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Emerging Molecular Therapeutic Targets for Cholangiocarcinoma

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Abstract

Cholangiocarcinomas (CCAs) are diverse epithelial tumors arising from the liver or large bile ducts with features of cholangiocyte differentiation, and are classified anatomically into intrahepatic (iCCA), perihilar (pCCA), and distal CCA (dCCA). Each subtype has distinct risk factors, molecular pathogenesis, therapeutic options, and prognosis. CCA is an aggressive malignancy with a poor overall prognosis and median survival of less than 2 years in patients with advanced disease. Potentially curative surgical treatment options are limited to the subset of patients with early stage disease. Presently, the available systemic medical therapies for advanced or metastatic CCA have limited therapeutic efficacy. Molecular alterations define the differences in biological behavior of each CCA subtype. Recent comprehensive genetic analysis have better characterized the genomic and transcriptomic landscape of each CCA subtype. Promising candidates for targeted, personalized therapy have emerged including potential driver *FGFR* gene fusions and somatic mutations in *IDH* 1/2 in iCCA, *PRKACA* or *PRKACB* gene fusions in pCCA, and *ELF3* mutations in dCCA/ampullary carcinoma. A precision genomic medicine approach is dependent on an enhanced understanding of driver mutations in each subtype and stratification of patients according to their genetic drivers. We review the current genomic landscape of CCA, potentially actionable molecular aberrations in each CCA subtype, and role of immunotherapy in CCA.

Keywords

Distal cholangiocarcinoma; intrahepatic cholangiocarcinoma; immunotherapy; perihilar cholangiocarcinoma; targeted therapy

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INTRODUCTION

Cholangiocarcinoma (CCA) is a heterogeneous hepatobiliary malignancy with a dismal prognosis. CCAs are epithelial tumors with markers of cholangiocyte differentiation (1). On the basis of their anatomic location, CCAs are classified into intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA) subtypes (1). iCCAs arise above the second order bile ducts, whereas pCCAs are located between the insertion of the cystic duct and the second order bile ducts, and dCCAs are located below the insertion of the cystic duct (1). Each anatomic subtype has a distinct biologic behavior, therapeutic options, and prognosis (2). In addition to the anatomic definition of CCA, stratification by histopathologic and growth type patterns has also been proposed (3–5). CCA arising from large bile ducts, predominantly but not exclusively pCCA, is characterized by well/moderately differentiated mucin producing cylindrical cells and a periductal infiltrating growth pattern. In contrast, CCA developing from small ducts and or hepatocytes, largely iCCA, are characterized by cuboidal non-mucin producing cells with a mass forming growth pattern. The clinical implications of this classification have yet to be realized and therefore, we will use the anatomic definition.

CCA is a devastating malignancy with an abysmal overall 5-year survival rate of less than 10% (6). Surgical resection and liver transplantation are potentially curative treatment option for early stage disease in all three subtypes. However, the median 5-year survival after R0-resection is approximately 30% (7). Liver transplantation as a potentially curative treatment option is limited to iCCAs and pCCAs. Neoadjuvant chemoradiation followed by liver transplantation has been established as definitive therapy for a subset of carefully selected pCCA patients (1). Five-year survival rates following liver transplantation are 70% for patients with pCCA in the setting of primary sclerosing cholangitis and 55% for sporadic or de novo pCCA patients. Until recently, the presence of iCCA had been considered a contraindication for liver transplantation. According to an international multicenter study, patients with “very early” iCCA (tumor size < 2 cm) had a 5-year survival rate of 65% following liver transplantation compared to 45% in the “advanced” group (tumor size > 2 cm) (8). Despite these encouraging results, the preponderance of patients have advanced disease at diagnosis. For patients who are not candidates for surgical resection or liver transplantation, the practice standard is systemic chemotherapy with gemcitabine and cisplatin (9). However, this combination chemotherapeutic regimen confers a median overall survival of only 11.7 months (9). Currently, there are no potentially curative medical therapies for CCA. Furthermore, no targeted molecular therapies have been approved for use in CCA. Development of potentially curative medical treatment strategies for CCA has been limited by the molecular and genetic heterogeneity of these tumors. The advent of next-generation sequencing has made the discovery of possible targetable or actionable molecular alterations in CCA feasible. Precision therapy for CCA is dependent on an enhanced understanding of molecular and genetic aberrations, including driver mutations, for each CCA subtype. Herein, we review the evolving mutational landscape of CCA and summarize novel targeted therapies that will help build a precision approach for treatment of this devastating malignancy.

GENOMIC AND TRANSCRIPTOMIC LANDSCAPE OF CHOLANGIOCARCINOMA DIFFER BY ETIOLOGY

It is well-known that genomic alterations in CCA vary by anatomic subtype. However, different etiologic exposures can also significantly influence the pattern of somatic mutations leading to a distinct mutational landscape. This varying impact of carcinogenic etiologies was initially demonstrated in a whole-exome sequencing analysis of eight liver fluke-associated CCAs (10). Opisthorchis viverrini (O. viverrini)-related tumors had 206 somatic mutations which included mutations in known cancer-related genes, such as tumor protein 53 (*TP53*) which was mutated in 44.4% of cases, Kristen ras sarcoma viral oncogene homolog (*KRAS*) and SMAD4, both mutated in 16.7% of cases (10). *KRAS* mutations were associated with a poor overall survival (10). Somatic mutations were also identified in several newly implicated genes with distinct biological functions. Genes involved in genomic stability and G-protein signaling which had somatic mutations included *GNAS, ROBO2, RNF43*, *PEG3*, *XIRP2*, *RADIL*, *NDC80*, *PCDHA13* and *LAMA2* (10). Inactivating mutations in *MLL3*, which has a role in histone modification, were also identified (10). Whole-exome sequencing of 108 cases of O. viverrini related CCA and 101 cases of non- O. viverrini highlighted the impact of different causative etiologies (11). Somatic mutations in *BAP1*, *IDH1*, and *IDH2* were a more frequent occurrence in non-O. viverrini CCAs, whereas *TP53* mutations were observed more frequently in O. viverrini CCAs. The detection of recurrent somatic mutations in *BAP1* and *ARID1A* was a novel finding in this study. Moreover, a role of chromatin remodeling in CCA carcinogenesis was ascertained by functional assays demonstrating a tumor suppressive function for *BAP1* and *ARID1A* (11). A subsequent whole-exome sequencing of 32 intrahepatic CCAs also identified inactivating mutations in multiple chromatin-remodeling genes including *BAP1*, *ARID1A* and *PBRM1* (12). Together, these studies underscored the importance of dysregulated chromatin remodeling in non-O. viverrini CCA carcinogenesis.

MOLECULAR ABERRATIONS AND TARGETED THERAPIES IN CHOLANGIOCARCINOMA

Molecular Aberrations and Targeted Therapies in Intrahepatic Cholangiocarcinoma (Figure 1, Table 1)

FGFR Gene Fusions and Inhibitors—FGF signaling regulates a multitude of biological processes including cell proliferation, differentiation, survival, wound repair, angiogenesis, and migration (13). The integral role of the FGF-FGFR axis in essential cellular processes fosters the oncogenic potential of aberrant FGF signaling. Deregulation of FGF signaling with consequent carcinogenesis has been implicated in various malignancies including CCA. *FGFR2* gene fusions have been detected in iCCA in several recent studies (14–17). In three different CCA cohorts, *FGFR2* gene fusions were identified in 11–14% of iCCAs with report of several different *FGFR2* gene fusions including *FGFR2-BICC1*, *FGFR2-AHCYL1, FGFR2-TACC3,* and *FGFR2-KIAA 1598* (15, 16, 18). A higher frequency of *FGFR* gene fusions was noted by Sia et al. in a cohort of 107 iCCA patients (45%, 17/107) (19). *FGFR2* gene fusions are rare in pCCA/dCCA. Mechanistic studies indicate that the FGFR binding

partners mediate oligomerization and resultant activation of the respective FGFR kinase in tumors harboring *FGFR* translocations (17). Moreover, *FGFR2* fusion proteins activate FGFR signaling with mitogen-activated protein kinase (MAPK) activation and confer anchorage-independent growth (18). Uncovering of these gene fusions is significant as these are often driver mutations and potentially targetable. For instance, targeting of the *BCR-ABL* gene fusion with Imatinib in chronic myelogenous leukemia has been one of the earliest instances of effective precision medicine (20).

Intrahepatic cholangiocarcinomas harboring *FGFR2* gene fusions appear to have distinct clinical and pathologic features (15). Morphologically, cases harboring *FGFR2* gene fusions appear to have a prominent intraductal growth pattern, anastomosing tubular structures, and desmoplasia (15). Immunohistochemically, iCCAs with *FGFR2* gene fusions have strong cytokeratin (CK)-7 positivity but only weak and patchy expression of CK-19 (15). In addition to describing the pathologic features of tumors with *FGFR2* gene fusions, Graham et al. also reported a survival advantage (median cancer specific survival 123 months versus 37 months) indicating that the presence of *FGFR2* gene fusions may have prognostic significance (15). However, in an Asian cohort the presence of FGFR2 gene fusions in iCCA did not appear to have an impact on overall survival, clinical stage or tumor differentiation (18).

FGFR-Selective Small molecule kinase inhibitors: Preclinical studies have demonstrated antitumor efficacy of FGFR inhibition selectively in cells harboring *FGFR2* gene fusions (17, 18). Moreover, BGJ398, a pan-FGFR inhibitor, significantly reduced tumor burden in a genetic murine model of CCA as well as a patient-derived xenograft model of iCCA (21). These promising data support the notion that patients who harbor *FGFR2* gene fusions may benefit from FGFR-directed therapy in a precision medicine approach. On this premise, several small molecule kinase inhibitors (SMKIs) of FGFR are currently in early phase clinical trials. Evaluation of BGJ398 in a phase I trial single-agent dose escalation and dose-expansion study in 132 patients with advanced solid organ malignancies harboring *FGFR* gene aberrations demonstrated antitumor activity in several tumor types as well as a manageable safety profile (22). Interim analysis from a phase II, multi-center study of BGJ398 in patients with advanced CCA with *FGFR2* gene fusions or other *FGFR* genetic alterations who have failed platinum-based chemotherapy (NCT02150967) demonstrated an impressive disease control rate of 82% (23). JNJ-42756493 or erdafitinib is another oral pan-FGFR selective SMKI being evaluated in clinical trials (24). Results of a phase I dose-escalation study of JNJ-42756493 in patients with advanced solid tumors and FGFR pathway aberrations indicated 4 confirmed responses, one unconfirmed partial response and disease stability in 16 patients (24). Notably, the 36 patients in this study without known FGFR alterations did not demonstrate any significant response (24). ARQ 087 is an ATP-competitive SMKI with anti-proliferative activity in cell lines driven by FGFR aberrations including amplifications, fusions, and mutations (25). ARQ 087 is currently being studied in a phase 1/2 trial in patients with advanced solid tumors with genetic alterations including iCCA patients with *FGFR2* gene fusions (NCT01752920). Other FGFR SMKIs which have demonstrated efficacy against FGFR-driven malignancies in preclinical studies and are

currently being assessed in early phase clinical trials include TAS-120, AZD4547, and CH5183284/Debio 1347 (NCT02052778, NCT01948297).

Non-Selective Small molecule kinase inhibitors: Borad et al. demonstrated early evidence of efficacy of the non-selective FGFR inhibitors ponatinib and pazopanib in advanced iCCA patients harboring *FGFR2* gene fusions (14). In a metastatic iCCA patient with the *FGFR2-MGEA5* fusion, ponatinib monotherapy as salvage treatment resulted in tumor necrosis, reduction in levels of the tumor marker carbohydrate antigen 19-9, and shrinkage in size of metastatic lymph nodes (14). In a patient with metastatic iCCA and *FGFR2-TACC3* fusion, partial response to pazopanib was noted. Ponatinib is currently being assessed in an active phase II clinical trial in patients with advanced biliary tract cancer harboring *FGFR2* gene fusions detected by next generation sequencing or FISH break-apart assay (NCT02265341). Combination chemotherapies are an attractive option in malignancies with marked molecular heterogeneity such as CCA. Deregulation of the Raf/MEK/ERK pathway is a well-established occurrence in CCA and the MEK inhibitor Selumetinib has been shown to have some benefit in advanced biliary tract cancer (26). A phase I trial to assess the combination of pazopanib and the MEK inhibitor trametinib in advanced solid organ malignancies including CCA is currently underway (NCT01438554).

A significant shortcoming of first-generation FGFR inhibitors including BGJ398 and AZD457 is the eventual emergence of drug-resistant tumors (27). Goyal et al. identified different *FGFR2* mutations in individual resistant clones in patients with acquired resistance to BGJ398 (28). Preclinical studies have indicated that mutations in the FGFR gatekeeper residue can confer resistance to FGFR inhibitors. Tan et al. described novel, next-generation covalent FGFR inhibitors, FGFR irreversible inhibitors 2 and 3, which can overcome the gatekeeper residue mutation and potently inhibit tumor cells dependent upon FGFR1 and FGFR2 gatekeeper mutants (27).

Monoclonal antibodies: FGFRs have a variety of isoforms through tissue-specific alternative splicing of their mRNAs (29). Borad et al. detected the FGFR-IIIb isoform in all of the identified *FGFR2* gene fusions in their study (14). The FGFR2-IIIb isoform has binding specificity for FGF7 and FGF10. Monoclonal antibodies directed against specific isoforms would theoretically be an attractive therapeutic option in advanced iCCA cases harboring *FGFR* gene aberrations as they would avoid the off-target effects of SMKIs. FPA144, a humanized monoclonal antibody directed against the FGFR2b isoform with demonstrated efficacy in preclinical tumor models of gastric cancer (29), is being evaluated in a phase I clinical trial in patients with advanced solid tumors with FGFR2b overexpression or amplification (NCT02318329).

MET-HGF—MET tyrosine kinase is a plasma membrane protein which is activated when its extracellular domain binds to hepatocyte growth factor (HGF) or scatter factor (30). HGF-MET signaling has a critical role in essential cellular behaviors including proliferation, resistance to apoptosis, increased cell motility, and angiogenesis (30). Tumors can harness these processes to promote tumor growth and invasion. MET overexpression occurs in iCCA and correlates with the degree of tumor differentiation (31). An integrated molecular analysis identified two distinct biological classes in iCCA, an inflammation class (38% of

iCCAs) and a proliferation class (62% of iCCAs) (32). The proliferation class featured activation of oncogenic signaling pathways such as MET, epidermal growth factor receptor (EGFR), and MAPK (32). Cross-talk between MET-HGF and ERRB family of receptors occurs and may account for resistance to MET and ERBB2 inhibitors (30). MET amplification has been detected in iCCA, albeit the incidence of these mutations is relatively low (16, 33).

A number of MET kinase inhibitors are being evaluated in clinical trials for various malignancies (34). Phase I results of a study of tivantinib, an oral MET inhibitor, in combination with gemcitabine in patients with solid tumors including CCA demonstrated partial response in 46% of patients and stable disease in 27% of patients (35). A phase II study of cabozantinib in patients with advanced CCA after progression on first or second line chemotherapy is currently ongoing (NCT01954745).

Mcl-1 and JAK/STAT Pathway—Myeloid cell leukemia sequence 1 (Mcl-1) is a potent antiapoptotic Bcl-2 protein. *MCL1* amplification occurs in iCCA (16–21%) (16, 33). Mcl-1 mediates tumor necrosis factor-related apoptosis ligand (TRAIL) resistance in CCA by blocking the mitochondrial pathway of cell death (36). Interleukin (IL)-6, an inflammatory cytokine implicated in CCA biology, upregulates Mcl-1 via an AKT-dependent mechanism (37). IL-6 inhibition overcomes apoptosis resistance via downregulation of AKT and Mcl-1 (37). IL-6 upregulation of Mcl-1 is also mediated via Janus kinase (JAK) and signal transducer and activator of transcription (STAT) signaling cascade (38). STAT3 directly regulates Mcl-1 transcription suggesting that STAT3 inhibition may have therapeutic utility in CCA (38). Epigenetic silencing of suppressor of cytokine signaling 3 (SOCS-3) mediates sustained IL-6/STAT-3 signaling and enhanced Mcl-1 expression in CCA (39). Furthermore, biliary transduction of constitutively-activated AKT and yes-associated protein and systemic IL-33 administration induces tumor formation in mice via an IL-6 sensitive mechanism, underscoring the role of inflammatory cytokines in CCA oncogenesis (40). *Mcl-1* amplification is a critical event in many cancers as cancer cells with *Mcl-1* amplification are dependent on this potent anti-apoptotic protein for survival (41). Detection of *Mcl-1* amplification utilizing FISH analysis is an essential component of a FISH probe set with high specificity for the detection of cholangiocarcinoma (42). Collectively, these findings provide several modes of therapeutic targeting in CCA. Novel, selective Mcl-1 inhibitors have been developed and preliminary preclinical studies have demonstrated evidence of their efficacy in pancreatic cancer (43, 44). Mechanistic studies have also demonstrated that S63845, a small molecule inhibitor of Mc-1 which binds with high affinity to the BH3-binding groove of Mcl-1, potently induces cell death in various solid cancer-derived cell lines (45). Preclinical studies have also demonstrated efficacy of the JAK2 inhibitor, AZD1480, in suppression of STAT-3 mediated oncogenesis in solid tumor cell lines (46).

IDH/IDH2 Mutations and Inhibitors—Isocitrate dehydrogenase 1 and 2 are enzymes that catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate. *IDH* mutations are heterozygous point mutations in catalytic amino acid residues and these alteration occur in Arginine 132 of *IDH1* and Arginine 140 or Arginine 172 of *IDH2* (47). *IDH1/IDH2* mutations results in elevated levels of 2-hydroxyglutarate, an oncometabolite which induces

widespread epigenetic changes. Consequently, *IDH* mutations have pleiotropic effect on differentiation, growth factor dependence, collagen processing and hypoxia signaling (47).

IDH1/IDH2 mutations, albeit a relatively common occurrence in gliomas, were previously thought to be rare in other solid organ tumors (48). However, several recent mutational profiling studies have demonstrated that *IDH* mutations are a relatively frequent genetic aberration in CCA (16, 33, 49). *IDH* mutations are more frequently observed in intrahepatic cholangiocarcinomas than pCCAs and dCCAs (23–28% versus 0–7%) (48, 50). Histopathologic analysis of 94 surgically resected primary cholangiocarcinomas demonstrated that *IDH* mutations are associated with clear cell change and poorly differentiated histology (48). In this study, patients with *IDH1/2* gene mutations also appeared to have a better overall survival a year after surgical resection compared to patients without the presence of *IDH* mutations (48). A positive prognostic association with *IDH* mutations was also observed in a study of 326 iCCAs which demonstrated that *IDH* mutations were associated with longer overall survival and longer time to tumor recurrence after iCCA resection (51). However, subsequent studies have demonstrated that the prognostic significance of *IDH* mutations remains unclear. In a smaller cohort of 34 iCCAs, Jiao et al. demonstrated that patients with *IDH* mutations had a 3-year survival of 33% compared to 81% for those with wild-type *IDH* genes, although patients with *IDH* mutations were somewhat older and had a higher tumor stage (12). Additionally, the sample size of this study (n = 32) was much smaller compared to the prior study. A subsequent mutational profiling of 200 resected iCCA specimens demonstrated that although *IDH*-mutant tumors were more frequently multicentric in the liver, there was no impact on long-term prognosis (49). Although these 4 studies had conflicting results regarding prognosis, they focused mainly on early-stage or resectable iCCA, whereas targeted therapy with IDH inhibitors would be considered in patients with unresectable or advanced iCCA (52). Accordingly, in a subsequent study Goyal et al. assessed the correlation of *IDH* mutations with prognosis in 104 patients with unresectable or advanced iCCA (52). The presence of *IDH* mutations did not have a significant impact on the median overall survival (52).

The finding that *IDH* mutations are a relatively frequent occurrence in several human malignancies logically lead to speculation whether inhibition of mutant IDH activity may have therapeutic benefits. Small molecule inhibitors of mutant IDH were assessed in two proof of concept preclinical studies (53, 54). AGI-5198, a selective IDH1 inhibitor, blocked the ability of this enzyme to produce the oncometabolite 2-HG with resultant impaired growth of IDH1-mutant cells (53). Likewise, AGI-6780, a selective mutant IDH2 inhibitor, induced differentiation in hematopoietic cell lines (54). AG-120 is a first-in-class, orally bioavailable inhibitor of mutant IDH1. Phase 1 results of AG-120 in solid organ malignancies (NCT02073994) including iCCA presented at the AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics in 2015 demonstrated that AG-120: i) is well-tolerated in the solid tumor patient population, ii) had encouraging evidence of clinical activity, iii) reduced intra-tumoral 2-HG levels. On the basis of these preliminary results, an advanced phase multi-center, randomized, double-blind, placebo-controlled study of AG-120 in previously treated subjects with unresectable or metastatic cholangiocarcinoma with an *IDH1* mutation is planned (NCT02989857). AG-221, an orally available, selective inhibitor of mutant IDH2, has received fast-track

designation from the Food and Drug Administration (FDA) and is currently being evaluated in multiple clinical trials including a Phase 1/2, multi-center study in subjects with advanced solid tumors including iCCA who harbor an *IDH2* mutation (NCT02273739). Saha et al. recently demonstrated that iCCA cells with mutant IDH have a unique dependency on SRC kinase (55). Consequently, the SRC inhibitor, dasatinib, had a significant anti-tumor effect in mutant IDH xenografts, signifying a potential new therapeutic strategy against mutant IDH iCCA (55)

Protein tyrosine phosphatases (PTPs)—Members of the protein tyrosine phosphatase (PTP) are enzymes which remove phosphate from tyrosine residues in proteins (56). Genetic aberrations of PTPs can result in disequilibrium of protein tyrosine-kinase phosphatase activity with consequent oncogenic potential (56). A prevalence screen of 124 pairs of iCCAs and nontumor samples demonstrated that 41.4% of iCCAs had gain of function mutations in *PTPN3* (57). Presence of these mutations conferred markedly enhanced pro-tumor activity and significantly increased postoperative tumor recurrence (57). This study has important therapeutic implications as it is well-known that gain of function mutations are potential therapeutic targets in various malignancies. Consequently, the high frequency of these mutations and their significant functional relevance makes these mutations an important potential therapeutic target.

Chromatin-remodeling Genes and HDAC Inhibitors—The role of dysregulated chromatin remodeling in iCCA was highlighted through exome sequencing of 32 iCCAs which detected genetic aberration of at least one chromatin-remodeling gene in 47% of cases (15 of 32) (12). Inactivating mutations were frequently identified in *BAP1*, *ARID1A* and *PBRM1. BAP1* encodes a nuclear deubiquitinase involved in chromatin remodeling, whereas *ARID1A* and *PBRM1* encode subunits of the SWI/SNF chromatin remodeling complexes (12). Subjects with mutations in any one of these genes tended to have shorter survival times and worse survival compared to subjects with wild-type genes, albeit these observations were not statistically significant (12). Frequent alterations of *ARID1A* (36% of iCCAs) were noted in next-generation sequencing of 28 iCCAs (16). *ARID1A* mutations appear to be more frequent in iCCA than pCCA/dCCA as indicated by a recent mutational profiling which identified *ARID1A* aberrations in 20% of iCCAs compared to 5% of pCCA/dCCAs (33). *BAP1* and *PBRM1* alterations were identified in 9% and 11% of iCCAs, respectively (33). *BAP1* mutations were also detected in 10% of pCCA/dCCA cases, and in these patients the presence of *BAP1* mutation was significantly associated with reduced progression-free survival (median 3 months vs. 8.8 months) and reduced overall survival (median 8.9 vs. 19.9 months) (33). Overall, in all cases harboring *BAP1* mutations in this study, progression after first-line chemotherapy was observed within 4 months (33).

Several small molecule inhibitors targeting chromatin remodeling have been approved by the FDA (58). These include the histone deacetylase (HDAC) inhibitors vorinostat and romidepsin and DNA methyltransferase (DNMT) inhibitors azacitidine and decitabine (58). As the role of chromatin remodeling in CCA carcinogenesis is being illuminated, these inhibitors may have increased therapeutic utility in CCA.

Molecular Aberrations and Targeted Therapies in Perihilar Cholangiocarcinoma (Figure 2)

EGFR/HER2—Aberrant expression and signaling of the epidermal growth factor receptor (ERBB) family of receptor tyrosine kinases has been reported in cholangiocarcinoma. Activation of this pathway leads to downstream oncogenic pathway activation including the MAPK pathway. The ERBB family has four distinct receptors including ERBB1 (EGFR), ERBB2 (Her-2/neu), ERBB3 and ERBB4 (59). In a cohort of surgically resected CCA cases, *HER2* amplification was identified using FISH analysis in a single iCCA case and two pCCA/dCCA cases (15). A recent mutation profiling of 75 CCA specimens identified *ERBB2* genetic aberrations in 20% of pCCAs/dCCAs and in only 1.8% of iCCAs (33). The pCCA/dCCA *ERBB2* alterations included one amplification and four mutations, the latter being a novel finding, as mutations had previously not been reported in CCA. These mutations included one in the kinase domain (V777L) and three in the extracellular domain (S310F) (33). The V777L mutation is an activating mutation with some demonstrated resistance to lapatinib, a reversible, dual inhibitor of EGFR and HER2 (60). However, the V777L mutation was sensitive to nertatinib, an irreversible, dual inhibitor of EGFR and HER2 (60). The presence of kinase domain mutations may explain the limited success of erlotinib, a reversible EGFR inhibitor, in human CCA clinical trials (61, 62). Irreversible EGFR inhibitors such as neratinib, afatinib, and dacomatinib may have greater utility in this setting (33).

Protein kinase cyclic AMP (cAMP)-activated catalytic subunit alpha PRKACA) and beta (PRKACB)—Protein kinase A (PKA) is a cAMP-dependent protein kinase. Both PRKACA and PRKACB are catalytic submits of PKA and are members of the serine/threonine protein kinase family (63). Novel gene fusions of the PKA signaling components and mitochondrial ATP synthase, α subunit (*ATP1B*) were recently detected in pCCA/dCCA (63). Nakumura et al. detected *ATP1B-PRKACA* and *ATP1B-PRKACB* fusions in pCCA/dCCA cases (63). These fusions stimulated significantly enhanced expression of the *PRKACA* and *PRKACB* genes with consequent downstream MAPK signaling activation (63). Interestingly, fusion of the same exon of the *PRKACA* gene to *DNAJB1* has been demonstrated in fibrolamellar hepatocellular carcinoma (64). Genetic aberrations in the PKA regulatory subunits, *PRKAR1A* (nonsense mutation) and *PRKAR1B (PRKAR1B- C7orf50* gene fusion), were also detected in two different cases of pCCA/dCCA. *PRKAR1A* expression is enhanced in CCA, and *PRKAR1A* knockdown induces growth inhibition and apoptosis of CCA cells with resultant decrease in MAPK, phosphatidylinositol 3-kinase (PI3K)/Akt, JAT/STAT, and Wnt/β-catenin pathway signaling (65). Isoquinoline H89, a small molecule inhibitor of PKA, significantly inhibited CCA cell proliferation, albeit this may be an off-target effect as H89 is known to have a number of PKA-independent effects (65).

Molecular Aberrations and Targeted Therapies in dCCA/ampullary carcinoma (Figure 3)

Distal CCA as an entity is not well-described. Ampullary carcinomas are rare neoplasms arising from the ampulla of Vater at the convergence of three different epithelia – pancreatic, biliary and duodenal (66). Intestinal and pancreatobiliary histological subtypes of ampullary carcinoma have been proposed (66). The pancreatobiliary subtype can be considered a subset of dCCA, and thus the genetic landscape of ampullary carcinoma can be extrapolated

to dCCA given their significant overlap. Moreover, preponderance of recent genomic analyses of CCA have grouped pCCA and dCCA as extrahepatic CCA. Therefore, there is substantial overlap in the molecular aberrations identified in these two subtypes and the potential targeted therapies.

ELF3—*ELF3* encodes an E26 transformation-specific transcription factor and hence is implicated in the regulation of several genes with essential roles in multiple cellular processes (67). By interacting with their promoter regions *ELF3* increases transcription of *TGFBR2* and *EGF1* activation, two known tumor suppressor gene (68). *TGFBR2* also has an integral role in TGF-β signaling. Two recent genomic analyses detected inactivating mutations of *ELF3* in ampullary carcinoma/dCCA. In a cohort of 98 periampullary carcinomas and 44 dCCAs, Gingras et al. identified inactivating frameshift or nonsense mutations in 10.6% of periampullary tumors (68). Similarly, *ELF3* mutations had been reported in 9.5% of extrahepatic CCAs (pCCA/dCCA) in a molecular characterization of biliary tract cancers (63). In a cohort of 172 ampullary carcinomas, driver mutations in *ELF3* were also identified in 13.3% of cases (69). *ELF3* mutations were present at high allele frequencies, indicating that *ELF3* mutation may represent a founder or driver mutation in ampullary carcinoma (69). Functional studies were subsequently carried out and demonstrated that ELF3 knockdown promotes motility and invasion in epithelial cells (69).

HER2 (ERBB2)/ERBB3—Molecular alterations in the ERBB family are well-described in pCCA (please see above) and have recently been reported in dCCA/ampullary carcinoma. *ERBB3* mutations were identified in 14% of pancreatobiliary-type ampullary carcinomas and *ERBB2* mutations were identified in 11.6% of ampullary carcinomas (69). *ERBB2* mutations overlapped with *ELF3* patients.

Molecular Aberrations and Targeted Therapies Common to all Cholangiocarcinoma Subtypes (Table 1)

Although each of the anatomic subtypes of CCA has a distinct mutational landscape, there are some molecular alterations that are observed with similar frequency in all of the subtypes. *KRAS* mutations and the activation of the PI3K-AKT-mTOR pathway are amongst the most prevalent aberrations noted in a number of malignancies and have a significant role in carcinogenesis.

KRAS—Activating mutations of the proto-oncogene *KRAS* are one of the most frequently encountered genetic mutations in CCA. The rate of *KRAS* mutations appear to be higher in pCCA/dCCA (40%) compared to iCCA (9%–24%) (16, 33, 49). The presence of these mutations has prognostic utility as patients harboring *KRAS* mutations have worse progression-free and overall survival (33, 70). Moreover, the *KRAS* mutant tumors are more likely to have adjacent organ involvement and R1 margin status (49). KRAS activation leads to upregulation of downstream effector pathways including PI3k-AKT-mammalian target of rapamycin (mTOR) and Raf/MEK/ERK. Presently, there are no effective direct inhibitors of KRAS and therefore the therapeutic approach for KRAS mutant tumors is inhibition of the downstream pathways. Selumetinib, a selective MEK 1/2 inhibitor, had demonstrated some efficacy in a phase II trial of advanced biliary tract cancers (26). Subsequently, phase I

results of a study with selumetinib in combination with gemcitabine and cisplatin in advanced biliary tract cancer demonstrated a median progression-free survival of 6.4 months and acceptable adverse event profile (71). Other early phase trials of MEK inhibitors with conventional chemotherapy or tyrosine kinase inhibitors in biliary tract cancer are ongoing (NCT02042443, NCT01438554).

PI3K-AKT-mTOR Pathway—The PI3K pathway is activated by stimulation of several different receptor tyrosine kinases (e.g. c-MET, FGFR) with consequent activation of AKT (72). This in turn phosphorylates the mTOR complex which is integral in regulation of several cellular processes including proliferation, survival, tumor invasion, metabolism and angiogenesis (72, 73). Consequently, the activation of the PI3K-AKT-mTOR pathway mediates oncogenesis in a multitude of malignancies including CCA (74–76). Exome sequencing has identified mutations in multiple family members of the PI3K pathway including inactivating mutations of *PIK3R1* and activating mutations of *PIK3CA*, *PIK3C2A*, *PIK3C2G* (12, 63). Activating mutations of *PIK3CA* have been reported in 4–8% of iCCAs and 4% of pCCA/dCCA cases (16, 49, 63).

Presently, there is a multitude of clinical trials investigation various inhibitors of the PI3K-AKT-mTOR pathway in different solid organ malignancies (77). Selective inhibition of mTOR can lead to feedback activation of AKT (78). However, preclinical data suggests that AKT inhibition can potentiate the anti-tumor efficacy of mTOR inhibition (78). The presence of functional redundancy within this pathway and extensive cross-talk with other pathways such as the RAF-MEK-ERK pathway makes combination chemotherapeutic approaches an attractive option. Preclinical data has demonstrated that dual targeting of the PI3K/AKT/mTOR and RAF-MEK-ERK pathways has synergistic effects in CCA (79). However, phase I trial of the PI3K inhibitor BKM120 in combination with mFOLFOX6 (a common chemotherapeutic regimen in gastrointestinal malignancies) revealed increased toxicity with the combination compared to both as single agents (80). Conversely, a phase I trial of the mTOR inhibitor everolimus in combination with gemcitabine and cisplatin, the current practice standard for metastatic CCA, in patients with biliary tract cancer demonstrated stable disease in 6/10 patients and an acceptable safety profile (81).

S100A4—S100A4, a cytoskeleton-associated calcium binding protein, has garnered recent attention for its role in metastasis of various malignancies including cholangiocarcinoma (82, 83). Depending on its subcellular localization, S100A4 contributes to the regulation of a multitude of cell biological processes including proliferation, survival, differentiation, and cytoskeletal rearrangement (82). S100A4 expression has prognostic implications as well, as it is has been identified in the subset of patients with worse outcomes in several malignancies including cholangiocarcinoma (83, 84). Fabris *et al.* identified enhanced nuclear expression of S100A4 in the CCA patients with a worse overall survival following surgical resection (83). Furthermore, nuclear expression of S100A4 was linked with increased CCA cell invasiveness and metastasization, suggesting that it may be a potential therapeutic target in CCA (83). A subsequent study demonstrated that low-dose paclitaxel down-regulated nuclear S100A4 expression. Consequently, a mouse xenograft model treated with low-dose paclitaxel had reduced lung metastasis, albeit no significant effect on local

tumor growth (85). Overall, these findings support the notion that therapeutic targeting of S100A4 may have utility in hindering metastatic progression of cholangiocarcinoma.

Hedgehog Signaling Pathway—The Hedgehog (HH) signaling pathway is an evolutionary conserved, developmental pathway which is known to be deregulated in a number of malignancies including cholangiocarcinoma (86, 87). Preclinical studies have demonstrated that hedgehog signaling inhibition with cyclopamine hinders CCA cell proliferation, migration, and invasion and promotes tumor suppression *in vivo* (86, 88). Hh signaling-induced apoptosis resistance in CCA is mediated by polo-like kinase 2 (PLK2), a cell division regulating kinase (89). Profiling of human iCCA as well as pCCA/dCCA has demonstrated the presence of PLK immunoreactive cells (89). Accordingly, Hh signaling inhibition by cyclopamine reduces PLK expression (89). The role of the non-canonical Hh pathway in CCA has been examined as CCA cells often do not express cilia, a prerequisite for canonical Hh signaling (90). Indeed, the non-canonical Hh pathway promotes tumor progression in CCA. Moreover, a small molecule antagonist of the Hh plasma membrane protein Smoothened, vismodegib, resulted in tumor suppression and inhibition of metastasis in a preclinical CCA model (90). Another small molecule Hh antagonist, BMS-833923, had a more profound tumor suppressive effect in CCA xenografts when combined with gemcitabine, compared to either treatment alone (91).

Mesothelin—Mesothelin, a cell surface protein expressed in normal mesothelial cells, has aberrant tissue expression in a several malignancies including pancreatic adenocarcinoma and CCA (92, 93). Moreover, mesothelin expression appears to have prognostic implications in CCA. In a cohort of 61 surgically resected pCCA and dCCA specimens, mesothelin expression was associated with intrahepatic metastasis, peritoneal metastasis, and worse overall patient survival (94). A series of surgically resected iCCAs also demonstrated an association of mesothelin expression with poor overall survival following surgical resection (95). On the basis of these findings, mesothelin has become a potential target for antibody-based cancer therapy (96). Consequently, several therapeutic agents are under investigation in preclinical and clinical studies. Amatuximab, a chimeric monoclonal antimesothelin antibody, combined with cisplatin was well-tolerated and had a stable disease rate of 90% in patients with advanced pleural mesothelioma (97). However, a phase I study of amatuximab in 17 patients with advanced solid tumors including pancreatic adenocarcinomas demonstrated that only 3 patients had stable disease (98). Phase I results of SS1P, a recombinant anti-mesothelin immunotoxin, in 33 patients with mesothelin expressing malignancies including pancreatic adenocarcinoma, 19 had stable disease and 4 had minor responses (99). A phase II trial of SS1P and pentostatin plus cyclophosphamide in mesothelin expressing malignancies including pancreatic adenocarcinoma is currently ongoing (NCT01362790).

IMMUNOTHERAPY IN CHOLANGIOCARCINOMA (TABLE 1)

Immune checkpoints are essential for the maintenance of self-tolerance and prevention of normal tissue damage during an immune response. However, these checkpoints are dysregulated in cancer and tumors can harness them as an important immune resistance

mechanism (100). Accordingly, activating therapeutic antitumor immunity via blockade of immune checkpoints holds significant potential in cancer therapy.

The immune system has several coinhibitory pathways for maintenance of T-cell tolerance (101). The finding of the critical role of cytotoxic t-lymphocyte associated protein 4 (CTLA-4) in T-cell downregulation was paradigm shifting and spurred the notion that coinhibitory signals block anti-tumor T-cell responses and removal of these signals would have an anti-tumor effect (101). This revolutionary concept ultimately resulted in the use of anti-CTLA-4 monoclonal antibody as effective cancer therapy in patients (102, 103). The same premise lead to therapeutic targeting of the programmed death 1(PD-1)/programmed death ligand-1 (PD-L1) pathway with significant response in human clinical trials for a number of malignancies (104).

A recent molecular characterization of 260 biliary tract cancers demonstrated that 45.2% of the cases had increased expression of immune checkpoint molecules (63). Interestingly, the cluster of patients with the worst outcomes had enrichment of genes involved in the immune system (63). Ye et al. reported significantly enhanced expression of PD-1 and PD-L1 in tumor tissue from surgically resected iCCA specimens compared to adjacent tissue (105). Cases with higher tumor tissue PD-L1 expression were characterized by poor histological differentiation and had more advanced pTNM staging (105). Moreover, the presence of higher PD-L1 expression was inversely correlated with CD8-positive tumor infiltrating lymphocytes (105). In another series of iCCA cases, a favorable clinical course was noted in cases with positive HLA class 1 antigen expression and negative PD-L1 expression, indicating that HLA expression defects in combination with PD-L1 expression may provide an immune escape mechanism for iCCAs (106).

Clinical experience with immune checkpoint inhibitors is very preliminary at this point. Early data from a phase I trial of pembrolizumab, a humanized monoclonal antibody against PD-1, in patients with PD-L1 positive advanced biliary tract cancer demonstrated encouraging evidence of efficacy (107). Overall, 4 patients (17%) had partial response, 4 patients (17%) had stable disease, and 12 patients (52%) had progressive disease (107). A phase II trial of pembrolizumab in advanced solid tumors is currently ongoing (NCT02628067). In another phase II trial, the combination of pembrolizumab and mFOLFOX6 is being investigated in patients with advanced gastrointestinal malignancies including CCA (NCT02268825).

FUTURE PERSPECTIVES

CCA, albeit an orphan disease, is beginning to attract attention from investigators and industry at an accelerated pace, given its lethality (108). Sophisticated clinical trials will require improved methods to stratify CCA patients and the time has come to abandon the inclusion of all patients with biliary tract cancers into a single trial. Future trials must stratify patients according to their anatomic subtype, genetic drivers, and stage of disease. Biomarker-guided information will also be imperative in the development of effective medical therapies for CCA. Towards this end, a well-developed clinical staging system for pCCA has been proposed (109); but even this staging system requires incorporation of

genetic and biomarker information. We await the results of the FGFR and IDH directed therapies described above; however, it is unlikely their effects will be durable even if efficacious in a subset of patients. Cancers are extremely heterogeneous even within a single mass and, unfortunately, efficacious single agent targeted therapy usually results in emergence of resistant clones over time. CCA contain a rich tumor microenvironment including cancer associated fibroblasts (CAF), tumor associated macrophages, and lymphocytes.(110–112). The tumor microenvironment can be exploited to treat CCA. For example, cancer associated fibroblasts can be therapeutically targeted in CCA using BH3 mimetics in preclinical models and such strategies should be applied to the human cancer (112). Immunotherapy to overcome T-cell exhaustion using so called check point inhibitors is also likely to be useful in a subset of CCA patients as described above. However, more attention must also be given to inducing modes of immunogenic cell death to aid immunotherapy strategies (113). In the future, one can envision a portfolio of therapies to treat this disease guided by genetic biomarkers (e.g., FGFR2 directed therapy for a CCA with FGFR2 fusion aberrations) coupled to a checkpoint inhibitor (if the CCA also expresses checkpoint modulators such as PD-L1), or a CAF-directed therapy (if the CCA is highly desmoplastic). We await with eagerness (and impatience) for such combination therapies targeting both CCA cell specific genetic abberations and the tumor microenvironment – our patients have little time.

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Abbreviations

2-HG	2-hydroxyglutarate
BAP1	BRCA1-associated protein 1
CCA	cholangiocarcinoma
dCCA	distal cholangiocarcinoma
EGFR	epidermal growth factor receptor
FDA	Food and Drug Administration
FISH	fluorescence in situ hybridization
FGFR	fibroblast growth factor receptor
HGF	hepatocyte growth factor
HDAC	histone deacetylase
iCCA	intrahepatic CCA

IDH	isocitrate dehydrogenase
IL	interleukin
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid cell leukemia sequence 1
mTOR	mammalian target of rapamycin
O. viverrini	opisthorchis viverrini
pCCA	perihilar cholangiocarcinoma
PI3K	phosphatidylinositol 3-kinase
PD-1	Programmed death 1
PD-L1	Programmed death ligand-1
PTEN	phosphatase and tensin homolog
SMKI	small molecule kinase inhibitor
SOCS-3	suppressor of cytokine signaling 3
STAT	signal transducer and activator of transcription
TP53	tumor protein 53
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand

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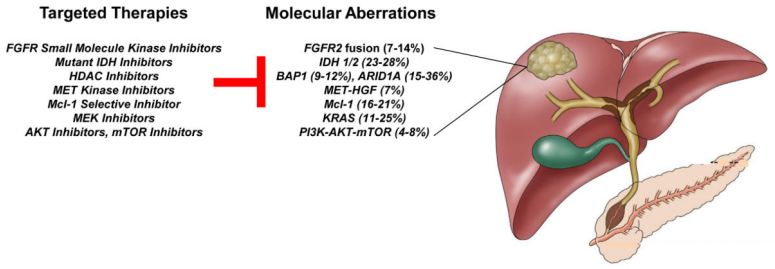

Figure 1.
Molecular aberrations and targeted therapies in intrahepatic cholangiocarcinoma (iCCA).

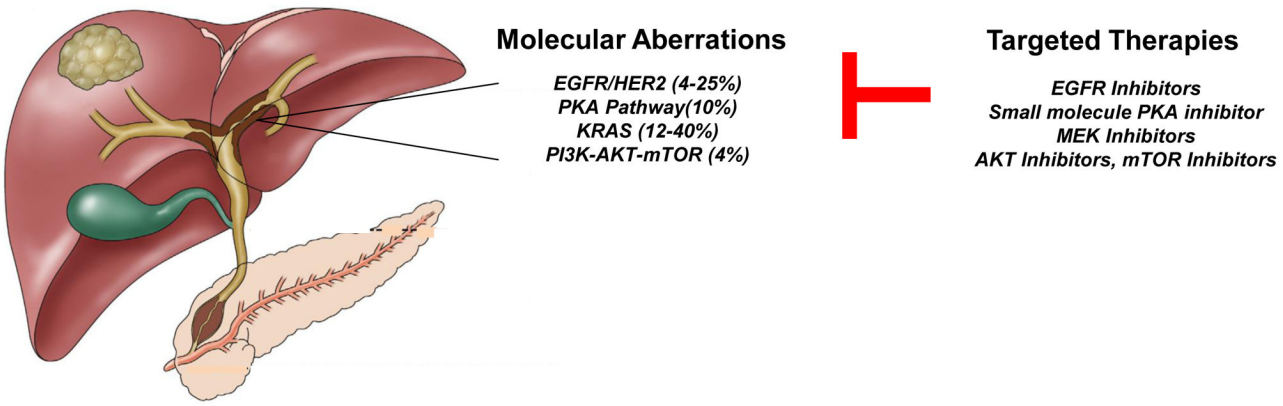

Figure 2.
Molecular aberrations and targeted therapies in perihilar cholangiocarcinoma (pCCA).

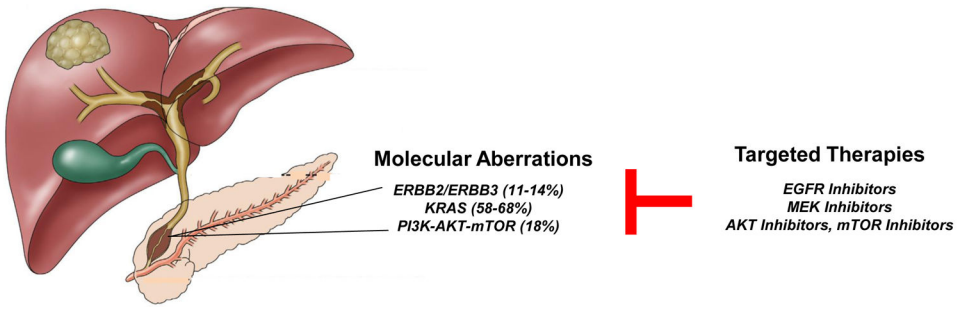

Figure 3.

Molecular aberrations and targeted therapies in ampullary carcinoma and distal cholangiocarcinoma (dCCA).

Table 1

Current Targeted Therapy Clinical Trials in Solid Organ Malignancies including Cholangiocarcinoma

Target	Agent	Trial Description	NCT Number
FGFR	NVP-BGJ398	Phase II trial in patients with advanced CCA harboring *FGFR* gene fusions/aberrations	NCT02150967
FGFR	JNJ-42756493	Phase I trial in patients with advanced solid organ malignancy or lymphoma	NCT01703481
FGFR	ARQ 087	Phase 1/II trial in patients with solid organ malignancy and FGFR genetic alterations (including iCCA patients with *FGFR2* gene fusions)	NCT01752920
FGFR	TAS-120	Phase I trial in patients with advanced solid malignancy or multiple myeloma with or without FGF/FGFR-related abnormalities	NCT02052778
FGFR	CH5183284/Debio 1347	Phase I trial in patients with solid organ malignancy and genetic aberration of *FGFR1*, *2*, or *3*	NCT01948297
FGFR2	Ponatinib	Phase II trial in patients with advanced cholangiocarcinoma harboring *FGFR2* gene fusions	NCT02265341
FGFR2	FPA144	Phase I trial in patients with advanced solid tumors with *FGFR2b* overexpression/amplification	NCT02318329
IDH1	AG-120	Phase I trial in patients with advanced solid tumors harboring an *IDH1* mutation	NCT02073994
IDH1	AG-120	Phase III, multi-center, double-blind, placebo-controlled trial in patients with advanced cholangiocarcinoma harboring *IDH1* mutations	NCT02989857
MEK	Trametinib	Phase II trial in patients with advanced CCA or gallbladder cancer	NCT02042443
MEK, VEGFR/PDGFR/Raf	GSK1120212 plus Pazopanib	Phase I trial in patients with advanced solid tumors	NCT01438554
PD-1	Pembrolizumab	Phase II trial in patients with advanced solid tumors	NCT02628067
PD-1	MK-3475 (Pembrolizumab)	Phase I/II trial in patients with advanced gastrointestinal tumors	NCT02268825