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Signaling networks in cholangiocarcinoma: molecular pathogenesis, targeted therapies and drug resistance

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ABBREVIATIONS

2-HG, 2-hydroxyglutarate; 5-FU, 5-fluorouracil; ABC, ATP binding cassette; AKT, AKT serine-threonine kinase; BTC, biliary tract cancers; CAF, cancer associated fibroblasts; CCA, cholangiocarcinoma; CK, cytokeratin; COX-2, cyclooxygenase-2; DCR, disease control rate; DDR, DNA damage response; DLL, delta-like; DSR, double-strand break repair; eCCA, extrahepatic CCA; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; ENT, equilibrative nucleoside transporter; ERK, extracellular signal-regulated kinase; F-TKI, FGFR-specific tyrosine kinase inhibitor; FDA, food and drug administration; FFs, FGFR2 fusions; FGFR2, fibroblast growth factor receptor 2; GSI, γ-secretase inhibitor; HCC, hepatocellular carcinoma; HH, hedgehog; HR, homologous recombination; HisR, histamine receptor; ICB, immune checkpoint inhibitors blockade; iCCA, intrahepatic CCA; IDH, isocitrate dehydrogenase; IL, interleukin; JAG, Jagged; JAK, Janus, kinases; MCL1, myeloid cell leukemia 1; miRNA, microRNA; MAPK, mitogen-activated protein kinases; MC, mast cell; MDR, multidrug resistance; miRNAs, microRNAs; MOC, mechanisms of chemoresistance; MRP, multidrug resistance associated protein; OCT, organic cation transporter; PARPi, poly ADP ribose polymerase inhibitor; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; PSC, primary sclerosing cholangitis; RIPK, receptor-interacting protein kinase; SCTR, secretin receptors; SMO, smoothened; SOX17, SRY-box 17; STAT, signal transducers and activators of transcription; TAM, tumor associated macrophages; TbRII, TGFβ type II receptor; TGFβ, transforming growth factor beta; TKI, tyrosine kinase inhibitor.

CONFLICT OF INTEREST

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ABSTRACT

Cholangiocarcinoma (CCA) is a deadly disease. While surgery may attain cure in a minor fraction of cases, therapeutic options in either the adjuvant or advanced setting are limited. The possibility of advancing the efficacy of therapeutic approaches to CCA relies on understanding its molecular pathogenesis and developing rational therapies aimed at interfering with oncogenic signaling networks that drive and sustain cholangiocarcinogenesis. These efforts are complicated by the intricate biology of CCA, which integrates not only the driving force of tumor-cell-intrinsic alterations at the genetic and epigenetic level, but also pro-tumorigenic cues conveyed to CCA cells by different cell types present in the rich tumor stroma. Herein, we review our current understanding of the

mechanistic bases underpinning the activation of major oncogenic pathways causative of CCA pathogenesis. We subsequently discuss how this knowledge is being exploited to implement rationale-based and genotype-matched therapeutic approaches that predictably will radically transform CCA clinical management in the next decade. We conclude by highlighting mechanisms of therapeutic resistance in CCA and reviewing innovative approaches to combat resistance at the pre-clinical and clinical level.

KEY POINTS

- Cholangiocarcinoma (CCA) is a deadly cancer worldwide as a result of limited therapeutic options and chemoresistance.
- CCA pathogenesis is associated with genetic and epigenetic alterations in tumor cells as well as important changes in the tumor microenvironment, which, collectively, lead to the activation of multiple signaling pathways responsible for driving tumor onset and progression. These pathways are linked to the control of cell proliferation, cell survival/death, metabolism, tissue morphogenesis and inflammation.
- A better characterization of the molecular mechanisms involved in CCA pathogenesis and chemoresistance is predicted to pave the way to the rational design of innovative therapies and to the prevention/bypass of chemoresistance.

INTRODUCTORY STATEMENT

Cholangiocarcinoma (CCA), the second most frequent primary liver cancer, is characterized by high mortality, clinical silence at early stages and rapid disease development and progression[1]. The unfavorable clinical history of the disease is largely caused by the aggressive biology of the malignancy, the nature and mechanisms of which are still largely

obscure[1]. A major consequence of our poor understanding of CCA molecular pathobiology is the limited range of therapeutic options currently available[1]. Risk factors for CCA are chronic inflammatory conditions of the biliary tree, such as primary sclerosing cholangitis (PSC)[1]. Initial investigations focused on the molecular links between the inflammatory milieu and CCA development. Those studies led to the identification of several cytokines and pathways that may have a relevant role in CCA initiation and progression. More recently, attention has also been drawn to genetic and epigenetic abnormalities as well as alterations of signaling pathways involved in cholangiocyte responses to physical, chemical or biological damaging agents. This knowledge is now being exploited to design novel, rationale-based therapeutic approaches to CCA clinical management. A vexing issue affecting CCA treatment is chemoresistance and strategies aimed at counteracting chemoresistance remain an unmet clinical need in CCA. The purpose of this manuscript is to i) provide an overview of our current understanding of the molecular pathogenesis of CCA; and ii) discuss present and future directions in the implementation of targeted therapies in CCA management. Immunotherapy will be discussed at length in another review in this special issue.

5.1. Molecular signaling map

Cholangiocarcinogenesis is associated with not only genetic and epigenetic alterations, but also with important modifications of the tumor microenvironment. These changes lead to the activation of multiple signaling pathways capable of driving tumor onset and progression.

5.1.1. Microenvironment and inflammation-related pathways

- **IL-6/STAT3 pathway**

Interleukin (IL)-6 plays a critical role in the context of acute phase response upon liver injury and in systemic inflammation. In the CCA tumor microenvironment, IL-6 is produced by activated Kupffer cells, tumor associated macrophages (TAM), cancer associated fibroblasts (CAF) and CCA cells, subsequently driving an iterative process that comprises cellular stress and damage, inflammation and compensatory proliferation[2]. IL-6 signals upon binding

to the IL-6 receptor via gp130 and intracellular activation of Janus kinases (JAK), signal transducers and activators of transcription (STAT), mitogen-activated protein kinases (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT serine-threonine kinase (AKT) pathways. STAT3 expression and pSTAT3 staining are increased in most intrahepatic CCA (iCCA) and correlate with worse prognosis in patients[3-5]. Stat3 is also activated in rat liver cells upon 3'-methyl-4 dimethylaminoazobenzene induced CCA formation[6]. These data indicate that the epithelial compartment is the predominant target of IL-6 in CCA.

Functional evidence for a tumor promoting role of IL-6 arises from STAT3 overexpression experiments, which resulted in increased proliferation and survival potential of CCA cell lines as well as faster growth of CCA xenografts in mice[4]. Mechanistically, IL-6/STAT3 and IL-6/p38 directly induce myeloid cell leukemia-1 (MCL-1) expression, a key antiapoptotic BCL-2 family member that inhibits cell death[7-9]. Further studies in CCA patients and cell lines indicated coexistence of MCL-1 expression and phosphorylated/activated (p)AKT. A functional relationship was shown by anti-IL-6 neutralizing serum, which reduced pAKT levels, as well as by AKT inhibitors that reduced MCL-1 expression and increased cell death[10].

Loss of negative feedback regulation of JAKs caused by hypermethylation of *SOCS3* promoter sequences and leading to oncogenic STAT3 activation was described in iCCA[11]. *Vice versa*, IL-6 signaling itself can trigger aberrant DNA methylation, resulting in up- or down-regulation of critical genes, as shown in detail for epidermal growth factor receptor (EGFR)[12] (Figure 1).

- **TGFβ/SMAD pathway**

Transforming growth factor beta (TGFβ) is a cytokine involved in multiple cell fate decisions that are strongly context-dependent. Nearly any cell type can produce and/or respond to TGFβ and there are multiple TGFβ receptors and co-receptors as well as multiple TGFβ family members. As a driver of liver fibrosis, TGFβ induces activation of hepatic stellate

cells. Stimulation of liver epithelial cells by TGFβ can produce either cytostatic or tumor promoting effects, therefore affecting CCA pathogenesis in a complex manner[13].

Mutational analysis of biliary tract cancers (BTC) highlighted frequent *SMAD4* mutations in extrahepatic CCA (eCCA)[14,15,16]. Loss of SMAD4 expression was reported in 45% of iCCA[17], with TGFβ-associated gene expression signatures being correlated to patient survival[18,19,20]. Besides exploiting SMAD4 loss, CCA cells may escape from TGFβ-mediated suppression of cell proliferation via upregulation of cyclin D1[21]. In a rat model of CCA, TGFβ and TGFβ type II receptor (TbRII) expression were induced in preneoplastic and fully transdifferentiated tumor cells[22]. As for its tumor promoting activity, TGFβ induces mesenchymal features in CCA cell lines, including decrease in E-cadherin and cytokeratin (CK) 19 expression, increase in vimentin, N-cadherin and S100A4 expression and nuclear presence of Snail. Epithelial-mesenchymal transition (EMT) enhances migration, invasiveness and peritoneal dissemination of eCCA cells[23,24]. Nuclear Snail immunoreactivity correlates with reduced CK19, increased vimentin, lymph node metastasis and poor survival. In addition, Twist was identified as a critical downstream target of TGFβ-induced EMT in CCA[25]. Interestingly, TGFβ participates in iCCA formation in the context of hepatocyte to cholangiocyte conversion in regeneration processes and in intermediate hepatocellular carcinoma (HCC)/CCA phenotypes[26]. In an elegant study delineating the consequence of TbRII depletion in hepatocytes or cholangiocytes, Schwabe *et al* found that loss of TGFβ signaling in either hepatocytes or cholangiocytes facilitates CCA formation by enhancing cholangiocyte proliferation upon carcinogenic damage[27] (Figure 1).

5.1.2. Cell survival/death related pathways

- **Oncogenic pathways linked to FGFR2 fusions**

RNA sequencing analyses led to the discovery of fibroblast growth factor receptor 2 (FGFR2) fusion transcripts in 10-15% of iCCA cases[28]. The predicted translation products of iCCA *FGFR2* fusion transcripts span aa. 1-762 of FGFR2IIIb joined C-terminally to sequences contributed by any of a long list of fusion genes (at least forty identified so far)[29-

[34]. FGFR2 fusions (FFs) display constitutive tyrosine kinase activity[29,34-36], which is caused by forced dimerization of the FGFR2 kinase domain imposed by protein-protein interaction motifs located in the fusion sequences[34,35]. FFs display transforming activity *in vitro* and *in vivo*, which was found to be kinase activity-dependent and as such subject to inhibition by pharmacological targeting of the FGFR2 kinase[29,34-36] (Figure 1). Activation of extracellular signal-regulated kinase (ERK)1/2 appears to be a major oncogenic pathway activated by FFs[29,36]. However, the routes of FF signaling which are necessary to maintain the oncogenic phenotype in iCCA have not been fully detailed as yet, because of lack of cellular and animal models of FF-driven iCCA.

- **Oncogenic pathways linked to BRAF, KRAS and TP53 mutations**

Mutations of *BRAF* occur mostly in iCCA, with a prevalence of 1-3%[37]. *BRAF* mutations affect most frequently the V600 position, thus generating class 1 mutants, i.e. BRAF oncoproteins that signal as monomers and are sensitive to currently licensed inhibitors, such as Vemurafenib and Dabrafenib[38]. Mutations generating class 2 (e.g. K601E, G469A and F595L) or class 3 (e.g. G469E) mutants have also been described in iCCA[37]. Class 2 and class 3 mutants are oncogenic, but insensitive to currently available BRAF inhibitors[38]. Regardless of the structural bases underpinning their signaling activity, all classes of BRAF mutants drive cell transformation through activation of the MEK/ERK module, which creates the opportunity of interfering with their activity through MEK1/2 blockade[38]. *KRAS* and *TP53* mutations occur in both iCCA and eCCA. Genetic experiments in mice have ascertained a role for *Kras* mutations in the development of iCCA, in cooperation with *Tp53* or *Pten* mutations[39], and eCCA, in cooperation with ablation of *Tgfbr2* and *Cdh1*[40]. Despite the availability of these models, mechanisms underpinning oncogenic RAS signaling have not been studied in detail in CCA cells. Thus, current modeling of KRAS biology in CCA is essentially built on assumptions which assign key roles to usual suspects acting downstream to RAS, i.e. MEK1/2 and the PI3K/AKT/mTOR axis.

- **EGFR pathway**

The ERBB/HER family of receptor tyrosine kinases comprises EGFR/ERBB1 (HER1), ERBB2 (HER2), ERBB3 (HER3), and ERBB4 (HER4). While mutations in ERBB family members are not frequent in CCA, overexpression of ERBB1-4 has been widely described, both in iCCA and eCCA, and frequently associated with poor prognostic features, especially in the case of EGFR and ERBB2[41]. While the pathophysiological mechanisms underlying the role of ERBB3 and ERBB4 in CCA are still unknown, multiple studies describe the impact of EGFR and ERBB2 in promoting CCA proliferation, migration and invasion through activation of downstream signaling pathways, including JAK/STAT, RAS/MEK/ERK and PI3K/AKT[42-45].

ErbB signaling is very complex because the four members can heterodimerize and be activated by different transmembrane pro-ligands (i.e. EGF, HB-EGF, Amphiregulin, Neuregulin 1-4, etc) that are released upon proteolytic cleavage by the ADAM family metalloproteinases. In addition, EGFR activation can be promoted indirectly by various compounds known to participate in CCA pathogenesis, such as conjugated bile acids, lipopolysaccharide and prostaglandin E2 (Figure 1). These molecules, through activation of their membrane receptors (TGR5, TLR4 and EP1, respectively), trigger intracellular signaling pathways that lead to metalloproteinase activation and the consequent release of different ErbB ligands[46,47] (Figure 1). Moreover, oxidative stress activates the MK2–dependent transduction pathway, which induces HB-EGF expression in CCA cells[48]. It was also reported that CAFs express EGFR ligands, including HB-EGF, which promotes activation of EGFR signaling in CCA tumor cells (Figure 1). In turn, EGFR activation induces the production of TGFβ by CCA cells, thereby generating a vicious cycle between CCA cells and CAFs[49]. Thus, EGFR acts as a hub by integrating multiple external signals, including its own ligands and other compounds such as bile acids, bacterial products, and inflammatory factors, promoting initiation and progression of CCA.

- **Secretin and histamine pathways**

The role of secretin receptors (SCTR) is poorly known in CCA[50,51]. While SCTR play fundamental functions in normal cholangiocyte physiology because they are exclusively expressed in biliary tree, the expression of SCTR is down-regulated in human CCA contrasting with its upregulation in proliferative cholangiocyte during cholestatic diseases. However, *in vitro* and *in vivo* studies show that secretin decreases CCA cell proliferation and tumor burden by inducing cell death[50]. CCA cells express histamine receptors (HisR) H1-H4[52], produce histamine and show up-regulated expression of histidine decarboxylase, the enzyme responsible for histamine synthesis via histidine decarboxylation, as well as reduced expression of monoamine oxidase B, the enzyme responsible for histamine breakdown[53]. In addition, mast cells (MC), i.e. the professional histamine-producing cell type, populate the iCCA stroma[53], possibly because iCCA cells produce stem cell factor, an established MC chemoattractant[54]. These observations have raised interest in the possibility that an autocrine/paracrine histamine circuit supports the malignant phenotype of iCCA cells. *In vitro* and *in vivo* experiments provide support to this hypothesis[53-57], although it remains unclear whether pharmacological manipulation of histamine signaling will ever gain relevance in CCA clinical management. Perhaps, a more viable approach is the use of HisR antagonists, which are used in medical conditions such as allergies and gastro-esophageal reflux, for iCCA chemoprevention in patients diagnosed with PSC. Thus, in the Mdr2(-/-) PSC mouse model, pharmacological blockade of H1/H2 HisR reduced cholangiocyte proliferation, fibrosis and inflammation[56,58]. These effects were the end result of direct inhibition of histamine activity on cholangiocytes as well as dampened MC activation, which, in turn, blunted the release of pro-inflammatory cytokines in the liver microenvironment[56]. It remains to be seen whether chronic H1/H2 HR blockade is capable of modifying PSC clinical course in humans.

- **PI3K/AKT pathway**

The PI3K/AKT pathway regulates several cellular processes, including proliferation, apoptosis and cytoskeletal rearrangement. AKT is a serine/threonine kinase, which, upon being activated downstream to PI3K, integrates various signaling cascades in a cell-context dependent manner. The oncogenic activity of AKT in liver depends on enhanced cell survival[59]. Ectopic expression of activated forms of AKT with Yap or Notch1 was found to promote CCA formation in mice[60,61]. Gain of function mutations in *PI3K* are evident in CCA[31] and AKT2 expression is found predominantly in pCCA[62]. AKT activation is induced in eCCA and correlates with phospho-mTOR, loss of PTEN and shorter patient survival[63] (Figure 1).

14-3-3ζ, which acts by binding to phosphorylated serine/threonine residues, is upregulated in CCA and correlates with poor survival and metastasis. 14-3-3ζ contributes to AKT activation and promotes cell cycle progression and chemoresistance in CCA[64]. In contrast, expression of PIP60, a catalytic subunit of the NuA4 acetyltransferase that is consistently downregulated in CCA, acts as a tumor suppressor via controlling the PI3K/AKT pathway, thereby predicting tumor progression and poor outcome[65]. The long non-coding RNA MALAT1, whose expression correlates with a poorer prognosis in CCA, is implicated in AKT regulation and was found to promote CCA cell proliferation[66].

- **Apoptosis and necroptosis pathways**

Apoptosis and necroptosis are two distinct forms of regulated cell death. Necroptosis was recently discovered as an immunogenic cell death subroutine that critically depends on receptor-interacting protein kinase (RIPK)1 and RIPK3 activities, and mixed lineage kinase domain-like oligomerization and translocation to cell membranes[67]. Necroptosis has been found to be triggered in liver parenchymal cells under acute and chronic injury in humans and experimental models of disease[68-71]. Importantly, mounting evidence suggests that necroptosis plays an intricate and often cell autonomous-independent role in carcinogenesis. In pancreatic ductal adenocarcinoma, necroptosis impinges on the tumor microenvironment by inducing the expression of the chemokine attractant CXCL1/Mincle pathway, thus

promoting macrophage-induced adaptive immune suppression[72]. Further, RIP3-dependent signaling promotes vascular permeability by both triggering necroptosis in vascular endothelial cells[73] and activating p38/heat shock protein 27[74]. Similarly, the necroptosis-associated hepatic cytokine microenvironment governs iCCA development from oncogenically transformed hepatocytes. Indeed, Seehawer et al. showed that *in vivo* electroporation of hepatocytes with transposon vectors co-expressing oncogenic mouse Myc and mouse *Nras*^{G12V}, or mouse Myc and human *AKT1* resulted mainly in iCCA due to necroptosis-driven epigenetic changes. Conversely, the delivery of the same oncogenic drivers by hydrodynamic tail-vein injection promoted liver apoptosis and solid or trabecular hepatocellular carcinomas. This lineage commitment was determined by decreased T-Box 3 (Tbx3) and increased PR Domain Containing 5 (Prdm5) mRNA levels in iCCA compared with HCC. Similar findings were conserved in human tumors. Likewise, using the same experimental models, pharmacological or genetic inhibition of necroptosis efficiently dampened necroptosis-associated hepatic cytokine microenvironment, also switching iCCA outgrowth towards HCC development[75]. Overall, necroptosis activation could dramatically impinge on hepatic microenvironment guiding lineage commitment towards iCCA (Figure 2).

5.1.3. Development related pathways

- **Notch pathway**

Notch signaling is implicated in differentiation of bipotent hepatoblasts towards the cholangiocyte lineage[76,77]. In mammals there are four Notch receptors (NOTCH1-4) and five ligands, Jagged (JAG1, 2) and Delta-like (DLL1, 3 and 4). Notch signaling is activated through cell-cell contacts, that lead to its interaction with cognate ligands expressed by adjacent cells. Following activation, proteolytic cleavage by the γ-secretase complex allows the release of the Notch intracellular domain from the plasma membrane, its translocation into the nucleus and the eventual activation of Notch target genes via the nuclear effector RBPJ. The signals exchanged between cells through these interactions determine cell fates, while its dysfunction is involved in developmental defects and post-natal pathologies,

including CCA[78]. Aberrant expression of NOTCH1-4 and their downstream target HES1 has been reported in eCCA, with NOTCH1 and 3 being correlated with a poorer histological differentiation[79]. In iCCA, NOTCH1 was associated with increased proliferation and survival of CCA cells, up-regulation of pro-survival MCL-1 and BCL-XL[80] and enhanced cell migration through RAC1 activation and EMT induction[81]. Over-expression of NOTCH2 was reported in well-differentiated iCCA. In mice, Notch2 drives hepatocyte-derived CCA formation[82]. Notch3 overexpression was shown to drive CCA onset and progression as well, through activation of the PI3K-AKT cascade, rather than through canonical Notch-RBPJ signaling[83]. NOTCH4 was up-regulated in iCCA as well and was associated with a poor prognosis[84]. In addition, JAG1 overexpression was observed in human iCCA concurrently with activated AKT. In mice, Akt/Jag1 overexpression in the liver induces iCCA exhibiting increased cell proliferation and extensive stromal reaction, confirming the importance of Notch signaling in iCCA[85] (Figure 1).

- **Hedgehog pathway**

The evolutionarily conserved Hedgehog (HH) pathway is implicated in tissue-patterning during embryonic development and carcinogenesis in post-natal life[78,86]. Its activation involves a family of ligands, named Sonic (SHH), Indian (IHH), and Desert (DHH) hedgehog, which interact with the Patched cell surface receptor. In response to HH binding, Patched inhibits Smoothened (SMO), thus initiating a downstream signaling pathway cascade that culminates in nuclear localization of the Glioblastoma (Gli) family transcription factors and the attendant transcriptional regulation of Gli-target genes[78] (Figure 1). HH pathway activation in liver progenitors expands the pool of cells available to restore liver integrity following acute or chronic liver damage. However, constitutive activation of the HH pathway promotes dysfunctional repair and results in chronic hepatic inflammation, fibrosis, and cholangiopathies[87-89]. Notably, SHH was found to be significantly expressed in iCCA[90]. It must be noted that canonical HH signaling requires that cells express cilia, yet CCA cells do not display cilia on their surface[91]. Interestingly, it was reported that non-canonical HH

signaling may be triggered in CCA cells via Gi-protein coupled receptors, as also reported in the fruit fly *Drosophila melanogaster*[92], thereby, promoting cytoskeletal remodeling and cell migration through RhoA and Rac activation[91,93].

- **Wnt/β-catenin pathway**

The Wnt/β-catenin signaling pathway regulates hepatobiliary development and promotes cell survival in CCA[94,95]. The function of β-catenin is central in the canonical Wnt signaling cascade that comprises a large family of Wnt ligands and Frizzled lipoprotein-receptors. While in normal epithelial cells β-catenin is mostly bound to the E-cadherin pool engaged in cell-cell junctions, in many transformed epithelia, including BTC cells, loss of E-cadherin promotes accumulation of β-catenin in the nucleus. Nuclear β-catenin associates with the LEF/TCF transcription factor to regulate the expression of target genes involved in cell proliferation, differentiation, migration and apoptosis (e.g. *CCND2*, *CDKN2A*, *BIRC5*)[96,97].

Numerous studies have shown that CCA has a high desmoplastic stroma in which inflammation influences tumor growth[98,99]. In a rat model of CCA and in human tumors, WNT7B was present in the stroma and often co-localized with a subset of CD68+ macrophages surrounding the tumor cells[96]. These macrophages were identified as a source of WNT signals that acted to enhance CCA cell proliferation via β-catenin[96] (Figure1). Wnt/β-catenin signaling regulates SRY-box 17 (SOX17) expression, a transcription factor which is key to the differentiation of pluripotent stem cells to cholangiocytes[100]. Down-regulation of SOX17 during CCA development promotes cholangiocyte de-differentiation and is correlated with worse outcomes after tumor resection. Additionally, overexpression of SOX17 in CCA cells decreased their tumorigenic capacity by increasing oxidative stress and apoptosis, also inhibiting cell migration and Wnt/β-catenin-dependent proliferation[100].

5.1.4. Metabolic and epigenetic pathways linked to IDH1/2 mutations

Recurrent mutations of the isocitrate dehydrogenase (IDH) genes *IDH1* and *IDH2* were reported exclusively in iCCA, with a prevalence of 15-20%. *IDH1/2* mutations generate

neomorphic IDH enzymes which convert α-ketogluturate, i.e. the normal end product of IDH1/2 activity, to 2-hydroxyglutarate (2-HG)[101]. In cells expressing mutant IDH enzymes (mIDH1/2) 2-HG accumulates at levels (5-30 mM) that are orders of magnitude higher than those detected in normal cells (100 μM) (Figure 3). In cancer cells, 2-HG appears to be a terminal metabolite, the accumulation of which has been shown to affect several metabolic pathways, with a major impact on epigenetic regulation[101]. Thus, 2-HG-dependent inhibition of histone N-methyl-lysine demethylases and ten-eleven translocation (TET) 5-methylcytosine hydroxylases has been linked to the markedly increased levels of histone and DNA methylation, respectively, in mIDH tumor cells[101]. In line with this, the mIDH subgroup showed the greatest level of DNA methylome alterations among iCCA samples classified on the basis of the three most frequently mutated genes, i.e. *TP53*, *KRAS* and *IDH1/2*[102]. A major consequence of the prominent epigenetic changes in mIDH cells appears to be altered cell differentiation[101]. IDH1/2 mutations were shown to block the differentiation of bipotent mouse liver cells towards the hepatocyte lineage, an effect ascribed to inhibition of hepatocyte nuclear factor 4α expression[103]. This, in turn, pushed oncogenic conversion of liver progenitors along the biliary epithelial lineage[91]. Additional potential roles of 2-HG in mIDH cells include disruption of HIF-1α regulation, altered collagen biogenesis and increased DNA damage[101].

5.1.5. Epigenetic and/or DDR-pathways linked to BAP1, PBRM1 and ARID1A mutations

Genes encoding proteins involved in the regulation of chromatin organization, including *ARID1A*, *PBRM1* and *BAP1*, are frequently mutated in CCA[104] (Figure 3). These mutations are predicted to be loss of function and causative of transformation[104]. ARID1A, which has DNA binding activity, and PBRM1, which binds to histones, are non-catalytic subunits of BAF and PBAF complexes (Figure 3), respectively[105]. BAF and PBAF complexes mediate chromatin remodeling and are involved in regulating transcription, DNA replication and DNA repair[105]. *Arid1a* deletion in mice is sufficient to initiate tumor development in some contexts,

while being implicated only in advanced stages of tumorigenesis in others[106]. ARID1A has been implicated in the control of cell cycle, possibly via regulation of p53 target genes[106], reactive oxidative species production, cell motility and DNA damage response (DDR) via double-strand break (DSR) and mismatch (MMR) repair[107,108]. A recent study has proposed a role for ARID1A in negative regulation of YAP/TAZ activity in the nucleus, linking this regulatory mechanism to mechanosignaling[109]. In that model, liver-specific *Arid1a* ablation was *per se* inconsequential, but led to the development of iCCA in the context of liver damage and was associated with tissue stiffening[109]. Loss of PBRM1 was reported to occur late in iCCA[110]. In line with its role in tumor suppression, PBRM1 was shown to be required for efficient DSR[111] and also for maintaining genome integrity[112]. BAP1 is a nuclear deubiquitinating enzyme, involved in chromatin remodeling, transcriptional regulation and DSR[105,113,114]. Inherited heterozygous *BAP1* mutations predispose to a wide range of malignancies[115], including CCA[116]. BAP1 tumor suppressor activity was linked to increased ERK and JNK activity in CCA cell lines[117].

5.2. Targeted therapies

5.2.2. Microenvironment and inflammation-related pathways

- **IL-6/STAT3**

In 2007, the utility of increased serum IL-6 values as a biomarker for CCA tumor burden and therapy response was reported. Therefore, targeting IL-6 was suggested as a promising therapy for CCA[118,119]. However, anti-IL-6 therapies have not been translated into the clinic as yet. Even though IL-6 can act through a membrane-bound receptor alpha-chain (mIL-6R, the so-called classic IL-6 signaling) or *via* soluble forms (sIL-6R, trans-signaling), Kleinegger and coworkers found that IL-6Rα expression is down-regulated in CCA, which was correlated with poor overall survival. Further, by discriminating classic and trans-signaling in CCA cell lines, it was found that the blockade of IL-6 trans-signaling and the

activation of IL-6 classic signaling are tumor promoting[120]. These findings suggested that an IL-6R directed therapy in CCA may facilitate tumorigenesis and were in keeping with the datum that IL-6Rα expression is rather a good prognostic marker.

On the other hand, many compounds in experimental cancer trials exert at least some of their tumor suppressing action by inhibiting the activation of STAT3, instead of directly targeting IL-6 and its receptors. For example, the EGFR inhibitor afatinib reduces proliferation of iCCA cell lines and sensitizes them to cell death signals concomitantly with pSTAT3 reduction[5]; SC-43, a sorafenib derivative, inhibits STAT3 phosphorylation by a Src homology region 2 domain-containing phosphatase-1 (SHP1) dependent mechanism, inducing cell cycle arrest/apoptosis in cultured CCA cell lines and growth inhibition of CCA xenografts in the mouse[121]. Other drug candidates with similar outcome are metformin, natural compounds from plants (berberine, cryptotanshinone, xanthohumol, matrine), genestein, and the synthetic sphingosine immunosuppressant FTY720[122-127]. Despite these data, the assessment of pSTAT3 expression has not been translated into the clinic as a biomarker for CCA management.

- **TGFβ/SMAD pathway**

Targeting TGFβ signaling via LY2157299, an inhibitor of the TGFβ receptor kinase, or CX4945, a Protein Kinase CK2 (formerly casein kinase II) inhibitor that blocks TGFβ-mediated EMT, resulted in reduction of CCA cell migration and survival[128]. Since TGFβ is a known driver of myofibroblast generation, this is also relevant regarding cancer feeding fibroblasts and in a rat model of thioacetamide (TAA)-induced fibrosis that progresses to CCA, the anti-TGFβ neutralizing monoclonal antibody 1D11, inhibited tumor formation, presumably by reducing pro-tumorigenic fibrosis/stroma[129].

5.2.2. Cell survival/death related pathways

- **FGFR2 fusions**

As discussed above, the transforming activity of FFs, assessed through their ectopic expression in a number of cellular models, was found to require FF catalytic activity[29,34-36]. In line with these preclinical studies, a seminal paper by Borad and co-workers reported encouraging clinical responses to non-selective FGFR inhibitors in FF positive patients carrying chemo-refractory iCCA[30]. Subsequently, the *ad hoc* analysis of a small group of BTC patients enrolled in the multi-cancer MOSCATO 01 trial revealed that iCCA patients carrying FF benefitted from the FGFR-specific tyrosine kinase inhibitor (F-TKI) therapy to which they were assigned based on the tissue-agnostic and genotype-matched therapeutic protocol informing the MOSCATO 01 trial design[130]. More recently, a phase II clinical trial tested the activity of the F-TKI BGJ398 in 61 advanced/metastatic chemo-refractory iCCA patients with FGFR genomic alterations (79% of which were *FGFR2* fusion genes). Focusing on FF positive patients, objective responses were documented in 18.8% of the cases, while disease control rate (DCR) was about 80%[131]. ARQ 087/derazantinib, another orally bioavailable small molecule F-TKI, was tested in a phase I/II trial that enrolled twenty-nine patients. Partial responses were observed in 20.7 % of patients, while the overall DCR was 82.8%[132]. Collectively, results from the MOSCATO 01, BGJ398 and ARQ 087 trials indicate that F-TKIs show promising activity in iCCA patients selected on the basis of FF expression. Additional F-TKIs are currently being tested in phase II clinical trials enrolling FF positive iCCA patients, namely Pemigatinib (NCT02924376) and TAS-120 (NCT02052778). The clinical development of BGJ398 in iCCA is also progressing. Thus, BGJ398 will be compared against the standard of care gemcitabine+cis-platinum combination in a phase III multicenter, open-label, randomized, controlled study (NCT03773302), that will enroll unresectable or metastatic iCCA patients.

- **BRAF, KRAS and ERK targeted therapies**

Oncogenic RAS proteins have been notoriously difficult to target. Consequently, signaling molecules acting downstream to RAS, such as MEK1 and PI3K-AKT-mTOR have been the focus of clinical investigations in RAS-mutated tumors. These studies have not been met by appreciable success in CCA and therefore genotype-matched therapeutic approaches remain problematic in *KRAS*-mutated CCA patients[133].

Although present at low prevalence and exclusively in iCCA to date, BRAF mutations at codon 600, mostly V600E, are of interest because they are potentially predictive of clinical response to BRAF kinase inhibitors. Disappointingly, responses to single agent vemurafenib were observed only in one out of twelve BRAF V600E iCCA patients enrolled in a Phase 2 basket trial[134]. Primary resistance to vemurafenib in iCCA might therefore recapitulate the paradigm observed in colorectal cancer, where feedback reactivation of EGFR upon BRAF V600E inhibition restores signal flow through the RAS-ERK pathway, thereby nullifying the effects of BRAF blockade[135]. In line with this model of primary resistance, two independent reports described impressive and durable responses to the dabrafenib and trametinib combination (i.e. dual BRAF/MEK blockade) in three BRAF V600E iCCA patients, who were assigned to this therapeutic protocol after being evaluated by an institutional molecular tumor board[136,137]. Thus, for the time being, double blockade of BRAF and MEK1/2, which is already approved in melanoma[138], appears to deserve consideration as a valuable off-label therapeutic option in BRAF V600E chemo-refractory iCCA.

- **EGFR pathway**

Two major classes of anti-ErbB therapies are used in cancer, i.e. monoclonal antibodies, which block ligand binding, and TKIs, which target the catalytic domain of the receptor. Treatment of CCA cell lines with anti-EGFR therapies inhibits cell proliferation[45,139] and induces G1-phase arrest and apoptosis[139,140]. ErbB2 inhibitors alone were also effective *in vitro* in CCA cell lines[141] and dual EGFR/ErbB2 inhibitors, such as lapatinib[141], afatinib[5] or NVP-AEE788[142], are even more efficient than anti-EGFR therapies alone. Besides cell

proliferation, EGFR TKIs, such as gefitinib, reduce the migratory and invasive properties of CCA cells[42,43] by interfering with EMT. In a mouse CCA xenograft model, gefitinib was efficient in reducing CCA tumor growth[43] and restoring E-cadherin membrane expression in CCA cells[43], implying that gefitinib can reverse EMT in CCA cells *in vivo*. Anti-EGFR therapies have been also tested in combination with other types of treatments, including chemotherapy (gemcitabine)[143], other anti-ErbB [144] and non-ErbB-targeted therapies (including MEK[145], mTOR,[146] or VEGFR[147] inhibitors). All these combinations showed enhanced inhibition both *in vitro* and *in vivo*. At the clinical level, anti-EGFR therapies have been the most studied, either as single agents or in combination regimens[41]. However, although they showed efficacy in preclinical studies, they did not provide significant improvement in overall survival in phases II and III clinical trials[41]. Interestingly, a recent phase Ib study showed longer median overall survival in CCA patients treated with pulsatile erlotinib combined with chemotherapy compared to patients treated with standard chemotherapy alone, suggesting an effect for pulsatile administration of anti-EGFR[148].

- **PI3K/AKT pathway**

In one clinical investigation, all tested CCA patient samples displayed AKT activity, as measured by *in vitro* kinase assays. Further, combined targeting of mTOR and AKT using RAD001 and MK-2206 small molecule inhibitors shows significant anti-tumor effects *in vitro* and in preclinical models[149-151], suggesting a promising potential for clinical use. When comparing the responses of HCC and CCA cell lines to sorafenib, the latter were found to be less sensitive, due to lower inhibition of both ERK signaling and cell proliferation. When compared to HCC, CCA cells showed also increased pAKT. Accordingly, combined inhibition of both ERK and AKT/mTOR pathways by sorafenib+everolimus (mTOR inhibitor) resulted in superior CCA cell proliferation inhibition[152]. Celecoxib, a cyclooxygenase-2 (COX-2) inhibitor, was found to inhibit the proliferation of CCA cells and to induce cell death *in vitro* and *in vivo* by reducing pAKT levels and subsequently facilitating pro-apoptotic events. This drug effect could be rescued by prostaglandin E2 treatment[153], which supported the rationale

underpinning the therapeutic strategy. Finally, the natural compound genestein showed experimental antitumor effects against CCA by interfering with AKT activation[126].

- **Apoptosis and necroptosis pathways**

The knowledge of the association between necroptosis, immune milieu, epigenetics and cancer [75] has not yet translated into a prophylactic pharmacological strategy against CCA. One of the reasons for this is the lack of specific pharmacological necroptosis inhibitors, further to eventual concerns regarding the safety of long-term inhibition of necroptosis. The first clinical trials with a specific necroptosis inhibitor GSK2982772, a RIPK1 kinase inhibitor, are ongoing for psoriasis (NCT02776033), rheumatoid arthritis (NCT02858492) and ulcerative colitis (NCT02903966)[154]. Ponatinib and pazopanib, multitarget TKIs clinically used in the treatment of cancer, were also reported to inhibit necroptosis at low doses; RIPK1 is the main functional target of pazopanib, whereas ponatinib directly binds and inhibits both RIPK1 and RIPK3[155]. Finally, dabrafenib, used for the treatment of BRAF(V600)-mutated metastatic or unresectable melanoma, selectively inhibits RIPK3 kinase activity, ameliorating early necroptosis and liver injury associated with acetaminophen-overdosed in mice[156].

Conversely, evasion from programmed cell death is also a cancer hallmark. In that regard, RIPK3 expression is often silenced through methylation of its promoter in cancer cells, including hepatoblastoma cell lines, and restoring RIPK3 expression through genomic demethylation could promote sensitivity to chemotherapeutics[157]. RIPK3 was weakly expressed but not silenced in a cohort of 42 CCA patients with no preoperative radiation or chemotherapy. The potential of the pharmacological induction of this immunogenic cell death pathway as an individualized approach to overcome chemoresistance in CAA was further highlighted by the ability of a natural alkaloid component to specifically induce necroptosis in two human CCA cell lines[158]. Overall, the modulation of necroptosis in CCA is a double-edge sword; the inhibition of necroptosis, as a chemopreventive approach, and its induction, as a therapeutic strategy, is simultaneously promising and challenging.

5.2.3. Development related pathways

- **Notch pathway**

Several Notch signaling inhibitors, different from each to other in terms of classification, molecular target and mechanism of action, are currently being tested in clinical trials. Monoclonal antibodies against Notch1 or Notch2 display anti-tumor and anti-angiogenic properties with limited gastrointestinal toxicity, while the simultaneous inhibition of Notch1 and 2 leads to gastrointestinal toxicity[159,160]. Likewise, mAbs targeting the DLL4 Notch ligand (i.e REGN421 and OMP-21M18) disrupt tumor angiogenesis, compromising solid tumor growth, in absence of intestinal toxicity *in vivo*[161]. Another class of drugs that is suitable for targeting the Notch pathway is that of γ-secretase inhibitors (GSI), which prevent the final proteolytic cleavage of Notch receptors[162]. Recently, a study on patients with advanced or metastatic solid tumors, including participants who have a histological prevalence of cholangiocarcinoma and mutations, amplification or alterations in the expression of genes/proteins related to the Notch pathway, was conducted using GSI LY3039478 (NCT02784795), which had been shown to inhibit Notch activation and downstream biological effects. LY3039478 was well-tolerated in heavily pre-treated patients. Ongoing studies are testing LY3039478 as single agent or in combination with a targeted agent or chemotherapy[163,164].

Further approaches to inhibit Notch signaling come from the use of proteins, fragments or peptides that have recently been discovered as a new class of small molecule inhibitors of protein-protein interactions (PPIs) capable of targeting the assembly of NOTCH transcription. These include CB-103 (NCT03422679), a first-in-class orally available small molecule with an excellent non-clinical safety profile[159,165]. CB-103 (NCT03422679) is being evaluated in ongoing clinical trials that enroll patients with advanced or metastatic solid tumors, including gastrointestinal cancers that include colorectal cancer, CCA carcinoma, gastric cancer) in phase I/IIA.

- **HH pathway**

Several studies suggest activation of the non-canonical HH signaling pathway is a potent mechanism for the initiation and maintenance of CCA[91,166]. As reported by Khatib et al., treatment with cyclopamine, a specific inhibitor of hedgehog signaling by direct binding to the heptahelical bundle of Smo, and human chimeric 5E1 (ch5E1) that binds Shh with enhanced calcium ions, inhibited the proliferation of human CCA cell lines, and down-regulated the Hedgehog target genes Gli1 and Gli2. The downregulation of these target genes was correlated with an increased number of apoptotic cells. *In vivo*, blockage of the Hedgehog pathway led to a significant inhibition of tumor growth[167 175]. However, Fingas and colleagues reported that secretion of platelet-derived growth factor (PDGF) by CCA-associated myofibroblasts promotes resistance to apoptosis in CCA cells and may prevent them from responding to cyclopamine. This is because CCA cells are able to activate the Hedgehog pathway in a HH-independent fashion *via* PDGF-mediated activation of SMO[168].

The SMO inhibitor vismodegib was tested in *in vivo* models and showed significant antitumor activity. The efficacy of vismodegib was also highlighted in the most advanced stage of cancer, demonstrating a reduction in migration and dissemination of CCA cells after the initial implantation of the tumor *in vivo*[91]. Going forward, another powerful SMO inhibitor, sonidegib has been tested in numerous clinical trials of several solid tumors including liver tumors[169]. Sonidegib has shown remarkable antitumor activity with a favorable clinical safety profile, therefore sonidegib and vismodegib have received Food and Drug Administration (FDA) approval as inhibitors of the Hedgehog pathway for the treatment of solid tumors including CCA (NCT02465060).

- **Wnt/β-catenin pathway**

Suppression of Wnt/β-catenin signaling could be a potential target for inhibition of CCA growth. Boulter et al.[96] showed that inflammatory macrophages are necessary to increase activation of WNT pathway in CCA cells. Accordingly, two specific inhibitors of the canonical Wnt pathway, ICG-001 and C-59, which act by inhibiting the CTNNB1-CTBP signal or WNT

ligand secretion, reduced CCA tumor growth *in vivo*. CGX1321, a small peptide that inhibits an O-acyltransferase necessary for the secretion of Wnt ligands is being evaluated in a phase I clinical trial (NCT02675946). Another ongoing clinical trial is on DKN-01, a humanized monoclonal antibody that inhibits DKK1. Although DKK1 is a WNT antagonist it appears to increase tumor growth and metastasis in preclinical models and its high expression correlates with poor prognosis in a series of tumors, indicating that DKK1 has more complex cellular and biological functions than those already investigated. In this regard it has been observed that DKN-01 inhibits invasion and migration in CCA[170]. DKN-01 is in a phase I trial in combination with gemcitabine and cisplatin in patients with hepatocellular carcinoma, CCA, or gallbladder cancer, amongst others (NCT02375880). Finally, Wnt-β -catenin is targeted in patients with other forms of advanced tumors in which only few of them show an activation of Wnt-β-catenin status and/or genetic mutations (NCT02013154, NCT02655952 and NCT02020291).

5.2.4 Metabolic and epigenetic pathways linked to IDH1/2 mutations

Several compounds capable of inhibiting mIDH1/2 enzymatic activity, and therefore curbing the accumulation of the pathogenic 2-HG oncometabolite in mIDH cancer cells, are in clinical development[101]. Among them, AG120 (ivosidenib), which has already gained FDA approval for the treatment of mIDH1 AML, is the most clinically advanced IDH inhibitor in iCCA and is being currently tested in a phase III clinical trial (NIH identifier: NCT02989857). As an alternative to direct IDH1/2 targeting, synthetic lethality screenings have been exploited as a strategy to discover vulnerable dependencies associated to the mIDH status. Using this approach, Saha and colleagues identified dasatinib, a multi-TKI that inhibits BCR-ABL and Src kinase amongst others, as a synthetic lethal drug in IDH1/2 mutated iCCA cells[171]. Notably, dasatinib scored poorly against non-iCCA mIDH1/2 tumors[1724], which again emphasizes the often cell context dependent nature of synthetic lethal interactions[173]. The tyrosine kinase Src was identified as the critical dasatinib target in iCCA cells, but the molecular mechanism underpinning this vulnerability was not clarified[171]. Preclinical studies

in glioma, AML and sarcoma cells identified a synthetic lethal interaction between mIDH1/2 and poly ADP ribose polymerase inhibitors (PARPi)[174,175]. Mechanistically, 2-HG inhibits histone lysine demethylases, which in turn inhibits homologous recombination (HR)-dependent DSR and therefore generates dependence on PARP activity[175]. Based on these results, the activity of olaparib against mIDH tumors, including iCCA, is being evaluated in a phase II clinical trial (NCT03212274).

5.2.4. Epigenetic and/or DDR-pathways linked to BAP1 and ARID1 mutations

As noted above, mutations of ARID1A and BAP1 may also inhibit DSR and therefore confer sensitivity to PARPi[108,113,114]. This notion informed the design of an ongoing phase II clinical trial that will evaluate the activity of the PARPi Niraparib in CCA and other solid tumors carrying mutations of HR genes, including *ARID1A* and *BAP1* (NCT03207347). *ARID1A* mutations may also sensitize cancer cells to inhibitors targeting Aurora kinase A[176] and ATR [177], although direct demonstration that this is actually the case in CCA models is still lacking.

The HR defect caused by BRCA1/2 mutations sensitizes tumor cells to therapies based on immune checkpoint inhibitors blockade (ICB)[178]. Although it is still to be proved that mutational inactivation of any HR gene suffices to cause a *bona fide* "BRCAness" phenotype, the question arises whether CCA patients carrying mutations of *ARID1A*, *BAP1, PBRM1* or any other HR gene, couldbenefit from ICB-based therapies. This appears to be relevant for two reasons. First, a recent study ranked BTC as the second malignancy, among 21 tumor lineages analyzed, for frequency of mutations of HR genes. Specifically, HR gene mutations were detected in 28.9% of 342 BTC samples, with 2/3 of the mutations affecting*ARID1A* and *BAP1*)[179]. Second, ARID1A and PBRM1 mutations were reported to be determinants of clinical responses to ICB in some tumor types and experimental models[108,180,181]. Clinical trials are currently evaluating ICB in unselected BTC patients (NCT03473574, NCT02834013, NCT03250273). Thus, it will be interesting to evaluate whether therapeutic responses to ICB in CCA patients correlate with mutations affecting HR genes. Remaining in the vein of putative "BRCAness", it will be important to assess whether

HR genes mutations predict responsiveness of CCA patients to platinum-based chemotherapy.

Finally, mutations in epigenetic regulators such as BAP1, ARID1A and PBRM1, may render tumor cells dependent on EZH2 activity and, consequently, highly sensitive to epigenetic drugs[182]. In line, pharmacological inhibition of EZH2 was reported to be detrimental to iCCA cell proliferation *in vitro*[183], an observation that needs to be further substantiated in genetically defined CCA models.

5.2.5. FXR and TGR5-mediated pathways

In previous studies, expression of the bile acid nuclear receptor FXR has been shown markedly reduced in iCCA[184]. This was accompanied by a reduction (from 80% to 50%) in the predominance of the, in general, more active isoform FXR-α1 *versus* FXR-α2[185]. In contrast, expression of the bile acid plasma membrane receptor TGR5 seems to be relatively well preserved in iCCA[186]. Based on data showing the ability of obeticholic acid (FXR agonist) and INT-777 (TGR5 agonist) to affect the biology of two CCA cell lines (EGI1 and TFK1), FXR and TGF5 have been suggested as potential therapeutic targets for the treatment of CCA[186]. In the same study, mice with orthotopic intrahepatic implant of EGI1 cells were treated with obeticholic acid or INT-777. Of note, FXR, but not TGR5 activation, inhibited tumor growth. Since the expression levels of FXR in implanted EGI1 cells was negligible, whereas TGR5 expression was relatively well preserved, the actual mechanistic implications of pharmacological activation of FXR and TGR5 remains uncertain. The question arises as to whether indirect effects through changes in bile acid homeostasis due to activation of FXR in surrounding hepatocytes might be involved in the inhibitory effect of obeticholic acid observed in this model. In addition, since FXR expression has been identified in hepatic stellate cells, one of the precursors of CAFs[187], other possibility is that the inhibitory action of obethicolic acid is mediated by a direct action on these stromal cells,

as it has been described in breast cancer[188]. Thus, further preclinical investigations are still needed to support a beneficial effect of obeticholic acid treatment on CCA outcome.

5.3. Mechanisms of chemoresistance

5.3.1. Molecular bases of multidrug resistance phenotype

The response of CCA to the currently available conventional and targeted chemotherapy is extremely poor due to the existence of complex and very efficient mechanisms of chemoresistance (MOC) that help cancer cells to escape from the effects of cytostatic drugs. The result of the combination of all MOC expressed by tumor cells characterizes the so-called multidrug resistance (MDR) phenotype. Although most genes involved in MDR are also expressed in normal cholangiocytes, where they play a variety of roles in the physiology of these cells, they are usually up-regulated (in some cases down-regulated) during carcinogenesis accounting for constitutive chemoresistance. Moreover, in response to pharmacological treatment their expression may be further altered contributing to acquired chemoresistance. More than one hundred genes involved in chemoresistance have been identified and classified into seven groups of MOC based on their mechanism of action[189,190].

5.3.2. Lack of response to conventional and targeted chemotherapy

The molecular targets of many antitumor drugs are located intracellularly, and therefore they need to be taken up to reach their sites of action inside the cell to carry out the desired pharmacological action. Accordingly, to become effective, these drugs must cross the plasma membrane by simple diffusion or more frequently through carrier proteins. Thus, changes in the expression and/or function of uptake transporters and export pumps can determine final intracellular concentrations of active agents and hence the overall response to the chemotherapy. These MOC have been included into the MOC-1 subgroup, which

includes MOC-1a (leading to impaired drug uptake) and MOC-1b (accounting for enhanced drug efflux).

Thus, the reduction in the expression levels of the organic cation transporter 1 (OCT1; *SLC22A1*) and 3 (OCT3; *SLC22A3*) in CCA can affect CCA response to cationic drugs. These transporters have been associated with uptake of the TKI sorafenib[191]. Accordingly, a reduction in their expression or the appearance of non-functional forms, by mutation or aberrant splicing, lead to lower sensitivity to the cationic drugs taken up by these transporters[191,192]. Also included in MOC-1a is the altered function of members of the families of concentrative nucleoside transporters CNTs (*SLC28*) and equilibrative nucleoside transporters ENTs (*SLC29*), which are involved in the uptake of nucleoside analogues, such as gemcitabine and 5-fluorouracil (5-FU). Studies on CCA cells have shown down-regulation of ENT1 in 5-FU-resistant cell lines[193]. Moreover, low ENT1 expression has been suggested as a predictive biomarker of chemoresistance to gemcitabine in patients with advanced CCA[194]. Low expression in CCA tumors and cell lines of the copper transporter CTR1 (*SLC31A1*), which is involved in cisplatin uptake, has been associated with the poor sensitivity of CCA cells to cisplatin[184].

On the contrary, up-regulation of ATP binding cassette (ABC) proteins involved in drug efflux leads to a reduced response to chemotherapy by reducing the intracellular content of chemotherapeutic agents (MOC-1b). A common case of ABC-mediated reduction in drug bioavailability in cancer cells is due to MDR1, previously termed P-glycoprotein (*ABCB1*). The expression of this protein has been detected in archival formalin-fixed paraffin-embedded gallbladder cancer tissues[195] and CCA cell lines[196]. MDR1 can play a role in the efflux of a large variety of drugs, such as doxorubicin, etoposide, paclitaxel and vinblastine, and its expression has been associated with poor prognosis in iCCA patients[197]. In addition, efflux transporters of the ABCC family of multidrug resistance associated proteins (MRP) MRP1 (*ABCC1*) and MRP3 (*ABCC3*) are the most abundantly expressed in CCA[184], where they could mediate the export of many drugs commonly used in CCA chemotherapy.

Among genes included in MOC-2 are those leading to a decreased ability of cancer cells to activate prodrugs or an enhanced detoxifying capability, in either event resulting in a lower proportion of active *versus* inactive agent inside the cells and hence to lower sensitivity to chemotherapy. The enzyme orotate phosphoribosyl transferase that participates in the biotransformation of 5-FU into its active metabolite, has been found upregulated in 5-FU-sensitive CCA tumors whereas it is poorly expressed in 5-FU-refractory cases[198]. The phase I detoxifying enzyme NAD(P)H-quinone oxidoreductase 1 (NQO1) plays important roles in chemoresistance and proliferation in several cancer cells lines including CCA where NQO1 has been described to be involved in chemoresistance to 5-FU, doxorubicin, or gemcitabine. Recent studies indicate that the use of the β-eudesmol (a compound that suppresses NQO1 enzyme activity) enhances chemosensitivity to 5-FU and doxorubicin in CCA cells[199]. Metallothioneins, which have been associated with the neutralization of platinum-derived drugs, are overexpressed in CCA and could be useful to predict the poor response of patients to chemotherapy based in platinum-derivatives[200].

Changes in drug molecular targets, which can also lead to poor response to chemotherapy, are classified into MOC-3. As an example, analysis of the expression levels and/or the detection of the presence of genetic variants of *EGFR* gene have been suggested to be useful to predict the pharmacological outcome of CCA patients treated with anti-EGFR therapy[201]. Although primary or secondary EGFR acquired mutations (such as T790M) are the most prevalent mechanism of resistance in other cancers, these mutations are not frequent in CCA and their impact is unknown. However, resistance to anti-EGFR therapies can also result from mutations in downstream signaling proteins, such as BRAF and KRAS, which are very frequent in CCA[202]. The recent development of a patient derived xenograft model of iCCA bearing the most frequent *KRAS* mutation (G12D) should provide answers on the role of this mutation in the efficacy of anti-EGFR and other targeted therapies[145]. In addition, tumor cells can use alternative signaling pathways through other growth receptors. In this sense, an upregulation of IGF2/IR/IGF1R signaling pathway has been recently

described in CCA cells after long term exposure to erlotinib[203]. Concerning resistance to F-TKIs in iCCA patients carrying FGFR2 fusions, it was observed that a major, albeit not unique, mechanism of resistance to BGJ398 was drug-induced selection of tumor sub-clones carrying mutations in the FF tyrosine kinase domain. These mutations inhibited binding of BGJ398 to the target[172]. Thus, further clinical development of F-TKIs in the management of iCCA will require to invest considerable efforts in understanding and counteracting molecular mechanisms of therapeutic resistance. Perhaps reassuringly, a few options already stand up at the horizon. For instance, F-TKIs capable of binding to kinase-mutated FFs are being developed[204]. HSP90 inhibitors have also shown promising activity against FFs[36]. This is because FFs are dependent on the HSP90-centered chaperone machinery for acquiring and maintaining a thermodynamically stable fold[36]. Accordingly, pharmacological inhibition of HSP90 caused precipitous FF degradation and consequent suppression of oncogenic signaling[36]. Of note, BGJ398-resistant FFs retained sensitivity to the HSP90 inhibitor ganetespib. Thus, the BGJ398+ganetespib combination might not only provide more efficient targeting of FFs but also delay/prevent BGJ398 resistance mediated by FF mutations[36].

The mechanism of action of many cytostatic drugs such as cisplatin or 5-FU is based on the direct or indirect alteration of DNA structure. Thus, mechanisms of DNA repair that preclude the effect of these drugs have been included in MOC-4. Some evidences indicate that p53R2, a ribonucleotide reductase that participates in the repair of damaged DNA, is upregulated in gemcitabine-resistant CCA tumors. Moreover, the excision repair cross-complementing 1 protein (ERCC1), which has been related with cisplatin resistance, has been suggested to have a prognostic value because better survival rates after cisplatin treatment have been observed in ERCC1-negative CCA tumors[193].

Changes in the balance between pro- and anti-apoptotic proteins that permit tumor cells to avoid drug induced apoptosis have been classified into MOC-5. Thus, down-regulation of pro-apoptotic mediators such as BAX, BAK, caspase-3 and caspase-9, has been associated

with drug-resistance, while the up-regulation or increase activity of anti-apoptotic factor, such as ERK and Bcl-2, or over-activation of the pathways PI3K-AKT and RAF/MEK/ERK have been found to play a role in the resistance of CCA cells to activate apoptosis in response to chemotherapeutic drugs. Thus, prevention of escape by AKT/mTOR signaling from the RAF/MEK/ERK pathway in sorafenib treatment by suppressing mTORC2 activity has been explored as a new approach in CCA therapy[152].

Finally, changes in tumor microenvironment (MOC-6), which typically include hypoxia and enhanced acidity, and modified phenotype transition (MOC-7) may also decrease the efficacy of antitumor drugs. Although these two types of MOC are less known, the fact that the carcinogenic process in CCA development includes stroma alterations, recruitment of fibroblasts, remodeling of the extracellular matrix and changes in angiogenesis suggest that MOC-6 and MOC-7 could have an important impact in determining the overall MDR phenotype of CCA tumors. In this respect, it has been reported that some factors, such as leukemia inhibitory factor, and proteins of the extracellular matrix, such as laminin-332 induce chemoresistance in CCA tumors. Moreover, alterations associated with epithelial-mesenchymal transition in these tumors also result in enhance resistance to chemotherapy[205].

5.3.3. Novel chemosensitization strategies

As treatment for cancer is moving towards personalized therapy, advances in knowledge of the molecular bases of chemoresistance and improvement in the detection of the dynamic changes in genetic signature characteristic of each tumor at each time point of its evolution, will increase the chances to develop novel therapeutic strategies and then select the best option for each CCA patient.

One of the promising fields concerns the investigation in non-translated RNA. Thus, microRNAs (miRNAs) are able to regulate multiple cellular functions, including drug

resistance, apoptosis and senescence. Increasing evidence suggests the importance of miRNAs in the regulation of MDR in CCA. Indeed, global changes in the expression of miRNAs have been reported in both CCA cells and tumor tissue. Aberrantly expressed miRNAs promote an anti-apoptotic and chemoresistant phenotype[206] and shows that miRNAs might be valuable biomarkers as well as potential targets for therapy in patients with CCA.

Regarding chemosensitizing strategies, a useful approach to improve the effectiveness of anticancer drugs is to enhance the amount of agent able to interact with its site of action usually located in intracellular compartment. One way is to use anticancer drugs encapsulated into nanoparticles, for instance liposomes or nanopolymers that are taken up by CCA cell by endocytosis leading to a higher intracellular concentration and enhanced anticancer drug efficacy (for details see[189]).

Additionally, some targeted strategies have been proposed to deliver the drug specifically to CCA cells. With this aim, bile acid derivatives have been used as “Trojan horses” to enhance the uptake by cancer cells of antitumor moieties in enterohepatic circulation, such as cisplatin, chemically bound to a bile acid-like moiety that is recognized and transported across the plasma membrane by efficient bile acid carriers, such as NTCP, OATPs and ASBT[207,208]. Thus, bile acid transporters ASBT and OATP1A2 expressed in cholangiocytes could be considered a potential target for these vectorized agents. Of note, functional ASBT expression is well preserved in CCA[208]. A good example of this strategy, with demonstrated efficacy was Bamet-UD2, synthesized by linking cisplatin to two ursodeoxycholic acid molecules. Both *in vitro* and *in vivo* assays have demonstrated better antitumoral effect of Bamet-UD2 than cisplatin alone, with less exposure of extrahepatic tissues together with non-detectable toxicity at therapeutic dose[208,209].

Gene therapy has also been envisaged as a potential tool to overcome drug resistance. One explored rational has been to use vectors that express a drug transporter or a tumor suppressor protein under the control of a specific promoter that is be up-regulated in the target tumor cell. In this sense some promoters such as those of TERT, CK19 or Cox-2 have been proposed for their potential utility in adenoviral gene therapy in CCA[210,211]. Using a xenograft model of CCA in mice, it has been recently demonstrated that the specific overexpression of OCT1 at the plasma membrane of CCA cells by an adenoviral vector carrying OCT1 open reading frame under the transcriptional control of the *BIRC5* promoter induced in a marked sensitization of otherwise highly chemoresistant CCA cells, which resulted in a strong antitumor effect of sorafenib[192].

A considerable effort has been employed in the development of chemosensitizers, i.e., non-toxic molecules able to inhibit drug export pumps with the aim of increasing intracellular drug accumulation and hence its chemotherapeutic efficacy. Although many compounds have been extensively studied[189] no clinical trials on CCA patients have been reported. A novel alternative that is being explored is the combination of drugs whose chemoresistance is due to MOC-1b. It has been recently recognized that MDR development in tumor cells is usually accompanied by specifically hypersensitive to other drugs, a phenomenon now termed collateral sensitivity[212]. Thus, the co-administration of serial treatments with antagonistic drugs regarding collateral sensitivity could be useful in order to reduce chemoresistance, for instance by inhibiting drug efflux. In this sense, some studies have provided evidence that TKIs can reverse MDR by blocking the function of ABC transporter and subsequently promote drug accumulation. Accordingly, co-administration of TKIs with other conventional chemotherapeutics has been proven as a feasible alternative in MDR cancer cells which is supported by *in vivo*, *in vitro*, and *ex-vivo* experiments and some clinical trials. Thus, some clinical trials have reported the potential of TKIs to reverse MDR: in pancreatic cancer patients erlotinib significantly enhanced the response to gemcitabine, and in breast cancer patients lapatinib improved the beneficial effect of capecitabin[213].

5.3.4. Perspectives in the fight against chemoresistance

A better understanding of the molecular bases of mechanisms involved in the poor response of CCA to chemotherapy is still needed to identify the genetic signature underlying the dynamic changes affecting the "resistome" during cancer development. This would permit us to predict the failure of a given pharmacological regime and decide the best option for each patient at each time, which would prevent suffering from unjustified side effects as well as the delay in using another therapeutic alternative with higher chance of beneficial response. In addition, the development of more efficient novel drugs and therapeutic strategies to overcome CCA chemoresistance will necessarily be based on the advance in our understanding of this problem.

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Figure legends

Fig 1. Major signaling pathways involved in CCA. The signaling pathways involved in CCA progression can be classified into three main types: i) microenvironment and inflammation-related pathways, including TGFß and IL6 signaling pathways; ii) proliferation/survival/death-related pathways, ignited by constitutive activation of receptor tyrosine kinases such as FGFR2 and ERBB receptors or components of downstream signaling modules, such as JAK/STAT, RAS/RAF/MEK/ERK and PI3K/; iii) development-related pathways, including Notch, Hedgehog and WNT/ß-catenin. Note that membrane receptors displayed by CCA cells may be activated by ligands provided by the tumor microenvironment including CAFs, mast cells and TAMs, that produce HB-EGF, histamine and WNT7b, which in turn activate EGFR, histamine receptor, Frizzled/ß-catenin, respectively. In addition, ERRB1/EGFR can be indirectly activated by other molecules, such as PGE2, BA and LPS. Several components of these signaling pathways can be targeted by monoclonal antibodies or small molecule inhibitors, as indicated. Stars indicate signaling molecules that may be affected by recurrent pathogenic mutations in CCA and are candidates for therapeutic targeting. Abbreviations: ADAM17, ADAM metallopeptidase domain 17; BA, bile acids; CAF, cancer associated fibroblast; CCA, cholangiocarcinoma; DLL, delta-like ligand; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FGFR2, fibroblast growth factor receptor 2; GLI, glioma-associated oncogene; HB-EGF, heparin-binding EGF-like growth factor; IL6, interleukin 6; IL6R, IL6 receptor; JAK, janus kinase; JAG, jagged; LPS, lipopolysaccharide; MMP, matrix metalloproteinase ;NICD, notch intracellular domain; PGE2, prostaglandin E2; PI3K, phosphatidylinositol 3-kinase; PTCH, patched receptor; PTEN, phosphatase and tensin homolog; SMO, smoothened; SOCS3, suppressor of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3; TAM, tumor associated macrophage; TGFß, transforming-growth factor-ß; TGF-ßR, transforming-growth factor-ß receptor.

Fig 2. Schematic model depicting the interplay between necroptosis, immune milieu and epigenetics in iCCA. During the execution of necroptotic cell death, phosphorylated receptor-interacting protein kinase 3 (RIPK3) recruits and phosphorylates mixed lineage kinase domain-like pseudokinase (MLKL), which oligomerizes and causes cell permeabilization with concomitant leakage of damage associated molecular patterns (DAMPs). Stimulation of toll-like receptors (TLR) in immune cells by danger signals induces a particular profile of cytokine secretion. In turn, the necroptosis-associated hepatic cytokine microenvironment may trigger intracellular signaling cascades in transformed hepatocytes, which regulate chromatin accessibility of T-Box 3 (Tbx3) and PR Domain Containing 5 (Prdm5) genes. The epigenetic regulation of Tbx3 and Prdm5 directs the lineage commitment in liver tumorigenesis towards iCCA.

Figure 3. Inactivation of epigenetic regulators may affect double strand break repair in iCCA cells, thus generating synthetic lethality with PARP inhibitors. The nuclear proteins ARID1A and PBRM1 (drawn as circles labeled by A and P, respectively) are subunits of the large BAF and PBAF multi-protein complexes (both drawn as an oval for the sake of simplicity), which regulate chromatin remodeling. BAP1 is a chromatin-associated deubiquinating enzyme. Loss of function mutations of ARID1A, PBRM1 and BAP1 (indicated by a yellow symbol) compromise the DNA damage response (DDR) involved in double strand break repair and therefore sensitize tumor cells to PARP inhibitors (PARPi). IDH1 and IDH2 are metabolic enzymes located in the cytosol and mitochondria, respectively. Neomorphic IDH1/2 mutations (dark grey symbol) lead to excess production of 2-KG. This oncometabolite is capable of inhibiting the histone demethylases KDM4A/B, which are involved in double strand break repair; thus, functional inactivation of KDM4A/B by excess 2-KG may be synthetic lethal with PARPi.