

Comparison of *KRAS* Mutation Analysis and FISH for Detecting Pancreatobiliary Tract Cancer in Cytology Specimens Collected During Endoscopic Retrograde Cholangiopancreatography

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Pancreatobiliary tract strictures result either from malignancies of the biliary tract and pancreas or from nonmalignant etiopathogenesis. The goal of this study was to determine whether *KRAS* mutations could be identified in residual pancreatobiliary stricture brushings and to compare the performance characteristics of *KRAS* mutation analysis to cytology and fluorescence *in situ* hybridization (FISH) for the detection of carcinoma. Residual brushing cytology cell pellets were retrieved from 132 patients with subsequent clinicopathologic follow-up of cholangiocarcinoma ($n = 41$), pancreatic adenocarcinoma ($n = 35$), gallbladder cancer ($n = 2$), and nonmalignant strictures ($n = 54$). All specimens had a prior cytology and FISH UroVysion results as part of clinical practice. *KRAS* mutation analysis was performed using the quantitative PCR DxS *KRAS* Mutation Test Kit. *KRAS* mutation analysis was successful in 130 of 132 specimens. *KRAS* mutations and polysomic (ie, positive) FISH results were identified in 24 (69%) and 22 (63%) pancreatic adenocarcinoma specimens, respectively, with a combined sensitivity of 86% (30/35). *KRAS* mutations and polysomic FISH results were identified in 12 (29%) and 17 (41%) cholangiocarcinoma specimens, with a combined sensitivity of 54% (22/41). *KRAS* mutations were identified in two patients with primary sclerosing cholangitis, and benign follow-up. Residual cytology specimens can be used to detect *KRAS* mutations by quantitative PCR. Combined *KRAS* mutation and FISH analysis appear to increase the cancer detection rate in patients with pancreatobiliary strictures. (*J Mol Diagn* 2010, 12:780–786; DOI: 10.2353/jmoldx.2010.100016)

malignancies such as cholangiocarcinoma and pancreatic adenocarcinoma. The diagnosis of malignancy in patients with pancreatobiliary tract strictures can be challenging, especially in patients with primary sclerosing cholangitis (PSC), and relies on clinical examination, biochemical testing (eg, CA 19–9), and endoscopic and imaging procedures such as endoscopic retrograde cholangiopancreatography (ERCP).¹ ERCP is a valuable tool for assessing pancreatobiliary tract strictures because it allows for visualization of the biliary tract, therapeutic interventions (eg, placement of biliary stents), and collection of brushing cytology specimens for cytopathologic evaluation. Biliary brushing cytology, although highly specific, has suffered from low to moderate sensitivity (15%–68%).^{2–6} As a result, ancillary diagnostic techniques including mutation analysis,^{7–11} DNA ploidy analysis,^{12,13} methylation analysis,^{14–17} and fluorescence *in situ* hybridization (FISH),^{2,4,18,19} have been studied to improve tumor detection.

FISH, using the UroVysion probe set, is a molecular cytology/cytogenetic test currently performed as an adjunct to routine cytology. Two types of chromosomal abnormalities are frequently identified with the UroVysion FISH probe set, polysomy and trisomy 7. Polysomy has been defined as a gain of two or more of the four probes in ≥ 5 cells. Trisomy 7 has been defined as a gain of a single probe targeting the pericentromeric region of chromosome 7 (three signals for the CEP7 probe) with no more than two signals in each of the other three probes in ≥ 10 cells. A large clinical study recently demonstrated that FISH was more sensitive than cytology for detecting pancreatobiliary tract cancer.² FISH was able to detect 49/227 (22%) cancers that routine cytology interpreted as nonmalignant without compromising test specificity. However, the overall sensitivity of polysomy FISH (43%) was only moderate. If a FISH-positive diagnosis included patients with a polysomy result or patients with a trisomy

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Pancreatobiliary tract strictures can result from nonmalignant etiopathogenesis or from pancreatobiliary tract

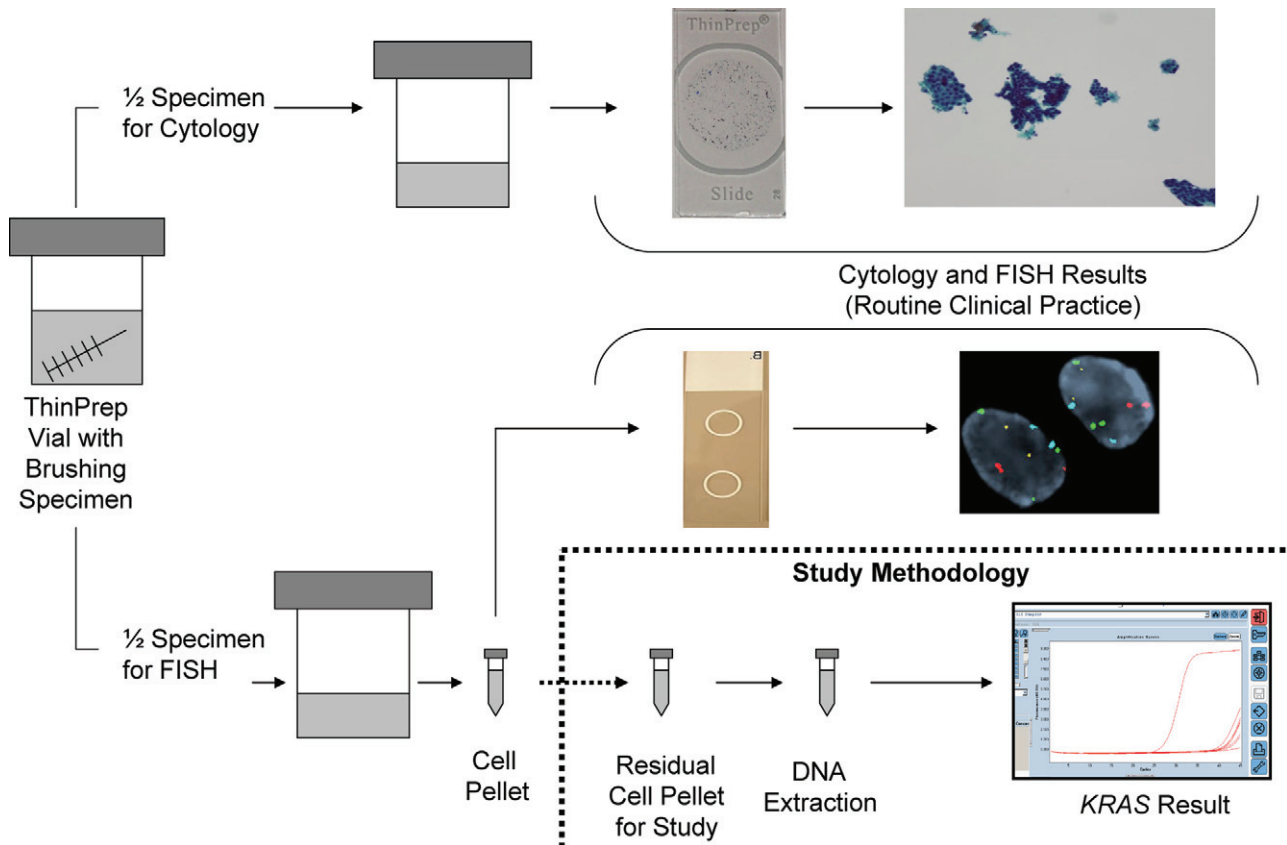


Figure 1. Specimen-processing flowchart, illustrating how pancreatobiliary samples were processed for routine clinical testing and *KRAS* mutation analysis.

7 result (without polysomy), FISH was able to detect 63% of all cancers. However, the inclusion of trisomy 7 as a criterion for a positive result significantly increases the false positive rate of FISH because only ~50% of patients with trisomy 7 results have cancer on clinicopathologic follow-up.² Consequently, although detection rates are improved by FISH testing, additional or substitute molecular markers are needed to further improve the detection of pancreatobiliary tract cancer.

The *KRAS* (Kirsten rat sarcoma viral oncogene homolog) gene is a member of the *RAS* family that encodes a G protein involved in signal transduction from cell surface receptors (eg, *EGFR*) to intracellular targets. Mutations in the *KRAS* gene (most commonly in codons 12 and 13 of exon 2) impair the GTPase activity of *KRAS*, which leads to constitutive activation of downstream signaling pathways that control cell proliferation, differentiation, and survival.^{17,20} Previous studies have shown that between 25%–100% of pancreatic adenocarcinomas harbor a *KRAS* mutation, depending on the type of specimen (eg, pancreatic tissue, pancreatic juice, etc) analyzed.^{8–10} *KRAS* alterations have also been identified in a wide range (21%–100%) of cholangiocarcinoma specimens.⁷ The most frequently observed *KRAS* mutations in both pancreatic adenocarcinoma and cholangiocarcinoma are mutations in codons 12 and 13, and less frequently, codon 61.^{7,21} There are no data comparing the performance characteristics of *KRAS* mutation analysis and aneusomy by FISH on clinical cytology specimens

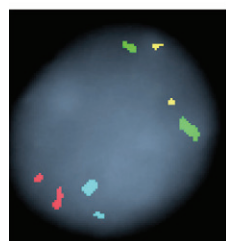
collected during ERCP. The goal of this study was to determine whether *KRAS* mutations could be identified in residual cytology pancreatobiliary tract specimens and to compare the performance characteristics of *KRAS* mutation analysis to cytology and FISH results for the detection of carcinoma.

Materials and Methods

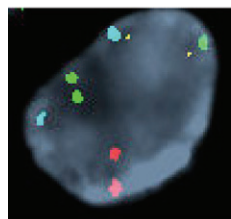
Patient Specimens

Residual cytology cell pellets were retrieved from 132 patients (86 males, 46 females) who underwent ERCP evaluation of a pancreatobiliary tract stricture with subsequent clinicopathologic follow-up. The ages of patients included in this study ranged from 15 to 87 years, with a mean and median age of 59 and 60 years, respectively. Specimens were selected from a larger study² by first subcategorizing patients based on the combination of their FISH and clinicopathology follow-up results. Specimens were included from each of the predetermined categorizations based on whether there was adequate (visible) residual cell pellet available for *KRAS* testing. This selection of patients was performed to assure that different FISH results were included from patients with nonmalignant (primary sclerosing cholangitis, $n = 26$; chronic pancreatitis, $n = 15$; autoimmune pancreatitis, $n = 9$; acute pancreatitis, $n = 1$; cholecystitis, $n = 1$;

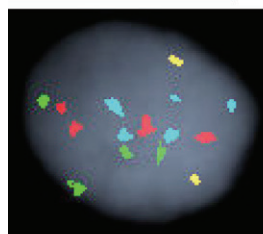
FISH



Disomy
(Negative)



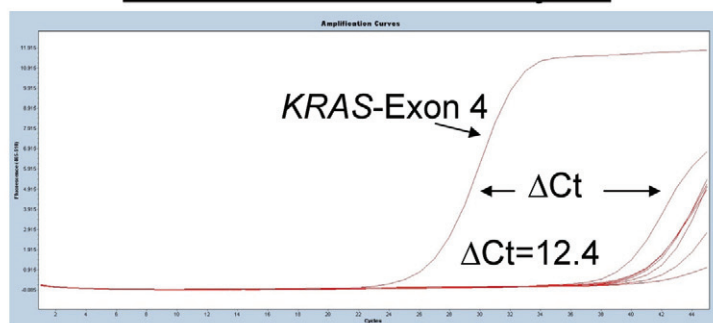
Trisomy 7
(Equivocal)



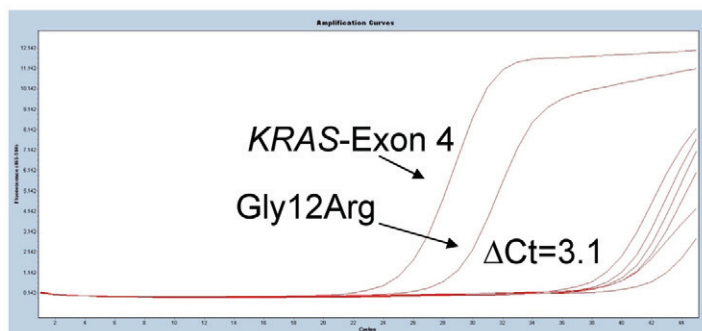
Polysomy
(Positive)

CEP 3 CEP 7 CEP 17 LSI 9p21

KRAS Mutation Analysis



KRAS Wild Type



KRAS Mutation

Figure 2. Representative examples of normal and abnormal FISH and *KRAS* testing results.

anastomotic biliary stricture, $n = 1$; and secondary sclerosing cholangitis secondary to choledochal cysts, $n = 1$ and malignant pancreaticobiliary tract strictures (pancreatic adenocarcinoma, $n = 35$; cholangiocarcinoma, $n = 41$; and gallbladder cancer, $n = 2$). Specimens used for this study were collected for clinical testing between October, 2004 and March, 2009.

All specimens had a prior conventional cytology and FISH (UroVysion probe set, Abbott Molecular Inc., Des Plaines, IL) result as part of routine clinical practice (Figure 1). Slides processed for FISH analysis were assessed by scanning for cytologically atypical cells (eg, nuclear enlargement, irregular nuclear shape) or cells with atypical staining patterns (eg, patchy and/or lighter nuclear DAPI staining), and the number of CEP3, CEP7, CEP17, and 9p21 signals in those cells were determined.⁴ FISH results were categorized as disomic (negative for malignancy), trisomy (equivocal for malignancy), or polysomy (positive for malignancy; Figure 2). As part of routine clinical testing at our institution,^{4,12} polysomy is defined as a gain of two or more of the four probes in ≥ 5 cells. Trisomy 7 has been defined as a gain of a single probe targeting the pericentromeric region of chromosome 7 (three signals for the CEP7 probe) with no more than two signals in each of the other three probes in ≥ 10 cells. Loss of FISH signal patterns (eg, loss of FISH probe(s) targeting 9p21) were not considered positive as part of routine clinical testing.

DNA Extraction

Residual cell pellets from clinical FISH testing (Figure 1) were retrieved from the -70°C freezer where they were stored in 3:1 methanol/glacial acetic acid. The pellets were centrifuged at 13,200 rpm for 5 minutes, followed by removal of the supernate, vortexing of the pellet, and addition of 1 ml of PBS. These steps were then repeated. This process was repeated a third time but 180 μl of ATL buffer (QIAGEN, Valencia, CA) and 20 μl of Proteinase K (QIAGEN) were added (instead of the 1 ml of PBS used in the prior 2 steps) and incubated at 55°C overnight with gentle agitation. The remainder of the DNA extraction procedure was performed using a QIAamp DNA Mini Kit (QIAGEN) as recommended by the manufacturer.

KRAS Testing

KRAS mutation analysis was performed using the quantitative PCR DxS *KRAS* Mutation Test Kit (DxS Ltd., Manchester, UK). The DxS *KRAS* Mutation Test Kit combines two technologies, ARMS and Scorpion to detect seven different mutations in codons 12 and 13 within exon 2 of the *KRAS* gene by real-time PCR. Allele-specific real-time quantitative PCR (qPCR) was carried out on a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Indianapolis, IN). The DXS kit assesses the number of PCR cycles necessary to detect fluorescent signal

above a background signal (exon 4 of the *KRAS* gene) to determine the cycle threshold (Ct) value. Control Ct values (exon 4 of the *KRAS* gene) between 20 and 35 were required to assure that there was an appropriate amount of DNA for analysis. The Δ Ct value was calculated by subtracting the Ct value from each of the seven assessed mutations by the background or control Ct value for each individual sample. Specimens were considered to have a particular *KRAS* mutation when the Δ Ct values were below the 1% Δ Ct value (Figure 2) published by the manufacturer for that mutation. The *KRAS* exon 2 (codons 12 and 13) mutations and respective Δ Ct thresholds included the following: G12A, ≤ 6.5 ; G12D, ≤ 8.0 ; G12R, ≤ 8.0 ; G12C, ≤ 7.0 ; G12S, ≤ 9.0 ; G12V, ≤ 6.5 ; and G13D, ≤ 9.0 . Specimens with all 7 Δ CT values above the aforementioned thresholds were interpreted as wild-type. *KRAS* testing was performed without knowledge of patients' previous testing results or clinicopathologic follow-up.

Statistics

Strictures were classified as benign or malignant based on surgical pathology findings when available or >6 months of clinical follow-up demonstrating either no progression (benign) or obvious neoplastic progression (mass lesion, metastasis, and/or death).² Equivocal FISH results (trisomy 7) were considered negative for statistical analyses. All *P* values were calculated using a two-tailed Fisher's Exact test with JMP 8.0 statistical software (SAS Institute Inc., Cary, NC). *P* values ≤ 0.05 were considered statistically significant.

Results

KRAS qPCR testing was successful in 130 of the 132 (99%) specimens. The control Ct values in the 130 specimens ranged from 21 to 32 with a mean and median value of 25. Cell pellets were dark brown and full of noncellular debris in the two specimens where qPCR failed, suggesting that bilirubin, bile salts, or noncellular debris interfered with the PCR reaction. In the 130 specimens where qPCR was successful, there was an identifiable *KRAS* mutation in 39 (30%) specimens. *KRAS* G12D (*n* = 16) and G12V (*n* = 13) mutations were the most prevalent, followed by G12R (*n* = 6), G12C (*n* = 2), G12S (*n* = 1), and G13D (*n* = 1) mutations (Figure 3).

The sensitivity of FISH, *KRAS*, and FISH + *KRAS* results combined are summarized in Table 1. Overall, FISH + *KRAS* analyses combined detected significantly (*P* < 0.001) more cancers (*n* = 53; 68%) than FISH alone (*n* = 39; 50%). Among patients with pancreatic adenocarcinoma, FISH + *KRAS* testing combined detected 86% of tumors which was significantly higher (*P* = 0.004) than FISH alone (63%). There were mixed results among patients with cholangiocarcinoma. In patients with non-PSC associated (ie, sporadic) cholangiocarcinoma, *KRAS* + FISH testing combined detected 56% of the cancers which was significantly higher (*P* < 0.001) than FISH alone which detected 37% of cancers. In PSC-

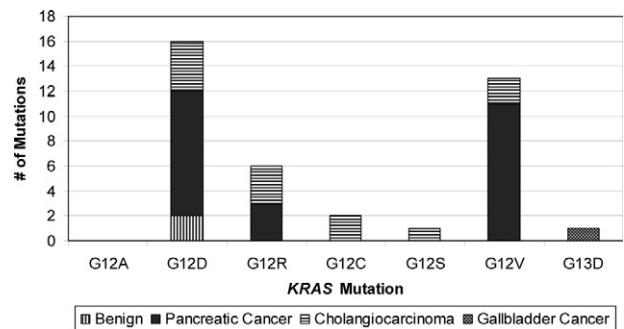


Figure 3. Frequency of specific *KRAS* mutations detected by qPCR.

associated cholangiocarcinoma, only four patients had an identifiable *KRAS* mutation and all four of these patients had a polysomic FISH result. Fifty-eight patients in this study had a disomy (ie, negative) FISH result. Within this group of patients, seven patients had an identifiable *KRAS* mutation and six of these patients had cancer. Thirty-nine patients had a polysomy FISH result with clinicopathologic evidence of cancer. A *KRAS* mutation was identified in 23 (59%) of these patients. Thirty-three patients had an equivocal FISH (trisomy 7). Nine of the 33 specimens (27%) had a *KRAS* mutation, and eight of nine (89%) were from patients with cancer. In contrast, nine of 24 patients (38%) with a trisomy 7 FISH and no *KRAS* mutation had malignancy.

The specificity of FISH and *KRAS* tests are summarized in Table 2. There were two patients who had false positive *KRAS* results (ie, a mutation was found in a patient without cancer). Interestingly, both of these patients had PSC and ulcerative colitis. There were 16 additional patients with PSC and UC and no identified *KRAS* mutations. There were no *KRAS* mutations identified in patients with chronic pancreatitis, autoimmune pancreatitis, acute pancreatitis, cholecystitis, or secondary sclerosing cholangitis.

The cytology interpretation for the 130 patients included negative (*n* = 85), atypical (*n* = 11), suspicious (*n* = 14), and positive (*n* = 20). Among the 85 patients with a negative cytology result, a *KRAS* mutation was identified in 15 specimens and 14 (93%) had clinicopathologic evidence of cancer. Eighteen of the 85 (21%) had a positive result by either FISH and/or *KRAS* analysis and 17 (94%) had clinicopathologic evidence of cancer. Eleven of the 25 patients with equivocal cytology (atypical or suspicious) had a *KRAS* mutation, and 10 (91%) of these patients had cancer. Nine of the 10 patients had subsequent pathological evidence (resection, *n* = 7; biopsy, *n* = 1; and fine needle aspiration, *n* = 1) of cancer whereas the remaining patient had obvious progression of disease and died from metastatic pancreatic adenocarcinoma. Seventeen of the 25 (68%) equivocal cytology specimens had a FISH and/or *KRAS* positive result, and 16 (94%) of these patients represented patients with cancer. Twenty patients with malignancy had a positive cytology result, and a *KRAS* mutation was detected in 13 (65%) of these specimens.

Table 1. Sensitivity of FISH and *KRAS* for Detecting Malignancy

Test	Pancreatic adenocarcinoma (n = 35)	Cholangiocarcinoma (n = 41)			All cancers* (n = 78)
		Sporadic	PSC-associated	Combined	
<i>KRAS</i>	24/35 (69%)	8/27 (30%)	4/14 (29%)	12/41 (29%)	37/78 (47%)
FISH†	22/35 (63%)	10/27 (37%)	7/14 (50%)	17/41 (41%)	39/78 (50%)‡
Combined	30/35 (86%)	15/27 (56%)	7/14 (50%)	22/41 (54%)	53/78 (68%)‡

*Includes two gallbladder cancers.

†Only polysomic FISH results were considered positive in this study.

‡Significant difference ($P < 0.001$) between FISH and *KRAS* + FISH results.

Discussion

In this study we performed qPCR *KRAS* mutation testing on residual pancreatobiliary tract brushing specimens and found that *KRAS* mutation analysis, in combination with FISH, could detect significantly more cancers than FISH alone. Previous reports suggest that *KRAS* mutation analysis can be used to detect cancer in pancreatobiliary tract brushings,^{10,22} bile fluids,^{9,23} and fine needle aspirates from pancreatic masses.^{8,24} Comparisons of *KRAS* mutation analysis to conventional cytology have generally shown that *KRAS* mutation analysis increases sensitivity, but often at the expense of test specificity.^{7,9,10,24,25} FISH testing of brushing specimens has been shown to be a valuable adjunct to routine cytology, and a statistical model that incorporates both of these test results with clinical information (eg, PSC status, age) can be used to predict the risk of malignancy in an individual patient.² The data from the present study suggests that *KRAS* mutation analysis combined with FISH testing could provide an additional increase in the diagnostic sensitivity over current testing algorithms. Our results suggest that *KRAS* testing may be most beneficial in patients with an equivocal FISH result or in patients suspected of having pancreatic adenocarcinoma or sporadic cholangiocarcinoma (Table 1). *KRAS* mutation analysis appears to be less sensitive for detecting PSC-associated cholangiocarcinoma.

In the present study, we identified *KRAS* mutations in 37 of the 78 (47%) ERCP brushing specimens from patients with cancer using the TheraScreen DxS quantitative real-time PCR assay. A wide variety of detection methods with varying analytical sensitivities (ie, limits of detection) have been used to assess tissue specimens for *KRAS* mutations. The most common methods that have been used include Sanger sequencing, pyrosequencing, qPCR, post-PCR fluorescent melting-curve analysis, single stranded conformation polymorphism analysis, and PCR clamping.^{26–28} A more sensitive technique called LigAmp has also been reported.²⁵ It has

been suggested that technologies such as LigAmp can detect a *KRAS* mutation in one of 10,000 cells, whereas the analytic sensitivities of qPCR and Sanger sequencing are approximately 1% and 20%, respectively.^{25–27}

The analytical sensitivity required of an assay may vary according to what the test is being used for. To date, *KRAS* mutation analysis has primarily been used clinically to assess paraffin embedded colorectal and lung tumors for determining whether patients are candidates for anti-EGFR therapies such as Cetuximab or Tarceva.^{26,27} It is generally easy for a pathologist to select areas within the paraffin-embedded specimen for microdissection that have a tumor percentage that exceeds the limit of detection of the assay being used in the lab.^{26,27} In this study, we used *KRAS* analysis to try to detect tumor cells in ERCP brush cytology specimens. The tumor cells generally comprise <20% of the cells in these samples and frequently as little as 1% of all of the cells. Consequently, we needed to use an assay that had an analytical sensitivity approaching 1%.

According to the Sanger Catalog of Somatic Mutation in Cancer Database (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>, last accessed on May 9, 2010), 58% (2784/4857 specimens) of pancreatic cancers and 31% (459/1463) of biliary tract cancers harbor a *KRAS* mutation. The most frequently observed *KRAS* mutations in both pancreatic adenocarcinoma and cholangiocarcinoma are mutations in codons 12 and 13 within exon 2, the mutations analyzed in this study. The data from our study are consistent with the Sanger database with *KRAS* mutations being detected in 69% and 29% of pancreatic and cholangiocarcinoma specimens, respectively.

The patients with cancer in this study who did not have a detectable *KRAS* mutation ($n = 40$) included 11 patients with pancreatic adenocarcinoma and 29 patients with cholangiocarcinoma. Possible explanations for false negative *KRAS* results include: i) low tumor percentage (ie, the fraction of tumor cells was lower than the analytical sensitivity of the assay); ii) the tumor cells within the specimen do not contain *KRAS* mutations; or iii) sample collection failure. The ability of *KRAS* testing, as well as FISH and cytology, to accurately detect cancer depends on the gastroenterologist's ability to brush malignant cells and place them into the vial for analysis. It is well known that the collection of biliary tract specimens is difficult and sampling failure occurs.² To determine whether sampling failure was a reason for some of the false negative *KRAS* results in this study, we performed *KRAS* and FISH testing on paraffin embedded cancer from three patients

Table 2. Specificity of *KRAS* and FISH Testing

Test	Fraction of patients without evidence of cancer and negative test result
<i>KRAS</i>	50/52 (96%)*
FISH	52/52 (100%)
Combined	50/52 (96%)

*False-positive *KRAS* results were identified in two patients with diagnoses of both primary sclerosing cholangitis, and ulcerative colitis.

(two patients with cholangiocarcinoma and one patient with pancreatic adenocarcinoma) who had negative biliary brushing FISH, *KRAS*, and cytology results. All three of these paraffin-embedded tumor specimens demonstrated polysomic cells by FISH and two specimens (one pancreatic and one cholangiocarcinoma) demonstrated a *KRAS* mutation. This suggests, at least in these three patients, that the malignant cells were either not present in the specimen collected during ERCP or present in quantities that were not detectable by either FISH or *KRAS* analysis.

Previous studies have shown that *KRAS* mutations are present in patients with PSC,²³ autoimmune pancreatitis,²¹ and chronic pancreatitis.²⁹ A current hypothesis suggests that increased Ras activity, either from elevated expression of mutant *KRAS* gene or by high levels of extrinsic Ras activators (eg, coexpression of TGF- α), may be a cause of inflammatory diseases such as chronic pancreatitis.²⁹ High levels of Ras activity that generate the inflammatory response are postulated to accelerate the genetic changes that promote tumorigenesis.^{29,30} In the present study, there were 26 patients with PSC, 15 patients with chronic pancreatitis, and 9 patients with autoimmune pancreatitis without clinicopathologic evidence of cancer. The *KRAS* results from these specimens were negative except for two patients, both of whom had PSC and ulcerative colitis. One of these patients underwent a liver transplant for PSC one month after the biliary brushing diagnosis. It is not possible to determine whether this patient would have progressed to cholangiocarcinoma. The second patient is a 49-year-old male with two years of clinicopathologic follow-up without evidence of cancer. More extensive human studies are needed to better understand possible relationships between *KRAS* mutations, inflammatory diseases such as PSC, and pancreatobiliary tract cancer.

In conclusion, the results of this study suggest that *KRAS* mutation analysis can detect cancers not identifiable by FISH. *KRAS* mutations appear to be more common in pancreatic adenocarcinoma than cholangiocarcinomas. The identification of *KRAS* mutations in patients with PSC without cholangiocarcinoma appears to be infrequent, but does occur. Additional data are needed to determine the significance of these mutations. This study, like others, demonstrates that the addition of *KRAS* testing may be beneficial for laboratories performing cytologic testing of pancreatobiliary tract specimens. Further prospective studies are needed to define the algorithm that would be most beneficial to clinicians, clinical laboratories, and patients with pancreatobiliary tract strictures.

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