Chapter 8
Pathological Diagnosis of Cholangiocarcinoma

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Abbreviations

AFP	Alpha-fetoprotein
BilIN	Biliary intraepithelial neoplasia
CEA	Carcinoembryonic antigen
CT	Computed tomography
DIA	Digital image analysis
ERCP	Endoscopic retrograde cholangiopancreatography
EUS	Endoscopic ultrasound
FISH	Fluorescent *in situ* hybridization
FNA	Fine needle aspiration
HCC	Hepatocellular carcinoma
IMP3	Insulin-like growth factor-I mRNA binding protein-3
IPNB	Intraductal papillary neoplasms of bile ducts
KRAS	Kristen rat sarcoma
MCN	Mucinous cystic neoplasm
NGS	Next-generation sequencing
PSC	Primary sclerosing cholangitis
pVHL	von Hippel-Lindau protein
TFF1	Trefoil factor 1

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Cholangiocarcinoma Diagnosis

Definitive histologic classification and staging of cholangiocarcinoma can be achieved by evaluation of surgically resected material. Given the advanced presentation of many patients with cholangiocarcinoma, radiologic guidance is commonly used to obtain small tissue samples for histologic or cytologic evaluation so that pathologists can reach a definitive diagnosis to guide therapy. There are many challenges to cholangiocarcinoma diagnosis on small biopsies. Noninvasive biopsy techniques require significant operator skill, and tumor cell yields can be low due to due to infiltrative tumor growth patterns, necrosis, and a relatively low tumor concentration compared to tumor-associated stroma. Furthermore, markedly reactive changes due to bile duct strictures or stenting can be difficult to distinguish from cancer. Nonetheless, the diagnosis can be established using morphologic criteria and, when indicated, immunohistochemistry, ancillary cytogenetics, and molecular-based techniques.

Tissue Acquisition Techniques

The tissue acquisition technique for diagnosis of cholangiocarcinoma (CCA) depends on the site of disease and the clinical features of individual patients. Tissue acquisition for intrahepatic CCA is typically obtained by percutaneous approach with radiologic guidance by computed tomography or ultrasound to obtain cores of tissue (FNB) and/or fine needle aspirate (FNA). Rapid onsite adequacy can be used to improve diagnostic yield.

Extrahepatic CCA can be sampled using several methods [1]. Intraductal forceps biopsy or fine needle biopsy can obtained by endoscopic retrograde cholangiopancreatography (ERCP) utilizing standard or mini-forceps with fluoroscopic guidance and/or specialized forceps under cholangioscopic; when retrograde access is not feasible due to anatomical or other factors, the same may be performed by percutaneous transhepatic cholangiography (PTC). The sensitivity and specificity of biopsy is 62–78% and 100%, respectively [2–4]. Cells in biliary fluid can be obtained for cytologic examination by direct aspiration during ERCP or via percutaneous drainage, and cytology techniques generally have near 100% specificity. Biliary fluid cytology has a low sensitivity (6–32%) for detecting malignancy and is commonly performed in conjunction with cytologic evaluation of bile duct brushings, which have a higher pooled sensitivity of 45% [1, 5]. Bile duct brushing obtained by ERCP or PTC involves scraping cells from the superficial biliary mucosa at the level of the bile duct lesion. The brush, charged with cellular material, is carefully smeared directly on a glass slide. The slide is reserved for air drying or fixed by very rapidly placing it in an alcohol-based fixative; any delay between the smearing and fixation creates artifactual distortion that hinders diagnosis. Alternatively, the cells can be dislodged from the brush using agitation into a container with a fixative appropriate

for liquid-based cytology preparation [6]. Liquid-based media is a flexible collection technique because the cells can be applied to slide using various proprietary techniques such as CytoSpin™ (Thermo Fisher Scientific, Waltham, MA), ThinPrep® (Hologic, Inc., Marlborough, MA), or BD SurePath™ prep (Becton, Dickinson and Company, Franklin Lakes, NJ). Also, tissue fragments can be centrifuged into a cell pellet and fixed with formalin into a cell block. Cytology diagnosis, DNA-based testing, fluorescence *in situ* hybridization (FISH), and immunocytochemistry, as indicated, can be performed on material placed in liquid-based fixative. As an alternative or compliment to ERCP, bile duct masses may also be sampled using endoscopic ultrasound (EUS)-guided FNA/FNB. Like bile duct brushings, FNAs can be prepared as direct smears, liquid-based preparations, and cell blocks. FNA has a high sensitivity and specificity for extrahepatic CCA (82% and 87.5%) [7, 8]. Because of the transduodenal approach of EUS, distal bile duct lesions are technically easier and safer to access compared to peri-hilar lesions, although overall complication rates are low in experienced hands [7].

Precursor Neoplastic Lesions

Three main precursor lesions exist: biliary intraepithelial neoplasia (BilIN), intraductal papillary neoplasm of the bile ducts (IPNB), and mucinous cystic neoplasm (MCN) These are each discussed in the forthcoming subsections (see also Chap. 3, Nakanuma et al., for complementary information).

Biliary Intraepithelial Neoplasia

Non-mass-forming dysplasia of the bile duct epithelium, termed "biliary intraepithelial neoplasia," is an incidental microscopic finding and putative precursor of CCA. The atypical epithelium is flat or micropapillary and confined to the lumen. There are two tiers in grade (low and high), and lesions are graded based on the highest degree of atypia [9]. Diagnosis of low grade reflects pseudostratification of nuclei, increased nuclear-cytoplasmic ratio, and nuclear hyperchromasia. High-grade BilIN lesions have increasing architectural complexity such as micropapillae, loss of cellular polarity, and marked nuclear atypia.

Due to the non-mass-forming nature of BilIN, it is rarely discovered prior to the development of carcinoma, and thus little is known about its natural history. However, patients with primary sclerosing cholangitis (PSC) are at markedly increased risk of CCA, with lifetime risk approaching 10% [10]. Retrospective studies in patients with PSC have shown strong associations between the presence of intestinal metaplasia, low- and high-grade BilIN, and CCA [11–13]. The inflammation-metaplasia-dysplasia-carcinoma model of progression in PSC is supported by the finding of increasing cytogenetic abnormalities as lesions progress

[14]. This model of progression in PSC is similar to that of inflammatory bowel disease, and there is also evidence it may be applicable to other clinical contexts, such as liver fluke-associated CCA [15].

Intraductal Papillary Neoplasm of the Bile Ducts

Single or multifocal grossly exophytic proliferations of neoplastic biliary epithelium within the bile ducts are termed intraductal papillary neoplasms of bile ducts (Fig. 8.1a). These premalignant neoplasms are seen in association with an invasive carcinoma in 74% of resected cases [16]. In East Asian populations, there is evidence of association between IPNB and hepatolithiasis, but many IPNB also arise in the absence of a predisposing condition [17, 18]. The histology comprises villous or finger-like branching fibrovascular cores lined by dysplastic cuboidal to columnar epithelium of biliary, intestinal, oncocytic, or gastric differentiation [19] (Fig. 8.1b). The mucin expression profiles are similar to those of their pancreatic counterparts; the pancreatobiliary type expresses MUC1, the intestinal type expresses MUC2, and while gastric and oncocytic types express MUC5AC and MUC6 [16].

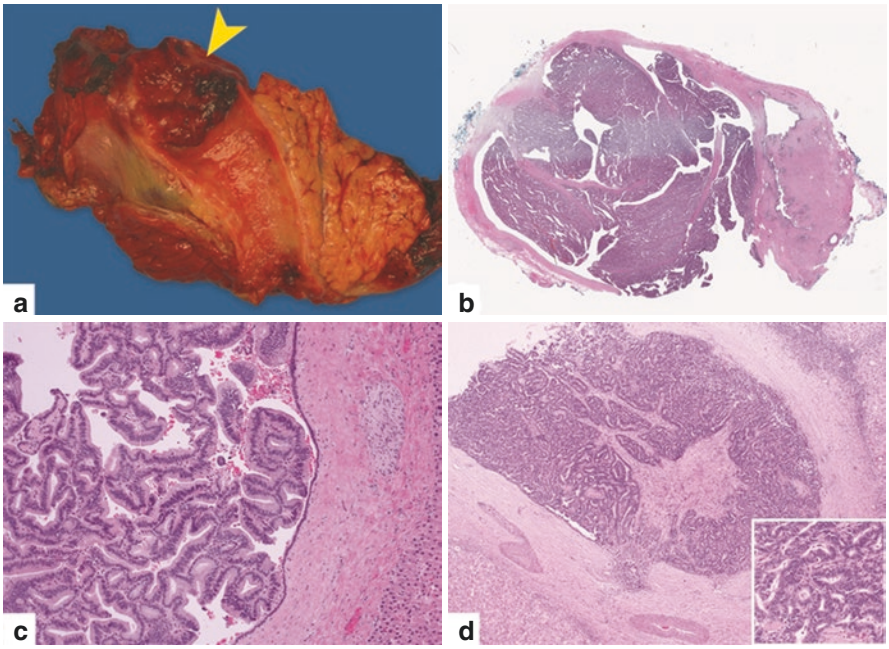

Fig. 8.1 (**a–d**) Grossly, an intraductal papillary neoplasm of the bile ducts (IPNB) is an exophytic and papillary lesion within the lumen of the bile duct (**a**). IPNB of the common bile duct fills and expands the duct lumen on low power histology (**b**). Branching and tubular architecture is typical of low-grade IPNB (**c**), while marked cytologic atypia and complex architecture are present in IPNB with high-grade dysplasia (**d**)

IPNB are graded in two tiers: low- and high-grade based on the highest level of cytological atypia and cellular organization in a given lesion (Fig. 8.1c, d). Recently, dividing IPNB into two types has been proposed due to clinical, pathologic, and genetic differences [20, 21]. Type 1 is similar to pancreatic intraductal papillary mucinous neoplasm and mainly in the intrahepatic bile duct, whereas type 2 is more architecturally complex with solid and tubular components, is more often associated with invasive adenocarcinoma at resection, and mainly involves the extrahepatic bile ducts [20].

A rarer and morphologically distinct mass-forming neoplasm exists that lacks the mucinous characteristics of IPNB. These lesions typically show predominantly compact tubular-glandular architecture with minimal papillae and are usually associated with high-grade dysplasia and invasive carcinoma (up to 80%) [22]. These lesions are designated "intraductal tubulopapillary neoplasm of the bile duct" and are also morphologically similar to their pancreatic analog [22].

Mucinous Cystic Neoplasm of the Liver and Biliary System

CCAs may arise, albeit rarely (approximately 6%), in association with mucinous cystic neoplasm of the liver and biliary system, placing it in the category of precursor neoplastic lesion [23]. MCN is a cystic neoplasm arising without clear communication with the bile duct. These neoplasms are well-demarcated grossly and contain fluid. The defining histologic feature is the combination of cystic glands and ovarian-type stroma. The neoplastic glands are lined by epithelial cells that are columnar (often mucinous), cuboidal (non-mucinous), or attenuated [23]. Invasive cholangiocarcinoma may be present in radiologically/grossly solid components of MCNs. Although typically flat, some neoplasms have papillary projections. The ovarian-type stroma must be identified for diagnosis but may only be focal and is highlighted by immunohistochemical stains for ER or PR. Rarely, MCN has high-grade dysplasia; the lining is typically low-grade.

Peri-hilar and Distal Extrahepatic Bile Duct Adenocarcinomas

Gross Evaluation

Peri-hilar CCA arises from the common hepatic duct, whereas distal CCA arises from the common bile duct. Resection specimens are evaluated by gross assessment of tumor size, appearance, location, relationship to adjacent structures, distance to margins, and the presence of lymph nodes. Most tumors have a firm white or tan appearance with poorly defined infiltrative margins. For peri-hilar tumors, the

macroscopic involvement of the common hepatic duct and its branches is important to document. Extension of these neoplasms along bile ducts leading to strictures is common. Peri-hilar resections usually include partial hepatectomy, and thus extension into liver parenchyma, branches of the portal vein, hepatic artery, or second-order biliary radicals can occasionally be seen grossly, and the documentation of tumor involvement is an element of tumor staging. The resection margins, proximal/distal bile ducts and soft tissue margins, are examined for the distance to tumor, with samples taken for microscopy.

For distal extrahepatic CCA, the resection is often a Whipple specimen. Likewise, the tumor is described in relation to the adjacent structures such as the pancreas, duodenum, and ampulla. The depth of invasion from the bile duct wall is key for pathologic T staging of distal CCA, which is assessed by gross measurement and confirmed with microscopy of the tumor at its widest invasive span. For distal CCA, the most important margin is often the proximal bile duct margin, but all other margins (uncinate, pancreatic neck, luminal gastrointestinal) are sampled, typically in a shave section, for microscopy.

Histology

The majority of extrahepatic CCAs have a histologic appearance similar to conventional pancreatic ductal adenocarcinoma. The infiltrating and irregularly angulated glands may appear scattered among residual biliary structures or occur within an obliterative desmoplastic stroma (Fig. 8.2). The cells are usually columnar and often contain intracellular mucin. Among the varied histologic subtypes described are intestinal, foveolar, mucinous, signet ring cell, clear cell, hepatoid, and micropapillary [24]. Rare CCA subtypes with a distinctive appearance include adenosquamous, sarcomatoid, and undifferentiated carcinomas. Lymphovascular invasion is common, which is reflected in the high proportion of resections with positive lymph nodes (39% to 76%) [25–27]. Perineural invasion is also common and, coupled with the tumors' tendency to extend along the existing ducts, results in high rates of positive resection margins (13–37%) [28–31]. Frozen section analysis with further resection on intraoperatively positive margins can result in improved survival [28, 29].

Differential Diagnosis of Extrahepatic Cholangiocarcinoma and Distal Bile Duct Carcinoma

The differential diagnosis of extrahepatic CCAs includes reactive peri-ductal glands in the setting of inflammation, metastatic lesions, and direct extension from primary pancreatic, ampullary, or duodenal tumors. Malignant glands are

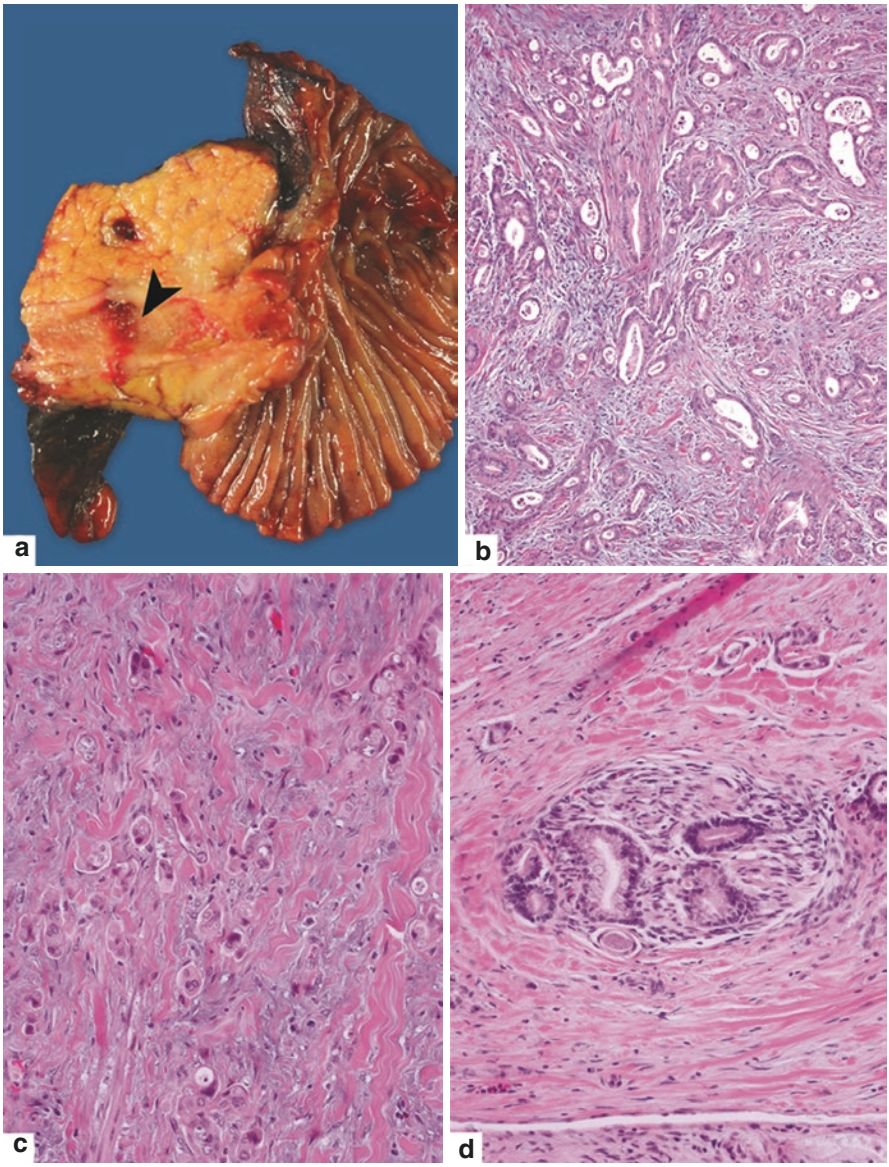

Fig. 8.2 Irregularly infiltrating glands and intra-tumoral desmoplastic stroma typify well-differentiated distal (**a**) and peri-hilar (**b**) CCAs. Poorly differentiated CCAs of the distal (**c**) and peri-hilar (**d**) bile ducts have poorly formed glands and single cell infiltration

distinguished from reactive glands by the irregular infiltration and degree of cytomorphologic atypia. This can be difficult in the setting of severe inflammation. Immunohistochemistry for p53 (abnormal overexpression or loss of expression) and/or SMAD4 (loss of expression) may be of value in distinguishing between

reactive versus neoplastic, but these stains are aberrant in only approximately half of extrahepatic CCAs [32, 33]. Therefore, non-aberrant staining does not exclude neoplasia. Another nonneoplastic mimicker is IgG4-related cholangitis, which can appear similar on cholangiography to PSC or cholangiocarcinoma [34]. Serum IgG4 is a useful ancillary test, but its sensitivity and specificity vary depending on the thresholds used [35]. Biopsies of IgG4 cholangitis may show lymphoplasmacytic inflammation and fibrosis, with significantly increased IgG4 plasma cells by immunohistochemistry [36].

With respect to neoplastic differential diagnoses, the extrahepatic bile ducts are uncommon locations for distant metastasis, but attention to the history of other prior malignancies is still important, particularly if the histomorphology is unusual. Far more commonly, the extrahepatic bile ducts may be involved by direct extension of adenocarcinoma from an adjacent organ. Extension of primary pancreatic ductal adenocarcinoma or ampullary adenocarcinoma into the bile duct may be morphologically and immunohistochemically indistinguishable from extrahepatic CCA. Therefore, the distinction is usually made based on gross and microscopic assessment of where the epicenter and/or bulk of the tumor is anatomically located.

Intrahepatic Cholangiocarcinoma

Gross Evaluation

Intrahepatic CCA is an adenocarcinoma arising from the second-order bile ducts and smaller branches. Resections for intrahepatic CCAs are typically partial hepatectomies. The macroscopic configuration can be mass forming (Fig. 8.3a), periductal infiltrating (Fig. 8.3b), or mixed. The gross appearance is firm, white, and fibrous. Gross assessment of tumor size, presence of multifocality, vascular involvement, capsular involvement, and extrahepatic extension are all important factors for pathologic T staging. There is a hepatic parenchymal margin, but distal biliary branches at the margin are important to evaluate due to the propensity for periductal tumoral extension.

Histology

There are two major histologic subtypes of intrahepatic CCA: small duct and large duct. Other rare subtypes include adenosquamous carcinoma, mucinous carcinoma, signet ring cell carcinoma, mucoepidermoid carcinoma, lymphoepithelioma-like carcinoma, and sarcomatous carcinoma [37].

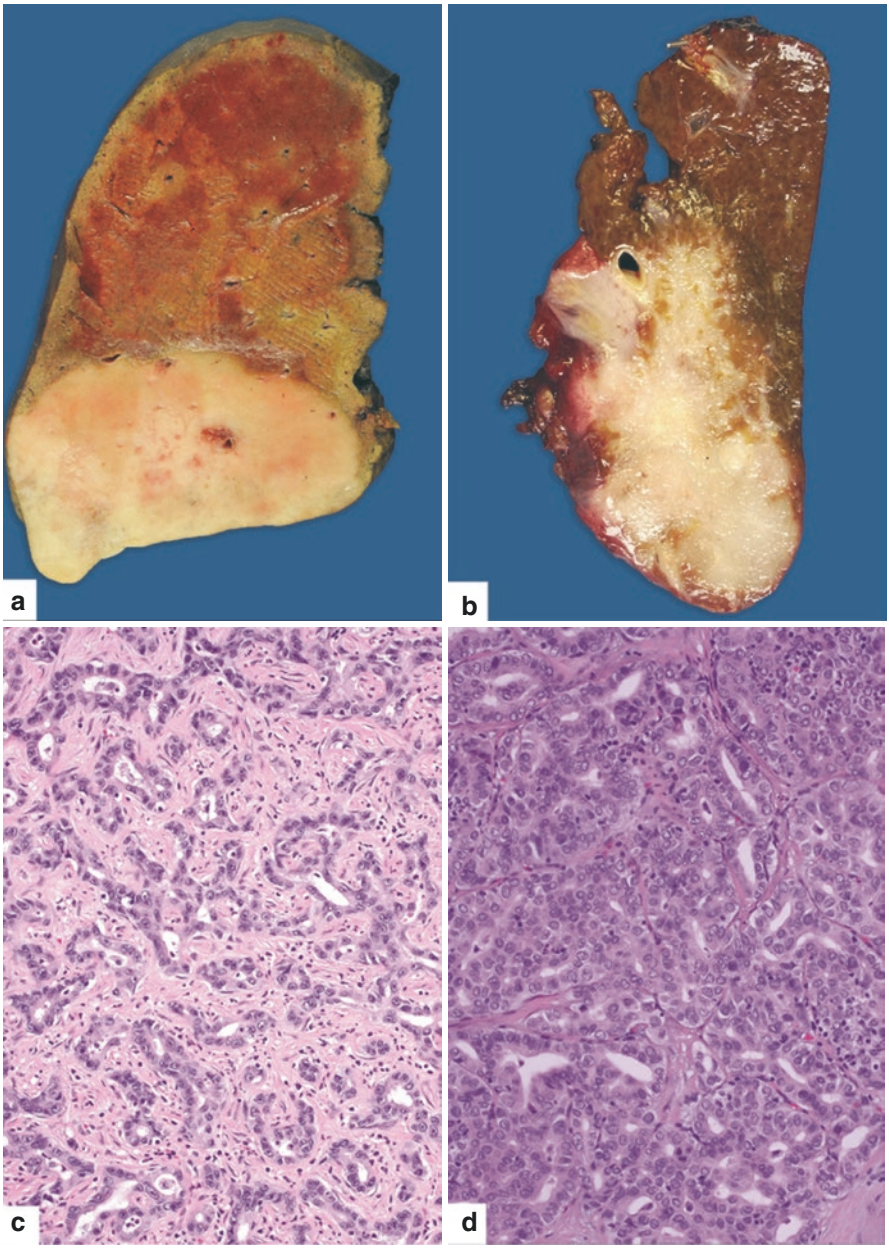

Fig. 8.3 (**a**–**f**) Mass-forming intrahepatic cholangiocarcinoma is well-circumscribed, firm, and fibrous in texture (**a**). A poorly circumscribed gross margin reflects peri-ductal infiltration of intrahepatic cholangiocarcinoma (**b**). Well-differentiated small duct type intrahepatic cholangiocarcinoma has distinct tubular or anastomosing glands, such as this cholangiolar pattern (**c**). Marked glandular complexity and sheets of cells are seen in moderately differentiated (**d**) and poorly differentiated (**e**) small duct type cholangiocarcinomas. The ductal malformation subtype of cholangiocarcinoma (**f**)

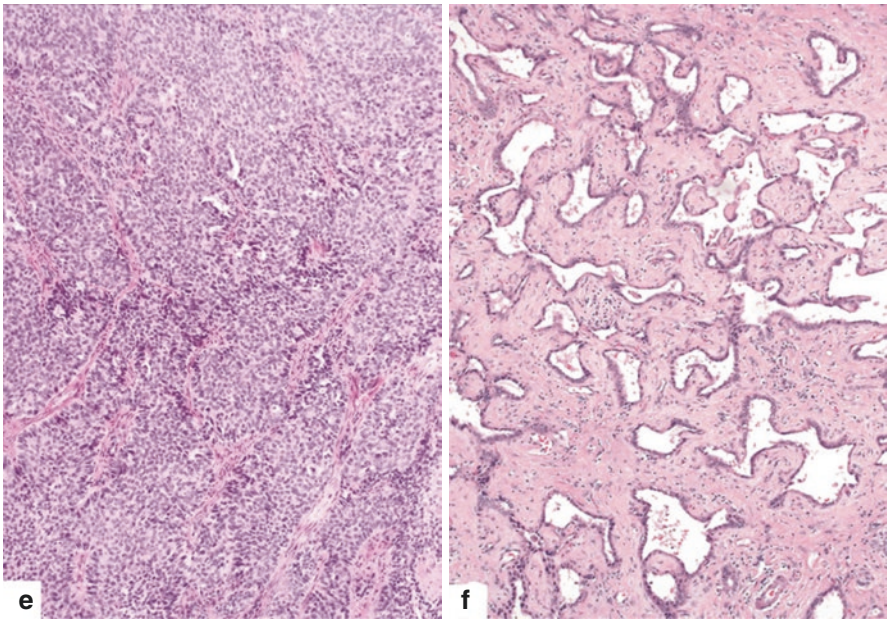

Fig. 8.3 (continued)

Intrahepatic Cholangiocarcinoma: Small Duct Subtype

Small duct subtype has also been called "peripheral," "cholangiolar," and "bile ductular," since they are more likely to present away from the liver hilum and resemble to reactive biliary proliferations. The prevalence of this phenotype is regionally dependent, comprising approximately 40–90% of intrahepatic CCAs [38, 39]. The predominantly tubuloglandular architecture shows remarkable inter-tumoral and intra-tumoral heterogeneity [38]. The patterns of the infiltrating glands include simple tubules, anastomosing tubules, confluent tubules with slit-like lumens, and dilated and solid sheets of cells (Fig. 8.3c–e). Micropapillary arrangements can be seen. The cells are cuboidal, polygonal, or low columnar with cytoplasm that can range from pale and amphophilic to plump and eosinophilic. The neoplastic cells may appear hepatoid but they do not express hepatocellular markers. Small collections of luminal mucin and intracellular mucin can be present in a minority of cases [38]. Many tumors have densely hyalinized intra-tumoral stroma. The tumor cells infiltrate and entrap hepatocytes at the tumor-liver interface. Some small duct type intrahepatic CCAs have architecture resembling ductal plate malformation or biliary adenofibroma (Fig. 8.3f) [40]. Very well-differentiated tumors with a uniformly anastomosing tubular pattern resembling the ductular reaction have been referred to as cholangiolocellular carcinoma, but they lack a unique genotype and may not be a distinct entity (Fig. 8.3c) [41].

Intrahepatic Cholangiocarcinoma: Large Duct Subtype

Large duct subtype intrahepatic CCAs have had prior descriptive labels including "hilar type," "peri-hilar type," and "bile duct type," which reflect the resemblance of this subtype to extrahepatic and peri-hilar CCAs. The histology consists of irregularly infiltrating glands with large-caliber lumens frequently containing mucin (Fig. 8.4a). Cells lining the glands are cuboidal to columnar and often contain intracytoplasmic mucin. The intra-tumoral stroma is characteristically desmoplastic and abundant. Higher-grade carcinomas have increasing architectural complexity and loss of glandular differentiation (Fig. 8.4b). Smaller infiltrating glands resembling the small duct subtype can be seen in variable proportion, and, in some instances, there is infiltration of single cells with signet ring cell appearance. The large duct subtype of intrahepatic CCA frequently exhibits perineural invasion [38].

Differential Diagnosis of Intrahepatic Cholangiocarcinoma

The diagnosis of intrahepatic CCA requires distinction from reactive biliary glands and benign biliary proliferations. Similar to extrahepatic bile ducts, IgG4-related cholangitis can also involve the intrahepatic ducts. Other malignancies such as hepatocellular carcinoma and metastasis from the lung, breast, and upper gastrointestinal tracts and extrahepatic pancreaticobiliary system also enter the differential. The *Immunohistochemistry of Cholangiocarcinoma* section in this chapter provides information on the use of stains in resolving the site of tumor origin.

Small biopsies containing well-differentiated CCA may present a challenge in diagnosis. Carcinoma is distinguished from bile duct adenomas and reactive biliary proliferations based on larger nucleus size, atypical cytological

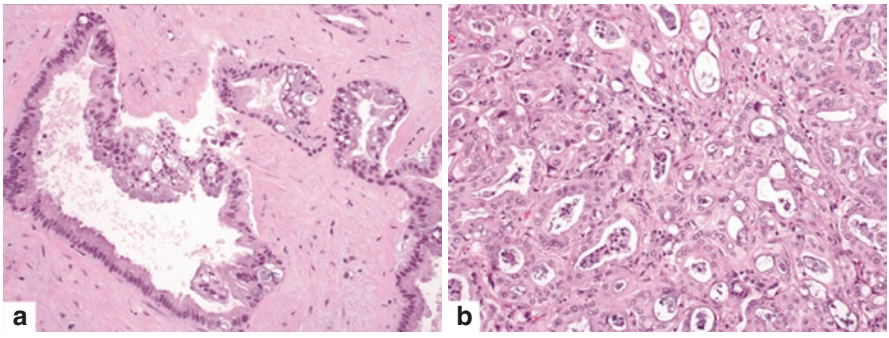

Fig. 8.4 (**a**, **b**) Large duct type intrahepatic cholangiocarcinoma resembles extrahepatic cholangiocarcinoma with widely spaced large-caliber infiltrating glands (**a**). Higher-grade tumors have a higher density of infiltrating glands with more complexity (**b**)

features, and irregular distribution of infiltrating glands. The Ki-67 proliferation index of adenomas is low compared to cholangiocarcinoma (average = 2% versus 23%) [42]. The immunohistochemical marker for p16 (*CDKN2A*) is expressed in most adenomas and bile ductular proliferations but less so in carcinoma [43].

Morphologic features are often sufficient for distinguishing CCA from hepatocellular carcinoma because CCA has tubuloglandular differentiation, mucin production, and intra-tumoral stroma. These features are absent in hepatocellular carcinoma (HCC), excepting the rare scirrhous or sclerosing variant of HCC [44, 45]. Poorly differentiated primary liver carcinomas require immunohistochemistry to exclude hepatocellular differentiation.

The histology of intrahepatic CCA overlaps with several extrahepatic adenocarcinomas. Fortunately, most well-differentiated intrahepatic CCAs have anastomosing glands and sclerotic stroma; this "cholangiolar pattern" of the small duct subtype has been shown to be specific for intrahepatic cholangiocarcinoma, particularly when combined with positive albumin RNA *in situ* hybridization [46]. Unfortunately, the large duct subtype of intrahepatic CCA resembles extrahepatic bile duct and pancreas adenocarcinomas both histologically and immunophenotypically. In the event of large tumors involving the liver hilum with a large duct phenotype, it can be impossible on a histologic basis to distinguish the large duct subtype of intrahepatic CCA from a peri-hilar CCA. Clinical and radiologic correlation plays a key role in these scenarios.

Combined Hepatocellular-Cholangiocarcinoma

Carcinomas containing areas with both hepatocellular and cholangiocytic differentiation are classified as combined hepatocellular-cholangiocarcinoma. Genomic studies have revealed that most cases of primary liver carcinoma with this bi-phenotypic morphology represent proliferations derived from the same clone [47, 48]. Tumors that show two distinct genomic profiles between the phenotypes may represent "collision tumors" which arose as separate primaries [48]. Collision tumors are currently excluded from the WHO classification of combined hepatocellular-cholangiocarcinoma, although there is still debate on this matter [37]. The two phenotypic components in combined hepatocellular-cholangiocarcinoma may be regionally distinct or intermixed. There is no defining proportion required for either component, but diagnosis is based on recognition of the two morphologies on routine hematoxylin and eosin (H&E)-stained slides (Fig. 8.5). Immunohistochemistry to demonstrate both hepatocellular (Arginase-1, HepPar1) and biliary (CK7, CK19) phenotype may be useful to support the diagnosis, but this technique is ancillary to the H&E morphology [37].

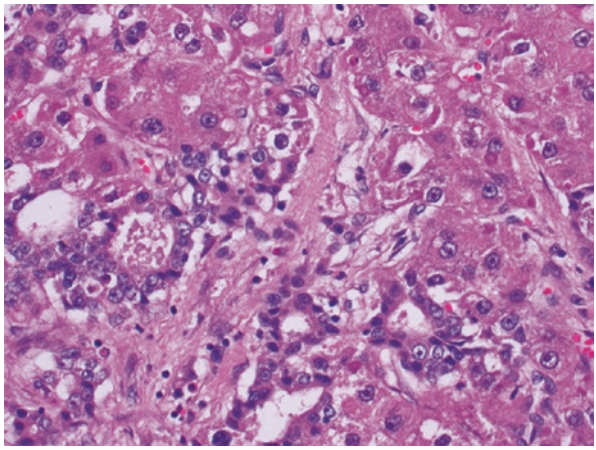

Fig. 8.5 Combined hepatocellular-cholangiocarcinoma has distinct histologic components

Table 8.1 Staining patterns in cholangiocarcinoma

Staining pattern	Intrahepatic cholangiocarcinoma, small duct type	Intrahepatic cholangiocarcinoma, large duct type	Extrahepatic cholangiocarcinoma
Positive	CK7 CK19 CK20 (−/focal positive) Albumin mRNA *in situ* Mucicarmine (focal) CD56 MUC1	CK7 CK19 CK20 (−/focal positive) Mucicarmine CA19–9 S100P TFF1 MUC5AC MUC6 MUC1	CK7 CK19 CA19–9 S100P IMP3 Maspin Methionyl-tRNA synthetase 1 Claudin-18 Mucicarmine
Negative	HepPar1 Arginase-1 Alpha-fetoprotein Polyclonal CEA (canalicular pattern) CD10 (canalicular pattern)	Albumin mRNA *in situ*	Albumin mRNA *in situ* Smad-4 pVHL

Abbreviations: *CEA* Carcinoembryonic antigen, *TFF1* trefoil factor 1, *IMP3* insulin-like growth factor-I mRNA binding protein-3, *pVHL* von Hippel-Lindau protein

Immunohistochemistry of Cholangiocarcinoma

Immunohistochemistry plays a larger role in assessing intrahepatic CCAs compared to their extrahepatic counterparts because of the differential diagnosis with hepatocellular carcinoma (HCC) and the propensity of a wide variety of other adenocarcinomas to metastasize to the liver. A summary of immunohistochemical labeling patterns is shown in Table 8.1. The distinction of CCA from non-hepatic

adenocarcinomas relies on the integration of morphology, ancillary tests, as well as clinical context and radiological findings. When a patient has a known prior extrabiliary adenocarcinoma, for instance, comparison should be made with prior histology to exclude metastasis.

CCAs of all types are positive for CK7 and CK19 while negative or only focally positive for CK20. This keratin labeling pattern is by no means specific for CCA over adenocarcinoma from another site but is supportive evidence that an established primary liver carcinoma is CCA (as opposed to HCC). Typical HCCs only rarely or weakly label with CK7, which is a marker of poor prognosis, while strong CK7 labeling is supportive of CCA [49]. The fibrolamellar variant of HCC is a clinically and genetically distinct variant which is strongly CK7 positive, but its morphology is so distinctive that it is unlikely to be mistaken for CCA. Mucicarmine is a histochemical stain that can be used to highlight intracellular mucin, which also supports glandular differentiation. Most CCAs are negative for hepatocytic lineage markers HepPar1, Arginase-1, and alpha-fetoprotein (AFP) [50–52].

Immunohistochemical approaches to evaluating intrahepatic tumors commonly involve excluding metastasis using a panel of markers given the keratin profile alone is nonspecific. In brief, these are generally useful ancillary tests for clarifying tumor origin, but interpretation requires an understanding of the sensitivity and specificity of these markers for their target sites. For example, TTF-1 and Napsin A are positive in the vast majority of lung adenocarcinomas and are generally negative in intrahepatic CCAs, but these stains have been reported positive in anywhere from 5–47% of extrahepatic CCAs [53–55]. This wide range may be related to the use of different antibody clones between different institutions. CDX2 is often positive in luminal gastrointestinal tract tumors, but it can stain CCA in roughly 30% of cases, albeit patchy or with weaker intensity [55–57]. Estrogen receptor (ER) and progesterone receptor (PR) have high specificity for breast and gynecologic origin but modest sensitivity [58, 59]. Other markers for mammary origin such as GATA-3, mammaglobin, and GCDFP-15 also show modest sensitivity and specificity [55, 60]. Some popular or emerging markers, such as PAX8 (renal, gynecologic, thyroid), NKX3.1 (prostate), and SATB2 (colon), have lower rates of cross-reactivity with CCA, in the range of 5–10% [55]. In summary, most popular immunohistochemical lineage markers are not entirely specific and may show staining in at least a subset of CCAs. Prudence dictates caution in drawing conclusions about site of origin based on immunohistochemistry without knowledge of the clinical and radiological setting.

Many markers have been evaluated for the differential expression in small versus large duct intrahepatic CCA. These stains are generally not employed in routine diagnosis. The large duct type is more likely to stain with CA19-9, S100P, and TFF1, while the small duct type labels with CD56. There is also differential mucin expression since MUC5AC and MUC6 label large duct while MUC1 labels both small and large duct types [38, 61, 62].

Albumin In Situ *Hybridization*

Until recently, there were no lineage-specific markers for CCA. While this remains the case for extrahepatic CCA, albumin mRNA expression has emerged as a relatively specific marker for primary liver cancers of both hepatocellular and cholangiocellular origin. Improvements in automated *in situ* hybridization staining methods have increased the availability of this marker for clinical use, but at the time of this writing, although commercial availability has improved, it is still not widely in use. Interpretation of albumin labeling requires familiarity with the possible range of staining patterns. The stain is often patchy and a positive result requires at least 5% of tumor cells to label [63]. Labeling of entrapped hepatocytes must be excluded. Albumin mRNA ISH has an 89% sensitivity for intrahepatic CCA [46]. It is also positive in almost all hepatocellular carcinomas. Albumin does not stain pancreatic adenocarcinomas, extrahepatic CCA, and gastric adenocarcinomas. The specificity is imperfect since it has been reported to occasionally label non-hepatic neoplasms such as acinar cell carcinoma of the pancreas, ductal breast carcinoma, gallbladder carcinoma, gastroesophageal junction carcinoma, lung carcinoma, and yolk sac tumors [46, 63–65]. The percentages of intrahepatic CCAs labeling for albumin in a given study are affected by the proportion of tumors of the large duct phenotype, which do not tend to express albumin [38, 66].

Cytology

Biliary brushings and drainage fluid are used to diagnose extrahepatic biliary lesions. For the diagnosis of malignant strictures, biliary brushings have variable sensitivity that ranges from 18% to 67%, with a pooled sensitivity of 45% by meta-analysis [5, 67]. The specificity is consistently high, with most studies approaching 99% [5]. Since the sampling utilizes an exfoliative technique, it is not possible to distinguish between a noninvasive intraductal carcinoma and an invasive carcinoma (Fig. 8.6). Fine needle aspiration may be performed for the diagnosis of both extra- and intrahepatic neoplasms. For extrahepatic cholangiocarcinoma, a direct comparison of FNA with brushing showed that FNA has a much higher sensitivity (73% vs 44%) [4].

The cytologic criteria for the diagnosis of cholangiocarcinoma are similar for both exfoliative and aspiration techniques [Table 8.2]. The diagnosis requires the identification of multiple atypical cytological features such as two distinct cell populations, cellular disorganization, cellular crowding and three-dimensionality, increased nuclear-cytoplasmic ratio, nuclear molding, nuclear size variation of >4:1 ratio in cellular clusters, coarse/clumped chromatin, irregular thickening and indentations of the nuclear membrane, and poor cellular cohesion leading to a background

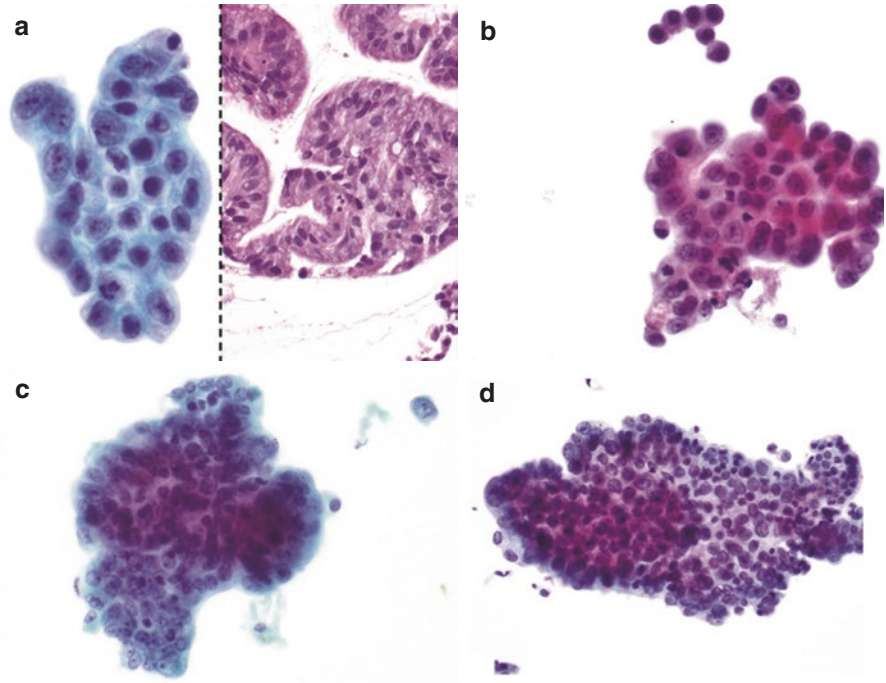

Fig. 8.6 (**a**–**d**) Clusters of ductal epithelium with reactive atypia in the setting of a stent (**a**, ThinPrep, 400× (left) cell block (right)) or primary sclerosing cholangitis (**b**, ThinPrep, 400×) is cohesive and lacks three-dimensional architecture. Intraductal papillary neoplasms with high-grade atypia demonstrate three-dimensional architecture and anisonucleosis, but cannot be distinguished from invasive adenocarcinoma (**c**, ThinPrep, 400×). Adenocarcinoma has crowded epithelial clusters with marked anisonucleosis and chromatin alterations (**d**, ThinPrep, 400×)

Table 8.2 Cytological features of reactive biliary mucosa and cholangiocarcinoma in bile duct brushing

Reactive biliary mucosa	Cholangiocarcinoma
Admixed inflammatory cells	Two distinct populations
Prominent nucleoli	Three-dimensional clusters
Lower nucleus-cytoplasmic ratio	Poor cellular cohesion and single atypical cells
Anisonucleosis up to 1:3 ratio	Increased nucleus-cytoplasmic ratio
Absent coarse chromatin	Nucleus molding
Smooth nucleus membranes	Anisonucleosis >4:1 ratio in clusters
Absent to rare single atypical cells	Coarse chromatin
	Irregularities of the nuclear membrane
	Marked cellular disorganization
	Marked cellular crowding

with single atypical cells [68–71]. The presence of inflammation due to primary sclerosing cholangitis or biliary stenting prior to endoscopic brush sampling of biliary disease creates significant diagnostic difficulties, yet specificity remains high even in this context (97%) [72]. A comparison of stent-associated changes with confirmed malignant cytology indicates that three-dimensional architecture, anisonucleosis (≥1:6), coarse chromatin, and single atypical cells are features significantly associated with malignancy (Fig. 8.6) [73].

The Papanicolaou Society classification system for the reporting of pancreaticobiliary cytology was published in 2015 [74] and provides useful terminology and criteria for the diagnosis of biliary cytology specimens. The system utilizes six diagnostic categories that include nondiagnostic, negative for malignancy, atypical, benign neoplastic, other neoplastic, suspicious for malignancy, and malignant.

Ancillary Techniques for Enhancing Biopsy Diagnosis

FISH, molecular analysis, digital image analysis, and immunohistochemistry have been investigated to improve the suboptimal sensitivity for extrahepatic CCA in biliary brushing specimens [Table 8.3]. Apart from FISH, few are widely used in practice [75]. FISH for CCA is available as a commercial kit that evaluates pericentromeric regions of chromosomes 3, 7, 17, and band 9p21 in biliary brushing cytology [76]. Cells are evenly spread onto a slide which is then incubated with hybridization probes that correspond to the areas of interest. Each probe has a different fluorescent marker and the stained cells are analyzed under a fluorescence

Table 8.3 Performance characteristics of biliary brushing and ancillary techniques for the diagnosis of cholangiocarcinoma

Test	Sensitivity (%)	Specificity (%)
Routine cytology [81, 101–103]	20.1–56	89–100
Biopsy [2–4]	62–78	100
FISH [83, 103]	41–45	95–100
KRAS mutation testing [83, 102]	29–38	96–100
TP53 mutation testing [102]	42	100
Digital image analysis for aneuploidy [67, 101]	39–45	77–89
Routine cytology + FISH [103]	57	89
Routine cytology + *KRAS* [102]	83	91
Routine cytology + DIA [67]	42.9	77
FISH + KRAS mutation testing [83]	54	96
Cytology + next-generation sequencing [81, 104]	56	97
Cytology + next-generation sequencing + FISH [81, 104]	66–73	97–100

Abbreviations: *DIA* Digital image analysis, *FISH* fluorescence *in situ* hybridization, *KRAS* Kirsten rat sarcoma

microscope. Aneuploidy or polysomy, which is defined as >2 copies in 2 or more probes, is considered a positive result if seen in more than 5 cells. FISH has a sensitivity of 34–52% for detecting malignancy in pancreatobiliary brushings [77]. The specificity of FISH is more variable and generally lower than cytology, reported at 89–100% [77, 78]. Combining FISH and cytology, particularly in equivocal cases, increases the sensitivity by roughly 20–30% in several studies without reducing specificity [79–81]. Digital image analysis for the detection of aneuploidy performs with a similar sensitivity to cytology and has high specificity [75].

Molecular techniques can enhance small biopsy diagnosis and potentially predict response to targeted therapy. For aiding diagnosis, testing is used in the context of the more prevalent genotypes of CCA at various anatomic sites. For extrahepatic CCA, the most common genetic alterations include *TP53* (47%), *KRAS* (37%), and *SMAD4* (30%) [82]. Options for molecular analysis on biliary brushings include single mutation testing or next-generation sequencing (NGS). *KRAS* testing is the most widely studied and reportedly increases the sensitivity of biliary brushing diagnostics to a degree roughly equivalent to the effect of combining cytology and FISH [83]. Limitations to *KRAS* testing include the lower prevalence of *KRAS* mutations in CCAs compared to pancreatic carcinomas and the fact that *KRAS* is an early genetic event in pancreatobiliary neoplasia and, therefore, the mutation can be detected in the absence of high-grade dysplasia or carcinoma [83–86].

There is less published experience with next-generation sequencing (NGS), but it seems to have similar sensitivity to FISH and equal specificity to cytology. NGS improves testing accuracy when used in combination with other methods [81]. An advantage of NGS is the possibility of testing cell-free DNA in exfoliative specimens [81]. One study showed combined NGS and cytology results achieved a sensitivity of 76%, elevated from 67% sensitivity of cytology alone, but it should be noted both suspicious and positive diagnoses were considered positive [81, 87]. An emerging technique is to perform NGS on the residual supernatant fluid after centrifugation of a liquid-based specimen [88].

For intrahepatic CCA, the most common mutations and prevalence estimates are *IDH1/2* (12–30%), *BAP1* (20–32%), *ARID1A* (20%), *TP53* (20%), PBRM1 (20%), and *FGFR2* rearrangements (14%) [89–91]. The hotspot mutation for IDH1 p.R132X is rarely seen in other epithelial neoplasms in the differential diagnosis, including extrahepatic CCA. A caveat is that rare HCCs have been reported with this mutation [90]. Histological features such as plump eosinophilic cells may suggest the genotype [92]. Currently there is no surrogate immunohistochemical testing available for the *IDH1* mutations found in CCA. Because intrahepatic CCAs are often amenable to core biopsies, sequencing of cytology aspirates is not commonly performed.

Several immunohistochemical markers have been reported to improve the sensitivity of biopsy and/or cytology for the diagnosis of extrahepatic CCA, such as S100 (expression), IMP3 (expression), pVHL (loss), CD10 (loss), SMAD4 (loss), Claudin-18 (expression), Maspin (expression), methionyl-tRNA synthetase 1

(expression), and p53 (expression), but published experience is limited, and these markers are not widely used in practice [93–99].

In summary, routine cytology and the ancillary techniques are all highly specific tests for CCA, but they are limited by low sensitivity such that negative results are of limited value. FISH is the most widely studied and utilized adjunct to cytology, while NGS is emerging to provide a similar improvement in test sensitivity and use in identification of patients eligible for targeted therapy.

Pathologic Grading and Staging

There is no specific grading system for CCA; most tumors are graded on a semiquantitative assessment of the proportion of tumor with gland formation. A tumor with ≥95% gland formation is well differentiated, between 50 and 95% gland formation is moderately differentiated, and less than 50% is poorly differentiated [100]. This system is similar to that of other gastrointestinal tumors. Pathologic staging of CCA is specific for tumors arising intrahepatic, extrahepatic, and distal bile duct as detailed by the Union for International Cancer Control eighth edition AJCC staging manual [100].

Conclusion

Routine histopathology and cytology remain the most definitive methods for diagnosing and classifying CCA. In resection specimens, histopathology provides not only the diagnosis but also crucial staging and prognostic parameters. In biopsies and aspirates, the technique and adequacy of tissue acquisition can have a significant impact on the sensitivity and specificity of the diagnosis. Well-established laboratory methods such as immunohistochemistry and *in situ* hybridization can provide valuable ancillary information to aid diagnosis, but pathologists and clinicians should be aware of existing caveats and limitations. Newer advances in molecular pathology and digital image analysis may become increasingly utilized in the near future and enhance clinical management.

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