Targeted therapies for extrahepatic cholangiocarcinoma: preclinical and clinical development and prospects for the clinic

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Abstract

Introduction.—Until recently, cholangiocarcinoma (CCA) was a largely overlooked disease, and among CCAs, extrahepatic CCA (eCCA) was even more neglected. Despite the growing impact of molecularly targeted therapies and immunotherapy, prognosis of eCCA is dismal. Therefore, unravelling the complex molecular landscape of eCCA has become an urgent need. Deep phenotyping studies have revealed that eCCA is a heterogeneous tumor, harboring specific alterations categorizable into four classes, "Mesenchymal", "Proliferation", "Immune", and "Metabolic". Molecular alterations convey the activation of several pro-oncogenic pathways, where either actionable drivers or outcome predictors can be identified.

Areas covered.—We offer insights on perturbed pathways, molecular profiling and actionable targets in eCCA and present a perspective on the potential stepping-stones to future progress. A systematic literature search in PubMed/ClinicalTrials.gov websites was performed by authors from different disciplines according to their specific topic knowledge to identify the newest and most relevant advances in precision medicine of eCCA.

Expert opinion.—eCCA is a distinct entity with unique features in terms of molecular classes, oncogenic drivers, and tumor microenvironment. Since more prevalent mutations are currently undruggable, and immunotherapy can be offered only to a minority of patients, international collaborations are instrumental to improve the understanding of the molecular underpins of this disease.

Keywords

cholangiocyte; cholangiocarcinoma; extrahepatic cholangiocarcinoma; epithelial-to-mesenchymal transition; immunotherapy; molecularly targeted therapies; oncogenic drivers; tumor microenvironment

1. INTRODUCTION

Cholangiocarcinomas (CCA) encompass a heterogeneous group of epithelial cancers with features of diverse degree of cholangiocyte differentiation that can arise from any segment of the biliary tree, except from the gallbladder, which represents a distinct type of cancer [1]. Currently, CCAs are classified as intrahepatic (iCCA), perihilar (pCCA), and distal (dCCA), based on the different anatomical location, each characterized by specific epidemiological and clinical features [1–3]. As shown in Figure 1, the point of demarcation between iCCA and pCCA is the confluence of the second order bile ducts, whereas the insertion of the cystic duct represents the border between pCCA and dCCA, which extends to the Vater's ampulla. Generally, pCCA and dCCA are grouped in a single extrahepatic CCA (eCCA) entity, although they represent subtypes with distinct clinicopathological features, prognosis and therapeutic options. Unfortunately, most clinical trials have been conducted considering all CCAs as a single disease, sometimes including even gallbladder cancer, thereby hindering the development of tailored therapies aimed at the specific type of biliary tract cancer [4].

Typically, eCCAs are mucin-producing adenocarcinomas presenting as poorly defined sclerosing nodules or, less frequently, papillary tumours with periductular and/or intraductal

growth (Figure 2) [5]. As cell origin, they may derive from transformation of columnar mucous cholangiocytes and/or of progenitor cells located in the peribiliary glands [6–8]. Biologically, eCCA is a highly aggressive: dedifferentiated tumor cells, a strong desmoplastic nature, with activation of cell survival and chemoresistance pathways, and a high genetic variability, are all factors contributing to early invasion and resistance to therapy [1,3,9]. Among all gastrointestinal cancers, eCCA accounts for 2–3% [3,10], with an apparent decrease in age-standardized incidence and mortality rates in the last decades. However, this trend must be interpreted with caution [11], since the nomenclature of eCCA has changed over time, sometimes including pCCAs and sometime not. This resulted in changes of incidence according to the inclusion or not of pCCA as eCCA. More recent studies indicate that indeed the worldwide incidence of both pCCA and dCCA is increasing in the last years [4]. CCA is considered a malignancy of the elderly population, however in the presence of risk factors, like chronic biliary inflammation [12], the disease may affect a younger population of patients [13,14] (Table 1). Diagnosis of eCCA is challenging; though painless jaundice and recurrent acute cholangitis may occur in the early phase, symptoms may be non-specific [15]. The diagnostic work-up includes laboratory, imaging, endoscopy, and pathology [3,4]. Contrast-enhanced computed tomography (CT) scan and magnetic resonance cholangiopancreatography (MRCP) are essential to select patients for curative surgery [16]. Endoscopic procedures provide both diagnostic and therapeutic potential [17], and the role of endoscopic ultrasound and cholangioscopy has expanded in the last decade [18] (Figure 1). Currently, diagnostic research aims to unveil tumor biomarkers enabling early detection of eCCA [19–26], since surgical resection with histologically negative margins, which remains the only potential curative options, can be performed in 35% of patients [27,28]. However, 5-years survival rates remain disappointing, ranging from 20 to 40% due to disease recurrence or metastasis [29,30]. Likewise, liver transplantation is an option only for a small subset of selected pCCA patients [31–35]. In advanced disease, systemic treatment has a limited impact on prognosis, with survival generally shorter than 12 months [36,37]. Thus, enrolling eCCA patients in clinical trials to test efficacy of new treatments is an urgent need [1,4]. Recent studies performed at different genetic, epigenetic, proteomic and microRNA levels have started to unravel the intricate molecular landscape underpinning the pathogenesis of CCA in relation to specific subtypes, and possible therapeutic targets are emerging, though none have been approved yet [38–40].

2. SIGNALING PERTURBATIONS, MOLECULAR PROFILING AND CLASSIFICATION IN eCCA

The understanding of the molecular landscape of eCCA is limited by the high heterogeneity of most studies conducted so far without distinction of the specific tumor subtypes. Recent studies based on next generation sequencing (NGS) have reported several genetic alterations in CCAs, including gene fusions, variations in copy number and point mutations [4]. Overall, these genetic alterations lead to profound changes in cell metabolism and functions, including Warburg effect, derangement of cell proliferation/survival ratio, overactivation of intracellular signals downstream of cell surface receptors including tyrosine kinase receptors (RTK), and epigenomic perturbations [4]. Table 2 summarizes the genetic changes detected in eCCA as compared with the other types of biliary tract cancers (BTCs). The differences in

genetic mutations across the different CCA subtypes was first highlighted by Nakamura et al. [41] who investigated a cohort of 239 BTC, composed by 137 iCCA, 74 eCCA, and 28 gallbladder cancers. Notably, 40% of BTC showed mutations behaving as putative targets for intervention. In this study, eCCA harbored specific genetic mutations, in particular gene fusions of *protein kinase cAMP-activated catalytic subunit (PRKAC)A* and *PRKACB*, two serin/threonine protein kinases acting as catalytic subunits of the protein kinase A. The authors also described two novel fusions, ATPase, Na + /K + transporting, b polypeptide (*ATPB1B)-PRKACA* and *ATPB1B-PRKACB*, the fusions *PRKACA-DNAJB1 (DnaJ heat shock protein family member B1)* and *PRKACB-C7orf50*, and nonsense mutations in *PRKACA,* all involved in the deregulation of cAMP-mediated pathways and potentially representing actionable targets. Compared with other BTCs, the presence of mutations occurring with higher frequency in but not exclusive of eCCA, such as *AT-rich interaction domain (ARID) 1A* and *1B, TP53, Kirsten rat sarcoma (KRAS), BRAF, Guanine nucleotide binding protein, alpha stimulating activity polypeptide (GNAS), small mother against decapentaplegic homolog 4 (SMAD4), Serine/Threonine kinase 11 (STK11),* and *E74 like ETS transcription factor 3 (ELF3)*, which is prevalent in ampullary carcinoma (a subtype of dCCA), was confirmed in other studies with smaller cohorts of patients [42,43]. Among the morphogenetic pathways, the overexpression of Notch receptor, initially described in iCCA, has been reported also in eCCA, including pCCA and dCCA, where it associated with gain of cancer stem cell-like properties [44]. A seminal paper by Montal and col. has been the first to focus solely on eCCA. By performing an integrative genomic profiling in a large cohort of 184 eCCA, 38 of which with paired non-neoplastic tissue, the authors found a differential expression of 174 genes that allowed to categorize eCCA into four classes: i) Metabolic; ii) Proliferation; iii) Mesenchymal; and iv) Immune [39]. The "Metabolic" class (18.7% of all cases) was characterized by alterations of genes involved in the metabolism of bile acids and fatty acids, as the peroxisome regulating glucose and lipid metabolism. A distinctive phenotypic trait of this class was the up-regulation of hepatocyte markers, in particular *HNF4A*, the master regulator of hepatocyte differentiation signals, and *HDAC6*, a tubulin deacetylase influencing the activity of bile acid receptors and the levels of acetylated alpha-tubulin in the primary cilium, leading to primary cilium alterations in CCA cells [45]. The "Proliferation" class (22.5%) was characterized by the activation of cell cycle checkpoints, cyclin-dependent kinase, and DNA repair pathways, with enrichment of *erb-b2 receptor Tyrosine kinase 2 (ERBB2)* mutations and amplifications. This type of eCCA showed a strong up-regulation of biliary epithelial genes (EpCAM and cytokeratins), and histologically, papillary lesions were prevalent. The "Mesenchymal" class (47.3%) displayed features of epithelial-to-mesenchymal transition (EMT), associated with activation of morphogenetic pathways, such as Hedgehog and transforming growth factor-β (TGF-β), a prototypal activator of EMT in cancer. An abundant stromal reaction enriched in cancer-associated fibroblasts (CAFs) and in the matrix component periostin, with a negligible contribution of macrophages was the histological hallmark of this class. The least frequent "Immune" class (11.5%) was dominated by the overexpression of genes regulating the adaptive immune responses, with pronounced lymphocytic infiltration and interferon-γ activation at the histological and biochemical levels, respectively. Of note, expression of immune checkpoints (PD-1/PD-L1) was also increased in the Immune class compared with other eCCAs. Interestingly, dCCA showed prevalence of the "Proliferation" and "Immune"

classes, whereas the "Mesenchymal" class was preponderant in pCCA. "Metabolic" tumors were more frequent moving closer to the intrahepatic biliary system. However, no specific patterns of genetic mutations could be related to the known risk factors of eCCA, likely because of the limited clinical information available, though it can be reasoned that several environmental and disease-related factors may cooperate to generate distinct molecular profiles. This integrative molecular classification seems to have strong prognostic significance, since the Mesenchymal class showed the poorest overall survival (OS), differing from the Immune class, which instead correlated with the best OS. Besides expanding the knowledge on the mutations of eCCA, with discovery of novel chromosomal amplifications (*YEATS domain-containing 4 (YETS4), E3 ubiquitin-protein ligase Mdm2 (MDM2), Cyclin E1 (CCNE1), Cyclin dependent kinase 4 (CDK4),* and *ERBB2*), this study wired genomic alterations to structural aberrations of four fundamental pro-oncogenic pathways, namely RTK-RAS-phosphatidylinositol 3-kinase (PI3K), TGF-β, histone modification, and TP53-RB, each harboring at least one putative actionable driver. Mutations activating the RTK-RAS-PI3K pathway were detected in 53% of eCCA and were associated to modifications in cell survival and proliferation. Aberrant epidermal growth factor receptor (EGFR), ERBB2 and MET RTK expression were described in the Proliferation class and correlated with advanced stage and poor prognosis. RTK modification triggers RAS/MAPK and PI3K/AKT/mammalian target of rapamycin (mTOR) cascade, to stimulate a hyperproliferative response and to inhibit cell apoptosis [4,46,47]. *EGFR* mutations and amplification could be found in eCCA with a slightly lower prevalence as respect to iCCA (5–19% vs 11–27%, respectively) [48–50]. Conversely, mutations in human epidermal growth factor 2 (*HER2)/ERBB2* is a common signature in 11–20% of eCCA, being instead rarely found in iCCA [51]. In patients with eCCA, *ex-novo* expression of *c-ErbB-2* correlated with higher histological grade (III and IV), perineural invasion, and metastatization [52]. Other clusters of signals commonly found to be deregulated in eCCA (47%) are those supervised by TP53-RB. Mutations in these pathways affect cell survival and apoptosis, as well as the progression through the cell cycle. In eCCA, alterations in the TGF-β pathway are less frequent (18%) and are typically enriched in the Mesenchymal class. Perturbations of the histone acetylation pathways (22%) is a feature typical of tumors classified in the Metabolic and Proliferation classes. They result in the modification of chromatin remodeling processes inducing the aberrant activation of a number of transcription factors, including yes-associated protein (YAP) [53], the main effector of the Hippo pathway.

3. ACTIONABLE DRIVERS AND OUTCOME PREDICTORS IN PERTURBED PATHWAYS OF eCCA

According to OncoKB [54], actionable drivers have been found in about 50–60% of cases of iCCA, whereas in eCCA they are present in only 25% of tumors [39] or even less [55]. Besides providing druggable targets, these pathways might be relevant also as outcome predictors, as currently, only parameters obtained by analysis of surgical samples (perineural invasion, margin clearing, node involvement, and advanced TNM stage) are used. In this context, a number of phenotypic changes have been identified as tool to discriminate tumor aggressiveness and to allocate patients to the more appropriate curative approach (surgical

resection, liver transplant, chemotherapy, and targeted therapies). In a large meta-analysis of 54 cohorts, that included 4454 patients, Wiggers and coll. [56] evaluated the differential expression of 102 immunohistochemical biomarkers, among which 18 with different and 16 with comparable expression between iCCA and eCCA. Specifically, Akt2 [57], Annexin II [57], Annexin 10 [58], c-ErbB-2 [52], CD10, CDX2, K20, mucin (MUC)1, MUC5A5, p53 [59], and S100P were found to be significantly more expressed in eCCA. In particular, when overexpressed, Annexin 10 behaved as a promising indicator of worst prognosis in patients with pCCA and dCCA, but not iCCA, and its up-regulation resulted in an over-activation of the PLA2G4A/PGE2/COX2/STAT3 pathway, a potentially druggable signaling [58]. A panel of tissue biomarkers, encompassing MUC2, MUC5AC, MUC6, CD10, CDX2 and K20, has been proposed to stratify patients undergoing resection for pCCA and dCCA in two subgroups, where subgroup 1, characterized by a lower expression of MUC5AC and MUC6, showed an impaired survival rate in pCCA, but not in dCCA patients [60]. Recent studies have pinpointed the strong pathobiological significance of S100A4 when expressed at the nuclear level. S100A4 is a cytoskeleton-associated calcium-binding protein that regulates several physiological and pathological processes, such as cell proliferation, migration and invasion, and it is usually expressed in the cytoplasm of mesenchymal lineage-derived cells [61]. In a cohort of 86 patients with surgical resection, the aberrant nuclear translocation of S100A4 in malignant biliary cells was an independent predictor of worst OS and metastatization in either iCCA or eCCA, similarly to what reported for other desmoplastic tumors of epithelial origin (breast, colorectal) [61]. Furthermore, in a xenograft mouse model generated by intraportal injection of human eCCA cells (EGI-1), the low-dose metronomic (LDM) treatment with paclitaxel reduced the hematogenous metastatization to the lungs, by interfering with SUMOylation mechanisms that directs the nuclear shuttling of S100A4 [62,63]. Overexpression of mesothelin (MSLN), a cell surface protein commonly present in mesothelial cells which expression is increased in several malignant tumors, associated with poor OS and relapse-free survival in patients with eCCA [64]. Table 3 summarizes the phenotypic biomarkers expressed at the tissue level in eCCA compared with iCCA. Studies devoted to circulating biomarkers, where eCCAs were kept distinct from iCCAs, are limited. In a mixed cohort of 45 CCAs, including 32 pCCA/dCCA, a panel of miRNA contained in extracellular vesicles (EVs) was used to discriminate CCA from non-tumoral controls, represented by primary sclerosing cholangitis (PSC), biliary obstructions and biliary leaks [65]. A recent study profiled miRNA expression, using nanostring technology, in a highly selected and clean cohort of 12 dCCAs. In particular, miR-451a and miR-144–3p were downregulated and, *in vitro* experiments demonstrated their ability to suppress both migration and invasion of CCA cells, and these effects were mediated by targeting activating transcription factor 2 (ATF2) and A disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) [66]. Moreover, in a cohort of 32 eCCAs and 55 iCCAs, investigated by a combined approach of immunohistochemistry and high sensitive *in situ* hybridization (RNAscope), expression of IL-6 and IL-33 was increased in eCCA and also in large, but not in small ducts of iCCA. Among them, overexpression of IL-33 was a predictor of better OS in patients with eCCA [67]. Within the multifaceted tumor cell population, even selective subsets may bear prognostic information and provide targets for therapeutic intervention. An interesting *in-vitro* study conducted in a very small cohort of eCCA, showed that organoids derived from $CD47^+/CD147^+/EPCAM^+/CD45^-$ circulating

tumor cells (CTC) immunopurified from portal venous blood, were able to recruit fibroblasts and to proliferate, indicating that CTCs may behave as vehicle for distant tumor spreading and recurrence [68]. Another study showed that CTCs isolated from blood samples using CellSearch System predicted a 10fold-reduced survival in patients with pCCA/dCCA [69]. Serum metabolites abnormally generated by CCA cells are the rational of metabolomics-based approaches that have been used to distinguish iCCA from PSC and hepatocellular carcinoma (HCC) [70] and to differentiate eCCA from iCCA. Thus, in a large cohort of Asian CCAs, 34 serum metabolites showed altered levels compared with healthy subjects. Among them, four molecules (21-deoxycortisol, bilirubin, lysoPC(14:0), and lysoPC(15:0)) were found to be deregulated in eCCA vs iCCA, though their prognostic impact was not assessed [71].

4. PROSPECTS FOR THE CLINIC: OPPORTUNITIES FOR ACTIVE DRUGS IN eCCA

Molecular classifications provide unique opportunities to devise novel therapeutic strategies, as recently shown also for eCCA, where multiple targetable mutations are being identified [39]. However, as a rare tumor, designing target-specific single trials in eCCAs is extremely challenging, and therefore, oncologists may focus on umbrella and basket trials to avoid the risk of including target-negative patients and to increase the number of patient cohorts.

Currently, there are 256 ongoing clinical trials including patients with eCCA, aimed at 131 different compounds belonging to the molecular targeted class (Supplemental Table 1) [72]. These studies are mainly designed as basket trials and the most specific ones include not only eCCA, but also gallbladder or iCCA. Targeted DNA-sequencing and whole-genome expression-based studies have pinpointed *KRAS, TP53, ARID1A* and *SMAD4* as the most frequent genetic alterations in eCCA [39]. As aforementioned, in about a quarter of eCCAs, these mutations result in at least a putative actionable driver (*BRCA1–2*, *EGFR*, *ERBB2*, *CDK4*, *IDH1–2*, *BRAF*, *NRAS*, *PI3K*, *MDM2*). Nevertheless, the only currently approved (Level 1) targeted therapy for use in eCCA is the PD-1 monoclonal antibody pembrolizumab, which show an improved outcome in tissue-agnostic DNA MMR deficient tumors including CCA [73], whereas it showed only a 7–13% objective response rate in BTCs [74]. Compared with iCCA, actionable molecular aberrations, with proven clinical benefit in different tumor types, are expressed in a minority of eCCA, such as *BRCA1/2* (3%), *EGFR* (1%), *IDH2* mutations (3%), *ERBB2* overexpression (5%), *CDK4* amplifications (1%), *BRAF* mutant (2%) [39]. However, the deep molecular phenotyping may open up interesting perspective on new targets amenable of intervention, with a certain specificity for each class (Supplemental Table 2).

The Metabolic class can be linked with treatment strategies aimed at targeting bile acid metabolism with nuclear receptor modulators (the farnesoid X receptor (FXR) agonist obeticholic acid (OCA)), or with inhibitors of the enzyme sphingosine kinase-2 (SphK-2) (ABC-294640, Opaganib) and of the histone deacetylase-6 (HDAC-6) (KA-2507). It is worth mentioning that in bile duct ligated (BDL) rats, the intrahepatic accumulation of bile acids is not sufficient *per se* to induce the development of CCA, which is instead stimulated

by challenging BDL rats with thioacetamide. Neoplastic transformation is then sustained by biliary cell proliferation, portal inflammation and reduction in FXR expression [75]. Interestingly, ability to inhibit CCA progression has been demonstrated for FXR agonists, though limited to *in-vitro/in-vivo* experimental models [76,77] (NCT03377179, NCT04186156).

The Mesenchymal class, the most prevalent in the Montal's study, is unfortunately, that with the worst prognosis and nowadays, with the fewest treatment chances [39]. Possible current approaches are hyaluronidase (PEGPH20), TGFβ inhibitors (M-7824) and Hedgehog antagonists (cyclopamine, 5E1, BMS-833923, BI6727). However, the development of PEGPH20 (NCT03267940) was prematurely stopped after negative data obtained in metastatic pancreatic cancer [78]. The bifunctional protein M-7824 targeting TGFβ along with PD-L1 has been testing in a phase II trial as second-line monotherapy in advanced BTCs (NCT03833661), whereas antagonism of the Hedgehog pathway showed encouraging results, but only in *in-vivo/in-vitro* studies [79–81].

On the other hand, the Proliferation and Immune classes provide the most promising targets for current clinical practice. In the Proliferation class, a number of druggable pathways can be envisaged, by inhibiting ERBB2/HER2 (with the monoclonal antibodies, trastuzumab and pertuzumab, or with the pan-ERBB inhibitors, lapatinib and varlitinib), or its downstream effectors CDK (abenaciclib, alvocidib), CK (silmitasertib) and mTOR (everolimus), with the potential to interfere in an early phase of tumorigenesis [82]. Multiple ongoing phase II clinical trials are studying ERBB2/HER2 inhibitors. Particularly, a phase II umbrella-trial of trastuzumab/lapatinib/everolimus/nivolumab plus GEMOX (NCT02836847) is conducted in eCCA and gallbladder cancer, and a phase II-III trial testing varlitinib plus Gem-Cis (NCT02992340) or capecitabine (NCT03093870, NCT03231176) is being conducted in advanced BTC. Lapatinib, as monotherapy, did not meet clinical outcomes in two phase II trials in the subgroup with advanced CCA (NCT00101036, NCT0107536). By acting on different pathways, an ongoing phase II basket trial (NCT03339843) and a more specific in BTC (NCT04003896) are testing abemaciclib and silmitasertib plus Gem/Cis in CCA (NCT02128282). Targeting BRAF is also emerging as possible therapeutic strategy in CCA, including eCCA. A very recent phase 2 multicenter basket trial (ROAR) has evaluated the effect of the combined treatment with dabrafenib and trametinib in 43 $BRAF^{V600E}$-mutated BTCs. Results hold promise, with 51% of patients showing partial response, 17% stabilization of the disease, and no treatment-related deaths (NCT2034110) [83].

Finally, the Immune class may benefit from anti-PD-1/PD-L1 inhibitors, and indeed, promising data come from pembrolizumab and nivolumab. In a phase II trial (KEYNOTE-016), 86 patients with mismatch-repair (MMR)-deficient tumors were treated with pembrolizumab, with disease control in all the 4 CCA included [84]. In the KEYNOTE-028 basket trial, 24 patients with BTC expressing PD-L1 (1% of tumor cells by immunohistochemistry) underwent pembrolizumab, with 8 patients (34%) showing partial response or stable disease [85]. The KEYNOTE-158, a phase II basket trial of pembrolizumab, conducted in patients with advanced solid tumors including CCAs with disease progression on standard-of-care therapy, is currently ongoing (NCT02628067). Moreover, in a cohort of 46 patients with refractory BTC [86], nivolumab showed modest

efficacy with durable response in 2 out of 5 patients (40%) with eCCA (NCT02829918). Multiple phase II-III clinical trials are now testing the effect of immune checkpoint inhibitors added to chemotherapy in advanced BTCs (nivolumab (NCT03101566, NCT04172402), pembrolizumab (NCT03111732, NCT03260712, NCT04003636), toripalimab (NCT03982680, NCT04027764, NCT04191343, NCT04217954, NCT03796429), durvalumab (NCT03046862, NCT03875235, NCT04308174), KN-035 (NCT03478488)). Furthermore, similar clinical trials to evaluate combination of targeted therapies as second-line treatment in advanced BTCs (nivolumab (NCT02866383, NCT03250273, NCT03639935), pembrolizumab (NCT03797326, NCT03895970, 04550624), toripalimab (NCT04010071, NCT04211168)) are under way.

5. CONCLUSIONS

Dissecting the specific molecular alterations harbored by the tumor of a single patient is the pre-requisite to pursue a precision medicine-based approach [87]. This need is becoming even more relevant for those types of cancer where prognosis remains gloomy despite the growing impact of targeted therapies and immunotherapy. Compared with HCC and other more common tumors, CCA has been largely overlooked until recently, and among CCAs, eCCA has been yet neglected, at least if considering the recent interest garnered by iCCA. Moreover, the understanding of the intricate tumor biology of eCCA has remained scarce, essentially because most studies have focused on iCCA, for which identification of clinically relevant targetable findings are more frequently identified. In this context, the comprehensive multi-platform molecular characterization performed in a very recent study [39] has laid the basis to delve into the multifaceted phenotype of eCCA, to identify the distinctive oncogenic fingerprints and possibly, to unveil some areas for targeted interventions. However, this is only the dawn of a new era, where multiple aspects, only partially addressed by the ongoing clinical trials, must be urgently addressed. To develop molecular diagnostic testing, possibly ascertaining the role of liquid biopsy, to implement specific phase II/III clinical trials with enrichment designs to expedite the approval, to dissect the intrinsic/acquired chemoresistance mechanisms, to test the efficacy of combination therapies, are among the issues to put next on the agenda.

6. EXPERT OPINION

Compared with other types of cancer, CCAs seem to miss a predominant and specific driver molecular alteration able to support the clinical decision-making process, as *RAS/BRAF* for colorectal cancer (CRC) [88], or *EGFR* for lung, breast and gastric cancers [89–91]. However, eCCA is gaining interest and steps forward are being made in looking for targetable alterations able to be utilized for "precision medicine" approaches in this setting. It has become clear that among primary liver malignancies, eCCA is indeed a "troublesome client". Despite the progress made in the identification of an increasing number of genetic mutations and perturbed signal pathways, enabling a first molecular classification of eCCA (that we will be discussing below), there are several gray areas and challenges faced by the scientific and medical community that impinge on the current clinical management of this rare cancer. The conventional chemotherapeutic protocols are largely unsatisfactory in eCCA patients, in terms of both survival and quality of life. Of note, first-line treatments are

limited to the combined use of gemcitabine and cisplatin, while data regarding second-line treatments are not strong enough. This reflects the limited understanding of the molecular mechanisms that sustain tumorigenesis in eCCA. A small percentage of eCCA (<20%) derives from diseases known to be prodromal of CCA, while the vast majority develop on a healthy biliary tree. Moreover, according to OncoKB targets [54], actionable drivers can be detected in only 25% of cases of eCCA, less than half with respect to iCCA (50–60%), making the application of targeted therapies more difficult and less impactful in eCCA,. In fact, at the time of this writing, just one targeted therapy using pembrolizumab has been approved for the treatment of eCCA. Use of immunotherapy is currently limited to the context of MMR deficiency or MSI high tumours, which represent only around 2% of eCCA [39]. Another important observation is the low prevalence in eCCA of the potentially targetable *FGFR* aberrations (fusions/mutations/amplifications) and *IDH* mutant-enriched subtypes, (1% and 4.7%, respectively). These molecular traits are more common in iCCA (20% for *FGFR*, 15% for *IDH*) [92,93], further confirming that oncogenic drivers of eCCA and iCCA are different, and actually only iCCA are included in clinical trials assessing efficacy of FGFR and IDH inhibitors [94]. Among FGFR inhibitors, pemigatinib, an oral inhibitor of FGFR2, has recently received the approval of Food and Drug Administration (FDA), for the treatment of iCCA harboring *FGFR2* fusions or rearrangements [95–97]. Furthermore, a multicenter, phase III clinical trial (ClarIDHy) [NCT02989857] showed encouraging results in the treatment of iCCA characterized by *IDH-1* mutations, using ivosidenib, a small molecule inhibiting IDH-1 [98]. To evaluate the response to therapy, the identification of easily achievable and assessable circulating biomarkers, indicating the disease progression stage and predicting the patient prognosis, would also be of great value, and recent studies dealing with this goal have been less informative in eCCA than in iCCA [4]. Finally, a further issue limiting the study of eCCA is the lack of suitable animal models. Although different rodent models of CCA are in use, generated by toxic or fluke infestation, genetically engineered, and transplantation approaches [99], they recapitulate only in part the complex genetic landscape and the intricate interactions between the different cell types populating the tumor microenvironment (TME), as observed in human eCCA.

According to Montal [39], eCCA can be categorized into four distinctive molecular classes (Mesenchymal, Proliferation, Immune, and Metabolic) converging into four main signaling perturbations (RTK-RAS-PI3K, TGF-β, histone modification, and TP53-RB). Unfortunately, despite the high frequency of *KRAS* mutations in eCCA (36.7%), there are no approved drugs that target mutated KRAS proteins directly, and also targeting *KRAS* indirectly by blocking its downstream effectors has been an ineffective strategy in other cancer such as pancreatic ductal adenocarcinoma (PDAC), the prototypical cancer harboring *KRAS* activation [100,101].

Among the four eCCA molecular classes, the Mesenchymal type is by far, not only the most prevalent (47.3%), but also the most aggressive, with the poorest response to conventional chemotherapy, similar to what reported for other desmoplastic cancers, as PDAC and CRC. The Mesenchymal eCCA is characterized by an intense fibrotic reaction, enrichment in EMT features, and abundant recruitment of CAF, which prevail on tumor-associated macrophages (TAM), which anyway are rarely observed in all eCCA classes, at variance with what observed in the TME of iCCA. However, given the extent and the likely impact of

the TME on the marked aggressiveness of this class, we could imagine some alternative strategies. A first possibility is the use of BH3 mimetics (small compounds that antagonize anti-apoptotic BCL-2 family proteins, as navitoclax) based on their ability to induce selectively apoptosis in CAF, with concomitant effects on tumor growth and metastatization in CCA [102,103]. Second, hedgehog (Hh) antagonists (i.e. cyclopamine) might revert the EMT program and potentially reduce tumor growth, as shown in experimental models of CCA. [104]. Third, it has been shown that CAF recruitment is associated with tumor lymphangiogenesis and lymph node dissemination [103], and thus, use of angiogenesis inhibitors is worth pursuing in CAF-enriched Mesenchymal eCCA. Although angiogenesis inhibitors have been often unsuccessful, also in different oncology settings, this should be dependent on the fact that the route mainly responsible for CCA dissemination (lymphatic vascular system) has not been selectively targeted yet. Angiogenesis inhibition may be useful in combination therapy, as shown recently in HCC [105].

The Proliferation class, the second most prevalent in eCCAs (22.5%), harbors activation of two common proliferative pathways, the Ras/MAPK and AKT/mTOR, with chances of therapeutic interference at multiple levels. ERBB2/HER2 overexpression (mutations and amplifications) is a defining feature of this molecular class, and actually, is higher in eCCA (17.4%) compared with iCCA (4.8%) [106]. Unfortunately, the results obtained so far in CCA, with the pan-ERBB inhibitor lapatinib, a dual-targeted small molecule, which blocks RTK phosphorylation by binding to the cytoplasmic ATP-binding sites of EGFR/HER1 and HER2 receptors (NCT00101036, NCT0107536) are disappointing. However, in principle, eCCAs belonging to the proliferation class may benefit also from the use of conventional cytotoxic drugs, potentially also in combination therapies, to add DNA damaging to anti-proliferative effects.

Extrahepatic CCAs belonging to metabolic class (18.7%) displays a hepatocyte-like phenotype pathophysiologically underpinned by an aberrant bile acid metabolism. It provides an intriguing set of molecular alterations that can be translated into actionable targets. For instance, harnessing bile acid metabolism deregulation thorough nuclear receptor modulators (OCA among others) may represent in theory a strategy of translational interest, as suggested by first experimental data [76].

The stratification of patients in molecular classes as proposed by Montal's study [39] could lead, in the next few years, to the implementation of clinical trials already ongoing or to new eCCA-specific clinical studies where such stratifications are planned from the beginning. This may increase the use of targeted therapies, at least in the quarter of eCCAs with putative actionable targets. With regard to the majority of eCCA patients, without current evidence of driver mutations or druggable pathways, it is desirable that the costs of cutting-edge techniques relevant for target discovery, such as single cell analysis, may become more affordable in the near future to encourage more translational studies. In fact, besides evaluating the presence of genetic mutations, single cell analysis perfectly suits to dissect, within the huge heterogeneity of TME, the intimate relationships between the various cell types herein hosted. Furthermore, it is conceivable that a new generation of experimental models, such as the humanized murine models or the tumor spheroids and organoids, may favor a more comprehensive and thorough study of cell-cell and cell-matrix interactions in

eCCA. This approach is of paramount importance to unveil new therapeutic targets in the large subgroup of patients that remain allocated to palliative care because of the lack of actionable alterations. The crosstalk involving tumor, TME cells (CAF, TAM, endothelial cells, along with innate and adaptive immune cells) and the extracellular matrix, is indeed a niche area deserving more consideration, especially by proposals devoted to cancer types featuring a rich desmoplasia. In fact, studies performed in breast and pancreatic cancer [107,108] have shown that these interactions are essential for both tumor development and metastatisation, though the effects exerted by the TME can be ambivalent [109,110]. In eCCA, another knowledge gap in the field of TME relates to tumor chemoresistance. Similar to iCCA and HCC, eCCA shows a poor response to pharmacological treatments, but at present mechanisms of chemoresistance in eCCA are poorly eludicated [111]. Therefore, developing novel strategies to overcome eCCA chemoresistance, by increasing selectivity of drug delivery or by enhancing the amounts of active agents inside the tumor cells, is an urgent need. In this context, it will take more efforts to uncover the biology of cancer stem cells, given their fundamental role in promoting chemoresistance, as well as tumor recurrence and metastasis to distant organs [112].

As we start to unravel the complexity of CCA, it has become clear that eCCA must be regarded as a distinct entity, with unique features in terms of molecular classes, oncogenic drivers, and TME. These concepts must be borne in mind by future studies aimed at developing treatment algorithms for eCCA. It is expected that the treatment opportunities will be improved through close international collaborations, as the European Network Study for CCA (ENS-CCA) has been boosting during the last few years [113].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding

Abbreviations

ADAM10	A disintegrin and metalloproteinase domain-containing protein 10
ATF2	activating transcription factor 2
ATPB1B	ATPase, Na+ /K+ transporting, b polypeptide
ARID	AT-Rich Interaction Domain
BTC	biliary tract cancer
CAF	cancer-associated fibroblasts
CDX2	Caudal Type Homeobox 2
CCA	cholangiocarcinoma
CTC	circulating tumor cells
CRC	colorectal cancer
CT	computed tomography
CDK4	cyclin dependent kinase 4
CCNE1	cyclin E1
dCCA	distal CCA
DNAJB1	DnaJ heat shock protein family member B1
ELF3	E74 like ETS transcription factor 3
EGFR	epidermal growth factor receptor
EpCAM	epithelial cell adhesion molecule
EMT	epithelial-to-mesenchymal transition
ERBB2	erb-b2 receptor tyrosine kinase 2
EVs	extracellular vesicles
eCCA	extrahepatic CCA
FXR	farnesoid X receptor
OCA	agonist obeticholic acid
FGFR	Fibroblast Growth Factor Receptor

GNAS	Guanine Nucleotide binding protein, Alpha Stimulating activity polypeptide
HCC	hepatocellular carcinoma
HNF4A	hepatocyte nuclear factor 4 alpha
HDAC6	histone deacetylase 6
IL	interleukin
iCCA	intrahepatic CCA
IDH	Isocitrate Dehydrogenase (NADP(+))
KRAS	Kirsten rat sarcoma
MDM2	E3 Ubiquitin-Protein Ligase Mdm2
MMR	mismatch-repair
mTOR	mammalian target of rapamycin
MRI	magnetic resonance imaging
MSLN	mesothelin
MUC	mucin
NGS	next generation sequencing
OS	overall survival
PDAC	pancreatic ductal adenocarcinoma
PI3K	phosphatidylinositol 3-kinases
pCCA	perihilar CCA
PSC	primary sclerosing cholangitis, PSC
PRKAC	Protein Kinase CAMP-Activated Catalytic Subunit
RTK	tyrosine kinase receptors, RTK
S100	S100 Calcium Binding Protein
SMAD4	small mother against decapentaplegic homolog 4
STK11	Serine/threonine kinase 11
SphK-2	sphingosine kinase-2
TGF-β	transforming growth factor-β
TAM	tumor-associated macrophages

TME	tumor microenvironment
YAP	yes-associated protein
YETS4	YEATS domain-containing 4

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Article Highlights

- Extrahepatic cholangiocarcinoma (eCCA) is a biliary epithelial malignancy defined by well-established anatomical criteria with unique phenotypic traits.

- eCCA is characterized by marked aggressiveness and poor prognosis, with scarce therapeutic options.

- Within the wide eCCA heterogeneity, four molecular classes, "Mesenchymal", "Proliferation", "Immune", and "Metabolic", have been recently identified by whole-genome analysis-based studies.

- Molecular alterations converge into stimulation of four main pro-oncogenic signalling, RTK-RAS-PI3K, TGF-β, histone modification, and TP53-RB, collectively resulting in the presence of actionable drivers in about 25% of eCCA.

- Compared with the intrahepatic variant, *FGFR* and *IDH* mutant-enriched subtypes are less common in eCCA.

- Phenotypic features associated with immunotherapy response are present in only 2% of eCCA.

- Among 256 clinical trials ongoing, the only one actually approved for eCCA treatment, involves the use of pembrolizumab, a PD-1 monoclonal antibody.
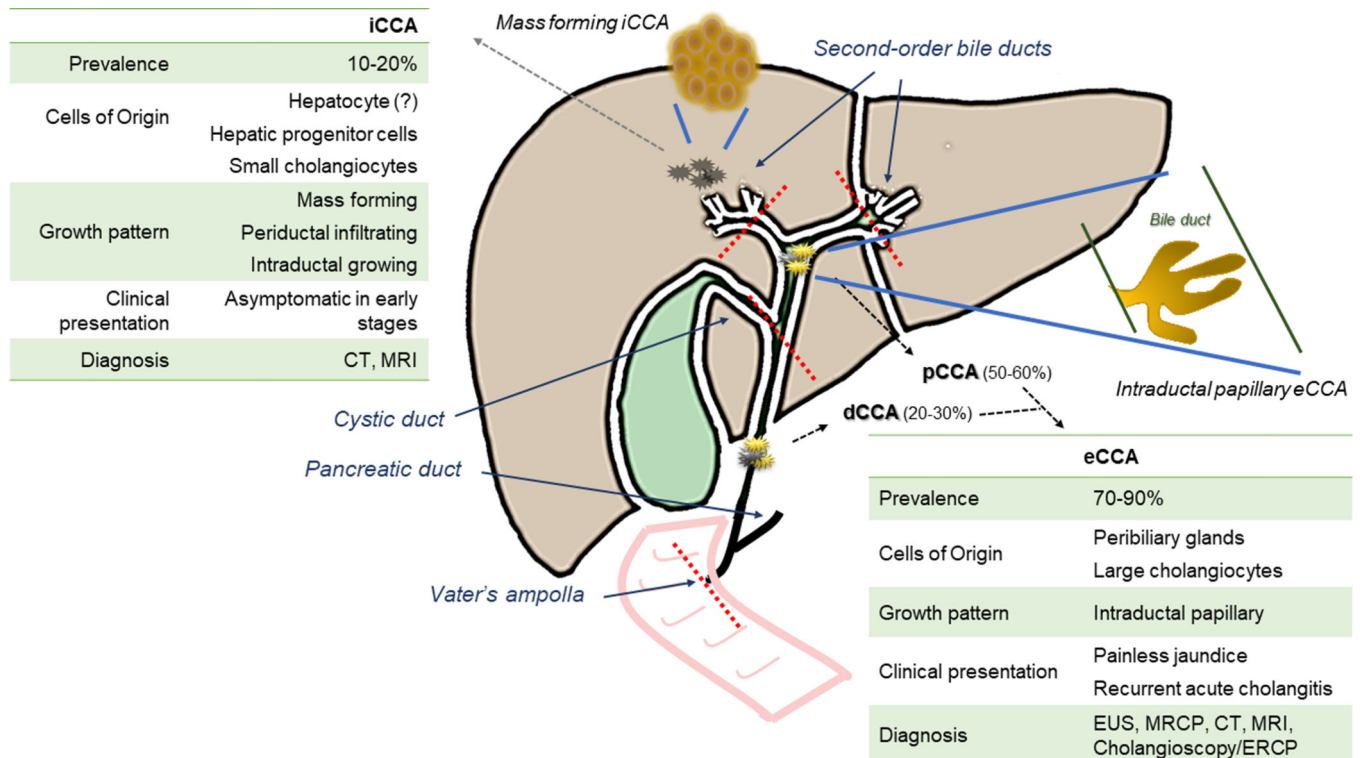
	iCCA
Prevalence	10-20%
Cells of Origin	Hepatocyte (?)
	Hepatic progenitor cells
	Small cholangiocytes
Growth pattern	Mass forming
	Periductal infiltrating
	Intraductal growing
Clinical presentation	Asymptomatic in early stages
Diagnosis	CT, MRI

	eCCA
Prevalence	70-90%
Cells of Origin	Peribiliary glands
	Large cholangiocytes
Growth pattern	Intraductal papillary
Clinical presentation	Painless jaundice
	Recurrent acute cholangitis
Diagnosis	EUS, MRCP, CT, MRI, Cholangioscopy/ERCP

Figure 1. Criteria classification and main clinical features of eCCA compared with iCCA.
Cholangiocarcinomas (CCAs) are classified as intrahepatic (iCCA), and extrahepatic
(eCCA), which includes the perihilar (pCCA) and distal (dCCA) variants. The central
diagram illustrates the lines of demarcation (red dotted lines) along with the pattern of
growth of each subtypes, mass forming, periductal infiltrating, intraductal growing (iCCA)
and intraductal papillary (eCCA). Mass forming and intraductal papillary patterns are shown
in enlarged cartoons (connected with the biliary system by blue lines). The boxes right- and
left-sided highlight the main clinical differences between eCCA and iCCA, with respect to
prevalence, cells of origin, pattern of growth, presentation and diagnosis. Of note, painless
jaundice and recurrent acute cholangitis caused by biliary obstruction may favour a
diagnosis earlier in eCCA than in iCCA. Moreover, diagnosis of eCCA harnesses imaging
techniques, as endoscopy ultrasonography (EUS) and magnetic resonance
cholangiopancreatography (MRCP), which allow a better visualization of the extrahepatic
bile ducts, though computed tomography (CT) and magnetic resonance imaging (MRI) show
the highest accuracy for both pCCA and iCCA, especially in the pre-operative setting. In the
diagnostic work-up of eCCA, beside endoscopic retrograde cholangiopancreatography
(ERCP), cholangioscopy is now a fundamental procedure, particularly in pCCA, as it can
increase sensitivity up to 80–90% by providing visual inspection and direct sampling of
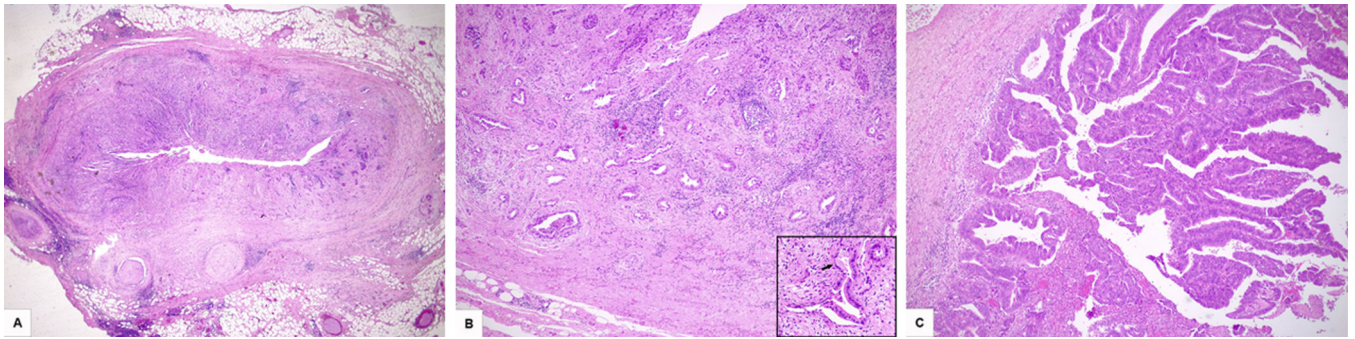tumoral lesions.

Figure 2. Histological features of eCCA.
A) Distal moderately differentiated cholangiocarcinoma arisen in the choledochus. The tumor extends into periductal soft tissues and shows diffuse peri-neural invasion (HE; original magnification 2x). B) Perihilar well differentiated cholangiocarcinoma (HE; original magnification 5x). The magnification in the lower right corner shows neoplastic glands lined by tall mucin-secreting cells (arrow) and immersed in a desmoplastic stroma (HE; original magnification 20x). C) Perihilar cholangiocarcinoma, papillary type (HE; original magnification 5x).

Table 1.

Risk factors for eCCA categorized according to odds ratio (OR).

Risk factor	Disease
OR > 10	Choledochal cyst [114]
	Choledocholithiasis [114]
	Primary sclerosing cholangitis [115]
	Caroli disease [115]
3 < OR < 10	Chronic pancreatitis [115]
	Cholelithiasis [114]
	Liver fluke infection (*Opisthorchis viverrini, Clonorchis sinensis*) [116]
	Cirrhosis [114]
OR < 3	Nonalcoholic fatty liver disease [117]
	Cholecystolithiasis [114]
	Alcohol-related disorders [114]
	Autoimmune hepatitis [114]
	Inflammatory bowel disease [114]
	HBV [114]
	HCV [114]
	Smoking [114]
	Type 2 diabetes [118]
	Hypertension [114]

Table 2.

Differences in genetic mutations in eCCA with respect to other biliary tract cancers (BTCs).

Genetic mutations		
eCCA > BTCs	eCCA = BTCs	eCCA < BTCs
ARID1B	*ARID1A*	*ARID2*
ELF3	*BRAF*	*BAP1*
PRKACA fusions (*ATPB1B-PRKACA; PRKACA-DNAJB1*)	*BRCA1*	*CDK4* amplifications
PRKACB fusions (*ATPB1B-PRKACB; PRKACB-C7orf50*)	*BRCA2*	*EGFR*
	GNAS	*EPHA2*
	KRAS	*ERBB3*
	MDM2	*FGFR2* fusion
	PIK3CA	*IDH1/2*
	SMAD4	*MLL2*
	TP53	*MLL3*
		PTEN
		TERT

Table 3.

Differential expression of immunohistochemical biomarkers in eCCA with respect to iCCA.

eCCA > iCCA	eCCA = iCCA	eCCA < iCCA
Akt2	Aurora-A	Aurora-B
Annexin II	β-catenin	bcl-2
c-erbB-2	Bax	EGFR
CD10	CK7	Filamin A
CDX2	CK8	Galectin-1
CK20	CK18	p16
MUC1	CK19	p27
MUC5AC	Cyclin D1	SMAD-4
p53	COX-2	VEGF-A
S100P	D10	
	E-cadherin	
	Fas	
	hENT1	
	Mdm-2	
	Metallothionein	
	MUC6	
	p21	
	S100A4	
	TNF-α	
	VEGF	
	Vimentin	