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ORIGINAL CONTRIBUTIONS

Pathology

Prospective Evaluation of Advanced Molecular Markers and Imaging Techniques in Patients With Indeterminate Bile Duct Strictures

Michael J. Levy, M.D.,[1] Todd H. Baron, M.D.,[1] Amy C. Clayton, M.D.,[2] Felicity B. Enders, Ph.D.,[3] Christopher J. Gostout, M.D.,[1] Kevin C. Halling, M.D.,[2] Benjamin R. Kipp, M.D.,[2] Bret T. Petersen, M.D.,[1] Lewis R. Roberts, M.D.,[1] Ashwin Rumalla, M.D.,[1] Thomas J. Sebo, M.D.,[2] Mark D. Topazian, M.D.,[1] Maurits J. Wiersema, M.D.,[1] and Gregory J. Gores, M.D.[1]

[1]*Division of Gastroenterology and Hepatology,* [2]*Department of Pathology, and* [3]*Division of Biostatistics, Mayo Clinic Foundation, Rochester, Minnesota*

BACKGROUND AND AIMS: Standard techniques for evaluating bile duct strictures have poor sensitivity for detection of malignancy. Newer imaging modalities, such as intraductal ultrasound (IDUS), and advanced cytologic techniques, such as digital image analysis (DIA) and fluorescence *in situ* hybridization (FISH), identify chromosomal abnormalities, and may improve sensitivity while maintaining high specificity. Our aim was to prospectively evaluate the accuracy of these techniques in patients with indeterminate biliary strictures.

METHODS: Cholangiography, routine cytology (RC), intraductal biopsy, DIA, FISH, and IDUS were performed in 86 patients with indeterminate biliary strictures. Patients were stratified based on the presence or absence of primary sclerosing cholangitis (PSC).

RESULTS: RC provided low sensitivity (7–33%) but high specificity (95–100%) for PSC and non-PSC patients. The composite DIA/FISH results (when considering trisomy-7 [Tri-7] as a marker of benign disease) yielded a 100% specificity and increased sensitivity one- to fivefold in PSC patients *versus* RC, and two- to fivefold in patients without PSC, depending on how suspicious cytology results were interpreted. For the most difficult-to-manage patients with negative cytology and histology who were later proven to have malignancy (N = 21), DIA, FISH, composite DIA/FISH, and IDUS were able to predict malignant diagnoses in 14%, 62%, 67%, and 86%, respectively.

CONCLUSIONS: DIA, FISH, and IDUS enhance the accuracy of standard techniques in evaluation of indeterminate bile duct strictures, allowing diagnosis of malignancy in a substantial number of patients with false-negative cytology and histology. These findings support the routine use of these newer diagnostic modalities in patients with indeterminate biliary strictures.

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INTRODUCTION

Gastroenterologists commonly evaluate patients with benign and malignant biliary strictures. Although transabdominal ultrasound, computed tomography, and magnetic resonance imaging are sensitive for detecting bile duct pathology, they do not reliably distinguish strictures as being malignant or benign (1). Invasive techniques, such as endoscopic retrograde cholangiography (ERC) with brushings for routine cytology (RC) and intraductal forceps biopsy, are often required to establish the diagnosis of malignancy (2–4). The sensitivity for diagnosing malignant biliary strictures using combined RC and forceps biopsy is only 20–65% (2, 5–8). It is important to determine the etiology of a biliary stricture in order to provide appropriate therapy. Patients with benign strictures can often be treated by endoscopic stent placement alone. Malignant strictures may be treated with surgical resection, endoscopic stent placement, photodynamic therapy, chemoradiation, or liver transplantation. Newer imaging techniques, such as intraductal ultrasound (IDUS), and molecular markers, such as digital image analysis (DIA) and fluorescence *in situ* hybridization (FISH), may improve the accuracy of nonsurgical

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diagnosis of biliary strictures. However, more data are needed before their use can be widely advocated.

IDUS probes operate at a high frequency, and produce detailed images of the bile duct wall and adjacent structures. During ERC, the IDUS catheter can be easily and safely placed into the bile duct via a transpapillary route (9–11). These small caliber probes are placed over a guidewire, and can be positioned in the bile duct in nearly 100% of patients without the need for sphincterotomy (9, 11). IDUS adds only 5–10 min to the duration of ERC, and is safely performed (9–12).

DIA is a specialized technique of cytologic analysis that quantifies abnormalities of nuclear DNA (aneuploidy or tetraploidy) by using spectrophotometric principals (13). FISH utilizes fluorescently labeled DNA probes to detect aneusomy of individual cells (abnormal loss or gain of chromosomes or chromosomal loci) (14, 15). Using these techniques, small numbers of tumor cells can be analyzed in contrast to flow cytometry where a larger number of cells are required for analysis (16). Although aneuploid/aneusomic cells are generally considered markers of malignancy, premalignant lesions, such as colonic adenomas, have also been shown to demonstrate these findings (17–19). In contrast, inflammatory processes that involve the bile ducts can increase cellular proliferation, but usually do not produce aneuploid/aneusomic cell populations (20). Furthermore, not all cancers display aneuploid/aneusomic cell populations, and it is estimated that only 80% of pancreatobiliary malignant strictures demonstrate these genetic aberrations, thereby setting an expected upper limit for diagnostic sensitivity of 80% for DIA and FISH.

Our aim was to compare the diagnostic accuracies of DIA, FISH, and IDUS to those of standard techniques (cancer antigen [CA] 19-9, cholangiography, RC, and biopsy) for determining the cause of indeterminate bile duct strictures. We also sought to assess the accuracy of the composite DIA/FISH result in distinguishing the stricture type.

METHODS

The institutional review board granted approval for the study, and informed consent was obtained for all procedures described in this report. Patients with indeterminate bile duct stricture were prospectively enrolled and underwent ERC with RC, intraductal biopsy, DIA, FISH, and IDUS. Among 147 patients initially screened for enrollment, 47 either declined enrollment or their procedures were performed on a day or time during which a physician who performs IDUS was unavailable. Among the 100 patients who were enrolled in the study, 14 were excluded because they were not found to satisfy all the enrollment criteria due to diagnostic uncertainty, loss to follow-up, or protocol violation. Thus, in total 86 enrolled patients were included in data analyses.

Patients were eligible for enrollment if they satisfied each of the following three criteria, including: (a) ERC demonstration of a biliary stricture accompanied by one or both of the following: clinical symptoms (right upper quadrant pain, jaundice, and pruritus) and cholestatic laboratory values (bilirubin and alkaline phosphatase); (b) indeterminate nature of the stricture based on presence of all of the following: no identifiable cause (*e.g.*, mass) for the bile duct stricture on transabdominal ultrasound or computed tomography performed within 30 days of enrollment, no distant metastases on transabdominal ultrasound or computed tomography, absence of common bile duct stones, and no history of traumatic or iatrogenic bile duct injury (including biliary surgery within 6 months); and (c) patients with primary sclerosing cholangitis (PSC) must have had evidence of a "dominant" stricture based on recent (<3 months) progression of clinical symptoms or cholestatic laboratory values. Patients were excluded if there was: (a) inability to provide informed consent, (b) anticipated unavailability or patients declined phone follow-up, (c) coagulopathy (international normalized ratio [INR] <1.5 and/or thrombocytopenia [platelets <50,000]), and/or (d) they had signs and symptoms of cholangitis necessitating emergent drainage.

Medical records were reviewed to document demographic information, presenting clinical, radiographic, and laboratory findings. The findings and techniques initially employed to verify patients' candidacy for enrollment were recorded. The findings of protocolized techniques were recorded. While findings of others studies, such as serum CA 19-9 values and follow-up noninvasive imaging, were documented, their performance was not mandated by the protocol.

A patient was considered to have a malignant stricture if there was: (a) cytologic and/or histologic evidence of malignancy based on material obtained via: EUS-guided fine-needle aspiration, ERC and tissue sampling, percutaneous biopsy, surgical exploration, or autopsy; or (b) clinical course (18 months following enrollment) suggesting malignancy based on presence of: new radiographic abnormality, including regional or distant mass (hepatic, pulmonary, or bone), mass infiltrating large blood vessels, or malignant-appearing lymphadenopathy determined by positron emission tomography; or death (death certificate diagnosis). Designation of a stricture as benign required at least 18 months of follow-up, and absence of any of the above criteria and/or follow-up imaging demonstrated complete resolution of the abnormality.

While trials evaluating the accuracy of new diagnostic tests should not include the results of the evaluated tests into the diagnostic gold standard, we did include a positive RC result, given the reported specificity of 99–100% (7, 8). This approach is widely adopted in the literature because there is no way to guarantee a true gold standard short of operative intervention in all patients. We recognize that doing so risks biasing the results in favor of RC. More importantly, DIA and FISH results were not included as components of the diagnostic gold standard.

ERC

ERC was performed (THB, CJG, MJL, BTP, and MDT) in standard fashion with the endoscopist unblinded to the clinical data. The cholangiographic appearance of the stricture

was designated as benign or malignant by the endoscopist prior to performing any interventions (dilatation, IDUS, or tissue sampling). Cholangiographic diagnosis of a malignant stricture was based on the presence of an irregular margin or shelf-like appearance, while benign processes were defined by a smoothly tapering and smooth-bordered stricture.

IDUS

Prior to tissue sampling, a guidewire was placed through the stricture. When necessary, severely narrowed strictures were dilated using a catheter (5–8 Fr; Wilson-Cook, Winston-Salem, NC) and/or balloon (4-, 6-, or 8-mm diameter/4-cm length balloon; MaxForce, Microvasive®, Boston Scientific Corp., Natick, MA). A 20-MHz US probe (Olympus® UM G20–29R, Olympus America Corporation, Inc., Center Valley, PA) was passed over the guidewire. Fluoroscopy was used to advance the transducer at least 2 cm proximal to the upper border of the stricture. The catheter was slowly withdrawn through the stricture with continuous imaging. This process was repeated at least once. The stricture was evaluated for malignancy using two separate methods. One was based on "formal" criteria (IDUS-f), for which a stricture was deemed malignant if any one of the following three criteria was met: (1) stricture hypoechoic and infiltrating (irregular outer margin); (2) stricture hypoechoic and noninfiltrating, and one or both of the following: (a) abnormal stricture morphology (asymmetry, notching, or shelf-like), or (b) suspicious lymph nodes (hypoechoic, round, and smooth-border); or (3) stricture intermediate echogenicity and infiltrating and one or both of following: (a) abnormal stricture morphology (asymmetry, notching, or shelf-like), or (b) suspicious lymph nodes (hypoechoic, round, and smooth border). The stricture was deemed benign if not satisfying any of the aforementioned criteria. Separately, the stricture was diagnosed as either benign or malignant according to the general "gestalt" of the endosonographer (IDUS-g) based on subjective and/or objective parameters and not by formal criteria. The IDUS exam was performed (MJL and MDT), as is routinely done in clinical practice, with the endosonographer unblinded to the clinical data and ERCP findings.

Sample Acquisition and Preparation

Two separate samples were collected from the biliary stricture using standard cytology brushes (DLB-35–1.5 or DLB-35–3.5 brushes; Wilson-Cook, Winston-Salem, NC). At least five passes were made through the stricture using a to-and-fro motion. To optimize the cellular yield, the brush was pushed from the end of the sheath, as opposed to pulling the brush from the sheath, and the cut brush was placed in a vial containing 20 mL PreservCyt solution (Cytyc Corporation, Marlborough, MA). In addition, the evacuated brush lumen was flushed with saline into the same solution to enhance recovery of cells. Care was taken to avoid sampling nonstrictured regions to avoid filling the brush fibers with normal mucosa, which can reduce diagnostic sensitivity. Given the varied data and uncertain benefit of initial stricture dilatation, we did not routinely dilate a stricture unless required to gain access. All specimens were transferred to the Mayo Clinic cytopathology department on the same day they were collected, and processed using the ThinPrep 2000 processor (Cytyc Corporation). A cytotechnologist equally divided the specimen, submitting half of the total sample for RC analysis and half for DIA and FISH analyses, resulting in 25% of the total sample designated for DIA analysis and 25% for FISH analysis. As per standard practice, a second gastrointestinal nurse assistant was present during tissue acquisition to help minimize time delays during tissue processing that can lead to air-drying artifacts.

RC, DIA, and FISH

Dedicated gastrointestinal (GI) cytopathologists with particular expertise for each diagnostic test independently reviewed the RC, DIA, and FISH specimens, and were blinded to the clinical records without knowledge of the other test results. RC specimens were interpreted as either positive for malignancy, suspicious for malignancy, atypical, negative for malignancy, or with inadequate cellularity for interpretation.

DIA is a form of cytologic analysis that uses spectrophotometry to quantify cellular constituents (13). Small foci of tumor cells can be analyzed as compared to flow cytometry (16, 21). DIA processing utilizes a Feulgen reaction that strips away nonnuclear material, and hydrolyzes DNA into constituent nucleic acids that stoichiometrically bind to the Feulgen dye (13) (Fig. 1A and B). ThinPrep specimens were prepared for DIA analysis as previously described (22). Up to 50 cells with the most nuclear atypia (irregular size, shape, hyperchromasia, etc.) were selected by a technologist for quantification using the CAS 200 image analyzer (Bacus Laboratories, Lombard, IL) (Fig. 2A and B). The CAS 200 captures these cells with a camera, quantifies the optical density readings, and compares these readings to the summed optical readings of rat hepatocytes (standard external control). A video camera captured the light transmitted through a glass slide specimen and converted the absorption values into pixels of variable color (white, gray, or black) (23). The absorption value was converted into an analog signal and

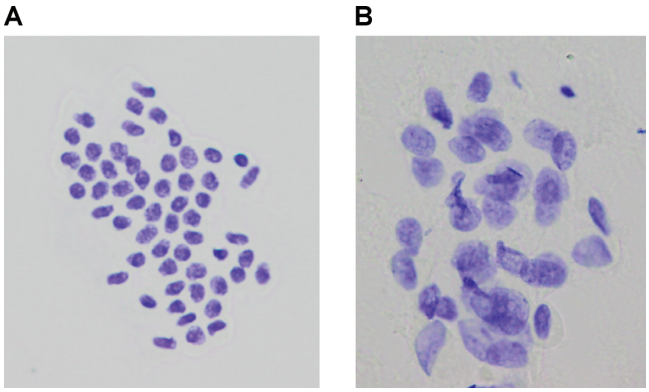

Figure 1. (*A*) Feulgen staining during digital image analysis demonstrates a benign sample. (*B*) Feulgen staining during digital image analysis reveals a malignant specimen.

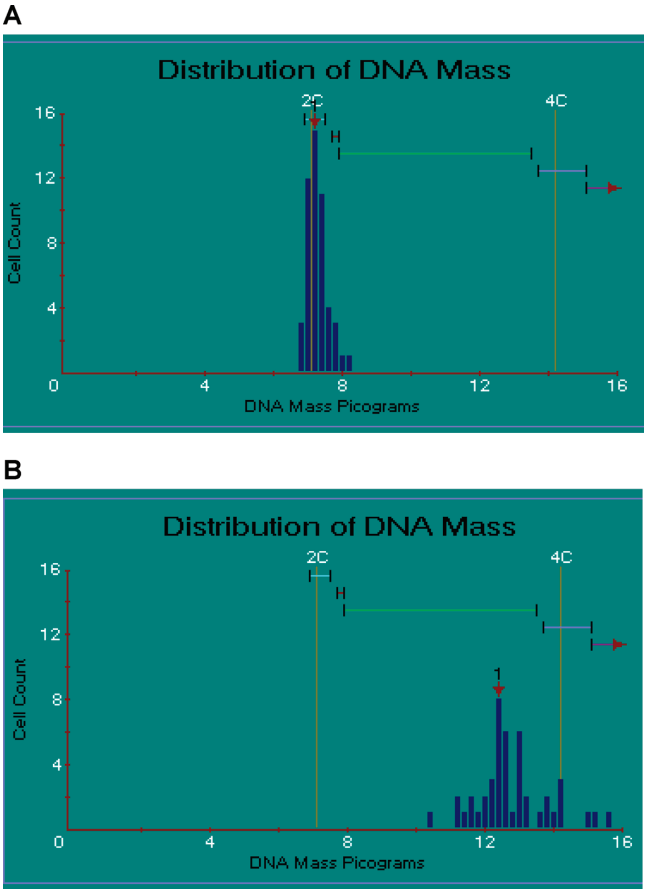

Figure 2. DNA histograms showing cell distributions based on nuclear DNA content. 2C represents cells in the diploid range, and 4C indicates tetraploid cells. Cells between 2C and 4C are considered aneuploid. (*A*) cells from a benign biliary stricture, and (*B*) cells from a malignant biliary tract stricture.

"digitized" into a series of tiny squares called picture elements or pixels. DNA ploidy status was then assigned to the collected cells based on a histogram generated by the Quantitative DNA Analysis program (Bacus Laboratories Inc., Lombard, IL). Results were categorized as diploid (DNA index between 0.95 and 1.10), aneuploid (DNA index between 1.11 and 1.89), or tetraploid (DNA index between 1.90 and 2.10). Aneuploid and tetraploid results were considered positive for malignancy (24).

FISH utilizes fluorescently labeled DNA probes to chromosomal centromeres or unique loci to detect cells that have numerical or structural abnormalities indicative of malignancy (Fig. 3A and B). The probe set used for FISH (UroVysion; Abbott Molecular, Inc., Des Plaines, IL) targets centromeres of chromosomes 3 (CEP3), 7 (CEP7), and 17 (CEP17), and band 9p21 (P16/CDKN2A gene). Slides were processed and hybridized with the probe set using the manual method as described previously (25). The slides were assessed by scanning for cytologically atypical cells, and by determining the number of CEP3, CEP7, CEP17, and 9p21 signals in those cells. In order to scan for atypical cells by FISH, the cells are assessed for patchy and lighter nuclear 4′-6-diamidino-2-phenylindole (DAPI) staining, nuclear enlargement, and irregular nuclear contour. Two general types of chromosomal abnormality were observed by FISH in this study: polysomy and trisomy of chromosome 7 (Tri-7) or 3. A patient's specimen was positive for malignancy if $\geq$5 cells showed gains of two or more of the four probes (polysomy) or if $\geq$10 cells showed three copies of chromosome 7 (or 3) and two or fewer copies of the other three probes. We set a higher cutoff for Tri-7 or Tri-3 because signal splitting can lead to false-positive trisomic signals being observed at low numbers even in normal specimens.

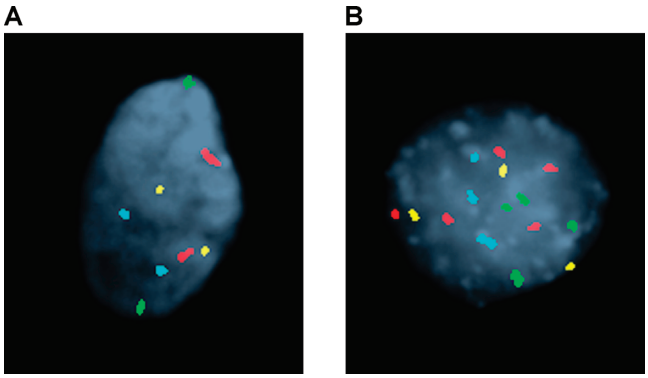

Figure 3. (*A*) Fluorescently labeled probes detect normal nuclear content (two signals per color), revealing a benign sample. (*B*) Fluorescently labeled probes detect abnormal nuclear content (>two signals per color), revealing a malignant sample.

Endoscopic Intraluminal Biopsies

Endoscopic biopsies were taken following IDUS and tissue acquisition for RC, DIA, and FISH analyses. Using fluoroscopic guidance, at least three forceps biopsies were collected from the stricture using standard biopsy forceps (FB24Q-1 or FB40Q-1; Olympus America Corporation).

STATISTICAL ANALYSIS

Each subject had six protocolized measurements of malignancy: ERC, RC, intraductal biopsy, DIA, FISH, and IDUS. In addition, CA19-9 levels, which were not protocolized, but were obtained in 80 of 86 patients, were considered as well. Each measurement was used as a diagnostic test to categorize strictures as malignant or benign. In addition to expressing these data as separate measurements, we provide findings for the composite results of DIA and FISH (DIA/FISH). Composite results for DIA and FISH were constructed by declaring the biopsy result malignant if either of the tests was interpreted as malignant, and by declaring the biopsy site benign only when both tests were interpreted as benign. Each patient served as his own control since each diagnostic modality was performed for each lesion sampled. Doing so allowed direct comparison of standard and new diagnostic techniques. For the purpose of statistical analysis, the test result was considered negative when a test was interpreted as any of the following: suspicious for malignancy, atypical, negative for malignancy, or the specimen was deemed inadequate. Data

Table 1. Patient Demographics

	Entire Group	PSC	Non-PSC
Number of patients	86	34	52
Age (mean ± standard deviation, range)	59 ± 15.6, 18–87	51 ± 13.7, 23–81	63 ± 16.4, 18–87
Gender (male/female)	51/35	20/14	31/21
Malignant stricture, N (%)	47 (55)	14 (41)	33 (63)

are provided when the cytologic interpretation of suspicious for malignancy was considered as positive for malignancy. Data were also separately analyzed by considering the presence of Tri-7 as an indicator of a benign process rather than a malignant process. We did so because our preliminary data have demonstrated that this chromosomal aberration is nearly equally indicative of benign or malignant strictures (24, 26–28).

Demographic features of study participants, as well as biopsy site features, were recorded. Continuous data are reported using descriptive statistics. Continuous variables are expressed as mean ± standard deviation (SD) or median (range). Each test was expressed in proportions in terms of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy. For each of these quantities, the exact 95% confidence interval (CI) is presented based on the binomial distribution. The statistical software package JMP Version 6 (SAS Institute, Inc., Cary, NC) was used for purposes of statistical analysis. Comparisons were performed by the Student's *t*-test. Comparisons between qualitative variables were performed using the χ^2 test or Fisher's exact test. A P value of ≤ 0.05 was considered to be statistically significant.

RESULTS

General information, patient demographics, and diagnoses are presented for the group as a whole and separately for patients with and without PSC in Tables 1 and 2, respectively. As expected, patients with PSC were younger and less likely to have malignancy than patients without PSC. The performance characteristics are provided for standard and investigational diagnostic techniques for the entire group (Table 3) and then separately for those with and without PSC (Tables 4 and 5). We should note that enrollment and variability of individual investigated factors led to a relatively limited precision of the 95% CI values.

Standard Diagnostic Techniques

CA 19-9 levels were obtained in 45 of 47 patients with malignancy, and in 35 of 39 patients with a benign stricture. The CA 19-9 level provided significantly greater sensitivity for non-PSC patients with similar specificity between groups. Similarly, the ERC findings were significantly less sensitive, but significantly more specific, for detecting malignancy in the group of patients with PSC.

As anticipated, for RC, when only unequivocally positive test results were considered diagnostic of malignancy, the sensitivity was quite low for both PSC and non-PSC patients. The sensitivity improved when suspicious cases were considered positive for malignancy. Similar to cytology, histologic review of intraductal biopsies provided very high specificity, albeit with relatively poor sensitivity. Intraductal biopsies could not be obtained in 7 patients (PSC N = 3, non-PSC N = 4). When excluding these patients, the sensitivity, specificity, and accuracy of histology for the group as a whole were 41%, 100%, and 67%, respectively. As expected, the sensitivities of cytology and histology were lower for patients with PSC, although this difference was not significant.

Investigational Diagnostic Techniques

The diagnostic sensitivity of DIA was comparable to RC for patients with PSC, and greater than RC for patients without PSC. Furthermore, the sensitivity of DIA in non-PSC patients was greater than that in the PSC group ($P < 0.05$). The specificity of DIA was excellent for PSC and non-PSC patients. FISH provided the greatest diagnostic sensitivity of any of the tissue sampling techniques, particularly when considering Tri-7 as indicative of malignancy. However, by deeming Tri-7 a marker of malignancy in PSC patients, the specificity substantially dropped, as all false-positives in this group resulted from the presence of Tri-7. Therefore, in the PSC group, it may be preferable to regard Tri-7 as a measure of benignity, which results in excellent FISH specificity, albeit with lower sensitivity. However, for patients without PSC, the consideration of Tri-7 as indicating malignancy marginally increased diagnostic sensitivity while minimally decreasing specificity. It appears that in the non-PSC group, Tri-7 results negligibly impact overall FISH results. While providing 100% specificity, the composite DIA/FISH result (when considering Tri-7 as a marker of benign disease) increased diagnostic sensitivity for PSC patients one- to fivefold over

Table 2. Stricture Diagnosis (N = 86)

Etiology	N (%)
Malignant (N = 47, 55%)	
Cholangiocarcinoma	37 (43)
Pancreatic cancer	4 (5)
Gallbladder cancer	3 (3)
Intraductal biliary tumor	2 (2)
Metastatic gastric	1 (1)
Benign (N = 39, 45%)	
Primary sclerosing cholangitis	21 (24)
Inflammation	9 (10)
Chronic pancreatitis	4 (5)
Autoimmune pancreatitis	4 (5)
Surgical trauma	1 (1)

Table 3. Performance Characteristics for Entire Group (PSC and Non-PSC)

			Cytology				FISH		DIA/FISH		IDUS	
Private	CA 19-9 ≥100 = M	ERC	M = M	S&M = M	Histology	DIA	Tri-7 = B	Tri-7 = M	Tri-7 = B	Tri-7 = M	(Formal)	(Gestalt)
Sensitivity (%)	42	77	11	32	38	38	45	64	55	70	89	87
(95% CI)	(0.28–0.57)	(0.65–0.89)	(0.02–0.20)	(0.19–0.45)	(0.24–0.52)	(0.24–0.52)	(0.31–0.59)	(0.50–0.78)	(0.41–0.70)	(0.57–0.83)	(0.81–0.98)	(0.78–0.97)
Specificity (%)	91	79	97	97	100	95	100	82	100	82	64	92
(95% CI)	(0.82–1.00)	(0.67–0.92)	(0.93–1.00)	(0.93–1.00)	(1.00–1.00)	(0.88–1.00)	(1.00–1.00)	(0.70–0.94)	(1.00–1.00)	(0.70–0.94)	(0.47–0.79)	(0.84–1.00)
PPV (%)	86	82	83	94	100	90	100	81	100	83	75	93
(95% CI)	(0.72–1.00)	(0.70–0.93)	(0.54–1.00)	(0.82–1.00)	(1.00–1.00)	(0.77–1.00)	(1.00–1.00)	(0.69–0.94)	(1.00–1.00)	(0.71–0.94)	(0.64–0.86)	(0.86–1.00)
NPV (%)	55	74	48	54	57	56	60	65	65	70	83	86
(95% CI)	(0.34–0.76)	(0.61–0.87)	(0.08–0.88)	(0.30–0.79)	(0.35–0.80)	(0.34–0.78)	(0.39–0.81)	(0.50–0.81)	(0.47–0.83)	(0.55–0.84)	(0.74–0.93)	(0.75–0.96)
Accuracy (%)	64	78	50	62	66	64	70	72	76	76	78	90
(95% CI)	(0.53–0.74)	(0.69–0.87)	(0.39–0.61)	(0.51–0.72)	(0.56–0.76)	(0.54–0.74)	(0.60–0.80)	(0.63–0.82)	(0.67–0.85)	(0.67–0.85)	(0.69–0.87)	(0.83–0.96)

B = benign; M = malignant; S&M = suspicious and malignant.

Table 4. Performance Characteristics for Primary Sclerosing Cholangitis (PSC) Group

			Cytology				FISH		DIA/FISH		IDUS	
Private	CA 19-9 ≥100 = M	ERC	M = M	S&M = M	Histology	DIA	Tri-7 = B	Tri-7 = M	Tri-7 = B	Tri-7 = M	(Formal)	(Gestalt)
Sensitivity (%)	14	36	7	29	29	21	29	64	36	64	71	64
(95% CI)	(0.00–0.33)	(0.11–0.61)	(0.06–0.21)	(0.05–0.52)	(0.05–0.52)	(0.00–0.43)	(0.05–0.52)	(0.39–0.89)	(0.11–0.61)	(0.39–0.89)	(0.48–0.95)	(0.39–0.89)
Specificity (%)	95	95*	100	100	100	95	100	70	100	70	55	95
(95% CI)	(0.85–1.00)	(0.85–1.00)	(1.00–1.00)	(1.00–1.00)	(1.00–1.00)	(0.85–1.00)	(1.00–1.00)	(0.50–0.90)	(1.00–1.00)	(0.50–0.90)	(0.33–0.77)	(0.85–1.00)
PPV (%)	67	83	100	100	100	75	100	60	100	60	53	90
(95% CI)	(0.13–1.00)	(0.54–1.00)	(1.00–1.00)	(1.00–1.00)	(1.00–1.00)	(0.33–1.00)	(1.00–1.00)	(0.35–0.85)	(1.00–1.00)	(0.35–0.85)	(0.30–0.75)	(0.71–1.00)
NPV (%)	60	68	61	67	67	63	67	74	69	74	73	79
(95% CI)	(0.5–1.00)	(0.31–1.00)	(0.35–1.00)	(0.21–1.00)	(0.21–1.00)	(0.16–1.00)	(0.21–1.00)	(0.51–0.96)	(0.28–1.00)	(0.51–0.96)	(0.53–0.93)	(0.54–1.00)
Accuracy (%)	61	71	62	71	71	65	71	68	74	68	62	82
(95% CI)	(0.44–0.77)	(0.85–1.00)	(0.45–0.78)	(0.55–0.86)	(0.55–0.86)	(0.49–0.81)	(0.55–0.86)	(0.52–0.83)	(0.59–0.88)	(0.52–0.49)	(0.45–0.78)	(0.70–0.95)

Table 5. Performance Characteristics for Non-PSC Group

	Cytology				FISH		DIA/FISH		IDUS	
Private	CA 19-9 ≥100 = M	ERC	M = M	S&M = M	Histology	DIA	Tri-7 = B	Tri-7 = M	Tri-7 = B	Tri-7 = M	(Formal)	(Gestalt)
Sensitivity (%)	55*	94*	12	33	42	45	52	64	64*,†	73	97*	97*
(95% CI)	(0.37–0.72)	(0.86–1.00)	(0.01–0.23)	(0.17–0.49)	(0.26–0.59)	(0.29–0.62)	(0.35–0.69)	(0.47–0.80)	(0.47–0.80)	(0.58–0.88)	(0.91–1.00)	(0.91–1.00)
Specificity (%)	88	63*	95	95	100	95	100	95	100	95	74	89
(95% CI)	(0.71–1.00)	(0.42–0.85)	(0.85–1.00)	(0.85–1.00)	(1.00–1.00)	(0.85–1.00)	(1.00–1.00)	(0.85–1.00)	(1.00–1.00)	(0.85–1.00)	(0.54–0.94)	(0.76–1.00)
PPV (%)	90	82	80	92	100	94	100	96	100	96	87	94
(95% CI)	(0.76–1.00)	(0.69–0.94)	(0.45–1.00)	(0.76–1.00)	(1.00–1.00)	(0.82–1.00)	(1.00–1.00)	(0.87–1.00)	(1.00–1.00)	(0.88–1.00)	(0.76–0.98)	(0.87–1.00)
NPV (%)	50	86	38	45	50	50	54	60	61	67	93	94
(95% CI)	(0.28–0.73)	(0.75–0.97)	(0.04–0.81)	(0.17–0.73)	(0.24–0.76)	(0.26–0.75)	(0.31–0.78)	(0.40–0.81)	(0.41–0.82)	(0.48–0.85)	(0.85–1.00)	(0.87–1.00)
Accuracy (%)	66	83	42	56	63	63	69	75	77	81	88	94
(95% CI)	(0.52–0.80)	(0.72–0.93)	(0.29–0.56)	(0.42–0.69)	(0.50–0.77)	(0.50–0.77)	(0.57–0.55)	(0.63–0.87)	(0.66–0.88)	(0.70–0.92)	(0.80–0.97)	(0.88–1.00)

Significance was calculated for each test in terms of their sensitivity and specificity between groups (*i.e.*, PSC *vs* non-PSC patients).
*Statistical significance ($P < 0.05$) present when compared to the same test in the alternate group of patients (*i.e.*, PSC *vs* non-PSC groups).
†Statistical significance ($P < 0.05$) for DIA/FISH compared to RC, regardless if suspicious for malignancy is considered a positive or negative result.

that for cytology, and in patients without PSC was two- to fivefold greater, depending on how suspicious cytology results were interpreted. In non-PSC patients, the sensitivity provided by the DIA/FISH composite result was significantly greater than that for RC, regardless of how a suspicious cytology interpretation is classified.

We evaluated IDUS using preset "formal" criteria (IDUS-f) and based on the endosonographers' general "gestalt" (IDUS-g) for distinguishing the stricture type. As one might expect, the use of preset criteria led to disparate results for the PSC and non-PSC groups. In patients with PSC, IDUS-f was significantly less sensitive for identifying malignant strictures, and provided lower specificity compared to that for the non-PSC group. However, even in non-PSC patients, the specificity of IDUS-f was suboptimal. On the contrary, IDUS-g provided moderate sensitivity and high specificity for patients with PSC. More importantly, IDUS-g demonstrated excellent sensitivity and specificity for patients without PSC.

Negative Cytology and Histology Group

Likely, the most important issue regarding these investigational studies is their ability to diagnose malignancy in patients with negative cytology (even when considering cytologically suspicious cases as benign) and histology who are later proven to have malignancy based on the diagnostic gold standard (N = 21). These data are presented for the entire study group in Table 6, and separately for patients with and without PSC (Tables 7 and 8). For the entire group (PSC and non-PSC), despite negative cytology and histology, a diagnosis of malignancy was established by DIA, FISH, composite DIA/FISH, and IDUS in 14%, 62%, 67%, and 86% of cases, respectively.

The CA 19-9 level was significantly more sensitive in patients without PSC, but failed to detect malignancy in patients with PSC. The ERC findings poorly distinguished the stricture type. Individual and composite DIA and FISH (with Tri-7 considered benign) results provided low-to-moderate sensitivity, but excellent specificity for patients with and without PSC. While IDUS-f results were inadequate, IDUS-g proved both sensitive and specific for patients with and without PSC.

DISCUSSION

Because of the relative inability of RC and intraductal biopsy to distinguish benign from malignant bile duct strictures, there has been a pursuit for improved cytologic techniques, such as DIA and FISH, and improved imaging studies, including IDUS (7, 8, 29, 30). We have previously studied these new tests independently, and demonstrated that DIA, FISH, and IDUS each improve the accuracy in diagnosis of bile duct strictures (11, 24, 26–28). The present study differed from our prior experience in that it is the first formal, prospective study directly comparing all these technologies. In addition, we restricted enrollment to a difficult-to-diagnose group of

Table 6. Performance Characteristics When Cytology and Histology Are Negative for "Malignancy" or "Suspicious for Malignancy" in Total Group

Private	CA 19-9 ≥100 = M	ERC	DIA	FISH Tri-7 = B	FISH Tri-7 = M	DIA/FISH Tri-7 = B	DIA/FISH Tri-7 = M	IDUS (Formal)	IDUS (Gestalt)
Sensitivity (%)	35	71	14	24	62	33	67	90	86
(95% CI)	(0.14–0.56)	(0.52–0.91)	(0–0.29)	(0.06–0.42)	(0.41–0.83)	(0.13–0.54)	(0.47–0.87)	(0.78–1.00)	(0.71–1.00)
Specificity (%)	94	79	95	100	79	95	74	63	92
(95% CI)	(0.85–1.00)	(0.66–0.92)	(0.88–1.00)	(1.00–1.00)	(0.66–0.92)	(0.88–1.00)	(0.60–0.88)	(0.48–0.79)	(0.84–1.00)
PPV (%)	78	65	60	100	62	78	58	58	86
(95% CI)	(0.51–1.00)	(0.46–0.85)	(0.17–1.00)	(1.00–1.00)	(0.41–0.83)	(0.51–1.00)	(0.39–0.78)	(0.41–0.74)	(0.71–1.00)
NPV (%)	70	83	67	70	79	72	80	92	92
(95% CI)	(0.40–0.99)	(0.68–0.99)	(0.25–1.00)	(0.30–1.00)	(0.62–0.86)	(0.43–1.00)	(0.64–0.96)	(0.83–1.00)	(0.81–1.00)
Accuracy (%)	71	76	66	73	73	73	71	73	90
(95% CI)	(0.59–0.84)	(0.65–0.87)	(0.54–0.78)	(0.62–0.84)	(0.62–0.84)	(0.62–0.84)	(0.60–0.83)	(0.62–0.84)	(0.82–0.98)

patients with indeterminate bile duct strictures based on strict enrollment criteria meant to exclude patients with easily diagnosed pathology.

Standard Diagnostic Techniques

Our finding of lower sensitivity in the PSC group is likely accounted for by the greater degree of fibrosis, inflammation, and relatively inaccessibility of strictures in this population relative to the non-PSC group. On the contrary, there is no clear rationale for why CA 19-9 levels provided lower sensitivity but higher specificity in patients with PSC, and this is contrary to that reported in the literature (31, 32).

The sensitivity of RC was very low when atypical and suspicious cytologic results were excluded and only an unequivocally positive test result was considered adequate for diagnosis of malignancy. The sensitivity of RC in our study is within the lower range of what has been published (8, 29, 33). There are a number of factors that may account for the discrepancy in reported sensitivities among different centers. Lower sensitivity rates are typically found in tertiary referral centers that tend to evaluate a select population, often following a negative initial evaluation elsewhere. In addition, the threshold for diagnosis likely varies among pathologists and medical centers. We recognize that the threshold for diagnosis may be greater in our center as evidenced by the fact that when we analyzed our results that were interpreted as suspicious for malignancy as positive for malignancy, the specificity was unchanged. In addition, our method of sample acquisition and processing may have impacted the findings. We collected and combined two samples from each biliary stricture. The specimen was then equally divided, submitting half of the total sample for RC analysis and half for DIA and FISH analyses, which resulted in 25% of the total sample designated for DIA analysis and 25% for FISH analysis. While many centers routinely obtain one brush specimen for RC, as we did, a few centers collect two brush specimens. It is possible that the sensitivity of RC would have been greater if a greater number of samples had been obtained. However, one would also expect that the sensitivities of DIA and FISH would have increased as well. Perhaps most significant in terms of impact on diagnostic sensitivity is that our study population was a highly selected group of patients with "indeterminate" strictures defined by strict enrollment criteria.

The inclusion of suspicious cytology cases as a marker of malignancy is expected to enhance diagnostic sensitivity, but risks compromising specificity. Practice varies regarding how to categorize RC specimens interpreted as suspicious for malignancy (29, 34). In deciding how best to interpret this finding (as malignant or benign), one must balance the impact of overdiagnosis resulting in unnecessary interventions

Table 7. Performance Characteristics When Cytology and Histology Are Negative for "Malignancy" or "Suspicious for Malignancy" in PSC Group

Private	CA 19-9 ≥100 = M	ERC	DIA	FISH Tri-7 = B	FISH Tri-7 = M	DIA/FISH Tri-7 = B	DIA/FISH Tri-7 = M	IDUS (Formal)	IDUS (Gestalt)
Sensitivity (%)	0	43	14	14	57	29	71	86	71
(95% CI)	(0.00–0.00)	(0.06–0.80)	(0–0.40)	(0–0.40)	(0.21–0.94)	(0–0.62)	(0.38–1.00)	(0.60–1.00)	(0.38–1.00)
Specificity (%)	100	90	95	100	71	95	67	52	95
(95% CI)	(1.00–1.00)	(0.78–1.00)	(0.86–1.00)	(1.00–1.00)	(0.52–0.91)	(0.86–1.00)	(0.47–0.87)	(0.31–0.74)	(0.86–1.00)
PPV (%)	–	60	50	100	40	67	42	38	83
(95% CI)		(0.17–1.00)	(0.00–1.00)	(1.00–1.00)	(0.10–0.70)	(0.13–1.00)	(0.14–0.70)	(0.14–0.61)	(0.54–1.00)
NPV (%)	–	83	77	78	83	80	88	92	91
(95% CI)		(0.49–1.00)	(0.19–1.00)	(0.00–1.00)	(0.60–1.00)	(0.35–1.00)	(0.69–1.00)	(0.78–1.00)	(0.68–1.00)
Accuracy (%)	77	79	75	79	68	79	68	61	89
(95% CI)	(0.61–0.93)	(0.63–0.94)	(0.59–0.91)	(0.63–0.94)	(0.51–0.85)	(0.63–0.94)	(0.51–0.85)	(0.43–0.79)	(0.78–1.00)

Table 8. Performance Characteristics When Cytology and Histology Are Negative for "Malignancy" or "Suspicious for Malignancy" in Non-PSC Group

Private	CA 19–9 ≥100 = M	ERC	DIA	FISH Tri 7 = B	FISH Tri 7 = M	DIA/FISH Tri 7 = B	DIA/FISH Tri 7 = M	IDUS (Formal)	IDUS (Gestalt)
Sensitivity (%)	50*	86	14	29	64	36	64	93	93
(95% CI)	(0.24–0.76)	(0.67–1.00)	(0.00–0.33)	(0.05–0.52)	(0.39–0.89)	(0.11–0.61)	(0.39–0.89)	(0.79–1.00)	(0.79–1.00)
Specificity (%)	83	65	94	100	88	94	82	76	88
(95% CI)	(0.62–1.00)	(0.42–0.87)	(0.83–1.00)	(1.00–1.00)	(0.73–1.00)	(0.83–1.00)	(0.64–1.00)	(0.56–0.97)	(0.73–1.00)
PPV (%)	78	67	67	100	82	83	75	77	87
(95% CI)	(0.51–1.00)	(0.45–0.88)	(0.13–1.00)	(1.00–1.00)	(0.59–1.00)	(0.54–1.00)	(0.51–0.99)	(0.56–0.97)	(0.70–1.00)
NPV (%)	59	85	57	63	75	64	74	93	94
(95% CI)	(0.27–0.91)	(0.70–1.00)	(0.01–1.00)	(0.16–1.00)	(0.49–1.00)	(0.26–1.00)	(0.49–0.99)	(0.81–1.00)	(0.82–1.00)
Accuracy (%)	65	74	58	68	77	68	74	84	90
(95% CI)	(0.47–0.84)	(0.59–0.90)	(0.41–0.75)	(0.51–0.84)	(0.63–0.92)	(0.51–0.84)	(0.59–0.90)	(0.71–0.97)	(0.80–1.00)

Significance was calculated for each test in terms of their sensitivity and specificity between groups (*i.e.*, PSC *vs* non-PSC patients).
*Statistical significance ($P < 0.05$) present when compared to the same test in the alternate group of patients (*i.e.*, PSC *vs* non-PSC groups).

against the benefit of earlier diagnosis, which may potentially lead to earlier therapy and potentially improved outcomes. Other factors must be considered, including the performance characteristics of a specific test, patient characteristics (*e.g.*, age, health status, and suspected pathology), and the invasiveness and risks of planned therapies, when deciding whether to consider a suspicious finding as benign or malignant. Our data support the practice of designating cytology interpreted as suspicious for malignancy as equivalent to a positive finding, given the improved sensitivity while maintaining specificity.

Investigational Diagnostic Techniques

The low sensitivity of DIA in the PSC group (and moderate sensitivity in the non-PSC group) but high specificity suggests that DIA has a role similar to that of RC. A positive result for either test can be viewed as reliable for diagnosing cancer, but a negative test is of little value. The FISH probe results, other than Tri-7, accurately distinguish malignant from benign strictures; the presence of Tri-7 in patients with PSC typically represents a false-positive finding. These findings suggest that Tri-7 has limited utility in patients with PSC. However, the moderately enhanced sensitivity when using Tri-7 as a marker of malignancy results in only a slight decrease in specificity. Therefore, depending on one's desired threshold for diagnosis and therapy, Tri-7 maintains an important diagnostic role in non-PSC patients. Importantly, in patients without PSC, the DIA/FISH composite result (when considering Tri-7 as a marker of benign disease) maintained 100% specificity while providing a significant increase in sensitivity over RC, regardless of how suspicious for malignancy findings on RC were interpreted.

In previous studies, the reported accuracy of IDUS in distinguishing benign from malignant strictures ranged between 76 and 90% (9–11). We evaluated IDUS in terms of "formal" predefined criteria (IDUS-f) and based on the endosonographers' general "gestalt" (IDUS-g). Other than the high diagnostic sensitivity for patients with PSC, we found that IDUS-f provided insufficient accuracy in distinguishing malignant from benign strictures. Given these data, IDUS-f finding are unlikely to be considered relevant to hepatologists and surgeons when managing patients with indeterminate strictures. However, IDUS-g was far more accurate, especially in the non-PSC group. As for many applications of ultrasound (including endoscopic ultrasound and IDUS), one cannot rely solely on specific criteria. Furthermore, individual IDUS-f criteria, such as echodensity, stricture thickness, infiltration, and presence of lymph nodes, did not accurately predict malignancy. It appears that the overall experience and general gestalt of an endosonographer are of more value than a set of formal criteria. While the IDUS-g findings may be of value within a particular center, the broad applicability and reproducibility of our findings to other centers are likely limited.

The superior accuracy of IDUS-g *versus* IDUS-f is likely accounted for by several factors. IDUS was performed with knowledge of the patients' clinical presentation and ERC findings, which is expected to influence the interpretation, and may introduce bias in favor of both ERC and IDUS-g. However, this is also the manner in which these procedures are performed in clinical practice. In contrast, pathologists were blinded to all clinical and laboratory data. In addition, one might expect the poor accuracy of IDUS-f for the PSC group, given the nature of this disease, which is often associated with diffuse stricturing of the biliary tree. Use of IDUS-f requires that the dominant stricture be considered in isolation without reference to other strictures. However, IDUS-g allows one to examine both the stricture of interest and other strictures to determine if they differ enough to distinguish the stricture type. In our experience, many of the strictures in PSC appear malignant based on IDUS-f, and only upon comparing strictures relative to one another may such a distinction be made. Use of formal criteria does not allow for this important assessment tool.

Negative Cytology and Histology Group

Because of the nearly 100% reported specificity of cytology and histology, one can regard a positive result of either as a reliable indicator of malignancy. In this setting,

investigational tests, such as DIA, FISH, and IDUS, are not needed and a negative DIA, FISH, or IDUS test result would be misleading. The more important question concerns the sensitivity and specificity of these investigational techniques in patients with presumed malignancy with negative histology and cytology. DIA and FISH results (with Tri-7 considered benign), when viewed in isolation or as part of a composite result, provided low-to-moderate sensitivities. However, when one considers the excellent specificity in this setting, the presence of a positive finding may be viewed as a reliable marker of malignancy. While IDUS-f results were unsatisfactory for both PSC and non-PSC patients, the accuracy of IDUS-g was superior, and the results may be of use in evaluating both groups of patients.

We now routinely perform DIA, FISH, and IDUS-g in all patients undergoing ERC for evaluation of an indeterminate bile duct stricture. As with any diagnostic study, one must consider the performance characteristics for a specific test, and interpret the findings with caution. In our practice, for both PSC and non-PSC patients, we view positive DIA and FISH results (with Tri-7 considered benign) as diagnostic for malignancy. Due to the risk of false-positive Tri-7 results in patients with PSC, we do not consider a positive Tri-7 result alone as a marker of malignancy. Although, the finding of Tri-7 positivity in non-PSC does increase sensitivity without a significant drop in specificity, the decrement in specificity is sufficient enough to raise concern for underlying malignancy. We use this finding of isolated Tri-7 as an indication for closer monitoring with repeat ERC and tissue sampling 1–6 months later, depending on the degree of clinical suspicion. When doing so, approximately 50% of patients with isolated Tri-7 positivity are diagnosed with a malignant stricture within the following year.

We view IDUS-g findings in a manner similar to that of ERC findings. While these imaging studies may suggest that a stricture is benign or malignant, many clinicians are reluctant to base management decisions on imaging findings alone. In our practice, when IDUS-g findings suggest malignancy, but all other diagnostic tests are negative, patients undergo close monitoring with repeat ERC and tissue sampling 1–6 months later depending on the degree of clinical suspicion. IDUS-f criteria are currently disregarded other than for investigational purposes.

In summary, our data suggest a lack of utility when using formal IDUS criteria for distinguishing benign from malignant biliary strictures. However, DIA, FISH, and IDUS-g enhance the accuracy of standard techniques in determining the cause of indeterminate bile duct strictures, allowing diagnosis of malignancy in a substantial number of patients with false-negative cytology and histology. These findings support the routine use of these newer diagnostic modalities in all patients with indeterminate biliary strictures. Future study is needed to determine whether these new diagnostic modalities impact patient prognosis, staging, and clinical outcomes.

STUDY HIGHLIGHTS

What Is Current Knowledge

- Standard techniques for evaluating bile duct strictures have poor sensitivity for detection of malignancy.
- Routine cytology (RC) and histology yield a high specificity, but low sensitivity, for determining the etiology of bile duct strictures.

What Is New Here

- Newer imaging modalities, such as intraductal ultrasound (IDUS), and advanced cytologic techniques, such as digital image analysis (DIA) and fluorescence *in situ* hybridization (FISH), identify chromosomal abnormalities and improve sensitivity while maintaining high specificity.
- Composite DIA/FISH results (when considering trisomy 7 [Tri-7] as a marker of benign disease) yielded 100% specificity and increased sensitivity one- to fivefold in patients with primary sclerosing cholangitis (PSC) *versus* RC and two- to fivefold in patients without PSC (depending on how suspicious cytology results were interpreted).
- For patients with negative cytology and histology who were later proven to have malignancy (N = 21), DIA, FISH, composite DIA/FISH, and IDUS allowed diagnosis of malignancy in 14%, 62%, 67%, and 86%, respectively.
- DIA, FISH, and IDUS enhance the accuracy of standard techniques in evaluation of indeterminate bile duct strictures, allowing diagnosis of malignancy in a substantial number of patients with false-negative cytology and histology.

Reprint requests and correspondence: Michael J. Levy, M.D., Director of Endoscopic Ultrasound, Mayo Clinic College of Medicine, Division of Gastroenterology and Hepatology, 200 First Street SW, Charlton 8, Rochester, MN 55905.

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CONFLICT OF INTEREST

Guarantor of the article: Michael J. Levy, M.D.
Specific author contributions: concept and design: Michael J. Levy, Amy C. Clayton, Felicity B. Enders, Kevin C. Halling, Benjamin R. Kipp, Ashwin Rumalla, Thomas J. Sebo, Maurits J. Wiersema, and Gregory J. Gores; data acquisition: Michael J. Levy, Todd H. Baron, Amy C. Clayton, Christopher J. Gostout, Kevin C. Halling, Benjamin R. Kipp, Bret T. Petersen, Ashwin Rumalla, Thomas J. Sebo, Mark D. Topazian, and Gregory J. Gores; and data analysis and interpretation and manuscript drafting and critical revising: Michael J. Levy, Todd H. Baron, Amy C. Clayton, Felicity B. Enders, Christopher J. Gostout, Kevin C. Halling, Benjamin R. Kipp, Bret T. Petersen, Lewis R. Roberts, Thomas J. Sebo, Mark D. Topazian, Maurits J. Wiersema, and Gregory J. Gores.
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