Identification of Malignant Cytologic Criteria in Pancreatobiliary Brushings With Corresponding Positive Fluorescence In Situ Hybridization Results

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Abstract

Cytologic evaluation of pancreatobiliary brushings is specific but poorly sensitive for malignancy. Detection of polysomic cells by fluorescence in situ hybridization (FISH) is significantly more sensitive than routine cytology with similar specificity. The purpose of this study was to identify cytologic criteria most associated with malignancy in specimens unaffected by sample failure. Endoscopic brushings were split equally for routine cytologic and FISH analyses per clinical practice. We retrospectively evaluated 16 cytologic criteria on Papanicolaou-stained slides. We assumed that the presence of polysomic cells by FISH indicated successful tumor sampling in specimens from patients with pathologic evidence of malignancy on follow-up. We compared cytologic criteria of malignant brushings with corresponding positive FISH results (positive control, n = 39) with those without evidence of malignancy and corresponding negative FISH results (negative control, n = 30). The presence of single abnormal cells, irregular nuclear membranes, and enlarged nuclei were independent predictors of malignancy by logistic regression ($P < .05$).

Cholangiocarcinoma and pancreatic carcinoma are the most common malignancies of the pancreatobiliary tract. More than 80% of patients with these cancers have unresectable tumors and/or metastatic disease at diagnosis. The associated prognosis is bleak, with 5-year survival rates of less than 5% owing to widespread disease and ineffective therapeutic options. Surgical resection is the only treatment shown to provide long-term survival benefit, but surgery is not offered to patients with late-stage disease or specific tumor characteristics that complicate resection. Early detection is, therefore, critical for maximizing survival.

The identification of malignancy in the pancreatobiliary tract is burdened by an anatomic location that is difficult to access and challenging to sample. Patients found to have a stricture on imaging studies are likely to undergo endoscopic retrograde cholangiopancreatography (ERCP), an invasive procedure that allows for visualization and pathologic sampling of suspicious areas within the biliary tree. Brush sampling during ERCP has become standard practice because forceps biopsy specimens tend to be small (ie, inadequate), may lack diagnostic tumor cells, or may not contain stroma for the determination of invasion. Cytologic evaluation of ERCP brushings can provide a diagnosis of malignancy when biopsy results are negative$^{3,4}$; however, the associated sensitivity is suboptimal and ranges widely from 4% to 61%.$^{5-13}$ Inadequate sampling is a major contributing factor to the low sensitivity of cytology owing to the lack of malignant cells within the sample. Causes of sample failure include desmoplastic lesions that do not readily shed tumor cells, fibrosis or benign epithelium overlying the tumor, and procedural challenges such as poor visualization or limited access to strictures.$^{14}$
Advanced cytologic methods have been investigated to improve the detection rate of pancreatobiliary malignancy, most notably fluorescence in situ hybridization (FISH). This technique uses fluorescently labeled DNA probes that target specific chromosomal locations for the detection of aneusomy (ie, gain of chromosomal material). Most tumors are aneuploid, and, therefore, the presence of cells exhibiting extra probe signals in pancreatobiliary brushings is very concerning for malignancy. FISH has been shown to be more sensitive than conventional cytology by 2-fold (20% vs 43%). The fact that FISH is approximately twice as sensitive as cytology suggests that even when tumor cells are present, cytology may be falsely negative. The goal of this study was to evaluate various nuclear and architectural characteristics of epithelial cells on Papanicolaou-stained conventional cytology slides that best differentiate benign from malignant strictures, with a focus on the specimens having corresponding positive FISH results such that cytologic criteria predicting pancreatobiliary tract malignancy might be further refined.

Materials and Methods

Sample Preparation and Analysis

Pancreatobiliary brushing specimens were collected during ERCP per standard practice and placed in a vial of PreservCyt (Hologic, Bedford, MA) preservative. Half of the specimen was used to make a Papanicolaou-stained ThinPrep (Hologic) slide for diagnosis by a pathologist as nondiagnostic, negative, atypical, suspicious, or positive for malignancy. A cell pellet was formulated from the other half of the specimen. As previously described, cells were manually dropped from the pellet onto a slide and subsequently hybridized with fluorescently labeled FISH probes that target the centromeric regions of chromosomes 3, 7, and 17 and the 9p21 band (CDKN2A; alias p16). A specimen was considered positive by FISH when 5 or more cells showed polysomy (defined as >2 signals in at least 2 of the 4 probes).

Identification of Study Specimens

Study specimens (n = 93) were retrospectively identified from a clinical database of specimens obtained by ERCP that underwent cytology and FISH testing in our laboratory between October 2003 and April 2006. Inclusion criteria were as follows: (1) available routine cytology slide with a diagnostic result, (2) corresponding FISH result of negative or positive (ie, polysomy), and (3) definitive clinical follow-up diagnosis of the stricture as benign or malignant. The “gold standard” for the diagnosis of carcinoma included pathologic evidence of high-grade dysplasia or malignancy within the pancreatobiliary tract (ie, biopsy, surgical resection, or liver transplant specimen) or pathologic evidence of metastatic pancreatobiliary carcinoma (ie, positive fine-needle aspiration or biopsy finding).

Cytopathologist Retrospective Review of Cytology Diagnosis

Each cytology specimen was blindly reviewed by 2 expert cytopathologists (M.R.H. and A.C.C.) who each formulated an independent cytologic diagnosis followed by a discussion to reach a consensus diagnosis.

Morphologic Assessment of Cytologic Criteria

Each cytology slide was retrospectively reviewed by one of a group of cytologists (J.E.B., K.D., L.F., B.K.M., and A.A.R.) without knowledge of the original cytology diagnosis or patient outcome. Overall cellularity, architectural group characteristics, and nuclear features were assessed and documented for each slide. Overall cellularity included quantitation (0-20 or >20) of cell groups (>10 cells), cell clusters (2-9 cells), and single cells. Architectural features included architectural disarray, depth of focus, anisonucleosis (size variation of at least 3 times within a cell cluster or group), and cell-within-cell arrangement. Nuclear features that were evaluated included increased nuclear/cytoplasmic ratio, enlarged nuclei (>3 times a normal cell or cell within the same...
The presence of a nuclear or architectural feature in at least 1 cell or group, respectively, was sufficient to classify the specimen as representative of the feature. If a specimen was considered abnormal for any architectural or nuclear feature, the presence of abnormal cell groups (≥10 cells), abnormal cell clusters (2-9 cells), and abnormal single cells was determined. Challenging cases were reviewed during a group scope session with an expert cytopathologist (A.C.C.), and a consensus diagnosis was made.

Statistical Analysis
The entire set of 93 specimens was used to calculate sensitivity and specificity of routine cytology and FISH. For statistical analysis of cytologic criteria, the 24 cases
Results

There were 93 specimens from 85 patients (48 men and 37 women) that fulfilled study criteria. Patient ages ranged from 25 to 89 years with a mean of 62.3 years. Patients with primary sclerosing cholangitis constituted 31% of the study population (26/85). Of the brushing specimens, 63 (68%) were from patients with malignancy (n = 56). The cancer types included cholangiocarcinoma (n = 34), pancreatic adenocarcinoma (n = 20), gallbladder adenocarcinoma (n = 1), and ampullar adenocarcinoma (n = 1). In 4 patients with negative FISH and pathologic evidence of malignancy were excluded because they were possibly affected by sample failure. Consequently, we compared brushing specimens with pathology-confirmed malignancy on follow-up in which corresponding FISH results were positive (positive control [PC], n = 39) with specimens without clinicopathologic evidence of carcinoma and negative corresponding FISH results (negative control [NC], n = 30). Statistical analyses were performed using JMP 8.0 (SAS Institute, Cary, NC). P values less than .05 were considered statistically significant.
cholangiocarcinoma, there was no evidence of invasion in the histologic material; however, high-grade dysplasia was identified. Of these 4 patients, 2 died of metastatic disease and 2 had imaging and clinical evidence of cholangiocarcinoma. The mean length of follow-up for patients with benign strictures was 1,002 days (range, 0-2,198 days).

The comparison of performance characteristics of routine cytology and FISH is shown in Figure 1. The original routine cytology analysis had a sensitivity of 19% with a specificity of 100%. Expert consensus review identified 3 additional specimens with carcinoma, but this identification was at the expense of 1 false-positive (sensitivity, 24%; specificity, 97%). When suspicious diagnoses were included as malignant, expert consensus review (sensitivity, 56%; specificity, 83%) outperformed the original diagnosis (sensitivity, 40%; specificity, 77%).

Polysomy FISH was detected in 39 of 63 specimens from patients with malignancy (sensitivity, 62%) and in no patients with benign strictures (specificity, 100%; Figure 1). Statistical analysis of cytologic criteria was performed by comparing specimens with a corresponding polysomy FISH result and pathologic evidence of carcinoma as the PC (n = 39) vs specimens without clinicopathologic evidence of carcinoma as the NC (n = 30) Table II.

Cytologic criteria that were significantly different include depth of focus (PC, 25.6% vs NC, 3.3%; P < .05), anisonucleosis (PC, 53.9% vs NC, 10.0%; P < .001), architectural disarray (PC, 79.5% vs NC, 36.7%; P < .001), presence of abnormal groups (PC, 94.9% vs NC, 70.0%; P < .01), presence of abnormal clusters (PC, 87.2% vs NC, 36.7%; P < .0001), presence of abnormal single cells (PC, 76.9% vs NC 10.0%; P < .0001), increased nuclear/cytoplasmic ratio (PC, 89.7% vs NC, 46.7%; P < .0001), enlarged nuclei (PC, 59.0% vs NC, 10.0%; P < .0001), irregular nuclear membranes (PC, 92.3% vs NC, 46.7%; P < .0001), and prominent nucleoli (PC, 69.2% vs NC, 33.3%; P < .01). The most discriminatory features for differentiating carcinoma (PC) from benign (NC) specimens include the presence of abnormal single
cells (sensitivity, 76.9%; specificity, 90.0%), enlarged nuclei (sensitivity, 59.0%; specificity, 90.0%), and anisonucleosis (sensitivity, 53.8%; specificity, 90.0%). The presence of abnormal single cells and anisonucleosis resulted in a sensitivity of 48.7% and specificity of 100%. Multiple logistic regression analysis revealed that abnormal single cells, irregular nuclear membranes, and enlarged nuclei were independently associated with malignancy. The only feature that was exclusively associated with malignancy in this study was the cell-within-cell architectural feature, but this feature was only rarely identified in malignant cases.

Discussion

Our study aim was to critically evaluate cytologic criteria that may lead to improved sensitivity in detecting malignancy in pancreatobiliary brush specimens. Abnormal single cells, irregular nuclear membranes, and enlarged nuclei were independently associated with malignancy by multiple logistic regression analysis of 16 cytologic criteria in pancreatobiliary brushings. Sensitive and specific cytologic criteria are important to identify because the evaluation of pancreatobiliary brushings is inherently challenging for cytologists. Complicating factors include absence of diagnostic cells (ie, sample failure), reactive atypia that may confound cell interpretation in patients with chronic inflammation or recent instrumentation, and well-differentiated tumor cells that may not be easily identifiable.8,9 The implications of a positive cytology diagnosis likely drive the sensitivity lower because pathologists understand that even without biopsy confirmation, surgical resection may be performed. However, the corresponding benefit of conservative interpretation is very high specificity of a positive diagnosis. Any improvement in the sensitivity of routine cytology for detecting pancreatobiliary malignancy without detriment to specificity would be beneficial for patient care owing to the importance of early and accurate detection.

Other authors have demonstrated the value of pathologist experience and expertise for optimal sensitivity when interpreting biliary cytology. Unblinded review of 20 false-negative specimens led Kocjan and Smith7 to reclassify 5 specimens as positive and 1 as dysplastic owing to the pitfalls of sampling, dysplasia, well-differentiated carcinoma, and smear background. Lograno et al9 similarly reviewed 36 false-negative specimens and found that 6 (17%) were interpretive errors. Both groups concluded that an understanding by pathologists of the nuances of biliary tract cytology may help improve sensitivity.7,9 Wight et al13 reported an increase in sensitivity from 49.4% to 89% after consensus review by 2 pathologists, with unaltered specificity (100%). Improved accuracy was reported by de Peralta-Venturina et al15 after revision of 7 (9%) of 74 specimens following blinded review. Likewise, in the present study, blinded review by 2 experienced cytopathologists increased the sensitivity of a positive diagnosis from 19% to 24%, but at the expense of...
1 false-positive. When a suspicious diagnosis was included as malignant, the review diagnosis detected 10 additional malignant specimens and produced 2 fewer false-positives compared with the original diagnosis. These data support the assertion that cytopathologist education and experience are valuable in the context of pancreatobiliary tract cytology and are likely to improve diagnostic accuracy.

Another prospect for improving the detection of pancreatobiliary malignancy is the application of other testing techniques, such as FISH, to ERCP brushings. Detection of specimens with cells showing polysomy (gain of multiple chromosomes) has approximately twice the sensitivity for malignancy compared with positive routine cytology. Routine cytology and FISH were recently shown to be independent predictors of malignancy by multivariable modeling in 498 cases of pancreatobiliary strictures; therefore, both tests are used in the clinical testing algorithm at our institution. Other techniques that might be used for tumor detection include mutation analysis (eg, KRAS), methylation analysis, and loss of heterozygosity.

By definition, polysomic cells are chromosomally unstable and, therefore, are highly correlated with malignancy. Like positive routine cytology, positive FISH (ie, polysomy) is likely to represent carcinoma and, hence, is a meaningful test result. Conversely, negative cytology and FISH results are not clinically helpful for gastroenterologists owing to suboptimal negative predictive value of these tests. Other authors have shown inadequate sampling to be a major culprit of false-negative routine cytology results. Logrono et al reported that 24 (67%) of 36 false-negative results in their study were due to sampling error. Technical problems during endoscopy (eg, poor visualization), benign epithelium over the invasive lesion, and severe fibrosis are recognized as issues that adversely affect successful sampling. The limitation of sample failure undoubtedly confounds a morphologic study that aims to identify features most associated with malignancy. This dilemma prompted us to consider corresponding positive FISH results as evidence of adequate sampling.

In our clinical practice, brushing specimens are placed into a vial of liquid preservative after endoscopic collection. In the laboratory, cells are scraped from the brush into the vial, and the specimen is split for routine cytology and FISH analysis. The specimen’s liquid nature allows for equal division into 2 parts, each of which presumably contains representative components of the sampled area. FISH has higher sensitivity with equal specificity compared with cytology, which suggests that malignant cells are present in some specimens falsely classified as negative by cytology. Targeting cytology slides for morphologic analyses that contain polysomic cells on the corresponding FISH slide offers a novel learning opportunity to improve the sensitivity of routine cytology.

Previous morphologic studies have been performed to evaluate diagnostic criteria for the detection of malignancy in pancreatobiliary brushings. Investigators from Japan determined loss of honeycombing, enlarged nuclei, loss of polarity, bloody background, flat nuclei, and cell-within-cell arrangement to be the best criteria for diagnosis of malignancy in bile specimens based on multivariate statistics. One of the first morphologic studies published on brushing specimens reported nuclear molding, chromatin clumping, and increased nuclear/cytoplasmic ratio (referred to as the Iowa criteria) to be most associated with malignancy by multiple logistic regression analysis. A rigorous study by Renshaw et al tested the Iowa, Japan, and Boston (developed from the Iowa and Japan criteria) criteria against each other. An overall assessment was also tested, which was loosely defined as the reviewer’s gestalt interpretation of the slide, thus allowing for the extent of atypia to be factored into the analysis. They concluded that an overall assessment was the most accurate and reproducible in the detection of malignancy compared with any specific set of criteria.

Results of previous studies and the present study vary, likely owing to differences in patient population (eg, number of patients, prevalence of cancer, proportion with primary sclerosing cholangitis), specimen processing techniques (eg, direct smears, cytopreparation, ThinPrep), specific criteria evaluated, and definitions of criteria. Furthermore, the present study evaluated only a subset of specimens (based on corresponding FISH results and follow-up), which may also contribute to variation in results with previous studies.

The presence of abnormal single cells (defined as single cells having at least 1 atypical nuclear feature) was the most significant finding by univariate and multivariate analyses in the current study. Perhaps the presence of single abnormal cells is an accurate feature because such cells simultaneously represent nuclear and architectural atypia. Other investigators have evaluated the presence of single naked nuclei and single isolated cells, but these criteria were not particularly sensitive. Association of abnormal single cells with malignancy has not been reported in a formal morphologic study to our knowledge, and, therefore, its significance is a new finding that should be investigated further.

Like other investigators, we have shown that expertise and experience improve accuracy of pancreatobiliary brushing interpretation, and, therefore, education is invaluable for cytopathologists. This study is unique in that corresponding ancillary cytologic test results were used to identify routine cytology specimens most likely to contain tumor cells, thereby enabling us to more reliably assess the cytologic features of malignant pancreatobiliary specimens. Our findings indicate that single abnormal cells, irregular nuclear membranes, and enlarged nuclei are important cytologic criteria for differentiating malignant from benign pancreatobiliary
specimens. An important future study would test the predictive value and reproducibility of these features in brushings from a separate and unselected cohort of patients to verify the robustness of these criteria.

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Dr Halling has a patent on and receives royalties from the sale of the FISH probe set (UroVysion, Abbott Molecular, Des Plaines, IL) mentioned in this article.

References
