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Comparison of Methods to Detect Neoplasia in Patients Undergoing Endoscopic Ultrasound-Guided Fine-Needle Aspiration

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See editorial on page 1055.

BACKGROUND & AIMS: Digital image analysis (DIA) and fluorescence in situ hybridization (FISH) can be used to evaluate biliary strictures with greater accuracy than conventional cytology (CC). We performed a prospective evaluation of the accuracy of CC, compared with that of DIA and FISH, in detection of malignancy in patients undergoing endoscopic ultrasonography (EUS) fine-needle aspiration (FNA). **METHODS:** We collected a minimum of 6 FNA samples from each of 250 patients during EUS. CC or DIA and FISH analyses were performed on every other specimen (from every other FNA pass); patients were randomly assigned to the first test performed. CC slides were reviewed by gastrointestinal cytopathologists who were blinded to all data. Findings from cytohistologic analysis, after a minimum 24-month follow-up period, were used as the standard (n = 202; median age, 65 years). **RESULTS:** Aspirates were collected from lymph nodes (n = 111), pancreas (n = 61), gastrointestinal lumen wall (n = 9), periluminal mass (n = 4), liver (n = 8), and miscellaneous sites (n = 9). Matched samples provided a mean of 3.2 passes for CC and 1.6 passes for DIA and FISH. The data indicate a potential lack of utility for DIA. The combination of CC and FISH detected malignancy with 11% greater sensitivity than CC alone (P = .0002), but specificity was reduced from 100% to 96%. **CONCLUSIONS: FISH analysis identifies neoplastic lesions with significantly greater sensitivity than CC in patients with diverse pathologies who underwent EUS with FNA, despite limited tissue sampling for FISH analysis.**

Keywords: Cancer Detection; Molecular Cytogenetic Marker; Diagnostic; Pathology.

Endoscopic ultrasonography (EUS) is routinely used to evaluate intraintestinal and extraintestinal mass lesions and lymphadenopathy. The diagnostic accuracy of EUS-guided fine-needle aspiration (FNA) with conventional cytology (CC) is 60%–90%.[1–7] By enhancing diagnostic sensitivity, staging accuracy, and prognostic determination,[1,8] EUS FNA helps guide patient care and improves outcomes.[8–10] As a result, EUS FNA has become an essential component in the evaluation of a variety of gastrointestinal and nongastrointestinal disorders.

CC has high specificity but poor sensitivity.[1,11,12] This has driven the pursuit of new technologies such as digital image analysis (DIA) and fluorescence in situ hybridization (FISH) with potentially higher sensitivity to detect malignancy/neoplasia by assessing nuclear DNA content and the presence of aneusomy (ie, abnormal chromosome copy number), respectively.[13,14] These tests have the ability to identify malignant cells in samples of limited cellularity and yield greater diagnostic sensitivity than CC alone.[14,15] DIA and FISH were initially investigated for the detection of bladder cancer at our institution. However, because most solid tumors are characterized by numerical and structural chromosomal abnormalities,[16,17] DIA and FISH should also be able to detect cells that have chromosomal abnormalities consistent with neoplasia in exfoliative and aspiration gastrointestinal cytology specimens. We tested this hypothesis on endoscopic retrograde cholangiography (ERC) brush biopsy samples collected to evaluate indeterminate bile duct strictures and found that DIA and FISH provided greater diagnostic accuracy than CC in distinguishing benign from malignant strictures.[14,18–22] FISH is now a standard test used in our practice to help guide clinical decision making.

We speculated that assessing for numeric chromosomal alterations may also enhance the diagnostic accuracy of CC for samples collected at EUS FNA, based on the fact that numerical chromosomal alterations (aneuploidy) are observed among different cancer types. In a pilot study

Abbreviations used in this paper: CC, conventional cytology; DIA, digital image analysis; ERC, endoscopic retrograde cholangiography; EUS, endoscopic ultrasonography; FISH, fluorescence in situ hybridization; FNA, fine-needle aspiration; QNS, quantity not sufficient.

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involving 39 patients who underwent EUS FNA, we showed enhanced diagnostic accuracy for the composite DIA/FISH results over CC alone.[23] In the current study, we sought to more rigorously evaluate these techniques and test the hypothesis that the accuracy of the composite DIA/FISH result is greater than that of CC when applied to EUS FNA specimens from patients with a diverse spectrum of disease processes.

Patients and Methods

Patients

We prospectively enrolled patients referred for EUS FNA (1) who had known or suspected luminal or extraluminal malignancy and (2) in whom the endosonographer deemed the target lesion safe and feasible to allow the necessary study passes. Patients were excluded for the following reasons: (1) inability to provide informed consent, (2) anticipated unavailability or patients declined follow-up, and/or (3) coagulopathy (international normalized ratio >1.5) and/or thrombocytopenia (platelet count $<50 \times 10^9$/L).

The institutional review board granted approval for this prospective study and informed consent was obtained for all procedures, including DIA and FISH. Information concerning the presentation, clinical course, and outcomes was abstracted from the medical records and patient interviews. A patient was considered to have malignancy if there was (1) cytological and/or histologic evidence of malignancy based on material obtained via EUS FNA, ERC and tissue sampling, percutaneous biopsy, surgery, or autopsy; (2) a clinical course ($\geq$24 months) suggesting malignancy based on the presence of a new radiographic abnormality, including regional or distant mass (hepatic, pulmonary, or bone), mass infiltrating large blood vessels, or malignant-appearing lymphadenopathy with positive positron emission tomography imaging; or (3) cancer-related mortality. Designation of a lesion as benign required at least 24 months of follow-up and absence of any of the previously described criteria and/or follow-up imaging showing complete resolution of the abnormality. DIA and FISH findings were excluded from the medical records and did not affect patient care.

EUS FNA and Sample Processing

EUS FNA and CC, DIA, and FISH processing were performed with a 22-gauge needle (Echotip; Cook Medical, Winston-Salem, NC) using approximately 5 mL of negative pressure and standard techniques as previously described.[1,2,18,20,24–26] Six FNA samples were obtained from each patient during EUS using a 22-gauge needle (Echotip; Cook Medical). Additional passes were obtained at the discretion of the endosonographer and in-room cytotechnologist. CC or DIA/FISH analysis were performed on every other specimen (ie, every other FNA pass) with patients blindly randomized to the first test performed. Additional DIA/FISH passes were matched with additional CC passes in a 1:1 fashion as previously outlined. Primary statistical analysis included only matched samples. Our study protocol mandated that at least 3 passes be obtained each for CC and DIA/FISH. The DIA/FISH specimens were evenly divided for subsequent FISH and DIA analysis. Therefore, twice as much material was available for CC review as compared with DIA and FISH. This protocol was adopted to determine the accuracy of DIA and FISH sampling in a manner that limited additional passes to maintain safety. However, this approach biased sample acquisition and analysis in favor of CC. To help evaluate the impact of limiting DIA and FISH sampling relative to CC, the sample adequacy was graded for each specimen.

Although the study mandated 3 study passes for CC and DIA/FISH, additional passes could be obtained at the discretion of the endosonographer and in-room cytotechnologist. Additional DIA/FISH passes were matched by CC passes in a 1:1 fashion as previously outlined. To optimize patient care, the study allowed additional unmatched passes for CC. Primary statistical analysis included only matched samples. Gastrointestinal cytopathologists with expertise in each diagnostic modality reviewed the specimens while blinded to DIA/FISH results and follow-up. Specifically, CC, DIA, and FISH were each interpreted independently and without knowledge of the result for the other evaluated diagnostic modalities. All pathology interpretations and the assessment of the gold standard were conducted by different physicians who were completely blinded to the alternate data.

Dia

DIA is a form of cytologic analysis that quantifies cellular constituents by using spectrophotometry.[13] Small foci of tumor cells can be analyzed, unlike the large number of cells required for flow cytometry.[15] DIA processing uses the Feulgen reaction, which strips away non-nuclear material and hydrolyzes DNA into its constituent nucleic acids, which stoichiometrically bind to the Feulgen dye.[13] ThinPrep specimens (Hologic, Marlborough, MA) were prepared as previously described.[27] Up to 50 cells with the most nuclear atypia were selected for quantification using the CAS 200 image analyzer (Bacus Laboratories, Lombard, IL), which captures these cells and quantifies the optical density and compares these readings with the summed optical readings of the standard control. A video camera captured the transmitted light and converted the absorption values into an analog signal and "digitized" pixels of variable color.[28] DNA ploidy status was assigned based on a computer-generated histogram (Figure 1*A* and *B*). Cases were diagnosed as positive for malignancy if the histograms showed a clonal population of cells beyond a DNA index of 1.10 as previously described.[29]

Fluorescence In Situ Hybridization

FISH uses fluorescently labeled DNA probes to chromosomal centromeres or unique loci to detect cells that have numerical or structural abnormalities indicative of malignancy. The probe set used (UroVysion; Abbott Molecular Inc, Des Plaines, IL) targets centromeres of chromosomes 3 (CEP3), 7 (CEP7), and 17 (CEP17) and band 9p21 (P16/CDKN2A locus). Slides were processed and hybridized with the probe set as previously described.[17] 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. Specimens were considered positive for malignancy if they showed gains of 2 or more chromosomes in 5 or more cells (ie, "polysomy"), homozygous chromosomal loss of the 9p21 locus in $>$20% of cells, a single copy of one chromosome (ie, "monosomy") in $>$20% of the cells, or gains of one chromosome in 10 or more cells. Hemizygous 9p21 (single copy) was equivocal and considered negative for data analysis (Figure 2*A* and *B*).

Statistical Analysis

We hypothesized that the diagnostic accuracy of the composite DIA/FISH result is greater than CC. Our specific aim was to determine the accuracy of CC versus the composite

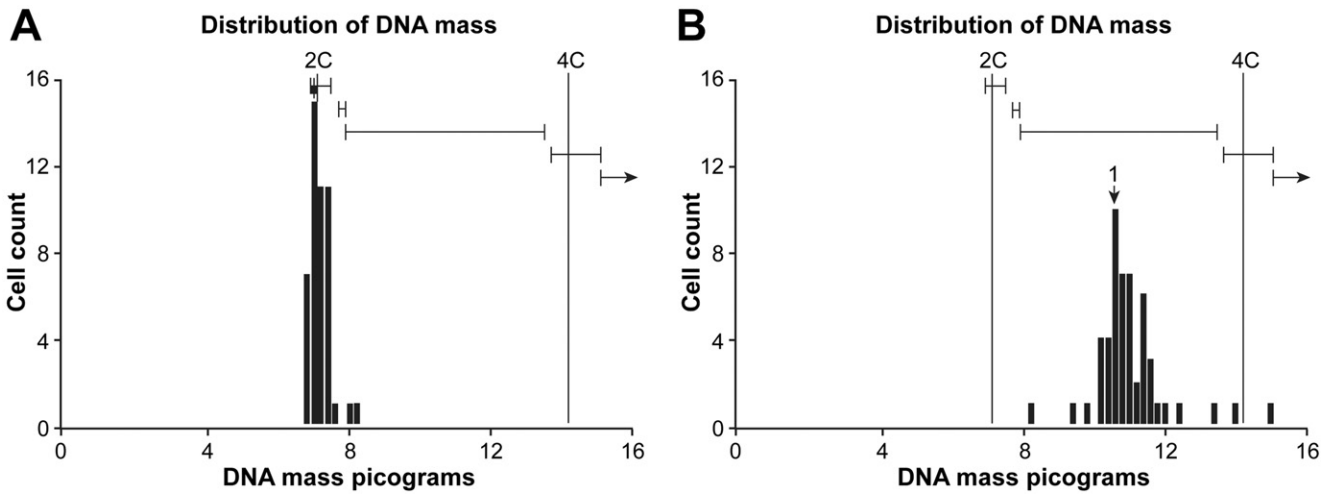

Figure 1. DNA histograms showing cell distributions based on nuclear DNA content. 2C represents cells in the diploid range, and 4C indicates tetraploid cells. Cells between 2C and 4C are considered aneuploid. (*A*) Negative (diploid) sample is shown. (*B*) Positive (aneuploid) results are shown.

DIA/FISH result in patients undergoing EUS FNA. Each subject had 3 measurements of malignancy: (1) CC, (2) individual DIA and individual FISH results, and (3) the composite DIA/FISH result. Composite DIA/FISH results were constructed by declaring the biopsy result malignant if either of the test results (DIA or FISH) was interpreted as malignant and by declaring the biopsy site as benign only if both tests were interpreted as benign. Each patient served as his or her own control because each diagnostic modality was performed for each lesion sampled. For CC, all nonpositive interpretations (ie, negative, atypical, and suspicious interpretations) were considered negative for malignancy. Additionally, a separate analysis in which either positive or suspicious CC sample was considered as indicative of malignancy was also performed. DIA and FISH specimens were interpreted only as positive or negative for malignancy. All samples deemed inadequate for review were considered negative for malignancy. Formal statistical analysis was based on a matched number of CC and DIA/FISH passes. We also calculated the performance characteristics of CC when considering the study and additional nonstudy passes. No nonstudy DIA/FISH samples were taken.

Continuous data are reported using descriptive statistics. Continuous variables are expressed as mean (SD) or median (range). Sensitivity, specificity, and positive and negative predictive values and accuracy with 95% confidence intervals were calculated. We used the statistical software package JMP Version 8 (SAS Institute Inc, Cary, NC). Given the matched pairs nature of this study, McNemar test was used for hypothesis testing. A P value $\leq .05$ was considered statistically significant.

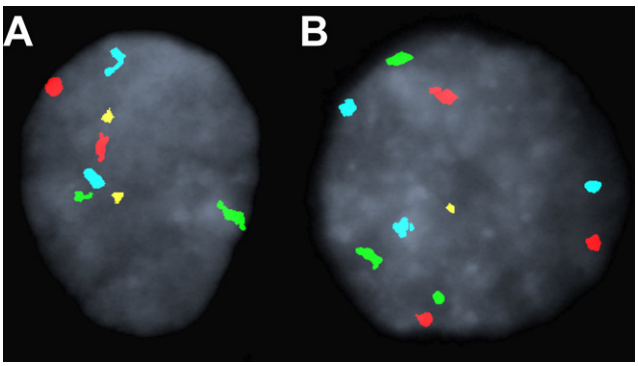

Figure 2. Cells from FISH specimens: CEP 3 (*red*), CEP 7 (*green*), CEP 17 (*aqua*), and LSI 9p21 (*gold*). (*A*) Negative specimen showing 2 signals for each probe. (*B*) Positive specimen (polysomy) showing ≥2 probes with ≥3 signals.

Sample Size Calculation

The reported accuracy of EUS FNA with CC is 60%–90%.[1–7] In our experience over the past 10 years, the accuracy of CC has approximated 80%.[4,7,8,10,23,30–33] This study was designed to detect a difference of 10% between our historically observed accuracy (80%) for CC and the expected accuracy (90%) for the combined results of DIA/FISH. Using the same method as Lachenbruch,[34] we estimated the need to enroll 107 patients with an available diagnostic gold standard to provide 90% power and a 2-sided type I error α level of 0.05. To account for patient dropouts, incomplete follow-up, and the inability to satisfy diagnostic gold standard criteria and to help assure that the statistical metrics could be met, we set the target enrollment at 250 patients. We intentionally set stringent criteria in regard to sample size calculation, evident by the fact that we assumed a difference of only 10% between the historical and anticipated accuracy rates. Our selection of a narrow measure of difference required enrollment of a greater number of patients but was chosen to optimize the chance of identifying any difference in the performance characteristics of CC versus DIA/FISH.

Results

Among the 250 patients enrolled between March 2007 and October 2008, a gold standard diagnosis was available for 202 patients (median age, 65 years; range, 24–87 years; 142 male/60 female). A diagnosis of malignancy was made in 148 patients (73%). The indications for EUS varied substantially, with a broad representation in tumor site, histology, and spectrum of disease (Table 1). The biopsy sites included lymph nodes (n = 111), pancreas (n = 61), gastrointestinal lumen wall (n = 9), periluminal mass (n = 4), liver (n = 8), and miscellaneous sites (n = 9) (Table 2). A mean of 3.2 ± 0.5 (range, 3–7) matched study samples per patient were submitted for CC

Table 1. Indications/Diagnoses

	Malignant (n = 148)	Benign (n = 54)	Total (n = 202)
Mediastinum/lung	7	2	9
Non–small cell lung cancer	5	1	6
Squamous cell carcinoma	1	0	1
Desmoid	1	0	1
Benign nodule	0	1	1
Esophagus	28	3	31
Adenocarcinoma	24	1	25
Squamous cell carcinoma	3	0	3
Adenosquamous	1	0	1
Benign wall thickening	0	2	2
Stomach	7	5	12
Adenocarcinoma	5	1	6
Mucosa-associated lymphoid tissue lymphoma	1	0	1
Gastrointestinal stromal tumor	1	0	1
Leiomyoma	0	2	2
Fundic gland polyp	0	1	1
Benign wall thickening	0	1	1
Liver	2	1	3
Hepatocellular carcinoma	2	1	3
Gallbladder	4	0	4
Adenocarcinoma	4	0	4
Bile duct	2	14	16
Adenocarcinoma	2	9	11
Primary sclerosing cholangitis	0	3	3
Immunoglobulin G4-associated cholangiopathy	0	1	1
Benign polypoid mass	0	1	1
Pancreas	64	13	77
Adenocarcinoma (primary)	58	3	61
Neuroendocrine (primary)	2	1	3
Plasmacytoma (primary)	1	0	1
Renal cell carcinoma (metastatic)	2	0	2
Thyroid papillary (metastatic)	1	0	1
Benign/inflammatory	0	3	3
Autoimmune pancreatitis	0	2	2
Acute/chronic pancreatitis	0	2	2
Intraductal papillary mucinous neoplasia	0	1	1
Serous cystadenoma	0	1	1
Colon/rectum	18	9	27
Adenocarcinoma	18	6	24
Polyp (adenoma)	0	1	1
Polyp (hamartomatous)	0	1	1
Perirectal soft tissue thickening (benign)	0	1	1
Anal	3	0	3
Squamous cell carcinoma	3	0	3
Lymphoma	6	1	7
Anaplastic large (null cell) lymphoma	1	0	1
Large B-cell lymphoma	2	0	2
Chronic lymphocytic leukemia	2	0	2
Follicular lymphoma	1	0	1
Small cell lymphoma	0	1	1
Other	7	3	10
Cancer unknown primary (adeno)	2	0	2
MEN I syndrome	1	0	1
Mastocytosis	1	0	1
Renal cell carcinoma	0	1	1
Cholecystitis	0	1	1
Accessory spleen	0	1	1
Ileal carcinoid	1	0	1
Bladder (transitional cell)	1	0	1
Endometrial (serous)	1	0	1
Lymphadenopathy	0	3	3

and DIA/FISH analysis. As noted in Patients and Methods, as a result of evenly dividing the DIA/FISH sample, twice as much material (average of 3.2 passes/patient) was available for CC analysis compared with an average of 1.6 passes/patient for DIA and FISH.

All Patients: Malignant and Benign

When considering all patients with a diagnostic gold standard, the sensitivity, specificity, and accuracy for CC when all nonpositive interpretations were considered negative for malignancy were 75%, 100%, and 82%, respectively (Table 3). The performance characteristics of DIA alone were significantly inferior to CC ($P = .0002$), whereas the performance characteristics of FISH alone were nonsignificantly superior to CC. The sensitivity of CC was 75% and significantly lower than the composite CC/DIA (82%; $P = .0044$), CC/FISH (86%; $P = .0002$), and CC/DIA/FISH (89%; $P = .0001$) results.

When considering all potential test combinations, the performance characteristics were optimized by either CC/FISH or CC/DIA/FISH. Although CC/DIA/FISH provided greatest overall accuracy (90%), the resulting specificity was 93%, which was less than the 100% specificity of CC alone even though the difference was not statistically significant. We instead view the CC/FISH result as providing the most clinical utility with a sensitivity, specificity, and accuracy of 86%, 96%, and 89%, respectively. The 11% increment in sensitivity over CC alone was significant ($P = .0002$) while maintaining high test specificity, although now 96% versus 100%. The addition of FISH to CC allowed detection of malignancy for another 16 patients, including pancreatic adenocarcinoma (n = 5), neu-

Table 2. Biopsy Sites

	Malignant (n = 148)	Benign (n = 54)	Total (n = 202)
Lymph node	76	35	111 (55%)
Pancreas	53	8	61 (30%)
Lumen wall	4	5	9 (4%)
Periluminal mass	3	1	4 (2%)
Liver	8	0	8 (4%)
Other	4	5	9 (4%)

Table 3. Performance Characteristics for Entire Group: Malignant (n = 148) Plus Benign (n = 54)

	CC	DIA	FISH	DIA/FISH	CC/DIA	CC/FISH	CC/DIA/FISH
Sensitivity	75 (0.67–0.81)	59 (0.51–0.67) $P = .0002^a$	77 (0.69–0.83)	81 (0.73–0.87)	82 (0.75–0.88) $P = .0044^b$	86 (0.79–0.91) $P = .0002^b$	89 (0.83–0.93) $P = .0001^b$
Specificity	100 (0.93–1)	94 (0.84–0.98)	96 (0.87–0.99)	93 (0.82–0.97)	94 (0.88–0.98)	96 (0.87–0.99)	93 (0.82–0.97)
Positive predictive value	100 (0.96–1)	97 (0.90–0.99)	98 (0.93–0.99)	97 (0.91–0.99)	98 (0.93–0.99)	98 (0.94–0.99)	97 (0.92–0.99)
Negative predictive value	59 (0.48–0.69)	46 (0.36–0.55)	60 (0.49–0.70)	64 (0.52–0.74)	66 (0.54–0.76)	71 (0.59–0.81)	76 (0.63–0.85)
Accuracy	82 (0.75–0.89)	69 (0.61–0.77) $P = .0002^a$	83 (0.62–0.91)	84 (0.78–0.91)	86 (0.79–0.92) $P = .0233^b$	89 (0.83–0.94) $P = .0005^b$	90 (0.85–0.95) $P = .0001^b$

NOTE. All values are expressed as percentage (95% confidence interval).
[a]Statistically significant ($P < .05$) decrease compared with CC.
[b]Statistically significant ($P < .05$) increase compared with CC.

roendocrine tumor (n = 2), lymphoma (n = 1), and metastatic renal cell carcinoma (n = 1), as well as lymphadenopathy secondary to metastatic adenocarcinoma (n = 4), adenosquamous carcinoma (n = 1), and lymphoma (n = 2). Two patients had a false-positive FISH result. One patient with a presumed pancreatic head cancer instead had autoimmune pancreatitis verified by core biopsy, response to corticosteroid therapy, and absence of malignancy over the subsequent 3.1 years. Another patient underwent a distal pancreatectomy for an intraductal papillary mucinous neoplasia with high-grade dysplasia, with 6 negative lymph nodes refuting the FISH interpretation that suggested malignant lymphadenopathy. There has been no evidence of disease recurrence over the subsequent 3.3 years.

CC and FISH were both falsely negative in 20 patients, including pancreatic adenocarcinoma (n = 3), neuroendocrine (n = 1), lymphoma (n = 1), and renal cell metastasis (n = 1), as well as lymphadenopathy secondary to adenocarcinoma (n = 7), squamous cell carcinoma (n = 1), lymphoma (n = 3), hepatocellular carcinoma (n = 1), and a primary lung adenocarcinoma (n = 1) and desmoid tumor (n = 1).

All Patients: Adenocarcinoma Versus Nonadenocarcinoma

Among all patients enrolled with a gold standard diagnosis of malignancy (n = 154), adenocarcinoma accounted for 116 patients (78%) (Table 4). Other tumor types included lymphoma (n = 9), squamous cell carcinoma (n = 7), neuroendocrine tumor (n = 4), and miscellaneous (n = 12). For adenocarcinoma, the sensitivity of CC was 82%, which was significantly lower ($P = .0009$) than the composite CC/DIA (89%; $P = .0133$), CC/FISH (90%; $P = .0077$), and CC/DIA/FISH (93%; $P = .0009$) results. The diagnostic sensitivity for all individual and combined tests was lower for nonadenocarcinoma tumor types than for adenocarcinoma. The addition of FISH to CC for this group of patients allowed tumor detection in another 7 of 32 patients (22%).

Pancreatic FNA

A total of 61 patients underwent pancreatic EUS FNA with the finding of malignancy in 53 patients (87%) (Appendixes 1 and 2). CC provided a sensitivity, specificity, and accuracy of 74%, 100%, and 76%, respectively. The addition of FISH to CC significantly ($P = .0077$) enhanced the sensitivity by 15%, enabling diagnosis in another 8 patients, including adenocarcinoma (n = 4), neuroendocrine tumor (n = 2), lymphoma (n = 1), and metastatic renal cell carcinoma (n = 1). The false-positive FISH result occurred in the aforementioned patient with autoimmune pancreatitis. The diagnostic sensitivity of the individual tests and all composite test results was greater for pancreatic adenocarcinoma compared with the same test(s) in patients for nonadenocarcinoma tumor types.

Table 4. Sensitivity Based on Tumor Type for Entire Group: Adenocarcinoma (n = 116) Plus Nonadenocarcinoma (n = 32)

	CC	DIA	FISH	DIA/FISH	CC/DIA	CC/FISH	CC/DIA/FISH
Adenocarcinoma (n = 116)	82 (0.73–0.88)	65 (0.55–0.73) $P = .00015^a$	82 (0.73–0.88)	86 (0.78–0.91)	89 (0.81–0.93) $P = .0133^b$	90 (0.82–0.94) $P = .0077^b$	93 (0.86–0.96) $P = .0009^b$
Nonadenocarcinoma (n = 32)	50 (0.31–68)	41 (0.23–0.59)	59 (0.40–0.76)	66 (0.46–0.81)	63 (0.43–0.78)	72 (0.53–0.86) $P = .0233^b$	75 (0.56–0.88) $P = .0133^b$
Lymphoma (n = 9)	11 (0–0.48)	33 (0.07–0.70)	44 (0.13–0.78)	44 (0.13–0.78)	33 (0.07–0.70)	44 (0.13–0.78)	44 (0.13–0.78)
Squamous cell carcinoma (n = 7)	86 (0.42–0.99)	43 (0.09–0.81)	71 (0.29–0.96)	86 (0.42–0.99)	86 (0.42–0.99)	86 (0.42–0.99)	86 (0.42–0.99)
Neuroendocrine (n = 4)	50 (0.06–0.93)	00 (0–0.60)	50 (0.06–0.93)	50 (0.06–0.93)	50 (0.06–0.93)	100 (0.39–1)	100 (0.39–1)
Other (n = 12)	58 (0.27–0.84)	50 (0.21–0.78)	67 (0.34–0.90)	75 (0.42–0.94)	75 (0.42–0.94)	75 (0.42–0.94)	83 (0.51–0.97)

NOTE. All values are expressed as percentage (95% confidence interval).
[a]Statistically significant ($P < .05$) decrease compared with CC.
[b]Statistically significant ($P < .05$) increase compared with CC.

Lymph Node FNA

Among the 111 lymph nodes sampled, malignancy was present in 76 (68%) (Appendixes 3 and 4). CC provided a sensitivity, specificity, and accuracy of 74%, 100%, and 82%, respectively. In contrast to the aforementioned data, the composite CC/DIA provided greater accuracy than CC/FISH, with a sensitivity approximating that for CC/DIA/FISH. The addition of DIA to CC enhanced the sensitivity by 12% (n = 9) ($P = .0077$), thereby establishing the presence of malignant lymphadenopathy secondary to metastatic adenocarcinoma (n = 6), adenosquamous carcinoma (n = 1), lymphoma (n = 1), and hepatocellular carcinoma (n = 1). The one false-positive DIA result occurred in a patient who underwent direct surgical intervention following polypectomy of a noninvasive rectal carcinoma. Surgical pathology revealed a residual tubulovillous adenoma with high-grade dysplasia and 28 benign lymph nodes. There has been no evidence of disease recurrence over the 3.4 years since surgery.

CC Count-by-Count Data and Impact of Suspicious Interpretations

The sensitivity of CC was 75% for the study passes (mean, 3.2), which increased to 79% when evaluating all passes (mean, 3.9), including the additional nonstudy passes (Table 5). To allow a more direct comparison of CC data (mean, 3.2 passes) with DIA and FISH (mean, 1.6 passes), 3 cytopathologists (A.C.C., M.R.H., J.Z.) reviewed every CC specimen for each patient in a blinded fashion. They collectively determined at what point the designation of a positive test result had been reached. After complete review, they constructed a pass-by-pass and overall consensus diagnosis. Each FNA was read one by one with a composite cytological interpretation assigned after each additional review, incorporating information obtained by the prior samples to determine the threshold number of passes at which a positive test result could be reached.

After a total of 1, 2, 3, 4, 5, 6, and 7 passes, the sensitivity of CC was 64%, 72%, 75%, 76%, 77%, 78%, and 79%, respectively. All positive interpretations were achieved within the first 7 passes, despite the fact that patients may have undergone additional sampling. When considering either a positive or suspicious CC sample as indicative of malignancy, the sensitivity of CC was 78% for the study passes and 79% when evaluating all passes. However, the inclusion of suspicious cytology decreased the specificity from 100% to 96% and 94% for study passes and total passes, respectively. Neither of these approaches (additional passes or inclusion of suspicious cytology) to enhance the sensitivity of CC approached the sensitivity achieved by CC/FISH. All positive interpretations were achieved within the first 7 passes, despite the fact that patients may have undergone additional sampling.

Inadequate DIA and FISH Samples

The use of an on-site cytotechnologist ensured CC sample adequacy for all patients. Because of the tech-

Table 5. Performance Characteristics of CC for Entire Group: Count-by-Count Data and Impact of Suspicious Interpretations

	CC M = M First pass	CC M = M First 2 passes	CC M = M First 3 passes	CC M = M First 4 passes	CC M = M First 5 passes	CC M = M First 6 passes	CC M = M First 7 passes	CC S&M = M Study passes (mean, 3.2)	CC S&M = M All passes (mean, 3.9)
Sensitivity	64 (0.55–0.71)	72 (0.64–0.79)	75 (0.67–0.81)	76 (0.68–0.82)	77 (0.69–0.83)	78 (0.70–0.84)	79 (0.71–0.85)	78 (0.70–0.84)	79 (0.70–0.84)
Specificity	100 (0.93–1)	100 (0.93–1)	100 (0.93–1)	100 (0.93–1)	100 (0.93–1)	100 (0.93–1)	100 (0.93–1)	96 (0.87–0.99)	94 (0.84–0.98)
Positive predictive value	100 (0.96–1)	100 (0.96–1)	100 (0.96–1)	100 (0.96–1)	100 (0.96–1)	100 (0.96–1)	100 (0.96–1)	98 (0.94–0.99)	97 (0.92–0.99)
Negative predictive value	50 (0.40–0.60)	57 (0.46–0.66)	59 (0.48–0.69)	61 (0.49–0.70)	61 (0.50–0.71)	62 (0.51–0.72)	64 (0.52–0.73)	62 (0.50–0.72)	61 (0.50–0.71)
Accuracy	74 (0.66–0.82)	80 (0.73–0.97)	82 (0.75–0.86)	83 (0.76–0.89)	83 (0.77–0.90)	84 (0.77–0.90)	85 (0.78–0.91)	83 (0.77–0.89)	83 (0.77–0.89)

NOTE. All values are expressed as percentage (95% confidence interval). Data in the last 2 columns were calculated when considering either a positive or suspicious CC sample as indicative of malignancy.
M, positive for malignancy; S, suspicious for malignancy.

niques required for DIA and FISH processing, it is not possible to evaluate sample adequacy at the time of EUS, so instead this was assessed at the time of DIA and FISH interpretation. By protocol, inadequate DIA and FISH samples are designated quantity not sufficient (QNS) or BUST, respectively. Our goal was to determine the potential impact of splitting the DIA/FISH samples, which provided only 1.6 passes for DIA and FISH versus 3.2 passes for CC. We questioned whether such a diminutive specimen may negatively impact the sensitivity of DIA and FISH. To do so, we recalculated the sensitivities including only those specimens considered adequate for review, thereby excluding the inadequate QNS (DIA) and BUST (FISH) specimens. These recalculated sensitivities were compared with the previously provided sensitivities that were calculated in standard fashion, including QNS and BUST specimens that were considered negative for malignancy. When considering all malignant study sites (n = 148), the sensitivity of DIA, FISH, and DIA/FISH increased from 59% to 77%, 78% to 80%, and 81% to 92%, respectively.

Discussion

EUS FNA is an essential tool for managing a diverse spectrum of disease processes because of its diagnostic and staging accuracy, enhanced prognostic capabilities, and effect on therapeutic planning and patient outcomes.[1,8,35–37] Cytological interpretation of FNA specimens is sometimes hindered by technical limitations and tumor-related factors that may decrease diagnostic sensitivity. This may result in the need for additional needle passes, prolonged procedure times, increased medication use, increased personnel demands, decreased room efficiency, and risk of adverse events. The use of novel tumor markers may overcome the limitations of CC by identifying structural and numerical chromosomal imbalances that have been found to commonly occur in a variety of cancers.[38,39] We have extensively evaluated DIA and FISH on tissue samples collected during ERC in patients with indeterminate bile duct strictures and found greater diagnostic accuracy for both DIA and FISH compared with CC.[14,18–21] FISH is now incorporated into our ERC practice, and we routinely rely on this to guide clinical management.

In this study, we evaluated these molecular cytology assays on EUS FNA specimens collected from a diverse group of malignant and benign disease processes. Our findings support the contention that several chromosomal regions are gained and/or lost across a spectrum of cancers irrespective of the histology, and although specific mutations may be unique to certain cancers, the use of a panel of markers usually permits diagnosis. These data suggest the presence of aneuploid cell populations in most tumors. It is known that most but not all tumors demonstrate numeric chromosomal abnormalities. Occasional tumors are diploid or near-diploid tumors that are driven by point mutations in oncogenes or tumor sup-

pressor genes, balanced translocations, or epigenetic alterations (eg, hypermethylation of promoters), and such tumors could lead to false-negative results with FISH and DIA testing. In addition, although abnormal DNA content is almost always indicative of malignancy, premalignant lesions and inflammation can sometimes show aneuploidy, risking a false-positive result.[14,21,40,41] It is unclear what percentage of tumors contain aneuploid cells and at what point in the process of malignant progression that tumors manifest aneuploidy. Therefore, an unspecified subset of tumors likely exists for which malignancy escapes detection when using techniques that rely solely on the presence of aneuploidy. The findings suggest the potential need for development of new or more sensitive genetic and epigenetic markers. FISH testing is limited to use of validated probe sets. Hence, there are several tumor types that will require development of new probe sets. This is a potentially important limitation that may impact widespread application.

The maximum test sensitivity is also influenced by inadequate tissue sampling, which we assessed by recalculating the sensitivities of DIA and FISH, including only those specimens that were deemed to have an adequate amount of tissue. The recalculated sensitivities further enhanced the already significantly improved sensitivity of DIA and FISH testing over CC alone, suggesting even greater utility for DIA and FISH were we to have not biased tissue acquisition in favor of CC. Although one must cautiously view the validity and relevance of the recalculated sensitivities, doing so provides some insight as to the impact of submitting only 1.6 FNA passes for DIA and FISH, which was half the CC sample and far less than typically obtained in clinical practice. Just as the sensitivity of CC increases with the number of FNA passes, as shown in our pass-by-pass review, the same gain in sensitivity is likely to occur with additional passes for DIA and FISH, with the understanding that a maximum sensitivity is achieved at some point without benefit of additional sampling. Further study is needed to define the tumor types likely to escape DIA and FISH diagnosis and to develop more sensitive DIA and FISH probe sets. Certain types of lymphoma are characterized by relatively few numerical chromosomal abnormalities; for example, follicular lymphoma tend to have a t(8;14) translocation but be diploid or near-diploid tumors. These would not be picked up with the probe set used in this study. Low-grade solid tumors tend to have fewer chromosomal abnormalities, and they also might be missed. Similarly, we need to determine the ideal specimen volume that optimizes DIA and FISH sensitivity while avoiding excess tissue sampling and the associated risk.

The scenarios in which these new molecular cytology assays might be applied in clinical practice need further exploration. Similar to previous findings for endoscopic retrograde cholangiopancreatography and brush cytology, our data suggest that the enhanced diagnostic utility of DIA and FISH is nearly fully captured by FISH alone. Limited FISH sampling (mean, 1.6 passes) provided comparable sensitivity to CC (mean, 3.2 passes), and the composite CC plus FISH results increased overall sensitivity by 11% (16 patients). The potentially greater accuracy of FISH must be balanced against the additional time, expertise, and cost of processing and interpretation. FISH is a moderately labor-intensive test. It typically requires 30 minutes of technologist time compared with approximately 10 minutes for CC. One must also consider the approximate $1000 cost of FISH testing. The cost is relatively nominal for those with nondiagnostic CC due to the avoidance of additional or repeat testing and ability to deliver patient care with a secure diagnosis. However, one should always consider how best to implement new and often expensive tests.

Considering the high specificity, relatively low cost, and ready availability of CC, we anticipate continued use of CC. An on-site cytological interpretation of malignancy will likely obviate the need for FISH for cases in which CC is found to be positive. However, the relatively low diagnostic sensitivity of CC (and resulting low negative predictive value) reported in most studies often leaves uncertainty as to the validity of a negative result. Therefore, an on-site cytological interpretation of benignity or sample inadequacy may suggest the need for FISH testing. FISH specimens can be banked and analyzed in a delayed manner if indicated by final cytology review. A review of our 202 patients provides some perspective as to the potential impact of the proposed algorithm. Additional sample collection and FISH analysis would be unnecessary for the 111 patients (55%) in whom CC was positive for malignancy assuming accurate in-room cytotechology interpretation. FISH testing would have been indicated for the remaining 91 patients (45%) to help clarify whether the negative CC interpretation represented a true- or false-negative result. The addition of FISH allowed detection of malignancy in another 16 patients (11%) over CC alone ($P = .0002$), but specificity was reduced from 100% to 96%.

The performance characteristics of CC must also be considered in individual practices. For instance, Turner et al recently reported the Massachusetts General Hospital experience with pancreatic EUS FNA over a 9-year period, in which the sensitivity of CC was 76%.[6] This is similar to our data in which the sensitivity of CC was 74% with the study (mean, 3.2) passes and 83% after all (mean, 4.1) passes. Knowledge of individual institutional and personnel experiences would likely affect the use of additional diagnostic markers.

New technologies such as comparative genomic hybridization continue to emerge. Although comparative genomic hybridization may identify any tumor cell chromosomal alteration as long as the alteration is large enough to be detected by comparative genomic hybridization techniques, this test does not have sufficient sensitivity to detect alterations when tumor cells constitute a small fraction of cells within the cytology specimen. In this regard, FISH is currently the optimal molecular technique

for assessing chromosomal abnormalities in cytologic specimens.

It is important to note a few study limitations. Ideally, trials evaluating new diagnostic tests should avoid use of the evaluated tests as part of the diagnostic gold standard. In this study we included a positive FNA result as part of the gold standard. This is the approach universally adopted in the EUS literature because there is no rational way to reliably provide a separate gold standard result short of direct operative intervention in all patients, which is neither plausible nor in keeping with practice standards. This approach likely led to a falsely increased specificity for CC and a falsely decreased sensitivity for DIA and FISH.

The large enrollment and diversity of patient pathologies in this study allowed us to address the clinical utility of DIA and FISH on EUS FNA specimens for common tumor types such as adenocarcinoma, but questions remain as to the utility of DIA and FISH for patients with less common tumor types. The diminished diagnostic accuracy for certain pathologies (eg, neuroendocrine tumors) requires focused investigation within these select cohorts and possible use of alternate FISH probe sets. We may also discover that for certain tumor types the relative paucity of malignant cells requires additional biopsies to obtain a critical threshold of malignant cells to allow diagnosis. This may be the case for neoplasia associated with intense inflammation, fibrosis, and/or vascularity. Further study is needed to determine if DIA and FISH findings correlate with tumor stage, prognosis, resectability, recurrence, and survival. The answers to these questions will ideally allow us to optimize diagnostic and therapeutic strategies to improve outcomes for patients with gastrointestinal cancers. Such discoveries may also apply to FNA specimens obtained via other routes, including percutaneous and surgical, as well as other organ systems and disease processes outside the field of gastroenterology. Any improvement in the performance characteristics provided by FISH has to be balanced against the additional time, necessary expertise, and associated cost. Given that the specificity of CC approaches 100%, on-site cytologic interpretation of malignancy would obviate the need for FISH for patients with a positive CC result. However, the relatively low diagnostic sensitivity of CC reported in most studies often leaves uncertainty as to the validity of a negative result. Therefore, an on-site cytologic interpretation of benignity or sample inadequacy may indicate a need for FISH analysis.

In a large cohort of patients with diverse pathologies undergoing EUS FNA, FISH analysis significantly enhanced the diagnostic sensitivity over CC alone, despite limited tissue sampling. Our data support the contention that the majority of tested cancers harbor chromosomal alterations, irrespective of the underlying histology. Although certain alterations may be unique to specific cancers, evaluation of a panel of FISH markers permits diagnosis of most cancers. Further study is needed to verify the clinical utility of FISH as an ancillary test for the detection of malignancy in EUS FNA specimens. However, as for ERC and brush cytology specimens in our center, these data suggest a likely role of FISH analysis for evaluating EUS FNA specimens.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2012.02.002.

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Conflicts of interest

The authors disclose the following: Dr Halling and the Mayo Clinic hold a patent on and receive royalties from the sale of the FISH probe set (UroVysion) discussed in this paper. The remaining authors disclose no conflicts.

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Appendix 1. Performance Characteristics for Pancreatic FNA: Malignant (n = 53) Plus Benign (n = 8)

	CC	DIA	FISH	DIA/FISH	CC/DIA	CC/FISH	CC/DIA/FISH
Sensitivity	74 (0.59–0.88)	53 (0.38–0.66) $P = .0153^a$	79 (0.65–0.89)	79 (0.65–0.89)	79 (0.65–0.89)	91 (0.79–0.99) $P = .0077^b$	91 (0.79–0.99) $P = .0077^b$
Specificity	100 (0.63–1)	100 (0.63–1)	88 (0.47–0.99)	88 (0.47–0.99)	100 (0.63–1)	88 (0.47–0.99)	88 (0.47–0.99)
Positive predictive value	100 (0.90–1)	100 (0.87–1)	98 (0.87–0.99)	98 (0.87–0.99)	100 (0.91–1)	98 (0.89–0.99)	98 (0.89–0.99)
Negative predictive value	36 (0.17–0.59)	24 (0.11–0.42)	39 (0.17–0.64)	39 (0.17–0.64)	42 (0.20–0.66)	58 (0.27–0.84)	58 (0.27–0.84)
Accuracy	77 (0.64–0.91)	59 (0.43–0.75) $P = .0153^a$	80 (0.68–0.93)	80 (0.68–0.93)	82 (0.70–0.94)	90 (0.81–0.99) $P = .0133^b$	90 (0.81–0.99) $P = .0133^b$

NOTE. All values are expressed as percentage (95% confidence interval).
[a]Statistically significant ($P < .05$) decrease compared with CC.
[b]Statistically significant ($P < .05$) increase compared with CC.

Appendix 2. Sensitivity Based on Tumor Type for Pancreatic FNA: Adenocarcinoma (n = 46) Plus Nonadenocarcinoma (n = 7)

	CC	DIA	FISH	DIA/FISH	CC/DIA	CC/FISH	CC/DIA/FISH
Adenocarcinoma (n = 46)	83 (0.68–0.92) $P = .0008^a$	59 (0.43–0.73) $P = .043^a$	83 (0.68–0.92)	83 (0.68–0.92)	87 (0.73–0.95) $P = .0027^a$	94 (0.82–0.98)	94 (0.82–0.98)
Nonadenocarcinoma (n = 7)	14 (0–0.57)	14 (0–0.57)	57 (0.18–0.90)	57 (0.18–0.90)	29 (0.03–70)	71 (0.29–0.96)	71 (0.29–0.96)

NOTE. All values are expressed as percentage (95% confidence interval). Nonadenocarcinoma tumor types include lymphoma (n = 2), renal cell carcinoma (n = 2), neuroendocrine (n = 2), and thyroid papillary (n = 1).
[a]Statistically significant ($P < .05$) when compared with the same diagnostic test for tumor types other than adenocarcinoma.

Appendix 3. Performance Characteristics for Lymph Node FNA: Malignant (n = 76) Plus Benign (n = 35)[b]

	CC	DIA	FISH	DIA/FISH	CC/DIA	CC/FISH	CC/DIA/FISH
Sensitivity	74 (0.62–0.83)	63 (0.51–0.73)	72 (0.60–0.82)	82 (0.71–0.89)	86 (0.75–0.92) $P = .0077$[a]	80 (0.69–0.88)	88 (0.78–0.94) $P = .0026$[a]
Specificity	100 (0.90–1)	97 (0.85–0.99)	94 (0.80–0.99)	91 (0.76–0.98)	97 (0.85–0.99)	94 (0.80–0.99)	91 (0.76–0.98)
Positive predictive value	100 (0.93–1)	98 (0.89–0.99)	96 (0.87–0.99)	95 (0.87–0.99)	98 (0.91–0.99)	97 (0.89–0.99)	96 (0.87–0.99)
Negative predictive value	64 (0.49–0.76)	55 (0.41–0.67)	61 (0.46–0.74)	70 (0.54–0.82)	76 (0.60–0.87)	69 (0.53–0.81)	78 (0.62–0.89)
Accuracy	82 (0.73–0.91)	80 (0.70–0.90)	79 (0.70–0.89)	85 (0.76–0.93)	89 (0.82–0.97) $P = .0133$[a]	85 (0.76–0.93)	89 (0.82–0.97) $P = .0133$[a]

NOTE. All values are expressed as percentage (95% confidence interval).
[a]Statistically significant ($P < .05$) increase compared with CC.
[b]Statistically significant ($P < .05$) decrease compared with CC.

Appendix 4. Sensitivity Based on Tumor Type for Lymph Node FNA: Adenocarcinoma (n = 56) Plus Nonadenocarcinoma (n = 20)

	CC	DIA	FISH	DIA/FISH	CC/DIA	CC/FISH	CC/DIA/FISH
Adenocarcinoma (n = 56)	79 (0.65–0.88)	68 (0.54–0.79)	79 (0.65–0.88)	86 (0.73–0.93)	89 (0.78–0.95)	88 (0.75–0.94) $P = .0416$[a]	93 (0.82–0.98) $P = .0174$[a]
Nonadenocarcinoma (n = 20)	60 (0.36–0.80)	50 (0.27–0.72)	60 (0.36–0.80)	65 (0.40–5)	70 (0.45–).88	65 (0.40–5)	70 (0.45–0.88)
Squamous cell carcinoma (n = 7)	86 (0.42–0.99)	43 (0.09–0.81)	71 (0.29–0.96)	86 (0.42–0.99)	86 (0.42–0.99)	86 (0.42–0.99)	86 (0.42–0.99)
Lymphoma (n = 6)	17 (0–0.64)	33 (0.04–0.77)	33 (0.04–0.77)	33 (0.04–0.77)	33 (0.04–0.77)	33 (0.04–0.77)	33 (0.04–0.77)
Other[b] (n = 7)	71 (0.29–0.96)	71 (0.29–0.96)	57 (0.18–0.90)	71 (0.29–0.96)	86 (0.42–0.99)	71 (0.29–0.96)	86 (0.42–0.99)

NOTE. All values are expressed as percentage (95% confidence interval).
[a]Statistically significant ($P < .05$) when compared with the same diagnostic test for tumor types other than adenocarcinoma.
[b]Other tumor types include neuroendocrine (n = 2), transitional cell bladder cancer (n = 1), non–small cell lung cancer (n = 1), serous (endometrial) (n = 1), hepatocellular carcinoma (n = 1), and adenosquamous (n = 1).