwent ERCP with endobiliary brush cytology to rule out or confirm a neoplastic cause of their bile duct stenosis. Brush cytology accurately diagnosed malignancy in 36% of the patients with a malignant etiology for their biliary stenosis, a

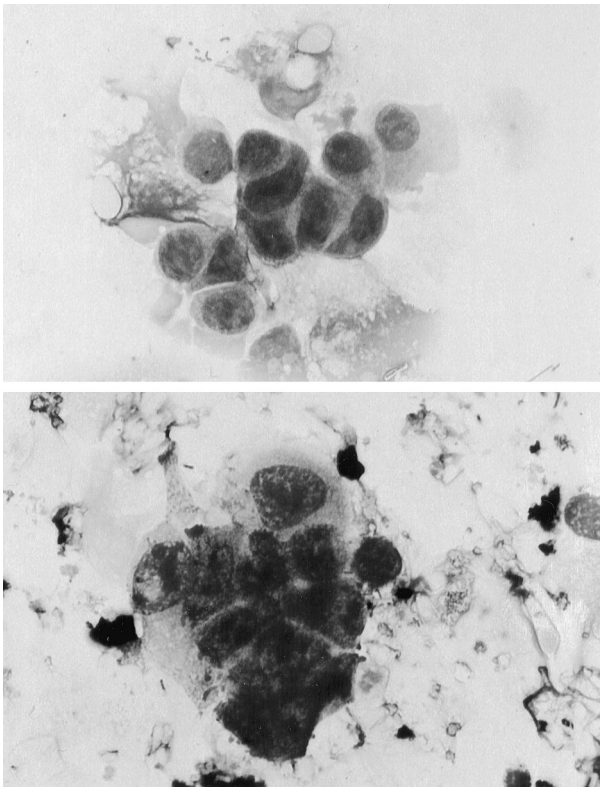

Fig. 2 *A* and *B*, cytology positive for carcinoma from the two patients with a diagnosis of postsurgical stenosis (Giemsa stained, ×132).

sensitivity comparable to two previous studies in which a large consecutive series of patients was analyzed (1, 2). These authors reported similar frequencies of biliary stenosis caused by malignant disease as in our study (57 and 66% *versus* 70%), and in these previous studies, pancreatic and bile duct carcinoma were also the most frequent carcinomas; the demographics in these two studies are comparable with this series. Thus, the study population in our series can be considered representative for patients undergoing ERCP with brush cytology for the evaluation of a potentially malignant biliary stenosis. Other studies that reported higher sensitivities of biliary cytology dealt with smaller groups of selected patients (28).

The K-*ras* mutational analysis was especially valuable in the diagnosis of patients with pancreatic carcinoma (63% sensitivity *versus* 27% in patients with malignancy other than pancreatic carcinoma). One would expect that the K-*ras* mutational analysis is particularly sensitive for a stenosis caused by pancreatic carcinoma, whereas light microscopic brush cytology is the more sensitive method for carcinomas arising from the bile duct epithelium. Endobiliary brush cytology samples the bile duct epithelium most efficiently, whereas the frequency of K-*ras* mutations is highest in pancreatic carcinoma (3–7, 9, 26, 28), and the PCR-based technique for detecting K-*ras* mutations is highly sensitive and thus, in contrast to cytology, less dependent on obtaining a large amount of tumor cells. Because cytology and K-*ras* mutational analysis have opposite sensitivities for the two most frequent causes of malignant biliary stenosis,

Table 4 Results of the cytology and K-*ras* mutational analysis in 71 patients with a precise etiology of the malignant bile duct stenosis based on histology of the stenotic lesion

Final diagnosis	Cytology				Total
	Positive	Negative	Suspect	Insufficient	
Pancreatic carcinoma	8	21	6	3[a]	38
K-*ras* positive	7	11	4	2	24
K-*ras* negative	1	10	2	1	14
Bile duct carcinoma	6	8	4		18
K-*ras* positive	3	1	1		5
K-*ras* negative	3	7	3		13
Gall bladder carcinoma	1	4			5
K-*ras* positive	0	1			1
K-*ras* negative	1	3			4
Ampullary carcinoma	2	4	2		8
K-*ras* positive	1	1	1		3
K-*ras* negative	1	3	1		5
Lymph node metastasis	1	1			2
K-*ras* positive	0	0			0
K-*ras* negative	1	1			2

[a] One specimen could not be amplified and was called K-*ras* negative.

Table 5 K-*ras* mutational spectrum in brush cytology specimens and corresponding carcinomas of 60 of the 71 patients with a precise etiology of the malignant stenosis based on histology of the stenotic lesion

Mutations in carcinomas[a]	Mutations in brush cytology[a]							
	Cys	Ser	Arg	Val	Asp	Ala	Gly[b]	Total
Cys	1						1	2
Ser								
Arg			2				1	3
Val				8			3	11
Asp					15		2	17
Ala						1		1
Gly[b]							26	26
Total	1		2	8	15	1	33	60

[a] The six possible mutations code for cysteine, serine, arginine, valine, aspartic acid, and alanine.

[b] Wild-type codon 12 (GGT) codes for glycine.

the methods nicely supplement each other. Indeed, the sensitivities of cytology (36%) and K-*ras* mutational analysis (42%) for the diagnosis of malignant stenosis were similar but, when their sensitivities were combined, increased to 62%.

K-*ras* mutations were detected in the brush cytology specimens of 8 of the 74 patients (11%) with benign disease. Performing all PCR analyses in duplicate independently minimized the chance of technical errors as a cause for false-positive results. Positive results of the K-*ras* mutational analysis in the absence of malignancy may be caused by the presence of noninvasive "hyperplastic duct lesions" containing K-*ras* mutations (23, 24, 29, 30). Hyperplastic duct lesions are frequently found together with cancer in the pancreas, and indeed, there is evidence that these duct hyperplasias can progress to infiltrating carcinoma with K-*ras* mutation as an early event (30–32). K-*ras* mutations are also found in hyperplasias in patients with chronic pancreatitis, a condition thought to be a risk factor for developing pancreatic cancer (23, 33). However, it is clear that not all duct hyperplasias progress to invasive carcinoma during the life span of an average patient (24). A longer follow-up would, therefore, be needed to better understand the meaning of the observations in the eight patients in our study without obvious neoplastic disease who harbored K-*ras* mutations in their brush cytology. A recent study found no cancer in 20 patients with pancreatitis and K-*ras* mutation in their pancreatic juice after a mean follow-up of 78 months (34). On the other hand, Brat *et al.* (35) reported three patients with hyperplastic duct lesions who developed pancreatic cancer after 17 months to 10 years, and Berthelemy *et al.* (13) reported two patients without evidence of cancer at the time of ERCP but with mutated K-*ras* in their pancreatic juice who developed clinically detectable pancreatic cancers after 18 and 40 months. Nonetheless, it seems best, at present, to consider the eight patients in our study false-positives until it is proven otherwise. Only two of these patients had chronic pancreatitis. Tissue for K-*ras* analysis was available from one of these patients with primary sclerosing cholangitis in which the resected biliary stenosis did not harbor a K-*ras* mutation.

As in our study, the specificity of cytology reported in the literature is often 100% or approaching 100% (1, 2). Interestingly, the two patients with false-positive cytologies also had K-*ras* mutations detected in their cytology specimens. One patient was a 62-year-old white male. During ERCP, a regular smooth stenosis of the distal common bile duct was seen. He had undergone a cholecystectomy for cholelithiasis in the past; hence, the stenosis was diagnosed as postsurgical. The stenosis was stented, and since then, he has not been jaundiced and had any other complaints. There was no evidence of bile duct obstruction 18 months after brush cytology. The other patient was a 71-year-old white female, also with a postsurgical stenosis. The mid-common bile duct stenosis was treated with a stent. Eighteen months after brush cytology, she had no complaints of extrahepatic bile duct obstruction. These two patients clearly did not meet our criteria for a clinical diagnosis of a malignant bile duct stenosis. Nonetheless, even in retrospect, the cytologies of these two patients were considered positive for carcinoma (Fig. 2, *A* and *B*, respectively). One could speculate that these cells came from pancreatic duct lesions with high-grade dysplasia or from an *in situ* carcinoma, which would also explain the K-*ras* mutations detected in these patients. Long-term follow-up may then provide a clue to their final diagnosis.

In 60 cases, we were able to directly compare the K-*ras* mutations identified in brush cytology specimens to those present in the corresponding surgical specimens. We found that the results were identical in 88% (53 of 60) of the cases with malignancy. The main cause for false-negative results was the absence of K-*ras* mutations in the tumor (26 of 33), mostly cancers other than pancreatic cancer. The discrepant results from the seven patients in which wild-type K-*ras* was found in the brush cytology but mutant K-*ras* was detected in the patients' carcinoma could be due to sampling error because the conventional cytology in these cases was also negative for carcinoma.

More direct sampling of the stenotic lesion could potentially improve the sensitivity of cytology but would diminish specificity of the K-*ras* mutational analysis. Van Laethem *et*

al. (22) examined the diagnostic value of K-*ras* in pancreatic duct brushings and bile duct brushings. Sensitivity of conventional light microscopy of endobiliary brush cytology was similar in their study. They also showed the additional diagnostic value of K-*ras* mutational analysis in these cytology specimens, especially in the diagnosis of patients with pancreatic cancer, and the high specificity. In contrast, they found that the sensitivity of conventional cytology of pancreatic duct brushings is higher (51%), but the diagnostic value of K-*ras* was impaired by a high percentage (25%) of patients with chronic pancreatitis who harbored K-*ras* mutations in their cytology. This lower specificity of K-*ras* mutational analysis of pancreatic duct brushings may well be attributed to the more direct sampling of the hyperplastic duct lesions that are frequent in the pancreas with chronic inflammation. It is likely that, in brush cytology specimens from the bile duct, the yield of cells from these hyperplastic duct lesions is lower compared to cells derived from carcinomas because the cells in carcinomas grow less coherently and are easily shed. Following this reasoning, colorectal neoplasms can be diagnosed specifically with the detection of K-*ras* mutations in the stool despite the frequent occurrence of K-*ras* mutations present in aberrant crypt foci and hyperplastic polyps, two nonneoplastic lesions that are prevalent in the colorectum without neoplastic disease (36, 37).

In conclusion, PCR-based tests for the detection of K-*ras* codon 12 mutations can be a valuable diagnostic adjunct to conventional light microscopy of endobiliary brush cytology specimens obtained from patients who have a suspicious stenosis of the extrahepatic bile duct, especially in patients with pancreatic carcinoma. The presence of a mutation favors malignancy, even when the cytology reading is negative or equivocal.

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