HHS Public Access

Author manuscript

Cancer Discov. Author manuscript; available in PMC 2018 March 01.

Published in final edited form as:

Cancer Discov. 2017 September ; 7(9): 943–962. doi:10.1158/2159-8290.CD-17-0245.

REVIEW | New horizons for precision medicine in biliary tract cancers

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Abstract

Biliary tract cancers (BTCs), including cholangiocarcinoma and gallbladder cancer, are poor-prognosis and low-incidence cancers, although the incidence of intrahepatic cholangiocarcinoma is rising. A minority of patients presents with resectable disease; however, relapse rates are high; benefit from adjuvant capecitabine chemotherapy has been suggested. Cisplatin/gemcitabine combination chemotherapy has emerged as the reference first-line treatment regimen; there is no standard second-line therapy. Selected patients may be suitable for liver-directed therapy (e.g. radioembolization or external beam radiation); pending confirmation of benefit in randomized studies. Initial trials targeting the epithelial growth factor receptor and angiogenesis pathways have failed to deliver new treatments. Emerging data from next generation sequencing analyses have identified actionable mutations (e.g. FGFR fusion rearrangements and IDH-1 and -2 mutations) with several targeted drugs entering clinical development with encouraging results. The role of systemic therapies, including targeted therapies and immunotherapy for BTC is rapidly evolving and the subject of this review.

Keywords

Biliary cancer; Targeted treatment; Mutation; Molecular biology; Advanced

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Conflict of interest disclosure statement

No potential conflicts of interest were disclosed by the other authors.

Introduction

Biliary tract cancers (BTCs), including cholangiocarcinoma (both intra- (ICC) and extrahepatic (ECC)) and gallbladder cancer, are low-incidence cancers carrying a poor prognosis (1). BTCs account for approximately 3% of all adult cancers (2). Incidence and mortality are increasing, largely due to a rise in ICC (3–5). Most patients (>65%) are diagnosed with non-resectable disease (1) and there is a high relapse rate in the minority of patients who undergo potentially-curative surgery (6, 7). The five-year survival is around 5–15% when considering all patients (8, 9); estimated five-year survival varies with stage: 50% for AJCC stage I, 30% stage II, 10% stage III and 0% stage IV (6, 10).

It is widely accepted by the BTC community that BTC malignancies are not one unique disease only but a group of different diseases with distinct demographics, molecular characteristics and treatment options (Figure 1). Such differences are worth taking into account at time of treatment planning, research, and clinical trials design. BTCs are more frequent in patients aged between 50–70 years; with a male preponderance for cholangiocarcinoma, and female for gallbladder cancers (2); >90% are adenocarcinomas (1). Several risk factors, mainly associated with chronic gallbladder or biliary tract inflammation, have been identified (11–13), *Opistorchis viverrini* is one of the three major liver trematodes (flukes) that infect humans; it is endemic in Thailand, Vietnam, Cambodia, and Laos, and accounts for a global “hot-spot” of intrahepatic cholangiocarcinoma in this region. Adult flukes can remain in the bile ducts for years stimulating a host immune response, leading to chronic biliary tract inflammation; these results in up to 15-fold increase in risk of developing intra/extra-hepatic cholangiocarcinoma (14). There are also differences in risk factors (15–17), and symptoms at presentation between the different BTC (gallbladder patients (in advanced stages) are more unlikely to present with jaundices and usually present with abdominal pain) (15, 18).

Clinical context

Patients with tumors arising in proximity to the bile ducts present with biliary obstruction, due to local infiltration of the biliary tract. A minority of patients will be diagnosed with early (resectable) disease; in which case treatment will be surgical with curative intent. For patients diagnosed with advanced disease (often presenting with non-specific, non-biliary obstructive symptoms), treatment options are non-curative and mainly chemotherapy-based.

Despite potentially-curative resection for localized disease, relapse rates are high (19); highlighting the need to optimize adjuvant strategies. Role of adjuvant treatment for BTC has been unclear for many years (20). A systematic review and meta-analysis found that adjuvant treatment did not improve survival when compared to surgery alone when considering all patients (21). However, there appeared to be benefit for patients with microscopically-involved margins (R1-resection) vs. R0-resection (clear resection margins) (odds ratio (OR) 0.36, 95%-CI (confidence interval) 0.19–0.68) and lymph node-positive disease (OR 0.49, 95%-CI 0.30–0.80). Two randomized phase III clinical trials exploring adjuvant chemotherapy were reported in 2017. Firstly, the results from the PRODIGE-12/ACCORD-18 clinical trial assessing the benefit of adjuvant combination chemotherapy

compared with observation alone were reported in January 2017 (22). This multicenter phase III trial randomized 196 patients within 3 months of resection of a localized BTC (intra-hepatic, perihilar, extra-hepatic cholangiocarcinoma or gallbladder cancer), to receive either adjuvant gemcitabine and oxaliplatin or surveillance; there was no significant difference in relapse-free survival between the arms (hazard ratio of 0.83 (95%-CI 0.58–1.19); p-value 0.31). Secondly, the BilCap clinical trial exploring the role of adjuvant capecitabine compared with observation alone was reported in ASCO 2017. A total of 447 BTC patients were randomized to capecitabine (n = 223) or observation (n = 224) (23). Sensitivity analyses by intention-to-treat adjusted to nodal status, grade of disease and gender (447 patients), this trial demonstrated benefit from capecitabine in terms of overall survival (OS) [Hazard Ratio (HR) 0.71 (95%-CI 0.55–0.92); p-value < 0.01; median OS 51 months (95%-CI 35–59) and 36 months (95%-CI 30–45) for capecitabine and observation arms, respectively]. There was also benefit from adjuvant capecitabine in terms of relapse-free survival (median 25 months (95%-CI 19–37) and 18 months (95%-CI 13–28) for capecitabine and observation arms, respectively). Based on these results, adjuvant capecitabine is likely to be considered standard of care following surgery for BTC.

Around 60–70% of patients will be diagnosed with advanced disease, which is defined as inoperable or metastatic disease. For these patients, palliative treatment, usually in the form of systemic chemotherapy, is the only option of treatment. Selected patients with liver-predominant disease may benefit from liver-directed therapies such as external beam radiation (24, 25) or radioembolization (26, 27). Unfortunately, data suggesting such benefit is based on retrospective series or small phase II trials rather than randomized studies; further data are awaited to confirm the incremental benefit of approaches involving liver radioembolization. Liver transplant has been suggested as a potential option of treatment for patients with small perihilar cholangiocarcinoma (28, 29). However, prospective studies are needed to ensure the most suitable patient selection and benefit due to challenges for organ allocation and living donation policies; currently, liver transplant remains controversial in this setting.

New options of systemic treatment are an urgent area of unmet need for this patient population.

Systemic therapy of advanced disease

First-line chemotherapy

The most active cytotoxic chemotherapy agents in the management of BTCs are gemcitabine and platinum agents (30, 31). The first study to suggest that palliative chemotherapy could improve survival and quality of life was reported back in 1996 and established gemcitabine as a treatment option for patients with advanced disease (32). These results increased the interest in the treatment of BTCs and over the past 20 years many studies have been performed (30) that have been negative or lacked the statistical power and rigour to change clinical practice.

In 2010 we showed, in the 410-patient, randomized, phase III, ABC-02 study a benefit from cisplatin/gemcitabine chemotherapy over single-agent gemcitabine; the doublet conferred an

advantage in OS over gemcitabine alone (11.7 vs. 8.1 months; HR 0.64 (95%-CI 0.52–0.80); p<0.001) (33). These results were replicated in the Japanese randomized phase II (BT22) study (34). Based on these results, cisplatin/gemcitabine has become the recognized reference regimen for first-line treatment of patients with advanced BTC. Although patients with jaundice were excluded from the ABC-02 study, we have since shown that the doublet may safely be used in patients with jaundice secondary to obstructing endoluminal disease (whilst not the case in patients with jaundice due to parenchymal replacement by metastatic disease) (35).

The lack of further practice-changing trials since these studies highlights the desperate need for new therapies for patients with advanced BTC.

Second-line chemotherapy

Patients progressing on first-line chemotherapy often have a rapidly-worsening performance status and only a small number of patients may be suitable for further treatment; in addition, patients often have the inherent problems of biliary obstruction and sepsis associated with BTC, which may preclude further treatment.

Currently, no quality evidence is available supporting the use of second-line chemotherapy (36). For most patients active symptom control (ASC) (e.g. biliary stenting and antibiotics, as appropriate) is considered the standard of care after progression to first line chemotherapy. However, small prospective and retrospective studies have shown potential signals of benefit in selected patients.(37–41) Based on the previously-reported benefit from 5-fluorouracil (5-FU) in BTCs (42, 43), the ongoing UK ABC-06 study is a randomized phase III study comparing oxaliplatin and 5-FU (FOLFOX regimen) with ASC vs. ASC alone following progression on or after first-line cisplatin/gemcitabine; recruitment is ongoing (NCT01926236 (44)).

In summary, robust (phase III) evidence is available for the use of first line chemotherapy in patients presenting with advanced disease. Cisplatin and gemcitabine has become the reference regimen; other regimens are sometimes considered by individual clinicians based on phase II studies. The role of second-line therapy is unclear; no single regimen has emerged. Ongoing trials are trying to address this lack of treatment options, highlighting the need of development of novel targeted therapies approaches.

Current genetic landscape and actionable signatures

Tumor sequencing data

There has been a great effort to apply new parallel sequencing technologies to gather more insight about the molecular biology of these malignancies. As with other studies with next generation sequencing (NGS) in cancer, the starting questions were if these malignancies share common anomalies with other cancers and which is the idiosyncratic pattern of anomalies associated with biliary tract cancers.

Main new findings for ICC and ECC—Several studies have identified different genetic alterations that occur in cholangiocarcinoma using different approaches, from whole exome

sequencing (WES) (45–47) to a focused approach on specific pathways (48–53). A WES study of 8 cases of *Opistorchis viverrini*-related cholangiocarcinoma revealed mutations in ***TP53*** (44.4%), ***KRAS*** (16.7%) and ***SMAD4*** (16.7%) (45). Loss-of-function mutations of tumor suppressor genes have been reported in cholangiocarcinoma with an overall frequency of 15% (54–59). Activating mutations of *KRAS* (22%, range 5–57%), mainly located in codon 12 hotspots, have been associated with a worse prognosis after radical surgery. The tumor suppressor gene *SMAD4*, located in the long arm of chromosome 18q 21.1, is a nuclear transcription factor of TGFβ (60, 61); it is usually inactivated when mutated. In the infection-driven cases, mutations in *TP53* and *SMAD4* were more common, 39.8% and 19.4% vs 9.3% and 5.8% respectively. Mutations in *CDKN2A/B* (p16) were identified in 7% of ICC patients (52).

In a sequencing study of 102 ICC patients from China, Zou and colleagues found *TP53* mutations are more likely to be HBsAg-seropositive, whereas *KRAS* mutations are nearly exclusively found in HBsAg-seronegative ICC patients (62).

Interesting findings include inactivating mutations in *MLL3* (14.8%), *ROBO2* (9.3%), *RNF43* (9.3%) and *PEG3* (5.6%); and activating mutations in *GNAS* (9.3%) (45). *RNF43* (a RING domain E3 ubiquitine ligase) interacts with *P53* suppressing P53-mediated apoptosis (63). In this study, *RNF43* was an independent prognostic factor in the multivariable analysis. Interestingly, *RNF43* is also a key molecule in the Wnt-β-catenin pathway and can inhibit Wnt signaling by interacting with the Wnt receptors of the Frizzled family (64). *RNF43* mutations may predict the sensitivity to porcupine inhibitors (65). ***PEG3*** **(paternally expressed gene 3)** is an imprinted gene that regulates apoptosis; when it is inhibited it blocks P53-induced apoptosis (66). Loss of *PEG3* activates **WNT signaling** leading to chromosomal instability (67). *MLL3* encodes a histone-lysine N-methyltransferase that is one of the histone modifiers implicated in numerous cancers such as pancreatic cancer (68–70). Most of the tumors harboring a mutation in *MLL3* did not contain mutations in *TP53, KRAS* or *SMAD4*, despite the fact these three genes were mutated together in 57% of cases. This finding suggested the possibility of a particular subtype of cholangiocarcinoma where the alterations in the **chromatin packaging** have driven the development of the disease.

ROBO2 is a receptor protein involved in activating the Slit-Robo pathway. These proteins are key components of the axon guidance signaling and have been recently implicated in pancreatic adenocarcinoma (71).

GNAS encodes a guanine nucleotide-binding protein alpha subunit (72), which is frequently mutated in intraductal papillary mucinous neoplasms of the pancreas and villous adenoma of the colon (73, 74).

IDH alterations—Mutations in the isocitrate dehydrogenase genes (***IDH1*** **and** ***IDH2;*** Figure 2 (75) (76)) were found more often in non-infectious cholangiocarcinomas (76). *IDH1* and *2* mutations were also found (19%) by the John Hopkins Group; these mutations were clustered in previously-identified hotspots (codons 132 and 172, respectively) (77, 78) and were associated with a worse prognosis in contrast to the previously-published report (47). These differences in prognosis may be accounted for by the differing sample size and

baseline characteristics of the two studies. In a Chinese study, only five (4.9%) ICC harbored *IDH1* mutations (62).

FGFR pathway—Genome-wide structural analyses showed recurrent translocation events involving the ***FGFR2*** locus (48). Wu, *et al.* published the first report of *FGFR* fusions in ICC in 2013 with a description of 2 cases of *FGFR2-BICC1* fusions (79). *BICC* is a negative regulator of the WNT signaling (80). Tumor profiling studies of ICC have reported multiple additional fusion partners with *FGFR2* including *AHCYL1, TACC1, MGEA5*, and *PPHLN* (48, 81–83) which all fuse at a consistent breakpoint within the *FGFR2* gene on chromosome 10 (48). The mechanism by which *FGFR2* fusions drive oncogenesis is under active investigation. Arai, *et al.* showed that in clones expressing *FGFR2-BICC1* and *FGFR2-AHCYL1*, the MAPK pathway was activated but not AKT or STAT, suggesting that FGFR2 fusion proteins activate canonical signaling events downstream of FGFR (81). A mutation in *ERRFI1* was found, a negative regulator of the EGFR family, that was not present in cases having alterations of the *FGFR* (84). ERBB receptor inhibitor-1 has a role as negative regulator of the **EGFR family** of receptors (85–88). Thus, patients harboring this mutation may be suitable for an anti-EGFR treatment approach. Another cooperative effort of sequencing intrahepatic cholangiocarcinoma tumor samples confirmed the presence of *FGFR2* fusions in 3 of 28 tumor samples (10.7%). The John Hopkins study also found four somatic mutations in the fibroblast growth factor receptor-2 (*FGFR2*) (13%) (47).

Nakamura et al conducted a comprehensive genomic analysis of 260 BTCs and found that 40% of cases harbored targetable genetic alterations (89). They found that gene fusions involving *FGFR2* and *PRKACA* or *PRKACB* preferentially occurred in ICC and ECC, respectively.

Chromatin modifiers—The Singapore group subsequently analyzed cases of infection- vs. non-infection-related cholangiocarcinoma (46). A new set of 15 non-infection-related cases were sequenced identifying mutations in chromatin-remodeling genes: ***ARID1A*** (19%) and ***BAP1*** (25%). *ARID1A* encodes an accessory subunit of the SWI/SNF chromatin remodeling complex. In cell lines, silencing *ARID1A* results in a significant increase in the proliferation of cholangiocarcinoma-derived cell lines compared to cells expressing the wild-type form (46); showing mechanistically the involvement of this alteration in cholangiocarcinoma proliferation. BAP1 is a deubiquitinase protein of the ubiquitin C-terminal hydrolase (UCH) family (90–92). Increased proliferation was shown after *BAP1* knock-down using an RNA-interference approach.

The John Hopkins group identified mutations in several of the chromatin-remodeling genes including the ones described by Chan-On *et al.* (46), *ARID1A* and *BAP1*. They also found alterations in *PBRM1* (17%), a gene that encodes a subunit of the ATP-dependent SWI/SNF chromatin-remodeling complexes. These findings reinforced the idea of a major role of the **chromatin remodeling process** in the carcinogenesis of this tumor (Figure 3) (93).

New gene fusions. NTRK—New gen fusions have been also identified in ICC such as *RABGAP1L-NTRK1*.(52) *NTRK1* encodes a protein of the neurotrophic tyrosine kinase receptor (NTKR) family; this kinase is a membrane-bound receptor that, on neurotrophin-

binding, phosphorylates itself and members of the MAPK pathway (94). Gene fusions involving the *NTRK1, NTRK2* and *NTRK3* genes result in constitutively-active TRKA, -B, and -C kinases. The presence of these kinases leads to cell differentiation and may play a role in specifying sensory neuron subtypes. The TRK inhibitor LOXO-101 has shown early promise in a phase I trial of patients with advanced solid tumors where 5 out of 6 (83%) patients evaluable for response and harboring *NTRK*-fusions achieved a partial-response (although no patients had diagnosis of cholangiocarcinoma/gallbladder cancer) (95). Other compounds targeting *NTRK* 1/2/3, *ROS*1, or *ALK* gene rearrangement have shown positive responses in selected population (96). TRK inhibition is being explored in cholangiocarcinoma in a selected cohort in the STARTRK-2 phase II basket study of Entrectinib (RXDX-101) in patients with solid tumors harboring an *NTRK* 1/2/3, *ROS*1, or *ALK* gene rearrangement (NCT0256867). Sequencing studies have identified the presence of *NTRK* fusions in 1/28 (3.5%) of patients diagnosed with ICC (52). *ROS1* and *ALK* fusions are also rare targets ICC, with a frequency of 0–8.7% (97, 98) and 2.6% (99), respectively.

Protein tyrosine phosphatases—Protein tyrosine phosphatases (PTPs) are a structurally diverse family of tightly-regulated enzymes. Gao *et al.* (49) found frequent mutations in *PTPN3* in ICC that were significantly correlated with tumor recurrence.

Gene profiling—Gene expression profile, high-density single-nucleotide polymorphism array, and mutation analyses using formalin-fixed ICC samples from patients diagnosed with ICC identified 2 main **biological classes of ICC** (100). The first group, the inflammation class (38% of ICCs), was characterized by activation of inflammatory signaling pathways, overexpression of cytokines, and STAT3 activation. In contrast, the proliferation class (62% of ICCs) was characterized by activation of oncogenic signaling pathways and was associated with shorter survival. Andersen and colleagues also characterized ICC based on the genomic as well as transcriptomes signatures and were able to classify ICC into subclasses with different prognosis (101). Recently, the study with The Cancer Genome Atlas (TCGA) in ICC was published and this integrated analysis of somatic mutations, RNA expression, copy number, and DNA methylation also led to a molecular classification scheme and identified an IDH mutant-enriched subtype with distinct molecular features including low expression of chromatin modifiers, elevated expression of mitochondrial genes, and increased mitochondrial DNA copy number (102).

Gallbladder subset findings—A later study sequenced **gallbladder** carcinoma and cholangiocarcinoma separately. The analysis of 57 tumor-normal pairs with a double approach using WES and ultra-deep sequencing of 283 gene panel gave a striking result; mutations in the ERBB family of proteins (including their downstream genes) were found in 35.8%; in the multivariable analysis these cases had a worse outcome (p=0.001). Amongst the 11.8% mutations of the *ERBB3*, the majority of mutations were found in a hotspot in codon 104 (103). This pattern is not shared with cholangiocarcinoma suggesting that although both tumors originate from the biliary epithelium, they are genetically distinct. No *IDH* mutations have been identified in gallbladder cancer (104). Regarding the classical

PI3K/AKT/mTOR pathway, activating mutations in *PIK3CA* have been identified (12.5%) (105).

In summary, the recent targeted and WES genomic analyses have enriched our understanding of the genetic landscape of BTCs and informed us on the most actionable signatures (Figure 4). They have highlighted 1) the genomic spectra vary significantly in different subtypes of BTCs; 2) *IDH* mutations and *FGFR2* fusions are the most common genetic alterations in ICC; 3) frequent mutations occur in chromatin-remodeling genes: *ARID1A, BAP1, PBRM1;* 4) mutation frequency in different genes vary by etiology and geographic regions.

Animal models

Some of the efforts to generate animal models were primarily focused on well-known oncogenes such as *KRAS* and *TP53* (106).

The Notch and IDH pathways seem to have an oncogenic role in cholangiocellular malignancies. A transgenic **mouse model** (*NotchIC::AlbCre*) expressing the intracellular domain of Notch receptor-1 (NICD) in the liver was able to generate cholangiocarcinomas (a similar approach was used by Sekiya et al. and Fan et al previously (107, 108)) derived from hepatic progenitors. This model describes a subtype characterized by the over-expression of the Notch pathway with a different genetic background to bile-duct derived cholangiocarcinomas.(109) Interestingly, a more recent mouse model expressing IDH2 mutant variants R140Q or R172K in adult mouse hepatocytes, generated ICCs when combined with $KRAS^{G12D}$ mice suggesting the need for additional hits after the mutation of the IDH genes.(76) *IDH1/2* mutants reduced the expression of HNF-4α (Hepatocyte nuclear factor 4 alpha) which is a master transcriptional regulator of hepatocyte differentiation. The Notch1 activation as a transdifferentiating factor has been also noticed in an animal model by Guest et al.(110)

A model of cholangiocarcinoma using **zebrafish** was generated after inducing the co-expression of viral hepatitis-B and -C core protein; the first animal model showing the involvement of these viral proteins in the pathogenesis of cholangiocarcinoma.(111) Classical models in **rodents** have used a carcinogen-induced model; usually diethylnitrosamine (DEN) and thioacetamide (TAA) and infection with *Opistorchis viverrini*. (112) Genetically-engineered mice models of cholangiocarcinoma were generated targeting *TP53*, neurofibromatosis type 2 (*NF2*), *PTEN, SMAD4* and *KRAS* (112–114). In a transgenic mouse model, constitutive overexpression of ERBB2 in the basal layer of biliary tract epithelium led to the development of gallbladder adenocarcinoma (115).

Unfortunately, some of the key features of human disease, such as the genetic landscape, the chronic inflammation and the cholestasis, are clearly under-represented. The latest published model, consisting in combining an activating mutation in *KRAS* and *PTEN* deletion, has not incorporated the new knowledge from NGS information as yet (116). Little information about the involvement of the microenvironment has been generated in animal models. Furthermore, there is no model of non-gallbladder extra-hepatic cholangiocarcinoma.

Emerging therapies

Targeting the Molecular Biology of BTC

A deeper understanding of the natural behavior and activated pathways involved in BTCs is required to guide development of new drugs, aiming to improve patient outcomes. A summary of the main pathways and potential targeted therapies is shown in Figure 5 and Table 1.

Isocitrate Dehydrogenase (IDH) metabolism—Results from the collective efforts of several groups to characterize *IDH* mutations have shown: 1) *IDH1* is more common than *IDH2*; 2) the "hotspot" *IDH1/2* mutations are point mutations located in the arginine 132 (R132) residue in *IDH1* or the arginine 172 (R172) residue in *IDH2* (104, 117–121); 3) These mutations are ubiquitously higher in ICC than ECC (121); 4) the prognostic significance of *IDH* mutations remains conflicting in cholangiocarcinoma (117–120) 5) The mutant IDH loses its normal enzymatic activity and gains a new ability to produce the oncometabolite 2-hydroxyglutarate (2-HG), which can be detected in the tumor and blood (104, 122).

Pharmacologic inhibitors highly specific to the individual IDH-mutant alleles (e.g. to IDH1-R132 and IDH2-R172) have been developed. These block the function of mutant IDH1 or IDH2 at nanomolar concentrations leading to reduced 2-HG levels (Figure 2). Rohle *et al.* found that a selective R132H-IDH1 inhibitor (AGI-5198) impeded the growth of *IDH*-mutant glioma cells (123). Similarly Wang and colleagues demonstrated that AGI-6780 could selectively inhibit leukemic cells harboring mutant IDH2-R140Q (124). In a phase I trial, AG120 (IDH1-inhibitor, Agios) was well tolerated among patients with advanced solid tumors with *IDH1* mutations (NCT02073994); there were no dose limiting toxicities; anemia was the most frequent grade 3 adverse event (5%). Of the 20 patients with advanced ICC, one patient (5%) achieved a partial response and 11 patients (55%) had stable disease. In all patients responding to AG120, a reduction in circulating 2-HG level ranging from 73% to 99% and reduction in Ki67 staining ranging from 22% to 96% from baseline were observed. The expansion phase with 500 mg once daily is underway. Other IDH1 and IDH2 inhibitors have entered clinical trials recently (NCT02273739, NCT02381886, NCT02481154) and are enrolling patients with ICC. Through a high-throughput drug screen of a large panel of cancer cell lines including 17 biliary tract cancers, we recently found that ICC cells harboring *IDH* mutations exhibited a striking response to the multi-TKI dasatinib (125). In addition dasatinib-treated *IDH*-mutant xenografts demonstrated pronounced apoptosis and tumor regression. A trial with dasatinib in patients with *IDH* mutant advanced ICC is ongoing (NCT02428855).

Fibroblast Growth Factor Receptor (FGFR)—The recent discovery of recurrent *FGFR2* fusions in 11–45% of patients with ICC, described previously, has opened a promising therapeutic avenue (52, 81–83, 126, 127). In genomic profiling studies, they are found concurrently with mutations such as *ARID1A, PBRM1*, and *TP53, among others* (79). Histologically, *FGFR2* fusions are associated with prominent intraductal growth and

anastomosing tubular glands (126); prognostically, they appear to be associated with an indolent disease course and prolonged survival (83, 126).

The discovery of *FGFR* aberrations in multiple tumor types has stimulated pharmaceutical and scientific interest in the development of FGFR inhibitors. The earliest reported data of selective FGFR inhibition in cholangiocarcinoma is with oral agent BGJ-398 (Infigratinib, Novartis), which has a half maximal inhibitory concentration (IC50) for FGFR2 of 1.4nM. Preliminary results of 34 patients in the ongoing phase II trial of BGJ-398 in advanced cholangiocarcinoma with FGFR aberrations after first-line chemotherapy (NCT02150967) included patients with FGFR2 fusions (n=28), FGFR2 mutations (n=2), FGFR2 amplification (n=3), or FGFR3 amplification (n=1); the median time on treatment was 188 days and the objective response rate was 22% (all 8 patients with a partial response had an FGFR2 fusion) (128). As seen with other oncogene addicted tumors treated with tyrosine kinase inhibitors, acquired resistance limited the durability of response in some patients. Goyal, *et al* reported the first evidence of clinically acquired resistance to FGFR inhibition in an analysis of three patients with FGFR2-fusion positive ICC who were treated with BGJ398 (129). Sequencing of cell-free DNA and biopsy samples collected at baseline and post-progression revealed polyclonal secondary mutations in the FGFR2 kinase domain, including the gatekeeper mutation FGFR2 V564F in all three patients. *In vitro* studies further identified structurally distinct FGFR inhibitors which may overcome the resistance, and these data may guide future treatment strategies in this scenario.

Other selective FGFR inhibitors including INCB54828 (Incyte, NCT02924376), BAY1163877 (Bayer, NCT01976741), and the irreversible FGFR inhibitor, TAS-120 (Taiho, NCT02052778), are currently being evaluated in early phase trials in patients with advanced solid tumors including ICC. Non-selective multi-TKIs that also target FGFR, including ponatinib and pazopanib, have demonstrated activity in individual patients with ICC who have developed resistance to chemotherapy (48). A third non-selective TKI, ARQ-087 (Arqule, NCT01752920) which inhibits RET, PDGFR, KIT, SRC, and FGFR1-3 (IC50 for FGFR2=0.68nM) is currently being evaluated in a phase II trial of previously-treated patients with FGFR aberrant tumors including *FGFR2* fusion-positive advanced ICC. Preliminary data from the phase I/2 basket trial indicate that 3 of the 12 patients with *FGFR2*-fusion-positive advanced ICC treated with ARQ-087 had a partial response (with a disease control rate of 75%) (130).

FGFR2 fusions appear to be driver alterations that predict sensitivity to FGFR inhibition in ICC, but the impact of the fusion partner and the sensitivity of *FGFR* mutations and amplifications to FGFR inhibition in ICC is yet unknown. Circulating levels of FGF ligands such as FGF19, FGF21, and FGF23 showed some correlation with response in the ARQ-087 trial (131) but further investigation into these biomarkers and others is warranted.

The safety profile of FGFR inhibitors is manageable, with hyperphosphatemia being one of the most common toxicities. This is a class-effect due to on-target blockade of FGF23 in the bone and kidney. FGF23 is a phosphaturic hormone that regulates phosphorus excretion in the proximal tubule of the kidney, and inhibition of this hormone leads to phosphate reabsorption (132). FGFR inhibitors can also cause nail changes with onycholysis, mucosal

dryness, ocular toxicity, nausea, anorexia, diarrhea, and constipation, and adequate management of the toxicities will be key to further development of this class of drugs.

Overall, the preliminary data for FGFR inhibitors in advanced ICC are encouraging.

Angiogenesis—Not only are the ligands regulating angiogenesis (particularly vascular endothelial growth factor (VEGF)-A) commonly present (40–75%) in BTCs (133–135), but expression is co-located with their receptors VEGFR-1 and -2 in endothelial cells adjacent to the tumors (136). This appears to be most evident at the invasive edge of the tumor (137). VEGF expression is associated with a number of adverse prognostic features: the presence of metastases in ICC (135) and increased microvascular density (MVD) in both cholangiocarcinoma (134) and gallbladder cancer (133). MVD is an independent prognostic factor for disease-free survival following resection of ECC(137) and for OS in lymph-node negative ICC (138) and gallbladder cancer (137). MVD is also an independent negative predictor of OS in ECC (139). Consequently, a number of clinical trials have evaluated VEGF-inhibition.

In a phase II trial of bevacizumab combined with gemcitabine and oxaliplatin our group demonstrated a significant decrease in standardized uptake values on FDG-PET scans after two treatment cycles, particularly in patients with a partial response or stable disease (140). However, the 6-month progression-free survival (PFS) (63%) was below the target rate of 70%. Combining bevacizumab with erlotinib (an anti-EGFR tyrosine kinase inhibitor [TKI]) achieved partial responses in 12% of patients, and 51% stable disease, with a median OS of 9.9 months; notable for the absence of concurrent chemotherapy (141).

Cediranib is an oral VEGFR1, VEGFR2, and VEGFR3 TKI, with activity against platelet-derived growth factor (PDGF) receptors and c-KIT. In the randomized phase II, placebo-controlled, ABC-03 study we observed an improved response rate in patients receiving cisplatin/gemcitabine with cediranib (44%) vs. placebo (19%; p=0.0036) and improved 6-month PFS (70.5% vs. 61·3%; p>0.05). However, the study did not meet its primary endpoint (improvement in median PFS); in part, due to the relatively-poor tolerability of cediranib (142).

Forays into VEGF inhibition with other TKIs have been disappointing. Single-arm, phase II studies of sorafenib in monotherapy (143, 144) or combined with erlotinib (145) or cisplatin/gemcitabine (146) have all failed to demonstrate sufficient activity in BTC. Most recently, sorafenib failed to improve PFS when added to gemcitabine in a randomized phase 2, placebo-controlled study (147). A phase II clinical trial of sunitinib including 56 BTC patients reported a median time to progression of only 1.7 months, an objective response rate of 8.9% and disease control rate of 50%.(148)

The VanGogh study failed to show an improvement in PFS in a 3-arm randomized phase II study of exploring the role of vandetanib in 173 patients (149). Results of ongoing studies with pazopanib (NCT01855724), regorafenib (NCT02053376, NCT02115542), and ramucirumab (NCT02711553) are awaited.

Human Epidermal Growth Factor Receptor (HER) family—Epidermal growth factor receptor (*EGFR*) amplifications and mutations have been described in around 6% and 13.6–15% of BTCs, respectively. However, the biological implication of these mutations is unclear (150–152).

Several phase II clinical trials have combined cetuximab, a monoclonal antibody targeting EGFR, with chemotherapy in treatment of BTCs: most of them with gemcitabine and oxaliplatin (153–156). Initial promising results reporting high tumor response rates (63%) from a small study (n=30) (154) were not confirmed in the randomized phase II BINGO study, in keeping with results from other negative phase II studies combining cetuximab with chemotherapy (156).

KRAS wild-type patients with advanced BTC were treated with panitumumab combined with gemcitabine, capecitabine and oxaliplatin (46 patients) (157) and with gemcitabine and oxaliplatin (31 patients)(158) in two separate phase II clinical trials. In each study the primary end-point was achieved (6-month PFS of 74% (95%-CI 58–84) (157) and response rate of 45% (158), respectively). A third phase II study (panitumumab with gemcitabine and irinotecan in non-selected patients) also supported further development of this compound in BTC with no difference in OS by *KRAS* status (7 of 31 patients harbored a *KRAS* mutation) (159). Unfortunately, these signals have not been confirmed in other studies (160, 161); including the largest randomized phase II study combining panitumumab with gemcitabine and oxaliplatin (the Vecti-BIL study)(161) which showed no differences in survival in 85 randomized patients.

Erlotinib, a TKI targeting EGFR has also shown varying results (162–164). In the largest (phase III) study 133 patients were randomized to receive gemcitabine and oxaliplatin chemotherapy with or without erlotinib (163); there were no differences in PFS or OS when all BTC patients were analyzed together. However, the cholangiocarcinoma patient subgroup did appear to derive benefit from adding erlotinib to chemotherapy (median PFS 5.9 months (95%-CI 4.7–7.1) vs. 3.0 months (95%-CI 1.1–4.9); p=0.049); further clinical trials are ongoing (NCT00832637; NCT00987766).

HER-2 (v-ERB-B2, erythroblastic leukaemia viral oncogene homolog-2) over-expression and gene amplification is also described in BTCs with a higher incidence in gallbladder cancer (19%) (165). Rate of *HER-2* over-expression was found to be higher in ECC (17.4%) compared to ICC (4.8%) in a recent systematic review and meta-analysis published by Galdy *et al.* in 2016 (165). Good correlation between overexpression and gene amplification (75%) has been shown (151). Two phase II trials have yielded disappointing results of first/second-line lapatinib monotherapy in an unselected population of patients with advanced BTC (166, 167). Case reports using trastuzumab in patients with gallbladder carcinoma with HER2-overexpression have suggested activity (168, 169); and a phase II clinical trial is underway (NCT00478140). Afatinib, has shown activity in one patient diagnosed with cholangiocarcinoma in a phase I clinical trial (170); a phase I study of afatinib combined with cisplatin and gemcitabine in patients with BTC is ongoing (NCT01679405).

Targeting WNT/β-CATENIN, HGF/c-MET and Hedgehog—The WNT/β-CATENIN pathway is involved in the regulation of cell invasion and migration. High nuclear expression with low membranous expression of β-CATENIN expression has been described in ICC (15%) (171); WNT signaling seems to be most relevant in hilar cholangiocarcinoma (172). WNT pathway activation is associated with chemo-resistance and metastatic spread in a cholangiocarcinoma xenograft tumor model (173) and WNT-inhibition has reversed chemo-resistance in cell lines (174). In contrast, its impact and the mutational status of this pathway's components is not completely understood in BTC (175, 176). Whole-exome sequencing of Opisthorchis viverrini-related cholangiocarcinoma identified mutations in the WNT pathway (RNF43 (9.3%) (45, 177)). β-CATENIN expression is associated with decreased apoptosis in gallbladder cancers (178). Yadav *et al.* showed that most of the genetic variants of WNT signaling pathways that were evaluated influenced gallbladder cancer susceptibility (179). Although multiple WNT pathway inhibitors are currently under development (180), only few clinical trial have been reported for BTC as yet. Eads and colleagues explored the safety of DKK1, an inhibitor of the canonical Wnt/β-catenin pathway, in combination with gemcitabine and cisplatin in a phase I clinical trial enrolling patients with BTCs (181). The combination was found to be safe, suggesting possible prolonged disease stabilization; further development is awaited.

The Hedgehog pathway may also be involved in the development of BTC (182–185). Among gallbladder cancer specimens, expression of Hedgehog pathway components by immunohistochemistry (IHC) has been described [SHH (81.7%), PTCH1 (75.3%) and GLI1 (70.0%)] with impact on stage and lymph node, venous and hepatic infiltration; patients with activated Hedgehog pathway had a more aggressive behavior and worse outcome.(186) Similar findings have been described in cholangiocarcinoma [SHH (87.8%), PTCH1 (89.2%), GLI1 (85.4%)].(187) Suppression of Hedgehog pathway in gallbladder (188) and cholangiocarcinoma (189) cell lines implanted in mice xenograft inhibited epithelial-mesenchymal transition and reduced tumor volume, suggesting this pathway as a potential new target.

c-MET tyrosine kinase plays an integral role in carcinogenesis by promoting angiogenesis, tumor invasion, and metastasis. Binding of this receptor by the ligand hepatocyte growth factor (HGF) activates multiple downstream signal transduction pathways, including the Grb2-Ras-mitogen-activated protein kinase (MAPK) cascade and the phosphatidylinositol-3 kinase (PI3K), EGFR, VEGF and RAC1-CDC42 pathways (190). c-MET overexpression, associated with a poor prognosis in CCA,(191) is seen in 12–58% of ICCs (191–193) and 0–16% of ECCs (193), a wide variation likely accounted for by differences in the c-MET antibody used, definition of positivity, analysis of resection vs. late-stage biopsy samples, and sample size per study. c-MET amplification is rare in cholangiocarcinoma, but has been reported at a frequency of 7% in one study of 26 cases of ICC analyzed by next generation sequencing (NGS) (52). In addition to the above mention effect in cholangiocarcinoma, HGF/c-MET pathway promotes proteolytic activity and induces cellular motility, essential for the invasive progression of gallbladder carcinoma cell lines (194). In human tissue, c-MET expression is higher in cancer cells than in normal gallbladder tissue (195), up to 74% in some series (196).

Significant crosstalk has been demonstrated between the c-MET pathway and other pathways such as the EGFR and VEGF pathways in other tumor types. c-MET amplification has been shown to drive resistance to EGFR inhibitors via ERBB3-dependent activation of PI3K (197). Similarly, tumor hypoxia, which can be a consequence of VEGF pathway inhibition, has been shown to up-regulate c-MET and enhance scatter and invasiveness (198). Thus dual inhibition of c-MET with other pathways may be a strategy in cholangiocarcinoma. Cabozantinib, which has potent activity against both c-MET (IC50=1.3nM) and VEGFR2 (IC50=0.035nM), was tested in a phase II trial in advanced cholangiocarcinoma patients; preliminary data showed minimal activity with early trial discontinuation after 12 of 19 patients failed to be progression-free at 16 weeks (199). A randomized phase II study with merestinib in addition to cisplatin/gemcitabine in first-line is ongoing (NCT02711553).

KRAS-BRAF-MEK-ERK pathway—As in many other cancers, the RAS/RAF/MEK/ERK signal transduction pathway is frequently dysregulated in cholangiocarcinoma (200). The binding of ligands including EGF and PDGF to the receptors trigger the cascade activation of downstream signaling molecules. Activated *RAS* triggers phosphorylation and activation of RAF kinase, leading to end-phosphorylation of MEK1 and 2. Activated MEK phosphorylates ERK-1 and ERK-2, the only known substrates. Phosphorylated-ERK (p-ERK) then dimerizes and translocates to the nucleus (201), where it regulates several important cellular functions. Gain-of-function *KRAS* mutation with a frequency of 9%–40% has been reported in cholangiocarcinoma (52, 83). *KRAS* mutation has been associated with perineural invasion, advanced stage, and poor prognosis (202). *KRAS* mutations have been found in up to 7.8% of gallbladder cancers (103, 203). *BRAF* mutations seem not to be significant in gallbladder cancer and appear to be restricted to ICC only (203, 204). However, other groups' results show mutation rates between 5.9%(103) and 33% (205).

Despite the recognized frequency of *KRAS* mutations, targeting this pathway remains challenging. *BRAF* is a proto-oncogene and is a key component of the RAS/RAF/MEK/ERK proliferation signaling pathway. The most common *BRAF* gene mutation found in human cancers is V600E, with varying frequency reported in cholangiocarcinoma (206). In a recent phase II basket study of vemurafenib in *BRAF* V600-mutated non-melanoma cancers, one patient with cholangiocarcinoma achieved a durable partial response of over a year (207).

MEK is an attractive target as ERK-1 and ERK-2 are the only known MEK substrates (208). Early evidence of efficacy of MEK inhibitor was reported in a single-arm study of selumetinib in advanced BTCs (209). Of 29 patients enrolled, 25 were evaluable for response: 3 patients (12%) had confirmed partial responses and 17 (68%) had stable disease. The median PFS was 3.7 months (95%-CI 3.5–4.9), and median OS was 9.8 months (95%-CI 5.97-not available). In this study, no *BRAF* V600E mutations were found but absence of pERK staining appeared to be associated with lack of response to selumetinib. Recently, in the ABC-04 phase Ib study we assessed the safety and tolerability of selumetinib in combination with cisplatin/gemcitabine in advanced BTC; 3 of 8 patients evaluable for

response had partial responses. Selumetinib-related toxicities were manageable and included grade 1-2 edema and rash (210).

Given well-known redundancy and crosstalk in this pathway, novel combined strategies targeting different molecules within this pathway or different pathways represent attractive approaches in cholangiocarcinoma.

The PI3K/AKT/mTOR pathway—This pathway is up-regulated in cholangiocarcinoma cells; moreover, activation of this pathway is associated with adverse prognosis in some BTC patients (211) and good prognosis in others. Some studies have shown that somatic PIK3CA mutations contribute to the frequent activation of the PI3K/AKT pathway in BTC (212).

In a study of 212 ECC cases, patients with high phosphorylated-AKT (p-AKT) expression group had shorter survival than those with low p-AKT expression (p=0.06). Cases with high phosphorylated-mTOR (p-mTOR) expression showed shorter survival (p=0.06). Patients with low PTEN expression (median survival, 18 months) had a significantly worse survival time than those with high PTEN expression (median survival 39 months; log-rank test p=0.004) (213). Conversely, a study on 101 ICCs showed the aberrant expressions of p-AKT1 and p-mTOR was associated with a favorable prognosis regardless of PTEN (214). PTEN overexpression was found as an independent favorable prognostic factor for patients with ICC. In addition, the overexpression of p-mTOR was more frequently observed in well- to moderately-differentiated tumors, and in tumors without metastasis. The comparison of these two studies underlined the difficulties in comparing different BTCs due to biological differences depending on their primary location and, ultimately, cell of origin. In addition, the redundancy and crosstalk involving this intracellular pathway makes targeting a single point/level unlikely to be a successful approach.

MK-2206 is an oral selective allosteric inhibitor of AKT that targets all three isoforms of human AKT (AKT-1, AKT-2 and AKT-3) with IC50 values of 8, 12 and 65 nM, respectively. An abbreviated phase II study using this compound in 8 patients with at least one prior systemic treatment was disappointing with a median PFS of 1.7 months and median OS of 3.5 months (215).

A first-line phase II study with everolimus showed evidence of antitumor activity with 14 out of 27 patients (56%, 95%-CI 35–76) achieving tumor control at 12 weeks; two of them achieving partial response. The median PFS was 6.0 months (95%-CI 2.1–11.2) and median OS 9.5 months (95%-CI 5.5–16.6). Correlative studies suggest that *KRAS* mutational status and basal p-AKT might be associated with resistance to everolimus treatment (216).

A phase II trial using a PI3K inhibitor, copanlisib (BAY 80-6946), in first-line in combination with gemcitabine and cisplatin is ongoing (NCT02631590).

Current status of emerging targeted therapies

Currently, the most promising targets under development due to a more solid preclinical research background are IDH inhibitors for IDH-mutant BTC and molecules targeting

FGFR2 gene fusions (Figure 4 and Figure 5). A window of opportunity is open with new drugs in development targeting chromatin remodeling gene mutations (*ARID1, BAP1* and *PBRM1*) such as bromodomain and extra-terminal (BET) inhibitors (217). Most of the remaining molecular targets that have been tested in clinical trials have been somewhat disappointing with conflicting data and negative trials, underlining the need for new models and new approaches to unravel the complex molecular biology of BTC (Table 1).

Is precision medicine regarding targeted therapies in BTC ready for the clinic?—As in other malignancies, the meaningful decrease in cost of next generation sequencing technologies has opened the door for more sophisticated trials where different molecular subtypes of a malignancy can be matched to targeted inhibitors. Obtaining tumor molecular profiling on patients who are fit to enroll in clinical trials beyond first line systemic therapy may offer these patients additional promising treatment options. However, obtaining sufficient tissue for such analyses in BTC can be difficult, making this approach more challenging. For this scenario, the use of liquid biopsies (circulating tumor cells (CTCs), cfDNA, exosomes, etc.), when validated, may lead the way to such approaches in these neoplasms.

Role of immunotherapy—The relationship between chronic inflammation and the development of BTC has led investigators to harness the immune response through vaccination, adoptive immunotherapy and check-point inhibition.

Immune cells (both innate and adoptive) are present in BTCs; this appears to be stage-dependent (for macrophages) and the presence of dendritic cells, CD4+ helper T-lymphocytes, CD8+ cytotoxic T-lymphocytes and B-lymphocytes/plasma cells is associated with improved survival (218).

Vaccination studies have yielded modest results in monotherapy; the commonest targets are Wilm's Tumor-1 (WT-1) and mucin protein1 (MUC-1). WT-1, a transcription factor, is also a tumor suppressor through interaction with PDGFR, EGFR, c-MYC and BCL-2. A phase I study in combination with gemcitabine showed that patients demonstrating a T-cell response to WT-1 vaccination had a longer OS than gemcitabine-only-treated patients (219). MUC-1, a glycoprotein forming a hydrophilic barrier to hydrophobic cytotoxic agents and immune surveillance, is highly overexpressed in gallbladder cancers (90%); less so in cholangiocarcinoma (59–77%), and is associated with advanced stage and impaired survival. An early study showed that MUC-1 vaccination did not translate into clinical benefit despite achieving an IgG-response (220). A dendritic cell-based vaccine targeting MUC-1 in patients with resected pancreatic and BTC (with adjuvant chemo- or radiotherapy as appropriate) saw 4 of 12 patients disease-free at four years (221). Expanding vaccination to target two (222), three (223) or four (224) peptides, or even "personalizing" the vaccination (225) hold promise but remain investigational. Defining the optimal target amongst heterogeneous entities within BTC, vaccination against single vs. multiple targets, and definition of optimal adjuvants is required.

Shimizu *et al.* vaccinated patients with resected ICC with autologous tumor lysate-pulsed dendritic cells plus ex-vivo activated T-cell transfer (adoptive immunotherapy). These

patients had a near-double OS (31.9 vs. 17.4 months, p=0.022) compared to surgery-alone patients, most marked in patients with prominent skin reactions (226).

Mutational load is known to be “high” in tumors in which immunotherapies have been shown effective, such as melanoma and lung cancer (227). Based on a similar rationale, efficacy of check-point inhibitors in tumors with mismatch-repair deficiency was proven to be successful in a phase II study achieving up to 40% of objective responses (228). Mutational load, has shown to be high in BTCs (89). In addition, mismatch-repair (MMR) and microsatellite instability (MSI) have been explored in BTCs. MMR and MSI have been suggested to be infrequent in BTCs without hereditary non-polyposis colorectal cancer (229). Results vary between series; high level MSI has been shown in 5% of gallbladder carcinoma (230), 5–13% of ECC (230, 231) and up to 10% of ICC (230). MMR status (hMLH1- and hMSH2 negativity) was shown in 51.3% and 59% of gallbladder carcinoma, and 57.1% and 65.7% of ECC, respectively (232). O(6)-Methylguanine-DNA methyltransferase (MGMT) methylation was identified in 59% of gallbladder carcinoma and 60% of ECC (232). Both, MGMT-methylation and MMR status, correlated with poor prognosis in gallbladder and ECC (232).

A case report of tumor-infiltrating lymphocytes from a patient with metastatic cholangiocarcinoma containing CD4+ T-helper-1 cells recognizing a mutation in ERBB2 interacting protein induced an impressive and durable response; moreover this effect was reproduced after subsequent disease progression (233). Adoptive immunotherapy studies in Thailand (NCT01868490) and the USA (NCT01174121) are ongoing.

Holcombe and colleagues explored a cohort of BTC samples (126 ECC, 434 ICC, 244 gallbladder cancer, 11 not specified) and identified high PD-1 (40%) and PD-L1 in (15%) expression (50). In the BTC cohort of KEYNOTE-28 [NCT02054806] 37 of 89 patients (42%) were PD-L1-positive (defined as ≥1% staining of cells in tumor nests or PD-L1-positive bands in stroma by IHC). Four of 24 patients (17%) treated with pembrolizumab, a highly-selective humanized monoclonal antibody targeting PD-1, had a partial response with another four achieving stable disease; five patients entered long-term treatment, including all 4 responders (234). These encouraging results suggest that this strategy is worth pursuing (a phase I study in combination with FOLFOX chemotherapy, with an expanded phase II cohort in BTC, is underway [NCT02268825]); along with validation of PD-L1 expression as a predictive biomarker; evaluation of the role of PD-L2 expression; and assessment of efficacy in the various BTC subgroups as well as in patients with mismatch-repair deficient tumors (228).

Conclusion

The treatment paradigm for patients with advanced BTC is evolving; through international collaboration, BTCs are no longer considered “too rare” for adequately-powered clinical studies. Emerging evidence suggests that biliary tract cancer encompasses subgroups with discrete driver mutations, some of which are targetable with novel therapies. The role of conventional therapies (chemotherapy and radiotherapy) has yet to be fully defined, particularly in the adjuvant and second-line settings. In addition, investigation of a number

of pathway-targeted therapies, as well as modulation of the immune environment, hold promise for patients with these diseases. Given the low-prevalence of BTC, clinical development must go hand-in-hand with sound basic and translational research.

Acknowledgments

JWV is consultant/advisory board member for Agios; AstraZeneca; Baxalta; Delcath Systems; Ipsen; Lilly; Merck; Midatech; Novartis; Pfizer; has received speakers' bureau honoraria from Celgene; Novartis; Pfizer; research funding from Novartis (Inst); and travel, accommodations or expenses from Lilly; Novartis. LG has received honoraria from Ribon Therapeutics; and research funding from Dicerna; Exelixis; Leap Therapeutics; Taiho Pharmaceutical. AZ is consultant/advisory board member for Bayer; Bristol-Myers Squibb; Eisai; Merck; Novartis; Sanofi; and has received research funding from Bayer (Inst); Lilly (Inst); Merck (Inst); Novartis (Inst).

Financial support

Juan W Valle received research funding from Cancer Research UK (CRUK) and The Alan Morement Memorial Fund (AMMF). Angela Lamarca received funding from the Spanish Society of Medical Oncology (SEOM) Fellowship, the Cholangiocarcinoma Foundation Research Fellowship and the Conquer Cancer Foundation Young Investigator Award. Jorge Barriuso is part-funded by the Spanish Society of Medical Oncology (SEOM) Fellowship. Lipika Goyal is supported by the MGH Executive Committee on Research Fund for Medical Discovery Award, the Cholangiocarcinoma Foundation Research Fellowship, the NIH Loan Repayment Program Grant, the Jonathan Kraft Translational Award, and NIH/NCI Gastrointestinal Cancer SPORE P50 CA127003. Andrew Zhu is supported by the Target Cancer Foundation, a Translational Research Grant Award from the V Foundation for Cancer Research, and the Jonathan Kraft Translational Award.

Authors would like to thank Stephanie L. Reyes for her assistance with Figures 2 and 4.

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Statement of Significance

Authors address genetic drivers and molecular biology from a translational perspective, in an intent to offer a clear view of the recent past, present and future of BTC. The review describes a state-of-the-art update of the current status and future directions of research and therapy in advanced biliary tract cancer.

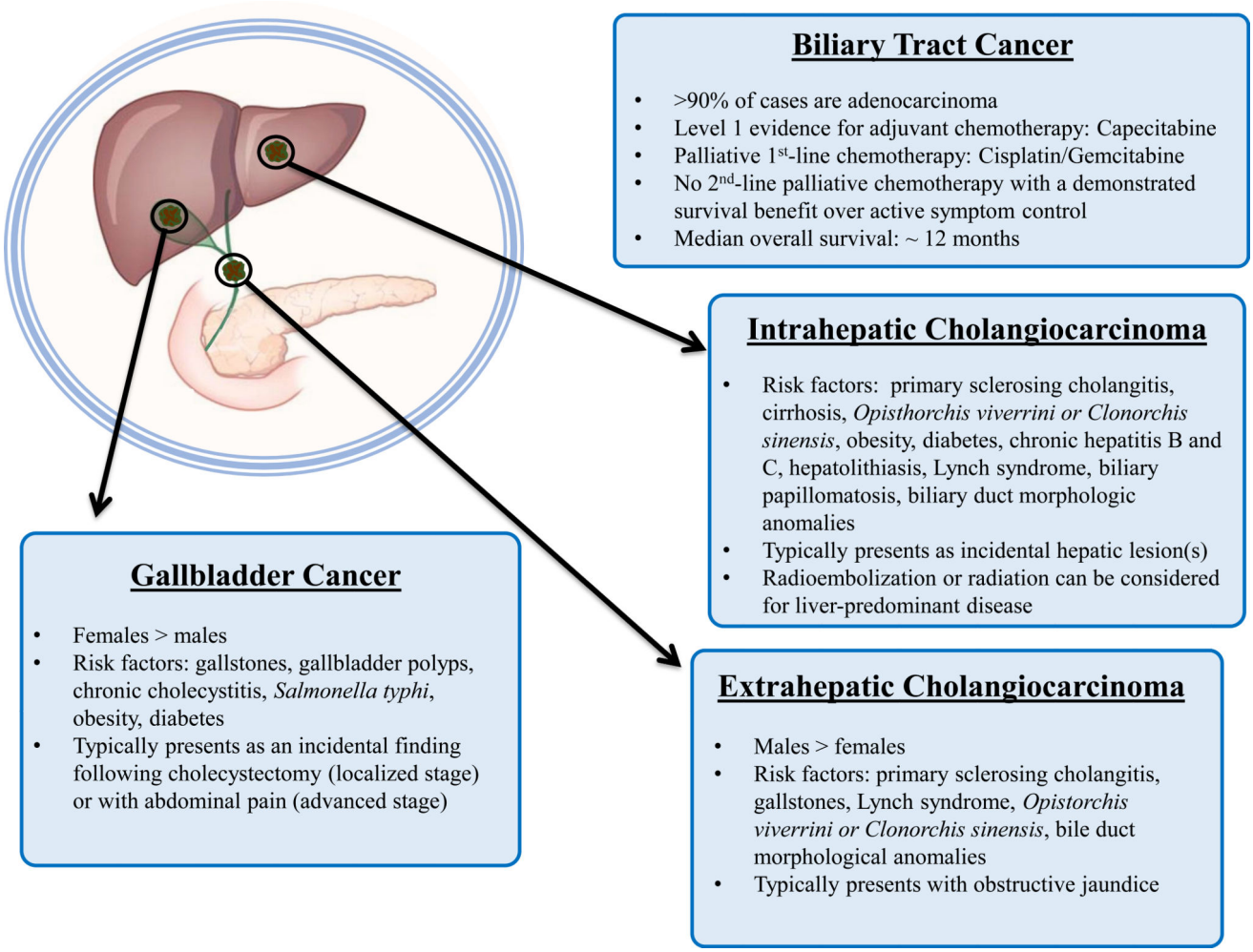

Figure 1.

BTC, are a group of different diseases, which includes ICC, ECC and gallbladder cancer. They differ in many aspects, such as anatomical location, demographics, clinical presentations and treatment options. BTC: biliary tract cancer; ICC: intrahepatic cholangiocarcinoma; ECC: extrahepatic cholangiocarcinoma; PEI: pancreatic exocrine insufficiency.

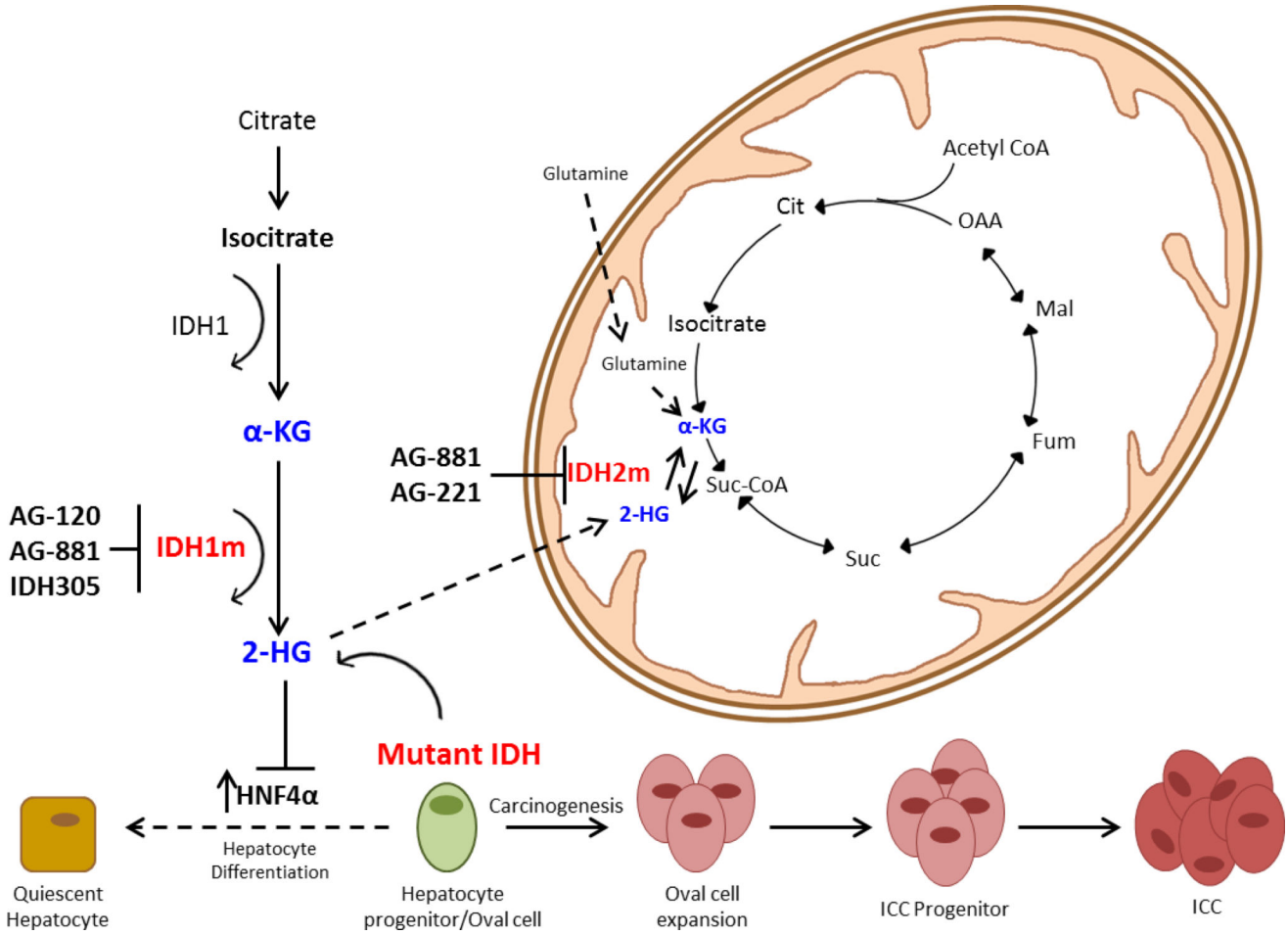

Figure 2.

IDH1 and IDH2 are metabolic enzymes found in the cytoplasm and mitochondria respectively, and catalyze the decarboxylation of isocitrate to alpha-ketoglutarate (α-KG), resulting in the reduction of NADP+ to NADPH. The oncometabolite2-hydroxyglutarate (2-HG) can competitively inhibit one or more members of the family of over 60 dioxygenases which require α-KG as a cofactor. The dioxygenases include the JmjC family of histone demethylases and the Ten-eleven translocation (TET) family of methylcytosine dioxygenase enzymes that catalyze the demethylation of DNA. IDH and Kras mutations can cooperate to drive the expansion of liver progenitor cells, development of premalignant biliary lesions, and progression to metastatic ICC. Agents targeting IDH1 and IDH2 are under development.

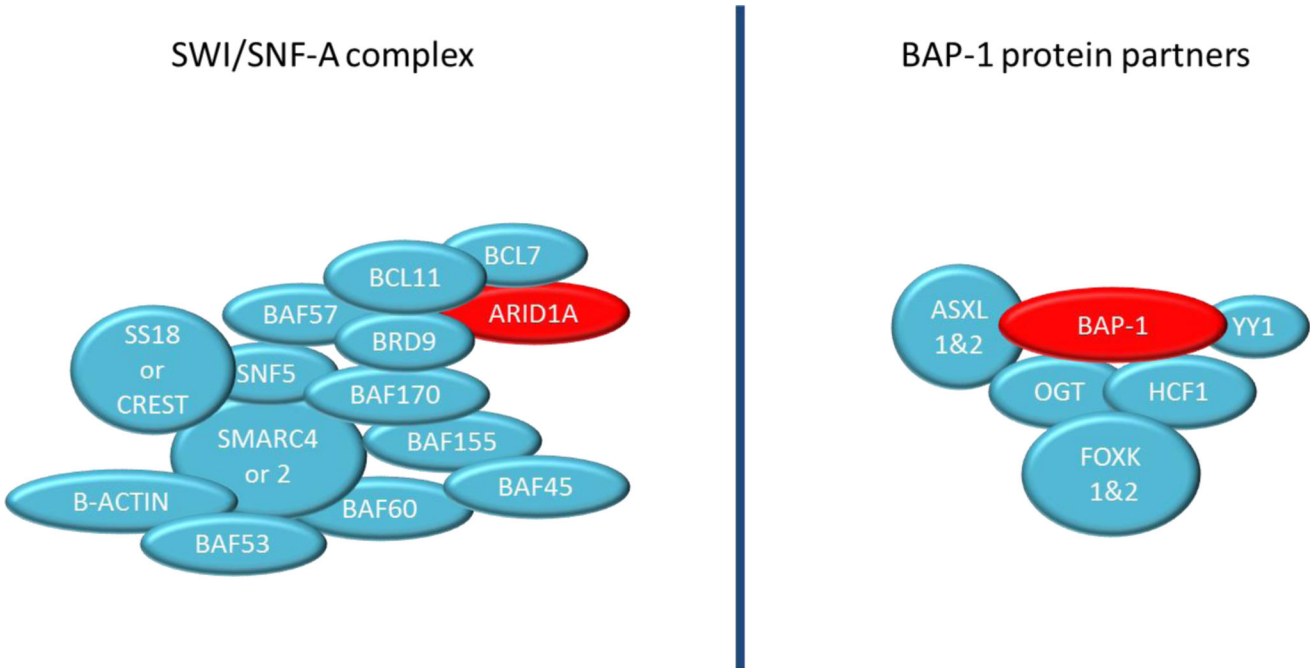

Figure 3.

Chromatin remodeling complex: DNA is packaged in chromatin to allow 1.8 meter-long human genome to fit in a single cell of the body. SWI/SNF (SWItch/Sucrose Non-Fermentable) complexes are evolutionary conserved, ATP-dependant, molecular machines that alter local chromatin structure. ARID1A encodes an accessory subunit of the SWI/SNF chromatin remodeling complex. ARID1A: AT-rich interactive domain-containing protein 1A. BAF: BRG1 associated factor. BRD: Bromo domain containing protein. SMARC: SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, also known as BRG: Brahma related gene. BAP1: BRCA1 associated protein-1 ASXL: additional sex combs-like. OGT:UDP-glucose-dependent O-glucosyltransferase. HCF1:host cell factor 1. YY1: Ying Yang 1. FOXK: Forkhead box protein K.

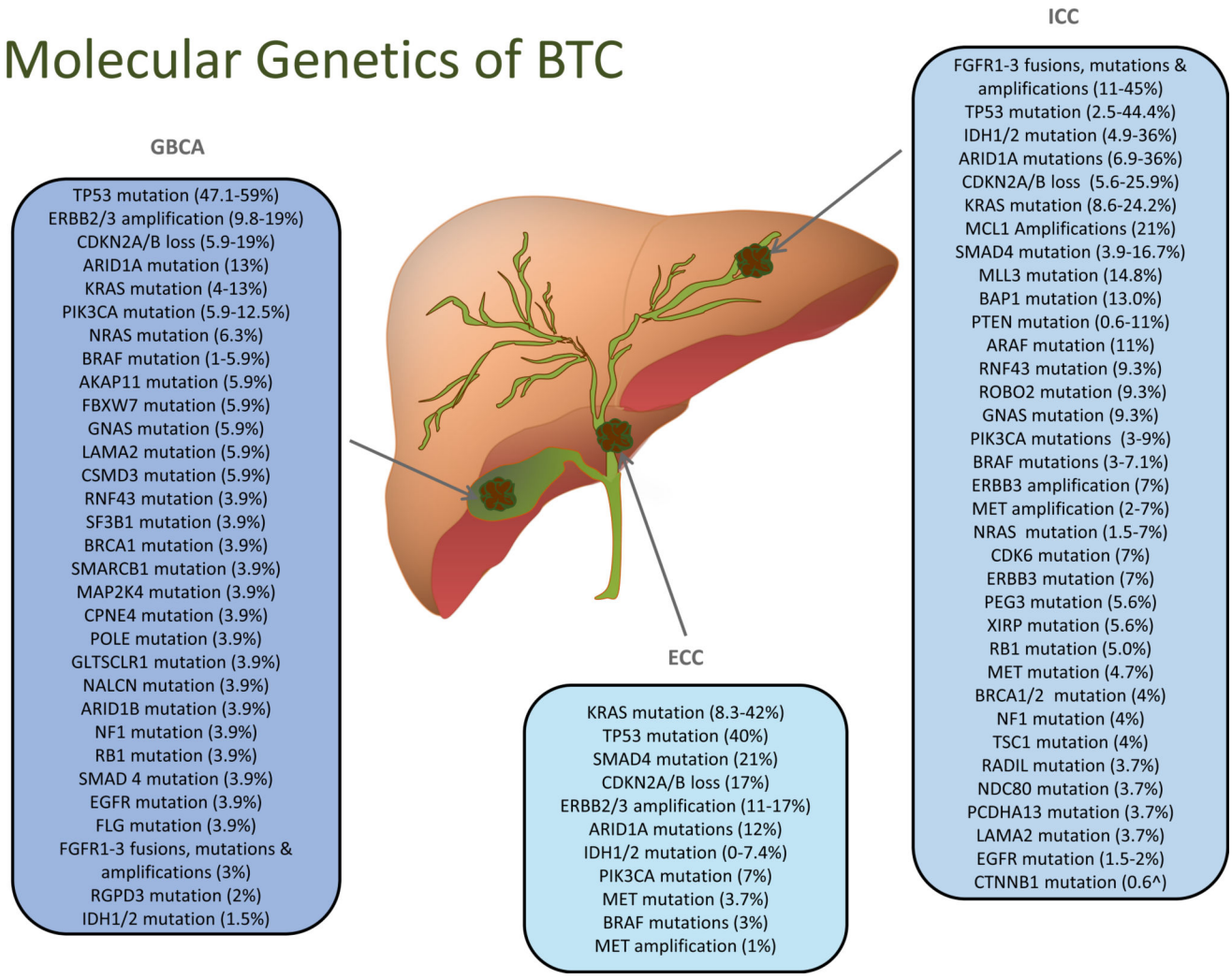

Figure 4.

Genetic landscape on BTC. Most frequent genetic aberrations in targetable pathways of interest in BTC. The mutation is quoted as the highest to lowest with range from different reports on each mutation. Those without range come from single reports. Extracted from: Desphande *et al* BMC Cancer 2011(105), Borger *et al* The Oncologist 2012(104), Voss *et al* Human Pathology 2013(208), Ross *et al* The Oncologist 2014(52), Ong *et al* Nature Genetics 2012(45), Graham *et al* Human Pathology 2014(126), Arai *et al* Hepatology 2014(81), Sia *et al* Nature Communications 2015(82), Javle *et al* Cancer 2016(53), Zou *et al* Nature Communications 2014(62), Li *et al* Nat Genet 2014(103), Zhu *et al* Ann Surg Oncol 2014(118), Sia Gastroenterology 2013(54), Jiao *et al* Nature Genetics 2013(119), Chan-on *et al* Nature Genetics 2013(46), Wang *et al* Oncogene 2013(117), Wu *et al* Cancer Discovery 2013(79), Ross *et al* Journal of Clinical Oncology 2015(51), Nakamura *et al* Nature Genetics 2015(89), Borad *et al* PLoS Genetics 2014(48), Randall *et al* Journal of Clinical Oncology 2015(50), Galdy *et al* Cancer and Metastases Reviews 2016(165), Churi *et al* PlosOne 2014(83), Turner *et al* Nature Reviews in Cancer 2010(127), Pai *et al* European Journal of Cancer Prevention 2011(203), Riener *et al* Genes Chromosomes and Cancer 2008(212).

ICC: intrahepatic cholangiocarcinoma, ECC: extrahepatic cholangiocarcinoma, GBCA gallbladder cancer.

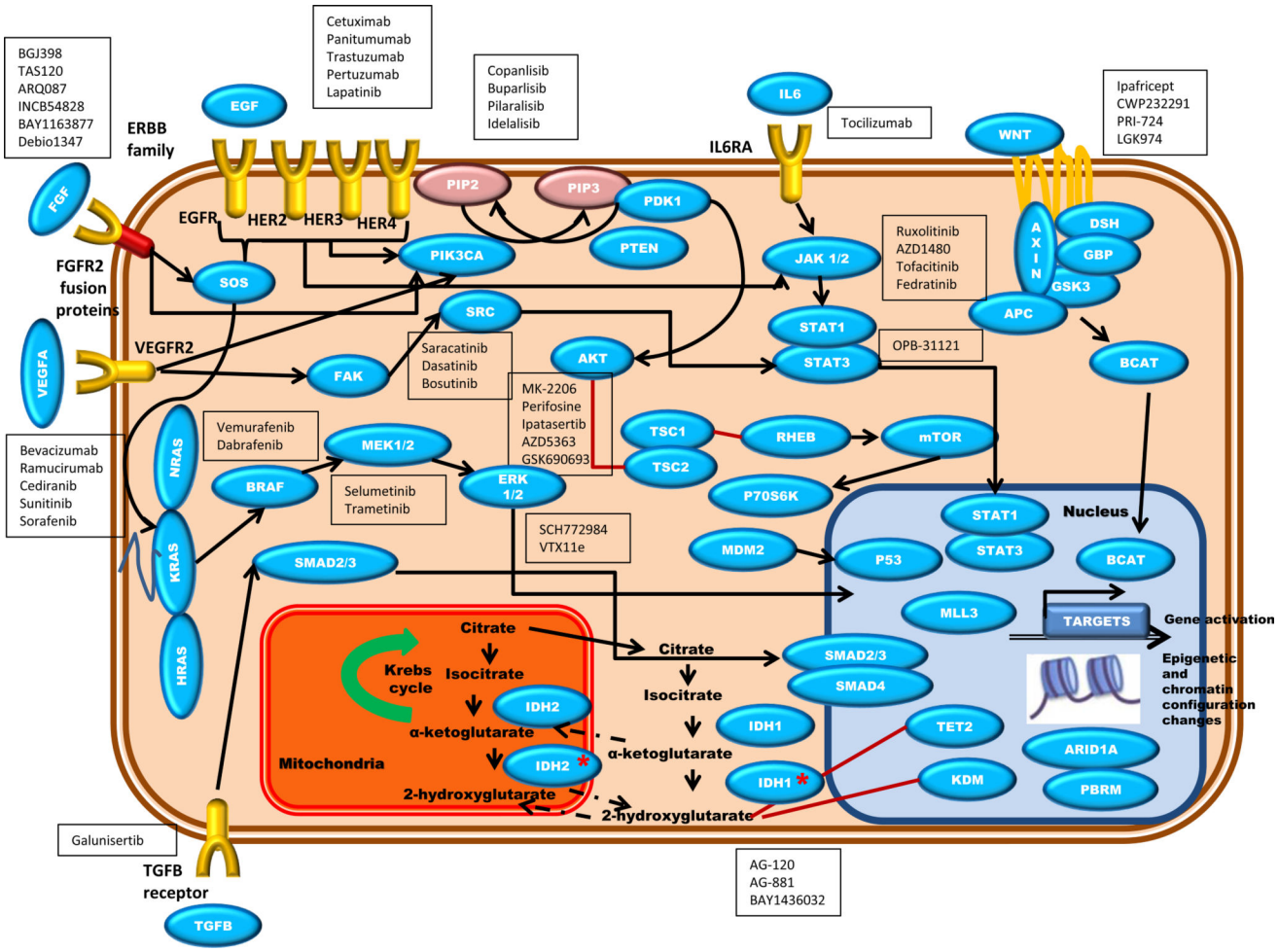

Figure 5.

Summary of the relevant pathways for biliary tract cancers. Activation links are described with black arrows. Negative links are described as red lines. Red asterix identifies the mutated variant of the protein. TGFB: Transforming Growth Factor Beta. VEGFA: Vascular Endothelial growth factor A. VEGFR2: Vascular Endothelial Growth Factor Receptor 2. FGF: Fibroblast Growth Factor. FGFR2: Fibroblast Growth Factor Receptor 2. ERBB: Avian Erithroblastic Leukemia Oncogene Homologue protein, previous name for EGFR: Epidermal Growth Factor Receptor. EGF: Epidermal Growth Factor. HER: Human Epidermal growth factor Receptor. SOS: Son Of Sevenless, Ras/Rac Guanine Nucleotide Exchange Factor. HRAS: Harvey Rat Sarcoma Viral Oncogene Homolog protein. KRAS: Kirsten Rat Sarcoma Viral Oncogene Homolog protein. NRAS: Neuroblastoma Rat Sarcoma Viral Oncogene Homolog protein. BRAF: v-Raf murine sarcoma viral oncogene homolog B. SMAD:Mothers against decapentaplegic homolog 1 (Drosophila) protein also know as Transforming Growth Factor-Beta Signaling Protein. MEK:Mitogen-Activated Protein Kinase Kinase. ERK: Mitogen-activated protein kinase. FAK: Focal Adhesion Kinase. PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha. PIP2: Phosphatidylinositol 4,5-bisphosphate. PIP3:Phosphatidylinositol (3,4,5)-trisphosphate. SRC:Avian Sarcoma (Schmidt-Ruppin A-2) Viral Oncogene Homolog protein. PDK1:

Pyruvate Dehydrogenase Kinase 1. PTEN: Phosphatase And Tensin Homolog. AKT: V-Akt Murine Thymoma Viral Oncogene-Like Protein. TSC: Tuberous sclerosis protein. RHEB: Ras Homolog Enriched In Brain protein. mTOR: Mechanistic or Mammalian Target Of Rapamycin. P70S6K: Ribosomal Protein S6 Kinase B1. MDM2: Human Homolog Of Mouse Double Minute 2, P53-Binding Protein. P53: Mutant Tumor Protein 53. IL6: Interleukin 6. IL6RA: IL-6 Receptor Subunit Alpha. JAK: Janus Kinase. STAT: Signal Transducer And Activator Of Transcription. WNT: Wingless-Type MMTV Integration Site Family proteins. AXIN: Axis Inhibition Protein. DSH: Dishevelled family of proteins. GBP: Guanylate Binding Protein. GSK3: Glycogen Synthase Kinase 3. APC: Adenomatous Polyposis Coli BCAT: Beta-catenin. MLL3: Mixed-Lineage Leukemia 3 protein. TET2: Tet Methylcytosine Dioxygenase 2. KDM: Histone lysine demethylase. ARID1A: AT Rich Interactive Domain 1A. PBRM: Polybromo 1 protein. IDH: Isocitrate Dehydrogenase

Table 1

Heat-map summary of the status of evidence supporting known molecular biology involved in BTCs. Available evidence is classified according to the type of research: basic/preclinical, translational or clinical research.

Pathway	Supported by basic / preclinical research (including sequencing or animal models)	Supported by translational research (including pathway status analysis by immunohistochemistry or other techniques)	Clinical research with available results or ongoing clinical trials pending data
Cell proliferation (FGFR)	Yes +++	Yes ++	Yes ++
Cell metabolism (IDH)	Yes +++	Yes ++	Yes ++
Angiogenesis (VEGF)	Yes +	Yes +++	Yes +++
Inflamation (IL6, TGFβ)	Yes ++	Yes ++	No
Stroma and stemness (Wnt/β catenin pathway)	Yes ++	Yes ++	Yes +
Stroma (cMET/HGFR)	Yes ++	Yes ++	Yes +
Stroma and stemness (Hedgehog pathway)	Yes +	Yes +	No
Stroma and stemness (Notch pathway)	Yes ++	Yes +	No
Cell proliferation (Kras-Braf-MEK-ERK pathway)	Yes ++	Yes +++	Yes ++
Cell proliferation (HER family growth factor receptors: EGFR, HER2)	Yes ++	Yes +++	Yes +++
Cell proliferation (PI3K-AKT-mTOR pathway)	Yes +++	Yes +	Yes +
Tumors suppressor genes (p53, p16 (CDKN2A/B), SMAD4)	Yes +++	Yes ++	No
Chromatin remodeling (ARID1, BAP1, PBRM1)	Yes +++	Yes +	No

The table indicates if there is any evidence available (Yes (Grey), No (White)); moreover, the evidence is ranked as follows: + (light grey; poor quantity/quality; retrospective data, absence of prospective/randomized studies), ++ (mid dark grey colour; medium quantity/quality; prospective

clinical trials) and +++ (dark grey; high quantity/quality; randomized trials). Please refer to the main text for references applicable for each pathway.