

REVIEW

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The biliary tree musketeers: cholangiocarcinoma – one name for all, but at least three different diseases with distinct targets

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Abstract

Cholangiocarcinoma, a malignant neoplasm of the biliary system, poses a significant challenge in clinical practice due to its increasing incidence and diagnostic and therapeutic complexity. This review addresses the epidemiological aspects, risk factors, and classification of this disease. We examine advances in histological diagnosis, highlighting essential criteria for accurate assessment. Additionally, we discuss standard treatment approaches and their efficacy, alongside the latest innovations in therapy, including emerging biomarkers and targeted therapies. By providing a comprehensive overview of these topics, this article aims to enhance understanding and guide the pathological diagnosis and clinical management of this devastating disease.

Introduction

General

Cholangiocarcinoma (CCA) arises from the biliary epithelium. Intrahepatic CCA (iCCA) originates within the liver from small ducts or ductules. Perihilar CCA (pCCA) occurs in the hilar region near the bifurcation of the common bile duct. Distal CCA (dCCA) is situated distal to the cystic duct insertion on the biliary tree. Unfortunately, the diagnosis of CCA most frequently occurs at advanced stages of the disease (Ilyas and Gores 2013).

Much like the unexpected revelation in Alexandre Dumas' novel where the three musketeers are four,

studies on CCA reveal that what appears to be one disease is, in fact, at least four distinct conditions, each with unique characteristics and therapeutic targets. Expanding the understanding of morphological, immunohistochemical, and molecular profiles is essential for advancing disease classification and developing targeted therapeutic interventions.

Epidemiology

CCA is considered a rare neoplasm, accounting for 3% of all malignancies arising in the gastrointestinal tract and 10–20% of all primary liver cancers. CCA is known for its aggressive nature, as the disease is often diagnosed in an advanced stage (Siegel et al. 2023).

Most CCAs are sporadic, with approximately 10–15% associated with inherited cancer predisposition syndromes. Currently, genetic testing for CCAs is not an established medical recommendation due to insufficient

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comprehensive data on the prevalence of germline mutations in this malignancy (Maynard et al. 2020).

Regarding CCA distribution, iCCA is the least common, accounting for 10–20% of all CCAs. The classic Klatskin tumor, represented by pCCA, corresponds to 50–60% of all CCAs. The remaining 20–30% of CCA cases are represented by dCCA. The term extrahepatic CCA (eCCA) encompasses both pCCA and dCCA (Fig. 1) (Vithayathil et al. 2022).

Misclassification is a significant issue that impacts CCA epidemiology and research, particularly in non-central health institutions, where pCCA (the majority of CCAs) is often incorrectly coded as iCCA. Similarly, iCCA can be misclassified as hepatocellular carcinoma (HCC), and eCCA can be mistakenly coded as gallbladder cancer (Cardinale 2019).

The incidence and mortality of iCCA are increasing globally while eCCA rates remain stable. In Western countries, this rising incidence of iCCA may be associated with obesity, diabetes, and steatotic liver disease. However, these trends may also be influenced by misclassification and should be interpreted with caution (Vithayathil et al. 2022).

The global distribution of CCA is heterogeneous, with higher rates observed in China and Thailand (Vithayathil et al. 2022). In most regions, the peak incidence of CCA

occurs in the seventh decade of life, affecting both genders with a slight male preponderance (male-to-female ratio of 2:1 to 3:1). Epidemiological studies focusing exclusively on CCA and its subtypes in Brazil are rare (Santos et al. 2019).

Risk factors and precursor lesions

Multiple studies have demonstrated that iCCA and eCCA share some risk factors, such as PSC, liver fluke infection, lithiasis, and tobacco and alcohol consumption. However, distinct risk factors have also been identified for each subtype. iCCA is additionally associated with hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, cirrhosis, and liver metabolic diseases, especially steatotic liver diseases. In contrast, eCCA is linked to choledochal cyst, and Lynch syndrome (Khan et al. 2019).

Common precursors of CCAs include intraductal papillary neoplasm of the bile duct (IPNB), biliary intraepithelial neoplasia (BillIN), and mucinous cystic neoplasm (MCN), particularly when they exhibit high-grade dysplasia (Nakanuma et al. 2022). These precursor lesions are more commonly associated with large duct intrahepatic iCCA (Nakanuma and Sudo 2017).

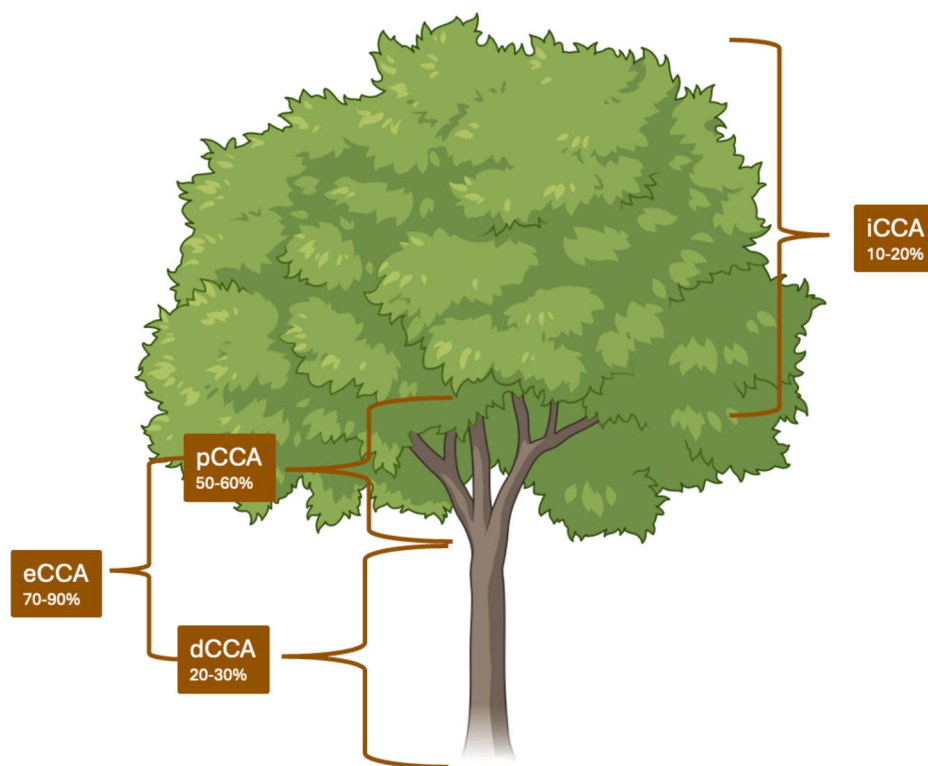


Fig. 1 Distribution of cholangiocarcinoma subtypes in the biliary tree and their frequency. iCCA: Intrahepatic cholangiocarcinoma; pCCA: perihilar cholangiocarcinoma; dCCA: distal cholangiocarcinoma (dCCA); eCCA: extrahepatic cholangiocarcinoma

Evolution of classifications used in CCA

The classification of primary liver neoplasms is a widely discussed topic in medical literature. Initially, anatomical localization served as the primary method for subclassifying CCAs. However, the classification has evolved to encompass variables such as tumor gross appearance, histologic findings, cell of origin, and more recently, molecular aspects.

In terms of gross appearance, iCCA typically presents as a solid, white, and compact mass-forming tumor (MF type). In contrast, pCCA and eCCA manifest in the hilar region and the extrahepatic biliary tree as either periductal infiltrating (PI type) or intraductal polypoid lesions (ID type). Macroscopic classification facilitates a close radiological correlation to morphological findings (Fig. 2) (Guedj 2022).

Histology demonstrates distinct differences between various types of CCAs (11). More often, dCCA, pCCA, and the large-duct type iCCA (located proximal to the hepatic hilar region) are mucin-producing adenocarcinomas characterized by large ducts and papillary morphologies with columnar cells. In contrast, iCCA located in the periphery of the hepatic parenchyma commonly exhibits tubular or acinar architectures with low columnar to cuboidal cells, organized in a small-duct pattern

associated with a desmoplastic stroma. The small duct type of iCCA includes two additional distinct subgroups: the ductal plate malformation pattern and cholangiolocarcinoma. The ductal plate malformation pattern of iCCA resembles ductal plate malformations (Nakanuma et al. 2012), while the cholangiolocarcinoma subgroup features a more primitive architecture that resembles immature cholangioles (Fig. 3) (Nakanuma and Kakuda 2015; Akita et al. 2017; Sempoux et al. 2011; Liau et al. 2014).

These tumors also have different cells of origin. Generally, peripheral and small-duct type iCCA arises from hepatic progenitor cells or cuboidal (mucin-negative) cholangiocytes, whereas pCCA, dCCA, and central iCCA originate from mature (mucin-producing) cholangiocytes that line the biliary tree (Sigel et al. 2018; Komuta et al. 2008, 2012).

Genomic profiling has identified several oncogenic alterations in CCAs. (Normanno 2022). Based on these molecular discoveries, the US Food and Drug Administration (FDA) has granted accelerated approval for tumor-target therapy independent of the type of the malignant neoplasm (pan-tumor therapy), and that encompasses patients with advanced CCA.

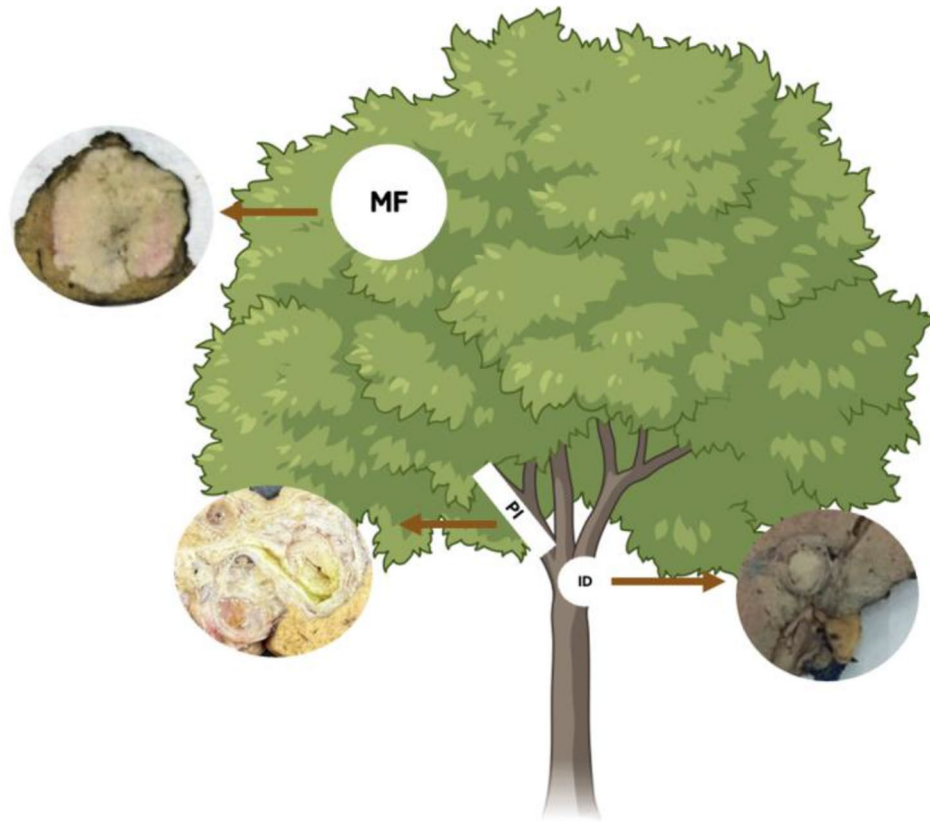


Fig. 2 Macroscopic appearance of cholangiocarcinoma subtypes. MF: mass-forming tumor, PI: periductal infiltrating tumor, ID: intraductal polypoid tumor

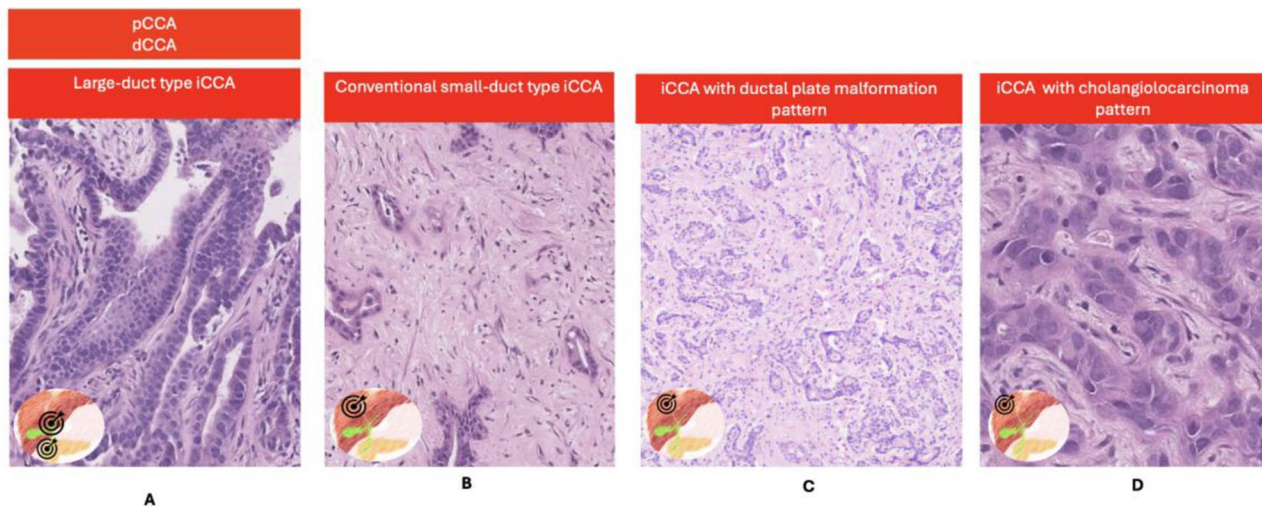


Fig. 3 Most common histological subtypes of cholangiocarcinoma and their location in the biliary tree: **A**) Peri-hilar cholangiocarcinoma, distal cholangiocarcinoma and intrahepatic cholangiocarcinoma, large-duct type (H&E, 200X); **B**) Intrahepatic cholangiocarcinoma, small-duct type (H&E, 200X); **C**) Intrahepatic cholangiocarcinoma with ductal plate malformation pattern (H&E, 100X); **D**) Intrahepatic cholangiocarcinoma with cholangiolocarcinoma pattern (H&E, 100X)

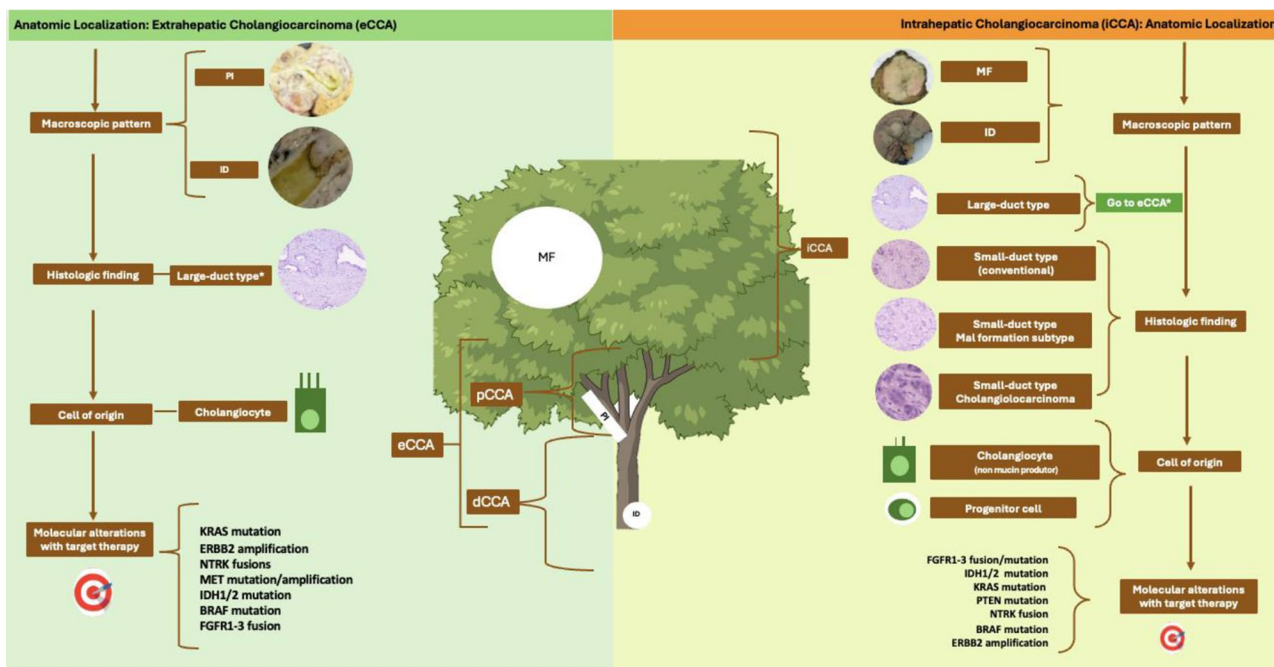


Fig. 4 Different aspects of cholangiocarcinoma including anatomic localization, microscopic pattern, main histologic finding, cell of origin and molecular alterations with target therapy. iCCA: intrahepatic cholangiocarcinoma, pCCA: perihilar cholangiocarcinoma, dCCA: distal cholangiocarcinoma (dCCA), eCCA: extrahepatic cholangiocarcinoma, MF: mass-forming tumor, PI: periductal infiltrating tumor, ID: intraductal polypoid tumor

The literature contains numerous efforts to develop comprehensive description of the three entities (iCCA, pCCA, dCCA) (Fig. 4). Many of these aspects are considered in the World Health Organization classification of digestive system tumors (5th edition) that accepts two additional subtypes for iCCA: a small-duct type, which shares many etiologic, pathogenetic, and imaging characteristics with HCC, and a large-duct type resembling

eCCA and pancreatic ductal adenocarcinoma (Nagtegaal et al. 2020).

Does the patient have CCA?

Confirming the presence of CCA is a critical diagnostic step before making therapeutic decisions. For the diagnosis of CCA, the exclusion of metastatic adenocarcinomas

is essential, as these represent the most prevalent malignant neoplasms in the liver parenchyma. (Fig. 5).

This diagnostic process integrates clinical history, imaging examinations, and histological analysis of both neoplastic and non-neoplastic tissue, including morphological evaluation and immunohistochemical (IHC) studies.

It is important to note that the histological findings and the immunohistochemical profile commonly observed in CCAs are not exclusive to this tumor group. The well-known pancreaticobiliary immunophenotype - characterized by positivity for cytokeratin 7 and 19 combined with negativity for cytokeratin 20 and markers indicative of primary sites outside the biliary system - is shared by adenocarcinomas originating from the intrahepatic and extrahepatic biliary tree, gallbladder, pancreas, and upper gastrointestinal tract (Bellizzi 2020).

Certain immunohistochemical markers, such as S100P, NCAM, N-cadherin, and CRP can aid in distinguishing different subtypes of CCAs. S100P is often expressed in large-duct type adenocarcinomas and can help differentiate these from other subtypes. NCAM (CD56) is typically expressed in cholangiolocarcinomas. Additionally,

N-cadherin and CRP are useful markers in conventional small-duct type of iCCA, helping to differentiate this tumor from metastatic adenocarcinomas (Akita et al. 2021; Hayashi et al. 2016).

Recently, in situ hybridization for albumin has emerged as a valuable tool for distinguishing between liver metastases of adenocarcinoma and iCCA (particularly of the small duct morphology, which shows positive staining for this marker). It is crucial to note that albumin expression is also observed in HCC and is described in other rare adenocarcinomas with hepatoid differentiation (Chung et al. 2023).

Predictors of survival in CCA

Predicting survival in individual patients is complex, requiring the evaluation of multiple factors. Early-stage tumors generally carry a more favorable prognosis compared to advanced-stage tumors. Presence of metastasis to distant organs is associated with poor prognosis.

Performance status is a relevant factor because it reflects the patient's overall health and ability to tolerate treatment. Concurrent liver diseases significantly impact

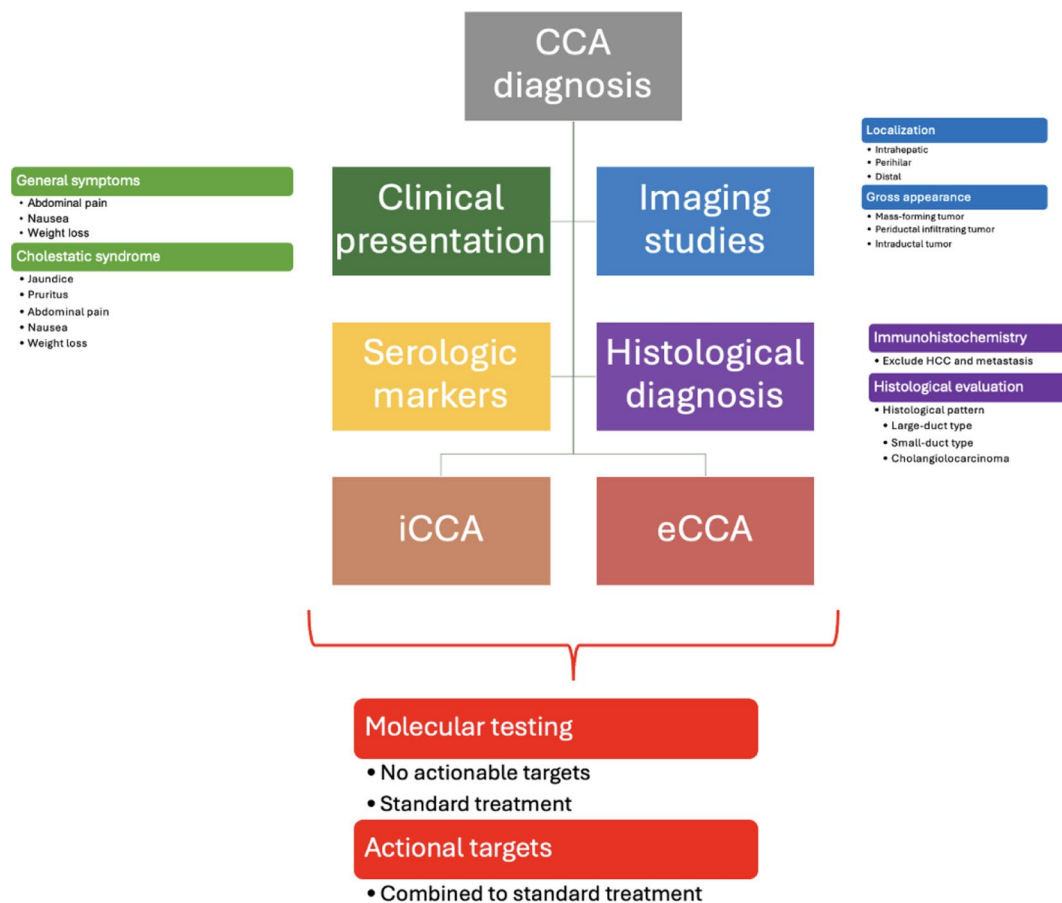


Fig. 5 Algorithm for diagnose and treatment of cholangiocarcinoma. CCA: cholangiocarcinoma, iCCA: intrahepatic cholangiocarcinoma; eCCA: extrahepatic cholangiocarcinoma

treatment modalities and prognosis in CCA patients. High levels of CA19.9 often indicate poorer outcomes.

The pathology report, particularly from surgical specimens, provides critical information about the tumor, including its localization, size, gross appearance, histological subtype, presence of lymphovascular and perineural invasion, surgical margins and regional lymph node status. For instance, patients with resectable tumors (with negative surgical margins) have better outcomes than those with unresectable tumors. It is also important to note that in the recent eighth American Joint Commission on Cancer (AJCC) edition, iCCA, pCCA, and dCCA have different staging systems (Amin et al. 2017).

Recent studies suggest that the histological subtype of iCCA also have an impact on survival. Patients with small-duct type (mass-forming) iCCA have better survival rates compared to those with large-duct type iCCA. Additionally, there is evidence that cholangiolocarcinoma is associated with even better survival outcomes. Patients with large-duct type iCCA have prognosis more similar to patients with pCCA (Akita et al. 2017).

Impact of pathology on treatment approach

The management of biliary tract cancers is rapidly evolving, driven by the exponential discovery of target therapies recently focused on fibroblast growth factor receptor-2 (FGFR-2) fusions, isocitrate dehydrogenase-1 (IDH-1) mutations, B-Raf proto-oncogene serine/threonine kinase (BRAF V600E) mutations, neurotrophic tyrosine receptor kinase (NTRK) fusions, human epidermal growth factor-2 (ERBB2/HER2) amplifications, and microsatellite instability. An early assessment of the molecular profile allows for adequate therapy planning (Lamarca et al. 2022; Tomczak et al. 2022).

Surgical resection followed by adjuvant chemotherapy with capecitabine is considered the primary treatment for early-stage CCA, particularly for patients with good liver function (Primrose et al. 2019). Hepatectomy is the main surgical approach for iCCA, while surgical options for eCCA include bile duct resection or pancreaticoduodenectomy (Whipple procedure) (Cillo et al. 2019). Chemoradiotherapy may be considered for patients with unresectable disease or lymph node involvement.

In selected centers, liver transplantation (LT) may be considered a treatment option for early stage iCCA or pCCA, especially for patients with underlying liver chronic disease or cirrhosis. LT is typically integrated with other treatment modalities (Mazzaferro et al. 2020).

Systemic chemotherapy is employed for advanced or metastatic disease. Since 2010, cisplatin in combination with gemcitabine has emerged as a standard of care based on findings from the open-label phase III trial (ABC-02) involving 410 patients with locally advanced or metastatic biliary tract tumors (64% CCA). This trial

demonstrated significant improvements in overall survival (OS) and progression-free survival (PFS) compared to treatment with gemcitabine alone (Valle et al. 2010).

Two phase III studies analyzed the addition of immunotherapy to the standard chemotherapy. TOPAZ-1 trial investigated the addition of durvalumab to gemcitabine plus cisplatin in 685 patients with treatment-naive advanced or metastatic biliary tract tumors (75% CCA) and no hyperbilirubinemia and has shown significantly improved patient OS. The estimated 24-month OS rate was 24.9% for durvalumab and 10.4% for placebo group, with durable responses (Rimini et al. 2023).

The KEYNOTE-966 trial evaluated the addition of pembrolizumab to gemcitabine plus cisplatin in 1069 patients with previously untreated locally advanced or metastatic biliary tract cancer (78% CCA) and showed improvements in OS, with a median overall survival of 12.7 months in the pembrolizumab group versus 10.9 months in the placebo group (Kelley et al. 2023). Both regimens are considered appropriate as first-line treatment (category 1 by NCCN 2023) (Benson et al. 2023).

Second-line therapy with short-term infusional 5-fluorouracil plus leucovorin and oxaliplatin (FOLFOX) after progression with gemcitabine plus cisplatin is considered the preferred regimen, based on ABC-06 trial. In this phase III trial, FOLFOX was associated with significantly better rates of OS compared with active symptom control (Lamarca et al. 2021).

Patients who are not candidates for surgical and systemic therapies are managed by palliative radiation therapy (RT), RT with concurrent fluoropyrimidine, and palliative care.

Multiple genomic alterations have been identified in CCAs, some of which are now targetable with newly discovered medications. These therapies have the potential to significantly alter the landscape of CCA treatment in the future (Longerich et al. 2024).

New tools in the management of CCAs

Breakthroughs in molecular alterations are linked to emerging tools that influence the management of patients suffering from advanced CCA. For every emerging tool, we elucidate the involved gene (nomenclature, chromosomal localization, type and frequency of molecular alterations; Table 1), its physiological function, detectable molecular alterations in CCA, the aberrant function resulting from genetic and epigenetic events, available molecular testing methodologies, and the corresponding approved target therapy.

Among available molecular testing methodologies, IHC studies are the most cost-effective, employing specific antibodies for initial screening of protein abnormalities. Polymerase chain reaction (PCR)-based testing can be utilized to detect well-known genetic alterations in small

Table 1 Comprehensive gene characteristics and molecular alterations in Cholangiocarcinomas (CCAs)

Gene symbol	Gene approved nomenclature*	Gene name	Chromosomal location	Type of molecular alterations in CCAs**	Frequency of molecular alterations in CCAs**
IDH1	IDH1	Isocitrate Dehydrogenase 1	2q33.3	Mutation	10–20% of iCCA***
IDH2	IDH2	Isocitrate Dehydrogenase 2	15q26.1	Mutation	~5%**
FGFR1	FGFR1	Fibroblast Growth Factor Receptor 1	8p11.23	Mutation > amplification > deep deletion	-
FGFR2	FGFR2	Fibroblast Growth Factor Receptor 2	10q26	Structural variation > mutation > amplification	95 – 15% of iCCA***
FGFR3	FGFR3	Fibroblast Growth Factor Receptor 3	4p16.3	Amplification > deep deletion > mutations	~3%**
FGFR4	FGFR4	Fibroblast Growth Factor Receptor 4	5q35.1	Mutation > amplification > deep deletion	-
NTRK1	NTRK1	Neurotrophic Tyrosine Receptor Kinase	1q23.1	Amplification (13.89%) > Mutation (2.78%)	< 1%***
NTRK2	NTRK2	Neurotrophic Tyrosine Receptor Kinase	9q21.33	Mutation	< 1%***
NTRK3	NTRK3	Neurotrophic Tyrosine Receptor Kinase	15q25.3	Mutation	< 1%***
PD-L1	CD274	Programmed Death-Ligand 1	9p24.1	Amplification > mutation	-
PD-1	PDCD1	Programmed Cell Death Protein 1	2q37.3	Mutation > amplification > deep deletion	-
CTLA-4	CTLA4	Cytotoxic T-Lymphocyte-Associated Protein 4	2q33.2	Mutation	-
MLH1	MLH1	MutL Homolog 1	3p21.31	Mutation	1-3%***
MSH2	MSH2	MutS Homolog 2	2p22.2	Mutation	1-3%***
MSH6	MSH6	MutS Homolog 6	2p16.3	Mutation	1-3%***
PMS2	PMS2	Postmeiotic Segregation Increased 2	7p22.1	Mutation	1-3%***
HER2	ERBB2	erb-b2 receptor tyrosine kinase 2	17q21.1	Amplification > mutation	5–20% of CCAs***
BRAF	BRAF	B-Raf proto-oncogene, serine/threonine kinase	7q34	Mutation > amplification	6.55% of iCCA**
RET	RET	ret proto-oncogene	10q11.21	Mutation = amplification	< 1%***
KRAS	KRAS	KRAS proto-oncogene, GTPase	12p12.1	Mutation > amplification	40% of eCCA****

*According to HUGO Gene Nomenclature Committee

**Based on cBioPortal for Cancer Genomics website

*** Based on NCCN guidelines, Biliary Trac Cancers, Version 2.2 (34)

**** (Subbiah et al. 2023)

panels. Fluorescence in situ hybridization (FISH) can demonstrate fusions and amplifications. Next-generation sequencing (NGS) panels enable simultaneous analysis of multiple genes. These molecular studies allow for comprehensive assessment across multiple targets, aiding oncologists in treatment decision-making and potentially facilitating patient enrollment in ongoing clinical trials.

The amount of viable tumor required for testing varies depending on the technique used. For IHC studies, as low as 10% tumor content is sufficient, as the analysis focuses on protein expression. For PCR-based testing, a minimum of 20% tumor content is often recommended to ensure sufficient DNA yield from the tumor cells. FISH analysis typically requires at least 50–60% tumor content to accurately detect chromosomal abnormalities. For NGS panels, which provide a larger genetic profile, the percentage of tumor cells should ideally be 20–30% or higher, depending on the sequencing platform's sensitivity. This ensures that the genetic material analyzed

predominantly represents the tumor, allowing for precise detection of mutations and other genomic alterations (da Cunha et al. 2021).

These tests can be performed on formalin-fixed, paraffin-embedded tissues. NGS is specifically recommended by NCCN and the European Society for Medical Oncology (Mosele et al. 2020).

It is important to emphasize that CCAs are not always amenable to biopsy because of their localization or involvement with anatomic structures. Additionally, even when a biopsy is feasible, it may not yield sufficient tumor samples for all necessary ancillary tests (Lamarca et al. 2020). In such cases, collaboration between pathologists and oncologists is essential to prioritize tests and optimize the use of available tissue.

IDH1/2 inhibitors

Normal function

IDH1 is found in the cytosol and peroxisomes, while IDH2 is a mitochondrial enzyme. Both IDH1 and IDH2 play crucial roles in the citric acid cycle, also known as the Krebs cycle, which is a central pathway for energy production in cells. IDH1/2 enzymes participate in the conversion of isocitrate to alpha-ketoglutarate, generating NADPH and maintaining the balance of reactive oxygen species in the cell. NADPH in the liver is also important for metabolic processes including synthesis of fatty acids and cholesterol (Molenaar and Wilmink 2022).

Molecular alteration and frequency in CCA

Mutations in IDH1 are hotspot mutations with a broader spectrum in CCA involving various amino acid substitutions. The most common IDH1 mutations in CCA are R132C, which accounts for approximately 50% of IDH1 mutations, followed by R132S (around 20%) and R132G (about 10%). Mutation in IDH2 involves substitution of arginine at position 172 originating the mutant protein IDH2 R172. IDH1 mutations are detected in approximately 10–20% of iCCA and are rare in pCCA and dCCA. IDH2 mutation is rare in all CCA subtypes (Lowery et al. 2018).

Abnormal function

The neomorphic, mutant enzyme, converts alpha-ketoglutarate to the oncometabolite 2-hydroxyglutarate (2HG) both in the cytosol and in the mitochondria. This metabolite causes inhibition of the activity of Ten-Eleven Translocation (TET) enzymes and histone lysine demethylases (KDM). The result is global epigenetic modifications on DNA and histones originating in a hypermethylated phenotype (Golub et al. 2019). The oncometabolite 2HG is particularly relevant for iCCA development because high levels of 2HG block the gene HNF4 alpha and, without a functioning HNF4 transcription factor, the hepatic progenitor cell cannot differentiate into hepatocytes. This phenomenon added to mutations in KRAS induce cell expansion and formation of CCA (Saha et al. 2014).

Molecular testing methods

(1) IDH1 immunohistochemistry is not recommended in CCA cases. The IDH1 R132H mutation is the most prevalent hot spot mutation in gliomas and is targeted by the commonly used IDH1 antibody. However, the spectrum of IDH1 mutations in CCA is broader and often involves different amino acid substitutions. The most common IDH1 mutations in CCA are R132C (approximately 50% of IDH1 mutations), R132S (around 20%), and R132G (about 10%). These mutations are not detected by the IDH1 R132H-specific antibody. Therefore, the use of this

antibody in CCA is not appropriate for accurate mutation detection and may lead to false-negative results (Rimini et al. 2022; Makawita et al. 2024), (2) PCR-based testing can be utilized to amplify and detect specific IDH1 or IDH2 mutation hotspots, and (3) NGS panels can simultaneously analyze multiple genes, including IDH1/2. Testing should be performed for unresectable or metastatic CCA (subsequent-line therapy if disease progression (Benson et al. 2023).

Target therapy

A multicentre, randomized, double-blind, placebo-controlled, phase III study (ClarIDHy) investigated the efficacy and safety of Ivosidenib (AG-120), a first-in-class, oral, small-molecule inhibitor of mutant IDH1 in patients with advanced CCA. This study included 187 previously treated patients with CCA and IDH1 mutation and resulted in numerically improved overall survival benefits vs. placebo (median OS 10.3 vs. 7.5 months), despite a high rate of crossover. Based on this trial, Ivonidenib has been approved by the FDA and Anvisa (Agência Nacional de Vigilância Sanitária in Brazil) for the treatment of adults with previously treated, locally advanced, or metastatic CCA (Abou-Alfa et al. 2020a).

FGFR inhibitors

Normal protein function

FGFRs belong to a family of cellular transmembrane receptors. All proteins share similar structures consisting of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. FGFRs act in various cellular processes such as cell proliferation, differentiation, migration, and survival. Upon ligand binding, FGFRs undergo dimerization and autophosphorylation, activating downstream signaling pathways such as the MAPK/ERK and PI3K/AKT pathways (Krook et al. 2021).

Molecular alteration and frequency in CCA

Molecular alterations include single-nucleotide variants, copy number amplifications, and gene fusions. FGFR2 fusions and rearrangements are found almost exclusively in iCCA, occurring in 10–16% of patients. In iCCA, the most frequent partner genes involved in FGFR2 fusions are BICC1 (Bicaudal C Homolog 1), AHCYL1 (Adenosylhomocysteinase Like 1), KIAA1217, PPHLN1, and TACC3 (Transforming Acidic Coiled-Coil Containing Protein 3) (Borad et al. 2014).

Abnormal function

The abnormal function allows constitutive activation of FGFR signaling pathways (MAPK/ERK and PI3K/AKT) leading to addictive proliferative advantage to the tumor cell, uncontrolled cell proliferation, survival,

and angiogenesis. The signaling for activation can occur through various mechanisms such as ligand-independent activation (gene fusion can directly affect the conformation of the FGFR protein, resulting in its activation without the need for binding to fibroblast growth factors (FGFs), the natural ligands for FGFRs), autocrine/paracrine signaling (tumor cells or neighboring stromal cells may produce and secrete FGF ligands, which can then activate FGFRs in an autocrine/paracrine manner), amplification and overexpression (increase the number of FGFR receptors on the cell surface, thereby enhancing the sensitivity of cells to FGF ligands present in the microenvironment).

Molecular testing methods

(1) Despite its utility as a screening tool, IHC is not suitable for detecting FGFR2 fusions in iCCA because robust antibodies for all targets are not always available, since different fusion partners may affect the result (Saborowski et al. 2020); (2) PCR-based testing can be utilized to detect specific FGFR fusion transcripts. However, the increasing number of fusion partners necessitates the design and use of numerous different primer pairs in a single assay to screen for FGFR2 fusions (Saborowski et al. 2020); (3) Break-apart FISH is suitable for detecting both known and novel gene fusions but does not identify the fusion partner (Saborowski et al. 2020); and (4) NGS panels can simultaneously analyze multiple genes, including FGFR genes. Testing should be performed for unresectable or metastatic CCA (subsequent-line therapy if disease progression) (Benson et al. 2023).

Target therapy

Targeting FGFR2 fusions and rearrangements with specific inhibitors has emerged as a promising therapeutic strategy in CCA treatment. In two single-arm, phase 2 studies (FIGHT-202 and NCT02150967), the use of Pemigatinib and Infigratinib respectively, oral inhibitors of FGFR 1–3 selective, have shown efficacy in objective response in patients previously treated and have been approved in this scenario (Abou-Alfa et al. 2020b). Phase III studies (FIGHT-302 and NCT03773302) are now ongoing to compare this targeted therapy versus standard chemotherapy. Futibatinib, a next-generation irreversible FGFR1–4 inhibitor, has shown clinical benefit and improved objective response rates and it has also been approved by the FDA for the treatment of locally advanced/metastatic iCCA after one or more systemic therapy (Goyal et al. 2023). The use of Erdafitinib, an oral selective pan-FGFR tyrosine kinase inhibitor, remains investigational in this setting.

NTRK inhibitors

Normal protein function

These proteins have important roles in neuronal development, cell survival, and proliferation

Molecular alteration and frequency in CCA

Mutations can be found, but fusions involving NTRK are the most common mechanism of oncogenic activation. According to available literature, the prevalence of NTRK gene alterations in CCA ranges is rare (<1%), necessitating physicians' understanding of managing 'rare-alteration-in-a-rare-disease' scenarios (Boilève et al. 2021). Specific data on the prevalence of NTRK gene alterations in iCCA, pCCA, and dCCA individually are not readily available nor consistent across studies.

Abnormal function

The abnormal function permits constitutive activation of signaling pathways (MAPK/ERK and PI3K/AKT), and alteration of cellular processes including differentiation, migration, apoptosis, tumor progression and metastasis since dysregulated NTRK function can promote epithelial-to-mesenchymal transition increasing tumor aggressiveness and dissemination to distant sites.

Molecular testing methods

(1) IHC may detect overexpression of the TRK protein and can be used as initial screening for NTRK fusions, but it can produce false-positive results necessitating confirmation with other techniques (Zhang et al. 2022); (2) PCR-based testing and FISH can demonstrate rearrangements, and (3) NGS panels can simultaneously analyze multiple genes, including NTRK genes. Testing should be performed for unresectable or metastatic CCA (primary treatment or subsequent-line therapy if disease progression) (Benson et al. 2023).

Target therapy

Larotrectinib, a highly selective tropomyosin receptor kinase (TRK) inhibitor has shown efficacy in a combined analysis of 55 patients with various TRK fusion-positive malignancies enrolled in three trials, including patients with CCA tumors, and is approved by the FDA for solid tumors with an NTRK gene fusion and without a known acquired resistance mutation that is either metastatic or irresectable and has progressed following treatment (Drilon et al. 2018). Similarly, Entrectinib, another TRK inhibitor, has received FDA approval for ten types of cancers with an NTRK gene fusion, including CCA, based on combined analysis from three single-arm clinical trials (Drilon et al. 2018).

Immune checkpoint inhibitors (ICIs)

Normal protein function

PD-L1 is a critical immune checkpoint protein involved in regulating the immune response by interacting with the PD-1 receptor on T cell lymphocytes, causing T cell inhibition and immune evasion by tumor cells. CTLA-4 is expressed in the activated T cells to inhibit T cell activation by competing for binding of the CD28 ligand (Zeng and Chen 2021).

Molecular alteration and frequency in CCA

Studies have reported varying levels of PD-L1 expression in iCCA (tumor or tumor plus immune cell PD-L1 expression), and the reported prevalence ranges from 10 to 40% depending on the cutoff criteria used for defining positivity. Data on PD-L1 expression in pCCA and dCCA are limited (Zeng and Chen 2021).

Abnormal function

PD-L1 is often upregulated in tumor cells and the cells of the microenvironment as a mechanism of immune evasion. When PD-L1 on tumor cells binds to its receptor PD-1 on T cells, it inhibits the cytotoxic activity of T cells and induces T cell exhaustion, reducing antitumor immune response. This strategy allows tumor cells to evade immune surveillance and promote tumor growth and metastasis.

Molecular testing methods

(1) IHC is a widely used method for assessing PD-L1 protein expression in different tumor tissue samples. Although PD-L1 expression is a potential biomarker for immunotherapy response, there is no specific companion diagnostic test approved for CCA. Various studies have employed different antibodies and scoring systems (CPS or TPS). The pathologist should consult the oncologist to select the appropriate antibody. The anatomical report should include a comment stating that the applied score is used in the absence of a specific protocol for PD-L1 testing in CCA (Fontugne et al. 2017; Sangkhamanon et al. 2017; Ahn et al. 2020; Matsumoto et al. 2022) (2) PCR-based testing can be utilized to detect specific PD-L1 alterations, such as mutations or gene rearrangements, (3) FISH can be employed to detect gene amplification or copy number alterations involving the PD-L1 gene, and (4) NGS panels can be used to analyze the entire genomic sequence of the PD-L1 gene and identify various types of alterations, including mutations, amplifications, deletions, and structural variants. Studies are insufficient to warrant a recommendation for testing (Benson et al. 2023).

Target therapy

Phase III studies have demonstrated the efficacy of checkpoint inhibitors like Durvalumab and Pembrolizumab in metastatic CCA, with benefits observed regardless of PD-L1 status. This suggests that additional biomarkers may be important for predicting response rates in CCA treatment (Rimini et al. 2023; Kelley et al. 2023).

Microsatellite instability-high (MSI-H), deficient mismatch repair (dMMR), and tumor burden high (TMB-H) profiles

Normal protein function

The respective proteins are essential in the DNA mismatch repair pathway

Molecular alteration and frequency in CCA

Molecular alterations in CCA that cause MSI-H/dMMR profile include: (1) silencing of MLH1 gene expression due to hypermethylation of its promoter region, (2) mutations in any of the MMR genes (MLH1, MSH2, MSH6, or PMS2), and (3) epigenetic mechanisms, such as histone modifications or altered expression of microRNAs. Reported frequencies of MSI-H/dMMR in CCA subtypes are relatively low compared to other cancer types. The frequencies of dMMR and MSI-H in CCA are 0 to 9.4 and 0-18%, respectively (Ando et al. 2022).

Abnormal function: dMMR causes

(1) accumulation of errors in repetitive DNA sequences known as microsatellites; (2) elevated mutation rate that contributes to tumor heterogeneity and progression; and (3) formation of neoantigens generated by frame-shift mutations that lead to immune recognition and activation.

Molecular testing methods

The tests commonly used to assess dMMR and MSI-H status include: (1) IHC demonstrating loss of expression of one or more MMR proteins; (2) PCR-based testing using the Bethesda panel which includes a set of five mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) and two pentanucleotide repeat markers (Penta C and Penta D). MSI-H is defined by the presence of novel or shifted bands in the tumor sample compared to the normal sample; and (3) NGS can detect mutations and alterations in MMR genes and assess MSI status by analyzing microsatellite regions across the genome. Testing should be performed for unresectable or metastatic CCA (primary treatment or subsequent-line therapy if disease progression) (Benson et al. 2023).

Targeted therapy

The efficacy of pembrolizumab in dMMR advanced CCA was shown in the phase II KEYNOTE-158 study.

Retrospective evaluation of 149 individuals with various solid tumors and DNA repair enzyme deficiency treated with pembrolizumab resulted in an objective response rate of 40.9% among 22 cases of CCA, confirming that immunotherapy is active in this histology. The Keynote-158 study led to the FDA approval of pembrolizumab for tumors with high mutational burden (≥ 10 mutations/megabase) or MSI-H/dMMR that had progressed following prior treatment, and for which there were no satisfactory alternative treatment options, the first such approval of a tissue-agnostic anticancer treatment (Marabelle et al. 2020). The combined regime with Nivolumab plus Ipilimumab was evaluated in a phase II nonrandomized clinical trial that included patients with advanced rare cancers, including 39 patients with biliary tract cancers. Responses were exclusively observed in patients with intrahepatic CCA and gallbladder carcinoma. Based upon this study, a trial of nivolumab plus ipilimumab is reasonable in the setting of second-line therapy for patients with iCCA who do not have dMMR, high levels of TMB, or PD-L1 overexpression, and who did not receive frontline immunotherapy (Klein et al. 2020).

ERBB2 (HER2) inhibitors

Normal protein function

ERBB2 (HER2) is a member of the epidermal growth factor receptor (EGFR) family. It functions as a receptor tyrosine kinase and activates intracellular signaling pathways upon binding with specific ligands, allowing cell growth, proliferation, and survival.

Molecular alteration and frequency in CCA

ERBB2 (HER2) was amplified in 3.9–8.5% and activating ERBB2 mutations was shown in 2% of CCAs (Czuderna et al. 2021). One study showed that alterations are present in 6.3% of eCCA cases and 0.6% of iCCA cases (Yan et al. 2015).

Abnormal function

Abnormal ERBB2 (HER2) protein signaling contributes to cancer progression by promoting cell proliferation, survival, and metastasis.

Molecular testing methods

(1) IHC may detect protein overexpression; it is important to note that there is no specific guideline to analyze ERBB2 (HER2) protein expression in CCAs, and in most circumstances, pathologists use the guideline developed for gastric and gastroesophageal junction adenocarcinoma as a reference (Fig. 6) (Shen et al. 2013; Galdy et al. 2017); (2) FISH can detect ERBB2 (HER2) gene amplification (Fig. 5); the detection of gene amplification by FISH follows guidelines similar to those used in breast cancer, as outlined by the American Pathologists (ASCO/CAP) guidelines (Wolff et al. 2018); and (3) NGS panels can simultaneously analyze multiple genes, including ERBB2 (HER2) genes. Testing should be performed for unresectable or metastatic CCAs (Benson et al. 2023).

Targeted therapy

For patients with advanced or metastatic ERBB2 (HER2) positive CCA who progress on chemotherapy, regimens based on trastuzumab plus pertuzumab or trastuzumab plus tucatinib have shown good objective response rates with an acceptable toxicity profile in two phase II basket

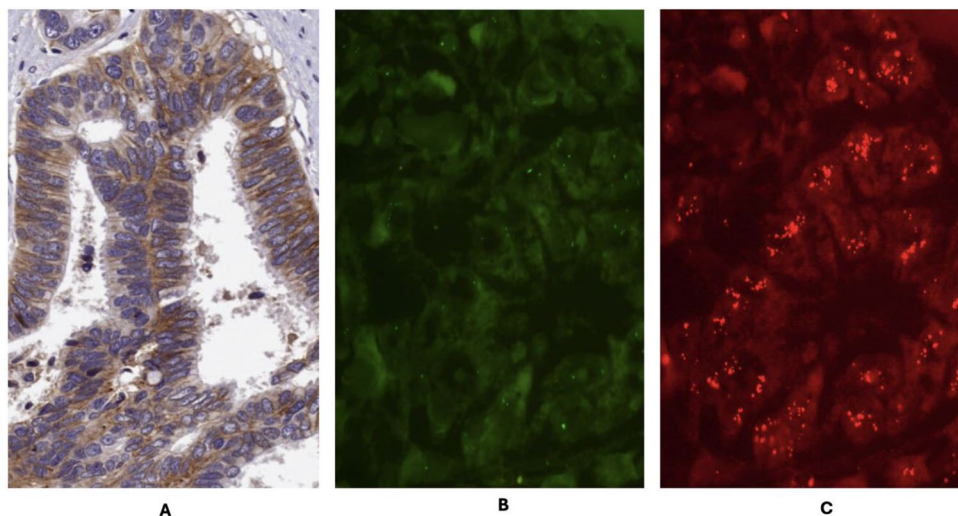


Fig. 6 Example of extrahepatic cholangiocarcinoma with amplification for ERBB2 (HER2) gene (ERBB2/CEP17): ≥ 2 . **A)** Score 3+ for ERBB2 (HER2) oncoprotein immunohistochemistry (IHC) pattern in biopsy specimen (following criteria used in the ToGA trial for scoring ERBB2 (HER2) oncoprotein expression by IHC in gastric and gastroesophageal junction adenocarcinoma; **B)** CEP17; **C)** ERBB2 (HER2) gene demonstrating amplification

studies (MyPathway and SGNTUC-019). These regimens are preferred options for late-line treatment in this setting (Javle et al. 2021). The efficacy of trastuzumab-deruxtecan (T-DXd), a ERBB2 (HER2)-directed antibody-drug-conjugate in treatment-refractory CCA has been demonstrated in two phase II clinical trials (HERB and DESTINY-PanTumor02). It has FDA approval for patients with unresectable or metastatic ERBB2 (HER2)-positive (IHC 3+) solid tumors who have received prior systemic treatment and have no satisfactory alternative treatment options (Ohba et al. 2022). However, patients should be cautioned about the risk of interstitial lung disease with this agent.

BRAF inhibitors

Normal protein function

BRAF encodes a protein called B-Raf, which is a serine/threonine kinase involved in the RAS-RAF-MEK-ERK signaling pathway. This pathway regulates cell growth, proliferation, and differentiation.

Molecular alteration and frequency in CCA

BRAF mutations are relatively rare in CCAs, occurring in approximately 1–4% of cases. The most common mutation is the V600E substitution (Chakrabarti et al. 2020; Li et al. 2020).

Abnormal function

The V600E mutation leads to constitutive activation of the BRAF kinase, resulting in dysregulated cell signaling and increased cell proliferation.

Molecular testing methods

(1) One study reveals that VE1 IHC is an approach to screen for BRAF V600E mutation in CCA, facilitating the detection of rare patients who may benefit from BRAF mutation-targeted therapies (Goepfert et al. 2014), and (2) PCR or NSG can simultaneously analyze multiple genes, including BRAF. Testing should be performed for unresectable or metastatic CCAs (Benson et al. 2023).

Target therapy

A phase II basket trial (ROAR) including 43 patients with BRAF V600E-mutated biliary tract showed that the combination of dabrafenib (BRAF inhibitor) and trametinib (MEK inhibitor) appears to be effective on objective response rate in the later line scenario. Based on this trial, the combination of dabrafenib and trametinib was approved from the FDA for the treatment of adult and pediatric patients one year of age and older with unresectable or metastatic solid tumors carrying mutations in BRAF V600E following prior treatment and with no satisfactory alternative treatment options (Subbiah et al. 2023).

RET inhibitors

Normal protein function

RET encodes a receptor tyrosine kinase involved in cell growth, differentiation, and survival, particularly in the development of neural crest-derived tissues.

Molecular alteration and frequency in CCA

RET fusions are found in a subset of CCAs, occurring in approximately 1–2% of cases (Adashek et al. 2021).

Abnormal function

RET fusions result in constitutive activation of the RET kinase, activating aberrant signaling pathways involved in cell proliferation and survival.

Molecular testing methods

Detection of RET fusions typically involves molecular techniques such as FISH, reverse transcription PCR (RT-PCR), or NGS. Testing should be performed for unresectable or metastatic CCAs (Benson et al. 2023).

Target therapy

The phase I/II basket trial LIBRETTO-001 analyses activity in the RET fusion-positive tumour-agnostic population, including CCA, using the RET tyrosine kinase inhibitor selpercatinib (Subbiah et al. 2022). Based on this trial the FDA approved Selpercatinib for adult patients with locally advanced or metastatic solid tumors, with a RET gene fusion and disease progression on or following prior systemic treatment who have no satisfactory alternative treatment options.

KRAS G12C inhibitors

Normal protein function

KRAS protein acts in cell signaling pathways such as signal transduction, cell growth and division, differentiation, apoptosis regulation, and integration between cell and environment.

Molecular alteration and frequency in CCA

In one study KRAS mutations were identified in almost 40% of eCCA (Montal et al. 2020).

Abnormal function

Mutant KRAS protein is associated with oncogenic activity, resistance to apoptosis, increased metastatic potential, altered cellular differentiation, and dysfunctional interaction with the microenvironment.

Molecular testing methods

(1) As molecular testing is performed on formalin-fixed paraffin-embedded samples, immunodetection appears to be an attractive alternative for detecting mutations. However, when tested on colorectal tumor samples with

known KRAS status, the KRAS polyclonal antibody showed poor sensitivity and specificity for detecting KRAS mutations (Piton et al. 2015). The literature on this technique for CCA is limited and IHC is not used for this investigation Akita, and (2) PCR-based assay or NGS is performed in this setting. In CCA, testing is not recommended (Benson et al. 2023).

Target therapy

There has been no approved therapy specificity targeting KRAS mutations in CCA until now. Patients with treatment-refractory metastatic CCA that harbors a KRAS G12C mutation should be encouraged to enroll in clinical trials, where available. Adagrasib, an agent target KRASG12C, was evaluated in a phase II trial (KRYSTAL-1) of 64 patients with KRAS G12C mutated, treatment-refractory solid tumors of various histologies, excluding non-small cell lung cancer and colorectal cancer, which includes eight patients with CCA, have shown promising results with an objective response rate of 50% (Pant et al. 2023).

Other molecular alterations

Molecular alterations in genes such as MET, ROS1, and ALK are linked to FDA-approved treatment in other tumor types. They are rare alterations but may be individually and multidisciplinary discussed as options for patients with progressive and refractory metastatic CCAs.

Conclusion

The evolution in CCA studies mirrors the intriguing complexity found in Alexandre Dumas' novel, where the introduction of a fourth musketeer parallels new CCA subtypes. This analogy underscores that, much like Dumas' tale, what initially appears to be a single entity often conceals deeper intricacies. Thus, the study of CCA reveals a multifaceted and dynamic landscape. Understanding this disease's complexity is essential for advancing classification and developing more effective, targeted therapeutic interventions.

Unlike Dumas' novel, however, the story of CCA is far from complete. New discoveries continuously add layers to our understanding of this disease. To forge a more hopeful future, ongoing debates about classification standards, integration of clinical, radiological, pathological, and molecular data, enhanced patient selection for clinical trials, and improved access to molecular studies at diagnosis are essential (Cardinale et al. 2012; Cardinale et al. 2013).

Author contributions

Lidiane Vieira Marins wrote de manuscript and designed the diagrams. Cecília Souza Freire, Camila Motta Venchiarutti Moniz, and Antonio Hugo José Fróes

Marques Campos wrote the manuscript. The authors reviewed and approved the final manuscript.

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Competing interests

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