

Preliminary experience comparing routine cytology results with the composite results of digital image analysis and fluorescence in situ hybridization in patients undergoing EUS-guided FNA

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Background: Studies indicate enhanced diagnostic accuracy for digital image analysis (DIA) and fluorescence in situ hybridization (FISH) versus routine cytology examination (RC) when biliary strictures are evaluated. These tumor markers have not been applied to EUS-guided FNA.

Objective: Our purpose was to determine the accuracy of RC versus the composite results of DIA/FISH.

Design: Patients enrolled with known or suspected malignancy. The final diagnosis was based on strict cytopathologic and imaging criteria and 12-month follow-up.

Settings: Tertiary referral center.

Patients: A total of 39 patients were enrolled in whom each diagnostic test was performed on samples from 42 sites to evaluate lymphadenopathy (n = 19), pancreatic mass (n = 19), esophageal or gastric wall mass (n = 3), and thyroid mass (n = 1).

Interventions: EUS-guided FNA with RC, DIA, and FISH.

Main Outcome Measurement: Diagnostic accuracy of RC, DIA, and FISH.

Results: Malignancy was diagnosed in 30 of 42 patients, including esophageal squamous cell carcinoma, esophageal adenocarcinoma, gastric adenocarcinoma, pancreatic adenocarcinoma, pancreatic mucinous cystic neoplasia, intraductal papillary mucinous neoplasia, metastatic forearm sarcoma, small cell and non-small cell lung cancer, thyroid carcinoma, malignant GI stromal tumor, melanoma, adenocarcinoma of unknown primary, and lymphoma. The sensitivity, specificity, and accuracy of DIA/FISH versus RC for detecting malignancy were 97%, 100%, and 98% versus 87%, 100%, and 90%, respectively.

Limitations: Single-center pilot study.

Conclusions: Our findings suggest that DIA and FISH processing of EUS-guided FNA specimens provides higher diagnostic accuracy than RC does. These data suggest that these tumor markers incorporate generic targets as suggested by the high diagnostic sensitivity in this patient cohort with diverse pathologic conditions. (Gastrointest Endosc 2007;66:483-90.)

EUS is a sensitive method for evaluating intrainestinal and extraintestinal mass lesions and peri-intestinal lymphadenopathy. The addition of FNA and routine cytologic

Abbreviations: DIA, digital image analysis; FISH, fluorescence in situ hybridization; IPMN, intraductal papillary mucinous neoplasia; RC, routine cytologic examination.

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analysis yields a diagnostic accuracy of 60% to 90%, depending on the site and lesion sampled.¹⁻⁶ EUS-guided FNA has not only been shown to improve diagnostic and staging accuracy^{1,7-9} but also guides clinical care and improves patient outcomes.^{7,10-12} As a result, EUS-guided FNA is an essential component in the evaluation of patients with luminal and pancreatic cancers, lung cancer, and subepithelial lesions. It is also often performed to biopsy abnormalities in the liver, adrenal gland, perirectal tissues, and for aspiration of peritoneal and pleural fluid.

Despite the utility of EUS-guided FNA, cytologic interpretation is often hindered by technical limitations and tumor-related factors leading to failed diagnosis.^{1,13,14} These limitations have driven the pursuit to develop new technologies, including digital image analysis (DIA) and fluorescence in situ hybridization (FISH) to enhance diagnostic accuracy. These techniques assess nuclear DNA content and the presence of aneuploidy to diagnose malignancy.¹⁵⁻¹⁷ These tests can identify malignant cells in samples of limited cellularity and provide greater diagnostic sensitivity than does routine cytologic examination (RC).¹⁷⁻¹⁹

DIA and FISH were initially designed to evaluate non-GI tumors. Given that most solid tumors are characterized by numeric and structural chromosomal abnormalities,²⁰⁻²⁶ we previously theorized that use of these molecular markers may also allow diagnosis of GI tumors. Our initial efforts focused on use of these molecular markers in patients with indeterminate bile duct strictures. We initially tested this hypothesis by prospectively analyzing brush biopsy samples collected at ERCP with DIA and FISH demonstrating greater diagnostic accuracy than RC for distinguishing benign from malignant biliary strictures.^{17,27-30} These results led us to wonder if, and to what degree, these biomolecular markers may enhance the diagnostic accuracy over routine cytologic study for non-biliary malignancies. As such, we sought to test our hypothesis that the composite results of DIA and FISH provide greater diagnostic accuracy than RC when applied to EUS-guided FNA specimens from patients with a diverse spectrum of malignancies. Our supposition is supported by the fact that genomic regions are routinely amplified among a spectrum of cancer types. Although specific mutations may be unique to certain cancers, use of a panel of markers may allow diagnosis of most forms of cancer. The ultimate goal is to enhance diagnostic and staging capabilities to guide clinical decision making and improve patient outcomes.

MATERIAL AND METHODS

Patients

We enrolled patients referred for EUS-guided FNA (1) who had known or suspected luminal or extraluminal malignancy and (2) for whom the endosonographer deemed the target lesions safe and amenable to FNA. Patients were excluded if there was (1) inability to provide informed consent, (2) anticipated unavailability or patients declined phone follow-up, or (3) coagulopathy (international normalized ratio >1.5 or thrombocytopenia platelets <50,000). Patients were nonconsecutively enrolled to ensure inclusion of a varied patient cohort. Although this approach has the potential to introduce bias in favor of a particular diagnostic modality, we are unaware of any rationale to indicate that selection of a diverse group of

Capsule Summary

What is already known on this topic

- Despite the diagnostic accuracy of FNA, cytologic interpretation and staging evaluation for various neoplasms is often hindered by technical limitations and tumor-related factors.

What this study adds to our knowledge

- In 39 patients with known or suspected malignancy, the sensitivity, specificity, and accuracy of digital image analysis and fluorescence in situ hybridization versus routine cytologic results for detecting malignancy were 97%, 100%, and 98% versus 87%, 100%, 90%, respectively.

pathologic conditions would favor any one of these techniques over another. However, this issue must be clarified through additional study.

The institutional review board granted approval for the study and informed consent was obtained for all procedures. Information concerning the presentation, clinical course, and outcomes were abstracted from the medical records and patient interviews. A patient was considered to have malignancy if there was (1) cytologic or histologic evidence of malignancy on the basis of material obtained by EUS-guided FNA, ERCP, tissue sampling, percutaneous biopsy, surgical exploration, or autopsy or (2) clinical course (12 months after enrollment) suggesting malignancy on the basis of presence of new radiographic abnormality including regional or distant mass (hepatic, pulmonary, or bone), mass infiltrating large blood vessels, or malignant-appearing lymphadenopathy with positron emission tomography or death (death certificate diagnosis). Designation of a lesion as benign required at least 12 months' follow-up and absence of any of the above criteria or follow-up imaging demonstrating complete resolution of the abnormality.

Generally, trials evaluating the accuracy of new diagnostic tests should not incorporate the results of the evaluated tests into the diagnostic gold standard; we did include a positive result for FNA given the reported specificity rate of 99% to 100%.^{1,5,7} Although this approach is widely adopted in the literature because there is no better way to provide a gold standard short of operative intervention in all patients, we recognize that doing so risks biasing the results in favor of FNA. More importantly, DIA and FISH results were not included as a component of the diagnostic gold standard.

EUS-guided FNA and initial tissue processing. EUS-guided FNA and tissue processing (RC, DIA, and FISH) were performed by standard techniques as previously described along with use of an on-site cytotechnologist with samples taken until an adequate specimen

was collected in all cases.^{1,2,27,29,31-34} Each FNA specimen was examined by either (1) RC or (2) DIA and FISH. When submitted for DIA and FISH analysis, the entire specimen was sent to the laboratory, which evenly divided and submitted half of the specimen for DIA and half for FISH interpretation. Dedicated GI cytopathologists with particular expertise in each of these diagnostic modalities reviewed the specimens while blinded to the clinical records and other test results. Specifically, RC, DIA, and FISH were each interpreted independently and without knowledge of the result for the other evaluated diagnostic modalities.

DIA. DIA is a form of cytologic analysis that quantifies cellular constituents by using spectrophotometric principles and is a sister technique to flow cytometry.¹⁵ Small foci of tumor cells can be analyzed, unlike the large number of cells required for flow cytometry.^{18,19} DIA processing uses the Feulgen reaction, which strips away all nonnuclear material and hydrolyzes DNA into its constituent nucleic acids, which stoichiometrically bind to the Feulgen dye¹⁵ (Fig. 1A and 1B).

In this study, ThinPrep specimens were prepared for DIA analysis as previously described.³⁵ Up to 50 cells with the most nuclear atypia (irregular size, shape, hyperchromasia, etc) were selected by a technologist for quantification with the CAS 200 image analyzer (Bacus Laboratories, Lombard, Ill). The CAS 200 image analyzer captured these cells with a camera and quantifies the optical density readings and compares these readings with 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)³⁶ (Fig. 2A). The absorption value was converted into an analog signal and “digitized” into a series of tiny squares called picture elements or pixels (Fig. 2B). DNA ploidy status was then assigned to the collected cells on the basis of a histogram generated by the Quantitative DNA Analysis program on the CAS 200 image analyzer (Fig. 3). 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.³⁷

FISH. FISH uses fluorescently labeled DNA probes to chromosomal centromeres or unique loci to detect cells that have numeric or structural abnormalities indicative of malignancy (Fig. 4A and 4B). Specifically, the probe set used in this study (UroVysion, Abbott Molecular, Des Plaines, Ill) 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 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 by FISH if the specimens demonstrated chromosomal loss of the 9p21 locus in

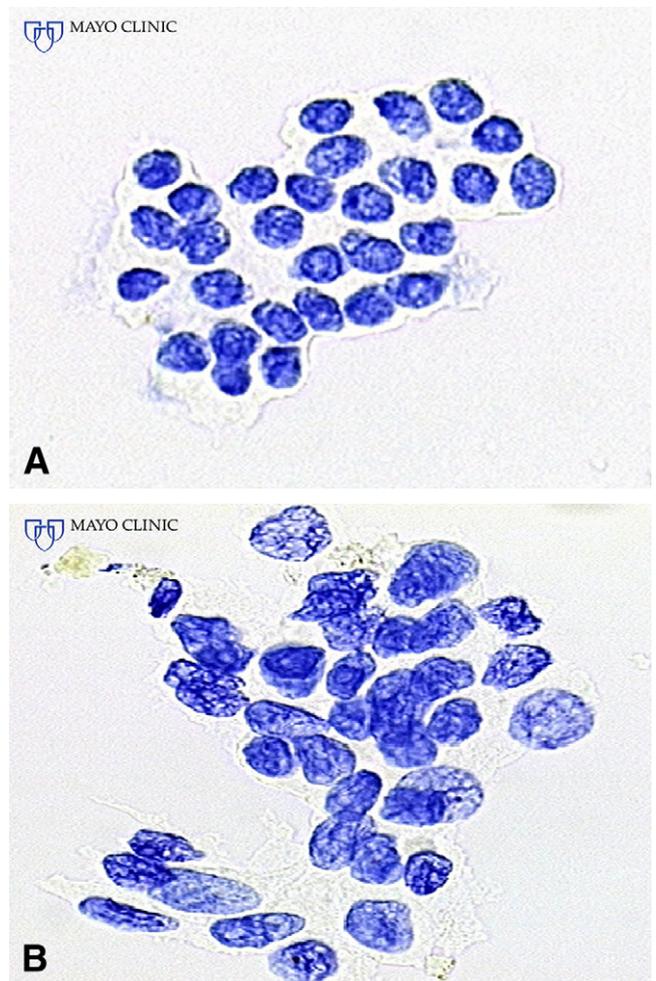


Figure 1. **A**, Feulgen staining during DIA demonstrates a benign sample (H&E, orig. mag. $\times 400$). **B**, Feulgen staining during DIA reveals a malignant specimen (H&E, orig. mag. $\times 400$).

$> 20\%$ of cells or gains of 2 or more chromosomes (aneuploidy) in more than 5 cells. All remaining specimens were diagnosed as negative.

Statistical analysis

We hypothesized that the diagnostic accuracy of the composite result of these new molecular markers (DIA and FISH) is greater than standard RC. To address this issue, our specific aim was, in patients undergoing EUS-guided FNA, to determine the accuracy (as assessed by the sensitivity and specificity) of RC versus the composite results of DIA and FISH as determined by the final cytopathologic diagnosis. Each subject had 2 measurements of malignancy: (1) RC and (2) the composite results of DIA and FISH. Composite results for DIA and FISH were constructed by declaring the biopsy result malignant if either of the tests were interpreted as malignant and by declaring the biopsy site 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. Doing so allowed direct comparison of

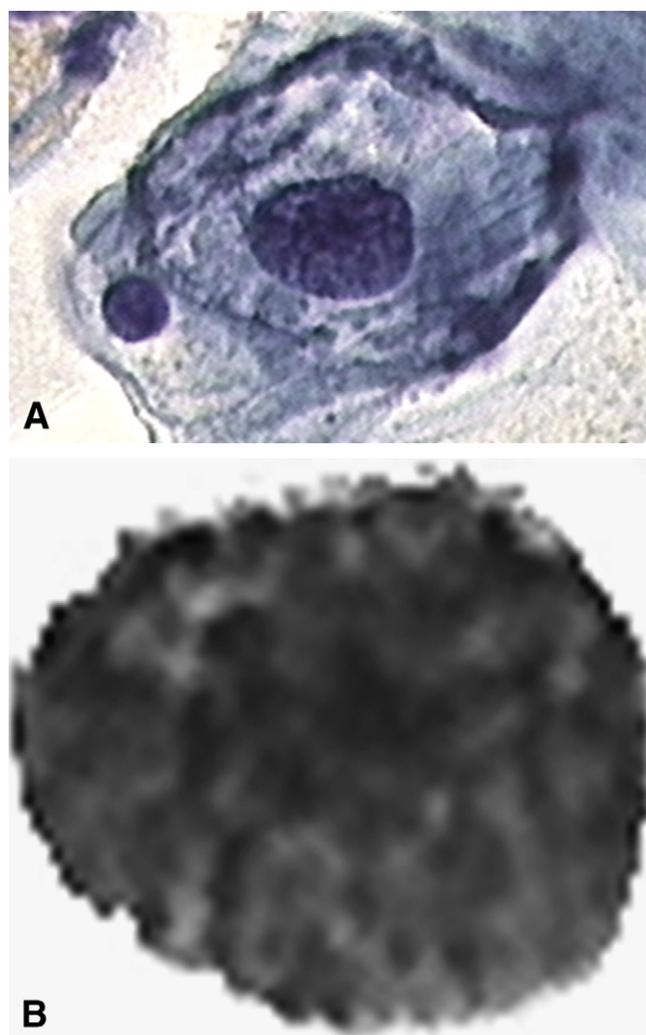


Figure 2. **A**, Light transmitted through a glass-mounted specimen is captured with a video camera. The digital signal is represented by pixels varying in color from white to black, corresponding to an absorption value (amount of light detected by the video camera). **B**, Computer analysis produces a digital image of the nucleus that is used to assess DNA content (Pap stain, orig. mag. $\times 1000$).

standard and new diagnostic techniques. For the purpose of statistical analysis, when a test was interpreted as atypical, suspicious, or indeterminate or the specimen was inadequate, the test result was considered negative. Demographic features of study participants and biopsy site features were recorded. Continuous data are reported by descriptive statistics.

Continuous variables are expressed as mean (SD) or median (range). Sensitivity, specificity, positive and negative predictive values, and accuracy with 95% CI were calculated. The statistical software package JMP Version 6 (SAS Institute, Cary, NC) was used for statistical analysis. Comparisons between them were performed by the Student *t* test. Comparisons between qualitative variables were performed with the χ^2 test or Fisher exact test. A *P* value $\leq .05$ was considered statistically significant.

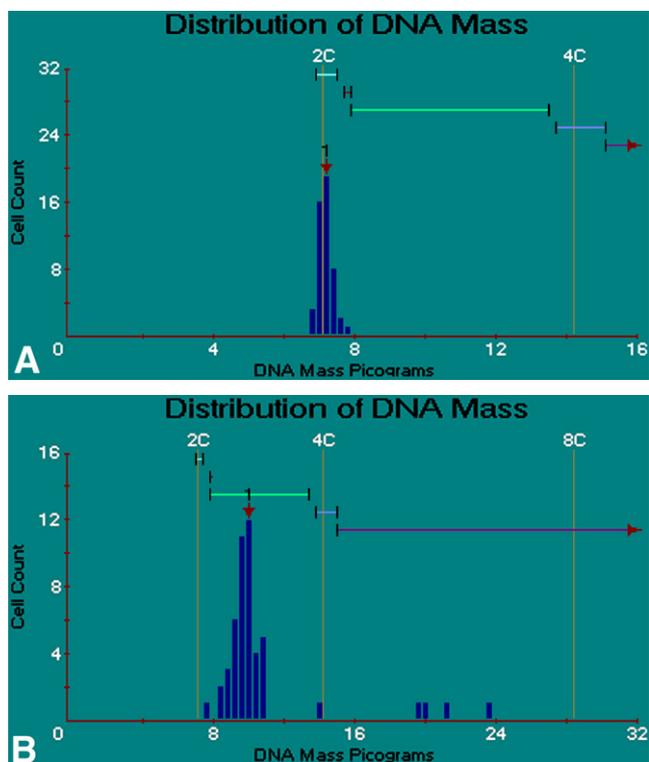


Figure 3. **A** and **B**, DNA histograms showing cell distributions based on nuclear DNA content. *2C*, Cells in the diploid range; *4C*, tetraploid cells. Cells between *2C* and *4C* are considered aneuploid. *3a*, Cells from a benign biliary stricture; *3b*, cells from a malignant biliary tract stricture.

RESULTS

A total of 42 sites from 39 patients were evaluated by EUS-guided FNA. The mean age was 68 years (SD 13.9 years, range 24-88 years), including 25 males and 14 females. Tissue samples from each site were evaluated with RC, DIA, and FISH. Target lesions included lymphadenopathy ($n = 19$), pancreatic mass ($n = 19$), esophageal or gastric wall mass ($n = 3$), and thyroid mass ($n = 1$). A final diagnosis of malignancy was made in 30 patients. A mean of 4.0 ± 2.94 (range 1-7) samples were submitted for RC analysis versus 3.8 ± 2.87 (range 1-7) samples for DIA/FISH. The primary tumor location and histologic diagnosis varied considerably and included esophageal squamous cell carcinoma, esophageal adenocarcinoma, gastric adenocarcinoma, pancreatic adenocarcinoma, pancreatic mucinous cystic neoplasia, intraductal papillary mucinous neoplasia (IPMN), metastatic forearm sarcoma, small cell lung cancer, non-small cell lung cancer, thyroid follicular carcinoma, malignant GI stromal tumor, melanoma, adenocarcinoma of unknown primary, and B cell (centrocyte-like cell lymphoma). All the patients with malignancy were undergoing their initial evaluations and none were evaluated for recurrent disease. In addition, none of the patients with malignancy had undergone prior chemoradiation or any other therapeutic intervention. For

the 3 patients who underwent tissue sampling from more than 1 site, the initial biopsy specimen was obtained from a pancreatic mass ($n = 2$) or esophageal wall mass ($n = 1$) with a final diagnosis of malignancy in each. For these 3 patients, the second biopsy specimen was taken from a lymph node ($n = 3$), which yielded a negative result in each patient by all 3 techniques.

Regarding the aim of this study to compare RC with the composite results of DIA/FISH for detection of malignancy; the sensitivity was 26 of 30 (87%; 95% CI 75%-99%) versus 29 of 30 (97%; 95% CI 90%-100%), the specificity was 12 of 12 (100%; 95% CI 100%-100%) versus 12 of 12 (100%; 95% CI 100%-100%), and the accuracy was 38 of 42 (90%; 95% CI 82%-99%) versus 41 of 42 (98%; 95% CI 93%-100%). The finding of malignancy with DIA or FISH was considered a positive result for the composite test. This is the primary means of analysis and use of these tests in our ERCP practice, which led to our consideration of the test results in this manner. However, to more clearly discern the value of DIA and FISH individually, the performance characteristics are listed separately below. The sensitivity for detection of malignancy of DIA and FISH was 21 of 30 (70%; 95% CI 54%-86%) and 23 of 30 (77%; 95% CI 62%-92%), respectively. The specificity of DIA and FISH was 12 of 12 (100%; 95% CI 100%-100%) for both techniques. These data provided an overall accuracy for DIA of 33 of 42 (79%; 95% CI 66%-91%) and for FISH 35 of 42 (83%; 95% CI 72%-95%). No false-positive results occurred for DIA or FISH. The 1 failed diagnosis for DIA/FISH was in a patient with a malignant GI stromal tumor. DIA/FISH correctly identified malignancy in 5 patients with cytologic results interpreted as benign, atypical, or suspicious. The final diagnosis in these patients was pancreatic adenocarcinoma ($n = 3$), esophageal squamous cell carcinoma ($n = 1$), and malignant transformation of IPMN ($n = 1$). For the 5 patients with only a positive DIA/FISH, 4 of the 5 died within 7 months and all deaths were attributed to their malignancies. In particular, for the 5 patients with a positive DIA/FISH and nonmalignant RC interpretation, 4 of the 5 patients died within 7 months, all attributed to malignancy. In addition, among these 4 patients, percutaneous biopsy demonstrated positive cytologic results in 1 patient, and radiographic disease tumor advancement was seen in the other 4 patients before their deaths. Malignancy was verified in the fifth patient on review of the resected specimen after pancreatoduodenectomy.

DISCUSSION

EUS-guided FNA is an essential component of the diagnostic and staging evaluation for a variety of neoplasms because of the enhanced diagnostic and staging accuracy and the impact on prognostic determination.³⁸⁻⁴⁰ Through these benefits, improved therapeutic planning and patient

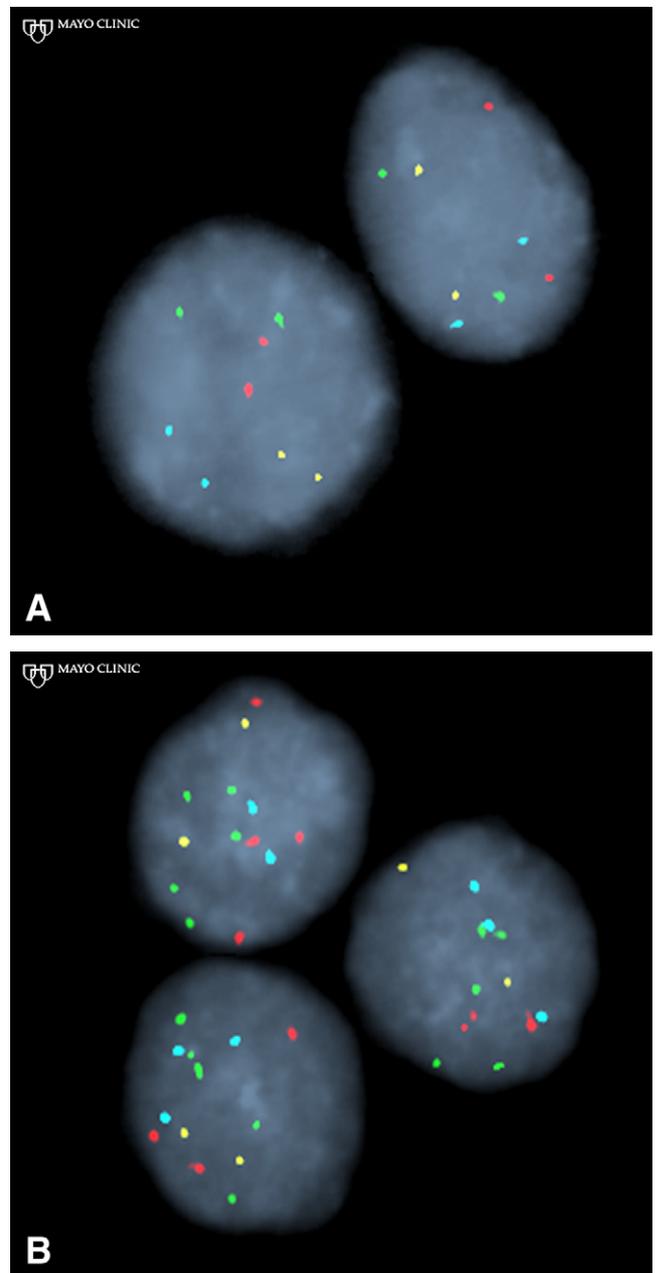


Figure 4. **A**, Fluorescent labeled probes detect normal nuclear content (2 signals per color) revealing a benign sample. **B**, Fluorescent labeled probes detect abnormal nuclear content (> 2 signals per color) revealing a malignant sample.

outcomes have been realized.^{1,7-9} Despite the improved diagnostic accuracy of FNA, cytologic interpretation is often hindered by technical limitations and tumor-related factors. Application of novel tumor markers may enhance analysis of tissue specimens obtained at FNA. The goal in applying these tumor markers to FNA specimens is to identify the structural and numeric chromosomal imbalances that have been found to commonly occur in a variety of cancers.^{21,41-46} We previously, and are currently, evaluating use of DIA and FISH on tissue samples collected

during ERCP in patients with indeterminate bile duct strictures. We have found greater diagnostic accuracy for both DIA and FISH compared with RC for distinguishing benign from malignant strictures.^{17,27-30} As a result of the often complementary information provided by each technique, we now perform all 3 in patients with indeterminate bile duct strictures.

In the current study, we evaluated these new molecular markers on EUS-guided FNA specimens collected from a variety of malignancies. Our findings support the contention that several genomic regions are amplified in most cancers irrespective of the histologic diagnosis and that, although specific mutations may be unique to certain cancers, use of a panel of markers usually permits diagnosis. These data also suggest the presence of aneuploidy in most tumors. Although the finding of aneuploidy is considered equivalent to malignancy,^{17,30,47,48} aneuploidy is not a prerequisite and may not be demonstrable in malignancy, potentially leading to failed diagnosis as can occur with cancers associated with the hereditary nonpolyposis colon cancer syndromes. In addition, although abnormal DNA content is almost always indicative of malignancy, premalignant lesions such as colonic adenomas may also demonstrate aneuploidy, risking a false-positive diagnosis.^{47,49,50} Furthermore, inflammatory processes usually do not produce aneuploid cell populations, but this occurrence has been reported.^{17,30,51} The percentage of tumors that contain aneuploid cells is unknown. It is also unclear at what point in the process of malignant transformation that tumors manifest aneuploidy. Therefore, an unspecified subset of tumors likely exists for which malignancy escapes detection when techniques that rely solely on the presence of aneuploidy are used. Additional study is needed to define this population and to establish the need for, and potential targets of, more sensitive probe sets for both DIA and FISH.

It is also unclear why DIA/FISH analysis of EUS-guided FNA specimens in this study yielded greater diagnostic sensitivity than samples collected by ERCP and brush cytology in our prior studies evaluating patients with indeterminate bile duct strictures. The most plausible explanation is greater tissue acquisition, and thereby an increased number of malignant cells, collected during FNA versus brush cytology.

The method by which these new diagnostic modalities may be applied in the future is speculative and can only be clarified through additional study. Subsequent data may indicate the lack of a role for these new diagnostic modalities. On the contrary, study findings may support discontinuation of RC in favor of DIA and FISH. We consider it more likely that RC and DIA/FISH will have complementary roles. If the anticipated results of greater diagnostic accuracy of DIA/FISH are realized, the improved performance characteristics will have to be balanced against the additional time, necessary expertise, and cost to process and interpret these studies. Considering the reasonable diag-

nostic accuracy and ready availability of RC, we predict continued initial application of cytologic analysis to EUS-guided FNA specimens. Given that the reported specificity of RC approaches 100%, on-site cytologic interpretation of malignancy will likely lead to no further diagnostic testing at EUS and no need for DIA/FISH. However, the relatively low diagnostic sensitivity of RC (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 cytologic interpretation of benignity or sample inadequacy would likely lead to subsequent DIA and FISH analysis. This proposed algorithm is one of conjecture and awaits prospective study in a large cohort of patients with diverse pathologic conditions before formal recommendations and implementation can be advised.

The greater accuracy of DIA/FISH versus RC was not statistically significant ($P > .05$). The lack of a significant difference may well be accounted for by the small sample size. Larger, better designed studies are needed to clarify the accuracy of these techniques. Also, in this study we did not control for the number of biopsy specimens taken. This methodologic limitation may certainly influence the results, and future studies should control for this feature. Further study is also needed to determine whether DIA/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 for patients with GI disease undergoing EUS-guided FNA. These findings also have potential bearing on the processing of FNA samples collected by other routes (including percutaneous and surgical) and for non-GI disorders. The ultimate goal is to apply these tests in a manner that improves patient survival and quality of life.

In conclusion, our preliminary data suggest that the composite result for DIA/FISH provides high accuracy for diagnosis of malignancy over a diverse spectrum of GI and non-GI malignancies. However, use of either test alone may provide insufficient sensitivity. Although these tests were initially designed to evaluate other tumor types, our data support the contention that several genomic regions are amplified in nearly all persons with cancer irrespective of the underlying histologic features. Our findings also suggest that, although certain mutations may be unique to specific cancers, that evaluation of a panel of markers permits diagnosis of most cancers. Despite the initial promise of these new diagnostic modalities, more data are needed to clarify the diagnostic accuracy and cost-effectiveness before their use can be widely advocated.

DISCLOSURE

Kevin Halling receives royalties from the sale of the UroVysion probe set (Abbott Laboratories). No other

authors have any financial interest in this or any related product.

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Results from *GIE* online polling

We asked and you answered. Here are the results of the latest *GIE* online poll. How often do you refer to the ASGE practice guidelines to aid you in your practice?

- (14 votes, 21%) Daily to weekly
- (31 votes, 46%) Weekly to monthly
- (19 votes, 28%) Monthly to yearly
- (4 votes, 6%) Never

To participate in the current online poll, go to www.giejournal.org or www.asge.org.