A Multivariable Model Using Advanced Cytologic Methods for the Evaluation of Indeterminate Pancreatobiliary Strictures

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Background & Aims: **Ancillary cytologic tests including digital image analysis (DIA) and fluorescence in situ hybridization (FISH) have been developed to improve the sensitivity of routine cytology (RC) for the diagnosis of malignancy in pancreatobiliary strictures. The goal of this study was to retrospectively compare the performance of RC, DIA, and FISH on clinical brushing specimens.** ***Methods:*** **Endoscopic retrograde cholangiopancreatography brushings were obtained from 498 consecutive patients with pancreatobiliary strictures and analyzed by RC, DIA, and FISH as per standard practice. RC diagnostic categories included negative, atypical, suspicious, or positive. Aneuploid/tetraploid histograms were considered positive for DIA. FISH was performed using UroVysion (Abbott Molecular, Inc, Des Plaines, IL) and classified as negative, trisomy, tetrasomy, or polysomy.** ***Results:*** **The sensitivity of polysomy FISH (42.9%) was significantly higher than RC (20.1%) when equivocal RC results were considered negative ($P < .001$) with identical specificity (99.6%). There was a significant difference in time for diagnosis of carcinoma between FISH diagnostic categories ($P < .001$) and between RC diagnostic categories ($P < .001$). Logistic regression analysis revealed that polysomy FISH, trisomy FISH, suspicious cytology, primary sclerosing cholangitis status, and age were associated with carcinoma ($P < .05$).** ***Conclusions:*** **Polysomy FISH had high sensitivity without compromise to specificity. DIA was not a significant independent predictor of malignancy. Multivariable modeling using RC, FISH, age, and primary sclerosing cholangitis status can be used to estimate the probability of carcinoma for an individual patient. We recommend including FISH as a routine test where available, along with RC, in the evaluation of indeterminate pancreatobiliary strictures.**

Pancreatobiliary tumors such as cholangiocarcinoma or pancreatic cancer often present as strictures. The accurate distinction between benign and malignant strictures of the pancreatobiliary tract frequently presents a dilemma to both clinicians and pathologists for a number of reasons. Benign and malignant strictures can be indistinguishable by imaging, especially in patients with primary sclerosing cholangitis (PSC).[1] It can be difficult for gastroenterologists to obtain adequate intraductal forceps biopsy specimens at the time of endoscopic retrograde cholangiopancreatography (ERCP) because of the narrow caliber of the bile duct and the scirrhous nature of many biliary tumors.[2–4] Tumor markers such as CA 19-9 have been used for tumor detection but appear to have low sensitivity for early stage tumors.[5–8]

Cytologic brushing specimens are often obtained during endoscopy and sometimes can provide a definitive diagnosis when biopsies are inadequate. However, the reported diagnostic sensitivity for routine cytology (RC) ranges from 15% to 68%[9–12] and therefore is regarded as low to moderate. This lack of sensitivity has led investigators to explore ancillary diagnostic procedures for improved tumor detection including immunohistochemistry,[13] mutational analysis (ie, *KRAS*, *P16*, *P53*),[14,15] DNA ploidy analysis,[16,17] and fluorescence in situ hybridization (FISH).[10,18] Digital image analysis (DIA) and FISH use cells obtained from ERCP brushing specimens. DIA is used to assess for aneuploidy by determining the DNA content of cells. FISH is a technique that utilizes fluorescently labeled DNA probes to examine cells for chromosomal abnormalities. The detection of nondiploid (DIA) or chromosomally abnormal (FISH) cells generally correlates with the presence of tumor.

Prior studies from our institution have compared DIA and FISH to RC and demonstrated that these methods increase the sensitivity for detecting pancreatobiliary tract cancers over RC.[10,18–21] Previous reports represent a subset of patient specimens collected during the time frame of our clinical database,[18,19,21] but the current

Abbreviations used in this paper: DIA, digital image analysis; ERCP, endoscopic retrograde cholangiopancreatography; FISH, fluorescence in situ hybridization; PSC, primary sclerosing cholangitis; RC, routine cytology.

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study is the most comprehensive analysis to date of cytologic and ancillary test results for patients with pancreatobiliary strictures. We report test results from ERCP brushings analyzed by RC, DIA, and FISH from approximately 500 patients in conjunction with clinical follow-up data. The findings of this study have influenced our current laboratory ancillary testing algorithm for analyzing and reporting pancreatobiliary brushings.

Material and Methods

Patients

Cytologic brushing specimens were obtained from pancreatobiliary strictures during ERCP from 540 patients at the Mayo Clinic between October 2003 and March 2006. After review of patient medical records, only patients with strictures that were definitively diagnosed as benign or malignant were included in this retrospective study (n = 526). A definitive diagnosis was defined as evidence from either surgery, surgical pathology, or clinical follow-up to ensure a benign or malignant course (such as progression of disease with obvious metastases, the presence of a tumor mass on imaging studies, and/or death from cancer).[19] The mean follow-up time for patients without carcinoma was 15 months. Patients with intraductal papillary mucinous neoplasm were considered negative for carcinoma unless the neoplasm showed high-grade dysplasia (n = 2). Patients with strictures possibly caused by nonprimary pancreatobiliary carcinoma (eg, colon carcinoma metastatic to the pancreatobiliary tract) (n = 20) and patients with a known history of primary pancreatobiliary carcinoma (n = 8) were not included, resulting in a total of 498 patients assessed in this study (Table 1). Histologic confirmation of malignancy was available for 174 of 227 patients with carcinoma (76.7%).

Table 1. Patient Characteristics

Characteristic	Result, n (%)
Total number	498
Males	299 (60.0)
Females	199 (40.0)
Median age (*range*), *y*	60.0 (7, 91)
Cancer	227 (45.6)
Cholangiocarcinoma	152 (67.0)
Pancreatic carcinoma	64 (28.2)
Gallbladder carcinoma	6 (2.6)
Ampullary carcinoma	4 (1.8)
Hepatocellular carcinoma	1 (0.4)
Primary sclerosing cholangitis	189 (38.0)
Cancer	59 (31.2)
No cancer	130 (68.8)
Nonprimary sclerosing cholangitis	309 (62.0)
Cancer	168 (54.4)
No cancer	141 (45.6)

Sampling and Specimen Processing

Specimens were collected during ERCP using 1 standard DLB-35-1.5 or DLB-35-3.5 brush (Wilson–Cook, Winston-Salem, NC). Caution was taken to sample only strictured areas to prevent normal epithelium from diluting the specimen. The stricture was brushed with at least 5 to-and-fro motions, and the brush was pushed from the end of the sheath to maximize cellularity. Each brush was cut and placed in a vial of PreservCyt solution (Cytyc Corporation, Marlborough, MA) and transported to the cytopathology laboratory at the Mayo Clinic. Once in the laboratory, each vial was gently agitated and split equally into thirds for processing.

RC

One third of the specimen was utilized to make a ThinPrep (Cytyc) slide and Papanicolaou-stained for review by a cytopathologist during routine practice. Diagnostic categories included negative, atypical, suspicious, and positive for malignancy (Figure 1) using accepted criteria as reported previously.[22,23] Specimens with cells demonstrating features most consistent with reactive changes but not unequivocally benign were considered atypical.[11,19,24] Specimens considered suspicious either (1) demonstrated an insufficient number of malignant-appearing cells to be considered unequivocally positive or (2) had a high number of abnormal cells, but these cells lacked sufficient morphologic criteria for malignancy.[11,19,24] Specimens were classified as positive if an adequate number of unequivocally malignant cells were present.

DIA

One third of the brushing fluid was utilized to make a ThinPrep (Cytyc) slide for ploidy analysis using DIA. Each slide was stained using the Feulgen dye and analyzed with a CAS 200 image cytometer (Bacus Laboratories, Lombard, IL).[25] Fifty of the most abnormal appearing cells were collected to produce a histogram, which was interpreted by a cytopathologist. Histograms were considered positive for malignancy if the peak DNA index was within the tetraploid (DNA index, 1.90–2.10) or aneuploid (DNA index, 1.11–1.89 or >2.10) range (Figure 2). Histograms were considered negative for malignancy if the peak DNA index was diploid (DNA index, 0.95–1.05) or near diploid (DNA index, 1.06–1.10), unless a genuine clonal peak of aneuploid or tetraploid cells was also present.[19]

FISH

One third of the ERCP fluid was processed for FISH analysis as previously published[10,19] and hybridized using the UroVysion probe set (Abbott Molecular, Des Plaines, IL). This assay utilizes fluorescently labeled probes that hybridize to the pericentric regions of chromosomes 3, 7, and 17 and the 9p21 locus of interphase

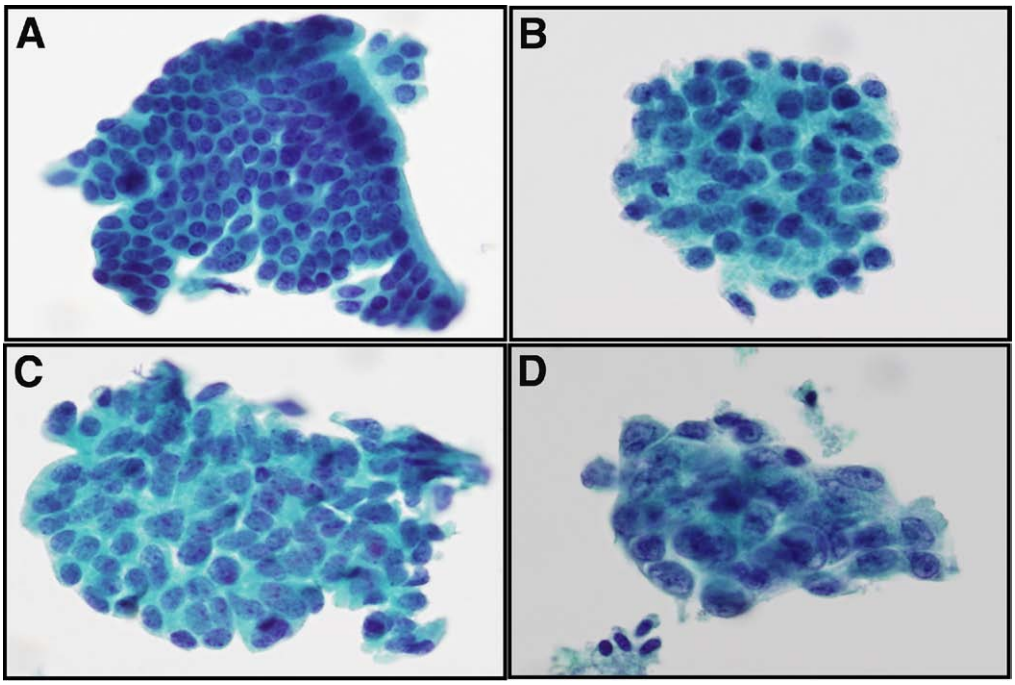

Figure 1. Cells from routine cytologic specimens representing (*A*) negative, (*B*) atypical, (*C*) suspicious, and (*D*) positive diagnoses.

cells. The signal patterns were visualized and recorded by experienced cytotechnologists using a fluorescence microscope. Specimens were diagnosed as positive based on the following criteria: (1) trisomy (≥10 cells with 3 signals representing chromosome 3 or 7 and ≤2 signals for the other 3 probes), (2) tetrasomy (≥10 cells with 4 signals for each of the 4 probes), or (3) polysomy (≥5 cells with ≥3 signals in at least 2 probes). Specimens were diagnosed as negative/disomy when none of the above criteria were fulfilled[18] (Figure 3).

Statistical Analysis

Statistical analyses were performed using Unix SAS 9.1.3 (SAS Institute Inc, Cary, NC). Test results were not available for all specimens because of insufficient cellularity (n = 43) or unsuccessful FISH hybridization (n = 14). Specimens with respective results for cytology (n = 493), DIA (n = 460), and FISH (n = 484) were used to calculate sensitivity and specificity. The test results of highest severity were used for statistical analysis if multiple sites from the same patient were sampled during endoscopy (ie, more than 1 brush submitted for cytologic analysis). Positive RC, polysomy FISH, and aneuploid DIA were considered the most severe diagnoses for each assay. The McNemar test was used to compare these calculated sensitivities and specificities.

Kaplan–Meier and multivariable analyses did not include FISH tetrasomy specimens because of the rarity of this diagnosis (n = 3). Kaplan–Meier curves were generated with specimens having results for all tests (n = 446). For the multivariable analysis, positive RC was not included in the model because of the high positive predictive value of this test (42/43 = 97.7%) and the small number of specimens (n = 43) in this category.

A logistic regression model (n = 403) was produced with DIA (using an indicator variable for positive DIA), FISH result (using indicator variables for trisomy FISH and polysomy FISH), RC result (with indicator variables for atypical RC and suspicious RC), PSC, and age (in years) as the independent variables and pancreatobiliary carcinoma as the dependent variable. We used odds ratios, along with their 95% confidence interval, to summarize the effect of each factor on the probability of getting

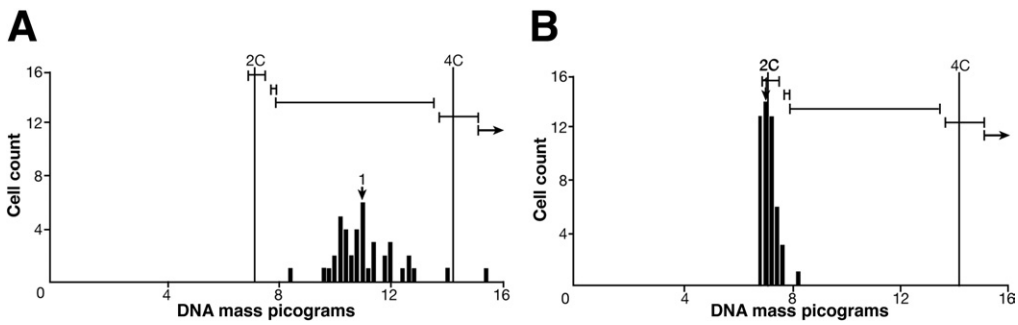

Figure 2. DNA histograms generated by digital image analysis (DIA) representing (*A*) aneuploid (positive) and (*B*) diploid (negative) specimens.

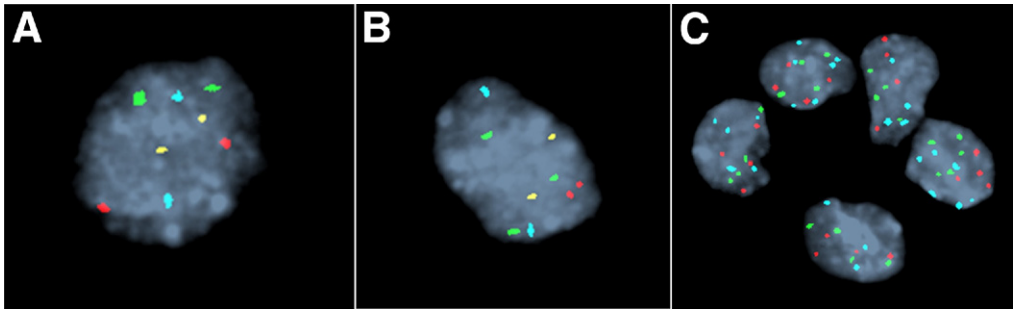

Figure 3. Cells from fluorescence in situ hybridization (FISH) specimens representing: (*A*) disomy (2 signals for each of the 4 probes), (*B*) trisomy 7 (3 signals representing chromosome 7 and 2 signals for the other probes), and (*C*) polysomy (≥3 signals for ≥2 probes). CEP 3 (*red*), CEP 7 (*green*), CEP 17 (*aqua*), LSI 9p21 (*gold*).

cancer. *P* values ≤ .05 were considered statistically significant.

Results

The sensitivity and specificity of RC, DIA, and FISH for the detection of carcinoma in pancreatobiliary strictures are depicted in Figure 4. Cytology 1, calculated using only the positive cytologic category as a positive test result, had the lowest sensitivity (20.1%) of all tests in this study but had a very high specificity (99.6%). Cytology 2 (positive and suspicious categories were considered a positive test result) and cytology 3 (positive, suspicious, and atypical categories were considered a positive test result) had increasingly higher sensitivities because additional cytologic categories were considered positive, but, as expected, this was accompanied by a decrease in specificity.

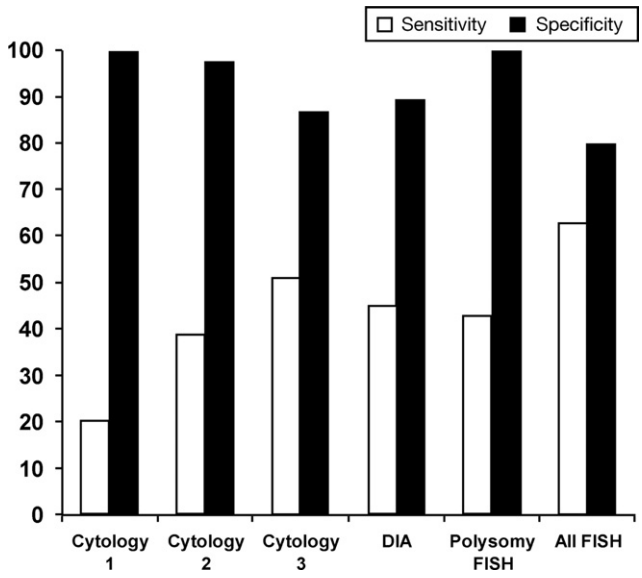

Figure 4. Sensitivities and specificities of routine cytology (RC), digital image analysis (DIA), and fluorescence in situ hybridization (FISH) for the detection of malignancy in pancreatobiliary strictures. *Cytology 1:* positive cytology results considered positive diagnoses; *Cytology 2:* positive and suspicious cytology results considered positive diagnoses; *Cytology 3*: positive, suspicious, and atypical cytology results considered positive diagnoses; *Polysomy FISH:* polysomic FISH results considered positive diagnoses; *All FISH:* polysomic, tetrasomic, and trisomic FISH results considered positive diagnoses.

The sensitivity of DIA was higher than cytology 1 (44.8% vs 20.1%, $P < .001$), but DIA had lower specificity (89.1% vs 99.6%, $P < .001$). The sensitivity of DIA was not significantly different from cytology 2 ($P = .12$), but DIA specificity was significantly lower (89.1% vs 97.4%, $P < .001$). The sensitivity of polysomy FISH (polysomic FISH results considered positive) was significantly higher than cytology 1 (42.9% vs 20.1%, $P < .001$) with the same specificity of 99.6%. Polysomy FISH had a higher sensitivity (42.9% vs 38.8%) than cytology 2, but this difference was not significant ($P = .21$); however, the specificity of polysomy FISH was significantly higher than cytology 2 (99.6% vs 97.4%, $P = .03$). Whereas the sensitivity of FISH when considering all FISH abnormalities (ie, polysomy, tetrasomy, and trisomy) as positive was the highest of all tests in this study at 62.6%; its specificity was the lowest at 79.6%. The performance characteristics of RC, DIA, and FISH in the current study are analogous to what has been previously reported from our institution.[10,18,19,21]

Kaplan–Meier analysis (Figure 5*A*) demonstrated a significant difference in time to progression to a definite diagnosis of carcinoma in the pancreatobiliary tract between the FISH diagnostic categories of negative, trisomy, and polysomy ($P < .001$). There was also a significant difference in time to a definite diagnosis of carcinoma among patients with negative, atypical, suspicious, and positive RC results ($P < .001$) (Figure 5*B*).

A logistic regression model revealed that FISH result (overall *P* value < .0001), RC result (overall *P* value = .001), PSC status, and age (in years) were significantly associated with presence of and/or progression to carcinoma (Table 2). Positive DIA was not significant in the multivariable model.

Patients with polysomy FISH results are over 77 times as likely to have carcinoma as those with normal FISH, whereas those with trisomy FISH are only 1.8 times as likely to have carcinoma. Patients with suspicious cytology are 6 times as likely to have carcinoma as those with normal cytology. The risk of developing carcinoma increased with age but decreased with the presence of PSC.

Discussion

For patients with a pancreatobiliary stricture, routine cytologic test results can be instrumental in detecting

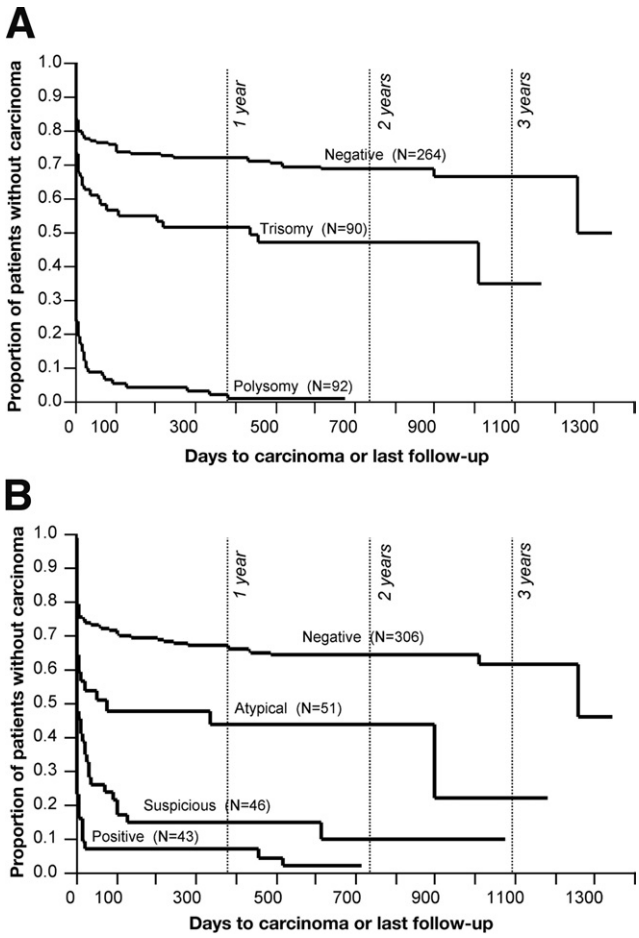

Figure 5. Kaplan–Meier demonstrates differences in time to carcinoma for patients by (*A*) fluorescence in situ hybridization (FISH) result ($P < .001$) and (*B*) routine cytology result ($P < .001$).

carcinoma, directing treatment decisions, and monitoring inflammatory conditions such as PSC. Histopathologic diagnosis of suspicious strictures is ideal for planning a surveillance strategy or the appropriate course of treatment, but unfortunately a tissue diagnosis is obtained on only a small proportion of all patients because of ineffective sampling or inability of the forceps biopsy to access the stricture.[4,18,26] Tissue fragments that are acquired tend to be small and distorted, factors that presumably contribute to the only moderate sensitivity of forceps biopsy for the detection of malignancy.[12,27] Therefore, clinical data (ie, imaging, biochemical or tumor serum levels) and routine cytologic diagnoses are used to augment histologic results for determining the presence of carcinoma in pancreatobiliary strictures.

Endoscopic brushing of pancreatobiliary strictures to obtain cells for routine cytology is commonly performed because of ease, speed, and safety of performance during ERCP.[12,28] Whereas the specificity of RC approaches 100%, its sensitivity is quite low.[3,4,11,29] Consequently, there is a need for novel diagnostic methods with improved sensitivity over RC for detecting pancreatobiliary carcinoma without compromising specificity.

In this study, we found that the ancillary cytologic techniques of FISH and DIA detected more patients with carcinoma than RC alone and that, in particular, polysomy FISH maintains the high specificity of RC. DIA and polysomy FISH each had a significantly higher sensitivity (44.8% and 42.9%, respectively) than RC when considering equivocal RC test results as negative (20.1%). A polysomic FISH result detected 49 more patients with cancer than RC without compromise to specificity (99.6% for both tests). DIA, however, had a significantly lower specificity (89.1%, $P < .001$) than RC or polysomy FISH. In our experience, a polysomic FISH diagnosis plays an important role in clinical practice for identifying additional patients with carcinoma over RC without additional false positives.

A relevant clinical question is the role of these tests for detecting malignancy relative to anatomic location (ie, proximal vs distal). A previous study from our institution evaluated a similar patient population and analyzed the performance characteristics of RC, DIA, and FISH for proximal vs distal strictures.[18] Interestingly, performance of the tests did not significantly differ with this stratification, and, therefore, the current study does not make such a distinction.

When evaluating the amount of time for patients to progress to carcinoma, Kaplan–Meier analysis revealed a significant difference between patients with negative, trisomic, and polysomic FISH results ($P < .001$) (Figure 5*A*). After 1 year, 98% of patients with a polysomic FISH result, 48% of patients with a trisomic FISH result, and 28% of those with a negative FISH result were diagnosed with carcinoma. There was also a significant difference in time to the diagnosis of carcinoma between routine cytologic diagnoses ($P < .001$) (Figure 5*B*). After 1 year, 94% of patients with a positive RC result were diagnosed with carcinoma, whereas 85% of suspicious patients, 56% of atypical patients, and 33% of negative patients were diagnosed with carcinoma. Whereas both polysomic FISH and positive RC results had positive predictive values approaching 100%, polysomy FISH detected 49 more patients with carcinoma 4.5 months earlier.

Table 2. Multivariable Model

Variable	Odds ratios (95% CI)	*P* value
Positive DIA	1.66 (0.81−3.41)	.17
FISH	NA	<.0001
Polysomy FISH	77.65 (9.93−607.18)	<.001
Trisomy FISH	1.76 (1.01−3.06)	.047
Cytology	NA	.001
Suspicious cytology	6.02 (2.11−17.19)	<.001
Atypical cytology	1.51 (0.72−3.18)	.27
PSC	0.43 (0.24−0.76)	.004
Age, *y*	1.02 (1.004−1.04)	.02

NOTE. N = 403. Positive cytology was not included in the model because of its very high positive predictive value (97.7%) and small sample size (n = 43).

Whereas a polysomic FISH result is indicative of carcinoma in the context of a biliary stricture, the interpretation of trisomy 7 remains uncertain because the finding of trisomy is not specific for the presence of malignancy, both in the current and previous studies.[18,19] Trisomy 7 has been observed in both tumors and benign conditions.[30] It is possible that, at least in some patients, trisomy 7 cells represent an early preneoplastic change in the development of pancreatobiliary malignancy. Our results suggest that patients with trisomy 7 should be treated conservatively but followed every 6 months for 2 years. Those with negative FISH and/or negative cytology should also be monitored because a proportion of these patients will be diagnosed with carcinoma over time as shown in Figure 5. It is recommended that such patients return within 3–6 months for repeat ERCP including brushings for cytology and FISH analysis.

Testing algorithms that provide gastroenterologists with accurate cancer probability data are valuable because of the need for early and aggressive intervention for pancreatobiliary malignancy. In this study, multivariable analysis revealed FISH, RC, PSC status, and patient age to be significant independent predictors of malignancy. For each additional year of life, a patient's odds of developing carcinoma increases (odds ratio, 1.02). This is substantiated by previous studies that have reported age to be associated with a higher risk for pancreatobiliary tract carcinoma.[5,31,32] Interestingly, PSC patients had a decreased risk of pancreatobiliary malignancy relative to patients without PSC. The Mayo Clinic is a referral center for PSC patients, and a large proportion of this study population (38%) had PSC. We attribute the lower hazard ratio (0.43) to the fact that PSC patients undergo regular ERCP surveillance for cholangiocarcinoma, and, therefore, the disease prevalence is lower in this population than that of non-PSC patients.

It is important to reiterate that positive RC was not included in the multivariable statistical analysis because of the infrequency of this diagnosis and its very high specificity. Undoubtedly, a positive RC result is a significant predictor of malignancy, although not statistically tested in this study. In multivariable analysis, a positive DIA result was not a significant independent predictor of carcinoma in the presence of FISH and RC results. We have therefore discontinued the use of DIA on pancreatobiliary brushings in our laboratory. We currently perform RC and FISH analysis on all patients who are being evaluated for pancreatobiliary malignancy. Furthermore, the model generated through multivariable analysis in this study is used in clinical practice to estimate a probability of malignancy for each patient based on their cytologic brushing test results (RC and FISH), age, and PSC status. This probability is then used to generate a report interpretation. Probabilities of greater than 98% are interpreted as consistent with malignancy, probabilities of 85%–98% are interpreted as suspicious for malignancy, and probabilities of less than 85% in which at least 1 test is not negative are interpreted as equivocal. A representative example of the report for a 50-year-old PSC patient with atypical cytology and polysomic FISH is shown in Figure 6. Whereas the predictive values of various scenarios are not within the scope of this manuscript, this practice is an example of personalized medicine that empowers clinicians with information to make customized management decisions based on the integration of individual test results and clinical factors. Identification of patients at greatest risk of pancreatobiliary malignancy is critical because surgical treatment at an early stage offers the best outcome.

Cytology	FISH	PSC	Age	Predicted	Lower C	Upper C
Atypical	Polysomy	Yes	50	96%	73%	99%

Interpretation: These results are suspicious for malignancy. An atypical cytology and polysomy FISH result in a 50 year-old patient with PSC resulted in a cancer rate of 96% (confidence interval, 73% to 99%) in a cohort of 498 patients undergoing evaluation for possible biliary tract malignancy at the Mayo Clinic, 46% of whom had a final diagnosis of malignancy. This risk estimate is our best appoximation given currently available data. The likelihood of cancer in a given patient will vary based on other factors.

Figure 6. Example of predicted risk of carcinoma and clinical report interpretation for a patient with a pancreatobiliary stricture based on routine cytology (RC) result, fluorescence in situ hybridization (FISH) result, PSC status, and age.

This study is the most comprehensive report to date on the role that ancillary cytologic testing can play in the detection of pancreatobiliary malignancy and includes information from approximately 500 patients with suspicious pancreatobiliary strictures evaluated by RC, DIA, and FISH. Our findings suggest that FISH testing is a valuable adjunct to RC for detecting pancreatobiliary carcinoma, and statistical models that integrate these test results with clinical parameters (eg, age and PSC status) can be used to predict the risk of malignancy in an individual patient. As a result of these data, it has become our routine practice to perform both RC and FISH on brushing specimens from indeterminate pancreatobiliary strictures when there is a degree of suspicion for carcinoma, and we recommend doing so in centers where available.

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Conflicts of interest

The authors disclose the following: Dr Kevin C. Halling has a patent on and receives royalties from the sale of the FISH probe set (UroVysion) discussed in this paper. The remaining authors disclose no conflicts.