Review

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Recent Advancement in Diagnosis of Biliary Tract Cancer through Pathological and Molecular Classifications

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Simple Summary: The development of various molecular techniques has led to the introduction of a new classification for biliary tract cancer and a better understanding of the clinicopathological features of the disease. Furthermore, as new diagnostic modalities and research findings have been published, they enable accurate diagnosis, differentiation, and clinical assessment based on the characteristics of each subtype. This article reviews the current imaging and histologic diagnosis, along with future perspectives of molecular diagnosis to approach precision medicine for biliary tract cancer.

Abstract: Biliary tract cancers, including intrahepatic, perihilar, and distal cholangiocarcinomas, as well as gallbladder cancer, are a heterogeneous group of cancers with distinct molecular characteristics in each anatomic and pathological subtype. The pathological classification of BTC includes grossly classifiable growth patterns, such as mass-forming, periductal infiltrating, and intraductal growing. The small duct and large duct types of intrahepatic cholangiocarcinoma have been recently introduced in the WHO classification. Typical clinical presentations and various radiological, endoscopic, and molecular modalities for diagnosis are described in detail. To overcome the limitations of traditional tissue acquisition, new diagnostic modalities are being investigated. In fact, the treatment landscape is evolving rapidly owing to the emergence of distinct subgroups harboring unique molecular alterations with corresponding targeted therapies. In addition, we highlight the key points in the diagnostic process in real-world clinical practice for biliary tract cancer.

Keywords: Biliary tract cancer; cholangiocarcinoma; gallbladder cancer; diagnosis; precision medicine

1. Introduction

Biliary tract cancer (BTC) encompasses a range of invasive adenocarcinomas, including cholangiocarcinomas (arising in the intrahepatic, perihilar, or distal biliary tree), and gallbladder cancers. Cholangiocarcinomas arising from the bile ducts proximal to the second-order ducts are classified as intrahepatic cholangiocarcinoma(iCCA), those originating between the second-order ducts and the insertion of the cystic duct are termed perihilar cholangiocarcinoma (pCCA; previously referred to as Klatskin tumors), and those arising from the bile ducts distal to the insertion of the cystic duct are termed distal cholangiocarcinoma (dCCA). Extrahepatic cholangiocarcinoma refers to pCCA and dCCA collectively [1]. Gallbladder cancer (GBC) originates either from the gallbladder itself or from the cystic duct.

BTCs exhibit heterogeneous clinical manifestations, molecular characteristics, and biological behaviors, depending on their anatomical, pathological, and molecular classifications. In recent years, increasing genomic research has begun to uncover the molecular underpinnings of BTC and offer

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many potential treatments, ushering in a new era in precision medicine. However, in addition to understanding the clinicopathologic development of each BTC subtype, there must be an individualized assessment of each subtype and an effort to overcome clinical diagnostic hurdles. Here, we review the current imaging and histologic diagnosis of BTC, along with future perspectives of molecular diagnosis to approach precision medicine in BTC.

2. Pathologic Classification

2.1. Pathoologic Classification and Precancerous Leions of Cholangiocarcinoma

iCCA is grossly classifiable in three growth patterns (Table 1): mass-forming (78% of cases) presents as a mass lesion within the hepatic parenchyma; periductal infiltrating (16% of cases) characterized by infiltration along the bile ducts and portal tracts, causing strictures and thickening of the affected bile ducts and dilatation of the peripheral bile ducts; intraductal growing (6% of cases) consists of a polypoid or papillary tumor within the dilated bile ducts [2]. pCCA and dCCA have similar growth patterns: flat or nodular sclerosing type (73% of cases, corresponding to features of periductal infiltrating) and intraductal papillary type (27% of cases) [3].

Table 1. Clinicopathological features of cholangiocarcinoma.

Title 1	Growth pattern	Precancerous lesion	Main etiology
iCCA – small duct type	Mass-forming	None	Chronic hepatitis Cirrhosis
iCCA – large duct type	Periductal infiltrating	BilIN	Hepatolithiasis Liver flukes PSC
	Intraductal growing	IPNB, *MCN, ITNB*	
pCCA - dCCA	Flat or nodular sclerosing Intraductal papillary	BilIN IPNB, *MCN, ITNB*	

iCCA, intrahepatic cholangiocarcinoma; BilIN, biliary intraepithelial neoplasia; IPNB, intraductal papillary neoplasm of the bile duct; MCN, mucinous cystic neoplasm; ITNB, intraductal tubular neoplasm of the bile duct; pCCA, perihilar cholangiocarcinoma; dCCA, distal cholangiocarcinoma; PSC, primary sclerosing cholangitis.

Conventional iCCA can be further categorized into two main histological types based on the level or size of the affected duct. Small duct type and large duct type iCCA have been recently introduced in the WHO classification [4]. Small duct-type iCCA (36-84% of cases) is composed of small-sized tubular growth of cuboidal or low-columnar tumor epithelial cells with little or no mucin production. Large duct-type iCCA (8-60% of cases) is characterized by mucin-producing columnar cells forming irregularly shaped and sized tubules or gland-like structures, which are usually accompanied by a highly invasive growth pattern and a desmoplastic reaction [4–6].

Different carcinogenesis and progression pathways may give rise to different morphological subtypes of cholangiocarcinoma. For example, canals of Hering and interlobular bile ducts can represent the cell-of-origin for small duct-type iCCA, which develops into a mass-forming pattern in the background of chronic hepatitis and cirrhosis [7], whereas the peribiliary glands are likely the origin of large duct-type iCCA, pCCA, and dCCA, which lead to periductal-infiltrating lesions related to biliary inflammation, such as hepatolithiasis, parasite infection in bile ducts, or primary sclerosing cholangitis (PSC) [8]. Notably, the intraductal growing pattern represents a distinct pathway from large bile ducts and is often associated with a more favorable prognosis.

The 2010 WHO classification proposed three types of precancerous lesions of the biliary tract: flat type (biliary intraepithelial neoplasia, BilIN), papillary type (intraductal papillary neoplasm of the bile duct, IPNB), and cystic type (mucinous cystic neoplasm, MCN). Recently, intraductal tubular neoplasm of the bile duct (ITNB) was proposed as another candidate for preneoplastic lesions; however, its advanced form remains unclear [9]. IPNB may be associated with intraductal growing type of iCCA and intraductal papillary type of pCCA and dCCA, whereas BilIN may be followed by

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a periductal infiltrating (iCCA) and a flat or nodular sclerosing (pCCA and dCCA) pattern [10]. MCN can result in MCN associated with an invasive carcinoma that may progress to a cystic lesion with a grossly surrounding nodular lesion. No precursor lesions have yet been defined for mass-forming iCCA [3].

2.2. Pathologic Classification and Precancerous Leions of Gallbladder Cancer

Adenocarcinoma is the main histological classification of GBC (approximately 90% of cases) [11]. Grossly, GBC can exhibit infiltrative, nodular, papillary, or a combination of these morphologies. In addition, there are three premalignant lesions of gallbladder adenocarcinoma: adenoma, BilIN, and intracystic papillary neoplasm (ICPN). BilIN is invisible on gross inspection, but can be microscopically identified around invasive tumors or chronic cholecystitis. ICPN is grossly identified as an exophytic polypoid mass or diffuse friable thickening of the mucosa and is composed of mucinous epithelial cells with papillary and tubular arrangements [12]. Dysplasia of the BilIN and ICPN is generally categorized using a three-tiered system, and high-grade dysplasia is placed in the same group as carcinoma in situ. The current definitions of Adenoma and ICPN are unclear and require revised diagnostic criteria to ensure consistency and accuracy of diagnoses.

3. Clinical Presentation

The presence and features of clinical symptoms depend on the anatomical location of the primary tumor and associated metastasis. Patients with extrahepatic cholangiocarcinoma typically become symptomatic when biliary obstruction caused by the disease results in jaundice. Patients with iCCA are less likely to experience jaundice and instead exhibit non-specific symptoms, such as dull right upper quadrant pain or unexplained weight loss. Approximately 20-25% of patients are asymptomatic, with the lesions detected incidentally [13]. Patients with early GBC are also usually asymptomatic and are often diagnosed incidentally through preoperative imaging studies or intra- or post-operative examinations.

Laboratory studies are generally non-diagnostic; elevated alkaline phosphatase or serum bilirubin levels may be related to biliary obstruction. Serum tumor markers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9) are frequently elevated, but do not provide diagnostically useful results due to their lack of specificity and sensitivity [14–16]. However, in the presence of an established diagnosis, these tumor markers can provide information on the response to treatment in addition to prognostic information.

Many patients initially undergo transabdominal ultrasonography to assess the biliary tree, and the results may aid in identifying the location of the lesion: an abrupt change in extrahepatic duct diameter with intrahepatic and extrahepatic biliary dilatation (dCCA case), intrahepatic ductal dilatation with normal-caliber extrahepatic ducts (pCCA case); mass lesions, occasionally in a non-cirrhotic liver, without radiographic characteristics of hepatocellular carcinoma (HCC) (iCCA case); or a protruding mass in the gallbladder, which sometimes extends directly into the liver bed (GBC case). Although BTC is frequently suspected based on ultrasound findings, it is crucial to conduct additional imaging studies, such as computed tomography (CT), magnetic resonance imaging (MRI)/ magnetic resonance cholangiopancreatography (MRCP), endoscopic retrograde cholangiopancreatography (ERCP), endoscopic ultrasound (EUS), and positron emission tomography (PET)-CT to verify the diagnosis and treatment plan. Imaging studies aim to rule out benign tumors or gallstones as potential causes, establish the extent and location of the primary tumor, and detect whether metastases have developed.

4. Diagnostic Tool

4.1. CT and MRI

Computed tomography (CT) is the standard modality for the diagnosis and staging of BTC. It provides a comprehensive evaluation of the primary tumor, its relationship with adjacent structures (specifically portal vein and hepatic artery involvement, determining resectability), and potential

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thoracic and abdominal spread [17]. MRI has a similar accuracy to CT for diagnosis and staging, but it offers specific sequences such as diffusion-weighted imaging (DWI) and the potential for performing MRCP, which is essential for pCCA staging [18].

4.1.1. Radiologic Findings of Mass-Forming Cholangiocarcinoma

The most common imaging pattern of mass-forming iCCA on both CT and MRI is arterial peripheral rim enhancement with centripetal progressive homogeneous contrast agent uptake until the delayed phase or stable uptake during the late dynamic phases [18]. In hepatobiliary phase of gadoxetic acid-enhanced MRI, most mass-forming iCCA exhibits 'EOB-cloud' described as cloud-like mild central hyperintensity and peripheral hypointensity in the tumor [19]. However, as no specific radiological pattern exists, histopathological or cytological results are mandatory to confirm the diagnosis.

The main differential diagnoses for mass-forming iCCA include HCC, metastatic adenocarcinoma, inflammatory pseudotumors, and angiosarcoma. HCC, the most common primary hepatic malignancy, should be differentiated due on different prognoses and treatments. Early arterial enhancement and washout of contrast are the main patterns in favor of HCC, and capsular retraction and peripheral bile duct dilatation are suggestive of iCCA. The target sign on DWI, defined as central hypointensity and a peripheral hyperintense rim, helps in the recognition of iCCA from HCC [20]. However, scirrhous HCC may closely resemble mass-forming iCCA on imaging, making it difficult to differentiate them and necessary for tissue diagnosis. Metastatic adenocarcinoma can show many typical findings of iCCA, including central hypointensity or intrahepatic bile duct dilatation. It can also be difficult to differentiate based on histopathology and requires special immunohistochemical studies [21]. Therefore, when approaching a suspected mass-forming iCCA, it is essential to exclude extrahepatic primary malignancies, especially colorectal cancer.

4.1.2. Radiologic Findings of Periductal-Infiltrating Cholangiocarcinoma

This is the most common growth pattern in pCCA and dCCA, which presents as a narrowed biliary duct with irregular circumferential wall thickening (usually ≥ 5 mm) and upstream biliary tree dilatation. These tumors slowly enhance to a peak in the delayed phase; however, they are rarely hypervascular and enhance in the arterial phase [22]. When infiltration is nodular, the bile ducts appear protuberant, whereas they appear narrowed and stretched when infiltrated diffusely [23].

PSC, Mirizzi syndrome, portal biliopathy, IgG4-related sclerosing cholangitis, benign idiopathic stricture, and hepatobiliary sarcoidosis or lymphoma can mimic periductal infiltrating cholangiocarcinoma. PSC typically presents as multiple intra- and extrahepatic biliary strictures with a beaded appearance on MRCP. These strictures are often short, with only mild dilatation of the intervening segments observed [24]. On MRCP of Mirizzi syndrome, an abrupt stricture of the common hepatic duct, along with a normal common bile duct, is evident, with an impacted gallstone located in the neck of the distended gallbladder [25]. Portal biliopathy refers to narrowing of the extrahepatic biliary tract secondary to extrahepatic portal venous obstruction, which results from the compressive effect of portal vein collaterals or ischemic damage. Imaging studies revealed a circumferential, long, and smooth stricture of the common bile duct accompanied by the presence of collaterals and choledochal varices. [26]. IgG4-related sclerosing cholangitis is a persistent inflammatory disorder of the biliary system that is most commonly associated with other manifestations of IgG4-related disease. IgG4-related sclerosing cholangitis shows circumferential symmetric wall thickening of the bile ducts, frequently involving the extrahepatic segments, with smooth outer and inner margins, visible lumen in the thickened segments, and delayed homogenous contrast enhancement [27]. Nine out of ten cases exhibit pancreatic involvement, which typically presents with diffuse or focal pancreatic enlargement, a peripheral capsule-like rim, and a pancreatic duct stricture [28]. The diagnosis of IgG4-related sclerosing cholangitis is established by a combination of characteristic imaging features, serum IgG4 antibody levels, histologic findings, and response to steroid therapy [29].

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4.1.3. Radiologic Findings of Intraductal-Growing Cholangiocarcinoma

The intraductal type of cholangiocarcinoma has been reported in 8-18% of all types of cholangiocarcinoma [18]. These tumors present as polypoid or sessile masses confined within the bile duct with proximal ductal dilatation due to occlusion or excessive mucin production. These lesions exhibit imaging characteristics similar to those of mass-forming types, displaying a heterogeneous enhancement that begins early and reaches its peak in the delayed phase. A markedly dilated intrahepatic bile duct segment may mimic a cystic mass such as cystadenoma, cystadenocarcinoma, or liver abscess [30].

4.1.4. Radiologic Findings of Gallbladder Cancer

GBC can appear as a polypoid mass protruding into the lumen or completely filling it, focal or diffuse wall thickening, or as a large mass in the gallbladder fossa with an unidentifiable gallbladder [31]. The presence of features such as an increased frequency of lymph node enlargement, more extensive wall thickness, focal irregularity in wall thickness, and less gallbladder distention are indicative of GBC complicated by cholecystitis rather than simple cholecystitis [32].

4.2. PET-CT

PET-CT can be used to complement CT and MRI to provide additional information about lymph node involvement, the presence of distant metastasis, and postoperative recurrence. In fact, preoperative PET scanning leads to a change in surgical management in approximately one-fourth of cases, mainly by detecting occult distant metastases. However, because of its low specificity, it is not sufficient for the diagnosis of primary lesions, and cytological or histological confirmation is still necessary [33].

4.2. EUS

EUS can visualize the local extent of the primary tumor and the status of the regional lymph nodes, particularly in cases where dCCA lesions are suspected. EUS-guided fine needle aspiration (FNA) of tumors and enlarged lymph nodes can also be performed. EUS-FNA has a higher sensitivity for detecting malignancies in distal tumors than ERCP with brushings [34]. EUS is less effective in imaging and staging proximal bile duct lesions than distal lesions, and clinical experience with this technique is limited [35].

EUS is also considered a useful modality both in the detection and differential diagnosis of gallbladder polyps and in staging early GBC. In particular, EUS is helpful in assessing the depth of tumor invasion in the gallbladder wall [36,37] and in defining lymph node involvement in the portal hepatis or peripancreatic regions). Although some authors reported accurate and safe results of EUS-FNA for GB wall lesions [38], this procedure poses a potential risk of bile leakage after gallbladder biopsy.

4.3. ERCP or Percutaneous Tranhepatic Cholangiography (PTC)

Preoperative cholangiography performed using ERCP or PTC may be indicated either diagnostically or therapeutically for patients with biliary obstruction. Recently, MRCP or CT scanning, which is non-invasive and highly accurate, has largely replaced invasive cholangiography for diagnostic purposes.

4.3.1. Intraductal Ultrasound (IDUS)

IDUS uses a small wire-guided ultrasound catheter that provides high-resolution images, enabling precise evaluation of the biliary tract during ERCP. IDUS is useful for characterizing malignant biliary strictures and for the local staging of cholangiocarcinoma. It can detect early lesions in the biliary tree, determine the longitudinal tumor extent, and identify tumor extension into adjacent organs (e.g., pancreas) and major vessels (e.g., portal vein and hepatic artery) [39–41]. In

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contrast to EUS, IDUS is often better able to evaluate the proximal biliary system and surrounding structures such as the right hepatic artery, portal vein, and hepatoduodenal ligament. However, IDUS limits the evaluation of more distant tissues or lymph nodes and cannot be used to perform FNA.

4.3.2. Peroral Cholangioscopy (POC)

POC (direct visualization of the bile ducts using a very thin cholangioscope during ERCP) can be used to evaluate indeterminate biliary strictures (e.g., strictures that cannot be diagnosed as benign or malignant using sampling techniques such as brush cytology or biopsy) by targeted biopsies of bile duct lesions [42–45]. It can also be used to investigate equivocal fluoroscopy findings during ERCP, assess the extent of cholangiocarcinoma prior to surgery, and identify invisible stones by conventional cholangiography. "Tumor vessels" may be observable during POC in patients with cholangiocarcinoma, which are characterized by irregularly dilated and tortuous blood vessels. Other characteristic findings suggesting malignancy include nodules or masses, infiltrative or ulcerative strictures, and papillary or villous mucosal projections [46]. A recent study reported 100% sensitivity and 89.5% specificity for visual impression during POC [47].

4.3.3. Tissue Acquisition

ERCP or PTC-guided biopsies or brush cytology are the traditional standard methods for the tissue diagnosis of periductal infiltrating or intraductal growing cholangiocarcinoma. Although brush cytology has high specificity, its low sensitivity is a major limitation (e.g., 97% specificity and 43% sensitivity for detecting cholangiocarcinoma in patients with PSC) [48]. The addition of endoscopic biopsy of malignant strictures increases the diagnostic accuracy to only 43–88 percent [49–51]. These tests may be useful in diagnostic evaluation if they are positive, but cannot rule out malignancy if they are negative.

Fluorescence in situ hybridization (FISH) is a cytological test using labeled deoxyribonucleic acid (DNA) probes to detect abnormal loss or gain of chromosomes or chromosomal loci on cells routinely collected by the brush technique, which may also improve the sensitivity of brush cytology [52]. A meta-analysis of FISH has demonstrated that this is highly specific (pooled 70%), but with limited sensitivity (68%) for the diagnosis of cholangiocarcinoma in patients with PSC [53].

Another auxiliary technique for improving the diagnostic ability of bile cytology is the implementation of a new scoring system for evaluating cytologic results. Hayakawa et al. proposed a scoring system based on four cytological findings, including abnormal chromatin, irregularly arranged nuclei, irregularly overlapped nuclei, and irregular cluster margins. The scoring system yielded an area under the receiver operating characteristic (ROC) curve (AUC) of 0.981 with a sensitivity of 87% and specificity of 98% [54]. Another study reported that the diagnostic sensitivity of bile cytology increased from 31.6% to 80.3% after combined p53 immunostaining [55].

Recently, various emerging analytical methods for extracellular vesicles (EVs), nucleic acids, proteins, and metabolites in bile have been developed as potential biomarkers for BTC diagnosis [56]. For example, circular RNA (Circ-CCAC1) in serum-derived or bile-derived EVs has a diagnostic role, with an AUC of 0.857 [57]. In a prospective study of bile samples, K-ras mutations detected in bile cell-free DNA indicated the possibility of cholangiocarcinoma in high-risk lesions such as PSC [58]. A study screened four DNA methylation biomarkers (COD1, CNRIP1, SEPT9, and VIM) based on DNA methylation analysis of ERCP brush samples and achieved 85% sensitivity and 98% specificity with an AUC of 0.944 [59]. Based on this study, the role of a four-gene methylation panel in bile was investigated to predict early diagnosis of BTC in patients with PSC. The results showed that the AUC for predicting the diagnosis of cholangiocarcinoma in PSC patients within one year was between 0.84 and 0.98, with a sensitivity of 67%–96% and a specificity of 93%–98% [60]. sB7-H3, a cancer-related immune protein, is upregulated in the bile of patients with malignant biliary obstruction including BTC and pancreatic cancer. The ROC-AUC for the diagnosis of malignant biliary obstruction was 0.878, and the sensitivity and specificity were 81.2% and 81.6%, respectively [61]. Another study revealed the potential of bile multi-omics analysis, including metabolomics, for molecular diagnosis

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of GBC, which combined lipidomics and metagenomics in bile to characterize microbial and lipid alterations associated with the development of GBC. Based on the random forest classifier model, this study established a diagnostic model containing eight lipid substances that can effectively be used to diagnose GBC from gallstones or healthy groups with an AUC of 1 [62]. Table 2 summarizes previous studies on bile EVs, nucleic acids, and protein detection for the diagnosis of BTC (Table 2).

Table 2. Diagnostic studies of bile EVs, nucleic acids, and proteins.

Biomarkers	*n*	ROC-AUC	Sensitivity (%)	Specificity (%)	Reference
Exosomal cargoes					
MicroRNA (miR-191, miR-486-3p, miR-1274b, miR-16 & miR-484)	*96*		0.67	0.69	[63]
	92	0.81, 0.74	0.811, 0.73	0.811, 0.865	[64]
MicroRNA (miR-483-5p, miR-126-3p)	*100*	0.757~0.869	0.63~0.83	0.6~0.867	[65]
MicroRNA (miR-141-3p, miR-200a-3p, miR-200c-3p, miR-200b-3p and ENST00000588480.1)	*91*	0.709	0.829	0.589	[66]
					[57]
LncRNA (ENST00000588480.1 & ENST00000517758.1)	*316*	0.857		0.875	[67]
Circle-RNA (circ-CCAC1)	*20*	0.945	0.875		
Protein (Claudin-3/CLDN3)					
DNA					
K-ras mutation				1	[68]
K-ras mutation				0.96	[69]
KRAS				1	[70]
K-ras mutation & p53 mutation	*20*	0.667	0.33	0.958	[71]
K-ras mutation & p53 mutation	*115*		0.25	0.848/ 0.970	[72]
K-ras mutation & p53 mutation	*46*	0.738	0.476	1, 1	[73]
TP53, ERBB2, and KRAS	*43*	0.742	0.526	1	[74]
KRAS, TP53, CDKN2A, SMAD4, BRAF	*109*	0.564/ 0.508	0.279/ 0.047	1	[70]
Promotor methylation INK4a/ARF	*50*	0.783, 0.750	0.567, 0.5	0.937/ 0.969	[75]
Promotor methylation of COD1,	*49*	0.733	0.467	0.93~0.98	[60]
CNRIP1, SEPT9 & VIM	*42*	0.955	0.909	0.778	[76]
Methylation of DKK3, p16, SFRP2,	*60*	0.737/ 0.715	0.536/ 0.462	0.94	[77]
DKK2, NPTX2 and ppENK	*243*	0.84~0.98	0.67~0.96	0.98	[78]
CCND2, CDH13, GRIN2B, RUNX3, and	*80*	0.775	0.773		
TWIST1	*125*		0.71~0.83	0.999	[79]
Gene mutations in KRAS, TP53,	*241*		0.92		[80]
SMAD4, and CDNK2A; Methylation changes in SOX17, 3-OST-2, NXPH1,	*10*		0.947	1	[81]
SEPT9 and TERT	*28*		0.955		[82]
150 tumor-related genes (Wildly target)				0.91	[83]
520 tumor-related genes (Wildly target)	*20*		0.833	0.89~0.92	[84]
RNA	*18*	0.765~0.975		0.605, 0.667	[85]
Human telomerase reverse transcriptase	*23*	0.856	0.67		
mRNA	*83*	0.78~0.81	0.5~0.67		
miR-9, miR-145, miR-944	*106*	0.730, 0.652	0.811, 0.657		
RNU2-1f					
miR-412, miR640, miR-1537 & miR-3189					
miR-30d-5, miR-92a-3p					
Protein					
CEACAM6	*73*	0.74	87.5	69.1	[86]
CEACAM6	*41*	0.92	83.3	93.1	[87]
SVV, CA199	*102*	0.78, 0.75	67.3, 96.4	80.9, 46.7	[88]

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MUC1	*68*	0.85	90.0	76.3	[89]
MUC4	*134*		27	93	[90]
MUC5AC	*46*	0.85	75	76.9	[91]
Mac-2BP	*78*	0.70	69	*67*	[92]
VEGF	*53*	0.89	99.3	88.9	[93]
MCM2, MCM5	*42*	0.80			[94]
HSP27 & HSP70	*20*	0.86, 0.81	90, 80	90, 80	[95]
SSP411	*67*	0.913*	90.0	83.3	[96]
NGAL	*40*	0.74	77.3	77.2	[97]
NGAL	*38*	0.76	94	55	[98]
LCN2/NGAL	*144*	0.81	87	75	[99]
S100P	*24*	0.861	92.9	70	[100]
sB7-H3	*323*	0.878	81.2	81.6	[61]
α-1-antitrpysin	*8*	0.833	80	75	[101]
Amylase	*239*	0.751	66	74	[102]
PE-3B/amylase	*68*	0.877	81.8	89.3	[103]
M2-PK	*167*		90.3	84.3	[104]
GSH, hydrogen peroxide, GPx, Fe2+, FNTA	*46*	0.683~0.852	67.9~100	52.9~76.5	[105]

* Serum samples for ROC analysis.

5. Pathologic and Molecular Diagnosis

5.1. Pathologic Diagnosis

Pathologic diagnosis can be assessed using a variety of methods in patients suspected to have BTC (ERCP or PTC-guided biopsy or brush cytology, EUS-FNA, ultrasonography/CT/MRI-guided biopsy), but obtaining tissue may be difficult, especially in patients with perihilar lesions. In cases of potentially resectable tumors with typical findings of malignant biliary obstruction, solitary intrahepatic mass, or early GBC confined to the gallbladder, surgery can be performed without preoperative pathologic diagnosis. In patients with biliary obstruction due to pCCA and dCCA without extraductal metastasis, ERCP or PTC-guided biopsies, or brush cytology are preferred and should be conducted to ensure adequate tissue for pathological diagnosis and molecular profiling. EUS-FNA may be an alternative method to obtain biopsies of regional lymph nodes (if enlarged) or distally located tumors and may be considered if ERCP or PTC-guided biopsies are negative or inconclusive. In addition, EUS-FNA or ultrasonography/CT/MRI-guided biopsy via the transperitoneal approach rarely results in seeding of the biopsy tract with tumor cells [106]. Therefore, it is necessary to establish tissue diagnosis prior to surgery in a multidisciplinary setting.

Pathological diagnosis is important in the following situations: clinically indeterminate strictures, patients requiring diagnostic documentation before nonsurgical treatment, or situations where a physician or patient is reluctant to undergo surgery without a tissue diagnosis [107]. Conversely, tissue diagnosis is not mandatory for unresectable patients who are scheduled to receive only palliative management, such as biliary drainage.

5.2. Molecular Diagnosis

Molecular profiling is recommended for advanced diseases and is considered suitable for systemic treatments [108]. Small duct-type iCCA are enriched for actionable targets, such as *IDH 1/2* mutations (15%–20%) and *FGFR-2* fusions (10%-20%). Large duct-type iCCA tumorigenesis frequently involves *KRAS* (15%–30%) and *TP53* mutations (10%–40%). GBC, pCCA, and dCCA are characterized by a high frequency of *KRAS* mutations (30–45%), *ERBB2* amplification (15–20%), and low frequency of *IDH 1/2* or *FGFR2* fusions. Although rare, gene rearrangements such as *NTRK, ROS1*, or *ALK* fusions have been identified in BTC. All subtypes of BTC harbor similar rates of *BRAF* alteration (3–5%), homologous recombination deficiency (5–15%), and microsatellite instability-high

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(MSI-H)/mismatch repair (MMR)-deficient (dMMR; 2–5%) [109–111]. Although distinct genetic and epigenetic profiles have been identified for each BTC subtype, subgroups with driver mutations amenable to targeted therapy are generally mutually exclusive from one another.

Parallel tests of several genes using focused next-generation sequencing (NGS) are preferred over single-gene sequencing. NGS can be performed on formalin-fixed and paraffin-embedded tumor tissues and is well suited for tissue biopsies. Alternatively, liquid biopsies using cell-free circulating DNA may be considered if insufficient tumor tissue is available for NGS [108]. MSI status can be evaluated by immunohistochemical (IHC) staining for MMR proteins, including MLH1, MSH2, MSH6, and PMS2. Instead, DNA-based assays can be used to analyze the composition and length of microsatellites. The preferred methods for NGS, IHC staining, or RNA sequencing depend on the target and availability of materials (tissue or circulating tumor DNA).

6. Approach to the Patient

6.1. Suspected iCCA

When an intrahepatic lesion is suspected, cross-sectional imaging (multiphasic contrast-enhanced CT or MRI) is performed to differentiate between HCC and mass-forming iCCA. However, the classical radiologic features of iCCA were present in only 70% of cases [19], and some small mass-forming iCCA may mimic HCC, which hyperehances during the arterial phase and demonstrates washout during the delayed phase. If the initial imaging test is non-diagnostic, other imaging modalities (CT or MRI) can be conducted. Biopsy or surgery of the lesion is performed if the diagnosis remains uncertain.

The issue is further complicated by the fact that some intrahepatic tumors can contain elements of both cholangiocarcinoma and HCC in the same nodule, termed mixed hepatocellular-cholangiocellular carcinomas [7]. Studies have suggested that these tumors have a distinct appearance in cross-sectional imaging studies. A strongly enhancing rim and irregular shape on gadoxetic acid-enhanced MRI are indicative of mixed hepatocellular-cholangiocellular carcinoma, while a lobulated shape, weak rim, and target appearance suggest a mass-forming iCCA [112]. The target appearance can also help differentiate mixed hepatocellular-cholangiocellular carcinomas from atypical hypovascular HCC [113]. Additionally, the presence of liver capsule retraction and biliary dilatation in the vicinity of the intrahepatic lesion can raise suspicion for a diagnosis of iCCA; however, biopsy may be needed to confirm the diagnosis. These mixed tumors were staged as iCCA and not as HCC.

IHC staining of tissue biopsies is required to differentiate iCCA from metastatic lesions and mixed hepatocellular-cholangiocellular carcinoma. Tumors negative for TTF-1 (lung), CDX2 (colon), and DPC4 (pancreas) and positive for AE1/AE3, CK7, and CK20 (biliary epithelium) were suggestive of iCCA [114].

6.2. Suspeected pCCA

Careful evaluation with cross-sectional imaging studies (enhanced MRI with MRCP was preferred over CT) and EUS helps delineate the tumor location, size, morphology, hepatic artery or portal vein involvement, volume of potential liver remnant, lymph node involvement, and presence of distant metastasis. If imaging studies and/or tissue samples are highly suggestive of pCCA, the tumor staging proceeds directly. In cases where the diagnosis is uncertain, we typically proceed with an ERCP procedure that includes brush cytology (with or without IDUS). Where available, POC can be performed to evaluate the bile ducts. Alternatively, MRI- or CT-guided biopsy can be performed if a mass lesion is observed on imaging, although there is a small risk of needle tract seeding. If the diagnosis remains doubtful, surgery may be required to confirm it.

Patients with PSC

PSC is one of the most common risk factors for BTC, and the lifetime incidence of cholangiocarcinoma in PSC has been reported to be 5%–10% [115,116]. Cholangiocarcinoma in PSC

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usually infiltrates and appears as the formation of progressive strictures in perihilar areas [116,117]. In such cases, patients may have a dominant benign biliary stricture that is difficult to differentiate from cholangiocarcinoma. Mass lesions are seldom identified on imaging, and patients often do not develop significant intrahepatic biliary ductal dilation. However, detection of a new parenchymal lesion adjacent to the bile ducts, appearance of a new bile duct dilatation in a short interval, and disproportionate regional/segmental bile duct dilatation are among the warning signs of developing cholangiocarcinoma in these patients.

In patients with PSC with suspected cholangiocarcinoma, CA 19-9 levels above 129 U/mL are 79% sensitive and 98% specific for confirming the diagnosis [118]. However, the positive predictive value (the likelihood that a patient with PSC and a CA 19-9 level ≥ 129 unit/mL has cholangiocarcinoma) was only 57 percent.

The initial step of work-up is MRCP to evaluate the segmental extent of ductal involvement, search for intrahepatic metastases, and identify aberrant ductal anatomy. If the MRCP is nondiagnostic or if a dominant stricture is identified, we will obtain an ERCP or PTC with brush cytology, which is almost 100% specific; however, even with FISH, only 40%–70% is sensitive for the detection of cholangiocarcinoma in patients with PSC [119,120]. In cases of negative cytologic results, MRI/MRCP and/or ERCP plus CA 19-9 should be repeated within 3-6 months.

6.3. Suspected dCCA

On cross-sectional imaging, dCCA may be seen as an abrupt stenosis with proximal biliary dilatation, where a nodular mass or concentric or asymmetric thickening of the bile duct with enhancement typically coexist. In uncommon cases of thickening or stricture of the distal bile ducts without a mass, it is difficult to differentiate them from benign strictures. While ERCP has traditionally dominated the initial workup of dCCA owing to tissue sampling for diagnosis and biliary decompression, EUS has recently become the preferred method for direct visualization and sampling of the distal bile duct. ERCP carries a risk of ascending cholangitis by injecting contrast, whereas EUS-FNA poses a risk of seeding the biopsy tract. If radiographic findings are sufficiently diagnostic for dCCA, such that a negative biopsy would be characterized as a potential false-negative and the tumor appears resectable, biopsy is not indicated.

7. Conclusion

BTC is a heterogeneous disease of cancer that arises from the biliary tree. Although they have historically been classified as a single disease, extensive molecular characterization has recently led to more informative anatomical, pathological, and molecular classification of BTC. The development of radiologic and endoscopic tools for accurate diagnosis strengthens our understanding of BTC carcinogenesis. Precision medicine for BTC patients is facilitated by pathological and molecular profiling. We anticipate that advancements in diagnostic and personalized strategies for BTC management will lead to improved patient outcomes in the near future.

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