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REVIEW

# Role of molecular genetics in the clinical management of cholangiocarcinoma

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The incidence of cholangiocarcinoma (CCA) has steadily increased during the past 20 years, and mortality is increasing. The majority of patients with CCA have advanced or metastatic disease at diagnosis, and treatment options for unresectable disease are limited, resulting in poor prognosis. However, recent identification of targetable genomic alterations has expanded treatment options for eligible patients. Given the importance of early and accurate diagnosis in optimizing patient outcomes, this review discusses best practices in CCA diagnosis, with a focus on categorizing molecular genetics and available targeted therapies. Imaging and staging of CCAs are discussed, as well as recommended biopsy collection techniques, and molecular and genomic profiling methodologies, which have become increasingly important as molecular biomarker data accumulate. Approved agents targeting actionable genomic alterations specifically in patients with CCA include ivosidenib for tumors harboring *IDH1* mutations, and infigratinib and pemigatinib for those with *FGFR2* fusions. Other agents currently under development in this indication have shown promising results, which are presented here.

**Keywords:** extrahepatic cholangiocarcinoma, FGFR2, genomic profile, IDH1, intrahepatic cholangiocarcinoma

## INTRODUCTION

Cholangiocarcinomas (CCAs) are cancers of the biliary tree comprising 10%-25% of primary hepatic and 3% of all gastrointestinal malignancies.[1] CCAs are often considered together with gallbladder cancer,[1] although the latter is not discussed in this review. CCAs occurring in the liver (arising from ductules or segmental ducts) are classified as intrahepatic cholangiocarcinoma (iCCA), and those occurring in the perihilar (pCCA) or distal portions of the biliary tract (dCCA) are classified as extrahepatic cholangiocarcinoma (eCCA).[2,3] iCCAs are the least common, representing 10%-20% of CCA tumors.[2]

Risk factors for CCA include parasitic infections and comorbid biliary/hepatic disorders, such as hepatolithiasis and chronic hepatitis B or C, as well as non-alcoholic steatohepatitis (NASH), non-alcoholic fatty liver disease (NAFLD), obesity, diabetes, cirrhosis, and alcohol consumption.[1,2,4] The prevalence of CCA is generally low in high-income countries, where CCAs usually fall into the category of rare tumors, while CCA is much more common in parts of Asia (China and Thailand), where hepatobiliary flukes and hepatolithiasis are more prevalent.[1,3] However, the incidence of CCA has been steadily growing in most countries around the world over the past 20 years,[5] particularly that of iCCA.[3,6] This is especially true in Western countries because of the growing rates of obesity and related conditions, such as diabetes and NASH/NAFLD.[7,8] In addition, and unlike most other types of cancer, mortality has been increasing in patients with CCA in recent decades,[9] and liver and intrahepatic bile duct cancers are estimated to become the third most common cause of cancer death in the United States by 2040.[10] The lethality of CCA may be because it is often asymptomatic in its early stages, such that ~70% of patients have advanced or metastatic disease at diagnosis.[2] For these patients, prognosis is poor (median survival of 2.5-4.5 months without treatment), and treatment options are limited since these patients are not candidates for surgery.[3] The 30%-40% of patients who present with resectable disease usually undergo potentially curative radical resection, followed by adjuvant chemotherapy in

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**Table 1. Clinicopathological and molecular features of different CCAs[2]**

| | iCCA—CLC | iCCA—small duct type | iCCA—large duct type | pCCA or dCCA |
|---|---|---|---|---|
| Bile duct type (diameter) | Bile ductules (<15 μm) | Small, interlobular bile duct (15-300 μm) | Large, peribiliary glands (300-800 μm) | Hepatic, cystic, choledochal ducts (>800 μm) |
| Putative cell of origin | Human pluripotent stem cell/ductular reaction | Cuboidal cholangiocyte | Mucous cells and/or columnar cholangiocyte | Mucous cells and/or columnar cholangiocyte |
| Gross appearance | Mass forming | Mass forming | Periductal infiltrating (± mass forming) or intraductal growing | Periductal infiltrating or intraductal growing |
| Precancerous lesions | None | None | Biliary intraepithelial neoplasia, IPNB, ITPN, mucinous cystic neoplasm | Biliary intraepithelial neoplasia, IPNB, ITPN, mucinous cystic neoplasm |
| Underlying disease | Viral, cirrhosis | Viral, cirrhosis | Primary sclerosing cholangitis, liver flukes | Primary sclerosing cholangitis, liver flukes |
| Tissue markers[a] | NCAM | NCAM, N-cadherin, SMAD4, BAP1$^{loss}$ | Mucin,[b] MUC5AC, MUC6, S100P, SMAD4$^{loss}$, BAP1 | Mucin,[b] MUC5AC, MUC6, S100P, SMAD4$^{loss}$, BAP1 |
| Common mutations | *IDH1/2, FGFR2* fusions, *BAP1, BRAF, ARID1A, KRAS, TP53, SMAD4*[15] Increased *IDH1* and *TP53* | *IDH1/2, FGFR2* fusions, *BAP1, BRAF, ARID1A, KRAS, TP53, SMAD4* Increased *IDH1*/2 and *FRFR2* fusions | *IDH1/2, FGFR2* fusions, *BAP1, BRAF, ARID1A, KRAS, TP53, SMAD4* Increased *KRAS* and *TP53* | *KRAS, TP53, SMAD4, ERBB3, PRKACA—PRKACB* fusions, *ELF3* |

CCA, cholangiocarcinoma; CLC, cholangiolocarcinoma; dCCA, distal CCA; iCCA, intrahepatic CCA, IPNB, intraductal papillary neoplasm of the bile ducts; ITPN, intraductal tubulopapillary neoplasm; pCCA, perihilar CCA.
[a]Markers from single-center experience.
[b]Mucin refers to histological stains periodic acid—Schiff (PAS) or Alcian PAS.

~75% of cases. However, >60% of treated patients relapse early after surgery and the median post-operative survival is 3 years.[11] Patients with iCCA have a worse prognosis than those with eCCA, with lower rates of overall survival (OS) and cancer-specific survival.[12]

Multiple genomic alterations have been identified in CCA, and a high proportion of patients harbor a targetable gene mutation.[13] As a result, eligible patients can receive targeted therapies for CCA, depending on their genomic profile. However, as with any treatment, early and accurate diagnosis is key to improving outcomes. The aim of the current review is to describe the diagnostic process for CCA and highlight best practice in CCA diagnosis, focusing on the optimal approach to categorizing molecular genetics, and the implications for targeted treatment.

## TYPES OF TUMORS

CCAs may be mass forming, periductal infiltrating, or intraductal growing (Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2022.100505), but the pattern differs depending on the size of the duct in which they arise.[14] iCCAs forming in small ducts or ductules (≤300 μm diameter) generally show a mass-forming pattern and are often preceded by viral hepatitis or cirrhosis, but not by pre-neoplastic lesions (Table 1).[2,14] These tumors are thought to arise from hepatic progenitor cells or cuboidal (mucin-negative) cholangiocytes. In contrast, pCCA, dCCA, and iCCA that arise in larger ducts (diameter >300 μm) show a periductal infiltrating or intraductal growth pattern, and often occur in the presence of primary sclerosing cholangitis or liver flukes.[14] These tumors may arise from precancerous lesions, such as biliary intraepithelial neoplasia or intraductal neoplasms. The putative cells of origin for these tumors are the mucinous cholangiocytes or the peribiliary glands lining the larger intrahepatic bile ducts.

## THE DIAGNOSTIC JOURNEY IN CAA

Treatment of CCA differs depending on the site and stage of the malignancy, as well as patient-related factors such as performance status and comorbidities and tumor-related factors such as its genomic profile.

Initial assessment should include evaluation of the patient's signs and symptoms (Figure 1) and medical history in order to identify risk factors and comorbidities, physical examination including establishment of performance status, liver function tests, and additional blood tests for differential diagnosis and/or identifying infection (e.g. serum carbohydrate antigen 19-9 or α-fetoprotein levels).[16]

### *Imaging*

Ultrasound is usually the first imaging modality in the diagnosis of CCA, used to rule out gallbladder disease and identify obstructions in the biliary tree or lesions in the liver. However, computed tomography (CT) and/or magnetic resonance imaging (MRI) and/or magnetic resonance cholangiopancreatography is required to determine the anatomical site and, therefore, identify the CCA subtype (iCCA, pCCA, or dCCA).[3,17] MRI with liver-specific contrast media and diffusion-weighted imaging may help differentiate iCCA from hepatocellular carcinoma,[3] although this may not be true in patients with cirrhosis.[17] Contrast-enhanced CT is used for staging,[16] assessing local invasion, and identifying distant metastases.[3] [$^{18}$F]2-fluoro-2-deoxy-D-glucose—positron emission tomography ($^{18}$FDG—PET) is not suitable for CCA diagnosis because of its low specificity, but can be useful when there is an equivocal finding on other imaging modalities.[16] $^{18}$FDG—PET can also help in staging,

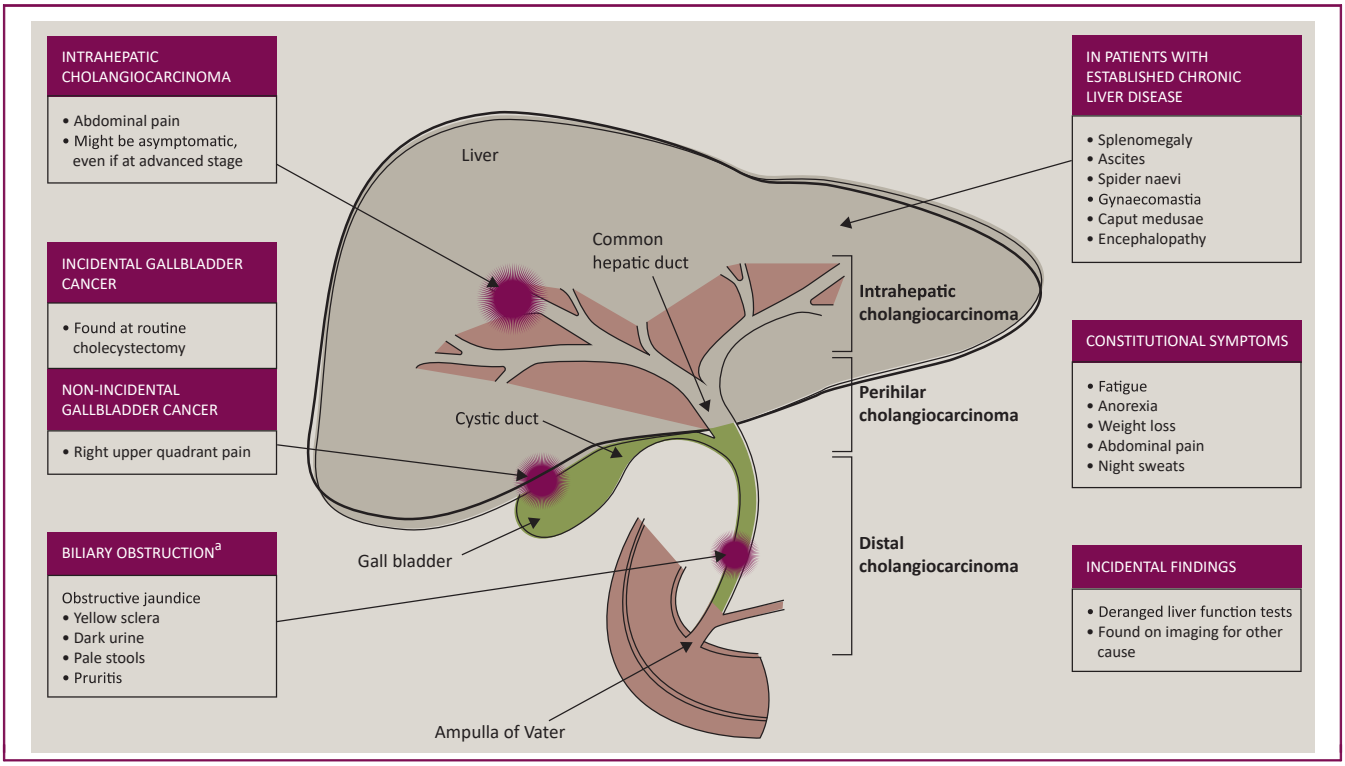


**Figure 1. Signs and symptoms of cholangiocarcinoma.**
[a]Biliary obstruction can occur from tumors in the major bile ducts (perihilar cholangiocarcinoma or distal cholangiocarcinoma), or because of lymph node compression at the hilum. Reprinted from Valle et al.,[3] with permission from Elsevier.

identification of lymph node involvement and distant metastases, and surgical planning.[17,18]

### *Staging*

Guidelines[16,17,19] recommend staging CCA using the American Joint Commission on Cancer (AJCC) staging system.[20] Definitions of these stages in the most recent (eighth) edition of the AJCC manual are shown in Table 2.[20] Imaging for staging should be undertaken before biopsy sample collection.[16]

### *Tumor specimen collection*

A biopsy is recommended wherever possible to obtain a tumor specimen for histopathologic diagnosis and molecular analysis. However, there are some exceptions. Biopsy sampling is not mandatory in eCCA patients who are candidates for curative surgery based on radiologic characteristics because the histopathologic diagnosis and molecular characterization will be conducted using the surgical specimen.[19] In contrast, a definitive diagnosis of iCCA based only on radiologic characteristics is not recommended. Collecting biopsy samples from the biliary tree is technically challenging, particularly obtaining sufficient tissue for molecular profiling.[21] According to one estimate, only ∼70% of biopsy tissue samples are suitable for molecular profiling because the samples do not contain enough cancer cells.[21] Most patients undergo endoscopic retrograde cholangiopancreatography (ECRP) to collect a biopsy specimen (preferred to biliary brush cytology that has low sensitivity).[3,19] If biopsy or tumor cell samples collected by ECRP are negative or inconclusive, fine-needle aspiration (FNA) guided by endoscopic ultrasound (EUS) can be useful.[19] EUS-FNA and biopsy may be the sampling method of choice in patients with suspected pCCA.[17] Other potential sampling methods include percutaneous transhepatic biliary drainage or biopsy collection guided by percutaneous transhepatic cholangioscopy, abdominal ultrasound, or CT.[17]

### *Histopathology*

As described above, histopathologic evaluation of biopsied tumor samples is the recommended method for diagnosing CCAs; however, cytologic evaluation of samples collected by FNA or brush cytology can be useful if histopathologic diagnosis is not possible.[17] Furthermore, there is the potential use of artificial intelligence, such as the deep-learning-based algorithm created by Lu and colleagues,[22] to determine the origin of the primary tumor using histology slides.

Histopathologic diagnosis is based on the detection of a typical biliary differentiation of cancer cells and its integration with immunohistochemical staining if histology is not univocal. Morphology usually shows the presence of neoplastic glands with empty lumen, composed of atypical cells with an increased nuclear/cytoplasmic ratio and macro-nucleoli. Immunostaining supporting the diagnosis of CCA mainly includes the positivity for the pancreatobiliary cytokeratin CK8/18 and the lack of expression of the intestinal cytokeratin CK20.[17]

**Table 2. AJCC (TNM) staging for CCA[20]**

| | iCCA | pCCA | dCCA |
|---|---|---|---|
| **Stage I** | **IA:** Solitary tumor ≤5 cm without vascular invasion (T1a), no regional lymph node metastasis (N0), and no distant metastases (M0)<br>**IB:** Solitary tumor >5 cm without vascular invasion (T1a), no regional lymph node metastasis (N0), and no distant metastases (M0) | Tumor confined to the bile duct with extension up to the muscle layer or fibrous tissue (T1), no regional lymph node metastasis (N0), and no distant metastases (M0) | Tumor invades the wall of the bile duct with a depth of <5 mm (T1), no regional lymph node metastasis (N0), and no distant metastases (M0) |
| **Stage II** | Solitary tumor with intrahepatic vascular invasion or multiple tumors with or without vascular invasion (T2), no regional lymph node metastasis (N0), and no distant metastases (M0) | Tumor invades beyond the wall of the bile duct to surrounding adipose tissue (T2a) or to adjacent hepatic parenchyma (T2b), no regional lymph node metastasis (N0), and no distant metastases (M0) | **IIA:** T1, metastasis in 1-3 regional lymph nodes (N1), and no distant metastases (M0), or Tumor invades the wall of the bile duct with a depth of 5-12 mm (T2), no regional lymph node metastasis (N0), and no distant metastases (M0)<br>**IIB:** T2 tumor, 1-3 regional lymph node metastasis (N1), and no distant metastases (M0) or Tumor invades the wall of the bile duct with a depth of >12 mm (T3), no regional lymph node metastasis (N0), and no distant metastases (M0) or T3 tumor, 1-3 regional lymph node metastasis (N1), and no distant metastases (M0) |
| **Stage III** | **IIIA:** Tumor perforating the visceral peritoneum (T3), no regional lymph node metastasis (N0), and no distant metastases (M0)<br>**IIIB:** Tumor involving local extrahepatic structures by direct invasion (T4) and/or regional lymph node metastasis (N1) and no distant metastases (M0) | **IIIA:** Tumor invades unilateral branches of the portal vein or hepatic artery (T3), no regional lymph node metastasis (N0), and no distant metastases (M0)<br>**IIIB:** Tumor invades the main portal vein or its branches bilaterally, or the common hepatic artery; or unilateral second-order biliary radicals bilaterally with contralateral portal vein or hepatic artery involvement (T4); no regional lymph node metastasis (N0), and no distant metastases (M0)<br>**IIIC:** Any T1-T4 tumor; 1-3 positive lymph nodes typically involving the hilar, cystic duct, common bile duct, hepatic artery posterior pancreatoduodenal, and portal vein lymph nodes (N1); and no distant metastasis (M0) | **IIIA:** Any T1-T3 tumor, metastasis in 4+ regional lymph nodes (N2), and no distant metastases (M0)<br>**IIIB:** Tumor involving the celiac axis, superior mesenteric artery, and/or common hepatic artery (T4), with no regional lymph nodes (N0) or ≥1 regional lymph nodes (N1 or N2), and no distant metastases (M0) |
| **Stage IV** | Any tumor (T1-T3), with or without regional lymph node metastasis (N0 or N1), and with distant metastases (M1) | **IVA:** Any T1-T4 tumor; 4+ positive lymph nodes from the sites described for N1 (N2); and no distant metastases (M0)<br>**IVB:** Any T1-T4 tumor, any N, and with distant metastases (M1) | Any tumor (T1-T4), with or without regional lymph node metastasis (N0, N1 or N2), and with distant metastases (M1) |

AJCC, American Joint Committee on Cancer; CCA, cholangiocarcinoma; dCCA, distal CCA; iCCA, intrahepatic CCA, pCCA, perihilar CCA; TNM, tumor—node—metastasis.

Molecular profiling using tumor multigene next-generation sequencing (NGS) is also recommended in patients with advanced CCA,[16,23] as described in the next section.

## MOLECULAR AND GENOMIC PROFILE OF CCA

Multiple genetic alterations (GAs) have been identified in patients with CCA, with at least 32 genes showing significant alterations (Table 3).[13,25,26] Data from a cohort of 260 patients with biliary tract cancer found that 40% of patients had targetable GAs; those with iCCA and eCCA had a median of 39 and 35 non-silent somatic mutations, respectively.[13] The presence of certain mutations has prognostic significance, and a number of them are now treatment targets. Thus, the European Society for Medical Oncology (ESMO) and United States National Comprehensive Cancer Network (NCCN) guidelines recommend molecular testing using NGS for patients with advanced CCA, because these findings can guide treatment decisions.[16,23]

The genomic profile of iCCA and eCCA differs, with fibroblast growth factor receptor (*FGFR)* GAs (fusions, mutations, or amplifications) and isocitrate dehydrogenase (*IDH)* mutations being much more common in iCCA than in eCCA, while *KRAS* mutations and *ERBB2* [(human epidermal growth factor receptor 2 (*HER2*)] amplification/overexpression are more prevalent in eCCA (Figure 2).[2,13,27-33] Based on the fact that there are actionable targets for some of these genes, ESMO recommends NGS testing for the following GAs (level I): *IDH1* mutations, *FGFR2* fusions, and neurotrophic receptor tyrosine kinase (*NTRK*) fusions, whilst acknowledging that other GAs (level II/III) have available targeted therapies that are not yet approved for use for CCAs [i.e. *BRAF* mutations, *ERBB2* (*HER2*) amplifications or mutations, *PIK3CA* hotspot mutations, *BRCA* 1 or 2 mutations, *MET* amplifications, and deficient mismatch repair (MMR)/microsatellite instability (MSI)].[23] Table 3 summarizes the prevalence of these GAs in CCA, the ESMO Scale of Clinical Actionability of molecular Targets (ESCAT), and the therapies that target these GAs, some of which are currently indicated in CCA.

**Table 3. Genomic alterations with actionable targets in advanced cholangiocarcinoma[23,24]**

| Gene alterations | Prevalence | ESCAT score | Available or potential targeted therapy[a] | Approved indication |
|---|---|---|---|---|
| *IDH1* mutations | 20% | IA | Ivosidenib | AML and CCA |
| *FGFR2* fusions | 15% | IB | Infigratinib, pemigatinib<br>Futibatinib, derazantinib<br>Erdafitinib | CCA<br>None<br>Urothelial carcinoma |
| MSI | 2% | IC | Pembrolizumab<br>Nivolumab | Tumor agnostic<br>Colorectal cancer |
| *NTRK* fusions | 2% | IC | Entrectinib, larotrectinib | Tumor agnostic |
| *BRAF*$^{V600E}$ mutations | 5% | IIB | Encorafenib<br>Dabrafenib<br><br>Vemurafenib | CRC, melanoma<br>Melanoma, NSCLC, anaplastic thyroid cancer<br>Melanoma |
| *ERBB2* (*HER2*) amplifications, mutations | 10%, 2% | IIIIA | Trastuzumab, pertuzumab, tucatinib, lapatinib, neratinib, trastuzumab deruxtecan, trastuzumab emtansine<br>Afatinib, dacomitinib | Breast cancer<br><br><br>NSCLC |
| *PIK3CA* hotspot mutations | 7% | IIIA | Alpelisib<br>Copanlisib | Breast cancer<br>Follicular lymphoma |
| *BRCA* 1/2 mutations | 3% | IIIA | Olaparib | Breast cancer, ovarian cancer, pancreatic cancer |
| *MET* amplification | 2% | IIIA | Crizotinib, capmatinib | NSCLC |
| TMB >10 mutations/megabase | — | — | Pembrolizumab | Tumor agnostic |
| *RET* rearrangement/mutation | — | — | Selpercatinib | NSCLC, thyroid cancer |

AML, acute myeloid leukemia; *BRCA1/2*, BRCA1/2 DNA Repair Associated; *BRAF*, v-Raf murine sarcoma viral oncogene homolog B; CCA, cholangiocarcinoma; CRC, colorectal cancer; *ERBB2*, Erb-B2 receptor tyrosine kinase 2; ESCAT, European Society for Medical Oncology Scale for Clinical Actionability of molecular Targets; *FGFR2*, fibroblast growth factor receptor 2; *HER2*, human epidermal growth factor receptor 2; *IDH*, isocitrate dehydrogenase; *MET*, MET proto-oncogene, receptor tyrosine kinase; MSI, microsatellite instability; NSCLC, non-small-cell lung cancer; *NTRK*, neurotrophic tyrosine receptor kinase; *PIK3CA*, phosphoinositide 3-kinase catalytic subunit alpha; *RET*, Ret proto-oncogene.
[a]May include agents studied/approved in indications other than CCA; see individual prescribing information for details.

## *Genetic mutations*

**IDH1.** *IDH* mutations are present in 10%-30% of patients with iCCA, but are less frequent in patients with eCCA (affecting ∼7%).[24,25,32,34,35] Up to 90% of these are *IDH1* mutations, with 10%-20% of *IDH2* mutations,[32,34] so *IDH1* mutations are present in between 10% and 20% of patients with iCCA.[27] Ivosidenib is an IDH1 inhibitor that was approved by the United States Food and Drug Administration (FDA) in August 2021 for the treatment of adult patients with previously treated, locally advanced, or metastatic CCA with a susceptible *IDH1* mutation.[36] In a randomized, double-blind, phase III trial ($n = 230$), ivosidenib significantly prolonged progression-free survival (PFS) in patients with *IDH1*-mutated advanced or metastatic CCA, all of whom had received up to two previous chemotherapy regimens.[37,38] Final OS analyses demonstrated a non-statistically significant improvement in OS

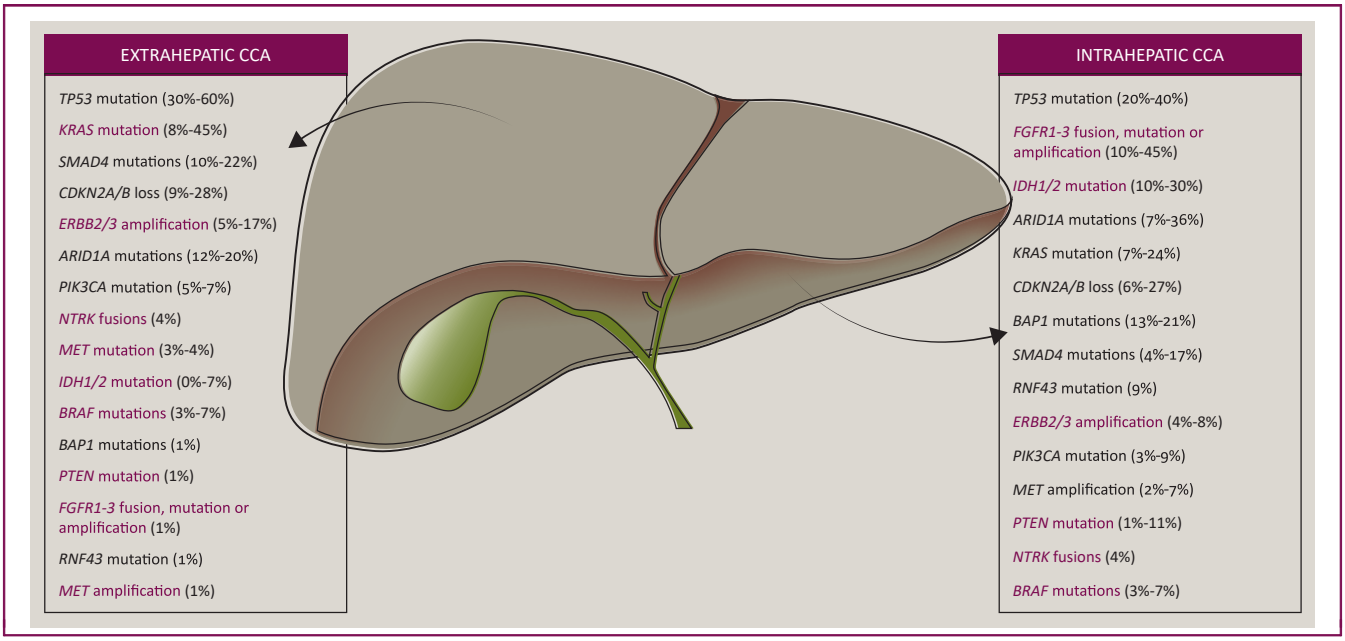


**Figure 2. Common genetic alterations in intrahepatic and extrahepatic cholangiocarcinoma (CCA).[13,24,27-29]** Genetic alterations in red can be targeted by available therapies.

with ivosidenib versus placebo despite a 70% crossover rate from the placebo group; the median OS was 10.3 months with ivosidenib compared with 7.9 months with placebo [unadjusted hazard ratio (HR) 0.79; 95% confidence interval (CI) 0.56-1.12; $P = 0.09$] or 5.1 months with placebo after adjustment for crossover (HR 0.49; 95% CI 0.34-0.70; $P < 0.001$). There were also significant differences in favor of ivosidenib for several quality-of-life domains, including pain, emotional and cognitive functioning, anxiety, and tiredness.[38] The most common all-grade treatment-emergent adverse event (TEAE) associated with ivosidenib was nausea (42%), and the most common grade ≥3 TEAEs were ascites (9%), anemia (7%), increased blood bilirubin level (6%), and hyponatremia (6%).[38] Based on these data, ivosidenib is now recommended in the United States NCCN guidelines for CCA patients with *IDH1* mutations.[16] There are currently limited data available regarding mechanisms of ivosidenib resistance in CCAs,[39] although *in vitro* studies suggest that resistance may develop from a switch of mutant *IDH* isoform (from mutant *IDH1* to *IDH2* or vice versa).[40]

**FGFR2.** The FGFRs are a family of tyrosine kinase receptors that include FGFR1, FGFR2, FGFR3, and FGFR4.[41] Aberrations in the genes for FGFRs are seen in a wide range of solid tumors,[42] and may include amplification, single-nucleotide variants, or gene fusions.[41]

Alterations in the genes *FGFR1*, *FGFR2*, and *FGFR3* may occur in CCA tumors, but the most commonly affected gene is *FGFR2,* with *FGFR1* or *FGFR3* GAs seen in ∼1% of CCA patients.[25] While *FGFR2* fusions have been associated with a better prognosis in iCCA,[43] *FGFR* alterations have also been reported to be prognostic of a poor response to gemcitabine + platinum chemotherapy.[44] *FGFR2* GAs are much more frequent in iCCA than in eCCA, and commonly co-occur with *BAP1* GAs,[25] and the prevalent types of *FGFR2* gene fusions differ between iCCA and eCCA. *FGFR2* fusions occur in ∼5%-7% of patients with any CCA and in 10%-20% of patients with iCCA,[27] with a wide diversity in fusion partners.[25,30,44] In 1202 patients with CCA screened for inclusion in the FIbroblast Growth factor receptor inhibitor in oncology and Hematology Trial (FIGHT-202) study, *FGFR2* gene fusions were detected in 113 patients, among which the most common *FGFR2* fusion or rearrangement (present in 27.9%) was a fusion of *FGFR2* and *BICC1*, followed by rearrangements in the *FGFR2* intron 17 or exon 18 fused to an intergenic region (present in 9.3%).[25] However, >150 *FGFR2* fusion partners in iCCA have been reported up to now.

Several FGFR inhibitors are currently being investigated in randomized clinical trials in patients with CCA harboring FGFR pathway alterations.

The ATP-competitive FGFR kinase inhibitor pemigatinib is the most advanced in its clinical development.[45] The FIGHT-202 study examined the efficacy and safety of pemigatinib in patients with advanced or metastatic iCCA, with or without *FGFR2* GAs, and found a marked difference in response rate between patients with *FGFR2* fusions or rearrangements, and those with other *FGF* or *FGFR* alterations or no *FGF/FGFR* GAs.[46] All patients had received at least one prior line of systemic therapy and 39.0% had received two or more prior lines of systemic therapy for advanced/metastatic disease. No objective responses were seen in patients with other or no *FGF/FGFR* GAs, whereas the response rate was 35.5% in the group with *FGFR2* fusions or rearrangements. Median PFS was 6.9 months in patients with *FGFR2* fusions or rearrangements compared with 1.7 and 2.1 months in patients with other or no *FGF/FGFR* GAs, respectively. The corresponding median OS was 17.5 months, 6.7 months, and 4.0 months, in these groups, respectively,[47] and 12-month survival rates were 68%, 23%, and 13%.[46] Updated analyses confirmed continued durable responses and sustained tolerability, with at least twofold longer OS in patients with *FGFR2* rearrangements/fusions who responded to pemigatinib compared with those who did not respond (median 30.1 versus 13.7 months).[47] In this study, pemigatinib was well tolerated and showed a favorable toxicity profile. The most common all-cause grade ≥3 TEAEs in patients treated with pemigatinib were fatigue (5.4%), diarrhea (3.4%), and nausea (2%), and 10.2% of patients discontinued pemigatinib due to TEAEs.[47] A *post hoc* analysis of European patients enrolled in FIGHT-202 ($n = 35$) reported efficacy and tolerability of pemigatinib that was consistent with the overall cohort, with an overall response rate (ORR) of 40.6% and a median PFS of 6.9 months.[48] Also, longitudinal analysis of quality of life according to best overall response showed maintenance of overall health status and emotional functioning, and decreases in pain and anxiety in patients with disease control compared with disease progression.[49] The ongoing phase III FIGHT-302 study is evaluating first-line pemigatinib versus gemcitabine plus cisplatin in patients ($n = 432$) with unresectable or metastatic CCA and *FGFR2* fusions/rearrangements (NCT03656536).[50] Preliminary data suggest that patients may develop resistance to pemigatinib through the acquisition of resistance mutations in residues that activate the kinase (pN549, p.K641, and p.E565) or that disrupt the binding of pemigatinib to the FGFR receptor (p.L617).[25]

Promising results have also been seen in phase II studies with the FGFR inhibitor infigratinib.[51,52] In a multicenter, open-label phase II study, infigratinib demonstrated an ORR of 14.8% and a disease control rate (DCR) of 75.4% in 61 patients with advanced or metastatic CCA harboring *FGFR* GAs whose disease had progressed after previous therapy.[51] Infigratinib had a manageable safety profile, with commonly reported TEAEs of hyperphosphatemia, fatigue, skin/fingernail/eye toxicities, and stomatitis.[51] Results in the cohort of patients specifically with *FGFR2* gene fusions or rearrangements ($n = 108$) reported an ORR of 23.1% after a median follow-up of 10.6 months and a median duration of response of 5.0 (range 3.7-9.3) months in infigratinib-treated patients.[52] A phase III study (PROOF 301; NCT3773302) evaluating infigratinib as first-line therapy is underway.[53]

Futibatinib is a third-generation, irreversible pan-FGFR inhibitor that blocks FGFR phosphorylation and

downstream signaling pathways.[54] In a multihistology phase I expansion trial in 197 patients with advanced solid tumors harboring a range of *FGFR* alterations (including 83 CCA patients), futibatinib showed antitumor activity and a tolerable safety profile.[55] In the phase I study, futibatinib 20 mg once daily resulted in an ORR of 15.6% and a DCR of 71.9% among patients with CCA, who mainly harbored *FGFR2* fusions/rearrangements or mutations.[55] In the phase II FOENIX-CCA2 trial with futibatinib, the results of which were presented at the American Association for Cancer Research conference in 2021,[56] ORRs of 43.8% in patients with *FGFR2* fusions and 34.8% in patients with other *FGFR2* rearrangements were reported. An earlier presentation at the American Society of Clinical Oncology meeting in 2020 reported a DCR of 76.1% and a median duration of response of 6.2 (range 2.1-14.2) months with futibatinib in 67 patients with previously treated iCCA and an *FGFR2* fusion/rearrangement with $\geq$6 months of follow-up.[57] The most commonly reported any-grade treatment-related adverse events were hyperphosphatemia (79.1%), diarrhea (37.3%), and dry mouth (32.8%).[57] The open-label, randomized phase III FOENIX-CCA3 trial (NCT04093362) is now evaluating futibatinib compared with gemcitabine plus cisplatin as first-line therapy in patients ($n = 216$) with advanced iCCA with an *FGFR2* rearrangement.[58] The FDA has recently granted Breakthrough Therapy Designation to futibatinib for the treatment of patients with previously treated locally advanced or metastatic CCA harboring *FGFR2* rearrangements, including gene fusions.[59]

Encouraging phase I/II data have also been reported for derazantinib in 29 patients with advanced or inoperable *FGFR2* gene fusion-positive iCCA (ORR 20.7, median PFS 5.7 months),[60] and a pivotal phase II trial (NCT03230318) is currently recruiting patients.[61,62] Preliminary data from this study, presented at the ESMO 2021 conference, reported an ORR of 21.4% in the subgroup of patients with *FGFR2* gene fusions ($n = 103$). Treatment with derazantinib was associated with a median PFS of 7.8 months and a median OS of 15.5 months in the $FGFR2^{fusion+}$ subgroup.[61]

Both pemigatinib and infigratinib have been approved by the FDA in previously treated CCA patients with an *FGFR2* fusion or rearrangement,[63,64] and pemigatinib has been approved by the European Medicines Agency (EMA).[65] Treatment with pemigatinib or infigratinib is a recommended option for patients with CCA and *FGFR2* fusions or rearrangements.[16] There is currently limited information regarding mechanisms of resistance to FGFR inhibitors in CCA, although co-occurring genomic alterations and acquired mutations in the kinase domain of FGFR2 have been associated with both primary and acquired resistance, as described for pemigratinib.[25,39]

**NTRK.** Genes for NTRK encode a range of tropomyosin receptor kinase (TRK) proteins. *NTRK* fusion genes are rare in patients with CCA,[66] but data from patients with a range of solid tumors suggest that patients with these GAs may benefit from treatment with TRK inhibitors (entrectinib or larotrectinib).[67,68] NCCN guidelines recommend these agents as first- or later-line therapy in patients with *NTRK* gene fusion-positive CCA.[16] Both entrectinib and larotrectinib are approved in the United States and Europe for the treatment of unresectable or metastatic solid tumors with an *NTRK* gene fusion progressing after previous therapy.[69-72] However, to date, data in patients with CCA are limited.

**BRAF.** Mutations in the gene for v-raf murine sarcoma viral oncogene homolog B1 (BRAF), including mutations in exon 11 (G469A and V600E), occur in between 1% and 7% of CCAs, most commonly iCCA.[29,30,32,73] Several BRAF inhibitors are available for the treatment of cancer (Table 3), but only dabrafenib and trametinib (anti-MEK) combination therapy has been investigated in patients with biliary tract cancers.[74,75] The phase II Rare Oncology Agnostic Research study reported an ORR of 47%, a median OS of 14 months, and a 12-month OS rate of 56% in biliary cancer patients with $BRAF^{V600E}$ mutations treated with dabrafenib in combination with trametinib.[75] Based on these data, dabrafenib + trametinib is included as a post-first-line treatment option for patients with $BRAF^{V600E}$-mutated CCA in the United States NCCN guidelines[75]; however, these agents are not currently approved in this indication.

**Others.** Other GAs that may be present in CCA include *ERBB2* (*HER2*) amplifications or mutations, *PIK3CA* hotspot mutations, *BRCA1/2* mutations, and *MET* amplifications; these are all considered to be level II or III actionable targets according to ESCAT. While ESMO recommends NGS testing for level I actionable genes (i.e. *IDH1*, *FGFR2*, *NTRK*), they do not yet recommend testing for level II or III genes in patients with CCA.[23] The United States NCCN guidelines note that testing for these GAs can identify patients with poor prognosis, but make no treatment recommendations for use of targeted therapies in patients with these GAs.[16]

Nevertheless, these GAs may be clinically relevant, and data are emerging on the use of agents targeting the level II/III GAs in patients with CCA. For example, *HER2* amplification and/or HER2 overexpression is present in about 13% of eCCA and 5% of iCCA.[29] The phase II MyPathway study examined the use of HER2-targeted therapy with pertuzumab + trastuzumab in patients with biliary tract cancers and *ERBB2* (*HER2*) amplification and/or HER2 overexpression.[76] Of the 39 patients in the study, 7 had eCCA and 7 had iCCA. In the eCCA group, two patients had a partial response and three had stable disease for >4 months, so the ORR was 29% and the DCR was 71% in this subgroup. The best response in the iCCA subgroup was stable disease for >4 months in two of the seven patients. Median duration of response was 7.5 months in the eCCA group and 10.8 months in the overall study population of patients with biliary tract cancer.[76] Treatment was generally well tolerated; the most common grade 3 TEAEs were elevated hepatic enzyme levels. The results were promising enough for more advanced clinical trials with HER2-targeted therapy to be conducted in this population.[76] Results are also expected soon from the phase II KAMELEON study with

the HER2-targeted antibody—drug conjugate trastuzumab emtansine in patients with pancreatic or biliary tract cancer including CCA (NCT02999672).

*BRCA* mutations are present in a small percentage of CCA: *BRCA1* mutations in 0.4% of iCCA and 2.6% of eCCA and *BRCA2* mutations in 2.0% and 2.5%, respectively.[77] Anecdotal reports indicate that CCA patients with *BRCA1* or *BRCA2* mutations can respond to treatment with poly(ADP) ribose polymerase (PARP) inhibitors.[78-81] A number of phase II studies are now underway to investigate PARP inhibitors in patients with biliary tract cancers including CCA (NCT04298021; NCT04306367; NCT04895046). Loss-of-function mutations in the ring finger protein 43 (*RNF43*) gene have been associated with aberrant Wnt signaling and poor prognosis in patients with iCCA.[82] A phase I study in patients with solid tumors (NCT01351103) and a phase II study in patients with advanced biliary tract cancer or pancreatic ductal adenocarcinoma (PORCUPINE2—NCT04907851) are currently investigating Wnt inhibitors in patients with tumors harboring *RNF43* mutations. In PORCUPINE2, only patients with pancreatic ductal adenocarcinoma were required to harbor the *RNF43* mutations.

*KRAS* mutations occur in ~9%-40% of CCAs,[83] with some of these being $KRAS^{G12C}$ mutations. The phase I/II KRYSTAL-1 study examined the use of adagrasib, a $KRAS^{G12C}$ inhibitor, in patients with advanced solid tumors harboring a $KRAS^{G12C}$ mutation. Of the 42 patients enrolled to date, 8 have biliary tract cancer. Initial results showed that adagrasib demonstrates encouraging clinical activity; among the patients with 'other' tumors, including the eight with biliary tract cancer, six (35%) achieved a partial response with a DCR of 100%.[84]

### *MMR/MSI/tumor mutational burden*

CCA is one of a number of solid tumors that may lack expression of MMR proteins, which leads to hypermutation during DNA replication or MSI.[85] MMR protein expression is identified using immunohistochemistry (IHC), and MSI or stability status is confirmed by molecular testing.[85-87] It is important to note that the terms MSI-high and MSI-low are no longer recommended, with MSI-low tumors being included in the microsatellite stable classification.[87] Samples that lack the coordinated expression of $\geq$1 MMR protein and show MSI at molecular testing are considered MMR-deficient (MMR-d), whereas samples are considered MMR-intact if they show intact MMR protein expression on IHC.[85,87] MMR-d occurs in ~6% of CCAs,[85] and MSI in 1%-2%,[86] but the presence of these tumor characteristics identifies a subgroup of patients who derive clinical benefit from treatment with the programmed cell death-ligand 1 (PD-L1) inhibitor pembrolizumab, according to data from the KEYNOTE-158 study.[88] In the United States, pembrolizumab is approved for any type of MSI-high or MMR-d solid tumor,[89] and the United States NCCN guidelines recommend pembrolizumab for first- or later-line treatment of these patients with unresectable or metastatic CCA.[16]

Tumor mutational burden (TMB), i.e. the number of mutations per megabase of coding DNA, is closely associated with MSI in some tumor types, but not necessarily in patients with biliary tract cancers.[87,90] Using a definition of >17 mutations per megabase for high TMB, Weinberg and colleagues found that 2%-3% of patients with CCA had high TMB.[30] The KEYNOTE-158 study found that patients with solid tumors and a high TMB (defined as $\geq$10 mutations per megabase, as assessed by the FoundationOne CDx assay) had a more robust response to pembrolizumab compared with those with <10 mutations per megabase,[91] which led to the approval of pembrolizumab in the United States for patients with high TMB solid tumors.[89] While these data indicate that PD-L1 inhibitor therapy may be a useful option in patients with high TMB, none of the patients in the KEYNOTE-158 study had biliary cancer, so the utility of this approach in CCA patients is still unknown.

Currently, these biomarkers (MMR, MSI, and TMB) and the matched therapies have yet to be approved by the EMA.

## TESTING MODALITIES

Because of the various different types of GAs, different testing methods are required to identify them, including NGS, IHC, fluorescent *in situ* hybridization (FISH), and liquid biopsy.

The availability of NGS techniques has allowed for the identification and discovery of many GAs.[41] DNA-based NGS tests are able to identify most types of genomic mutation and copy number alteration (e.g. single-nucleotide variants, indels, rearrangements, and amplifications).[24] However, it is important to note that specific capabilities of DNA tests depend on the size of available gene panels and the type of targeted sequence. RNA-based NGS can identify GAs in the transcriptome, including complex gene fusions and alternative splicing events, which may not be detected with DNA-NGS.[24] In particular, messenger RNA sequencing may be effective for identification of gene fusions in samples with a negative DNA-NGS result, so using a sequence of tests may be the optimal method for identifying actionable targets.[92] However, the sensitivity of RNA-NGS depends on the level of fusion expression.[41] Targeted NGS may be particularly useful for the detection of gene fusions in the clinical diagnostic process, and is the most clinically relevant sequencing method because it has lower costs, simplified workflow, easier data analysis, and shorter turnaround times compared with whole genome, exome, or transcriptome sequencing.[41] Available targeted NGS diagnostic panels include assays using hybrid capture, amplicons, and anchored multiplex PCR (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2022.100505).[24,41] Hybrid capture-based assays sequence target regions of DNA, as well as adjacent regions, using sequence-specific probes that are longer than PCR primers.[41] Amplicon-based assays allow for detection of gene fusions using very small amounts of DNA or RNA by

utilizing primers specific for known fusion partners, which may be appropriate for degraded samples. Anchored multiplex PCR-based assays allow for targeted amplification of RNA from known and unknown fusion partners using gene-specific primers anchored to an exon—intron boundary and universal reverse primers, enabling identification of any fusion partner, even when only one gene is known.[41] This targeted RNA-NGS technique has been used to identify various *FGFR* fusions in CCA of different etiologies, with *FGFR2* fusions being found almost exclusively in patients with non-fluke-associated CCA.[93]

Techniques such as IHC and FISH are inexpensive, readily available, and quick to carry out. However, although these techniques are suited to the identification of a specific GA in a sample, such as *HER2* or *BRAF*, they do not screen for multiple GAs and have limited clinical utility for identifying *FGFR2* GAs.[24] Therefore, the use of NGS is recommended by ESMO to ensure assessment of all relevant targets (level I recommendation according to ESCAT).[23]

Since poor sample quality may negatively impact NGS results, high-quality tissue samples are critical. In the absence of a high-quality biopsy sample, a liquid biopsy blood sample for circulating tumor DNA (ctDNA) may be an acceptable alternative. Although liquid biopsy is not yet available as a diagnostic test, early feasibility studies show promising results. A study of blood ctDNA and tumor tissue samples from patients with biliary tract cancer reported high blood/tissue concordance rates of genomic alterations of ∼74%.[94] In addition, ctDNA profiling has been found to significantly shorten genotype screening time during clinical trial enrollment compared with tissue molecular profiling, and may enable improved detection of clinically relevant biomarkers in patients with CCA.[95] Furthermore, the feasibility of detecting the emergence of acquired resistance to targeted therapy by serial ctDNA analysis has also been reported.[96]

## CONCLUSIONS

As data accumulate about molecular biomarkers in CCA and the number of targeted agents grows, genomic profiling will become an increasingly important element complementing CCA diagnosis. These data highlight the importance of obtaining a good tissue sample for histologic analysis, which can be technically challenging in patients with CCA. Taking a blood sample for analysis of ctDNA markers is a simpler process with a lower failure rate,[21] but not all NGS platforms are able to evaluate blood samples for ctDNA,[24] and these platforms may be less readily available. Since ∼45% of patients with CCA have a targetable GA, it is important to include genomic profiling in the diagnostic work-up, in order to identify treatments that may offer the best possible outcomes for patients. Three agents have already been approved in the United States for the treatment of CCAs with specific genetic GAs: ivosidenib for *IDH1* mutations and infigratinib and pemigatinib for *FGFR2* fusions; pemigatinib has also been approved in Europe, and it is likely that even more targeted therapies for level II or III actionable GAs will enter clinical use in the future.

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