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Review

Practical Applications of Molecular Testing in the Cytologic Diagnosis of Pancreatic Cysts

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Abstract: Mucinous pancreatic cysts are precursor lesions of ductal adenocarcinoma. Discoveries of the molecular alterations detectable in pancreatic cyst fluid (PCF) that help to define a mucinous cyst and its risk for malignancy have led to more routine molecular testing in the preoperative evaluation of these cysts. The differential diagnosis of pancreatic cysts is broad and ranges from non-neoplastic to premalignant to malignant cysts. Not all pancreatic cysts—including mucinous cysts—require surgical intervention, and it is the preoperative evaluation with imaging and PCF analysis that determines patient management. PCF analysis includes biochemical and molecular analysis, both of which are ancillary studies that add significant value to the final cytological diagnosis. While testing PCF for carcinoembryonic antigen (CEA) is a very specific test for a mucinous etiology, many mucinous cysts do not have an elevated CEA. In these cases, detection of a *KRAS* and/or *GNAS* mutation is highly specific for a mucinous etiology, with *GNAS* mutations supporting an intraductal papillary mucinous neoplasm. Late mutations in the progression to malignancy such as those found in *TP53*, *p16/CDKN2A*, and/or *SMAD4* support a high-risk lesion. This review highlights PCF triage and analysis of pancreatic cysts for optimal cytological diagnosis.

Keywords: pancreatic cytology; pancreatic cyst fluid; cyst fluid triage; molecular testing; mucinous cyst; intraductal papillary mucinous neoplasm; mucinous cystic neoplasm

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1. Introduction

Radiologic detection of pancreatic cysts in asymptomatic patients has increased in recent years, and distinguishing benign cystic lesions versus those with malignant potential is essential for patient management. Currently, endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) with pancreatic cyst fluid (PCF) analysis is the standard of care for the preoperative diagnosis of pancreatic cysts [1–4].

There are many different types of cysts in the pancreas, but the most common differential diagnosis of cysts that provide PCF includes non-neoplastic pseudocysts, benign neoplastic serous cysts, premalignant mucinous cysts, malignant mucinous cysts (such as those with invasive carcinoma), and secondarily cystic solid malignancies such as cystic pancreatic neuroendocrine tumors.

The Papanicolaou Society for Cytopathology (PSC) System for Reporting Pancreaticobiliary Cytology [5], published in 2015, promotes using a multimodal approach for the diagnosis of pancreatic cysts, whereby all the clinical, radiologic, cytologic, biochemical, and molecular information available are incorporated to provide the optimal preoperative risk assessment.

PCF combines cytology and biochemical analysis for carcinoembryonic antigen (CEA) and amylase to distinguish mucinous from non-mucinous cysts and to assess for high-risk features [6,7]. Cytologic evaluation provides high specificity but lower sensitivity for the detection of a mucinous cyst. The interpretation of CEA levels is also not without limitations: in one study, using a cutoff of 192 ng/mL resulted in the misdiagnosis of 39% of cystic mucinous neoplasms [8]. Furthermore, CEA levels have been shown to fluctuate in

repeated FNA samples [9]. At some institutions, molecular testing is routinely performed on all PCF with sufficient cyst fluid volumes to improve the detection of mucinous cysts as well as those at high risk for malignancy. With the addition of routine molecular analysis, where *KRAS/GNAS* mutations are highly specific for a mucinous etiology, the detection of mucinous cysts by PCF has been shown to have a sensitivity of 90% and specificity >90% [10–13].

The aim of this review is to summarize how cytopathologists approach the diagnosis of pancreatic cysts and how molecular testing impacts this diagnosis and ultimately patient care.

2. Tissue Management

Fresh, unfixed, and undiluted PCF is required for accurate evaluation and analysis. Fixing and diluting PCF in liquid-based preservatives or saline precludes the ability to obtain biochemical information. Rapid on-site evaluation does not aid in patient management, and all drained cyst fluid should instead be saved and appropriately triaged. If the lesion has a solid component, that area should be separately sampled and made into direct smears.

Even with minimal amounts of cyst fluid, PCF can be triaged for cytology, CEA/amylase analysis, and molecular testing (Figure 1) [6]. One study showed that using supernatant fluid for CEA/amylase testing is comparable to using neat fluid/cell block preparation [14]. If the PCF is very thick, a direct smear should be made, as the chemistry lab will reject the specimen for CEA analysis due to high viscosity. In these cases, the CEA level is not necessary because the thick, viscous nature of the cyst fluid indicates that the cyst is mucinous (and therefore CEA does not contribute additional information). Furthermore, the added value of testing for *KRAS* may be small if cytology and/or CEA levels are conclusive, as *KRAS* mutations do not add to the determination of a mucinous cyst and does not stratify the lesion by grade.

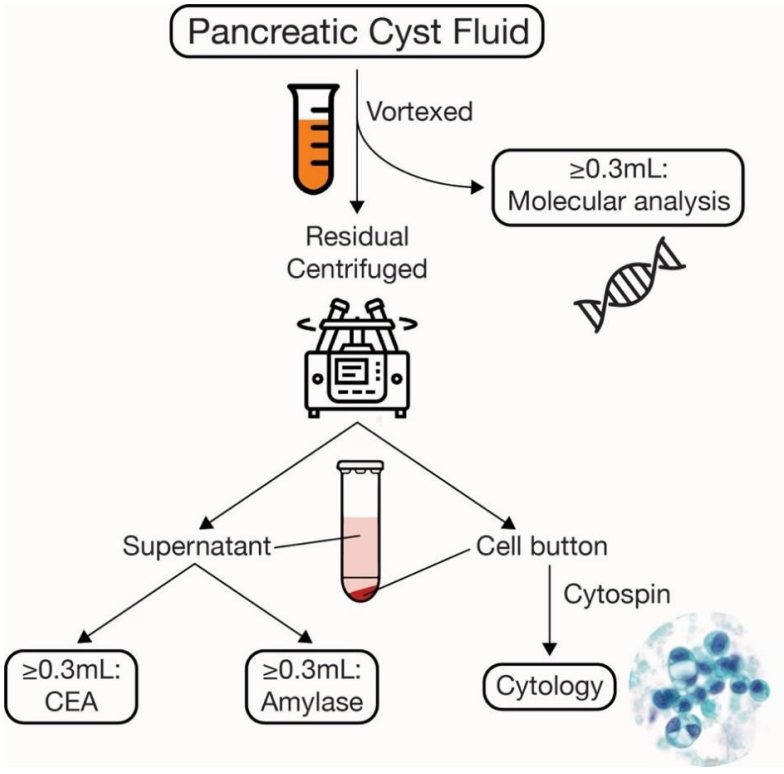


Figure 1. Pancreatic cyst fluid triage.

3. Benign Cystic Lesions

The benign pancreatic cysts have no malignant potential and can be conservatively managed. Grossly, the cyst fluid appears thin and brown or clear, and on cytology, these lesions tend to be sparsely cellular.

3.1. Pseudocyst

Pseudocysts are typically seen in the setting of acute pancreatitis and result from autodigestive damage of pancreatic parenchyma. These cysts lack a true epithelial lining and are collections of necrotic material surrounded by a thick, inflammatory fibrous capsule. PCF almost always shows a very high amylase level (≥250 U/L) and low CEA level (Table 1) [15]. Cytology shows an absence of epithelial cells (aside from possible gastrointestinal contamination) and can demonstrate mixed polymorphous inflammatory cells, histiocytes, crystalline debris, and yellow hematoidin-like pigment (Figure 2) [16]. The differential diagnosis includes serous cystadenomas and cystic mucinous neoplasms, which can slough the cyst lining and result in a very inflammatory cyst fluid without viable epithelial cells.

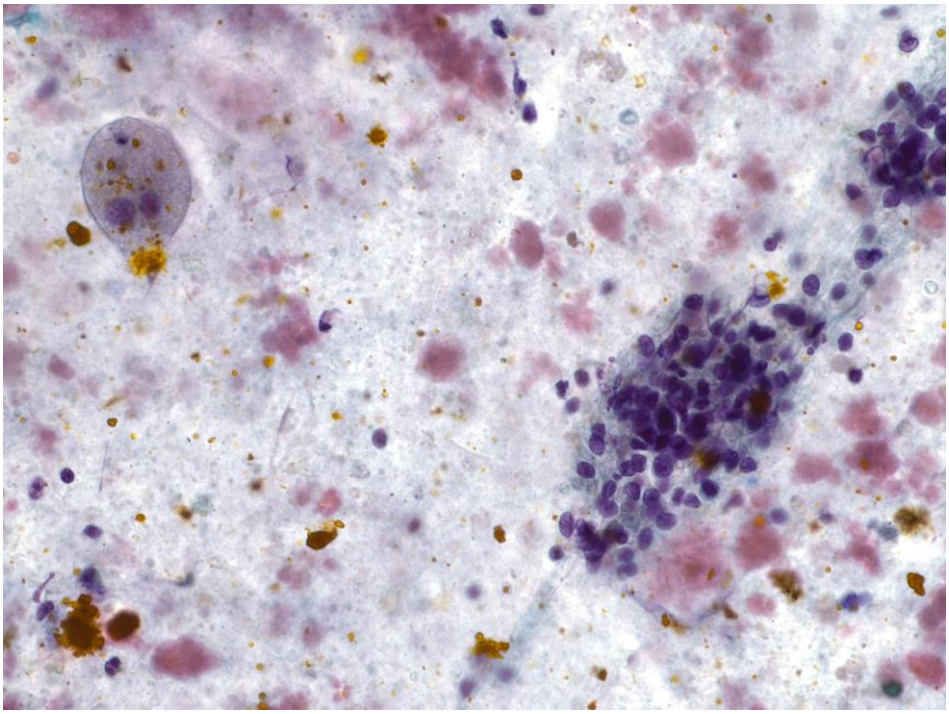


Figure 2. Pancreatic pseudocyst. Cytology shows inflammatory cells, macrophages, amorphous crystalline globular debris, and abundant hematoidin-like pigment (Papanicolaou stain).

3.2. Serous Cystadenoma

Serous cystadenomas (SCAs) are the most common benign cystic neoplasm of the pancreas and comprise 1–2% of pancreatic neoplasms [17,18]. As these cysts are benign, the goal of preoperative diagnosis is to distinguish them from neoplastic mucinous cysts, thus allowing optimal triage of patients to conservative management instead of surgical resection in the case of neoplastic cysts. However, the lack of intact cyst lining epithelium makes the diagnosis extremely challenging on cytology alone, with a sensitivity of 10% in a recent case series [19]. SCAs are lined by nonmucinous cuboidal cells with uniform round nuclei. Cytoplasmic clearing due to glycogen accumulation can be seen in some cases (Figure 3), but should not be mistaken for mucinous epithelial cells, which are columnar with abundant intracellular mucin. Most cytologic specimens are hypocellular, and epithelial cells are not always present. The background can be bloody with hemosiderin-laden

macrophages, which can act as a surrogate marker suggestive of the diagnosis [19]. SCAs have a low CEA level (<5 ng/mL) and a low amylase level [20].

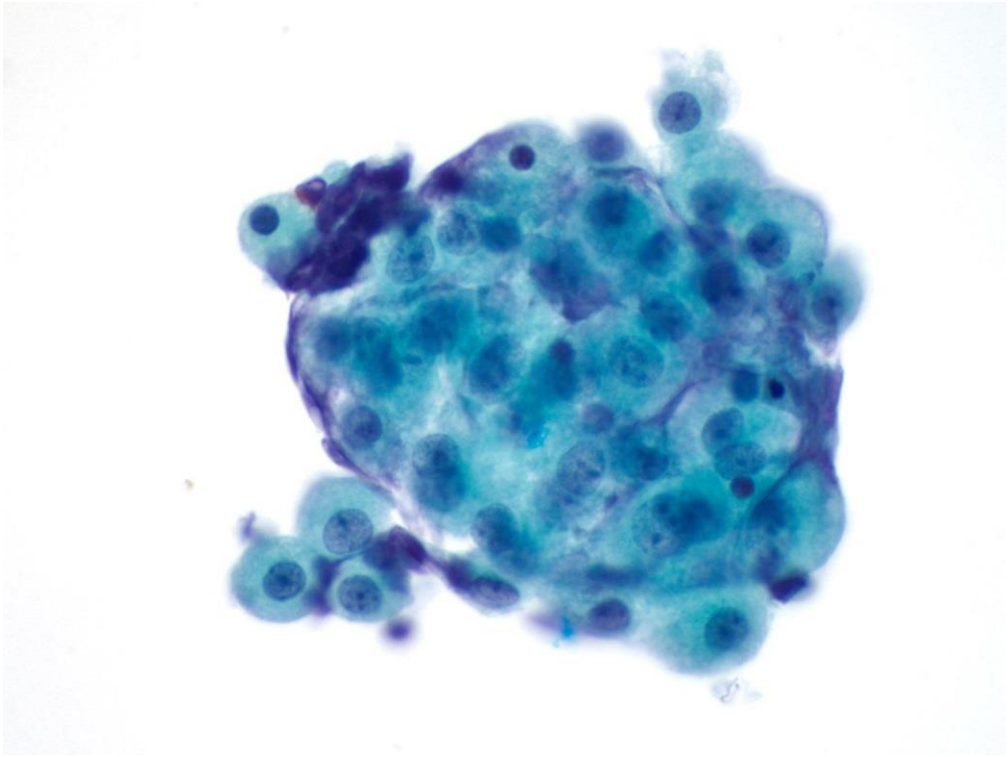


Figure 3. Serous cystadenoma. Cytology shows cuboidal cells with non-mucinous cytoplasm and uniform, bland centrally located nuclei. This finding may be a pitfall for mucinous epithelium (Papanicolaou stain).

Mutations in the von Hippel-Lindau (*VHL*) gene (3p25), loss of heterozygosity at the *VHL* gene locus on chromosome 3, and chromosome 3p aneuploidy have been identified in up to 67% of sporadic and hereditary SCAs but were not identified in mucinous cysts [21]. Thus, detection of one of the above genetic alterations in PCF supports a diagnosis of SCA (Table 1). Of note, cystic pancreatic neuroendocrine tumors can also harbor *VHL* mutations or promoter hypermethylation in up to 25% of sporadic cases [22].

3.3. Lymphoepithelial Cyst

Lymphoepithelial cysts (LECs) are benign cysts lined by mature squamous epithelium without atypia. The subepithelial stroma consists of dense, non-neoplastic lymphoid tissue with germinal center formation that may not be as well-represented on the FNA mateial as compared to the squamous epithelial component. Cytology shows anucleated and nucleated squamous cells with abundant keratinous debris, variable cholesterol crystals, lymphocytes, and histiocytes [23,24].

Table 1. Pancreatic cyst fluid analysis of cystic pancreatic lesions.

Cystic Entity	Biochemical Tests		Genetic Mutations					
	CEA	**Amylase**	***KRAS***	***GNAS***	**3p25 (*VHL*)**	***p53***	***p16***	***SMAD4***
Pseudocyst	↓	↑↑	−	−	−	−	−	−
SCA	↓↓	↓↓	−	−	+	−	−	−
LEC	↑↑	↓	−	−	−	−	−	−
IPMN	↑↓	↓↑	+	+	−	+(HR)	+(HR)	+(HR)
MCN	↑↓	↓↑	+	−	−	+(HR)	+(HR)	+(HR)
PanNET *	↓↓	↓↑	−	−	+/−	−	−	−
PDAC *	↑↓	↓↑	+	−/+ **	−	+	+	+ (55%)

Abbreviations: CEA, carcinoembryonic antigen; SCA, serous cystadenoma; LEC, lymphoepithelial cyst; IPMN, intraductal papillary mucinous neoplasm; MCN, mucinous cystic neoplasm; PanNET, pancreatic neuroendocrine tumor; PDAC, pancreatic ductal adenocarcinoma; HR, high-risk.* Cystic variants. ** GNAS mutations have been reported in PDACs with associated IPMN (~8% of cases) [25].

Importantly, there are pitfalls to consider in the differential diagnosis between LECs and mucinous cysts. More often than not, LECs produce a thick aspirate for direct smears where the keratinous debris and admixed lymphocytes are recognized and no liquid fluid is available for CEA testing (Figure 4). However, rarely, the keratinous debris is so degenerated and liquified that the PCF does get sent for CEA, which typically results in a very high CEA level (in the thousands), leading to a false classification of the cyst as mucinous (Table 1).

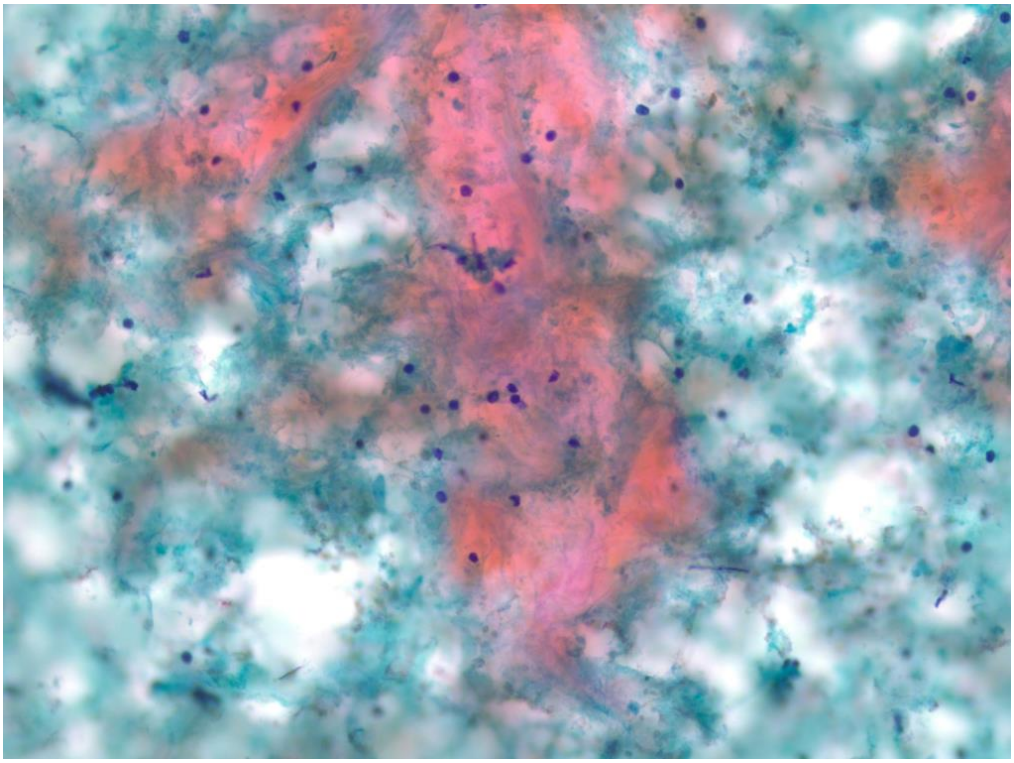


Figure 4. Lymphoepithelial cyst. Cytology shows thick keratinous debris and scattered admixed lymphocytes (Papanicolaou stain).

4. Premalignant/Malignant Cystic Lesions

Premalignant/malignant pancreatic cysts include the neoplastic mucinous cysts, which are diagnosed using the proposed standardized terminology system for pancreaticobiliary specimens from the Papanicolaou Society of Cytopathology (PSC) based on having

one or more of these features: thick, colloid-like extracellular mucin (Figure 5), mucinous cyst lining epithelium, and/or elevated CEA $\geq$ 192 ng/mL [15,26,27]. The two types of neoplastic mucinous cysts—intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN)—share common cytomorphological features but have differing clinical and biological characteristics. Both are stratified into low/intermediate-grade dysplasia or high-grade dysplasia. Both cyst types may be associated with an invasive carcinoma component, which is the most important negative prognostic factor. Cytology alone cannot distinguish between IPMN and MCN; however, this distinction has important implications, as surgical resection is recommended for all MCNs regardless of grade, while most low-grade branch-duct IPMNs can be managed without surgery.

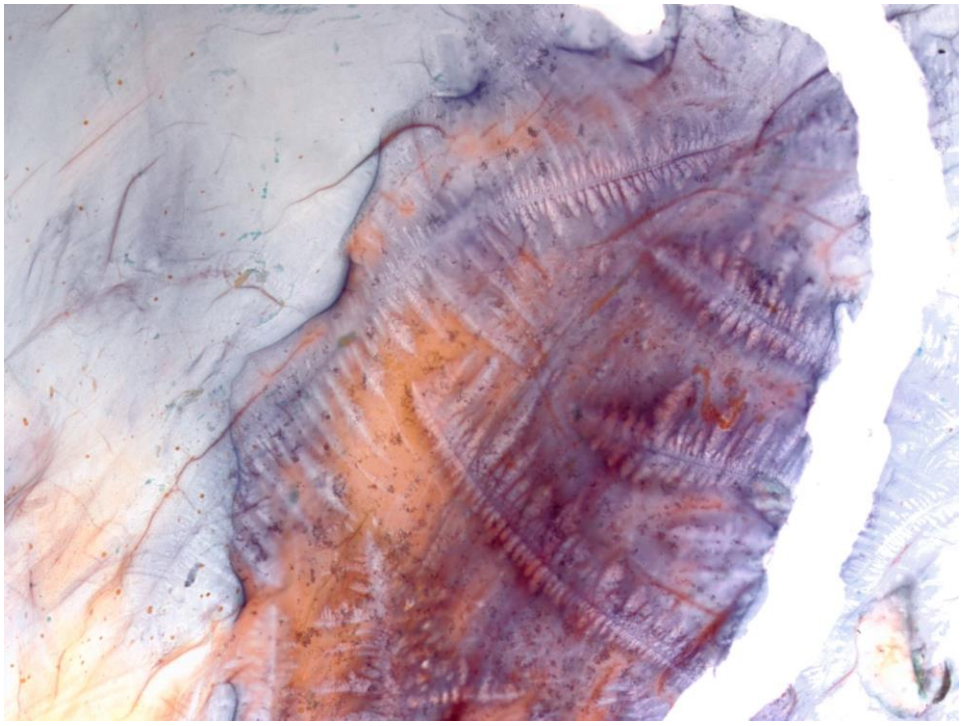


Figure 5. Mucinous cyst with thick colloid-like extracellular mucin (Papanicolaou stain). Even without the presence of mucinous epithelium in the sample, this finding is diagnostic of a mucinous cyst.

Cyst fluid CEA levels have been shown to be the most accurate method for identifying a mucinous etiology, while cytologic evaluation of the cyst lining epithelium is the best modality for identifying high-risk cysts [15,26,28]. A CEA $\geq$ 192 ng/mL has an overall accuracy of ~80% (74% sensitivity and 84% specificity). However, a CEA level below 192 ng/mL does not exclude a mucinous cyst. In addition, increasing the cutoff CEA level increases the specificity but lowers the sensitivity for the diagnosis of a mucinous cyst [20]. Amylase levels are highly variable in mucinous cysts and do not reliably distinguish between the mucinous cysts.

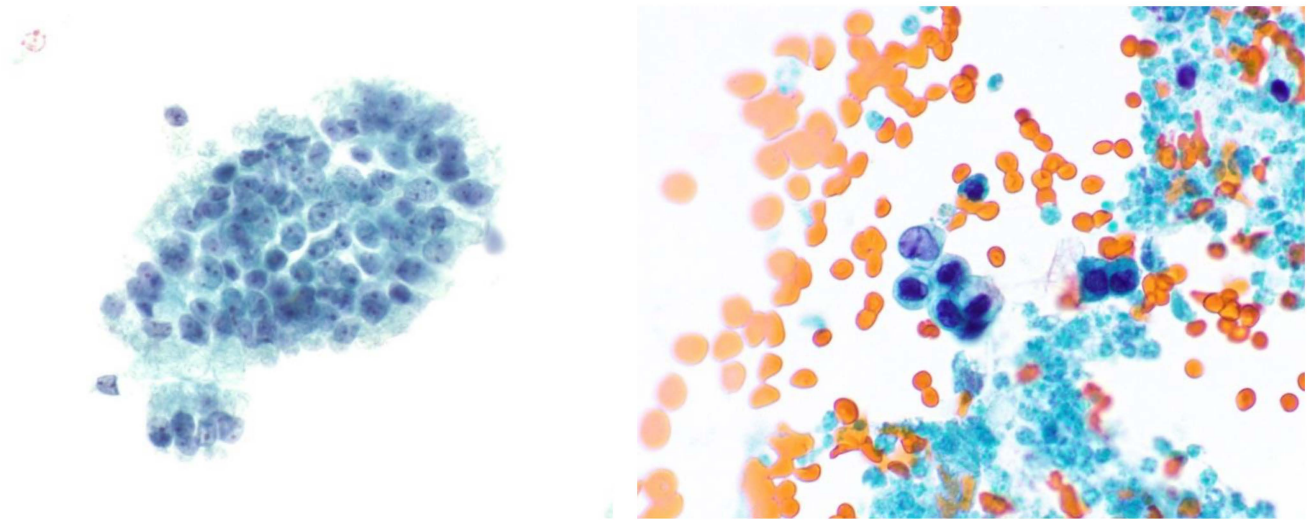
Pancreatic cancer arising from mucinous cysts is one of two major pathways; the other and more common pathway is from pancreatic intraepithelial neoplasia (PanIN). PanIN is not visible on imaging in its early noninvasive stages, whereas mucinous cysts are visible. Mutations in *KRAS* are shared by both pathways and are an early marker of neoplasia. Molecular studies have shown that *KRAS* mutations are highly specific but only moderately sensitive for the identification of a mucinous pancreatic cyst [10,29]. *GNAS* mutations were detected in 66% of cases, while *KRAS* or *GNAS* mutations were detected in 96% of cases [30]. A large prospective study performed next-generation sequencing (NGS) on 626 PCF specimens and found *KRAS* and/or *GNAS* mutations in 100% of IPMNs; *GNAS*

mutations were 100% specific for an IPMN. *KRAS* mutations were detected in 30% of MCNs. Overall, *KRAS/GNAS* mutations were found to have a sensitivity of 89% and specificity of 100% for the detection of a mucinous cyst [31], but these mutations did not stratify the cysts by grade. *RNF43* mutations were also detected in both IPMNs and MCNs [21,32].

Late mutations in the development of carcinoma include *TP53* mutations and deletions in *p16/CDKN2A* and *SMAD4*; thus, the presence of any of these mutations indicates a high-risk lesion [12]. In addition, mutations in *TP53*, *PIK3CA*, and/or *PTEN* with mutant allele frequencies equivalent to those for *KRAS* and/or *GNAS* mutations were highly sensitive and specific for IPMNs with advanced neoplasia (i.e., high-grade dysplasia and invasive adenocarcinoma) [31]. For *GNAS* mutations, mutant allele frequencies >55% were also correlated with IPMNs with high-grade dysplasia. These findings are promising for the possibility of detecting high-risk cysts and may become more relevant as molecular testing becomes more integrated into routine clinical practice.

NGS can have a significant impact on the clinical impression of the cyst etiology. One study of 92 PCFs found that NGS changed the clinical diagnosis in 12% of cases and importantly supported the clinical diagnosis in 78% of the cases, often when little other information was available from cytology and CEA analysis [10]. Additional studies showed that NGS was positive for a significant mutation in 59% and 38% of PCF cases, respectively, with some cases diagnosed as neoplastic mucinous cysts solely on the basis of molecular results [12,33].

The cytological evaluation of PCF aims to determine if the cyst is mucinous and, if mucinous, if the cyst is high-risk for malignancy. A mucinous etiology is supported by thick colloid-like extracellular mucin, mucinous epithelium that is clearly not contaminating gastrointestinal epithelium, CEA $\geq$ 192 ng/mL, or the detection of mutations in *KRAS/GNAS/RNF43*. A mucinous cyst at high-risk for malignancy is one with high-grade epithelial atypia (Figure 6b) or late mutations in the dysplasia-to-carcinoma progression (see below).



(**a**) (**b**)

Figure 6. Mucinous epithelium with (**a**) low-grade atypia versus (**b**) high-grade atypia (Papanicolaou stain). Features of high-grade atypia include small cell size, high nuclear-to-cytoplasmic ratios, hyper- or hypochromasia, and nuclear membrane irregularities.

4.1. Intraductal Papillary Mucinous Neoplasm

Intraductal papillary mucinous neoplasms (IPMNs) comprise 3–5% of pancreatic tumors and 20% of neoplastic pancreatic cysts. The majority arise in the head of the pancreas (~70%). Overall, the prognosis for low-grade IPMNs is excellent, and most can

be conservatively managed by surveillance. High-risk features that may prompt surgical resection include the presence of a mural ndodule, involvement of the main duct, and identification of high-grade dysplasia or invasive carcinoma [34]. Main duct IPMNs have a high rate of malignancy, with 40% of cases showing invasive carcinoma at the time of detection. On the other hand, branch duct IPMNs have a low rate of malignancy, and their management depends on other high-risk features. The Fukuoka Guidelines, first introduced in 2012 and then revised in 2017, provides an algorithm for the management of IPMNs [4,35]. EUS-FNA is recommended for patients with worrisome/high-risk imaging features, and suspicious or positive cytology warrants surgery. Thus, cytopathologists have important roles in guiding patient management with the goal of determining whether the cyst is non-mucinous or mucinous, as well as whether the cyst is low-grade or high-grade (and therefore high-risk for malignancy) based on evaluation of the epithelium and molecular testing.

4.2. Mucinous Cystic Neoplasm

Mucinous cystic neoplasms (MCNs) comprise 5–6% of pancreatic tumors and almost always occur in middle-aged women; 90% arise in the body or tail of the pancreas. In contrast to IPMNs, surgical resection is recommended for all patients with MCN, as their location in the distal pancreas lends them to a less morbid distal pancreatectomy or laparoscopic procedures, and resection removes the need for lifelong surveillance in these relatively young patients.

The characterstic histologic feature of MCN is the presence of subepithelial ovarian-type stroma. This feature can only be evaluated on a biopsy specimen. The ovarian-type stromal cells express estrogen and progesterone receptors, and immunohistochemistry may be performed if the morphology is ambiguous [36,37].

There are no known genetic mutations specific to MCN [38,39]. *KRAS* mutations are found in 30% of MCNs with low-grade dysplasia and 80% of MCNs with high-grade dysplasia or invasive carcinoma [40]. Mutations in *TP53*, *p16/CDKN2A*, and *SMAD4* have been detected in MCNs with malignant transformation (i.e., high-grade dysplasia or invasive adenocarcinoma), which comprises <15% of MCNs.

5. Molecular Testing in Practice

As previously mentioned, molecular testing can help to determine a mucinous etiology for a pancreatic cyst. This information is typically not available at the time of sign out; however, when a *KRAS* or *GNAS* mutation is subsequently detected in an otherwise nondiagnostic or indeterminate aspirate, it can impact the final clinical impression of the cyst and affect patient management. On the other hand, molecular testing may be limited by scant cyst fluid volumes: in previous studies, insufficient material precluded NGS analysis in 7–31% of cases [31,33]. It is important to note that mutations in *KRAS*, *GNAS*, and *RNF43* simply support a neoplastic mucinous cyst (and *GNAS* additionally supports an IPMN), but none of these mutations defines a high-grade cyst. When late mutations—primarily in *TP53*, *SMAD4*, and *p16/CDKN2A*, but also others—are found, they do indeed define a high-risk cyst, which warrants surgical intervention.

If NGS is not an option at your institution, immunohistochemistry for p53 and SMAD4 can be used as surrogate markers for their respective genes. These stains can be very helpful if there is sufficient tissue for evaluation in the cell block. Aberrant p53 staining suggestive of a mutant phenotype can show either strong, diffuse nuclear staining (in contrast to the patchy weak staining of wildtype p53) or a null staining pattern with no nuclear staining at all (Figure 7). In one study of 32 cases, increased p53 expression was seen in 31% of all mucinous cysts (all with at least "atypical epithelium") and 70% of those with high-grade dysplasia [41]. For SMAD4, loss of nuclear staining supports a high-grade lesion (seen in up to half of cases) (Figure 8).

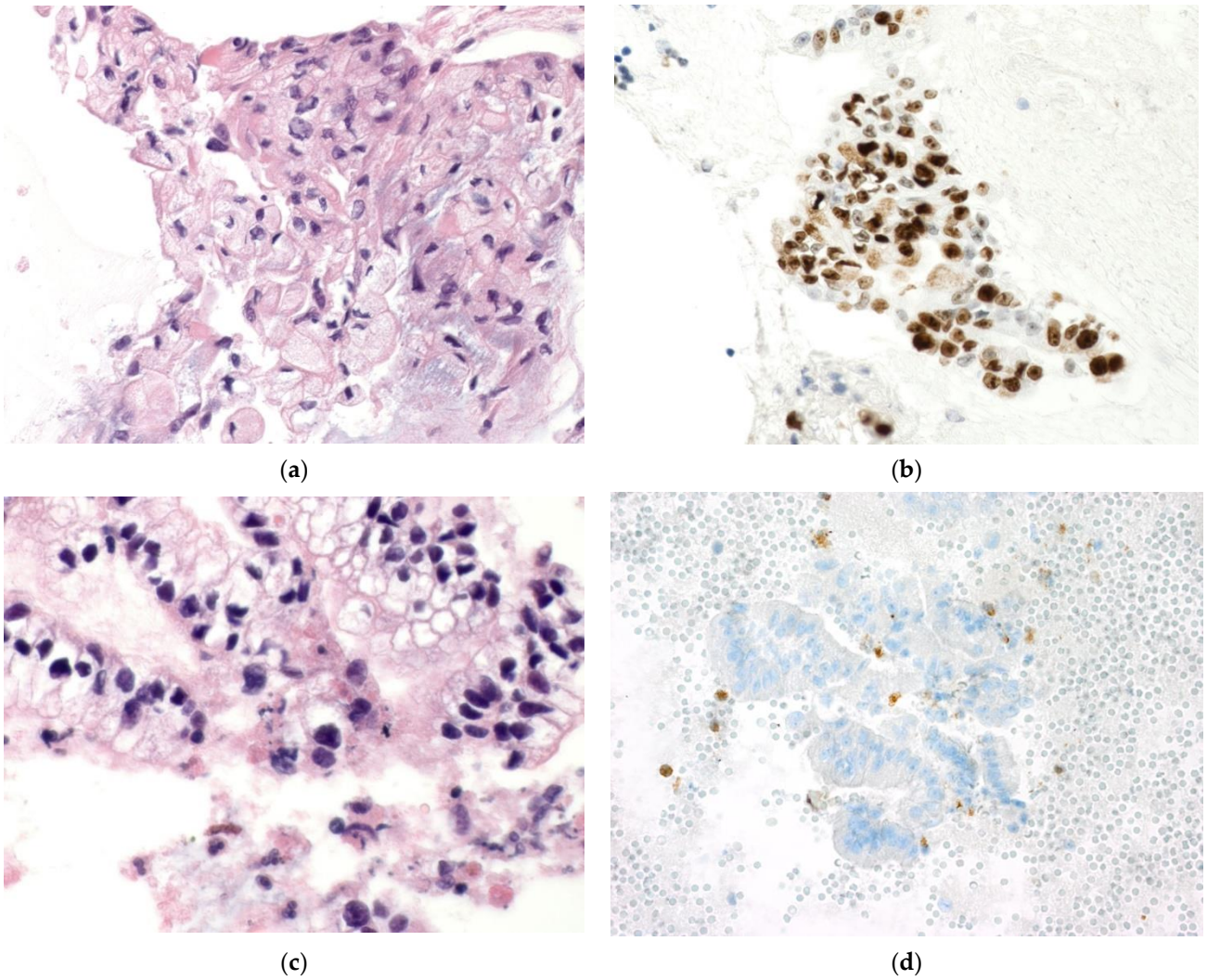


Figure 7. Pancreatic ductal adenocarcinoma. (**a**) Cell block preparation (H&E) and (**b**) corresponding p53 immunostain showing diffuse strong nuclear overexpression. (**c**) Cell block preparation (H&E) and (**d**) corresponding p53 immunostain showing complete loss ("null pattern") of nuclear expression.

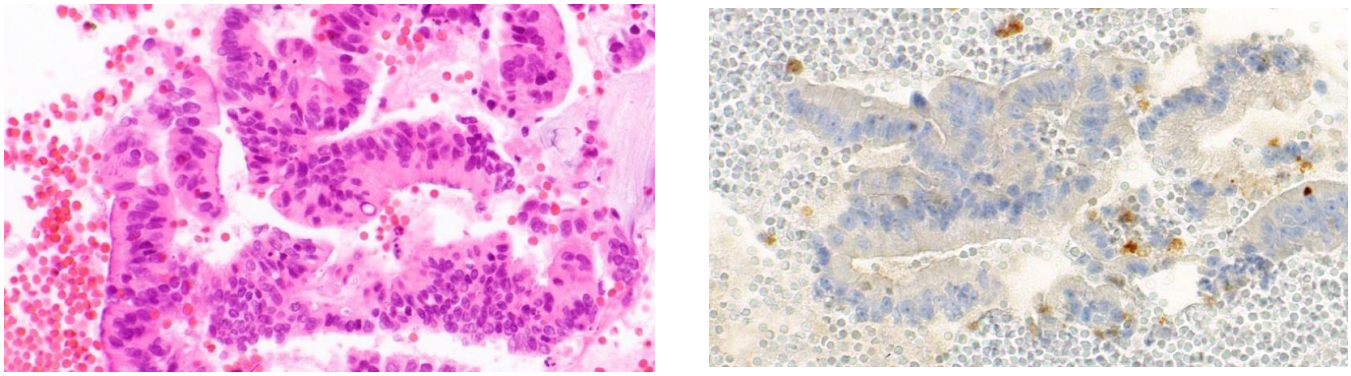


Figure 8. Well-differentiated pancreatic ductal adenocarcinoma. (**a**) Cell block preparation shows strips of adenocarcinoma with loss of nuclear polarity/disarray and nuclear membrane irregularities (H&E). (**b**) SMAD4 immunostain shows loss of nuclear staining within adenocarcinoma cells. Note the background inflammatory cells that are positive and serve as an internal control.

In summary, performing NGS in conjunction with biochemical testing and cytology improves the sensitivity for the detection of premalignant/malignant pancreatic cysts. Although molecular analysis is not a replacement for cytopathology, reflex co-testing provides additional valuable information for a more accurate preoperative diagnosis, helping to classify neoplastic mucinous cysts and high-risk cysts that require surgical resection, thereby impacting patient management.

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