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Morphologic and molecular diagnostic criteria of malignancies in biliary strictures

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Summary. The differential diagnosis of benign and malignant biliary strictures is not always feasible and still represents a major diagnostic challenge, mainly due to the scarcity of the tissue retrieved for proper cytological or histopathological diagnosis. The present review focuses on morphological criteria in the diagnosis of biliary strictures, in the course of primary sclerosing cholangitis and other pathologies, starting from the limits of the cytological and histological evaluation, as well as the ancillary methodologies currently available in Pathology laboratories The current guidelines suggest fluorescence *in situ* hybridization for the analysis of chromosomes 3, 7, and 17 polysomies and deletion of the 9p21 locus; however, other more promising techniques are on the horizon for both patient care and research purposes, such as Next-Generation Sequencing, able to analyze multiple genes simultaneously in a cost-effective fashion.

Lastly, the most recent approaches proposed in the literature for the differential diagnosis of biliary stricture are described, such as circulating tumor DNA, miRNAs, and DNA methylation, among others.

Key words: Ancillary techniques, Biliary strictures, Cholangiocellular carcinoma, FISH, Histopathology, Next-generation sequencing, Primary sclerosing cholangitis

Introduction

A biliary stricture is defined as an abnormal narrowing of the biliary tree, eventually leading to a cholestatic clinical pattern, characterized by obstructive jaundice, pruritus, cholangitis, and increased serum levels of cholestasis markers (i.e., alkaline phosphatase, γ-glutamyl transferase, and bilirubin). Strictures, however, can also be asymptomatic and diagnosed incidentally. Biliary strictures can be classified based on etiology, including malignancies, or anatomical location (extrahepatic, perihilar, and intrahepatic) and the structures involved, with significant implications for diagnosis and eventual drainage (Bismuth and Maino, 2001; Elmunzer et al., 2023).

Sclerosing cholangitis is classified as primary (PSC) or secondary sclerosing cholangitis (SSC), depending on the occurrence of identifiable etiologies (Ludwig et al., 2023). According to the identifiable causes, SSC can be grouped into major categories: ischemic damage (hepatic arterial thrombosis, ischemic-type biliary lesions), infection-related cholangitis (pyogenic, AIDS-related, COVID-associated cholangiopathy), toxic insults (including liver-directed arterial therapy or hepatic artery infusion pump), immunological causes (IgG4-related cholangitis, eosinophilic cholangitis), congenital diseases (e.g. Caroli's disease), post-surgical and other miscellaneous causes (Ludwig et al., 2023). On the other hand, PSC is a rare and progressive cholestatic liver disease characterized by inflammation and fibrosis, causing alternating narrowing and dilation of both intra- and extrahepatic bile ducts, eventually leading to biliary cirrhosis. Although the causes of PSC are still unknown, it is believed to be an autoimmune disorder, caused by immunological priming in a genetically susceptible individual, as proven by the identification of some well-known genotypic associations. The immunological component of its etiology might also explain the strong association with inflammatory bowel disease (IBD) (Ludwig et al., 2023).

At imaging, strictures in PSC patients with a diameter ≤1.5 mm in the common bile duct and/or ≤1.0 mm in a hepatic duct within 2 cm of the main biliary confluence, are defined as dominant stenosis (DS) (Dumonceau et al., 2020). The eventual development of DS has been observed in 10-62% of patients with PSC.

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It is important to stress that the presence of a DS may have a negative impact on prognosis: patients with PSC and DS have a shorter survival time (about 13.7 years from diagnosis) compared with those without DS (23 years): the increased risk of cholangiocarcinoma (CCA), amounting for 26% of patients with DS, is the reason for this survival difference (Chapman and Williamson, 2017).

The differentiation between benign and malignant causes of biliary strictures, and DS in particular, is a priority, because the therapeutic approach differs completely. Moreover, a confirmed cancer diagnosis significantly impacts the surgical and oncological approach and determines the possibility of a biliary stent placement as a treatment option. However, distinguishing between the two is not always easy and represents a significant diagnostic challenge (Bowlus et al., 2016). As proof of this, post-operative pathological evaluation proved that more than one-quarter of cases initially suspected to be malignant, were benign (Clayton et al., 2003; Wakai et al., 2012). In addition, it is not always possible to define whether a stricture is malignant or benign by laboratory testing, imaging, and endoscopic retrograde cholangiopancreatography (ERCP) with tissue sampling. In these cases, the term *indeterminate* biliary strictures (IDBS) is recommended (Bowlus et al., 2016). IDBS account for 20% of all newly diagnosed biliary strictures: notably, it was observed that >80% of IDBS are malignant (Martinez et al., 2020).

To improve diagnostic sensitivity, some techniques have already been integrated into specific algorithms and guidelines, and new techniques are still under development. The present review focuses on morphological criteria in the diagnosis of biliary strictures, including the many limits of the cytological and histological evaluation, as well as ancillary methodologies currently available in Pathology laboratories. The future perspectives for the improvement of the diagnostic yield of biliary pathology will also be addressed.

The current diagnostic approach to biliary stenosis

Surgical resection for a benign biliary disease implies a substantial morbidity and mortality rate, with a reduction in long-term survival (Ahlawat and Al-Kawas, 2022). Therefore, an accurate preoperative diagnosis with high sensitivity and negative predictive power is essential to identify those patients who should benefit most from surgery, and thus avoid unnecessary surgery (Martinez et al., 2020). Several diagnostic tests are available but none are accurate enough *per se* (Vlajnic et al., 2014; Bowlus et al., 2016) (Fig. 1). For this reason, some techniques have already been integrated into specific algorithms and guidelines, and new techniques are still under development, to improve diagnostic sensitivity, risk stratification, prognosis, and management of patients with biliary stenosis (Chaves et al., 2023). Specifically, once a biliary stricture has been diagnosed by imaging techniques, such as transabdominal ultrasonography (US) or contrast-enhanced computed tomography (CT), it is crucial to further characterize its nature through MRI or magnetic resonance cholangiopancreatography (MRCP) (Dumonceau et al., 2020). MRCP is the most accurate technique for the examination of the biliary tree, with 97% sensitivity and 98% specificity in detecting biliary obstruction (Martinez et al., 2020). Moreover, it represents a relatively helpful diagnostic tool in the differential diagnosis of benign and malignant strictures, with a

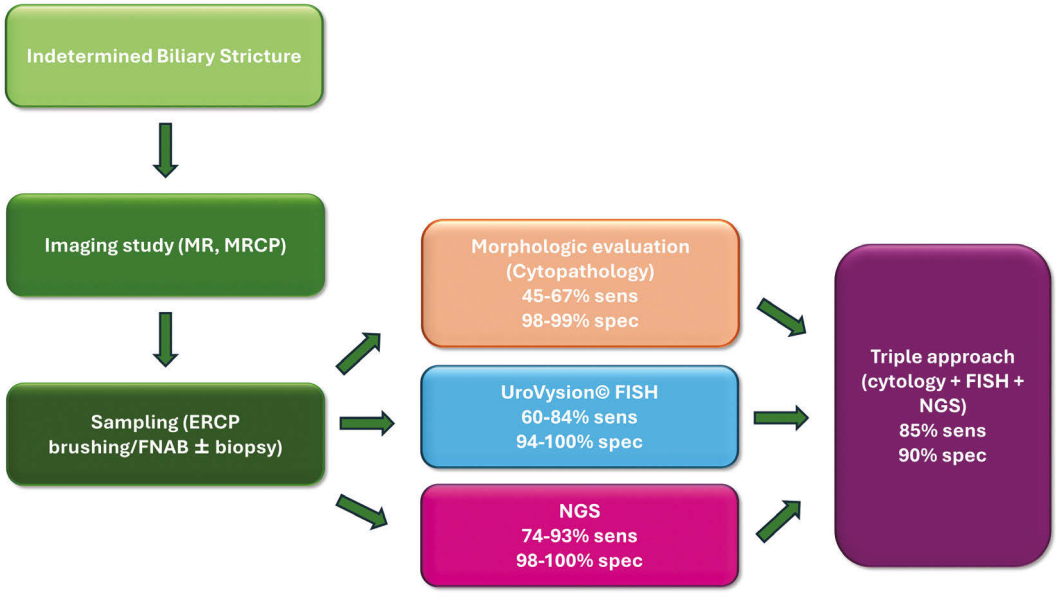

Fig. 1. Flowchart representing the current diagnostic algorithm of patients with indeterminate biliary stricture. The ranges of sensitivity and specificity are reported for each technique applied to brush cytology samples, according to literature results and for the combination of all techniques. References: Gonda et al., 2012; Vlajnic et al., 2014; Salomao et al., 2015; Dudley et al., 2016; Nguyen and Harada, 2016.

reported sensitivity ranging from 38 to 90%, and a specificity of 70-85% (Singh et al., 2015) (Fig. 1). The limited sensitivity of MRCP leads to many IDBS diagnoses. In such cases, current guidelines recommend assuming malignancy until proven otherwise, and to further characterize the strictures, measuring the levels of serum tumor markers, and using invasive techniques to obtain tissue samples through biopsy or brush cytology (Dumonceau et al., 2020).

Among the serum tumor markers, carbohydrate antigen 19-9 (CA 19-9) is the most widely used to diagnose malignant pancreaticobiliary diseases. However, CA 19-9 elevation may arise during the course of other liver diseases, including hepatocellular carcinoma, bacterial cholangitis, or cholestasis (Sinakos et al., 2011). Various cut-offs have been proposed to improve the diagnostic yield of CA 19-9. For example, it was observed that a cut-off of ≥44 U\mL implies 62% sensitivity and 44% specificity for the detection of malignancy (Singhi et al., 2020), while some authors found that a serum CA 19-9 level >85.5 U\ml is highly suggestive of neoplastic stenosis (cut-off calculated by building a ROC curve, with an Area Under the Curve of 0.630, however, sensitivity and specificity were not reported) (Barroso Márquez et al., 2022).

Endoscopic evaluation of indeterminate biliary stenosis aims to obtain a histopathological diagnosis while treating the biliary obstruction by stenting (Xu and Sethi, 2015). Current guidelines recommend the use of ERCP as the first-line diagnostic and treatment approach to obtain brush cytology and/or forceps biopsy and drain the bile ducts (Sato et al., 2022).

Morphological diagnostic criteria

Currently, pathological examination is still essential to distinguish benign/inflammatory from malignant lesions (Nguyen Canh and Harada, 2016). According to the latest guidelines from the World Health Organization (WHO, 2022), the key diagnostic cytopathological features for CCA diagnosis include nuclear pleomorphism, hyperchromasia, anisonucleosis, increased nuclear-to-cytoplasmatic ratio (≥0.6), prominent nucleoli and loss of polarity. The WHO Reporting System for Pancreaticobiliary Cytopathology (WHO System) encompasses seven categories:

1. Insufficient/inadequate/not diagnostic,
2. Benign/negative for malignancy,
3. Atypical,
4. Pancreatic neoplasm-low grade (pan-low),
5. Pancreatic neoplasm-high grade (pan-high)
6. Suspicious for malignancy,
7. Positive for malignancy.

This system classifies the risk of malignancy on cytopathological specimens from both the pancreatic and extrahepatic bile ducts. Since specific criteria for premalignant biliary neoplasms are lacking, the authors anticipate that most bile duct brushings will not fall in the PaN-Low or PaN-High categories (Pitman et al., 2023).

The main issue is that, despite the high specificity of CAA diagnosis (99%), brush cytology and intraductal biopsies obtained by ERCP have an extremely low sensitivity, no higher than 45% (Nguyen Canh and Harada, 2016). In addition, the sensitivity slightly increases, but remains low (59.4%), even when the two techniques are used together (Nguyen Canh and Harada, 2016).

The low sensitivity of cytological and histological diagnosis is due to several factors. The main problem is represented by the inability to obtain adequate samples (low cell count) in most cases because of the difficult access to the biliary tree and the need to perform a biliary sphincterotomy to obtain a biopsy sample, with an increased risk of complications (Dumonceau et al., 2020; Zhang et al., 2021). Other issues in the diagnostic approach include previous stentings, recurrent cholangitis, and the anatomical position of the stenosis (biliary biopsy has a higher yield in distal biliary stenosis than in proximal) (Lindberg et al., 2002; Xu and Sethi, 2015). In addition, the decreased sensitivity may be due to the histopathological and pathogenetic characteristics of CCA, including the association with dysplastic precursor lesions, such as biliary epithelial neoplasia (BilIN) and intraductal papillary neoplasms of the bile duct (IPNB) of low and high grade (WHO, 2022). Precursor dysplastic lesions are associated with CCA in up to 40-60% of cases, as well as in one-third of patients affected by PSC, nonetheless, they might not always be detected and diagnosed (Hennedige et al., 2014; Kendall et al., 2019). Based on cytopathological features, the differential diagnosis between a high-grade BilIN and CCA cannot be made due to the lack of the histological invasion parameter (WHO Reporting System for Pancreaticobiliary Cytopathology, 2022). Furthermore, distinguishing low-grade dysplasia from reactive atypia is often difficult, considering that an inflammatory *milieu* is frequently present. In these cases, where a definitive diagnosis of dysplasia is not possible, the terms “atypical” or “indefinite for dysplasia” have been proposed (Kendall et al., 2019; WHO, 2022) (Fig. 2). The morphologic overlap between inflammatory and neoplastic features is the main reason why the risk of malignancy in the various categories of bile duct brushing specimens is higher than in their pancreatic counterparts (Rosenbaum et al., 2020). Other features decreasing the sensitivity of the pathological analysis include small tumor size, the presence of a diffuse desmoplastic intratumoral component, and the pattern of tumor growth (Qin et al., 2004), especially periductal infiltrating type. This growth pattern is particularly misleading, complicating sampling of the neoplastic cell, which grows in single elements or small clusters infiltrating the stroma (WHO Digestive System Tumors 2019).

The overall low sensitivity and high rate of false negative results with morphology alone make it necessary to repeat the sampling procedure (with relative

complications) or to introduce ancillary techniques.

Fluorescence *in situ* hybridization (FISH)

FISH was the first ancillary technique proposed to increase the diagnostic accuracy of pathological evaluation in biliary strictures. In the previous Papanicolau Society of Cytopathology guidelines (now revised and updated in the WHO reporting system for Pancreaticobiliary Cytopathology), FISH was recommended as the ancillary technique yielding the highest sensitivity, without reducing specificity (Layfield et al., 2014). It has been observed that when atypical or positive cytology is further evaluated with FISH, the sensitivity increases to >70% (Nguyen Canh and Harada, 2016). Moreover, when all three approaches (cytology, biopsy, and FISH) are used together, sensitivity reaches 82% and specificity 100% (Nguyen and Harada, 2016).

FISH is a viable diagnostic option because biliary tract tumors are genetically unstable, causing changes in the number of chromosome copies (Kamp et al., 2021). Indeed, FISH is a molecular technique that detects aneuploidy and structural chromosomal abnormalities, using fluorescent-labeled polynucleotide probes that bind the target DNA sequences (Gonda et al., 2012). The research can be carried out either on mitotic preparations or on the interphase nucleus and the probe-target hybridization can be visualized under a fluorescence microscope (Vasilieva et al., 2012). This analysis is performed on brush samples and can provide an accurate diagnosis even when the sample contains only a few neoplastic cells (Gonda et al., 2012). The latter is the main advantage of FISH as the bile duct cytological specimens obtained by ERCP often contain scarce diagnostic material and a heterogeneous cell population; FISH is a powerful technique to overcome this limitation (Singhi et al., 2020). Moreover, FISH can be applied successfully to different types of cytopathological samples, without stringent restrictions for the pathologist or the endoscopist who retrieves the sample (Roh, 2019) (Table 1).

Polysomy of the centromeric regions of chromosomes 3, 7, and 17, as well as homozygous or heterozygous deletion of the 9p21 locus, are the most specific signs of malignancy (Salomao et al., 2015). Notably, chromosome region 9p21 contains the *CDKN2A* gene, encoding p16, a tumor suppressor protein that regulates cell cycle entry; p16 is often inactivated in CCA due to allelic loss of 9p21. Therefore, detecting the deletion of the 9p21 locus can increase the diagnostic yield. With the addition of 9p21 evaluation to 3, 7, and 17 chromosome polysomies, FISH sensitivity increases from 47% to 84% (Gonda et al., 2012) without compromising specificity. A positive FISH result in a lesion identified as "suspicious for malignancy" supports the diagnosis of CCA, while a

Table 1. Schematic table of the advantages (PROs) and disadvantages (CONs) of the FISH technique in the diagnostic algorithm of bile duct histo-cytopathologic analysis.

FISH			
PROs	Feasible with any cell enrichment High diagnostic sensitivity and specificity together with morphology	CONs	Highly operator-dependent Expensive, needs dedicated lab spaces and equipment May be positive also in BilIN, IPNB, and patients with PSC. FISH positivity in PSC patients has a controversial significance regarding cancer development

References: Levy et al., 2008; Gonda et al., 2012; Eaton et al., 2015; Nguyen and Harada, 2016; Adler and Witt, 2018; Roh, 2019; Kaura et al., 2020; Singhi et al., 2020.

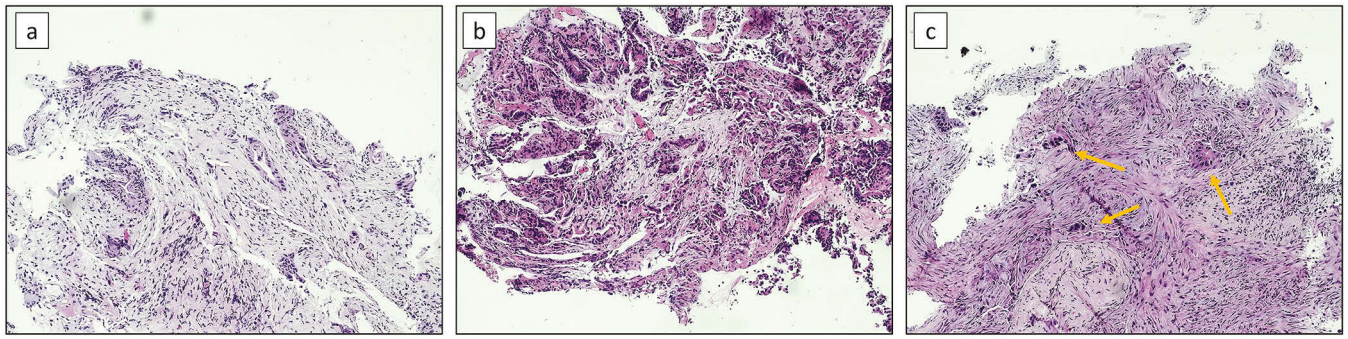

Fig. 2. Three cases of bile duct biopsies from our clinical routine. In case **a**, sparse glands are seen within a small fragment of fibro-muscular tissue, with a bland benign-looking appearance and non-atypical polarized cells; in cases **b** and **c**, the glands acquire an infiltrative pattern, a higher nuclear-to-cytoplasmic ratio, and nuclear hyperchromasia. Note the scarcity of malignant-looking glands in case **c** (arrows). Hematoxylin-Eosin stain, x 200.

negative FISH result is more likely to be associated with a benign or reactive lesion (Adler and Witt, 2018). It is also important to note that chromosomal anomalies can be present in both CCA and premalignant lesions. Because of this, FISH should not be used as the sole diagnostic tool but rather in conjunction with morphological evaluation to avoid false positive results. Any positive FISH results with negative cytology should, therefore, be interpreted and treated with caution (Adler and Witt, 2018). Other important aspects to be considered concern patients with PSC. First, chromosome trisomy 7 in PSC patients without malignancy is not uncommon and should be kept in mind to avoid false positive FISH results (Levy et al., 2008). Second, it has been observed that PSC patients who have polysomy at multiple locations along the biliary tree are more likely to develop CCA than those with unifocal polysomy. These polysomies are frequently found in areas not associated with DS, while DS is not more common in patients with multiple polysomies (Eaton et al., 2015). Conversely, other studies found that one-third of patients with FISH polysomy may never actually develop CCA (Kaura et al., 2020) (Table 1). These observations highlight two important points: (i) in patients with PSC, more areas of the biliary tree should be sampled, not just those around a DS (Eaton et al., 2015); and (ii) the development of CCA in patients with PSC is likely due to a *field cancerization*, meaning that larger areas of premalignant tissue, bearing molecular and chromosomal aberrations, may extend beyond the primary site of malignancy, in histologically normal areas (Eaton et al., 2015). However, the most recent guidelines from the European Association for the Study of the Liver recommend the use of FISH when brush cytology and/or histological evaluation are inconclusive (European Association for the Study of the Liver, 2022).

Next-generation sequencing

Next-generation sequencing (NGS) is another ancillary technique used to improve the diagnostic yield of biliary strictures sampling; it allows several genes to be analyzed with a single test (Layfield, 2020). The study of molecular profiling and gene alterations of a specific tumor facilitates the accurate diagnosis and optimal selection of target treatments based on the genetic variant (Stenzinger et al., 2024). NGS combines high analytical sensitivity with multigene analysis, enabling the identification of recurrent genomic alterations, particularly relevant for those genes commonly mutated, amplified, and/or deleted in CCA, including *AKT1*, *ALK*, *ATM*, *BRAF*, *CDKN2A*, *CTNNB1*, *EFGR*, *ERBB2*, *ERBB4*, *FGFR1*, *FGFR2*, *FGFR3*, *GNA11*, *GNAQ*, *KRAS*, *IDH1*, *IDH2*, *KIT*, *KRAS*, *MET*, *NRAS*, *PDGFRA*, *PIKRCA*, *PTEN*, *SMAD4*, *TP53*, and *VHL* (Singhi et al., 2020). The most frequent changes typically involve a limited number of oncogenes and tumor suppressor genes (the so-called driver *mutations*), including alterations in *CDKN2A*, *KRAS*, *TP53*, and *SMAD4* (Singhi et al., 2020) (Table 2).

KRAS mutations appear to be an early molecular event in CCA pathogenesis, occurring in about 40% of BilIN precursor lesions and a considerable percentage of CCAs (Hsu et al., 2013; Nakamura et al., 2015), such that specific molecular tests (i.e., PCR) for the identification of *KRAS* mutations were proposed to enhance the cytology diagnostic yield, alone or in combination with other techniques (Kipp et al., 2010; Layfield et al., 2014).

The *TP53* tumor suppressor gene has been found altered in about 50% of extra-hepatic CCAs, and it is considered a late event in neoplastic progression (WHO Digestive System Tumors, 2019). The application of immunocytochemistry for p53 protein expression has been deemed useful in biliary brush specimens. An abnormal expression pattern (indirectly suggestive of *TP53* mutation) can be found both in high-grade BilIN and in overt CCA (Sato et al., 2013; Xue et al., 2019), making the detection of *TP53* mutations a powerful tool to support a diagnosis of malignancy.

The implications of *SMAD4* gene alterations are like those of *TP53* from many points of view. They are late events in CCA pathogenesis, frequently found (the third most common mutation in most studies), and can be suggested based on SMAD4 loss of expression at immunocytochemistry (Tang et al., 2002; Nakamura et al., 2015; Wardell et al., 2018).

Genomic alterations of the *CDKN2A* gene, particularly allelic losses of 9p21, are well known to be among the most common alterations in biliary tract cancer. Their frequency and sensitivity in detecting malignancy warranted their inclusion in the multiprobe FISH analysis (*see above*). Recent data suggested a prognostic role for *CDKN2A* mutations, even in a cohort of intrahepatic-only CCAs (Takada et al., 2022).

Table 2. Schematic table of the advantages (PROs) and disadvantages (CONs) of the NGS technique in the diagnostic algorithm of bile duct histo-cytopathologic analysis.

Next-Generation Sequencing			
PROs	Less expensive and not operator-dependent High diagnostic sensitivity and specificity Possibility of identifying targetable mutations	CONs	Requires at least 10% of specimen cellularity

References: Dudley et al., 2016; da Cunha Santos et al., 2018; Wardell et al., 2018; Singhi et al., 2020; Stenzinger et al., 2024; Fassan et al., 2024.

In addition to helping in the diagnosis of malignancy, NGS is likely to provide prognostic information. Mutations in *ARID1A* and *KRAS* have been observed to have negative effects on overall survival, while the novel deletion of *MUC17* in the 7q22.1 region has been shown to worsen both overall and disease-free survival (Wardell et al., 2018). Furthermore, germline mutations in cancer-predisposing genes, such as *BRCA1*, *BRCA2*, *EAD51D*, *MLH1*, or *MSH*, have been detected in 11% of non-neoplastic biliary tract from patients with CCA. This discovery opens the possibility of tailoring therapeutic and follow-up strategies for specific patients (Wardell et al., 2018). According to this, NGS is a very promising technique; applying NGS to biliary brushing or biopsy increases sensitivity up to 77% and 83%, respectively (Singhi et al., 2020). Nevertheless, while NGS is effective in improving the diagnostic sensitivity of biliary strictures, as well as in evaluating patients' prognosis and predictivity, it still has limitations, including the inability to obtain suitable samples for NGS in some cases. Pathologists play a critical role in assessing the feasibility of biopsy specimens for NGS since the morphological assessment of specimen adequacy is decisive (Stenzinger et al., 2024). The location and close integration of CCA with other anatomical structures can complicate biopsy access and the retrieval of sufficient tumor tissue for NGS, representing a technical challenge. Unlike other neoplasms, a threshold number of cells for successful analysis has not been established as yet, however, in everyday practice the common threshold of 200-400 cells could be utilized (Fassan et al., 2024) (Table 2). Insufficient cellular content and neoplastic cell enrichment of 10% per sample (i.e., 5% of the mutated allele), among other factors, may contribute to sampling failure. An overall sample failure rate of 26.8% was observed (Stenzinger et al., 2024). To improve diagnostic sampling, current guidelines recommend considering different biopsy techniques depending on tumor locations and accessibility, e.g., percutaneous fine needle biopsy, endoscopic ultrasound (EUS) brush cytology, EUS-guided fine needle aspiration (FNA), or fine needle biopsy (Vogel et al., 2023). The type of cytopathology sample chosen does not significantly affect the molecular analysis; nucleic acid can be extracted from all routine specimen preparations (da Cunha Santos et al., 2018).

The global data already available in the literature and the multipurpose use of NGS suggest an advantage over FISH, such that NGS will most likely become the ancillary technique of choice (Dudley et al., 2016; Fassan et al., 2024).

Future outlook

Due to the objective difficulties in this field of diagnostic pathology, great efforts are being made toward the research of new techniques, enhancing the diagnostic power of tissue sampling from the biliary tree. One of these techniques is liquid biopsy, which has emerged as a promising new approach, particularly when the sample does not meet the quality requirements for tissue NGS (Stenzinger et al., 2024). Liquid biopsy aims to analyze tumor markers in non-solid biological tissues; the presence of these biomarkers in biological fluids (particularly peripheral blood) is due to the release of vesicles, proteins, exosomes, and nucleic acids by tumor cells, especially during necrotic events (Lin et al., 2021). Among the blood biomarkers, the NGS analysis of circulating tumor DNA (ctDNA) has gained interest in recent years. In particular, it has been observed that mutations detected by NGS in tissue biopsies, the commonest being *KRAS* and *TP53* mutations, as well as *APC*, *SMAD4*, *GNAS*, *FBXWT*, and *BRAF*, are also detectable in ctDNA with a sensitivity of 92% and a specificity of 100% (Zill et al., 2015). For the same authors, the ctDNA test detected mutations in 85% of patients, compared with 62% detected by the biopsy-based test (mainly due to difficulties in retrieving sufficient genomic material). This means that a larger group of patients can benefit from the ctDNA test (Zill et al., 2015), in addition to the obvious advantage represented by the ability to obtain material through a simple and non-invasive blood sample (Stenzinger et al., 2024). Additionally, since ctDNA has a short half-life (from 15 minutes to a few hours), its analysis reflects a snapshot of the actual tumor genomic state in a non-invasive fashion (Han et al., 2021; Lin et al., 2021). Therefore, ctDNA analysis can provide information on tumor heterogeneity and clonal changes before and during therapy, as well as information on therapy resistance, recurrence, and therapeutic outcomes (Stenzinger et al., 2024). While ctDNA analysis is minimally invasive and feasible in all patients, on the other hand, it is crucial to make sure that the alterations identified in the blood truly reflect what is present in tumor tissue (Astier et al., 2024). In this sense, the main limit of blood analysis of ctDNA is that it reflects only a small fraction of the total cell-free DNA (cfDNA), limiting the sensitivity of the method (Stenzinger et al., 2024). For example, it has been observed that clonal hematopoiesis, (a common age-related phenomenon in which non-malignant mutations accumulate in hematopoietic cells, resulting in genetically distinct subpopulations of cells), can be a potential source of false positives in liquid biopsy (Driescher et al., 2020).

Another issue is that early-stage tumors usually do not release enough detectable ctDNA into the bloodstream, compared with advanced cancers, limiting the application of liquid biopsy to advanced cases (Zill et al., 2015). Therefore, a further limitation of the mutational analysis of plasma ctDNA is the lack of knowledge regarding its efficacy in early-stage disease. To overcome this limitation, the effectiveness of a new type of liquid biopsy based on mutational NGS analysis of cfDNA present in the bile has been proposed. The bile was observed to contain genetic tumor material, even in early-stage cancer and precancerous lesions, suggesting

a potential for early diagnosis (Arechederra et al., 2022). One study showed that *KRAS* mutations in the bile fluid of PSC patients are common early events in CCA; however, not all PSC patients with *KRAS* variants developed cancer during follow-up. Therefore, the presence of this mutation should be considered a risk factor for the development of CCA (Kubicka et al., 2001). Additionally, bile-based liquid biopsy outperformed plasma-based liquid biopsy; less than 50% of the pathogenic mutations detected in bile cfDNA were detected in plasma cfDNA (Driescher et al., 2020). On the other hand, the main limitation of liquid bile biopsy is the need for ERCP or other invasive diagnostic procedures to collect bile (Arechederra et al., 2022).

The latest diagnostic techniques were not limited to the study of tumor DNA but they were extended to the study of RNA, particularly microRNA (miRNA), and long non-coding RNA (lncRNA). miRNAs are a group of non-coding RNAs of approximately 20 nucleotides, which control gene expression at the post-transcriptional level binding to the 3' UTR region of the target mRNA; lncRNA are 200-nucleotide-long RNAs that regulate gene expression by directly interacting with DNA. Both miRNA and lncRNA play a significant role in the carcinogenesis of CCA (Zheng et al., 2017). For this reason, in recent years, miRNA and lncRNA have been hypothesized to be potential biomarkers for characterizing biliary tract strictures and diagnosing CCA. Studies have shown that some miRNAs are significantly overexpressed in CCA, e.g., miR-21, miR-141, and miR-200b, while the downregulation of mi-21 and miR-200b increases sensitivity to Gemcitabine (Meng et al., 2006). Dysregulated expression of lncRNA involved in cell proliferation and apoptosis, namely lncRNA AFAP1-AS1, was found to be significantly overexpressed in CCA tissues compared with precancerous tissue (Zheng et al., 2017; Shi et al., 2017).

The discovery of the significance and the potential diagnostic role of miRNAs and lncRNAs in CCA led to a new type of bile fluid analysis, based on the assessment of exosome concentrations in the bile (Shu et al., 2024). Exosomes are cell-derived vesicles with a single-layer membrane, secreted by all cell types, which can be found in all biological fluids. Exosomes have a crucial role in cell-to-cell communication and are likely to play a role in carcinogenesis, for instance acting as transporters of miRNAs (Doyle and Wang, 2019; Xu et al., 2022). The analysis of exosomal components in the bile through microRNA sequencing (miRNA-seq) showed that miR-182-5p and miR-183-5p, secreted by CCA cells, are upregulated in CCA bile exosomes, and that elevated miR-182\183-5p levels in both CCA tissues and bile indicate an unfavorable prognosis (Shu et al., 2024).

DNA methylation represents another approach deserving attention among the new promising complementary techniques. Both malignancies and precancerous lesions have been linked to DNA hypermethylation of gene promoters, a constant and early occurrence in carcinogenesis (Laird, 2003). Differences in CpG island hypermethylation were described in different human tumors, depending on the type of gene involved and methylation frequency among different tissues. These characteristics result in distinct hypermethylation profiles in different cancers, meaning that certain gene alterations are cancer-specific (Laird 2003). Several authors found various specific hypermethylated genes in CCA, as well as in premalignant lesions such as BilIN, suggesting that DNA methylation is an early event in biliary carcinogenesis (Koga et al., 2005; Yang et al., 2005; Kim et al., 2009; Andresen et al., 2012). In addition, DNA methylation is readily detectable using PCR-based technologies, in a less expensive and technically simpler way in comparison with other molecular approaches. The relatively low costs and availability of the technique, together with the requirement of a small sample of a wide range of materials such as cells, bile, and blood have led to promising results in the past years (Pixberg et al., 2017; Vedeld et al., 2020).

A recent paper by Prachayakul and colleagues investigated the "methylation index (MI)" of two gene promoters (HOXA1 and NEUROG1) in CCA, determined by quantitative methylation-specific PCR, revealing that the MI of both genes had higher sensitivity (95.1% and 90.2%, respectively) than brush cytology (Prachayakul et al., 2022). Other works separately studied the potential of methylation markers in bile for early detection of CCA. Shin and colleagues developed a five-gene panel for the detection of extrahepatic CCA with a sensitivity of 75.6% and a specificity of 100% (Shin et al., 2012), while Vedeld and colleagues applied a previously validated methylation biomarker panel with a sensitivity of 85% and a specificity of 98% in biliary brush series to the analysis of bile samples from PSC patients (Vedeld et al., 2020). In the latter paper, authors found two interesting results: that methylation changes can be found up to 12 months before conventional CCA detection techniques, and that patients with biliary dysplasia show a greater methylation frequency compared with PSC patients without dysplasia (Vedeld et al., 2020). These studies demonstrated that good sensitivity can be achieved in a small and relatively accessible sample (bile). However, the authors themselves advised caution because few studies have been performed so far and further validation is needed (Vedeld et al., 2020).

Conclusions

In conclusion, the cytopathological and histopathological diagnosis of biliary strictures is still burdened by a lack of diagnostic accuracy, mainly due to the difficult access and, sometimes, the impossibility of obtaining sufficient material. The only ancillary technique approved by all guidelines is FISH, however, it is an operator-dependent technique, not available at all Centers. For this reason, recent research has been

primarily aimed at improving the diagnosis of biliary tract strictures, integrating innovative approaches for the best use of the scant tissue obtained, among which NGS is likely to be the main player in the next years. The liquid biopsy approach, which aims to analyze tumor DNA and RNA without invasive procedures, seems to represent another milestone in this field.

These future perspectives reflect the promise of facilitating an early diagnosis and a reliable prognostic and therapeutic evaluation of bile duct cancer, which is still burdened by late diagnosis and adverse prognosis.

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