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Utilization of Ancillary Studies in the Cytologic Diagnosis of Biliary and Pancreatic Lesions:

The Papanicolaou Society of Cytopathology Guidelines for Pancreatobiliary Cytology

Lester J. Layfield, M.D.[1,*], **Hormoz Ehya, M.D.**[2], **Armando C. Filie, M.D.**[3], **Ralph H. Hruban, M.D.**[4], **Nirag Jhala, M.D.**[5], **Loren Joseph, M.D.**[6], **Philippe Vielh, M.D., Ph.D.**[7], and **Martha B. Pitman, M.D.**[8]

[1]Department of Pathology and Anatomical Sciences, University of Missouri, Columbia, Missouri [2]Department of Pathology, Fox Chase Cancer Center, Philadelphia, Pennsylvania [3]Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland [4]The Sol Goldman Pancreatic Cancer Research Center, The Johns Hopkins University School of Medicine, Baltimore, Maryland [5]Department of Pathology and Laboratory Medicine, the Hospital of the University Of Pennsylvania, Philadelphia, Pennsylvania [6]The Department Of Pathology, the University of Chicago, Chicago, Illinois [7]Institut Gustave Roussy, Villejuif, France [8]Department of Pathology, the Massachusetts General Hospital, Harvard University, Boston, Massachusetts

Abstract

The Papanicolaou Society of Cytopathology has developed a set of guidelines for pancreatobiliary cytology including indications for endoscopic ultrasound-guided fine-needle aspiration, terminology and nomenclature of pancreatobiliary disease, ancillary testing, and post-biopsy management. All documents are based on the expertise of the authors, a review of the literature, discussions of the draft document at several national and international meetings, and synthesis of selected online comments of the draft document. This document presents the results of these discussions regarding the use of ancillary testing in the cytologic diagnosis of biliary and pancreatic lesions.

Currently, fluorescence in situ hybridization (FISH) appears to be the most clinically relevant ancillary technique for cytology of bile duct strictures. The addition of FISH analysis to routine cytologic evaluation appears to yield the highest sensitivity without loss in specificity. Loss of immunohistochemical staining for the protein product of the SMAD4 gene and positive staining for mesothelin support a diagnosis of ductal adenocarcinoma. Immunohistochemical markers for endocrine and exocrine differentiation are sufficient for a diagnosis of endocrine and acinar tumors. Nuclear staining for beta-catenin supports a diagnosis of solid-pseudopapilary neoplasm. Cyst fluid analysis for amylase and carcinoembryonic antigen aids in the preoperative classification of pancreatic cysts. Many gene mutations (KRAS, GNAS, VHL, RNF43, and

[*]Correspondence to: Lester J. Layfield, MD, Professor and Chair, Department of Pathology and Anatomical Sciences, M263 Medical Science Building, One Hospital Drive, Columbia, MO 65212, USA. layfieldl@health.missouri.edu.

CTNNB1) may be of aid in the diagnosis of cystic neoplasms. Other ancillary techniques do not appear to improve diagnostic sensitivity sufficiently to justify their increased costs.

Keywords

biliary tract; pancreas; EUS; fine-needle aspiration; ancillary studies; molecular diagnosis

Ancillary testing of pancreatobiliary cytology specimens is becoming increasingly important as we learn more about the molecular mutations associated with pancreatobiliary disease and develop clinically useful tests that aid in the diagnosis and management of patients. Management algorithms for pancreatic neoplasia are evolving: some are shifting from surgical intervention to conservative surveillance, whereas others recommend preoperative chemoradiation prior to surgery to improve the yield of R0 resections. As an invaluable member of the patient care team, the pathologist is at the forefront of diagnosis, both pre- and post-operatively. Ancillary tests are invaluable tools that assist in refining our diagnoses and include a wide range of biochemical and molecular tests with variable diagnostic and prognostic utility.

These proposed guidelines on ancillary testing of pancreatobiliary cytology specimens stem from the expertise of the authors, review of the literature, and discussions with pathologists at several national and international meetings over an 18-month period and synthesis of selected online comments of the draft document on the *Papanicolaou Society of Cytopathology* website [www. papsociety.org].

Ancillary Testing of Pancreatobiliary Strictures

Cytological evaluation of bile duct brushings is known to have suboptimal sensitivity ranging from 40 to 61%.[1–11] Many approaches have been developed to augment the purely cytologic analysis of bile duct brushing specimens. These have included protocols to improve diagnostic sensitivity of brushing specimens designated as inconclusive, indeterminate, or negative following on-site cytopathologic examination.[12–15] Ancillary procedures potentially useful in the interpretation of negative and indeterminate cytological results include intraductal ultrasound examination, digital image analysis (DIA), immune-labeling, fluorescence in situ hybridization, genetic analysis for neoplasia-specific mutations, and sequential mutational analysis.[1–14,16] Table I demonstrates markers that have been used in ancillary testing of brushing specimens.

Digital Image Analysis of Samples from Duct Strictures

At the level of the biosample, DIA has been used to identify abnormalities of nuclear DNA content using spectrophotometric techniques.[17–19] DIA utilizes Feulgen reactions that hydrolyze DNA into constituent nucleic acids that stoichiometrically bind to the Feulgen dye. Using this technique, DNA ploidy can be assessed by a variety of commercially available image analyzers.[17,20] DNA ploidy status is assessed on the collected cells using a histogram generated by commercially available quantitative DNA analysis programs. Results are characterized as diploid, aneuploid, or tetraploid. Aneuploid and tetraploid results are more likely supportive of malignancy.[21]

The sensitivity of DIA does not appear to improve diagnostic accuracy beyond that achievable with routine cytology for patients with primary sclerosing cholangitis. However, in patients without primary sclerosing cholangitis, the technique does appear to improve diagnostic sensitivity. DIA has been reported to have excellent specificity for the diagnosis of carcinoma but only moderate sensitivity.[18,19] Thus, DIA has diagnostic characteristics similar to routine cytology in that a positive test is highly accurate but a negative result is of little clinical value.

Immunocytochemistry

A number of immunocytochemical markers have shown promise distinguishing benign from malignant biliary epithelium. These include S100P, von Hippel–Lindau gene product (pVHL), IMP3, and CD10. Tretiakova et al.[22] demonstrated loss of CD10 immunopositivity in most examples of high-grade dysplasia and all cases of invasive carcinoma. CD10 positivity was present in the overwhelming majority of benign lesions. In a separate study, Levy et al.[23] demonstrated strong staining for S100P in 90% of adenocarcinomas with 83% of adenocarcinoma showing diffuse staining for S100P. IMP3 staining was present in 77.5% of adenocarcinomas. Total loss of pVHL staining was seen in 37 of 40 adenocarcinomas. Seventy percent of adenocarcinomas displayed the staining pattern S100P+/IMP3+/pVHL −.[23] Benign biopsies were invariably negative for S100P but 94% were pVHL+.[23] As discussed in the section on solid pancreatic lesions, adenocarcinomas frequently stain for MUC4 and mesothelin but demonstrate an absence of staining for clusterin-beta.[24]

Molecular Analysis of Duct Stricture Brushing Specimens

KRAS Mutational Analysis

KRAS gene mutation analysis has been used as an ancillary testing procedure for analysis of pancreatic juice, bile duct brushings, and fine-needle aspiration (FNA) of solid and cystic pancreatic masses.[25–27] The majority of studies involving *KRAS* mutations have investigated their relationship with pancreatic adenocarcinoma[28–31] and genetic progression in pancreatic duct lesions and intraductal papillary mucinous neoplasms.[32–34] *KRAS* mutational analysis of pancreatic juice has even been used as a potential method for the early diagnosis of pancreatic carcinoma.[35–37] Although *KRAS* mutations can be found in pre-invasive dysplastic lesions and invasive carcinomas of the pancreas,[25–32] several studies have shown that *KRAS* mutation analysis is a sensitive test for pancreatic adenocarcinoma.[38–40] Sturm et al.[26] studied 312 consecutive patients with extrahepatic biliary stenosis and found that conventional cytology combined with *KRAS* mutational analysis was more sensitive than conventional cytology alone. Kipp et al.[41] studied 35 brushing cytology samples collected during ERCP and demonstrated a combined sensitivity of 86% for *KRAS* mutation and fluorescence in situ hybridization (FISH) analyses in the diagnosis of pancreatic adenocarcinoma. Hruban et al.[33] have described a progression model for pancreatic carcinoma in which both *KRAS* and telomere abnormalities are seen in low-grade dysplasias as well as malignancies. *KRAS* mutations are, therefore, not specific for invasive cancer and have been described in chronic pancreatitis.[29,30] Reicher et al.[25] demonstrated some utility for *KRAS* mutational analysis in the evaluation of EUS-FNA specimens. They described *KRAS* mutations in approximately 9% of benign specimens and 56% of malignant

specimens. Importantly, *KRAS* mutational analysis was helpful in dividing atypical specimens into benign and malignant categories. Neither of the two cases in Reicher's study was considered cytologically atypical but on follow-up demonstrated to be benign, contained *KRAS* mutations.[25] On the contrary, in cases considered atypical with malignant follow up, 20% of cases (2/10) demonstrated *KRAS* mutations. Additional studies will be necessary to determine the value of *KRAS* mutational analysis in both bile duct stricture cytology samples and FNA of solid pancreatic masses, but current data do not support *KRAS* testing of solid pancreatic masses and bile duct strictures as a useful ancillary test for diagnosis. The utility of *KRAS* mutational analyses in the evaluation of cystic lesions of the pancreas is discussed below.

The potential application of analytic techniques for evaluating sequential mutation accumulation in bile duct brushing specimens has been studied. Lapkus et al.[16] showed that there is considerable overlap in the spectrum of mutational markers in pancreatic duct and biliary brushings, but the temporal profile of accumulation of these mutations differs significantly between pancreatic and biliary neoplasms. These authors studied the time course of mutation accumulation in pancreatic and biliary tract lesions by microdissecting cell clusters on the basis of cytomorphologic features and analyzed the cells for the loss of heterozygosity with a panel of fifteen markers (1p, 3p, 5q, 9p, 10q, 17p, 17q, 21q, 22q) as well as point mutations in *KRAS* using PCR/capillary electrophoresis. The prevalence of loss of heterozygosity (LOH) and *KRAS* mutations in these lesions varied.[16] Although distinctive prevalences for sequential mutation accumulation were demonstrable, diagnostic utility of this approach is still to be determined.

Fluorescence In Situ Hybridization

FISH analysis of bile duct brushing specimens for polysomy using a commercially available DNA probe set (Uro-Vysion; Abbot Molecular, Des Plaines, IL) has been reported as a useful technique by a number of authors.[11–13,21,42–44] This commercial kit utilizes probes targeting the pericentromeric regions of chromosomes 3 (CEP3), 7 (CEP7), and 17 (CEP17) as well as chromosomal bands 9p21 (LSI 9p21). The method can be automated using the Bioview Imaging Duet system (Bioview, Ltd., Nes Zionu, Israel). Others[21] developed probe sets of their own based on known chromosomal alterations in genes associated with pancreatic carcinoma including *TP53*, *CDKN2A*/*p16*, and *EGFR*. In a series of 93 pancreaticobiliary brushings, Barr, Fritcher et al.[43] demonstrated a specificity of 100% and a sensitivity of approximately 60% for the identification of carcinoma using FISH probes targeting centromeric regions of chromosomes 3, 7, 17, and 9p21 band. They considered a specimen as positive for malignancy by FISH when five or more cells showed polysomy (>2 signals in at least 2 of the 4 probes). In that study, FISH analysis outperformed routine cytology and review consensus cytology of the brushing specimens. Boldorini et al.[42] also reported similar findings with FISH of brushing specimens outperforming routine cytology. In that study, the sensitivity of FISH was 90% with 94% specificity, whereas the positive predictive value was 98% and negative predictive value was 75%. Levy et al.[12] reported similar success with FISH. In that study, the authors considered trisomy for chromosome 7 to be benign. In a series of morphologically negative cytology samples, FISH was able to suggest malignancy in 62% of cases.[12] Barr, Fritcher et al.[43] investigated the utility of

ancillary studies including DIA and FISH in a series of 498 consecutive patients with pancreaticobiliary strictures. They found that FISH had a sensitivity of 43% and was significantly better than the sensitivity of routine cytology (20%) when equivocal cytology samples were considered negative. They concluded that FISH had a higher sensitivity than cytology without compromising specificity.[13]

Of all the ancillary techniques currently available for analysis of cytology specimens obtained by brushings from pancreaticobiliary strictures, FISH appears to improve diagnostic sensitivity the most over that achievable by routine cytology.[12,13,15,25,42–44] Although not directly addressing bile duct brushings, Kubiliun et al.[14] recommended the use of FISH for the diagnosis of pancreatic carcinoma in inconclusive cytologic evaluations. It appears that a similar approach is successful for the evaluation of negative and inconclusive pancreaticobiliary tract brushing specimens.

Additional markers of malignancy in bile duct brushing are under investigation. Although experience is limited, RNA-binding protein-3 (IMP3) shows promise as a marker for adenocarcinoma in bile duct brushings. In one study, IMP3 immunohistochemical expression demonstrated a sensitivity of 64% with a specificity of 100% for adenocarcinoma.[45]

Ancillary Testing for Pancreatic Cystic Lesions

A number of ancillary tests have been proposed to aid in the diagnosis of cystic lesions of the pancreas. These vary from measuring carcinoembryonic antigen (CEA) and amylase levels in cyst fluid to histochemical stains for mucin on cytologic smears, to mutational analysis (*KRAS*, *GNAS*, *TP53*, *VHL*, *CTNNB1*, and *RNF43*) and measures of polysomy.[34,39,46,47] Table II. Proposed Ancillary Testing for Cystic Pancreatic Lesions lists markers that have been used in the diagnosis of pancreatic cystic lesions.

Gross Cyst Fluid Evaluation

Visual examination of the gross characteristics of the cyst fluid is diagnostically helpful. A gross description such as “thick, white, viscous, sticky fluid” and cyst fluid that is difficult to pull into the needle and express from the needle clearly indicates a mucinous cyst fluid. These descriptions act as a surrogate marker for viscosity, a test that is not readily available in the biopsy suite. Leung et al.[48] examined the role of the “string sign” as a marker of viscosity. By placing the fluid between the thumb and index finger and gently pulling the fingers apart, the fluid would “string” to 3.5 mm if mucinous. Ancillary testing adds little to this simple visual test. Similarly, thin, nonmucoid, serosanguinous, or frankly bloody cyst fluid is typical of serous cystadenoma due to the high vascularity of the septae in these cysts, which causes them to bleed internally and during aspiration.

Histochemical Stains for Mucin

Staining of direct smear preparations for mucin by a variety of techniques including the mucicarmine stain for neutral mucin and alcian blue pH 2.5 for acid mucin can be performed on direct smear preparations, but it is not diagnostically valid when performed on liquid-based preparations. Staining of smear preparations for mucin aids in the establishment of

mucinous differentiation of the lining epithelium; however, it cannot separate benign from malignant lesions, and standardization of how much staining constitutes a positive result in EUS-FNA specimens with contamination from the gastrointestinal tract has not been determined. Gastrointestinal contamination of mucin can lead to a false-positive interpretation. Thick colloid-like mucin with cellular debris floating in the mucin is consistent with mucinous cyst contents and not gastrointestinal contamination.[49] Documentation of mucinous differentiation in cystic pancreatic lesions is clinically helpful in that it points to either a mucinous cystic neoplasm or an intraductal papillary mucinous neoplasm.

Biochemical Tests of Cyst Fluid

Cyst fluid amylase—Amylase testing quantifies α-amylase using an enzymatic colorimetric assay to measure the formation of degradation products saccharogenically or kinetically with the aid of enzyme-catalyzed subsequent reactions. The color intensity of the degradation product formed is directly proportional to the α-amylase activity, which is determined by measuring the increase in absorbance. Cyst fluid amylase is elevated in pseudocysts (typically in the thousands) and in cysts that communicate with the pancreatic ductal system, such as intraductal papillary mucinous neoplasms, but not in cystic lesions that do not, such as serous cystadenoma and cystic neuroendocrine tumors.[49] Although MCNs do not communicate with the pancreatic ductal system, amylase levels can be quite elevated in MCNs making the specificity of this test for distinguishing IPMN and MCN of little value. High amylase combined with low CEA is consistent with a pseudocyst; low amylase and low CEA are typical of a serous cystadenoma and high amylase and high CEA are consistent with cystic-mucinous neoplasm with rare exceptions. A pancreatic cyst fluid amylase below 250 u/L is associated with a low risk for a pseudocyst.[50]

Cyst Fluid CEA—Cyst fluid CEA level is a reliable indicator of mucinous differentiation in a cyst, but unfortunately it does not reliably predict the presence or absence of malignancy.[51] Suggested cut-off values for distinguishing between non-neoplastic and neoplastic cysts vary, with early reports suggesting 192 ng/mL as a useful cut point.[51] More recent data suggest a cut point of 110 ng/mL. Increasing the cut-off point for support of a mucinous cyst increases specificity but at the expense of sensitivity. Early studies suggest that CEA level was helpful in separating benign neoplastic cysts from malignant neoplastic cysts,[46] but subsequent studies have made it clear that an elevated CEA is not a reliable test for malignancy.[50,51] In addition, a low CEA level does not exclude a mucinous cyst in general, nor malignant cyst in particular. However, in a single study, mean CEA levels of cyst fluid demonstrated a striking difference between benign and malignant cystic lesions.[46] CEA greater than 693 ng/mL predicted malignancy with a sensitivity of 80% and a specificity of 90%.[50]

CA 125 in Pancreatic Cyst Fluid

A few studies have investigated cyst fluid CA 125 levels to assess the usefulness of this marker in determining the cyst type and discriminating between benign and malignant neoplasms.[52–55] These studies have demonstrated that while the CA 125 levels are generally low in a pseudocyst and high in a cystic neoplasm, significant overlap exists between serous

cystadenoma and mucinous cystic neoplasm. Furthermore, low levels of CA 125 have been reported in cystic neuroendocrine tumors[56] and high levels in ciliated enteric duplication cyst[57] and lymphoepithelial cyst.[58] For these reasons, cyst fluid CA 125 assay does not have an important role in the evaluation of pancreatic cysts.

MUC Protein Expression Patterns in Pancreatic Cyst Fluid

Mucin expression patterns demonstrate a correlation with the presence of dysplasia and carcinoma. Several studies have demonstrated that analysis of pancreatic cyst fluid or serum can predict the presence of dysplasia or malignancy in the surrounding pancreatic cyst lining.[24,59,60] Jhala et al.[24] showed that 91% of pancreatic ductal adenocarcinomas expressed MUC4, but no evidence of staining was found in reactive ductal epithelium. In a study of 90 patients, Carrara et al.[59] investigated the expression of MUC1, MUC2, MUC3, MUC4 MUC5A, MUC5B, MUC6, and MUC7 and found that MUC7 expression was a strong marker for adenocarcinoma and borderline for IPMN. However, MUC7 was expressed in 37% of cases of chronic pancreatitis.[59] Maker et al.[60] showed that high-risk IPMNs demonstrated elevated concentrations of MUC2 and MUC4 in pancreatic cyst fluid. From these data, it appears that evaluation of cyst fluid for MUC2, MUC4, and MUC7 may be helpful in the recognition of dysplasia and malignancy in cystic neoplasms of the pancreas.

Immunohistochemical Analysis of Pancreatic Cyst Samples

Most cyst fluids are usually very scant and of insufficient volume and cellularity for cellblock preparation. Exceptions to this are secondarily cystic neoplasms such as PanNET and SPN where ancillary testing is often vital to a specific diagnosis. See discussion of immunohistochemical testing of solid pancreatic lesions.

DNA Analysis of Cyst Fluid

DNA analysis may also aid in the separation of non-neoplastic and neoplastic, and benign from malignant neoplastic cystic lesions. The success of DNA analyses depends on the amount of recoverable DNA.[9] Measurements of cyst fluid DNA can be quantitated using a variety of commercially available techniques.[61] The concentration of DNA is correlated with optical density (OD) as measured at a wavelength 260/280. The mean concentration of DNA present within a fluid from a pancreatic cystic lesion documented by OD ranges from a low of 6.5 in benign cysts to 16.5 in malignant cysts.[61]

Molecular Testing of Cyst Fluid and Tissue

Molecular analysis for *KRAS* mutations and for LOH has been reported to be helpful in the separation of benign from malignant cysts.[38,39,51] Khalid et al.[39] studied cyst fluid aspirates from 36 pancreatic cysts with histologically confirmed pathology. *KRAS* gene mutations were not observed in the benign cysts (a mix of pseudocysts and benign cystic neoplasms such as serous cystadenoma), whereas 40% of the cysts in the “premalignant” group (neoplastic cysts with low- or intermediate-grade dysplasia) had a *KRAS* mutation, as did the majority of “malignant” cysts (neoplastic cysts with high-grade dysplasia or an associated invasive carcinoma). Similar percentages were reported in an analysis of a larger number of

pancreatic cysts in the "PANDA study."[61] Of interest, in this latter study, all malignant cysts (those with either high-grade dysplasia or an associated invasive carcinoma) with negative cytologic evaluation could be diagnosed as malignant using DNA analyses.[61] Others[34] have had less success in using mutational analysis for the distinction of benign from malignant cystic lesions. Chadwick et al.[34] demonstrated that while *KRAS* point mutations were more common in malignant lesions than in benign lesions, they also could be found in both benign and malignant intraductal papillary mucinous tumors. Others have had a similar experience with solid pancreatic neoplasms.[28,29]

Shen et al.[62] studied the utilization of a commercially available test that combines the detection of *KRAS* mutation, LOH, and DNA quantity/quality in the diagnosis of pancreatic cystic lesions. The concordance between the clinical consensus diagnosis and the commercial test was high with the commercial test showing a sensitivity of 83% and specificity of 100% for a malignant cyst and a sensitivity of 86% and specificity of 93% for a benign mucinous cyst. The authors concluded that the molecular analysis of pancreatic cyst fluids adds diagnostic value to the preoperative diagnosis.[62]

From the available data, it appears that the analysis of pancreatic cyst fluid for mucin by histochemical stains on direct smears and CEA level are helpful diagnostic adjuncts for the recognition of a cystic lesion showing mucinous differentiation. Cyst fluid amylase levels appear to be of great value for the recognition of pseudocysts. *KRAS* and other mutational analyses may aid in the characterization of pancreatic mucinous cysts.[41,46,51]

Recent whole exome sequencing of the four most common cystic neoplasms of the pancreas (serous cystadenoma, solid-pseudopapillary neoplasm, mucinous cystic neoplasm, and IPMN) has identified a specific mutational profile in each cyst type. *VHL* mutations are seen in serous cystic neoplasms, *CTNNB1* (beta-catenin) in solid-pseudopapillary neoplasms, *RNF43*, *KRAS*, *TP53* and *SMAD4* in MCN, and *KRAS*, *RNF43*, *GNAS TP53*, and *SMAD4* in IPMN.[63,64] It has therefore been suggested that mutational analysis for *GNAS*, *KRAS*, *VHL*, *CTNNB1*, *RNF43*, *TP53*, and *SMAD4* may aid in the differential diagnosis of cystic lesions of the pancreas.

Mutations in the *GNAS* gene appear to be an important marker for IPMNs. The *GNAS* gene encodes for stimulatory G-protein alpha subunit, which is a component of many transduction pathways.[65] *GNAS* gene protein product is defective in McCune–Albright Syndrome and some forms of fibrous dysplasia.[65,66] *GNAS* mutations appear to be specific for intraductal papillary mucinous neoplasms,[67,68] while *KRAS* and *RNF43* mutations can also be seen in MCNs.

The von Hippel–Lindau gene (*VHL*) is a tumor suppressor gene linked to sporadic hemangioblastomas and clear cell renal cell carcinomas.[69] This gene is somatically mutated in serous cyst adenomas.[63,70] Mutations in *VHL* gene are not seen in other cystic lesions of the pancreas.

RNF43 (ring finger protein 43) product is a HAP95-binding ubiquitin ligase that promotes cell growth.[71] RNF43 has recently been linked to the beta-catenin pathway. *RNF43*

mutations have been shown to occur in MCN along with IPMN.[72] Unlike IPMN, MCN do not typically harbor *GNAS* mutations.[72]

Virtually all solid-pseudopapillary neoplasms (SPNs) harbor a *CTNNB1* gene mutation, and these mutations, in the absence of other mutations, appear to be relatively specific for SPNs in the pancreas. Because the betacatenin protein abnormally accumulates in the nuclei of cells with *CTNNB1* gene mutations, immunolabeling for the beta-catenin protein is also a useful aid in establishing the diagnosis on limited cytology samples.[73]

MicroRNA Analysis

The expression of selected microRNA is disregulated in cystic neoplasms of the pancreas, and Matthaei et al[74] and others[75,76] have shown that the patterns of microRNA expression can help classify cyst type and, in some instances, even suggest the degree of dysplasia. Some groups have used panels of microRNA, whereas others have used a more focused approach using selected microRNA such as microRNA 21 and micro-RNA 221.[75,76]

Ancillary Testing for Solid Pancreatic Neoplasms

A number of ancillary procedures have been investigated for the detection of malignancy in solid pancreatic masses. Table III lists markers that have been used in the diagnosis of solid pancreatic lesions.

Immunohistochemical Testing

Immunohistochemical markers for adenocarcinoma—*SMAD4* loss is observed in PanIN-3 high-grade lesions and over half of pancreatic ductal adenocarcinomas.[77,78] *SMAD4* loss also appears to be associated with a poor prognosis for pancreatic ductal adenocarcinoma.[79] Immunolabeling for Smad4, the protein product of the *SMAD4* gene, is a good surrogate for *SMAD4* genetic alterations and is useful in cytologic material processed as cellblocks allowing for easy testing of atypical to suspicious cellular groups. Loss of *SMAD4* staining supports a malignant diagnosis.

Mesothelin (MSLN) is a 40-kDa glycosylphosphatidyl inositol-linked protein that has been demonstrated to be highly overexpressed in pancreatic adenocarcinoma,[80] a finding corroborated in a subsequent study utilizing three platforms.[81] Differential expression of mesothelin in pancreatic adenocarcinoma has also been documented in resected samples.[24] Three independent studies have shown that when applied to FNA material, immunolabeling for mesothelin can serve as a useful marker for supporting the diagnosis of pancreatic adenocarcinoma.[24,82]

Immunohistochemical Testing for Pancreatic Neuroendocrine Tumor

A variety of immunohistochemical markers are useful in the diagnosis and grading of pancreatic endocrine tumors (PanNET). These include general markers of endocrine differentiation (chromogranin, synaptophysin, CD57, CD56, and neuron-specific enolase), cytokeratins CK8 and CK18 and CA 19-9.[83] The proliferation marker Ki-67 is of importance in the histologic classification of Pan-NET into low- and high-grade tumors, but its utility in cytologic preparations including cell block material remains to be determined.

Functional pancreatic endocrine neoplasms produce a number of hormones, which can be demonstrated by immunohistochemical techniques including insulin, glucagon, somatostatin, gastrin, vasoactive intestinal protein, and pancreatic polypeptide.[84–88]

The separation of PanNET into high- and low-grade neoplasms based on mitotic count is proposed by the World Health Organization.[89] Some authors have shown that Ki-67 score appears to be a superior predictor of outcome than degree of tumor differentiation.[89,90] Application of Ki-67 staining to cell block material may have value in stratifying tumors into low- and high-grade forms.[91]

Immunohistochemical Testing for Acinar Cell Carcinoma

Immunohistochemical staining for enzymes produced by acinar cell aids in the distinction of these neoplasms from ductal carcinoma and PanNET. Immunolabeling for amylase, trypsin, chymotrypsin, and lipase is most helpful in the recognition of these neoplasms in cellblock material.[92,93] Expression of CK7 appears to aid in the separation of acinar cell neoplasms (positive) from normal acinar cells (negative).[94] Abnormalities of beta-catenin can also be seen focally in acinar cell carcinomas.[95]

Immunohistochemical Testing for Pancreatoblastoma

Pancreatoblastoma displays diverse directions of differentiation as demonstrated by multiple patterns of antigen expression. The various components of differentiation—ductal, acinar, and endocrine—will label with markers for these lines of differentiation as described above. The most common line of differentiation is along acinar cell differentiation.[96,97]

Immunohistochemical Testing for Solid-Pseudopapillary Neoplasm

Solid-pseudopapillary neoplasms express some markers seen in PanNET and acinar cell neoplasms including alpha-1-antitrypsin, neuron-specific enolase, and CD56.[75,98,99] CD10 is also expressed in solid-pseudopapillary neoplasms.[99] Beta-catenin abnormalities are also characteristic of solid-pseudopapillary neoplasms and diffuse nuclear positivity for this markers usually suffices for the confirmation of the diagnosis.[100]

Immunohistochemical Testing in Pancreatic and Biliary Tract Lymphoma

Both B-cell and T-cell lymphomas arise within the pancreas and biliary tract. The majority of these neoplasms will react with antibodies directed against common leukocyte antigen.

Immunohistochemical testing through either flow cytometry analysis or antibody panels performed on cellblock specimens is necessary for definitive classification of these neoplasms.[101] In-depth discussion of these panels is beyond the scope of this document.

Molecular Analysis

Molecular Testing for Pancreatic Ductal Adenocarcinoma

Mutational analysis—*KRAS* and p16 point mutation analysis, FISH, and DNA ploidy analysis have been used as potential methods for separating benign and malignant masses by FNA.[13,28,51,64,73,74,102–107] Invasive pancreatic adenocarcinoma is believed to develop from

two noninvasive precursor lesions, IPMN and pancreatic intraepithelial neoplasia (PanIN), by the accumulation of a series of mutations.[33] In this progression model for pancreatic carcinoma, activating point mutations in the *KRAS* oncogene and telomere shortening appear to be early events.[73–80,104–109] The prevalence of *KRAS* mutations in IPMN and PanIN increases along with increasing degrees of dysplasia.[102] Additional mutations affecting other genes occur later in the course of progression with increasing dysplasia, with *p16/CDNK2A* loss occurring in intermediate to high-grade lesions, and *TP53* and *SMAD4* inactivation occurring in high-grade precursors and in invasive cancers.[37,78,104,110] *KRAS* gene mutations can be found in 95% of invasive ductal adenocarcinomas of the pancreas, and *p16/CDKN2A* is inactivated in 90%, *TP53* in 75%, and *SMAD4* in 55%.[34]

A retrospective study by Khalid et al. demonstrated that testing of EUS-FNA material for LOH and *KRAS* mutations is useful in differentiating autoimmune pancreatitis from pancreatic cancer.[111] *KRAS* mutations were detected in 10 of 11 (91%) pancreatic cancer cases that yielded DNA amplification, and in none of the autoimmune pancreatitis cases suggesting that *KRAS* mutation in a pancreatic mass FNA is associated with malignancy and may aid in the distinction from benign processes such as autoimmune pancreatitis.[111] However, *KRAS* mutations have been shown to occur in ductal hyperplasias, ductal metaplasias, and chronic pancreatitis.[102,105–107]

Fluorescence in Situ Hybridization

FISH performed for evaluation of solid lesions has been shown to significantly improve diagnostic sensitivity without loss of specificity for ductal adenocarcinoma.[14,42] The probes utilized are directed against chromosomes 3, 7, and 17 as well as 9p21. Loss of 9p21 or alterations in copy number for 3, 7, and 17 are diagnostically important. The technique can be applied using commercially available probe sets. Disadvantages include cost, and that the method is time consuming and requires a fluorescence microscope. FISH analysis when combined with routine cytology has a sensitivity of approximately 85%.[14] Because routine cytology has an excellent specificity, FISH is most useful in improving diagnostic accuracy for cases reported as cytologically negative or inconclusive. Kubiliun et al.[14] have recommended the use of FISH for the evaluation of cases assessed on site as inconclusive or negative.

microRNA

Additional techniques for determining malignancy in pancreaticobiliary lesions include microRNA (miRNA) analysis. miRNAs are short 20–25 nucleotide RNA molecules containing regions complementary to various messenger RNAs (mRNA). Specific binding of an miRNA to an mRNA blocks translation. miRNAs can be robustly quantified using real-time PCR and localized using ISH. Steele et al.[112] have reviewed profiling studies of pancreatic adenocarcinoma. Some miRNAs, such as miR-21 and miR-155, are highly expressed in early lesions.[113–115] Most studies have analyzed resection specimens and one study analyzed FNA specimens,[116] but none have analyzed brushings. The clinical utility remains to be more firmly established, especially in comparison and in conjunction with other techniques.

Molecular Testing for Pancreatic Neuroendocrine Tumors

Loss of Heterozygosity—Nodit et al.[117] explored the utility of analysis of microsatellite loss in the diagnosis of PanNET. They concluded that microsatellite loss analysis of EUS-FNA material obtained from PanNET can be performed reliably and that losses of chromosome arms 3p, 6pq, and 10pq along with gains of 5q, 12q, 18q, and 20q were associated with malignant behavior.[117–120] LOH studies appear to have some technical limitations when using cytologic techniques, because these techniques require microdissection of small numbers of neoplastic cells followed by amplification of DNA and resultant stochastic effects may alter the validity of test results. Additionally, the presence of polysomy or amplification in a cell population being analyzed by LOH may result in allelic imbalance due to other issues than loss of tumor suppressor genes. Polysomy of chromosome 17 might result in one *TP53* allele appearing more prominent than the other in some of these assays. Thus, techniques must be used that normalize overall signals and control for neoplastic cellularity to assure validity.

A number of additional techniques have been investigated for their use in determining the presence of malignancy in pancreaticobiliary lesions. These include investigation of microRNAs.

Mutational Analysis

A number of chromosomal alterations occur in PENs but do not appear to play a role in the etiology of these neoplasms.[120–122] PENs arising in association with MEN1 syndrome demonstrate germline mutations in the MEN1 gene characterized by losses at the 11q13 locus.[123] PENs do not characteristically harbor mutations in *KRAS*, TP53, p16, and *SMADA4*/DPC4.[123]

Molecular Testing of Acinar Cell Carcinoma

Acinar cell carcinomas appear to be associated with losses in the chromosome arm 11p.[96,97] Mutations in the *APC* gene have also been reported.[96,97] The diagnostic significance of these alterations is not yet clear.

Molecular Testing of Solid-Pseudopapillary Neoplasms

The majority (>95%) of solid-pseudopapillary neoplasms have a somatic point mutation in the beta-catenin gene (exon3).[98–100,124] Mutations in *KRAS*, p16, and *SMAD4*/DPC4 genes have not been reported in solid-pseudopapillary neoplasms.

Summary

Currently, FISH is the most clinically relevant ancillary technique applicable to FNA material from pancreatic lesions, because the addition of FISH analysis to routine cytologic evaluation appears to yield the highest sensitivity without loss in specificity. Loss of immunohistochemical staining for the protein product of *SMAD4* and positive staining for mesothelin support a diagnosis of ductal adenocarcinoma. Immunohistochemical markers for endocrine and exocrine differentiation are sufficient for a diagnosis of endocrine and acinar tumors, respectively. Nuclear staining for beta-catenin supports a diagnosis of solid-

pseudopapilary neoplasm. Cyst fluid analysis for amylase and CEA also appears of diagnostic utility for classification of pancreatic cysts. A number of gene mutations (*KRAS*, *GNAS*, *VHL*, *RNF43*, and *CTNNB1*) may be of aid in the identification of specific cystic neoplasms. Other ancillary techniques do not appear to improve diagnostic sensitivity sufficiently to justify their increased cost for the evaluation of EUSFNA and brushing specimens.

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Table I

Proposed Ancillary Testing for Pancreatobiliary Strictures

Marker	Purpose	Diagnostic finding	Utility
Digital image analysis	Separation of benign from malignant strictures	Aneuploid and tetraploid results support malignancy	Does not improve diagnostic accuracy above that achievable by cytology alone
KRAS	Separation of benign from malignant strictures	Mutation present	Insufficient specificity for malignancy to warrant usage
Sequential mutational analysis	Separation of benign from malignant strictures	Loss of heterozygosity	Diagnostic utility to be determined by future studies
FISH	Separation of benign from malignant strictures	Presence of copy number abnormalities in CEP3, CEP7, CEP17, and abnormalities of 9p21 favor malignancy	Diagnostically useful. It is the preferred test to complement routine cytology

Marker	Purpose	Diagnostic finding	Utility
Mucin (mucicarmine, alcian blue ph 2.5)	Identification of mucinous lesions	Positive stain	Diagnostically helpful
Cyst fluid amylase	Identification of pseudocysts and serous cystadenomas	Diagnosis of pseudocyst (level in 1000s, but not <250 U/L) and SCA (low levels, generally <1000 U/L); IPMNs have variable but elevated levels	Differential diagnosis of pancreatic cysts
Cyst fluid CEA	Identification of cystic mucinous lesions	CEA levels above 110 ng/mL support the diagnosis of a mucinous cyst	Distinction between mucinous and nonmucinous cysts
DNA analysis	Separation of benign from malignant cysts	Aneuploid and tetraploid results favor malignancy	Does not significantly improve diagnostic accuracy over routine cytology in majority of studies
KRAS mutations	Identification of mucinous cystic lesions	Presence of *KRAS* mutations supports diagnosis of a mucinous cyst	Distinguishes mucinous from nonmucinous cysts
CA 19-9	Separation of benign from malignant cysts	CA 19-9 level may be elevated in malignant cysts	Not generally useful in the diagnosis of pancreatic cysts
VHL gene mutation	Identification of SCA	Mutation present	Support the diagnosis of SCA
CTNNB1 (beta-catenin) mutation	Identification of SPN	Mutation present	Supports the diagnosis of SPN
GNAS mutation	Identification of IPMN	Mutation present	Supports the diagnosis of IPMN
RNF43 mutations	Identification of cystic mucinous lesions	Mutation present	Distinguishes mucinous from nonmucinous cysts

Table III

Proposed Ancillary Tests for Solid Pancreatic Lesions

Marker	Purpose	Diagnostic finding	Utility
KRAS mutations	Identification of adenocarcinoma	Mutation present	Insufficient specificity for malignancy to warrant usage
SMAD4	Identification of adenocarcinoma	Mutation present [IHC shows loss of staining]	Supports the diagnosis of adenocarcinoma
FISH	Identification of adenocarcinoma	Presence of copy number abnormalities in CEP3, CEP7, CEP17 and abnormalities of band 9p21 favor malignancy	Most reliable test for confirming adenocarcinoma in conjunction with routine cytology
Mesothelin	Identification of malignancy	Overexpression of mesothelin by IHC	Supports the diagnosis of adenocarcinoma
Loss of heterozygosity	Identification of adenocarcinoma	Losses of chromosome arms 3p, 6Qp and 10pq along with gains of 5q, 12q, 18q, and 20q supports a diagnosis adenocarcinoma	Clinical importance to be determined
microRNAs	Identification of adenocarcinoma	Presence of miRNA including miR-21 and mi-155 supports a diagnosis of adenocarcinoma	Clinical utility to be determined