# Molecular Cytology Applications on Pancreas and Biliary Tract

# 11

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## 11.1 EUS-FNA of Pancreas for Molecular Cytopathology

Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) of pancreatic lesions was introduced in the early 1990s [1]. Since then, the technique has become the standard of care for obtaining a tissue diagnosis from the pancreas, particularly pancreatic ductal adenocarcinoma (PDAC). This minimally invasive technique allows for real-time imaging of the pancreatic parenchyma as well as evaluation of peripancreatic lymph nodes. In patients who are not surgical candidates, whether due to comorbidities or advanced disease, samples obtained by EUS-FNA may be the only material available for ancillary testing including molecular studies. Aspiration of cells and/or fluid from solid and cystic pancreatic lesions may yield material for evaluation by a combination of modalities, including cytomorphology, immunocytochemistry, fluid chemistry analysis, and molecular testing.

### 11.1.1 Molecular Landscape of Solid Pancreatic Neoplasms

The gradual discovery of distinct subtypes of pancreatic cancer has resulted in a new molecular classification of pancreatic neoplasms, and these molecular signatures have been reviewed in the context of histologic subtypes [2–5]. Molecular

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alterations have been described in most solid pancreatic neoplasms, though testing for these alterations has not attained routine use.

Pancreatic ductal adenocarcinoma (PDAC), the most common primary pancreatic malignancy, is believed to progress along a morphologic and molecular pathway from pancreatic intraepithelial neoplasia (PanIN) to invasive carcinoma, given their shared genetic changes. The most commonly associated genetic alterations associated with PDAC are the oncogene *KRAS* and the tumor suppressor genes *TP53*, *p16/CDKN2A*, and *SMAD4* [6]. Somatic (acquired) mutations found in a minority of pancreatic cancers include those in *ARID1A*, *ATM*, *AKT2*, *MAP2K4*, *MLL3*, *TGFβR2*, and *FBXW7*, while germline (inherited) alterations that can predispose to the development of pancreatic cancer include *BRCA2*, *BRCA1*, *PALB2*, *p16/CDKN2A*, *STK11*, *ATM*, *PRSS1*, and the DNA repair genes (such as *MSH2*) [6].

Activating *KRAS* mutations and telomere shortening play an early role in PDAC development, with other gene mutations including *p16/CDKN2A*, *TP53*, and *SMAD4* implicated in progression [7]. Pancreatic neuroendocrine tumors, acinar cell carcinomas, solid pseudopapillary neoplasms, and pancreatoblastomas generally lack the most common abnormalities of PDACs, including mutations in *KRAS*, *TP53*, *DPC4*, and *p16/CDKN2A* [5].

Pancreatic neuroendocrine tumors (PanNETs) have been associated with microsatellite instability and chromosomal losses and gains. In these tumors, loss of 3p, 6pq, and 10pq along with gain of 5q, 12q, 18q, and 20q has been associated with malignant behavior [8]. These tumors may arise in patients with hereditary syndromes including MEN1 and von Hippel-Lindau (VHL) and may show *MEN1* gene mutations and inactivation of the *VHL* gene, respectively [5]. Some factors that are reportedly predictive of more aggressive behavior (at least in univariate analyses) include loss of progesterone receptor expression, aneuploidy, increased Ki67 labeling index, loss of heterozygosity (LOH) of chromosome 17p13, LOH of chromosome 22q, increased fractional allelic loss, upregulated CD44 isoform expression, and immunohistochemical expression of cytokeratin 19 [5]. Somatic mutations of the death-domain-associated protein (*DAXX*) and alpha-thalassemia/mental retardation syndrome X-linked (*ATRX*) genes have been found in sporadic PanNETs, whereas pancreatic neuroendocrine carcinomas (PanNECs) have been shown to have *TP53* and retinoblastoma (*RB-1*) mutations [4].

The development of solid pseudopapillary neoplasms (SPNs) has been linked to Wnt signaling associated with *CTNNB1* mutations, imparting cytoplasmic and nuclear accumulation of β-catenin [9]. Almost all SPNs have a somatic point mutation in exon 3 of the β-catenin gene, implicating the same pathway that is abnormal in acinar neoplasms [5, 10]. Pancreatic acinar cell carcinoma is rare, making it difficult to study. Studies have shown losses on chromosome arm 11p, alterations in *APC*/β-catenin pathway, and loss of DCC expression [11]. *BRAF* mutations may rarely be found, with the most prevalent fusion being *SND1-BRAF*, which may impart sensitivity to treatment with MEK inhibitors [12].

Pancreatoblastoma shows predominantly acinar differentiation and is the most common pediatric pancreatic neoplasm, although it may also occur in adults. These tumors have arisen in patients with Beckwith-Wiedemann syndrome, and a case has

been reported in a patient with familial adenomatous polyposis [5]. Molecular alterations are similar to those found in hepatoblastoma and acinar cell carcinoma; the most common genetic alteration is LOH of 11p, but alterations in the *APC*/β-catenin pathway have been reported [5].

Undifferentiated "medullary" carcinoma, defined as pushing borders, syncytial growth, and necrosis, has been shown to demonstrate microsatellite instability and be associated with a better prognosis as compared to classic PDAC [6]. Other genes that have been linked to pancreatic neoplasia include the following: *TGFBR1*, *ACVR1B*, and *RNF43*. Loss of heterozygosity and polysomy has also been identified in pancreatic carcinomas.

### 11.1.2 Molecular Testing of Solid Pancreatic Neoplasms

EUS-FNA enabled the diagnosis of various solid pancreatic tumors by cytopathologic evaluation, sometimes with rapid on-site evaluation (ROSE) to ensure that adequate material is obtained. In cases of PDAC, only one or two passes could be sufficient for diagnosis, whereas neuroendocrine neoplasms or acinar cell carcinomas may require additional passes to obtain material for immunocytochemical stains. Although cytomorphology is still paramount for the patient's diagnosis and management in cases of solid pancreatic neoplasms, immunocytochemical stains and molecular tests can be used to support the morphologic impression.

If ROSE is requested, the first drop or two of material from each EUS-FNA needle pass may be applied to a glass slide to produce Diff-Quik-stained and/or alcohol-fixed Papanicolaou-stained smears, with the remaining material rinsed into a balanced salt solution. If the needle and device are to be used for subsequent passes, a balanced salt solution is preferable to any alcohol-based preservatives. Molecular testing may be performed using cells from a variety of preparation methods, including cytology smears and touch preps, liquid-based slides, needle rinses, and cell block material. Needle rinses and cell block material are generally preferable to ensure preservation of diagnostic smears or liquid-based slides. Furthermore, molecular testing on needle rinse specimens rather than cell block or surgical material may yield better DNA quality and reduce turnaround time, since this obviates the need to cut additional slides. As with any test, laboratories must run the appropriate validation studies to ensure diagnostic accuracy.

KRAS mutation analysis performed on EUS-FNA specimens combined with cytomorphology does appear to improve overall diagnostic accuracy when distinguishing pancreatic carcinoma and pseudo-tumorous chronic pancreatitis [13, 14]. Indeterminate cytologic specimens obtained by EUS-FNA of pancreatic tumors have been evaluated for tumor suppressor gene-linked microsatellite markers for allelic loss analysis and *KRAS* point mutations to improve diagnostic yield [15]. If material is limited, *KRAS* testing could be prioritized in cases of suspected PDAC. However, multiplex polymerase chain reaction (PCR) "hot spot" mutation testing and next-generation sequencing (NGS) are available in a growing number of laboratories. These tests require a small amount of DNA to

query multiple genes of interest. *KRAS* testing could also be performed with a fully automated PCR detection system, which could help streamline testing of EUS-FNA specimens [16].

RNA extraction with real-time gene expression quantification is feasible in EUS-FNA specimens of advanced PDAC, although only samples with high-quality RNA were selected in this study [17]. MicroRNA (miRNA)-based testing in conjunction with cytology can also predict which preoperative pancreatic EUS-FNA specimens contain PDAC, thus reducing the number of indeterminate FNAs and repeat procedures [18]. As with other pancreatobiliary specimens, FISH analysis for loss of 9p21 or changes in copy number for chromosomes 3, 7, and 17 could be considered in cases that are inconclusive or negative by cytology [19].

### 11.1.3 Immunocytochemistry in Solid Pancreatic Neoplasms

Cell blocks of solid pancreatic neoplasms often suffer from scant cellularity but can be extremely helpful when lesional material is present. A panel of special or immunocytochemical stains can help in differentiating neuroendocrine neoplasms (i.e., chromogranin, synaptophysin, CD56), acinar cell carcinoma (i.e., PAS-D, trypsin, chymotrypsin, lipase), solid-pseudopapillary tumors (i.e., CD10, nuclear β-catenin, cyclin D1), and metastatic malignancies (i.e., differentiation- or organ-specific markers). Loss of SMAD4 immunocytochemical staining has been observed in approximately 55% of PDACs, reflecting genetic inactivation of the *SMAD4* gene [6]. Loss of staining supports a diagnosis of PDAC rather than reactive atypia, suggests a pancreatic primary in cases of metastasis, and is associated with worse prognosis and more widespread metastases [6]. E-cadherin has been used as a marker for poor prognosis in PDAC [20]. Mucin (MUC) expression profiles could also be helpful for diagnosis of PDAC, with MUC16 cytoplasmic expression potentially predicting a poor prognosis [21].

### 11.1.4 Evaluation of Pancreatic Cystic Lesions

The number of patients diagnosed with pancreatic cysts has increased in the last decades as a result of continuously improving abdominal imaging modalities and their growing use in an increasingly older population [22–24]. The incidence of pancreatic cysts increases with age; some report that pancreatic cysts may be as common as 25% in those older than 70 years. A significant number of cystic lesions in the pancreas are neoplastic. They include a range of benign neoplasms such as serous cystadenomas with almost zero risk of malignant transformation to the other extreme of malignant carcinomas that undergo cystic degeneration. Within this spectrum are low-grade neoplasms such as solid pseudopapillary neoplasms and mucinous neoplasms including mucinous cystic neoplasm (MCN) and intraductal papillary mucinous neoplasm (IPMN), both harboring at least some potential for malignant transformation.

Recent evidence suggests that the great majority of pancreatic cysts are benign on resection. Thanks to a better understanding of the natural history of these lesions, a shift to a more conservative approach has occurred. In contrast to pancreatic surgery, which carries a greater risk of long-term complications and mortality, regular surveillance with imaging studies could be an alternative approach. The overall risk of malignancy in an incidental pancreatic cyst is very low. In some neoplastic lesions such as main duct intraductal papillary mucinous neoplasm (IPMN), a surgical approach may be acceptable.

Although a minority, a proportion of PDACs is known to develop from these preneoplastic mucinous lesions. Early diagnosis of those cysts with early invasive cancer or high-grade dysplasia, along with appropriate surgical management, could reduce mortality from pancreatic adenocarcinomas. It is crucial to correctly triage and manage these patients. Commonly used diagnostic modalities have suboptimal sensitivity and specificity to accurately stratify and manage this population. These modalities include clinical features, CT, MRI, and EUS-FNA for cytologic and chemical analysis. EUS is particularly useful to detect structural alterations of the cyst and evaluate communication with the pancreatic duct, as well as provide the unique opportunity to aspirate fluid in real time for cytologic, biochemical, and molecular analysis.

The role of carcinoembryonic antigen (CEA) levels from pancreatic cyst fluid to determine the mucinous nature of a cyst has been well established. An elevated cyst fluid CEA (>192 ng/mL) is the most accurate (79%) test to distinguish a mucinous cyst [25, 26]. Although this answers an important question in the evaluation of a cyst, mucinous versus non-mucinous, it does not resolve the presence or absence of high-grade dysplasia or malignancy. Cytologic examination appears to be the more specific tool to determine the presence of high-grade dysplasia or malignancy; however, cytology is not very sensitive, mainly due to scarce cellularity in cysts lacking a solid component [26].

### 11.1.5 Molecular Testing of Pancreatic Cystic Lesions

Molecular analysis, performed on a minimal amount of fluid, smears, or deparaffinized sections of cell blocks, has been proposed to aid in the diagnosis of cystic lesions, based in part on a growing understanding of the molecular mechanisms involved in pancreatic carcinogenesis. As mentioned earlier, common alterations seen in PDAC include mutations in *KRAS*, *p16/CDKN2A*, *TP53*, and *DPC4*. Other molecular changes include those in retinoblastoma-interacting zinc finger (*RIZ*) on 1p36, *VHL* on 3p25-3p26, *APC* on 5q23, *MTS-1* on 9p2, and aberrant expression of the patched gene (*PTCH*) on 9q22 [27–33] (see Table 11.1).

On the assumption that some of these biomarkers may be altered in mucinous cysts with intermediate- to high-grade dysplasia and could be used to identify patients at risk for cancer development, molecular analysis has been proposed to aid in the diagnosis of pancreatic cystic lesions. Tests include mutation analysis (*KRAS*, *GNAS*, *TP53*, *VHL*, *CTNNB1*, and *RNF43*), DNA cyst fluid analysis (quality and quantification), loss of heterozygosity analysis, and microsomal analysis.

**Table 11.1** Molecular alterations and testing in pancreatic and biliary neoplasia

| Molecular alteration(s) | Molecular test(s) | Diagnostic finding(s) | Neoplasm(s) |
|---|---|---|---|
| *KRAS*, telomere shortening, *CDKN2A*, *TP53*, *SMAD4*, *BRAF*, *STK11/LKB1* Loss of heterozygosity at microsatellites linked to tumor suppressor gene loci known to be affected in pancreatic carcinogenesis | *KRAS* and other mutation analysis Loss of immunohistochemical staining for the protein product of the SMAD4 gene Loss of heterozygosity analysis | Mutation present in PDCA Loss of IHC staining for protein product of SMAD4 gene supports a diagnosis of ductal adenocarcinoma Losses of chromosome arms 3p, 6pq, and 10pq along with gains of 5q,12q, 18q, and 20q support a diagnosis of adenocarcinoma | PDAC |
| Selected miRNAs | MicroRNA analysis | Presence of miRNAs such as miR-21 and mi-155 supports a diagnosis of adenocarcinoma | PDAC |
| *VHL* (3p) mutation | VHL gene mutation analysis | Mutation present in SCA | SCA |
| *KRAS*, *RNF43*, *TP53*, *SMAD4* | Mutation analysis | Mutations seen in both IPMN and MCN | MCN, IPMN |
| High levels of DNA in cyst fluid, aneuploid, and tetraploid | DNA analysis of cyst fluid | High levels of intact DNA are associated with actively dividing cell; aneuploid and tetraploid favors malignancy | May aid in separation of benign from malignant cysts |
| *GNAS* | GNAS mutation analysis | *GNAS* mutation is the second most frequent mutations seen in IPMN, distinguishing it from MCN | IPMN |
| *CTNNB1* | *CTNNB1* (beta-catenin) mutation analysis | Mutation present in nearly all SPN | SPN |
| Polysomy | FISH for polysomy | Copy number abnormalities in CEP3, CEP7, CEP17, and abnormalities of 9p21 favor malignancy | CC |
| MSI, microsatellite alterations (loss of 3p, 6pq, 10pq, and gain of 5q, 12q, 18q, 20q) | Microsatellite loss analysis | Loss of 3p, 6pq, and 10pq along with the gain of 5q, 12q, 18q, and 20q have been associated with malignant behavior in PanNET | PanNET |

*PDAC* pancreatic ductal adenocarcinoma, *SCA* serous cystadenoma, *MCN* mucinous cystic neoplasm, *IPMN* intraductal papillary mucinous neoplasm, *SPN* solid pseudopapillary neoplasm, *PanNET* pancreatic neuroendocrine tumor, *MSI* microsatellite instability, *CC* cholangiocarcinoma

Activating *KRAS* mutations in codon 12 of exon 1 are common in both intraductal papillary mucinous neoplasms and mucinous cystic neoplasms, supporting a mucinous etiology; however, its presence is not specific for malignancy. *KRAS* mutation analysis has an additive value to CEA measurements for distinguishing non-mucinous and mucinous cysts [34]. *RNF43* (ring finger protein 43) encodes a protein with intrinsic E3 ubiquitin ligase activity that promotes cell growth and has recently been linked to the β-catenin pathway. *RNF43* mutations have been shown to occur in MCN and IPMN [35]. The *GNAS* gene encodes for stimulatory G-protein alpha subunit, which is a crucial component of many transduction pathways. In the pancreas, *GNAS* codon 201 mutations appear to be highly specific for intraductal papillary mucinous neoplasms, while *KRAS* and *RNF43* mutations can also be seen in MCNs [36, 37].

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* (β-catenin) in solid pseudopapillary neoplasms; *RNF43*, *KRAS*, *TP53*, and *SMAD4* in MCN; and *KRAS*, *RNF43*, *GNAS*, *TP53*, and *SMAD4* in IPMN. 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 [35]. The *VHL* tumor suppressor gene is somatically mutated in serous cystadenomas and is not seen in other cystic lesions of the pancreas [36].

DNA analysis may also aid in the separation of nonneoplastic and neoplastic lesions, as well as benign from malignant neoplastic cystic lesions. High levels of intact DNA are associated with actively dividing cells. The concentration of DNA is correlated with optical density (OD) as measured at a wavelength 260/280 nm. 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 [34].

Loss of heterozygosity (LOH) analysis identifies loss of heterozygosity at microsatellites linked to tumor suppressor gene loci known to be affected in pancreatic carcinogenesis. An assessment of allelic loss of these tumor suppressor gene-linked microsatellite markers [9p21 (*MTS-1*), 17p (*TP53*), 18q (*DPC4*), 9q22 (*PTCH*), 1p36 (*RIZ*), 3p25-3p26 (*VHL*), 5q23 (*APC*), 10q23 (*PTEN*)] is performed on extracted DNA subjected to polymerase chain reaction (PCR). Products from each PCR are analyzed by capillary electrophoresis on a genetic analyzer. Informative samples with a polymorphic allelic imbalance ratio <0.5 or >2.0 are considered evidence of allelic imbalance and LOH [34]. A buccal brushing sample can be used as a normal control.

MicroRNAs are small (18–24 nucleotide) noncoding RNA molecules whose principal function is to regulate the stability and translation of nuclear mRNA transcripts. Selected panels of dysregulated miRNAs previously identified in invasive pancreatic cancer have also shown aberrant expression in neoplastic mucinous cysts, adding another tool to distinguish mucinous versus non-mucinous cysts [38]. Matthaei et al. [39], using a logistic regression analysis, developed a 9-miRNA model that allowed subclassification of the degree of dysplasia in most instances.

### 11.1.6 Limitations to Molecular Testing in Pancreatic Lesions

Barriers to implementing molecular analysis include reliance on the presence of substantial diagnostic material (immunohistochemistry, FISH, digital image analysis), imperfect specificity (all tests), and cost (mutation analysis, LOH testing, FISH, digital image analysis), although testing may be helpful in some, usually atypical, cases [40]. While testing for *KRAS* in pancreatic cytology specimens will often yield positive results, the presence of *KRAS* mutations is not entirely specific for malignancy. Although *KRAS* mutation analysis on EUS-FNA samples can support the diagnosis of PDAC and is often associated with worse prognosis, no significant clinical benefits have been derived from therapies targeting KRAS in PDAC [41]. Multiple molecularly targeted therapies have been tested in metastatic pancreatic cancer but have not achieved widespread use in clinical practice [42]. Although there is insufficient evidence of clinical utility at this time to support widespread adoption of molecular testing in solid and cystic pancreatic neoplasms, active investigation may someday offer more targeted therapeutic options.

### 11.1.7 Recommendations

Overall, cytomorphology is still critical to the diagnosis of solid pancreatic neoplasms, and there is insufficient evidence to indicate that any molecular test should be used as a definitive method of evaluating these neoplasms. Loss of *SMAD4* and positive staining for mesothelin support a diagnosis of PDAC, nuclear staining for beta-catenin supports a diagnosis of solid pseudopapillary neoplasm, and immunocytochemistry for endocrine and exocrine differentiation are helpful for diagnostic purposes in solid pancreatic tumors [8]. FISH for copy number abnormalities can be used to support a cytologic impression of adenocarcinoma. Outside of these general guidelines, molecular testing does not currently have a routine clinical role for diagnostic, prognostic, or therapeutic purposes. Loss of heterozygosity and the presence of certain microRNAs could be used to support the diagnosis of adenocarcinoma, but the clinical utility of these tests remains to be seen. As the diagnosis can usually be made by cytomorphology alone, the addition and costs of these tests may not be warranted.

A multidisciplinary approach for pancreatic lesions with incorporation of all relevant ancillary data to arrive at a cytologic diagnosis is recommended [43–45]. In terms of molecular analyses for predicting malignancy in a mucinous cyst, there are commercially available assays with promising results but which may not be as accurate in classifying smaller (<3 cm), uncomplicated cysts. There is currently insufficient data to warrant their usage in routine practice [45–47].

Once molecular results are obtained, reporting of the results may be issued as an addendum to the cytopathology case and/or reported separately. The advantage of issuing an addendum to the cytopathology case is an opportunity to integrate the molecular findings with the cytomorphology and any other ancillary tests.

### 11.1.8 Future Directions

There are very few molecular alterations that will change patient management or inform prognosis, and molecular testing is not commonly applied to solid pancreatic neoplasms. Despite limited clinical utility, the hope of personalized medicine and targeted therapies continues to drive research in this area. Testing for certain molecular alterations could enable patients to enroll in clinical trials with targeted therapy, particularly when other therapeutic options have been exhausted. As our understanding of the molecular landscape improves, there may be opportunities to alter the tumor microenvironment. Genes overexpressed in the desmoplastic stroma of PDAC could be a target for chemotherapeutic agents [6]. Patient genetic factors could also influence the effectiveness of therapeutic regimens.

There is still a need for ancillary biomarkers in cyst fluid material that can reliably provide additional distinction between clinically insignificant cystic lesions and mucinous cysts with high-grade dysplasia. Examination of miRNAs could provide the needed test to identify malignant potential in cystic neoplasms of the pancreas. One of the advantages of the short mature miRNAs is the lack of propensity for degradation in biospecimens. Validation with prospective studies of large series with clinical or surgical follow-up is needed before adoption in clinical practice. Looking toward the future, cytopathologists could and should take an active interest in advancing the forefront of molecular cytopathology with regard to solid and cystic pancreatic neoplasms.

## 11.2 Biliary Tract Sampling for Molecular Cytopathology

The main indication for morphologic evaluation of the biliary tree is a duct stricture as the result of inflammatory or neoplastic disorders. Epithelial tumors originating from the biliary tree usually present a longitudinal growth pattern along the biliary duct rendering their detection more difficult by noninvasive imaging techniques such as ultrasound or computed tomography. On the contrary, the assessment of the biliary tree by endoscopic procedures allows tissue collection for cytologic or histologic diagnosis. Endoscopic retrograde cholangiopancreatography (ERCP), coupled with brush cytology or forceps biopsy, is routinely performed to detect malignancy in patients with biliary strictures [48, 49].

Brush cytology performed during ERCP is simple and safe. Contrary to the simple aspiration of the bile juice, the brush scrapes different sites of the biliary tract mucosa retrieving a cellular material [50]. Usually the samples are well preserved, providing an adequate specimen for cytologic examination. Indeed, the rate of unsatisfactory samples is low, around 5%, and mainly related to air-drying artifact if the samples are not properly fixed [49, 51].

### 11.2.1 Evaluation of Biliary Tract Specimens

A cytologic diagnosis of malignancy achieved by brush cytology is reliable, and the literature shows a very high specificity for this method, reaching 100% in several series [49, 52–54]. This means that brush cytology has a high positive predictive

value and a low rate of false-positive results. In a large series of 406 patients with pancreaticobiliary strictures, Stewart et al. [51] detected only three false-positive cases. Most of the false-positive results are attributed to misinterpretation of atypia in degenerated or reactive epithelial cells, mainly in the context of primary sclerosing cholangitis (PSC), a chronic liver disease causing inflammatory changes and fibrosis of the biliary tract [51, 55].

Despite the reported high specificity, the main limitation of brush cytology is the low sensitivity for detecting malignancy of the biliary tract. In their series of 406 patients, Stewart et al. [51] demonstrated that brush cytology correctly identified neoplastic diseases in 59.8% of the cases. Other series showed sensitivity rates varying from 48% [56] and 54.7% [49] to 68% [52] and 68.6% [54]. The main cause of false-negative results is due to sampling error, probably related to cases where the tumor spreads predominantly to the submucosa of the biliary duct or when the biliary stricture is secondary to an extrinsic compression.

Furthermore, some series reported an atypical or equivocal diagnostic category for which a conclusive cytologic diagnosis is not possible, varying from 4.9% [49] to 10.1% [51] of the cases. According to the terminology proposed by the Papanicolaou Society of Cytopathology, the "atypical" category includes a large spectrum of cytologic or architectural abnormalities that are not compatible with benign reactive changes but, on the other hand, are insufficient to be classified as suspicious or positive for malignancy [44]. Of the 41 cases reported as "atypical" by Stewart et al. [51], 29 were proved to be malignant on clinicopathologic follow-up, while 12 were benign, corresponding mostly to chronic pancreatitis and calculous disease.

Because of the limitations of biliary tract cytology, different complementary approaches have been developed to improve the diagnosis of biliary tract disease. The application of ancillary procedures such as in situ hybridization techniques or gene mutation analysis can improve the accuracy for detection of malignancy in biliary tract brush specimens [8].

### 11.2.2 Molecular Testing of Biliary Tract Specimens

The commercially available UroVysion FISH probe set (Abbott Molecular, Des Plaines, IL), originally developed for detecting urothelial carcinoma, has been applied to biliary tract specimens, including bile fluid, brushings, and aspirates of the pancreaticobiliary tree [57]. The FISH test can detect chromosome copy number gains and/or chromosome deletions. Aiming to detect cells with chromosome copy number gains (polysomic cells), which can have an association with malignancy, the FISH test employs probes to target the centromeric regions of the chromosomes 3, 7, and 17. According to Kipp et al. [57], a diagnosis suggestive of malignancy is obtained with a polysomic result, defined as five or more cells showing gains in at least two or more FISH probes. The detection by FISH of 9p21 loss (which results in the loss of the tumor suppressor p16) is another criterion that favors a diagnosis of malignancy in pancreatobiliary cytology specimens [58, 59]. Accordingly, 12 or

more cells with deletions of 9p21 should be interpreted as a positive FISH result [59] (see Fig. 11.1).

Currently, FISH is considered the most reliable complementary technique to cytology for the detection of biliary tract malignancies [8]. Using biliary brush specimens for the detection of malignancy in 131 patients with biliary tract

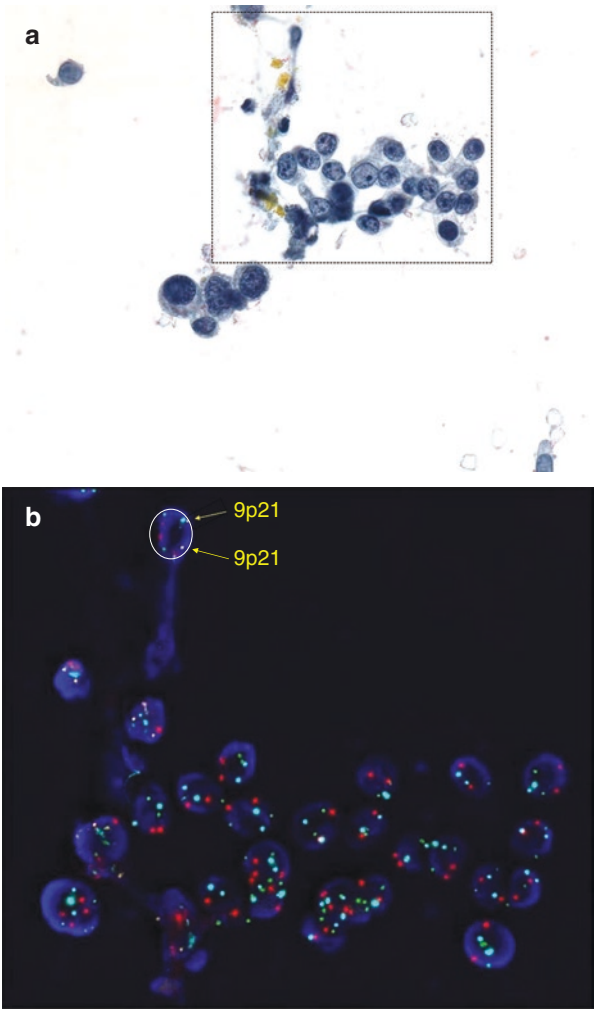


**Fig. 11.1** (**a**) Micrograph shows an example of a biliary brush cytology specimen from a 30-year-old female patient. One cluster of mildly atypical epithelial cells are mixed with rare small lymphocytes (Papanicolaou stain, 400×). (**b**) The same cells (inset of **a**) are shown after multi-target fluorescent in situ hybridization (FISH) UroVysion (magnification 600×). The atypical cells demonstrate a complete loss of the 9p21 (FISH-positive). These cells show 2–4 signals for the centromeric probes of chromosomes 3, 7, and 17. The encircled cell correspond to a small lymphocyte with normal two 9p21 signals (yellow signals). Courtesy of Dr Spasenija Savic from the Institute of Pathology of the University Hospital Basel, Switzerland

strictures, Kipp et al. [60] observed that the FISH test was significantly more sensitive in comparison to cytology alone. An analysis of 498 patients with pancreaticobiliary strictures corroborated the results of Kipp et al. [60], demonstrating that FISH testing had a significantly higher sensitivity compared to cytology alone (42.9 vs 20.1%) while retaining the same specificity as cytology (99.6%). Interestingly, the authors showed that the probability of having carcinoma was 77 times higher for patients with polysomic FISH results as compared to patients with normal FISH, whereas the probability of having carcinoma was six times higher for patients with suspicious cytology in comparison to patients with normal cytology [61].

In a small series of 50 patients with biliary strictures, a sensitivity of 89% was achieved when 9p21 loss detected by FISH was added to FISH polysomy + cytology as compared to FISH polysomy + cytology (58%) or cytology alone (21%) [58]. Using the FISH technique to analyze a series of 90 cases of pancreas and extrahepatic biliary tract cytology, Vlajnic et al. [59] found an overall sensitivity of 79% and an overall specificity of 100%. For cases with inconclusive (atypical or suspicious) cytology, sensitivity and specificity of 61.3 and 100% were achieved, respectively. In their study, the authors observed that 74% of FISH-positive results comprised both chromosome copy number gains and 9p21 loss, while 14% corresponded only to copy number gains and 12% consisted only of 9p21 deletion [59].

Patients with PSC may develop strictures of the biliary tract. Based on their symptoms (abdominal pain, jaundice, weight loss), laboratory tests (elevated levels of CA 19-9 and serum liver markers), and a high risk for developing cholangiocarcinoma, ERCP and brushing cytology are frequently applied for the evaluation of biliary tract strictures in such patients. However, cellular abnormalities as a result of inflammatory changes may pose difficulties in the interpretation of the cytologic specimens, resulting in equivocal (atypical, suspicious) or false-positive diagnoses [51, 55]. In a study that evaluated 102 patients with PSC and equivocal brush cytology, 76% of the patients with polysomy detected by FISH developed a pancreaticobiliary tract malignancy within 2 years [62]. The authors also demonstrated that patients with a combination of polysomy and elevated serum levels of CA 19-9 had a 10.92 times higher probability for developing cancer of the pancreaticobiliary tract in comparison to patients without polysomy and low levels of CA 19-9 [62].

The *KRAS* gene mutation is a common genetic alteration in pancreaticobiliary tumors, especially in carcinomas of the pancreas and, less frequently, in cholangiocarcinomas. Most of the *KRAS* mutations occur in codon 12 of exon 2 of the *KRAS* gene [6] (see Fig. 11.2). In an attempt to improve the detection of malignancy of the pancreaticobiliary tract, several studies have investigated the role of molecular techniques in detecting *KRAS* mutations in biliary brush specimens.

Using a PCR-based method to detect mutations of codon 12 in the *KRAS* gene, Sturm et al. [63] compared the sensitivity of brush cytology and molecular testing in a series of 312 patients with bile duct stenosis. Although the sensitivity of both methods were quite similar (36% for cytology and 42% for the molecular testing), it increased to 62% when cytology and *KRAS* mutation analysis were combined. In another study where the same series of patients were evaluated with real-time and quantitative PCR, a sensitivity of 71% was obtained with the combination of


**Fig. 11.2** Representative example of the molecular output showing a KRAS G12D mutation detected in a cytologic specimen from the pancreas. Courtesy of Jennifer Morrissette, PhD, from the Department of Pathology and Laboratory Medicine, Hospital of the University of Pennsylvania, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania, USA

cytology and molecular testing [64]. In a study that analyzed 129 pancreaticobiliary brushings and pancreatic fine-needle aspirations with an indeterminate (atypical or suspicious) cytologic diagnosis, the sensitivity and specificity for the detection of malignancy based on the presence of *KRAS* mutations were 57% and 94%, respectively [65].

Molecular techniques to identify *KRAS* mutations can detect pancreaticobiliary cancers not established by FISH. Kipp et al. [66] studied a series of 132 benign and malignant cytologic brush specimens assessed by FISH and a quantitative PCR-based method for *KRAS* mutations: out of 58 patients with FISH-negative results, 7 had *KRAS* mutations, and 6 of these had a carcinoma in the final histologic diagnosis. Out of 33 patients with FISH-equivocal results, 9 had *KRAS* mutations, including 8 patients with proven malignancy by histology. Although the sensitivity of the molecular technique (47%) and the FISH test (50%) were similar, the combination of both methods increased overall sensitivity to 68% [66].

In these studies, most of the detected *KRAS* mutations in the brush specimens involved codon 12 of the *KRAS* gene. More specifically, the G12D and G12V mutations were the two most prevalent, both resulting in a change from a glycine to an aspartic acid (G12D) or a valine (G12V) amino acid. These are followed in frequency by the G12R mutation, which changes a glycine to an arginine amino acid. Mutations in codon 13 of the *KRAS* gene were also found and resulted from the change of a glycine to an aspartic acid amino acid [63, 64, 66]. In a detailed analysis of 60 cases of brush cytologic samples and the corresponding carcinomas on histology, Sturm et al. [63] verified that when present, all detected mutations were

identical in both specimens. However, no mutation was found (wild-type *KRAS*) in 7 of the 60 cytologic samples, in contrast to *KRAS*-mutated matched histologic samples.

Indeed, false-negative *KRAS* results have been reported in the literature. In a study by Sturm et al. [63], a comparison of *KRAS* status between the cytologic brush samples and the corresponding histologic specimens showed that 53 of 60 cases (88%) had concordant results. According to the authors, the discrepant results could be the result of sampling error, because the cytologic evaluation of these cases was also negative [63]. Certainly, the low percentage of tumor cells in some biliary brush specimens may result in an insufficient sample, below the limit of detection of the molecular test, leading to a false-negative result [66].

### 11.2.3 Limitations

In spite of the reliability of multiprobe FISH in diagnosing malignancy in biliary tract brush specimens, there are some limitations that can result in equivocal and false-positive results. For instance, misinterpretation of FISH signals can lead to false-positive results. According to Kipp et al. [57], cells with weak or absent signals secondary to poor hybridization may be misinterpreted as having a deletion of 9p21. In a study by Barr Fritcher et al. [62], 6 of the 25 patients with FISH-positive results were not found to have a pancreaticobiliary tract malignancy on clinicopathologic follow-up. The authors postulated that some false-positive FISH results may result from detection of chromosomal abnormalities in dysplastic cells that do not progress to cancer [62]. Finally, we have to consider that a negative FISH result does not exclude a malignancy of the biliary tract. Vlajnic et al. [59] stated that if not correctly pre-evaluated, FISH slides with no or few tumor cells may result in false-negative results.

Furthermore, tumor cells with chromosomal abnormalities other than those potentially detected by the UroVysion FISH test may occur [59]. Indeed, other types of chromosomal abnormalities such as tetrasomy (four copies of all four probes) and trisomy (single probe gains) are considered equivocal FISH results and do not contribute to the diagnosis [57]. In their series of 102 patients with PSC, Barr Fritcher et al. [62] detected 33 cases (32% of the total) with non-polysomic FISH results comprising 3 cases with tetrasomy, 29 cases with trisomy 7, and just 1 case with trisomy 3. The majority of these abnormalities (88%) were found in patients without cancer on follow-up, suggesting that these patients may have a similar outcome as compared to patients with FISH-negative results. However, in their study, there were four patients (12%) with non-polysomic FISH abnormalities that developed biliary tract malignancy within 2 years of follow-up [62].

Unfortunately, *KRAS* mutations can occur in nonneoplastic pancreaticobiliary diseases. In one study, *KRAS* mutations were detected in brush specimens from 8 of 74 patients with histologically proven benign bile duct stenosis, including 3 cases of chronic pancreatitis, 3 cases of PSC, and 2 cases of "post-surgical stenosis" [63]. In another study with surgical follow-up, *KRAS* mutations were found in brush

specimens from patients with chronic pancreatitis (1 of 8 cases) and with "unremarkable histology" (1 of 28 cases) [65]. In a study by Kipp et al. [66], the *KRAS* test was negative for mutations in 50 of 52 patients with benign diseases of the pancreaticobiliary tract, corresponding to a specificity of 96%. Two patients had false-positive *KRAS* results: one patient with PSC and one patient with ulcerative colitis [66]. It has been speculated that *KRAS* mutations can precede overt histologic changes of malignancy [65] and that increased or uncontrolled RAS activity as a result of an inflammatory process can induce genetic changes leading to tumorigenesis [66].

### 11.2.4 Recommendations

In general, *KRAS* mutation analysis has a fair sensitivity for the identification of malignancy in the pancreaticobiliary tree, as demonstrated by the studies described above. Currently, there is no consensus to support the use of *KRAS* testing as an ancillary technique for the diagnosis of biliary duct strictures [8].

### 11.2.5 Future Directions

Recent advances in molecular techniques, such as next-generation sequencing (NGS), could provide a more comprehensive knowledge of the molecular genetics underlying pancreaticobiliary tumors and may result in the discovery of potential biomarkers. A recent study evaluated the role of targeted NGS in pancreaticobiliary brushing specimens in a series of 74 patients who underwent ERCP [67]. Among the 24 cases that had a positive NGS result, the most commonly mutated gene was *KRAS* (21 cases), followed by *TP53* (14 cases), *SMAD4* (6 cases), and *p16/CDKN2A* (4 cases). In this study, the NGS technique was the most sensitive test with a sensitivity of 74% when compared to cytology (67% sensitivity) and FISH (55% sensitivity). Adding the FISH test to cytology increased the sensitivity to 76%. When NGS was added to cytology, the sensitivity increased to 85%. This sensitivity (85%) remained the same when FISH was added to NGS and cytology, meaning that the FISH test had no impact on the sensitivity of NGS + cytology. Considering these results and that FISH is a labor-intensive and challenging technique, the authors concluded that NGS could be an alternative to FISH as an ancillary test for pancreaticobiliary brushing specimens [67].

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