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CURRENT CHALLENGES TO UNDERPINNING THE GENETIC BASIS FOR CHOLANGIOCARCINOMA

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Abstract

Cholangiocarcinoma (CCA) is an aggressive cancer type with a dismal prognosis. CCA typically presents at an advanced stage when surgical resection is not feasible, and systemic chemotherapy is generally of limited benefit. Thus, developing effective therapies against this deadly tumor is imperative and remains an unmet need. To provide more effective treatment options, high-throughput approaches have been employed, leading to a better delineation of each CCA subtype's genomic and transcriptomic landscape as well as the identification of promising candidates for targeted, personalized therapies. In this scenario, the recent approval of pemigatinib, a pan-Fibroblast Growth Factor Receptor (FGFR) inhibitor, for the treatment of the CCA subtype characterized by FGFR2 mutations, represents the first of (presumably) numerous novel therapeutic approaches against this aggressive disease.

Areas covered: This review provides an overview regarding the current scenario and knowledge of the genomic landscape occurring in CCA and the potentially actionable molecular aberrations in each CCA subtype.

Expert opinion: The establishment and advances of high-throughput methodologies applied to genetic and epigenetic profiling are changing the therapeutic landscape of many cancer types, including CCA. These approaches have led to the generation of a large body of data that must be interpreted appropriately and eventually implemented in clinical practice. The following advancements towards precision medicine in CCA management will require designing better clinical trials with improved methods to stratify biliary tumor patients.

Keywords

Cholangiocarcinoma; Genomic landscape; Epigenetics; Next-Generation Sequencing; Molecular pathogenesis; Molecular profiling; Targeted therapies; Driver mutations; Predictive biomarkers; Precision medicine

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1. Introduction

Cholangiocarcinoma (CCA) is a group of highly heterogeneous and aggressive epithelial malignancies emerging at any point of the biliary tract and affecting both genders, with a slight male preponderance. CCAs represent an estimated 3% of all gastrointestinal cancers and can be anatomically classified as intrahepatic (iCCA) and extrahepatic (eCCA) [1,2]. Additionally, eCCAs are divided in perihilar (pCCA) and distal cholangiocarcinoma (dCCA) [3]. In clinical practice, pCCA accounts for approximately 50%-60% of all CCAs, followed by dCCA 20%-30%, and iCCA 10%−20%. Moreover, iCCA comprises about 10% of all primary liver cancers, making it the second most common primary hepatic malignancy after HCC [4,5]. Concerning iCCA, mixed HCC–CCA tumors were recently acknowledged as a distinct subtype of CCA, showing features of both HCC and iCCA [6–9].

1.1 Epidemiology and risk factors

CCA subtypes differ by epidemiology, etiology, pathogenesis, and thus they require different management and therapeutic options. Over the past three decades, a gradual increase in the incidence and mortality rates, primarily iCCA, has been reported worldwide, while treatment options remain limited [10]. Globally, the geographical disparity in the prevalence of risk factors, ethnic variations, and genetic predisposition reflects the discrepancy in sporadic CCA occurrence. Asian countries show the highest incidence globally, accounting for 85 cases per 100000 people. Hepatobiliary flukes due to *Clonorchis Sinensis* and *Opisthorchis Viverrini* infections are considered predominant risk factors in Southeast Asia, where CCA is recognized as common cancer [11,12]. The situation is different in Western countries, where the incidence can be as low as 0.4 cases per 100000 [10,13], and CCA is still considered a rare disease (<6 patients per 100000). Several risk factors have been identified, some shared by all CCA forms, others more specific for a tumor subtype. They include hepatitis B virus, hepatitis C virus, primary sclerosing cholangitis (PSC), biliary lithiasis, cirrhosis, alcohol abuse, obesity, diabetes, and metabolic syndrome. Even though several CCA cases can be associated with a specific etiologic factor, an obvious risk factor cannot be identified in most patients, especially in Western countries. Thus, targeted surveillance in a population with predisposing conditions is unfeasible for this disease. A common characteristic amongst many of these risk factors is that they all contribute to generating a pro-inflammatory environment in the biliary tract [13,14].

1.2 Management

CCAs are typically asymptomatic in the early phase of the disease. No clinical molecular markers for the diagnosis are available, leading to the patient's presentation at an advanced stage when therapeutic options are limited. The prognosis remains poor, with a 5-years overall survival less than 10% [15]. Surgical resection and liver transplantation following neoadjuvant chemoradiation are restricted to patients who present with early-stage disease. However, survival at 5-years is associated with a high risk of recurrence [16], together with the fact that the patient's diagnosis at early-stage remains a compelling challenge. For unresectable advanced CCAs, overall survival is below a year; therefore, patients undergo palliative systemic chemotherapy as standard of care [17,18]. The latter consists of the

combination of cisplatin and gemcitabine, based on results reported a decade ago [19,20]. However, this pharmacological combination confers a median overall survival of about 11.7 months [9]. Other studies followed to evaluate the association with other agents, such as the nanoparticle albumin-bound (nab)–paclitaxel [21]. Only recently, the phase III ABC-06 study established the advantage of modified FOLFOX (5-fluorouracil, folinic acid, oxaliplatin) as a second-line therapy compared to active symptom control [22–24]. The lack of potentially curative medical therapies and its increasing incidence make CCA a growing health concern worldwide. Notably, a possible breakthrough in CCA treatment has been made in early 2020 with the FDA approval of pemigatinib as the first targeted therapy for patients harboring FGFR2 mutations [25,26].

1.3 Histopathology and genomic characterization

CCA arises from the biliary epithelium in eCCA, while hepatic progenitors are believed to play a role in iCCA. The tumor is characterized by a dense stromal component resulting from the recruitment of fibroblasts, remodeling of the extracellular matrix, altered immune cell migration, and angiogenesis. The tumor stroma surrounds the malignant ducts and glands and comprises most of the tumor mass [27]. Although an anatomical distinction is widely used and helped standardize the diagnosis and treatment, CCA heterogeneity supports a different classification considering other parameters such as tumor growth and the cell of origin, which may better predict tumor behavior and facilitate its management [28,29]. Based on the growth pattern, iCCA can be further classified as mass forming, periductal-infiltrating, or intraductal-growing, and eventually, mixed growth patterns can also occur [30]. Instead, pCCA and dCCA appear as flat or poorly defined nodular sclerosing tumors or, less frequently, as intraductal papillary tumors [31]. Histologically, the vast majority of pCCA and dCCA are pure mucinous adenocarcinomas or papillary tumors [1,31]. As concerns iCCA, two main subtypes related to the affected duct’s level or size can be detected: a mucin-producing adenocarcinoma and a mixed subtype, in which areas of adenocarcinoma coexist with areas of hepatocytic differentiation and neoplastic ductular proliferation [30,31]. According to the high intra- and inter-tumor heterogeneity among CCA subgroups, it is highly plausible that CCAs originate from different cell types, including hepatic stem or progenitor cells, cholangiocyte, and hepatocyte lineages at various stages of differentiation, and stem cells from peribiliary glands [28,32].

Interestingly, this histological differentiation matches the high molecular heterogeneity of CCAs and can be ascribed to different cells of origin and pathogenesis. Indeed, small bile duct iCCA are characterized by isocitrate dehydrogenase (IDH1, IDH2) mutations or FGFR2 fusions. By contrast, large bile duct iCCA, similar to pCCA and dCCA, shows a high frequency of mutations in KRAS and/or TP53 genes [1]. Furthermore, several studies have shown that iCCA from the small bile duct is positive to tissue markers such as neural cell adhesion molecule (NCAM), N-Cadherin, SMAD4, and BAP1 loss, while large bile duct in both iCCA and eCCA are positive to mucin, MUC5AC, MUC6, S100P, SMAD4 loss, and BAP1 [1,31]. Notably, a wealth of evidence supports the origin of iCCA from biliary epithelial cells (BECs) (cholangiocytes) of the intrahepatic biliary tract, hepatic progenitor cells (HPCs), or even mature hepatocytes. All liver cells share a common embryonic origin, from bipotential progenitors known as hepatoblasts [32,33]. Recent

findings from genetic lineage–tracing experiments indicate that differentiated (mature) hepatocytes have the potential to promote iCCA by directly changing their fate to a biliary lineage [34,35]. These studies, interestingly, hint at the simultaneous activation of Notch signaling during the transformation of hepatocytes into malignant BECs. Furthermore, other studies have proposed that some hepatocytes may behave like progenitor cells, and upon Notch activation may enable the production of biliary lineage cells [36,37].

On the other hand, large bile duct iCCA, pCCA, and dCCA presumably derive from columnar mucous cholangiocytes or peribiliary glands, which are also implicated in the origin of precursor lesions (such as intraductal papillary neoplasm). Activated cholangiocytes engage in a myriad of cellular processes, including hepatocellular proliferation, apoptosis, angiogenesis, and fibrosis. Cholangiocytes can also regulate the recruitment of immune cells, mesenchymal cells, and endothelial cells that participate in tissue repair and destruction in the context of persistent inflammation [38]. These preneoplastic lesions mainly develop in ducts affected by chronic inflammation as in PSC or following liver fluke infection [39,40]. Chronic inflammation is critical for cancer development and progression since it causes somatic mutation and/or epigenomic alterations and promotes the growth of those cells/clones best adapted to the new inflamed microenvironment.

Despite the abundance of histological, epidemiological, and genetic data collected, the lack of a global picture addressing the cellular and molecular alterations occurring in CCA accounts for an unfavorable clinical outcome. Consequently, there is an imperative need for a better understanding of CCA mechanisms of pathogenesis, and eventually, for the development of effective therapies based on precision medicine and the identification of biomarkers for early detection. In this scenario, the existing CCA stratification based on the anatomical location or pathological features is not suitable to provide insights into the characteristics of tumorigenesis, molecular heterogeneity, or yet to define new patient-tailored target therapy approaches aiming at improving patient management and outcome. Accumulating evidence suggests that the CCA phenotype depends on the crosstalk between cancer cells and the surrounding microenvironment and genetic and epigenetic alterations in the cancer cells. Lately, next-generation sequencing (NGS), exploring genetic and epigenetic alterations, helped to unravel the molecular complexity and heterogeneity of the different subtypes of CCA. Herein, we provide an overview of the recent advances in understanding the mutational landscape of CCA and summarize novel targeted therapies that will promote precision approaches for treating this malignancy.

2. Genetic and epigenetic landscape of cholangiocarcinoma

2.1 Genomic profiling of CCA

In the past, several clinical trials were designed, including patients with different subtypes of CCA based mainly on a broad definition of the disease rather than stratifying them according to gene-specific carcinogenic drivers. Nowadays, with the advent of large-scale genomic profiling, several studies have delineated, in detail, the genomic and transcriptomic phenotypes and described specific mutations for each CCA subtype. Indeed, the efforts in applying integrative genomics showed an increase in anti-apoptotic signaling, angiogenesis,

signal transduction, and transcriptional control [41,42]. The most common genetic variants in CCA (Table 1) affects key oncogenic networks, such as DNA and genomic instability (*TP53*, *CDKN2A*, *CCND1*, *ATM*, *ROBO2*, *BRCA1*, and *BRCA2*), *c-MYC* amplification, kinase signaling (*KRAS*, *BRAF*, *ERBB1–3*, *PIK3CA*, *SMAD4*, and *FGFR1–3*), de-ubiquitination (*BAP1*), SWI–SNF complex (*PBRM1*, *ARID1A*, *ARID1B*, *ARID2*, *SMARCA2*, *SMARCA4*, and *SMARCAD1*), epigenetic regulation including NADPH metabolism (*IDH1* and *IDH2*), and histone (de)methylation (*MLL2*, *MML3*, *KMT2C*, *KDM4A*, *KDM5D*, *KDM6A*, and *KDM6B*), immune dysregulation (JAK–STAT3 signaling); FGFR2 and PRKCA–PRKCB fusions, the WNT–CTNNB1 pathway, Hippo signaling (*NF2*, *SAV1* deletion), and deregulated Notch signaling [43–52]. Of note, it seems that the predominant genomic alterations in CCA are associated with epigenetic processes [53,54]. Also, the hotspot IDH mutations, $IDH1^{R132}$ and $IDH2^{R172}$, which cause accumulation of the oncometabolite 2-hydroxyglutarate (2-HG) [55–57], as well as the gene fusion events occurring between FGFR2 and several different partners, with the most frequent being the *BICC1*, *PPHLN1*, *TACC3*, and *MGEA5* genes, appear a peculiar molecular feature of iCCA lesions [44,46,47,49]. Although FGFR2 fusion events are prominent in iCCA, other fusions can also occur, as described by Yu et al. [58]. In particular, they reported a female iCCA patient with an EHBP1-MET fusion and multiple intrahepatic metastases responding to crizotinib treatment. These alterations are important, as they are the object of investigation in phase III clinical trials testing specific inhibitors that may be the first to transform iCCA clinical management, displaying prospective efficacy in fusion-positive FGFR2 or IDH mutant patients. The results shown in all these studies emphasize the need to include genomic analyses of the tumor samples when making a clinical decision. However, only a few of these studies have investigated the distinct molecular features among the biliary tract cancer subtypes. For instance, Nakamura et al. performed exome and transcriptome sequencing on samples from 260 patients diagnosed with hepatobiliary malignant diseases (iCCA, eCCA, and gallbladder carcinoma). Interestingly, nearly 40% of cases with biliary cancer proved to bear alterations in putative driver genes. The results showed that recurrent mutations in *IDH1–2*, *FGFR1–3*, specifically FGFR2 gene fusions, and *BAP1* were primarily associated with iCCA. In contrast, the novel gene fusions involving PRKACA and PRKACB, which encodes for the catalytic subunits of PKA, and mutations in *ARID1B* and *ELF3* genes occurred specifically in eCCA. Likewise, *ERBB3* and *EGFR* mutations were detected only in gallbladder cancer. Importantly, significant enrichment of hypermutated tumors was associated with poor overall survival and a characteristic increment in the expression of immune checkpoint molecules and anti-apoptotic signatures, suggesting an important role of the neoplastic immune environment [48]. Nepal et al. recently applied stratification of iCCA patients based on the occurrence of the three most frequently mutated genes, *IDH1/2*, *KRAS*, and *TP53*. The study revealed unique oncogenic programs, including mutational and epi-mutational signatures, structural alterations, and deregulated pathways. To test their results' clinical implications, the authors used a drug repositioning approach and screened a library of more than 500 drugs in patient-matched cell models. The findings highlighted the capability of individual mutations to induce molecular heterogeneity, which could facilitate the advancement of pharmacological and therapeutic responses [52]. Jiao et al. performed exome sequencing on iCCA specimens and detected inactivating mutations in chromatin remodeling genes, including *ARID1A*,

PBRM1, and *BAP1* [59]. In other studies, targeted sequencing was performed on cancer-related genes, including *IDH1–2*, *FGFR2*, and *CDKN2A*. The most significant changes were reported in *ARID1A*, *IDH1/2*, and *TP53* genes (each mutated in ~36% of the tumors) and *MCL1* (amplified in 21% of iCCA specimens) [47,48,60]. Lowery et al. conducted another prospective analysis of 195 patients, consisting of 78% of iCCA and 22% eCCA. Tumors, profiled for somatic genomic alterations, showed that the most recurrent altered genes were *IDH1* (30%), *ARID1A* (23%), *BAP1* (20%), *TP53* (20%), and *FGFR2* fusions (14%). The study also demonstrated that alterations in *CDKN2A/B* and *ERBB2* genes were associated with reduced survival and adverse prognostic outcome. Genetic alterations with potential therapeutic implications were identified in 47% of patients, leading to biomarker-directed therapy or clinical trial enrollment in 16% of patients [61]. Comprehensive genomic profiling by Javle et al. was performed on 554 patients, of which 412 iCCA, 57 eCCA, and 85 gallbladder carcinomas. The study highlighted that *TP53* (27%), *CDKN2A/B* (27%), *KRAS* (22%), *ARID1A* (18%), *IDH1* (16%), and *FGFR* (11%) mutations were most prominent in iCCA; *KRAS* (42%), *TP53* (40%), *CDKN2A/B* (17%), and *SMAD4* (21%) in eCCA, and *TP53* (59%), *CDKN2A/B* (19%), *ARID1A* (13%) and *ERBB2* (16%) in gallbladder tumors. *FGFR* and *IDH* mutations were mostly limited to iCCA but appeared to be mutually exclusive. In the iCCA group, *TP53* and *KRAS* mutations were associated significantly with poor prognosis, while *FGFR2* mutations were correlated with improved overall survival [62]. Sia et al. identified, through whole-genome analyses, two unique subclasses in iCCA: an inflammatory class with a predominant activation of inflammatory pathways and a proliferation class with predominant activation of RAS and MET oncogenic pathways, mutations in *KRAS* and *BRAF,* and other oncogenes that correlates with worse patient outcome [63]. Wardell et al. analyzed 412 biliary tract cancer from Japanese and Italian populations. They identified 32 significantly and commonly mutated genes, including *TP53*, *KRAS*, *SMAD4*, *NF1*, *ARID1A*, *PBRM1*, and *ATR*, some of which negatively affected the patient's prognosis. They also identified a novel deletion in the *MUC17* gene at 7q22.1, influencing the patient's outcome [64]. On a cohort of 80 Chinese patients bearing eCCA tumors, Xue et al. reported that the most frequently altered genes were *TP53* (68%), followed by *KRAS* (46%), *SMAD4* (22%), *ARID1A* (20%), and *CDKN2A* (19%). The top three actionable alterations included *CDKN2A*, *BRAF*, and *ERBB2* [65]. Montal et al. identified *KRAS* (36.7%), *TP53* (34.7%), *ARID1A* (14.0%), and *SMAD4* (10.7%) as the prevalent mutations in a cohort of 189 US and European patients with eCCA. The integrative genomic analysis defined four different subclasses of eCCA: metabolic (19%), proliferation (23%), mesenchymal (47%), and immune (11%). They also observed recurrent chromosomal amplifications in *YEATS4* (6.0%), *MDM2* (4.7%), *CCNE1* (2.7%), *CDK4* (1.3%), and *ERBB2* genes (1.3%) [66]. Genomic profiling was also applied by Yang et al. on a cohort of 108 Chinese and 107 US patients with gallbladder cancer. The most frequent alterations were *TP53* (69.4%), *CDKN2A/B* (26%), *ERBB2* (18.5%), *PIK3CA* (17%), and *CCNE1* (13%) in the Chinese cohort, whereas *TP53* (57.9%), *CDKN2A/B* (25%), *SMAD4* (17%), *ARID1A* (14%), *PIK3CA* (14%), and *ERBB2* (13.1%) were the predominant changes in US patients. The study indicates that most Chinese and US patients have actionable alterations that could potentially guide and influence personalized treatment options [67]. Furthermore, germline mutations appeared to be equally distributed among the iCCA, eCCA, and gallbladder subtypes, as reported by Maynard et al. after performing

germline variant analysis on a panel of up to 88 genes associated with an increased predisposition to cancer. Pathogenic germline alterations were most commonly observed in *BRCA1* and *BRCA2* genes (33.3%), followed by *PALB2*, *BAP1*, and *PMS2* [68]. Cao et al. performed comprehensive molecular profiling of 164 Chinese and 283 US patients with iCCA to explore genomic heterogeneity between populations, discovering important differences between the two ethnic groups. Specifically, Chinese patients had a significantly higher frequency of *TP53* and *KMT2C*, *BRCA1/2*, *DDR*, *TERT*, *TGFBR2*, *RBM10*, *NF1*, *SPTA1*, and *RB1* genetic aberrations. In the Western cohort, *IDH1/2*, *BAP1*, and *CDKN2A/B* were instead more dominant, ascribing the genetic diversity to variations in the underlying disease risk factors [69].

Eventually, the potential involvement of inherited CCA predisposition, which exogenous risk factors might modulate, renders the whole picture even more complex. However, these data are minimal. In the next future, extensive and integrated studies will be necessary to achieve personalized diagnostics and therapy in patients with different CCA subtypes. The little evidence of genomic association with aetiological risk factors investigated by genome sequencing has been demonstrated concerning liver fluke infection [70]. Noticeably, fluke-positive tumors showed an overall higher mutational rate with prevalent mutations in *SMAD4* and *TP53* as well as *ERBB2* amplification [43,71,72]. Furthermore, although not in a high proportion, *KRAS* mutations have been recurrently found in all CCA subtypes [71,72]. A statistically significant association has also been observed between *TP53* mutation and HBV infection [45,52].

2.2 Epigenetic alterations of CCA

Genetic aberrations may not fully explain the rapid progression and high chemoresistance of CCA. It has recently become apparent that epigenetic dysregulation, including DNA methylation, histone modification, chromatin remodeling, and non-coding RNAs (ncRNAs), may play a relevant role in tumorigenesis without modifying the DNA sequence [54]. Interestingly, all these epigenetic events are interconnected during cholangiocarcinogenesis [73–75]. Indeed, epigenetic perturbations have been proposed to function as oncogenic drivers and influence cancer heritability [76]. DNA methylation is a critical event with essential roles in gene regulation during normal development. At the same time, DNA methylation changes are one of the earliest alterations that characterize tumor development [77,78]. Besides, hypermethylation occurring in tumor suppressor gene promoters and global hypomethylation of the genome are key determinants causing transcriptional inactivation of a gene and malignancy. Yang et al. reported that CpG island methylation of tumor suppressor gene promoters is a frequent epigenetic event in this disease. It occurs in approximately 85% of all CCA cases, regardless of the anatomical site [79]. One of the most relevant epigenetic studies in CCA revealed an association between CpG hypermethylation and liver fluke-related tumors, increased mutation rate, elevated levels of EZH2 (a histone methyltransferase), and decreased expression of the TET1 demethylase. Hypermethylation of CpG sites was also observed in an iCCA cohort characterized by the enrichment of FGFR translocations and *IDH1/2* and *BAP1* mutations [71]. The situation becomes more complex when interpreting the therapeutic effects of mutant IDH inhibitors. Indeed, uncoupling the global consequences due to *IDH1/2* mutation in epigenome regulation from the additional

effects of metabolic regulation induced by the oncometabolite 2-hydroxyglutarate (2-HG) remains a task to accomplish [51,57,80–82]. Many other candidate genes exhibiting altered CpG methylation belonged to WNT, TGFβ, PI3K, MAPK, and NOTCH signaling pathways [83]. The promoter of *SOCS3*, the upstream regulator of the JAK/STAT cytokine signaling pathway, is frequently hypermethylated in CCA as reported by Isomoto et al. SOCS3 loss results in the sustained activation of the IL-6/STAT-3 signaling and the enhanced *MCL1* gene expression in CCA [84]. Similarly, *SFRP1*, a Wnt signaling modulator, is hypermethylated in CCA in approximately 85% of the patients analyzed [85]. More recently, the CpG site methylation profile of 172 iCCA unraveled that methylation of the *DLEC1* locus was associated with a better clinical outcome for both cancer-specific survival and recurrence-free survival [86]. On the contrary, the *SOX17* promoter was found hypermethylated in CCA, and its expression inversely correlated with the methylation grade [87]. Dysregulated expression of epigenetic regulators and histone post-translational modifications represent additional mechanisms mediating epigenetic perturbations in CCA. Histones are highly basic proteins that pack and order the genomic DNA into structural units called nucleosomes. Several histone deacetylase (HDAC) enzymes affecting the chromatin conformation are upregulated in CCA. Therefore, they have been investigated as potential targets for treatment [88,89]. Recent evidence suggests that various HDAC inhibitors can block CCA growth alone or in combination with chemotherapeutic agents both in vitro and in vivo [90–93]. Also, it seems that HDAC inhibitors could exert their activity in IDH mutated cells [94,95]. Moreover, the histone methyltransferase *EZH2* is overexpressed in CCA and associated with poor prognosis of the patients. Overexpression of *EZH2,* especially in both the nucleus and cytoplasm, can be used as a prognostic marker for CCA [96]. Inhibition of *EZH2* resulted in reduced proliferation, cell cycle arrest, and survival in vitro and impaired tumor growth in vivo [97,98]. Recently, another study by Nakagawa et al. demonstrated that high *EZH2* gene expression was associated with activation of the tumor angiogenesis pathway, predicting iCCA patients' prognosis [99]. Recurrent inactivating mutations or deletions in multiple chromatin-remodeling factors, such as *BAP1*, *ARID1A*, *PBRM1*, and *SMARCB1,* have been reported in about one-third of CCA cases [100]. The tumor suppressor *BAP1* is a deubiquitinase, whereas *ARID1A* and *PBRM1* are both subunits of the SWI/SNF complex [59]. Importantly, the SWI/SNF complex mediates nucleosomes repositioning to allow an efficient DNA repair while regulating transcription and DNA replication [53]. Notably, mutations inducing *ARID1A* and *BAP1* loss of function may also inhibit double-strand repair (DSR) [101,102], conferring hypersensitivity to poly ADP ribose polymerase (PARP) inhibitors alone and also in combination with radiation [103]. With this in mind, patients with *ARID1A* or *BAP1* mutations may constitute an ideal subgroup to evaluate this treatment option further. The inactivation of these chromatin remodelers by mutations makes it difficult or even impossible to restore them. Therefore, it will be crucial a further evaluation of their biological roles in CCA with a better understanding of the downstream signaling events induced by *BAP1*, *PBRM1*, and *ARID1A* loss of function, and hopefully being able to take advantage of the vulnerabilities these tumors may acquire [104].

Non-coding RNAs (ncRNAs) are single-stranded RNA molecules, which are not translated into proteins. ncRNAs include microRNAs (miRNAs) and long ncRNAs, among others, and

can regulate multiple cellular pathways affecting all the aspects of the cancer phenotype from increased proliferation, invasion, migration, and chemoresistance to epithelial/mesenchymal transition (EMT), inflammation, and the regulation of the primary cilium in cholangiocytes [105–107]. Several miRNAs are deregulated during CCA development, thus being able to affect different cancer-related pathways. Gene expression profiling studies have identified miRNAs with tumor-promoting (e.g., miR-21, miR-25, miR-26a, miR-181c and miR-191) or tumor-suppressing (e.g., miR-29, miR-200b/c, miR-203 and miR-214) functions [107]. For instance, overexpression of miR-21, miR-25, miR-421, and miR-221 in CCA have been classified as markers of poor prognosis. Likewise, miR-181c overexpression represses N-myc downstream-regulated gene 2 (*NDRG2*) gene, leading to a significant decrease in overall survival in CCA patients due to increased proliferation chemoresistance and EMT promotion [108].

Conversely, many tumor suppressor miRNAs, such as miR-122 and miR-605, are downregulated in CCA cells in which tumor growth is enhanced. Overexpression of these miRNAs may represent a mechanism-based therapy for CCA [109,110]. Similar to mRNA and miRNA, also long ncRNA signatures were recently reported in CCA. They are negatively correlated with CCA's prognosis promoting proliferation, migration, invasion, and inhibit apoptosis of CCA. Moreover, they may contribute to CCA development via modulating gene transcription, sponging microRNA, regulating CCA-related signaling pathways or protein expression. Long ncRNAs are thought to be potential diagnostic markers and therapeutic targets for CCA [111]. Several lncRNAs, such as ASAP1-IT1, CCAT1, CCAT2, CPS1-IT1, H19, HOTAIR, MALAT1, NEAT1, PANDAR, SPRY4-IT1, PVT1, TP73-AS1, CASC15, HULC, TUG1, and UCA1, are elevated in CCA and associated with increased proliferation, migration, enhanced metastasis and poor overall survival [111–114]. From an epigenetic point of view, CCA still requires more focused studies. Although ncRNAs' role has received increasing consideration in the last few years, little is known about their function(s). Given the high heterogeneity of CCA, they may be functional only in a subset of tumors. However, a more profound comprehension of these molecules' functionality may hold translational potential for diagnosis and prognosis and the improvement in designing new therapeutic drugs.

2.3 Alterations in precursor and pre-invasive lesions

Recent studies have shown the existence of at least two types of pre-invasive neoplasms of the bile ducts preceding frankly invasive CCA: the microscopic biliary intraepithelial neoplasm (BilIN) and the macroscopically visible intraductal papillary or tubulopapillary neoplasms of the bile duct, IPNB and ITPN, respectively. Historically, IPNBs have been studied with reference to intraductal papillary mucinous neoplasm of the pancreas (IPMN) based on their histological similarities. However, there is rising evidence that IPMN and IPNB are not identical regarding morphology, molecular biology, and clinical course [115].

In 2014 Schlitter et al. [116] suggested that the oncogenetic profile of IPNB follows the stepwise progression from low-grade intraductal papillary dysplasia to invasive adenocarcinoma. They identified *TP53, KRAS,* and *CDKN2A* as the most commonly affected genes associated with low-grade intraepithelial neoplasia, implying that these

molecules' mutational changes are among the events initiating the intraductal epithelial proliferations. The high-grade intraepithelial neoplasia to invasive carcinoma was characterized instead by an increased expression of nuclear TP53 and SMAD4 loss. Among other genes, whose changes appeared to be less relevant in the carcinogenesis process, they found *GNAS, CTNNB1, HER2,* and *EGFR* alterations [116].

Recently, by next-generation sequencing, Yang et al. [117] and Aoki et al. [118] identified frequent mutations in IPNBs. Yang et al. evidenced the molecular change in 37 IPNBs and discovered frequent mutations of *KRAS* (49%), *GNAS* (32%), *RNF43* (24%), *APC* (24%), *TP53* (24%), and *CTNNB1* (11%). Furthermore, a hierarchical analysis identified three distinct groups within IPNB: group 1, showing tumors with macroscopic mucin, old age, and frequent *KRAS, GNAS*, and *RNF43* mutations; group 2, exhibiting intestinal differentiation and frequent KRAS mutation but rare *GNAS* mutation, *MUC2* expression, and macroscopic mucin; and group 3, characterized by *CTNNB1* mutation, extrahepatic location, lack of expression of intestinal markers, and lack of mutations in *KRAS, APC, RNF43*, and *GNAS* [117]. In his work, Aoki et al. studied 36 IPNBs retrospectively. The team was able to identify several recurrent mutations in *TP53* (34.3%), *KRAS* (31.4%), *STK11* (25.7%), *CTNNB1* (17.1%), *APC* (14.3.%), *SMAD4* (14.3%), *GNAS* (11.4%), *PBRM1* (11.4%), *ELF3* (8.6%), *KMT2C* (8.6%), *NF1* (8.6%), *PIK3CA* (8.6%), *ARID1A* (5.7%), *ARID2* (5.7%), *BAP1* (5.7%), *BRAF* (5.7%), *EPHA6* (5.7%), *ERBB2* (5.7%), *ERBB3* (5.7%), *KMT2D* (5.7%), and *RNF43* (5.7%). The specimens were also classified into two groups based on a collaborating consensus study by Japanese and Korean pathologists. Type 1 was defined as a neoplasm showing well-organized and relatively uniform papillary growth with thin fibrovascular stalks. Type 2 was defined as a lesion exhibiting complex papillary growth with thick papillae or irregular branching. This classification of IPNB has helped uncover characteristic clinicopathological phenotypes and molecular tumorigenic mechanisms relevant for better clinical management of patients with IPNB [118]. Singhi et al. [119] analyzed 20 Intraductal oncocytic papillary neoplasms (IOPNs) of the pancreas and bile duct using a broad RNA-based targeted sequencing panel to detect cancer-related fusion genes. They found that all IOPNs had recurring fusions of ATP1B1–PRKACB, DNAJB1–PRKACA, or ATP1B1–PRKACA. These fusions also were found in corresponding invasive PDACs and intrahepatic CCAs, as well as in matched pancreatic cyst fluid and bile duct brushings. This work helped establish these recurrent fusion genes as genetic drivers concerning IOPNs. Because these rearrangements do not occur in other tumor of the pancreatobiliary system, the study supports the classification of IOPN as a distinct neoplasm from IPMNs and IPNBs [119].

Goeppert et al. [120] analyzed the genetic and epigenetic alterations in a well-characterized cohort of 54 patients with high-grade intraductal papillary (IPNB) or tubulopapillary (ITPN) neoplastic precursor lesions of the biliary tract. They correlated the results with an independent non-IPNB/ITPN associated CCA cohort (n=294). Their data confirmed well-known candidate genes such as *TP53, KRAS,* and *CDKN2A*. Furthermore, they could build a genetic evolution plot presenting distinct mutations that emerge in IPNB/ITPN and vanish during transformation to invasive CCA. For instance, *CTNNB1* and *CDKN2A* mutations occurred in many precursor lesions and were then commonly lost in invasive CCA. In

contrast, *ROBO1, ROBO2,* and *FBXW7* gene alterations were increasingly detected in invasive CCA but generally absent in IPNB/ITPN [120].

In conclusion, all these studies confirm IPNB and IPTN as unique forms of cholangiocarcinogenesis. These precursor lesions are involved in the stepwise progression from non-neoplastic biliary epithelium to invasive CCA, but also their presence could indicate a better prognosis and patient survival.

3. Targeted therapies based on genomic alterations in cholangiocarcinoma

As discussed above, recent studies have identified critical carcinogenic drivers as promising druggable targets in this disease. To date and according to these targets, several tailored compounds have been tested in clinical trials, and the most favorable results have been reported for patients harboring FGFR2 fusions or IDH mutations. Moreover, as indicated in Table 2, several other clinical trials using targeted therapies or combination therapies are ongoing with the scope to unravel new effective agents or to improve the available treatments.

3.1 FGFR2 fusions

The mammalian fibroblast growth factor (FGF) has evolved as an extraordinarily complex pathway regulating many physiological processes. It comprises 18 ligands, which exert their actions through 4 highly conserved transmembrane tyrosine kinase receptors, namely FGFR1, FGFR2, FGFR3, and FGFR4. A fifth receptor, FGFR5 (also known as FGFRL1), binds to FGFs but has no tyrosine kinase domain, and it is supposed to regulate the signaling pathway negatively [115]. Receptor activation by FGFs initiates a cascade of intracellular events that activate major survival and proliferative signaling pathways, mediating crucial physiological mechanisms, such as tissue and metabolism homeostasis and endocrine functions, and wound repair [121]. FGF ligands are secreted glycoproteins, which are readily stored within the extracellular matrix (ECM), and the cell surfaces by heparan sulfate proteoglycans (HSPGs), a cofactor. Cell-surface HSPGs stabilize the FGF ligand-receptor interaction by protecting FGFs from protease-mediated degradation [122]. Enzymatic cleavage releases the ligands, which bind to FGFRs, leading to FGFR dimerization and intracellular domain tyrosine residue phosphorylation. The subsequent recruitment, binding, and phosphorylation of adaptor proteins FGFR substrate 2 (FRS2), son of sevenless (SOS), and GRB2, is followed by the activation of several downstream transduction pathways, including mitogen-activated protein kinase (MAPK), PI3K/Akt/mTOR, Ca^{2+}/calmodulin-dependent protein kinase (CaMK), and signal transducer and activator of transcription 3 (STAT3) (Fig. 1). Activation of the MAPK pathway may also occur independently of FRS2 via binding of Src homology 2 of phospholipase C-gamma (PLC-γ) and subsequent protein kinase C (PKC) activation [123–128]. It has been established that FGF/FGFR signaling cascades are strongly regulated by feedback mechanisms. However, these attenuation and negative feedback control mechanisms are still poorly understood and depending on the cell type. Downstream signaling can be attenuated by the induction of the MAPK phosphatase MAPK3, Sprouty (SPRY) proteins, and Similar Expression to FGF (SEFs) family members

that modulate receptor signaling at several points in the signal transduction cascade. Following activation, FGFRs are internalized and then degraded or recycled according to ubiquitination levels [128–132]. In the CCA context, the FGF pathway plays a pivotal role in multiple biological processes, including the regulation of cell proliferation, differentiation, survival, wound repair, angiogenesis, and migration [122]. FGFR fusions, rearrangements, translocations, and gene amplifications are closely correlated with various cancers' development. As reported above, FGF signaling alterations, specifically FGFR2 gene fusions, occur in 11% to 45% of all iCCA [49]. In April 2020, the FDA approved pemigatinib (Pemazyre, INCB054828) (Fig. 1) for the treatment of adults with previously treated, unresectable locally advanced, or metastatic cholangiocarcinoma with an FGFR2 fusion or other rearrangements [25,26]. The FDA approval evaluated data from the FIGHT-202 study, a multi-center, open-label, single-arm study. It included 146 patients, 107 with FGFR2 fusions or rearrangements, 20 with other FGF/FGFR alterations, 18 with no FGF/FGFR alterations, and one patient had an undetermined FGF/FGFR alteration. After a median follow-up of 17.8 months, the overall response rate (ORR) was 36%, out of which three patients had a complete response (CR) and 35 had partial responses (PR). Of the 38 patients who had a response, 24 patients (63%) had a response lasting at least 6 months, and seven patients (18%) had a response lasting at least 12 months, with a median of 9.1 months for the duration of response. Pemigatinib is also currently under review by the European Medicines Agency (EMA), which granted provisional marketing authorization to the drug. The most adverse events associated with pemigatinib include serious dry or inflamed eyes, cornea, and disorders of the retina. Pemigantinib can also induce hyperphosphatemia and an increased risk in pregnant women of harm to the fetus or miscarriages [26]. Based on these promising results, FDA approved the follow-on FIGHT-302 clinical trial to evaluate the efficacy of pemigatinib in the first-line setting versus gemcitabine plus cisplatin chemotherapy [133]. Infigratinib (NVP-BGJ398), an oral pan FGFR antagonist, in a single-arm phase II trial was used with patients harboring FGFR2 fusion as a second-line treatment. ORR was 31% with a median of progression-free survival PFS of 6.8 months. The toxicity profile was similar to pemigatinib with hyperphosphatemia being the most common adverse event (72%) [134]. These promising preliminary results suggested that infigratinib has activity in heavily pretreated FGFR2 fusion-positive cholangiocarcinoma and supports assessing the role of this drug in the first-line setting. As a result, infigratinib is being investigated in the ongoing PROOF-301 trial compared with standard of care chemotherapy in cholangiocarcinoma patients harboring FGFR2 gene alterations [135]. There are several other FGFR selective inhibitors in clinical development, including derazantinib (ARQ087; pan-FGFR inhibitor) [136,137], erdafitinib [138], futibatinib (TAS-120; irreversible FGFR1–4 inhibitor) [139,140], ponatinib [46], and Debio 1347 (FGFR1–3 inhibitor) [141]. Of note, erdafitinib has been approved by the FDA for the treatment of urothelial cancer in patients with FGFR2/3 alterations [142].

3.2 IDH1 and IDH2 mutations

Isocitrate dehydrogenase (IDH) promotes the conversion of isocitrate to α-ketoglutarate and participates in the citric acid cycle and other metabolic processes [143–145]. IDH1 mutations are more common than IDH2 mutations and are point mutations located in the arginine 132 (R132) residue in the *IDH1* gene or the arginine 172 (R172) residue in *IDH2*.

These mutations are present at a higher frequency in iCCA than extrahepatic CCA. They result in dysfunctional enzymes and lead to increased intracellular levels of 2-hydroxyglutaric acid (2-HG), which causes extensive epigenetic changes and affecting cell differentiation, growth, and hypoxia signaling [146] (Fig. 2). At present, several clinical trials are evaluating the efficacy of several IDH inhibitors for the treatment of iCCA, such as ivosidenib, IDH305 or dasatinib (IDH1), and AG221 (IDH2). The pan-IDH1/2 inhibitor (AG881) is at the moment used in a study recruiting participants with residual or recurrent grade 2 Glioma with an *IDH1* or *IDH2* mutation (INDIGO) (Table 2). Promising results arose from phase I clinical study with ivosidenib, showing disease stabilization in 56% of the patients and a partial response in 6 % [147]. In the following phase III ClarIDHy trial, 185 patients were enrolled with metastatic IDH1 mutated CCA and treated with ivosidenib or placebo. Progression-free survival was significantly improved with ivosidenib compared with placebo. The most common grade 3 or worse adverse event in both treatment groups was ascites, followed by nausea, diarrhea, and fatigue [82].

3.3 Other tailored therapies currently in clinical trials

Numerous additional targets are currently investigated for CCA treatment (some depicted in Fig. 3; ongoing clinical trials summarized in Table 2). In particular, potential targeted therapies against BRAF and MEK, EGFR, MET, ERBB2, and VEGFR proteins are under clinical evaluation. The MAPK pathway is being targeted using BRAF and/or MEK inhibitors. The limited efficacy of these inhibitors alone has encouraged the use of an alternative dual inhibition of BRAF (dabrafenib) and MEK (trametinib) in a study that is part of an ongoing, phase II, open-label, single-arm, multi-center, Rare Oncology Agnostic Research (ROAR) basket trial in patients with $BRAF^{V600E}$-mutated rare cancers [148]. Dabrafenib plus trametinib combination treatment showed promising activity in patients with $BRAF^{V600E}$-mutated biliary tract cancer, with a manageable safety profile and a median follow-up of 10 months. The most common grade 3 or worse adverse event was increased γ-glutamyltransferase, followed by pyrexia [148]. In a phase 2 MATCH screening trial, the responsiveness to afatinib, crizotinib, and ado-trastuzumab emtansine inhibitors is tested in tumors harboring mutations in *EGFR*, *c-MET*, and *ERBB2* gene, respectively. In a recent study, ERBB2 amplification or mutations were identified in 28 (5.4%) of 517 patients with biliary tract cancer. Frequent co-altered genes in this cohort were *TP53*, *PIK3CA*, and *CDKN2A*, while *KRAS* alterations were less common. One patient with *ERBB2*-amplified eCCA enrolled in a basket trial had a partial response to the HER2-targeted antibody-drug conjugate ado-trastuzumab emtansine [149].

Studies using the PARP inhibitors niraparib and olaparib are currently ongoing in CCA and solid tumors carrying mutations of genes that regulate the DNA damage response, including ARID1A and BAP1. Two active different phase-II trial studies are underway to evaluate the efficacy of olaparib and olaparib plus ceralasertib (an oral inhibitor of the serine/threonine-protein kinase ATR) in treating patients with glioma, cholangiocarcinoma, or solid tumors with IDH1 or IDH2 mutations that are metastatic and refractory to other treatments. Olaparib may hamper the proliferation and expansion of tumor cells by blocking some of the enzymes needed for cell growth. Also, multikinase inhibitors targeting VEGFR such as regorafenib, sorafenib, apatinib, and cediranib displayed anti-tumor activity against CCA.

Regorafenib is an oral multikinase inhibitor that predominantly targets VEGFR (1–3), PDGFR-β, and FGFR1 proteins, which are involved in tumor angiogenesis and metastasis. Recently, the Reachin trial, a randomized phase II study, evaluated the safety and efficacy of regorafenib in patients with nonresectable/metastatic biliary tract cancer that progressed after gemcitabine/platinum chemotherapy. Regorafenib significantly improved the progression-free survival and tumor control, encouraging the evaluation of regorafenib in phase III studies and identifying patients who may benefit from regorafenib [150]. However, all patients progressed, and the overall survival was only 5.3 months with regorafenib treatment. Even though significant progress in understanding CCA pathogenesis has been made in the last decade through NGS platforms, most results from clinical trials using targeted therapies have been short-lived. This observation underlines the high heterogeneity of these tumors and the necessity to advance our knowledge of tumor biology and mechanisms of drug resistance and improve treatment outcomes by using a combination of targeted therapies.

3.4 Targeted therapies in the absence of mutations: The case of CDK4/6 inhibitors as a potential novel therapy for CCA

The mammalian cell cycle is driven by a complex interaction of cyclins and their associated cyclin-dependent kinases (CDKs), and its dysregulation is a hallmark of cancer-inducing unconstrained proliferation [151,152]. D-type cyclins and their associated CDKs (CDK4 and CDK6) are key components of cell cycle machinery in driving G1 to S phase transition via phosphorylating and inactivating the retinoblastoma protein (pRb) [153]. In physiologic conditions and under the stimulation of extracellular signals, cyclin D-CDK4/6 phosphorylates the pocket proteins, namely the pRb, p107 (also known as RBL1), and p130 (also known as RBL2), which leads to unrestrained E2F transcription factor activity, induction of E2F-dependent transcriptional programs and progression through G1 to S phase [154]. In particular, unphosphorylated (active) pRb binds to the E2F transcription factors, recruits co−repressors, and represses the transcription of E2Fs target genes (Fig. 4). Therefore, it is unsurprising that the vast majority of cancers subvert the CDK4/6–RB–$p16^{INK4A}$ axis to promote uncontrolled cell proliferation [155,156]. In light of this body of evidence, cell cycle inhibitors and more specifically CDK4/6 inhibitors have emerged as a powerful class of agents with clinical activity in several different tumors, including human breast, colon, lung, and bladder cancers, as well as hepatocellular carcinoma and CCA [157–159]. So far, three CDK4/6 specific inhibitors have been approved by the FDA to treat hormone receptor-positive/HER2-negative advanced breast cancer: palbociclib, ribociclib, and abemaciclib [160–162]. All three inhibitors are orally available with different pharmacokinetics and clinical toxicities. Palbociclib and ribociclib are similar in chemical structure, exhibit similar kinase inhibitory activities against CDK4 and CDK6, and show a similar toxicity profile [163,164]. By contrast, abemaciclib has a different spectrum of inhibitory activity, with a preference for CDK4 [165]. In CCA, pRb is often hyperphosphorylated (a reversible event) but rarely downregulated or mutated. From this perspective, we might assume that CCA could positively respond to treatments to suppress the CDK4/6 proteins. Following this hypothesis, it has been shown that palbociclib administration significantly hinders CCA lesions' growth induced by the simultaneous overexpression of AKT and YAP protooncogenes in the mouse liver [151]. However,

abemaciclib is the only drug in a clinical trial as a phase 2 screening program for its efficacy in multiple platinum-resistant tumor types, including CCA. In another phase II trial, the goal is to determine the effectiveness and safety of abemaciclib in patients with advanced or metastatic BTC that has progressed or intolerant following one line of chemotherapy.

4. Conclusion

Except for pemigatinib, approved in 2020 for the patients harboring FGFR alterations, the available therapies for advanced/unresectable CCA are limited and associated with minimal success. Indeed, most CCA patients present at an advanced stage and are not amenable to surgical intervention. One important question would be if the early stage's molecular targets are expressed in the advanced disease and if they still represent the main lethal drivers associated with tumor progression. From this perspective, it is noteworthy to observe that targeted therapies have given limited benefits even in highly selected subpopulations, indicating that advanced CCA is driven by multiple forces, most of which are still unknown or underestimated. It is accepted that these various forces push the tumor to evolve under the selective pressure of systemic therapy, thus making the understanding of the molecular landscape in CCA even more challenging. New advances in comprehensive genomic profiling are helping to elucidate the landscape of molecular alterations underlying cholangiocarcinogenesis and the high heterogeneity of this malignancy. Further studies are mandatory to improve our knowledge on the molecular pathogenesis of CCA and to target deregulated signaling pathways to develop a personalized medicine also supporting clinical evaluation of combination therapy.

5. Expert Opinion

Advanced CCA remains a deadly malignancy with few treatment options. In recent years, we witnessed a new era in the medical management of this tumor. Genomic profiling led to the identification of molecular alterations in different CCA pathways, increasing and improving our understanding of this heterogeneous disease. To date, the so-called precision or personalized medicine age finds its application restricted to iCCA patients harboring FGFR rearrangements or IDH mutations. Pemigatinib (a pan FGFR inhibitor) in 2020 opened the targeted therapy epoch in biliary tract cancer and represents the first target agent approved by the FDA. As expected, based on the clinical trials conducted and the fact that FGFR alterations cover only a small fraction of all iCCA patients and are rarely present in the other subtypes, the therapeutic efficacy of pemigatinib is limited. Strategies coupling this drug with other compounds should be developed to improve patient outcomes and avoid anti-FGFR drug resistance. In accordance with this hypothesis, the mutational landscape of CCA indicates that many different pathways are concomitantly activated in this tumor type. Consequently, some of these signaling cascades should be simultaneously targeted by specific agents. For this purpose, many different drugs are currently under evaluation in clinical trials, and their combination with chemotherapeutic agents or immune checkpoint inhibitors could improve their efficacy. Over the next five or ten years, presumably, tailored therapy approaches could become of major importance in CCA patients' standard of care. To achieve this goal, further basic and translational research should be implemented to increase our understanding of the functional interplay between signaling pathways and the

mechanisms leading to drug resistance and disease recurrence and to identify reliable biomarkers for patients' stratification and treatment. For this purpose, the development and use of preclinical disease models that faithfully recapitulate the human disease should be implemented. Besides mouse models, primary patient-derived cell lines, normal ductal liver organoids, and CCA organoids could be of significant help in this task. Due to CCA's high heterogeneity, we hypothesize that several models will be necessary to mimic the whole spectrum of CCA subsets. Also, significant efforts should be made to improve the drug-development technology toward poorly druggable oncogenic programs. Moreover, drug repositioning screen studies of compounds already known to be safe and well-tolerated could exploit therapeutic vulnerabilities in tumors. Acquired resistance is still a significant concern that shortens the duration of benefit for the patient. Liquid biopsy and circulating tumor cell DNA (ctDNA) analysis can help identify these mechanisms of resistance. Also, our knowledge of how primary and secondary resistance emerge should be significantly improved to develop better combination and next-generation drugs to overcome and/or delay resistance.

Furthermore, in the next decade, we expect a profound improvement in early detection strategies, recurrences reduction following surgery, including better use of adjuvant therapies. Finally, future implementation and increased performance of techniques such as mass cytometry and single-cell transcriptomics will delineate the role of innate and adaptive immune cell subsets in CCA. A better understanding of CCA immunobiology will help design new therapies, combining immune checkpoint blockade with molecularly targeted therapy, chemotherapy, and other agents.

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Article highlights

- Cholangiocarcinoma (CCA) is an aggressive cancer type with an overall poor prognosis due to limited therapeutic options and intrinsic chemoresistance.
- CCA pathogenesis is associated with genetic and epigenetic alterations in tumor cells as well as significant changes in the tumor microenvironment, resulting in the alteration of various signaling pathways.
- There is a pertinent need to advance our understanding of the molecular landscape in advanced CCA and integrate these data with targeted therapies and immunotherapies in novel combination strategies.
- Integrative genomics analysis of CCA, regarding mutational landscape, copy number variations transcriptome, and epigenetic modifications, allows molecular stratification of patients and tumor subtypes for precision-targeted therapy.
- Encouraging results and hopes come from the FDA approval of the pan-FGFR inhibitor pemigatinib for the treatment of CCA patients harboring FGFR2 fusions or other rearrangements.
- Several novel drugs for the advanced-stage disease are continuously developed, hitting potentially driver genetic aberrations.

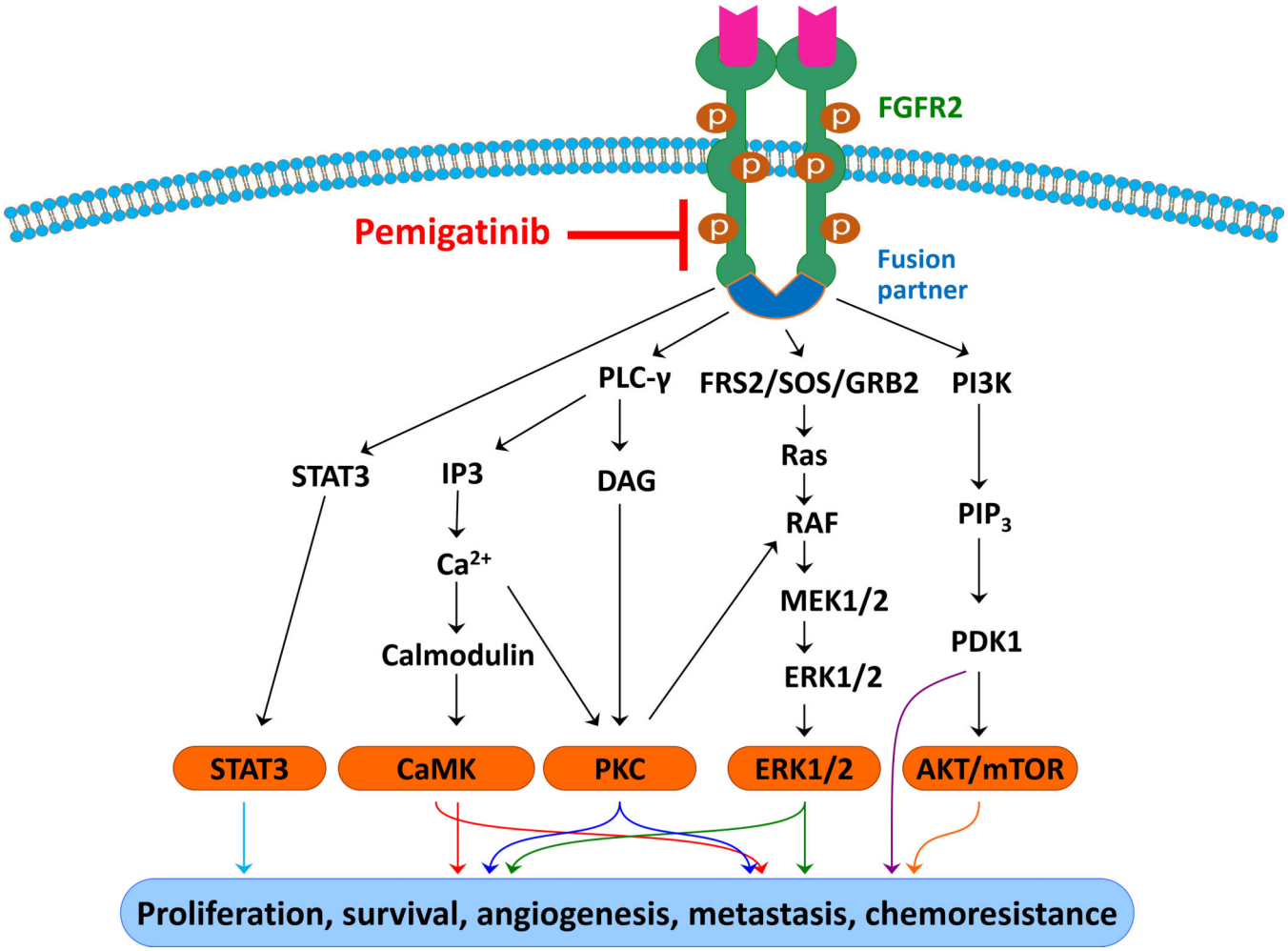

Figure 1.

Schematic representation of the mechanism of action of pemigatinib, a pan-FGFR inhibitor, in cholangiocarcinoma. Once activated by mutations (consisting of gene fusion or other rearrangements), FGFR2 triggers a plethora of downstream pathways and effectors inducing several biologic effects on the cholangiocarcinoma cells (proliferation, survival, migration, chemoresistance, etc.). The activity of activated FGFR2 is blunted by the pan-FGFR inhibitor pemigatinib. Black, red, blue, green, and purple arrows indicate activation, whereas red blunted arrows indicate inhibition.

Abbreviations: AKT, protein kinase B; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; DAG, diacylglycerol; ERK, Extracellular Signal-Regulated Kinase; FGFR2, Fibroblast growth factor receptor 2; FRS2, Fibroblast growth factor receptor substrate 2; GRB2, Growth factor receptor-bound protein 2; IP3, inositol 3-phosphate; MEK, ERK Activator Kinase; PKC, protein kinase C; mTOR, mammalian target of rapamycin; PDK1, Pyruvate Dehydrogenase Kinase 1; PI3K, phosphoinositide 3-kinase; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PLC-γ, phospholipase C-Gamma; RAF, Raf proto-oncogene kinase; Ras, Rat sarcoma; SOS, son of sevenless; STAT3, Signal Transducer And Activator Of Transcription 3.

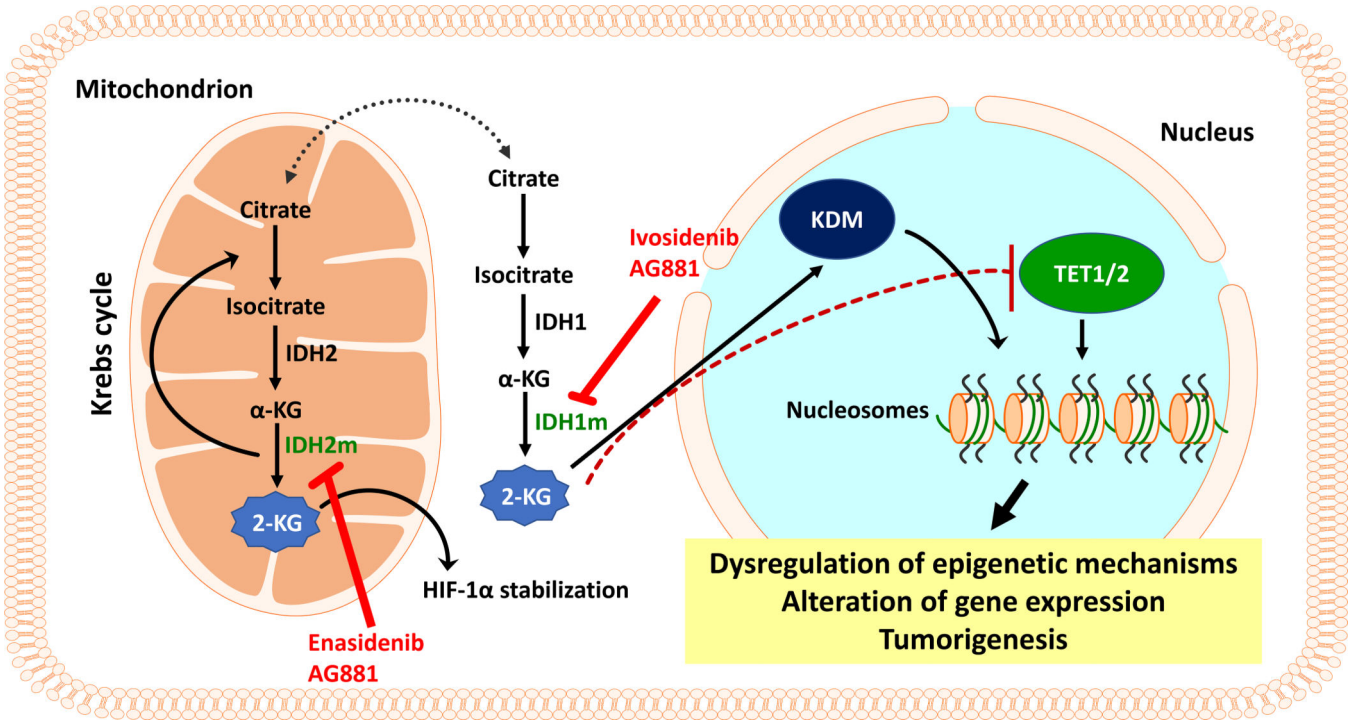

Figure 2.

Overview of the consequences of IDH1/2 mutations in cancer cells. Mutant (m) IDH1 (cytoplasmic) and IDH2 (mitochondrial) enzymes convert α-KG into 2-HG, a small oncometabolite. The presence of mutant IDH1 or IDH2 proteins leads to augmented amounts of 2-HG, with the consequent alteration of various cellular processes. Specifically, 2-HG inhibits α-KG binding to several histone demethylases (KDM), resulting in altered histone modification profiles. Also, 2-HG inhibits the TET1 and TET2 hydroxymethylases, thus decreasing 5-hydroxymethylcytosine levels. Aberrantly regulated KDM and TET1/2 proteins trigger extensive epigenetic dysregulation, leading to alteration of gene expression and, ultimately, tumorigenesis. Furthermore, 2-HG stabilizes the pro-angiogenic and pro-glycolytic factor HIF-1α. Mutant IDH1 and IDH2 forms can be inhibited by specific drugs, such as Ivosidenib (IDH1 inhibitor), Enasidenib (IDH2 inhibitor), and AG881 (acting on both proteins). Regular arrows indicate activation; blunted red arrows indicate inhibition. Abbreviations: IDH1, isocitrate dehydrogenase 1; IDH2, isocitrate dehydrogenase 2; α-KG, α-ketoglutarate; 2-HG, 2-hydroxyglutarate; HIF-1α, hypoxia-inducible factor 1, alpha subunit; TET1, Tet oncogene 1; TET2, Tet oncogene family member 2; KDM, lysine-specific demethylase.

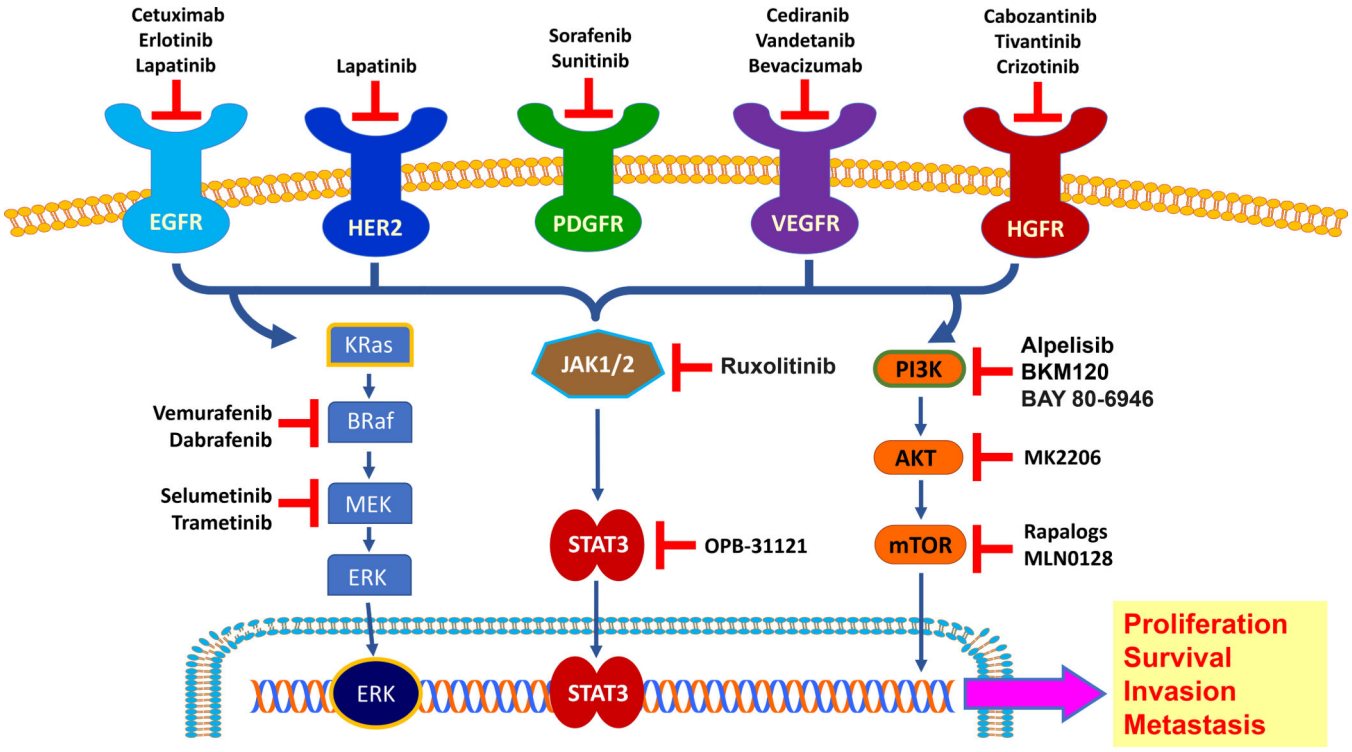

Figure 3.

Scheme depicting some of the relevant signaling pathways deregulated in cholangiocarcinoma and the available inhibitors of these cascades. Regular arrows indicate activation; blunted red arrows indicate inhibition.

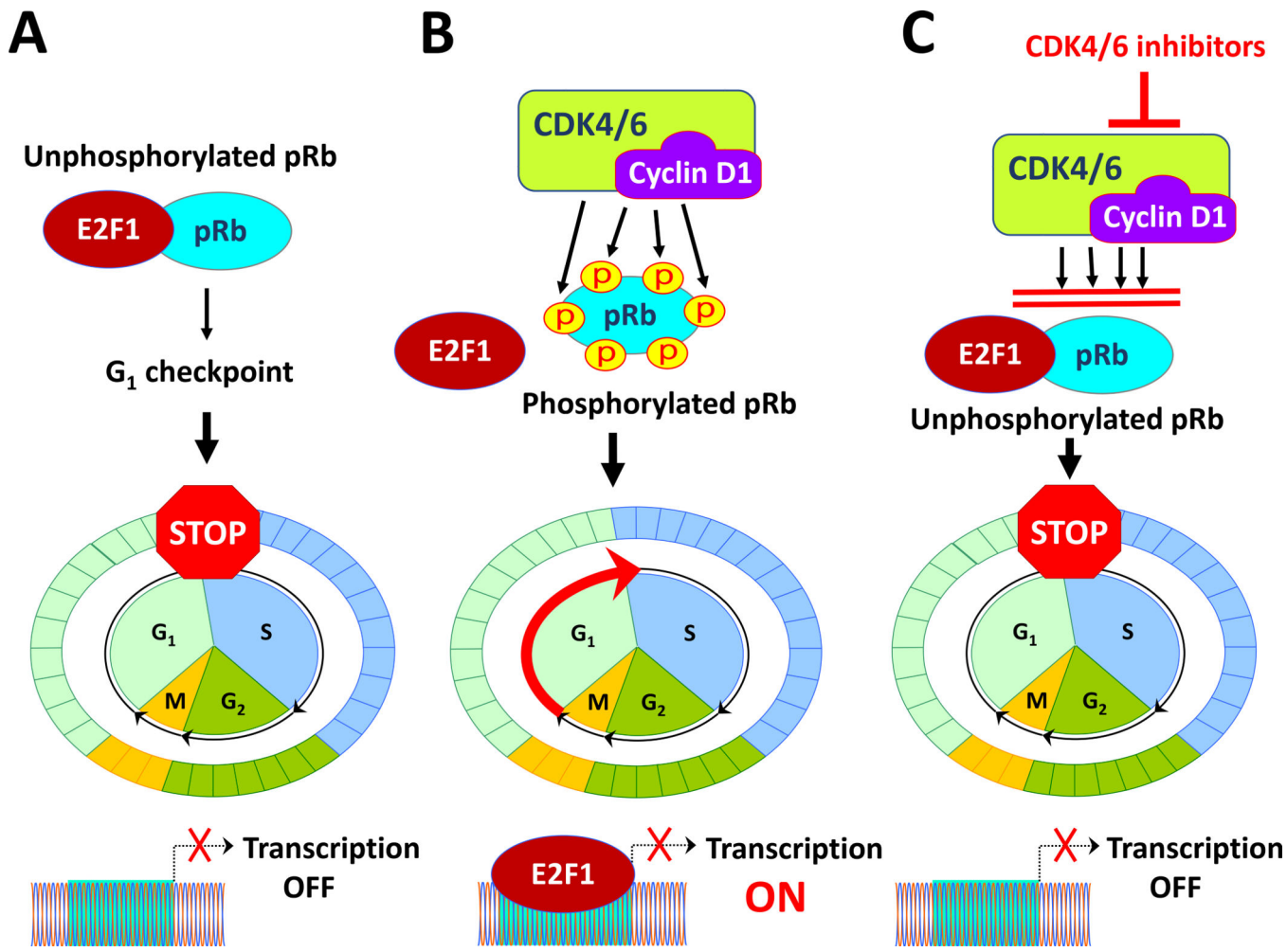

Figure 4.

Schematic representation of the mechanism of action of CDK4/6 inhibitors. (A) In normal, quiescent cells, the unphosphorylated/activated retinoblastoma protein (pRB) inhibits the activity of the E2F Transcription Factor 1 (E2F1) by direct binding. Specifically, the pRb/E2F1 physical interaction results in the inhibition of cell cycle progression from G1 to S phase (STOP), leading to the suppression of the E2F1-dependent transcriptional program (transcription OFF). (B) In cholangiocarcinoma cells, the aberrant upregulation of cyclin-dependent kinases 4 and 6 (CDK4/6), in association with the Cyclin D1 protein, phosphorylate (P), and inactivate pRb. The phosphorylation/inactivation of pRb is a reversible event. Due to pRb phosphorylation, E2F1 detaches from pRb and induces transcription of its target genes (transcription ON), leading to the G1→S transition of the cell cycle and promoting cell proliferation. (C) The administration of CDK4/6 inhibitors suppresses the activity of CDK4/6 proteins over pRb, thus reactivating the latter protein. Consequently, E2F1 is inactivated and is unable to drive its transcriptional program (transcription OFF).

Table 1:

Genomic alterations in biliary tract cancer

	Prevalence	References
Intrahepatic		
FGFR1–3 fusions, amplifications, and mutations	11–45%	41,45,57
IDH1 or IDH2 mutation	23–28%	42,45,48,52
TP53 mutation	2.5–44%	40,59,61
ARID1A mutation	15–36%	42,43,44,56,59
MCL-1 mutation	16–21%	44,56
EGFR expression	11–27%	44
CDKN2A or CDKN2B loss	6–30%	44,59
KRAS mutation	11–25%	59,61
SMAD4 mutation	4–17%	42,59
MLL3 mutation	15%	56,61
BAP1 mutation	13%	45,58
MET overexpression	7–21%	44,55
HER3 amplification	7%	61
FBXW7 mutation	6%	44
CDK6 mutation	6%	61
PIK3CA mutation	4–6%	44
BRAF mutation	4–22%	46
Extrahepatic		
TP53 mutation	40%	40,59,61
KRAS mutation	8–42%	59,61
SMAD4 mutation	21%	42,59
CDKN2A or CDKN2B loss	17%	44,59
FBXW7 mutation	15%	44
HER2 amplification	11–17%	44,45
ARID1A mutation	12%	42,43,44,56,59
EGFR expression	5–9%	44
PIK3CA mutation	7%	44
BRAF mutation	6%	46
Gallbladder cancer		
TP53 mutation	47–59%	40,59,61
HER2 amplification	10–19%	44,45
CDKN2A or CDKN2B loss	6–19%	44,59
ARID1A mutation	13%	42,43,44,56,59
PIK3CA mutation	6–12.5%	44
KRAS mutation	6%	59,61
BRAF mutation	6%	46
GNAS mutation	6%	63,64

Table 2.

Active clinical trials of agents targeting genomic alterations in patients with cholangiocarcinoma[†].

Agent	Target	Condition	Phase	NCT Number
Pemigantinib	FGFR	CCA/Solid tumors	I/II	NCT02393248
Pemigantinib	FGFR2 fusion/translocations	CCA	II	NCT02924376
Pemigantinib	FGFR2 rearrangement	CCA	III	NCT03656536
Infigratinib (NVP-BGJ398)	FGFR2 fusion/translocations	CCA	II	NCT02150967
Infigratinib (NVP-BGJ398)	FGFR2 fusion/translocations	CCA	III	NCT03773302
Derazantinib (ARQ 087)	FGFR2 alterations	CCA/Solid tumors	I/II	NCT01752920
Derazantinib (ARQ 087)	FGFR2 alterations	iCCA/cHCC-CCA	II	NCT03230318
Derazantinib (ARQ 087)	FGFR alterations	iCCA	expanded access	NCT04087876
Erdafitinib	FGFR alterations	CCA/Solid tumors	II	NCT02699606
Futibatinib (TAS-120)	FGFR alterations	CCA/Solid tumors	I/II	NCT02052778
Futibatinib (TAS-120)	FGFR2 rearrangement	CCA	III	NCT04093362
Ponatinib	FGFR2 alterations	Hepatobiliary Neoplasm	II	NCT02265341
Debio 1347	FGFR alterations	CCA/Solid tumors	II	NCT03834220
Ivosidenib (AG120)	IDH1 mutations	CCA	III	NCT02989857
IDH305	IDH1 mutations	CCA/Solid tumors	I	NCT02381886
Dasatinib	IDH1 mutations	CCA	II	NCT02428855
Enasidenib AG221	IDH2 mutations	iCCA/Solid tumors	I/II	NCT02273739
AG881[‡]	IDH1/2 mutations	Glioma	III	NCT04164901
Dabrafenib plus Trametinib	BRAF V600E	CCA/Neoplasms	II	NCT02034110
Afatinib	EGFR mutations	CCA	II	NCT02465060
Crizotinib	MET, ALK, ROS1	CCA/Neoplasms	II	NCT02034981
Trastuzumab emtasine	HER2 (ERBB2) amplifications	CCA/Solid tumors	II	NCT02675829
Niraparib	BAP1 and other DDR	CCA/Solid tumors	II	NCT03207347
Olaparib	IDH1/2 mutations	CCA/Solid tumors	II	NCT03212274
Olaparib	DNA repair gene mutations	CCA/Solid tumors	II	NCT04042831
Olaparib plus ceralasertib	IDH1/2 mutations	CCA/Solid tumors	II	NCT03878095
Regorafenib	VEGFR	CCA	II	NCT02162914
Abemaciclib	CDK4/6	CCA	II	NCT03339843
Abemaciclib	CDK4/6	CCA	II	NCT04003896

[†]Table is focused on genes that are frequently altered in CCA.

[‡]AG881 at present is evaluated only in patients with Glioma.

ClinicalTrials.gov searched on 01 February 2021.