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Emerging Technologies for the Diagnosis of Perihilar Cholangiocarcinoma

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Abstract

The diagnosis of malignant biliary strictures remains problematic, especially in the perihilar region and in primary sclerosing cholangitis (PSC). Conventional cytology obtained during endoscopic retrograde cholangiography (ERC)-guided brushings of biliary strictures is suboptimal due to limited sensitivity, albeit it remains the gold standard with a high specificity. Emerging technologies are being developed and validated to address this pressing unmet patient need. Such technologies include enhanced visualization of the biliary tree by cholangioscopy, intraductal ultrasound, and confocal laser endomicroscopy. Conventional cytology can be aided by employing complementary and advanced cytologic techniques such as fluorescent in situ hybridization (FISH), and this technique should be widely adapted. Interrogation of bile and serum by examining extracellular vesicle number and cargo, and exploiting next-generation sequencing and proteomic technologies, is also being explored. Examination of circulating cell-free deoxyribonucleic acid (cfDNA) for differentially methylated regions is a promising test which is being rigorously validated. The special expertise required for these analyses has to date hampered their validation and adaptation. Herein, we will review these emerging technologies to inform the reader of the progress made and encourage further studies, as well as adaptation of validated approaches.

Keywords

cell-free DNA; differentially methylated regions; FISH

Cholangiocarcinoma (CCA) represents a heterogeneous group of epithelial cell malignancies with features of cholangiocyte differentiation.[1] CCA is the most common biliary malignancy and the second most common hepatic malignancy (after hepatocellular carcinoma). CCAs are most commonly classified based on their anatomic location within the biliary tree into intrahepatic, perihilar (pCCA), and distal CCA (dCCA). pCCA involves the large bile ducts within the hepatic hilum and arises between the second-order biliary ducts proximally and the cystic duct insertion distally.[2] pCCA is the most common subtype, representing 50% of CCA cases in a large series.[3] Primary sclerosing cholangitis (PSC) is the most well-

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established risk factor for pCCA. The 10-year cumulative incidence of CCA in PSC patients is 6 to 11% with a 30-year risk of 20%.[4,5]

CCAs are highly aggressive malignancies with a 5-year overall survival of less than 10%.[6] Diagnosing pCCA at an early stage poses a significant challenge and contributes to the poor outcomes associated with this disease. Themajority of pCCA patients present with advanced-stage disease precluding curative treatment options such as surgical resection or neoadjuvant chemoradiation followed by liver transplantation.[7] A significant factor in delayed diagnosis is that most patients do not become symptomatic until the cancer is advanced. Other diagnostic challenges include the remote anatomical location of pCCAs which often reside in difficult-to-access areas within the biliary tree, and the desmoplastic, paucicellular nature of these tumors. These factors limit the sensitivity of conventional cytology and biliary biopsy. For example, conventional cytology obtained by endoscopic retrograde cholangioscopy (ERC)-guided brushings only has a sensitivity of 20 to 40%.[8,9] Moreover, the presence of inflammatory epithelial cell alterations in the setting of biliary infection or underlying PSC represents a diagnostic challenge on cytologic evaluation as these reactive cells often mimic cancer cells.[10] Advanced cytological techniques (►Fig. 1) such as fluorescence in situ hybridization (FISH) have improved sensitivity in combination with conventional biliary cytology.[11] However, FISH analysis also has suboptimal sensitivity (~60%).[8,12] Emerging techniques such as next-generation sequencing (NGS) and proteomic analysis have the potential to be viable adjunctive diagnostic tests for pCCA detection. Novel biomarkers such as circulating tumor cells (CTCs), circulating cell-free deoxyribonucleic acid (cfDNA), and extracellular vesicles (EVs) may overcome the sampling issues associated with direct biliary cytology and biopsy techniques. Herein, we review these novel biomarkers and advanced cytologic technologies in pCCA, along with advances in endoscopic techniques.

Noninvasive Imaging

Imaging is an indispensable tool for the detection of CCA. Ultrasonography, computed tomography (CT), positron emission tomography (PET), and magnetic resonance imaging(MRI)/magnetic resonance cholangiography (MRC) have been investigated as diagnostic imaging modalities.

In the absence of a mass, ultrasonography may only delineate intrahepatic ductal dilation, a common finding in PSC without a concomitant biliary cancer, without providing further details.[13] Indeed, the sensitivity of ultrasound was only 10% among those with definitive evidence of CCA. Hence, it is an insufficient screening modality to detect early stage biliary cancers.[14] CT scans may detect mass lesions and investigate invasion into adjacent structures or metastases. The chief disadvantages of CT scans are the need for radiation as well as the limited ability to visualize the biliary tree and associated features which can be seen in early pCCA. PET scan rarely provides additive value when MRI/MRC and biliary brushings are inconclusive and false positive areas of 18-fluorodeoxyglucose (FDG) avidity due to inflammation are common.[13,15] Recent data have suggested that standard uptake max may have prognostic implications and cut-offs may be able to distinguish between benign and malignant strictures among those with and without PSC.[16,17] Larger cohorts comprised of

PSC patients are needed to determine if these observations can be applied in this unique population. Among these modalities, MRI/MRC is the diagnostic technique of choice. Among patients with symptoms or laboratory tests suggestive of a biliary obstruction, MRI/MRC can provide useful information to the endoscopist prior to an ERC. The sensitivity and specificity of MRI/MRC to detect CCA is 88 to 89% and 75 to 85%, respectively, and is superior to the sensitivity and specificity of CT (75–79% and 79–80%, respectively).[14,18] A mass lesion with venous enhancement seen on MRI is very specific for CCA.[19] However, a discrete mass is often absent in early stage disease. Imaging features that are indeterminate for CCA which should prompt an ERC include bile duct wall thickening, a bile duct stricture with or without proximal dilatation, duct wall irregularity, or contrast enhancement.[20]

Advanced Endoscopic Techniques

Endoscopic Ultrasound

Endoscopic ultrasound (EUS) allows for a detailed exam of the extrahepatic bile duct and surrounding structures from the duodenum, making it a valuable tool for the diagnosis and accurate staging of pCCA (►Fig. 2). EUS is able to detect the primary tumor in ~94% of cases; however, in a large series, tumor detection was higher in dCCA (100%) compared with proximal tumors (83%).[21] Whether or not to perform EUS-guided tissue acquisition of a pCCA has been debated in the literature. Sensitivity of EUS fine-needle aspiration (FNA) has been demonstrated to be lower for pCCA compared with dCCA (59% vs. 81%);[21] however, a recent report suggests a higher diagnostic yield with EUS-guided tissue acquisition (82%) in patients with suspected pCCA.[22] A meta-analysis including 196 patients in 6 studies of EUS-FNA for the diagnosis of extrahepatic CCA demonstrated an overall pooled sensitivity of 66% (95% confidence interval [CI]: 57–74%).[23] The benefits of EUS-FNA of primary tumors must be weighed against the risk of tumor dissemination. This risk was highlighted in a study of 191 patients with locally advanced CCA being evaluated for liver transplantation.[24] Sixteen patients underwent transperitoneal FNA with peritoneal metastases identified in 5 of the 6 patients (83%) with positive FNA cytology for malignancy compared with 14 of 175 who did not undergo transperitoneal FNA (8%; $p = 0.0097$). In a retrospective, single-center study of 150 consecutive patients with CCA, 61 patients underwent preoperative EUS-FNA of a primary biliary mass or stricture.[25] In this study, performance of FNA did not adversely impact overall or progression-free survival; however, none of the patients underwent liver transplantation. The authors concluded that although tumor seeding is a theoretical risk with EUS-FNA, the clinical significance in patients with CCA is likely to be small. In that study, only 21/79 (27%) with pCCA underwent FNA compared with 40/53 (75%) with dCCA. FNA of dCCA does not carry the same concern for tumor seeding as the duodenal wall is part of the resection field for curative-intent operations, whereas the duodenal bulb is not typically part of the resection field for pCCA. Hence, EUS-FNA of a pCCA is still believed to increase the risk of tumor dissemination and is considered an absolute contraindication to liver transplantation at the Mayo Clinic.[26]

Lymph node metastasis portends a poor outcome and therefore accurate staging is critical for individuals being considered for curative surgery. The role of EUS for nodal staging was investigated in a series of 47 patients with pCCA being considered for liver transplantation. EUS identified regional lymph nodes in all patients, of which 8 patients were confirmed to have malignant lymph nodes by FNA, whereas CT and/or MRI identified malignant lymphadenopathy in only 2 patients.[27] Subsequent staging exploratory laparotomy confirmed the presence of benign lymph nodes in 20 of 22 patients with negative FNA (91%). Morphological and echo features, including size, shape, echogenicity, and homogeneity, were of poor predictive value. Therefore, it is our practice to sample all visualized lymph nodes in patients with pCCA being considered for liver transplantation or curative resection.[26]

ERC and Associated Technology

ERC has played an important role for the anatomic delineation and tissue acquisition from suspected pCCA. ERC with brushing for cytology has been the standard approach for suspected malignant biliary strictures, but has been associated with low sensitivity when only positive for malignancy results (15%) or when positive and suspicious for malignancy results (38%) have been used.[10] Various modifications of brush design and sampling technique have resulted in similarly suboptimal results. ERC with biliary biopsy has been associated with higher cancer detection rates, but a recent meta-analysis showed that this benefit was only demonstrated when biopsy was combined with brushing (59% sensitivity) as the pooled sensitivity was similar between brushing (45%) and intraductal biopsy (48%).[28]

Intraductal Ultrasound

Intraductal ultrasound (IDUS) is performed with a thin high-frequency probe that is advanced over a wire into the bile duct at the time of ERC. IDUS has been shown to improve diagnostic accuracy over traditional ERC-sampling techniques (e.g., conventional cytology has a sensitivity of 19–43%).[8,9] In a study of 264 patients with biliary strictures that underwent surgical resection, IDUS was found to have a sensitivity, specificity, and accuracy of 93, 89.5, and 91%, respectively.[29] The main limitation of IDUS is that it is a visual diagnosis and lacks the ability to acquire tissue for confirmatory diagnosis. In addition, previously placed stents decrease the diagnostic yield of IDUS.[30] IDUS provides limited nodal staging as the depth of radial penetration is ~2 cm. Although most endosonographers prefer conventional EUS as it provides superior locoregional staging and the ability to acquire tissue for cytology, IDUS may have a diagnostic role in patients with suspected pCCA not well visualized with prior imaging (including EUS) who do not have indwelling biliary stents.

Cholangioscopy

The development of single operator cholangioscopy (SOC) (►Fig. 2) allowed for an easier platform for direct visualization of the biliary epithelium compared with percutaneous approaches and mother–daughter platforms that required two operators. In addition to providing visual information, SOC was believed to allow for targeted biopsies of biliary lesions thereby increasing the diagnostic yield of tissue sampling. In a large prospective

multicenter study, Chen et al demonstrated that SOC visual impression had a sensitivity, specificity, positive predictive value, and negative predictive value of 78, 82, 80, and 80% respectively.[31] SOC-directed biopsies, however, demonstrated a lower overall sensitivity of 49% for malignancy, although this was higher for intrinsic bile duct malignancies (66%). A recent systematic review of SOC-targeted biopsies for indeterminate biliary strictures identified 6 studies that reported an overall 66% sensitivity and 97% specificity to detect CCA.[32] SOC with targeted biopsy sampling has been advocated as a cost-effective strategy for evaluating CCA in patients with PSC as compared with ERC with brushing for FISH with an incremental quality-adjusted life year gain of 0.22 at an additional cost of $8,562.[33] Data using newer generation SOC systems with digital imaging are limited. A recent multicenter observational study that included 44 patients with indeterminate biliary strictures demonstrated a sensitivity and specificity of SOC digital visual impression for the diagnosis of malignancy of 90 and 96%, respectively, and 85 and 100%, respectively, for SOC-targeted biopsies.[34] Similar digital imaging advancements have been achieved in choledochoscopes that are directly advanced into the bile duct after biliary sphincterotomy. An added benefit with these devices is that they allow for digital chromoendoscopy. In a study of 109 patients undergoing peroral video cholangiopancreatoscopy with narrow-band imaging that included 20 patients with biliary neoplasia, visual impression was associated with 85% sensitivity and 84% specificity.[35] However, imaging directed sampling resulted in a lower sensitivity (43%). Imaging features that were associated with neoplasia included tortuous and dilated vessels, infiltrative stricture, polypoid mass, and the presence of fish-egg lesions. While newer digital cholangioscopy platforms appear to have improved the ability to make a visual diagnosis of malignancy, it remains to be seen if these technologies can improve tissue diagnostic yield with targeted biopsy sampling.

Confocal Laser Endomicroscopy

Probe-based confocal laser endomicroscopy (CLE) allows for in vivo microscopic evaluation of the biliary epithelium, providing cellular and subcellular views by illuminating the tissue using a low-power laser and then detecting the reflected fluorescent light after administration of intravenous fluorescein. The CLE probe can be introduced through a catheter into the bile duct at the time of ERC. The probe must be in contact with the tissue for adequate imaging. In an effort to standardize imaging findings, the Miami classification system, which included the color and size of visualized bands as well as presence of dark clumps or epithelial structures, was proposed.[36] Combining two or more criteria suggestive of malignancy provided a sensitivity, specificity, positive predictive value, and negative predictive value of 97, 33, 80, and 80%, respectively, compared with 48, 100, 100, and 41% for standard tissue acquisition.[36] Although the study authors reported moderate interobserver variability for most variables, subsequent studies demonstrated poor to fair interobserver agreement for individual criteria and poor to slight agreement for final interpretation of benign versus malignant lesions with CLE.[37,38] The low specificity associated with the Miami classification was felt to be due to false-positive cases from benign inflammatory conditions. Consequently, the revised Paris criteria were developed that incorporated a third classification for findings associated with inflammatory biliary strictures.[39] A prospective, international, multicenter study of 112 patients with indeterminate biliary strictures demonstrated that CLE had sensitivity, specificity, and accuracy of 89, 71, and 82%,

respectively, compared with 56, 100, and 72%, respectively, for standard tissue sampling.[40] Given the high level of expertise required to interpret CLE in real time along with concerns regarding standardizing training to improve interobserver agreement, it remains to be seen if these results can be replicated in other centers.

Future Considerations

Advanced endoscopic techniques are able to provide a more reliable visual assessment of the indeterminate biliary strictures; however, it remains to be seen if these technologies can aid in improved tissue acquisition for a diagnosis of pCCA. As current technologies applied in other areas of the digestive tract miniaturize, there may be an opportunity to better characterize biliary strictures with improved microscopic imaging platforms, computer-aided imaging analysis, and methods for assessing stiffness including EUS-based elastography or impedance planimetry.

Advanced Cytologic Techniques

Biliary Cytology

Biliary cytology is obtained by passing a wire brush across the biliary epithelium at the time of ERC or percutaneous cholangiography. Biliary biopsies can also be obtained and enhance the diagnostic yield.[41] Biliary cytology can be classified into five categories: nondiagnostic, normal, atypical, suspicious, or positive for adenocarcinoma.[42] Nondiagnostic cytology occurs when there is an insufficient amount of cellular material to review and should prompt resampling if there is a concern for CCA. Atypical cytology, which is often the product of biliary inflammation, is a common finding especially in PSC and by itself should not raise concern.[43] In contrast, suspicious cytology is an independent predictor for CCA, and even in PSC nearly one-third of patients without a mass and suspicious cytology were eventually diagnosed with CCA.[43,44] Cytology positive for adenocarcinoma is diagnostic of CCA.[9,45] While the chief strength of cytology is its high specificity, the primary limitation is its limited sensitivity (19–43%).[8,9] This is secondary to the desmoplastic, paucicellular nature of CCA which can be difficult to sample. Therefore, the absence of a positive cytology does not exclude malignancy.

Fluorescence In Situ Hybridization

FISH was developed as an objective test to identify aneuploidy, a marker of chromosomal instability. Approximately, 85% of biliary tract cancers display aneuploidy and, therefore, may be expected to be diagnosed by FISH. Because FISH samples a limited number of chromosomes, the term polysomy is used instead of aneuploidy. FISH polysomy in a de novo or sporadic perihilar stricture is virtually diagnostic of CCA (►Fig. 3). However, in PSC FISH may indicate dysplasia rather than invasive cancer. Hence, the role of FISH in PSC will be further discussed here.

The original UroVysion FISH assay utilizes three centromeric probes that target chromosomes 3,7, and 17 and a locus-specific probe to 9p21 from samples obtained by biliary brushings. FISH detects abnormal gains or losses of chromosomes and the results can be categorized as normal, trisomy (10 or more cells with 3 copies of chromosome 7 or

chromosome 3, and 2 or fewer copies of the other 3 probes), tetrasomy (10 or more cells show 4 copies of all probes), or polysomy (5 or more cells show gains of 2 or more of the 4 probes).[42]

Polysomy (in contrast to a normal FISH result, trisomy or tetrasomy) is strongly associated with a diagnosis of CCA.[43,44,46,47] A meta-analysis examined the performance of FISH testing among 690 PSC patients and the pooled sensitivity and specificity for polysomy and CCA was 51 and 93%, respectively.[12] A second-generation locus-specific FISH probe set (targets 1q21, 7p12, 8q24, and 9p21) was recently derived and validated.[8] Among those with PSC, the second-generation probe set had greater sensitivity than the UroVysion assay (65% vs. 44%) and a high specificity (~90%) for the detection of CCA.[8]

FISH results should be interpreted in the context of each individual patient. The presence of other risk factors heightens the probability of harboring biliary cancer when polys-omy is detected. These additive risk factors include the presence of a “dominant stricture,” elevated serum carbohydrate antigen 19–9 (CA 19–9), presence of suspicious cytology, multifocal, or serial polysomy (►Table 1).[43,44,46–48] The largest study (n = 371) that examined the natural history of FISH in PSC found that polysomy detected in multiple areas of the biliary tree (i.e., multifocal polysomy) was the strongest predictor of CCA. This study also noted that 71% of patients with CCA had polysomy detected at another region of the biliary tree where adenocarcinoma was not detected by routine cytology, which suggests that it would be helpful to sample multiple locations of the biliary tree.[43]

While the application of FISH to biliary brushings is a step forward, the test is hampered by limited sensitivity and suboptimal specificity (when compared with cytology) as not all patients with polysomy will develop CCA as they likely have nonprogressive low-grade dysplasia. Consequently, a new generation of biomarkers is needed to enhance clinicians’ abilities to distinguish benign from malignant strictures in PSC.

Application of Existing Technologies for Early CCA Detection and Screening

Many experts in the field of PSC routinely screen their patients for CCA.[20] There is some evidence to suggest that screening for CCA in PSC is associated with improved survival and detection at an earlier stage.[49] The likelihood of CCA among asymptomatic pediatric patients or those with small duct PSC is low and they are not screened routinely.[50,51] We have previously published our approach to CCA screening and early detection in PSC.[52] If there is a concern for underlying CCA after imaging, an ERC with biliary brushings for routine cytology/FISH studies and intraductal biopsies are recommended. Suspicious cytology is an independent predictor of CCA and needs follow-up.[43,44,53] Indeed, an unpublished subgroup analysis from the aforementioned multifocal polysomy paper revealed that 14% (3/22) of patients with suspicious cytology alone (i.e., lack polysomy or a mass lesion) may have CCA diagnosed after a median follow-up of 1.5 years.[43] The presence of polysomy should be confirmed on subsequent testing provided definitive evidence of CCA is absent. It may take more than 1 year for definitive evidence of CCA to manifest after polysomy is detected even among patients in an intensive surveillance program.[8,43,44,47] Despite a lack of supporting or refuting evidence, we believe that this is a pragmatic

approach with the potential to increase early detection and deepen the pool of subjects eligible for curative therapy.[42,52]

Evolving Techniques Including NGS, Extracellular Vesicles, and Proteomics

NGS

As conventional biliary cytology has limited sensitivity for the detection of pancreaticobiliary malignancy, adjunctive molecular testing such as FISH is necessary to improve on the sensitivity of biliary cytology. NGS is another adjunctive approach (►Fig. 1) which has substantially improved the sensitivity of massive parallel sequencing by probing large panels of genes and identifying relatively rare mutations present in a small fraction of DNA templates.[54] In an effort to compare the performance characteristics of NGS with FISH as adjunctive tests for the detection of pancreaticobiliary malignancy, bile duct (n = 73) and main pancreatic duct (n = 8) specimens from 74 patients who underwent endoscopic retrograde cholangiopancreatography (ERCP) were subjected to conventional cytology, FISH using the UroVysion probe set, and targeted NGS.[55] On the basis of clinicopathologic follow-up, 33 specimens (41%) were high-risk neoplasia/malignant strictures and 48 specimens (59%) were benign. NGS combined with cytology had a sensitivity of 85% (compared with 67% for cytology alone), whereas FISH combined with cytology had a sensitivity of 76% for the detection of high-risk neoplasia or malignancy.[55] NGS also revealed driver mutations in 24 cases (30%). These findings indicate that NGS has the potential to be a viable adjunctive test for the detection of pancreaticobiliary malignancy. Moreover, NGS may have reduced cost and complexity compared with FISH as brushing samples subjected to NGS can be batched together with solid tumor specimens.

EVs

EVs are membrane-bound, heterogeneously sized vesicles released by diverse cell types which play an essential role in cell-to-cell communication.[56] EVs are released under both physiologic and pathologic conditions and carry cargo including proteins, lipids, and nucleic acids. Human biliary EVs also contain micro-ribonucleic acids (RNAs) (miRs) and long noncoding RNAs (lncRNAs).[57,58] Li et al identified a miR-based panel in biliary EVs with a sensitivity of 67% and specificity of 96%.[57] The same investigators identified EV-mediated trafficking of miR-195, downregulated in both cancer and stromal cells, between cancer cells and stromal cells.[59] EVs containing lncRNAs have been implicated in tumor progression.[58] Exosome sequencing analysis uncovered two lncRNAs with significantly increased expression in bile specimens of CCA patients compared with controls.[58] The combination of these two lncRNAs had an area under the curve (AUC) of 0.709 in CCA detection.[58] Tumor-associated microparticles (taMPs) are large EVs which may have a role in CCA detection.[60] Annexin V + EpCAM + CD147+ taMPs enabled distinction between hepatic malignancy and cirrhosis in patients without malignancy.[60] However, it is unclear if Annexin V + EpCAM + CD147+ taMPs can distinguish between hepatocellular carcinoma and CCA as both malignancies were grouped together in this study.[60] EVs produced by cancer cells may carry oncogenic factors which promote tumorigenesis.[61,62] A recent proteomic analysis demonstrated a higher abundance of oncogenic proteins in EVs obtained from human CCA cells compared with EVs obtained from normal human cholangiocytes.[63] In this analysis,

oncogenic proteins of particular interest included epidermal growth factor receptor, mucin-1, and integrin β-4 as these are upregulated in CCA and associated with CCA tumor growth and metastasis.[63] Similar oncogenic proteins were noted in serum EVs of mice which had undergone orthotopic implantation of human CCA cells.[63] Proteomic analysis using mass spectrometry demonstrated 95 differentially expressed proteins between healthy controls and CCA patients and 50 differentially expressed proteins between CCA patients versus PSC patients.[63] From a functional standpoint, these proteins were primarily related to wound healing response, inflammatory response, and immune activation.[63] Fibrinogen gamma chain, α-1-acid glycoprotein 1, and S100A8 were the three proteins with the best differential diagnosis capacity between CCA and PSC patients with AUC values of 0.796, 0.794, and 0.759, respectively.[63] The median concentration of EVs is also higher in bile specimens of patients with malignant biliary strictures compared with controls or nonmalignant biliary strictures (2.4×10^{15} vs. 1.6×10^{14} nanoparticles/L in the discovery cohort; $p < 0$ 0.0001, and 4.0×10^{15} vs. 1.3×10^{14} nanoparticles/L in the verification cohort; $p < 0$ 0.0001).[64] Moreover, a bile EV threshold of 9.46×10^{14} was able to distinguish malignant common bile duct stenoses from nonmalignant common bile duct stenoses with high diagnostic accuracy.[64]

Proteomics

Other advanced technologies with the potential to enhance early CCA diagnosis include proteomic analysis by mass spectrometry or gel electrophoresis. Such analyses can detect novel biomarkers such as peptide panels in biological specimens including bile, serum, urine, and stool. For instance, a bile proteomic analysis by capillary electrophoresis mass spectrometry identified a 22-peptide panel in a training cohort of PSC ($n = 18$) and CCA ($n = 16$) patients.[65] In a subsequent validation cohort, this peptide panel accurately detected 14 of 18 bile specimens from PSC patients and 21 of 25 specimens from CCA patients (78% specificity and 84% sensitivity).[65] Similarly, a urine proteomic analysis using mass spectrometry identified a 42-peptide panel which differentiated CCA from PSC and benign biliary disorders with 83% sensitivity and 79% specificity.[66] These data suggest a possible role for proteomic analysis in the detection of CCA in PSC patients, particularly when combined with noninvasive imaging studies such as MRI. However, prospective multicenter studies are necessary to validate these findings and to determine whether such peptide panels have utility in diagnosing CCA at an early stage.

Evolving Techniques including Liquid Biopsy and CCA

With recent advances in molecular technologies, genomics and epigenomic biomarkers display wide utility for early detection of various types of cancers.[67] CTCs and cfDNA (►Fig. 1) are novel biomarkers which may lead to improvements in patient outcomes by facilitating early detection of cancer.[68] This new technology can reveal cancer-specific genetic and epigenetic changes directly from CTC and cfDNA in the blood stream.[69] Circulating tumor biomarkers can overcome the sampling issues associated with cancer tissue biopsy as they reveal the whole spectrum of DNA mutations in cells released from heterogeneous cancer tissues, whereas tissue biopsy only reveals a snapshot of complex biology of tumors with extensive intra- and interlesional diversity.[70,71] Genomic and

epigenomic analyses from the peripheral blood will therefore help to develop a noninvasive accurate diagnostic approach in the era of individualized medicine. While CTC are typically detected in patients with advanced stage cancer, a role for CTC in screening and early detection of cancer was reported in other cancers such as lung, breast, and pancreatic cancer.[72-74] Detection of CTC was associated with a 10-fold increased risk of having shorter survival among patients with pCCA/dCCA.[75] Diagnostic role of CTC in early detection of CCA remains to be elucidated and a prospective study is ongoing to address this question.

An accumulating body of literature confirms an excellent correlation between DNA mutations found in cancer tissues and mutations detected in cfDNA from the same patients. In one study of 17 patients with pancreatobiliary malignancy reported by Zill et al, 90.3% of mutations detected in tumor biopsies were also detected in cfDNA.[76] While excellent concordances in tissue and cfDNA mutations were reported, all patients had advanced stage disease. Hence, the sensitivity of cfDNA mutations for early stage tumor detection is currently unknown. The sensitivity of detection of methylated DNA changes in cfDNA has improved; consequently, differentially methylated regions (DMRs) in cfDNA are often measurable in patients without detectable CTC and have the potential to be an excellent diagnostic biomarker for CCA. Andresen et al reported a four-gene DNA methylation biomarker panel from biliary brush samples, which achieved a sensitivity of 85% and a specificity of 98% for CCA detection, with an AUC of 0.944.[77] A similar pilot study using cfDNA methylation as a diagnostic biomarker identified the top nine DNA methylation makers using tissue methylome analysis.[78] These methylation markers were tested on cfDNA obtained from 2 mL of plasma of CCA patients ($N = 69$: 48 de novo, 21 PSC-associated) and age-sex matched healthy controls ($N = 95$).[78] The recursive partitioning decision tree method identified a 4-DMR panel, which classified CCA with a sensitivity of 83% and a specificity of 93% with AUC of 0.90.[78] Model calls were not significantly influenced by comorbid PSC or tumor stage. A prospective study is ongoing to confirm the diagnostic performance of cfDNA methylation marker in a larger number of samples.

Conclusion and Future Directions

To date, conventional cytology via ERC-guided acquisition approaches is most widely practiced for the diagnosis of malignant strictures. However, it is imperative that this approach be supplanted by improved techniques. Too often, decision making is deferred because the cytology is non-diagnostic. The diagnosis of pCCA will always require a cellular and/or genetic indicator of malignancy along with imaging evidence compatible with a cancer. Therefore, surrogate endpoints such as EVs without genetic cargo analysis, proteomics, and/or imaging technologies in isolation are unlikely to be diagnostic. It is also unlikely that further advances in cytologic techniques (e.g., FISH, machine learning employing conventional cytologic specimens, etc.) will be sufficiently more diagnostic than current assessments. This relates, in part, to the paucicellular nature of the specimens, and the highly desmoplastic nature of these cancers. Furthermore, sample acquisition following placement of biliary stents also confounds these diagnostic approaches. Likely, easy-to-employ biliary imaging techniques along with cfDNA technology from the bile and/or blood will improve our ability to diagnose this enigmatic cancer. We encourage clinical

investigators in partnership with diagnostic pathologists to develop protocols along these lines to address this unmet clinical need.

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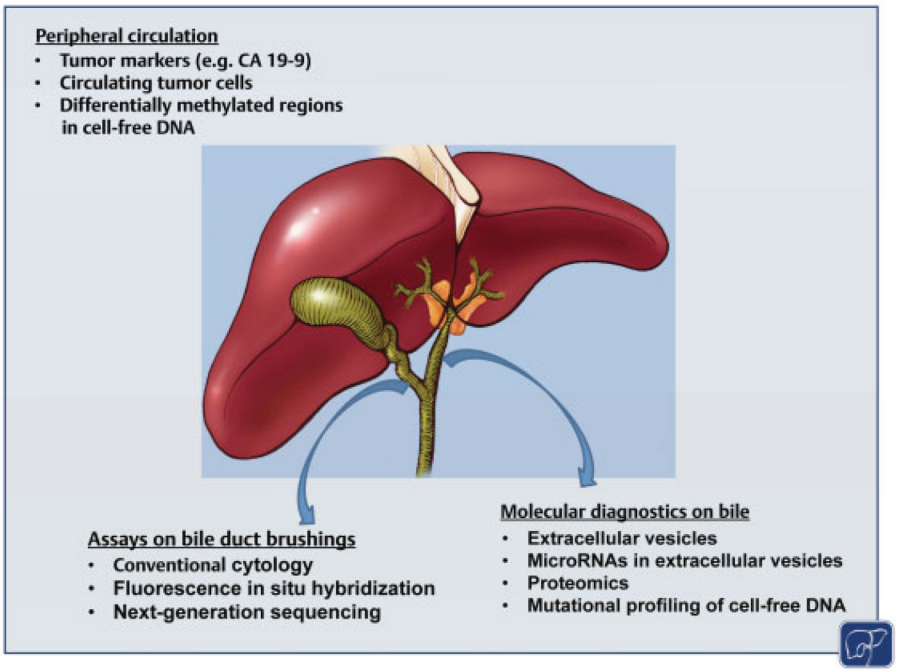

Fig. 1.

Diagnostic modalities in perihilar cholangiocarcinoma (pCCA). Conventional and emerging diagnostic techniques performed on serum, bile duct brushings, and bile specimens for the detection of pCCA.

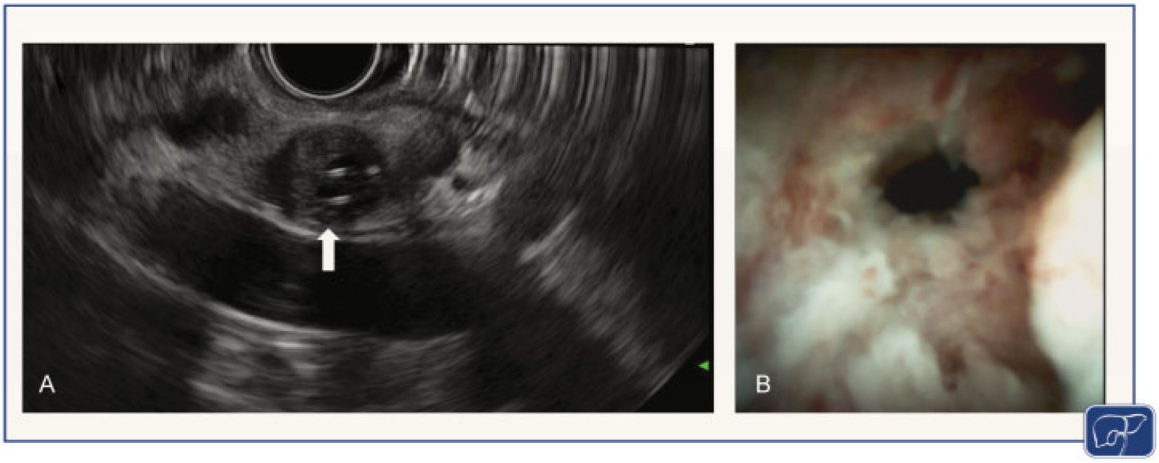

Fig. 2.

Advanced endoscopic techniques for the diagnosis of perihilar cholangiocarcinoma (pCCA). (**A**) Digital cholangioscopy demonstrating an infiltrative stricture with dilated vessels in a patient without a mass seen on cross-sectional imaging. Cholangioscopy-targeted biopsies confirmed the presence of adenocarcinoma. (**B**) Endoscopic ultrasound (EUS) image demonstrating a 1-cm mass (white arrow in **A**) of the common hepatic duct, corresponding with a perihilar cholangiocarcinoma with a plastic stent traversing within the bile duct.

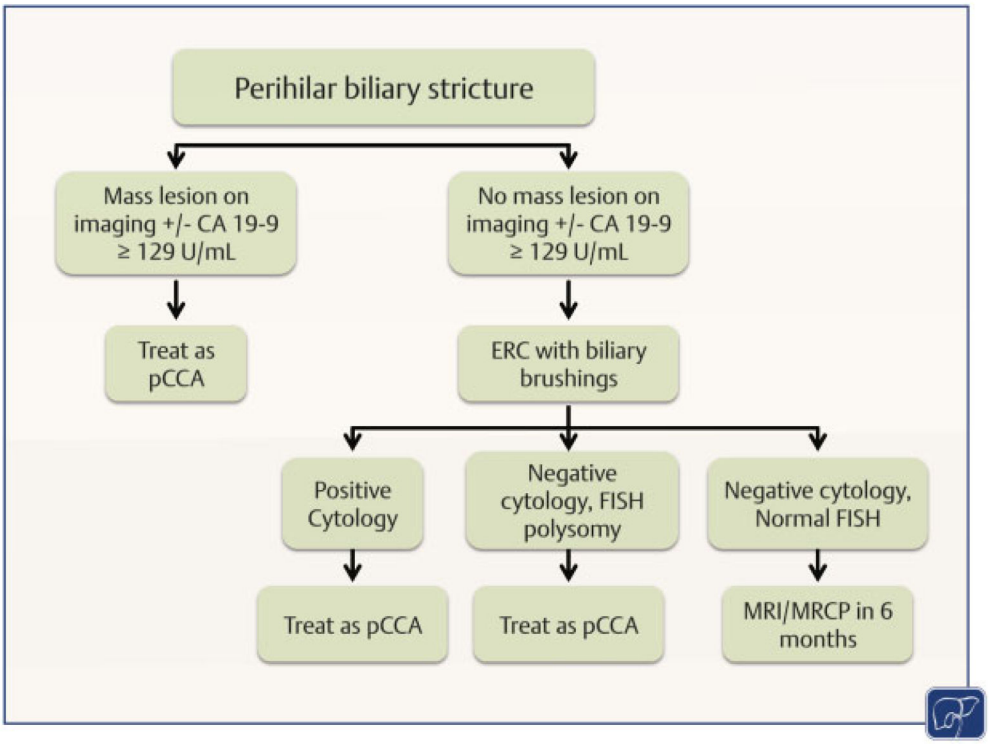

Fig. 3.

Approach to a sporadic (de novo) perihilar biliary stricture. Management algorithm for patients with a sporadic or de novo perihilar biliary stricture.

Table 1

Additive cholangiocarcinoma risk factors when polysomy is present in PSC

Risk factor	Follow-up (y)	Proportion with CCA
Dominant stricture[46]	1	73% (19/26)
No dominant stricture	1	43% (9/21)
Suspicious cytology[43]	1	61% (11/18)
Normal or atypical cytology	1	23% (8/35)
CA 19-9 ≥ 129 U/mL[44]	2.7	100% (10/10)
CA 19-9 < 129 U/mL	2	67% (10/15)
Serial polysomy[a,47]	2	69% (9/13)
Nonserial polysomy	2	18% (3/17)
Multifocal polysomy[43]	1	75% (23/31)[b]
Unifocal polysomy	1	19% (6/32)

Abbreviations: CA 19-9, carbohydrate antigen 19-9; CCA, cholangiocarcinoma; PSC, primary sclerosing cholangitis.

[a]80% (four-fifths) patients with serial polysomy + suspicious cytology were diagnosed with CCA.

[b]All but one surviving patients with continued follow-up ultimately developed CCA 2 years after this study was published (unpublished data).