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Signaling and molecular networks related to development and inflammation involved in CCA initiation and progression

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Abstract

Intrahepatic cholangiocarcinoma (iCCA) is a rare neoplasm of the bile ducts with a low survival rate, whose incidence is continuously increasing and is associated with a rich and varied tumor microenvironment (TME). Although the main mutations characterizing iCCA are known, there are several unresolved issues regarding the processes leading to the accumulation of mutations in the normal cholangiocyte. The inflammatory mediators and the molecular pathways involved in cholangiocarcinogenesis, which regulate the transition from normal to dysplastic cells, resulting in neoplastic cholangiocytes, are poorly understood. Moreover, once the tumor is established, it is unclear which effects of the interaction between the tumor and TME constituent cells, in particular cancer-associated fibroblasts (CAFs), are responsible for stimulating the malignant behavior of iCCA. In this review, we described the main mutations affecting the bile ducts leading to iCCA development as well as the putative inflammatory mediators and morphogenetic pathways involved in the establishment of the malignant transition of the bile ducts. We also described the main signaling pathways involved in TME-tumor cell interactions, with particular emphasis on the effect of CAFs in cancer. Finally, we wanted to analyze possible new therapeutic approaches aimed at modifying the composition of TME and the possible role of immunotherapy in improving the treatment of this cancer.

Keywords: Tumor microenvironment, cancer-associated fibroblasts, tumor-associated macrophages, YAP, Notch, immunotherapy, immune checkpoints

INTRODUCTION

Cholangiocarcinoma (CCA) is a tumor that arises from the neoplastic transformation of cholangiocytes, i.e., of the biliary epithelial cells, of each portion of the biliary tree, including the smaller intrahepatic to the larger collecting bile ducts and the extrahepatic structures[1]. CCA is the most pervasive malignant pathology of the biliary network, representing 3% of all primary tumors of the gastrointestinal tract and, in frequency, is preceded only by hepatocellular carcinoma (HCC) among primary malignant hepatic tumors[2]. Although considered a rare cancer, CCA is responsible for more than 10% of liver malignancy-related deaths. Globally, the average age of presentation of CCA is over 65 years. Only rarely does diagnosis occur in subjects under the age of 40, except for patients with primary sclerosing cholangitis (PSC) and fibropolycystic disease[3]. From an anatomical point of view, CCA can be classified into intrahepatic (iCCA, 10%-20% of cases), and extrahepatic CCA (eCCA), further subclassified as perihilar, also called "Klatskin tumors" (pCCA, about 50% of cases) or distal (dCCA, about 30% of the cases) CCAs[4]. These tumor subtypes differ from each other not only in terms of anatomical location, but also from an epidemiological, etiopathogenetic and therapeutic point of view[5]. Microscopically, CCA is characterized by the presence of the tumor reactive stroma (TRS), composed of an abundant stromal deposition[6] populated by different cell populations. The most frequent are cancer-associated fibroblasts (CAF), which promote tumor progression as well as fibrosis deposition, tumor-associated macrophages (TAM), and blood and lymphatic vessels which provide nutrients to and enhance the metastasis of the neoplastic cells[7].

Notably, the incidence and mortality of CCA, and in particular of iCCA, has been shown to steadily increase in the last three decades[5], but there are geographical variations in incidence, with areas at higher risk, such as South-East Asia and areas with lower incidence, such as Australia. This is a direct consequence of the lack of homogeneity of both the environmental risk factors and the genetic differences between the different populations[8]. The survival rate from the time of diagnosis of CCA is very low, less than 24 months on average, and the 5-year survival is less than 5%. This demonstrates the lack of knowledge of the mechanisms driving the development of this neoplasm, and the lack of effective therapies for its treatment. These unsatisfactory outcomes are due to the marked aggressiveness and tendency of CCA to metastasize early via the neighboring lymph nodes. Currently, the only curative therapeutic options are limited to surgical resection and, in selected cases and in specialized centers, by liver transplantation, but this treatment can be offered only to a minority of the patients (< 30% of cases), while those who are not candidates for surgery undergo palliation[5].

As previously mentioned, the molecular mechanisms and mutations in CCA tumors are poorly understood. The development of CCA is fueled by alterations in oncogenes and signaling pathways involved in the regulation of the inflammatory response, as well as genetic, epigenetic, and chromosomal aberrations. In-depth studies regarding these mechanisms have been carried out mainly for iCCA[9-11], while for eCCA, the data are still nebulous and are supported by only a few studies[12]. In this regard, we will focus our attention on the pathogenesis and carcinogenesis of iCCA.

CLINICAL PRESENTATION AND DIAGNOSIS OF ICCA

iCCA usually presents with weight loss and abdominal pain and less frequently with jaundice[13]. Laboratory workup usually reveals raised alkaline phosphatase, while total bilirubin is often normal or only mildly elevated. The main differential diagnoses of malignant intrahepatic masses in patients with and without

cirrhosis are hepatocellular carcinoma and metastatic lesions, respectively. In the absence of cirrhosis and extrahepatic solid malignancy, intrahepatic cholangiocarcinoma is the most common etiology of malignant intrahepatic mass lesions[4]. It is important to note that some intrahepatic tumors may demonstrate features of both iCCA and HCC[14]. In cross-sectional imaging, iCCA appears as a hypodense lesion that can be either well defined or infiltrative with associated ductal dilatation. In contrast to hepatocellular carcinoma, intravenous contrast injection usually reveals progressive contrast uptake in the arterial, venous, and delayed venous phases[15]. Less commonly, lesions smaller than 2 cm in diameter might exhibit arterial enhancement and venous washout, mimicking HCC.

Serum-based biomarkers have a role in differentiating HCC from CCA. While none are entirely specific, a combination of alpha-fetoprotein (AFP), AFP-L3, Carbohydrate antigen 19-9 (CA19-9) and cancer embryonic antigen (CEA) can often influence diagnostic decisions. The sensitivity and specificity of an elevated CA19-9 alone are 62% and 63%, respectively[16], while the combination of CA19-9 and CEA have shown a sensitivity and specificity of 90.2% and 88.24%, respectively[17]. In the absence of classic imaging features of cholangiocarcinoma or HCC, percutaneous biopsy may be needed for confirmation of the diagnosis. The significance of biopsies or, to an increasing degree, characterization of the mutational burden from circulating tumor DNA is increasingly relevant not only in the context of diagnosis but also for therapeutic purposes.

Molecular alterations of iCCA

CCA is characterized by the presence of wide genetic variability, which is only partially able to discriminate between the different forms of CCA, even if some mutations are more typical, as we will see, of some forms of this neoplasia. Furthermore, recent studies have raised the possibility to sub-stratify patients with iCCA, mixed hepatocellular cholangiocarcinoma (HCC-CCA) and eCCA into subgroups characterized by the stimulation of specific signaling pathways or by differences in the cellular composition, such as CAFs, cells of the immune response or of the tumor microenvironment (TME). Fundamental work by Sia *et al.*[9] analyzed a cohort of 153 iCCAs and, as subsequently confirmed by other studies[18,19], demonstrated in iCCA the presence of a large number of genetic mutations and the deregulation of several signal pathways potentially amenable to therapeutic intervention. These peculiar molecular signatures allowed the authors to discriminate between two subclasses of iCCA, proliferation class and inflammation class. These two classes differ in terms of patient outcome, with a worse outcome and a higher recurrence rate for the proliferation class. The proliferation class is poorly differentiated and characterized by neural invasion, in contrast to the inflammation class that is well differentiated and associated with specific transcriptional features. The proliferating iCCA presents mutations affecting various driver genes such as *Kirsten rat sarcoma virus (KRAS), epidermal growth factor receptor (EGFR), Isocitrate dehydrogenase (IDH) 1/2, ephrin type-A receptor 2 (EPHA2), BRCA1 Associated Protein 1 (BAP1)* and *Fibroblast growth factor receptor (FGFR)2*-fusions, while the inflammatory class is characterized by the expression of different cytokines including interleukin (IL)-3,-4,-6,-10 and -17A and C-C Motif Chemokine Ligand (CCL) 2 and 19[9,20,21]. The in-depth analysis of 38 patients, most of whom had iCCA (n = 32), allowed Farshidfar *et al.*[22] to identify an enriched IDH-mutant class with very homogeneous biological and phenotypic characteristics. This subpopulation is characterized by high expression of mitochondrial genes and by hypermethylation which likely silences the promoter of the AT-Rich Interaction Domain 1A (*ARID1A*) gene. This feature is probably responsible for the low chromatin modifier signature of this class of iCCA. The clinical readout of this observation is that it would allow for the stratification of patients based on IDH mutations, leading to potential pharmacological treatment of patients using IDH1-mutant-specific inhibitors[22].

Finally, a recent and original work has proposed a new classification of the iCCAs based on the composition of the TME, dividing this neoplasm into five TME-related subclasses, each with extremely peculiar biological characteristics[23]. Similar approaches have also been attempted for the subclassification of iCCA resulting from fluke infestation, in particular by *Opisthorchis viverrini*. Integrative analysis allowed the subdivision of these CCAs into 4 clusters, characterized by 1: an enrichment of *Erb-B2 Receptor Tyrosine Kinase 2* (*ERBB2)* amplifications in cluster 1; 2: of *TP53* mutations in cluster 2, the one with the worst prognosis; a high percentage of copy-number alterations and increased expression of Programmed cell death protein 1 (PD-1); 3: PD-L2 and B- and T-lymphocyte attenuator (BTLA) characterize the cluster 3; 4: an increased presence of mutations of *IDH1/2, BAP1* and *FGFRs* typical of cluster 4, the least aggressive phenotype[24].

A particular type of iCCA is HCC-CCA, characterized by the co-presence of both neoplasias, characterized by great heterogeneity and may derive both from the biliary epithelia, from the activation of tumor stem cells and from the dedifferentiation of mature hepatocytes[25]. This CCA subfamily carries different aberrations *IDH1, TP53, BRAF* mutations, *FGFR2-protein bicaudal C homolog 1 (BICC1)* fusions, and *Telomerase Reverse Transcriptase (TERT)* promoter modifications[25].

Targeted therapies

The therapeutic implications of an accurate diagnosis and the identification of targetable alterations are increasingly relevant in iCCA. In Table 1, we summarize the current targetable mutations and clinical trials, or approved therapies based on these alterations.

MECHANISMS OF NEOPLASTIC TRANSFORMATION IN ICCA

The actual knowledge regarding the neoplastic transformation of CCA, as well as CCA proliferation and metastatic potential, is closely linked to the alteration of signaling pathways related to the mutations mentioned in the previous section. iCCA generally develops in healthy livers, and there are few studies investigating which mutations are responsible for the initiation of the neoplasm and the causative mechanisms of these aberrations. These observations were usually performed studying pathologies prodromal to the development of CCA, such as PSC or fibro-polycystic liver diseases Autosomal Recessive Polycystic Kidney disease (ARPKD), Congenital Hepatic Fibrosis (CHF) or Caroli's Syndrome (CD)[26-28].

Inflammatory mediators in cholangiocarcinogenesis

The neoplastic transformation of normal bile ducts to iCCA involves, in addition to predisposing genetic mutations as described above, the concurrent deregulation of morphogenetic pathways and the accumulation of an inflammatory infiltrate resulting in the hypersecretion of local inflammatory mediators. These factors can induce the accumulation of genomic damage leading to the formation of dysplastic structures and, finally, overt tumors. One of the main inducers of carcinogenesis is the onset of chronic tissue inflammation, as typically occurs in chronic liver disease (CLD), which prompts the activation of proteins involved in the secretion of cyto- and chemokines. In particular, the persistent inflammatory state of CLDs induces the overexpression of numerous cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)γ, and IL-6 by cholangiocytes, resident inflammatory cells and fibroblasts. These cytokines are all able to stimulate the production of inducible nitric oxide synthase (iNOS). iNos is an enzyme that acts in a pro-tumorigenic manner[29], exerting its function both by the overproduction of nitric oxide (NO) in cholangiocytes and by stimulating mechanisms of cell proliferation and escape from cell death. NO, at low concentrations, has an antimicrobial effect, but at high concentrations is capable of inhibiting apoptosis, inducing DNA damage and inhibiting DNA repair. It can also stimulate the production of a large number of cyto/chemokines, such as CCL1, CCL2, CCL3, chemokine (C-X-C motif) ligand (CXCL)1, CXCL10, IL-6,

Table 1. Mutations with current treatment implications in CCA

Mutated gene	Percentage in iCCA	Drug name (if available)	Clinical trial code (phase)	Ref.
BAP1	13%-21%	Niraparib	NCT03207347 (II)	[21,164]
FGFRs fusion, mutations, and amplifications	11%-45%	Infigratinib (BGJ398)	NCT04233567 (II) NCT03773302 (III) NCT02150967 (II)	[20,23,165]
		Derazatinib (ARQ 087)	NCT03230318 (II) NCT04087876 NCT05174650 (II) NCT01752920 (I/II)	
		HMPL-453	NCT04353375 (II)	
		RLY-4008	NCT04526106 (I/II)	
		KIN-3248	NCT05242822 (I)	
		Bemarituzumab	NCT05325866 (I)	
		Futibatinib (TAS-120)	NCT02052778 (I) NCT04093362 (II) NCT04507503 (I)	
IDH1	10%-20%	Ivosidenib (AG-120)	NCT02073994 (I) NCT02989857 (III) NCT04088188 (I) NCT02073994 (I)	[166]
		IDH305	NCT02381886 (I)	
IDH2	Sporadic	Enasidenib (AG-221)	NCT02273739 (I/II)	[166]
KRAS	7%-25%	Selumetinib Trametinib	NCT02151084 (II) NCT04566133 (II)	[5,20,167]

BAP-1: BRCA1-Associated Protein 1; FGFR: fibroblast growth factor receptor; IDH: isocitrate dehydrogenase 1; KRAS: Kirsten rat sarcoma virus.

IL-8, IFNβ, transforming growth factor (TGF)-β, and TNF-α[7,30]. Those mediators, in turn, stimulate cell proliferation of neoplastic cells and fibroblasts, enhance fibrosis deposition due to myofibroblast activation and mediate the accumulation of infiltrating inflammatory cells. Intriguingly, the inflammatory microenvironment of PSC[31] and CLD[32] is characterized by the accumulation of macrophages which are the main cell type responsible for the generation and local diffusion of reactive oxygen and nitrogen species, ROS and RNS, respectively[33]. These families of highly reactive tumorigenic compounds cause locally high concentrations of NO and peroxynitrite, responsible for the synthesis of two potentially mutagenic compounds, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), and 8-Nitro guanine[33]. A study by Thanan *et al.*[34] demonstrated that 8-oxodG concentration, an indirect index of DNA damage, correlates with the expression of stem markers such as octamer-binding transcription factor 4 (Oct4) and CD133. Recent work has also confirmed that the rising expression of stem markers OCT4A, sal-like protein 4 (SALL4) and SRY-box transcription factor 9 (SOX9) characterize the transition from PSC to dysplastic foci of cholangiocytes and to iCCA, indicating that their detection may be a useful biomarker able to predict the neoplastic evolution of preneoplastic diseases[35]. Notably, the accumulation of neoplastic cells with stem-like traits is responsible for the overt chemoresistance and for the tendency of iCCA[36] to metastasize. Furthermore, high concentrations of NO are able to directly induce damage to double-stranded DNA[37]. It is important to remember that NO and peroxynitrite not only induce the formation of mutagenic products but are able to inhibit the action of important proofreading enzymes involved in maintaining the genetic stability of cells, such as 8-oxo-deoxiguanine DNA glycosylase 1 (hOGG1)[38]. The overexpression of iNOS is also responsible for the activation of cyclooxygenase (COX)-2, which, by generating high concentrations of prostaglandins (PGs), in particular of PGE2, induces the Src-mediated activation of the phosphatidylinositol-3 kinase (PI3K)/AKT pathway which in turn stimulates both cell proliferation and escape from apoptosis[39]. Confirming the importance of the accumulation of mutations as a driver of carcinogenic transition, recent evidence demonstrated that von Meyemnburg complexes (VMCs), histological structures derived from the dedifferentiation of the biliary epithelia typical of fibropolycystic liver diseases, can progress from a benign lesion to a neoplasm. This hypothesis was supported initially only by histopathological findings[40,41], then it was shown that genetic abnormalities, such as loss of heterozygosity (LOH) in loci presenting key

oncogenes (including p53, p16 CDKN2A, and others), are conserved through the different stages of dysplasia characterizing the evolution of VMCs[42].

Morphogenetic signal pathways involved in iCCA initiation

As previously mentioned, the neoplastic transformation of cholangiocarcinoma is also induced by the deregulation of some fundamental morphogenetic pathways, such as Hippo and NOTCH [Figure 1].

Hippo signal pathway

The Hippo pathway is an evolutionarily preserved signaling pathway and is critical for both normal physiological responses, such as cell proliferation, stem cell stabilization, organ size[43], cell fate determination[44], and angiogenesis[45], but it is also involved in various pathological responses. The pathway drives the pathogenesis of chronic diseases such as inflammatory bowel disease[46] and neurodegenerative diseases[47] and in various tumors (breast, colon, and lung)[48,49]. The main effectors of this signaling pathway are the transcriptional coactivators Yes-associated protein (YAP) and Transcriptional coactivator with PDZ-binding motif (TAZ), which, once dephosphorylated, shuttle into the nucleus to perform their specific functions[48]. The nuclear translocation of YAP[50] and TAZ[51] in iCCA has been reported as an important modulator of the aggressiveness of this tumor because it stimulates neoplastic cell proliferation/cell viability, but it is also associated with chromosomal instability[52]. Indeed, there is mounting evidence that YAP expression is a key step in the neoplastic transformation leading to the formation of iCCA. In fact, diseased cholangiocytes of PSC, as well as VMCs of Caroli's disease, both diseases known to be prodromal to iCCA development, exhibit an increased intranuclear expression of YAP[52,53]. Interestingly, rodent models of fibro-polycystic liver diseases (PCK rats and PKHD1$^{del4/del4}$ mice)[54,55] show, over time, an increase of the nuclear entry of YAP and TAZ demonstrating its importance in sustaining their pathogenesis. Another typical common trait of both CD and iCCA is the overexpression of β-catenin[32,56]. Notably, YAP and β-catenin are part of the same destruction complex[57], whose destruction allows not only the nuclear shuttling of YAP, but also that of β-catenin, which can exert its profibrogenic and proinflammatory function. Furthermore, in both CD and iCCA, β-catenin is aberrantly phosphorylated at the ser675 residue. This phosphorylation stabilizes β-catenin preventing its ubiquitination[56,58].

The importance of YAP and TAZ in inducing cholangiocarcinogenesis starting from a healthy liver has also been demonstrated in *in vivo* models of hydrodynamic tail vein injection (HTVI) of plasmids containing the active form of YAP accompanied by other oncogenes or morphogens. For the induction of iCCA, HTVI required the injection of plasmids containing AKT and the Notch Intracellular domain (NICD), the active form of Notch[59], but more recently, it has been demonstrated that the presence of the active form of YAP is able to give a boost to the formation of tumors induced by Notch2[60]. From these observations, there has been a plethora of experiments aimed at investigating the role of these morphogens in the iCCA context. In recent years it has been discovered that the oncogenicity of YAP in inducing iCCA has negative modulators, such as F-box and WD repeat domain containing 7 (FBXW7)[61], the G9a-derived characterize histone H3 lysine 9 (H3K9me2)[62] or positive, such as β-catenin[63] or DNA methyltransferase 1 (DNMT1)[64]. The data regarding TAZ are less numerous, but still demonstrate that the overexpression of its active form is directly involved in the formation of iCCA[51]. Notably, HTVI experiments generate iCCA by neoplastic reprogramming of hepatocytes but not cholangiocytes. These data are important because they can explain the mechanisms of neoplastic transformation present in mixed HCC-CCA tumors, a rare but particularly aggressive form of iCCA[5]. Moreover, these experiments demonstrate that YAP is necessary but is not sufficient by itself to induce neoplastic transformation, but that it requires one or more co-actors, among which the activation of the Notch pathway appears particularly important.

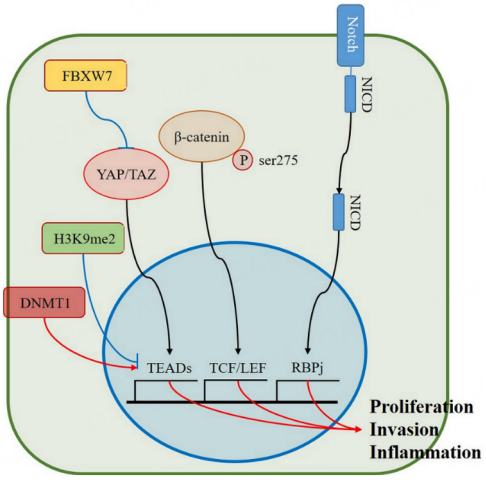

Figure 1. Molecular modulatory signaling involved in iCCA initiation. The figure represents the main known morphogenetic mechanisms involved in cell transdifferentiation towards iCCA. The interaction between YAP/TAZ, β-catenin and NOTCH signaling pathways stimulate the activation of malignant mechanisms in hepatic epithelial cells. These signaling pathways can be modulated positively by DNMT1 expression or negatively by FBXW7 and H3K9me2. The transcription factors, once transported into the nucleus, can bind to the specific binding sites TEADs, TCF/LEF and RBPj, for YAP/TAZ, β-catenin and NICD, respectively, activating the transcription of downstream genes involved in tumor proliferation and invasiveness and fibroinflammation. blue lines: inhibition; DNMT1: DNA methyltransferase 1; FBXW7: F-box and WD repeat domain containing 7; H3K9me2: G9a-derived dimethylated histone H3 lysine 9; NICD: Notch Intracellular domain; P: phosphorylation; Red arrow: activation; RBPj: recombination signal binding protein for immunoglobulin kappa J region; YAP: yes-associated protein; TAZ: transcriptional coactivator with PDZ-binding motif; TEAD: transcriptional enhanced associate domain; TCF/LEF: T cell factor/lymphoid enhancer factor family; YAP: yes-associated protein.

The NOTCH signaling

Notch is a key regulator of hepatic embryogenesis[65], in modulating the pathogenesis of different CLDs (Alagille Syndrome, liver-related cystic fibrosis disease or steatohepatitis)[66-68], and in the pathogenesis of iCCA[69], but its role in healthy liver-neoplasm trans-differentiation is not yet clear. In addition to the evidence previously discussed and obtained through HTVI experiments, little more is known about the possible induction effect of iCCA at the level of preneoplastic lesions. Few available works demonstrate that the reprogramming of hepatocytes to iCCA precursors can occur either through NICD-mediated activation of the proto-oncogene cyclin E[70] or through the activation of phosphatase and tensin homolog (PTEN)/p53 pathway[71].

ICCA-ASSOCIATED TUMOR MICROENVIRONMENT

The increasing knowledge of the molecular landscapes of iCCA highlights how this cancer may arise through different extrinsic and intrinsic processes. The TME can be described as a dynamic scaffold in which the surrounding tumor develops. TME is characterized by a multitude of cells, including tumor cells, infiltrating cells, secretory factors, proinflammatory cytokines, and growth factors embedded by the extracellular matrix (ECM) and surrounded by proteins such as collagen, laminin, fibronectin, and blood vessels. Notably, the presence of a variety of many nonimmune and immune cell types, such as CAFs, TAMs, tumor-associated neutrophils (TANs), regulatory T lymphocytes (Tregs), and natural killer cells (NK) within TME are considered negative prognostic factors for iCCA[7,72]. The complex interplay orchestrated by all these components determines the oncogenic role and development of iCCA through increased expression or aberrant activation of intracellular signaling pathways promoting iCCA occurrence via cell proliferation, survival, and genetic and epigenetic alterations. Interestingly, evidence from *in vivo* rodent models has shown that TME is able to stimulate not only the malignant features of the tumor but also to direct cell differentiation towards a specific tumor type, HCC or iCCA. A necroptotic

microenvironment is able to stimulate the generation of iCCA rather than HCC due to the epigenetic regulation of *T-box 3* (*Tbx3*) and *PR/SET Domain 5* (*Prdm5*)[73]. However, further studies are needed to better define which are the specific mediators involved in this mechanism. In a recent report[74], four TME-based subtypes were identified, associated with different patient outcomes. These subtypes were related to mechanisms inducing immune dysfunction, the most prominent of which was the so-called "immune desert phenotype" displaying few immune cells inside the tumor lesion. In other words, confirmation of why iCCA and CCA, in general, are considered an immune cold tumor. By contrast, in the "immunogenic subtype", a high number of infiltrating innate and adaptive immune cells were detected, comprising immune checkpoint molecules, such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and PD-ligand 1 (PD-L1) characterizing a better survival rate. The identification of a "myeloid subtype" with a M2-macrophages signature was also reported and a "mesenchymal subtype" characterized by the worst prognosis due to the presence of an activated pro-tumorigenic CAF population.

Cancer-associated fibroblasts in iCCA

CAFs are a heterogeneous group consisting of fibroblasts and myofibroblast-like cells that differ in origin, phenotype, morphology, and function. It is now well established that their presence has important implications for tumor growth and survival[75,76]. CAFs probably derive from hepatic stellate cells, periductal or portal fibroblasts, mesenchymal stem cells, and others[77,78]. Moreover, the expression of mesenchymal markers in iCCA cells is not sufficient for their trans-differentiation into CAF, but more likely they secrete platelet-derived growth factor D (PDGF-D) to stimulate fibroblast migration[79,80]. A variety of different markers expressed by activated fibroblasts is used for their identification. These include alpha-smooth muscle actin (α-SMA), platelet-derived growth factor receptor β (PDGFRβ), fibroblast specific protein-1 (FSP-1), alpha 1 collagen type I (COL1a2), vimentin and fibroblast activation protein alpha (FAP)[81].

In order to better elucidate the pivotal role played by activated CAFs in iCCA progression, the group of Zhang *et al.*[82] applied single-cell transcriptomic analysis to identify the different CAF subsets interacting with human iCCA cells. The first and most relevant sub-cluster was the "vascular CAF" (vCAF), in which a vasculature signature with the expression of specific genes and inflammatory chemokines such as IL-6 and CCL8 occurred. Specifically, vCAFs expressing CD146 and IL-6 were found surrounding the tumor core and determined the upregulation enhanced of zeste homolog 2 (EZH2) in iCCA cells, with increased malignancy as a consequence. In the second cluster, the "matrix CAF" (mCAF), high expression of typical ECM molecules was detected. Subsequently, we found the "inflammatory CAF" (iCAF), with low α-SMA but high levels of fibulin 1 (FBLN1), Insulin-like growth factor 1 (IGF1), insulin-like growth factor binding protein 6 (IGFBP6), secretory leukocyte peptidase inhibitor (SLPI), Serum amyloid A (SA A1), C3, and C7 implying immune modulation. Finally, other clusters identified were the "antigen-presenting CAFs", the "epithelial-mesenchymal transition (EMT) CAFs" (eCAFs) and the "lipofibroblast-c5-FABP1", characterized by alteration in lipid metabolism. Affo *et al.*[83] in a similar approach, discovered that hepatic stellate cell (HSC)-derived CAFs were the main component of TME interacting with the tumor cells. Using single-cell RNA sequencing, based on distinct ligand-receptor interactions, they were able to discriminate inflammatory and growth factor-enriched (iCAF) and myofibroblastic (myCAF) subpopulations. myCAF displayed hyaluronan synthase 2 expression and absence of type I collagen, while iCAF enhanced iCCA tumorigenesis via hepatocyte growth factor (HGF)-MET signaling.

These studies strongly corroborate that multiple CAF subpopulations are linked to different features of iCCA, such as immunomodulation, invasiveness, and metabolisms [Figure 2]. Indeed, immunopathology and immunohistochemistry helped to establish the ability of CAF to promote invasiveness, localizing them in lymph node metastases and describing CAF maturity phenotypes.

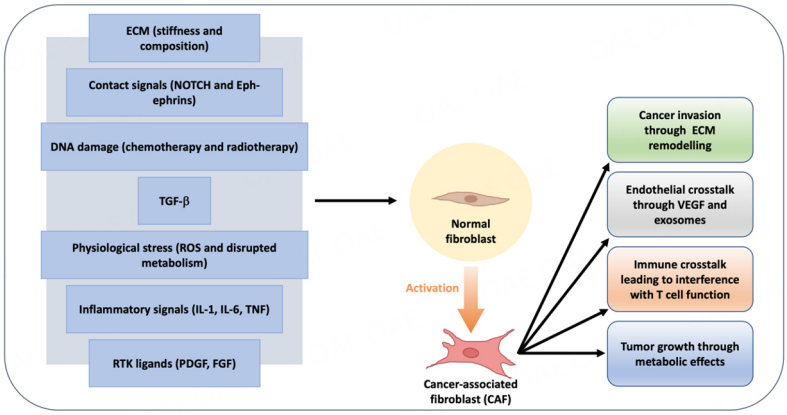

Figure 2. Mechanisms influencing cancer-associated fibroblast activation. This graphic highlights the multiple mechanisms that can contribute to cancer-associated fibroblast (CAF) activation. Also, in summary, the inducing tumor growth and development are depicted. FGF: fibroblast growth factor; PDGF: platelet-derived growth factor; ROS: reactive oxygen species; RTK: receptor tyrosine kinase; TGF-β: transforming growth factor-β; TNF: tumor necrosis factor. Modified from Sahai*et al.*[99].

Cancer-associated fibroblasts and tumor crosstalk

CAFs are permanently activated, resulting in continuous communication with tumor cells and, in general, with the surrounding environment through the release of different biochemical signals. Specifically, activation and/or alteration of TGF-β, HGF, epidermal growth factor (EGF), connective tissue growth factor (CTGF), stromal cell-derived factor-1 (SDF-1), and other components may affect the progression of iCCA by enhancing proliferation, survival, chemotaxis, and angiogenesis[84,85]. Indeed, TGF-β, fibroblast growth factor, and PDGF, released by TAMs and CCA cells, are the main agents responsible for the activation of CAFs. Specifically, the pivotal role played by TGF-β in promoting iCCA with a desmoplastic phenotype was elucidated using a rat-derived organotypic model[86], and targeting this pathway with a TGF-β neutralizing antibody in a rat model of thioacetamide-induced hepatic fibrosis was suggested to reverse pre-existing fibrosis and reduce iCCA burden[87].

Recruitment of CAFs to the tumor reactive stroma also depends on the activation of SDF1/C-X-C chemokine receptor type 4 (CXCR4) axis. SDF-1 secretion by cultured HSCs leads to up-regulation of the anti-apoptotic protein Bcl-2 and concomitantly activation of intracellular signaling including mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways, eliciting iCCA cell survival and invasiveness[88]. Another altered pathway in activated CAFs is the Wnt signaling, which may be involved in regulating the stem cell niches.

Multiple factors, genes but also microRNAs (miR), and cell-cell communication via exosomes clearly affect the activity of CAFs. In this respect, Qin *et al.*[89] found that downregulation of tumor-derived exosomal miR-34c can induce fibroblast activation, converting them to CAFs via targeted modulation of Wnt signaling pathway in iCCA. The exosomes isolated from iCCA cells demonstrated increased secretion of IL-1β, IL-6, and IL-8, promoting fibroblast activation, migration, and invasiveness of cancer cells. Moreover, NOTCH3[90] and Hedgehog[91] have been linked to CAF promoting iCCA growth through cell-cell morphogenetic signals. In addition, the PDGF-BB CAF-secreted isoform has been shown to protect iCCA cells from apoptosis affecting tumor necrosis factor-related apoptosis inducing ligand (TRAIL). PDGF-BB exerted its cytoprotective effects by a Hedgehog-signaling dependent pathway[92]. CAFs can promote an immunosuppressive TME engaging TAMs[93,94] through the secretion of immunomodulatory factors such as periostin. In particular, they can activate innate immunity supporting M2 macrophages and limit NK cell activation boosting cancer aggressiveness via signal transducer and activation of transcription 3 (STAT3)

activation[95]. In addition, M2 macrophages expressing CD163 may be useful in predicting clinical outcomes for iCCA patients[95]. CAFs might also constrain adaptive immunity at different levels resulting in the impairment of dendritic cells and cytotoxic T cells function[93].

Consistent data suggest that CAFs are able to communicate with lymphatic endothelial cells. Malignant cholangiocytes, upon hypoxic stimulus, release PDGF-D through the activation of Rho GTPases [Ras-related C3 botulinum toxin substrate 1 (Rac1), cell division control protein 42 homolog (Cdc42)] and the c-Jun N-terminal kinase (JNK) pathway, promoting fibroblast migration[85]. PDGF-D is also critical for vascular endothelial growth factor (VEGF)-C and VEGF-A production triggering the expansion of lymphatic vasculature and tumor cell intravasation. These early events in the metastatic iCCA process may be blocked or at least delayed, inducing CAF apoptosis or inhibiting the PDGF-D-induced axis[96]. Lymphatic network in turn is able to influence the biology of CCA cells through the secretion of CXCL5. This chemokine binds its cognate receptor CXCR2 present on the surface membrane of tumor cells and stimulates the production of lactate, the uptake of glucose and the increase of ROS. Furthermore, the activation of the CXCL5/CXCR2 axis induces the acquisition of an EMT phenotype on CCA cells through the extracellular signal-regulated kinase 1/2 (ERK1/2)-mediated signaling and leads to the secretion of matrix metalloproteinases (MMPs), responsible for tumor migration and invasiveness. Furthermore, CXCL5 is able to induce lymphatic tubule formation in vitro through an autocrine loop[97]. However, it is not only growth factors and chemokines involved in crosstalk among the different cell milieu of TME that stimulate the metastasis of CCA. In fact, it has recently been demonstrated that in CCA, metastasis is also enhanced by the action of circular RNAs (circRNAs) on endothelial cells. CircRNAs are covalently closed segments of RNA that can be secreted into the bloodstream alone or within extracellular vesicles (Evs). Recent work by Xu *et al.*[98] has in fact demonstrated that the cholangiocarcinoma-associated circular RNA1 (circ-CCAC1) from Evs originating from CCA can be transferred to endothelial cells and induce angiogenesis and disrupt the vascular barrier integrity, a key step for hematogenous metastasis of CCA. CircRNAs not only have major potential biological value, but there are early reports regarding CCA, which hypothesize an important role as a non-invasive biomarker[99].

The presence of FAP positive-CAFs characterized an inflammatory phenotype in which STAT3 activation led to CCL2 upregulation. In this setting, iCCA growth is orchestrated by the recruitment of myeloid-derived suppressor cells (MDSC) and the expression of the correlated genes promoting immunosuppression[100]. Another study identified reciprocal crosstalk between iCCA cells and CAFs through the heparin-binding (HB)-EGF/EGFR axis. EGFR, through its downstream effectors, mainly ERK1/2 and STAT3, caused disruption of adherens junction complexes with E-cadherin internalization and nuclear localization of β-catenin, thereby enhancing the ability of tumor cells to migrate, thus increasing motility and invasion[101]. Moreover, the aberrant expression of ERK5 by tumor cells stimulates the secretion of growth factors, such as VEGF and angiopoietin 1, involved in the crosstalk between tumor and cellular components of TME. In *in vitro* studies, its silencing significantly decreases the migration of macrophages and myofibroblasts and reduces angiogenesis and *in vivo* reduces the weight and volume of tumor masses in a xenograft mouse model[102]. The transcription factor Zinc finger E box binding homeobox 1 (ZEB1) is another player coordinating the crosstalk between iCCA dedifferentiation and CAF activation[103]. Another recent study showed the interactions between iCCA cells and their supportive environment via a chemical messenger, tumor necrosis factor-like weak inducer of apoptosis (TWEAK) and its receptor fibroblast growth factor-inducible 14 (Fn14). TWEAK/Fn14 through NF-kB alters the recruitment and type of immune cells in tumors and increases the growth of CAFs[104].

One of the major roles in which CAFs are involved within the TME is the deposition and remodeling of ECM[105]. The ECM is gradually altered, forming a compact and stiff scaffold, enabling cell communications via paracrine signals. CAFs, together with TAMs and malignant cholangiocytes, contribute to the increased stiffness of tumor tissue producing typical ECM proteins like tenascin C, osteopontin and periostin, in tandem with MMPs (MMP-1, -2, -3 and -9) thereby allowing the tumor ECM to be continuously remodeled, favoring cancer cell invasion[106-108]. All these phenotypic changes promote pro-invasiveness in malignant cells. In prostate cancer was demonstrated that contact mediated Eph receptor-ephrin ligands influence cancer cell migration, allowing the switch from restrained to invasive phenotype[109]. Using an experimental model, a correlation was shown between the ability of CAFs to shape the ECM and metastatic development[110,111]. After cancer cells have colonized at secondary sites, *"de novo"* fibroblast activation induced macro-metastases via several different mechanisms, including matrix components such as tenascin and periostin that support the growth of cancer cells[112,113]. Together with collagen I, tenascin C and integrins, periostin mainly produced from stromal fibroblasts, help malignant cholangiocytes proliferate, altering PI3K-AKT pathway in vitro[114]. Regarding the invasiveness of CAFs in iCCA, Itou *et al.*[78] analyzed metastatic lymph nodes (Met-LNs) positive for α-SMA. They found that α-SMA-positive CAFs were present in invasive areas and around cancer cells. ECM stiffness was also functional in the activation of YAP and TAZ as intracellular mechanosensors and was involved in iCCA initiation and progression. Modification in ECM stiffness promoted YAP-TAZ activity by favoring binding to transcriptional enhanced associate domain (TEAD) transcription factor, promoting cell proliferation, CSC traits, plasticity, and reprogramming[48,115]. Clearly, the alterations in ECM production have direct consequences for tumors, affecting tissue growth and morphogenesis by modulating cell contractility, triggering survival and proliferation signaling in cancer cells[116].

A crucial effect of increased mechanical stress is indeed the ability to reduce perfusion rates in blood and lymphatic vessels, concomitant with hypoxia, thereby contributing to immune evasion, promoting more aggressive cancer phenotype, and reducing the efficacy of administered therapies and drug delivery[117,118]. More recently, some data also suggested that changes in ECM organization may adversely affect the migration of infiltrating leukocytes resulting in a change in the immune microenvironment, thus having important implications for the immune surveillance of tumors[119].

THERAPEUTIC TARGETING OF TME

The signaling networks that govern iCCA development and progression depend not only on the genetic and epigenetic alterations affecting cholangiocytes, but also on the complex relationship between CAFs, immune cells and ECM that is constantly occurring and could offer potential new targets for the benefit of patients. Except for some targeted therapies (see Table 1), such as IDH1/2 and FGFR inhibitors[120-122], iCCA therapeutic treatments remain inadequate and show low responses to conventional chemotherapy. Indeed, a decade after the ABC-02 trial, the combination of gemcitabine and cisplatin (GemCis) as first-line treatment is still the standard treatment for the management of patients not presenting targetable alterations. Additionally, the addition of different cytotoxic drugs failed to provide better clinical outcomes[123]. Moreover, desmoplastic and hypovascularized stroma impairs the delivery of available therapies; therefore, the development of new therapeutic strategies and the discovery of new drugs is mandatory. Indeed, the clinical benefit may be achieved without removing reprogramming CAFs, but just by blocking signals originating from these cells. For example, targeting chemokines may impair the functionality of CAFs, and similarly, targeting ECM components may serve to hamper CAFs-cancer cell communication.

Mertens *et al.* investigated the sensitivity of liver CAFs to navitoclax, a BH3 mimetic[124]. Navitoclax induces selective apoptosis in CAFs and α-SMA positive-HSC compared to iCCA cells and non-activated fibroblasts.

Navitoclax-mediated apoptosis of CAFs was related to a higher expression in Bcl-2 associated X-protein (Bax) protein levels and was linked to the absence of Myeloid leukemia 1 (Mcl-1), which is a resistance factor for this drug[125]. In a rat model, navitoclax induced an improvement in survival by triggering the apoptosis of CAFs, diminishing the levels of matrix protein tenascin C. Finally, in vivo navitoclax was detrimental to tumor growth and metastasis suggesting that targeting stroma may be an attractive strategy for combination therapy, and specifically navitoclax maybe a relevant therapeutic agent.

Other targeting mechanisms, such as TGF-β signaling that modulate the tumor phenotype, are under investigation[126,127]. Recently a phase I clinical trial was started to evaluate the best dose and possible benefits/side effects of hypofractionated radiation therapy and bintrafusp alfa in patients with advanced iCCA. Bintrafusp alfa is a bifunctional fusion protein targeting TGF-β receptor II and PD-L1, that has shown clinical efficacy in multiple solid tumors[128]. Another strategy could be to make CAFs revert to a dormant state, limiting their aggressiveness. An example of this strategy is provided by targeting the vitamin D receptor in pancreatic cancer[129,130].

Many receptor tyrosine kinase (RTK) inhibitors are able to modulate the function of CAFs, exerting an effect on FGF and PDGF receptors[131]. An example is the repurposing of nintedanib (BIBF1120) which was initially developed for the treatment of idiopathic pulmonary fibrosis[132]. Nintedanib, with its action on the inhibition of PDGFR, FGFR and VEGFR, was able to suppress liver fibrosis in mice, blocking HSC activation[133]. In iCCA, Nintedanib impaired the activity and proliferation of CAFs and the production of cancer-promoting cytokines. Thus, administered together with gemcitabine might result in a new promising therapy for refractory iCCA[134].

In recent work based on a thioacetamide (TAA)-induced tumor model, upregulation of IL-6 levels and strong STAT3 activation were accentuated. EGFR signaling and mutant $KRAS^{G12D}$ can both activate IL-6 production in iCCA cells. The authors were able to delineate an intracellular mechanism led by mutant KRAS in which phosphoglycerate dehydrogenase (PHGDH), the rate-limiting enzyme in serine-glycine pathway, was upregulated in human iCCA. They also found a correlation with G9a expression, an epigenetic protumorigenic inducer. In this setting, iCCA cells showed increased viability and inhibition of G9a may suggest therapeutic application[135]. Another study found that low stromal expression of IL-6 and active autophagy flux in tumor cells showed the best prognosis for patients bearing iCCA, correlating with a more effective response compared with postoperative chemotherapy. Specifically, the authors observed that IL-6 production by CAFs impaired the autophagic-associated apoptotic response to 5-fluorouracil in cholangiocarcinoma cells[136].Another interesting study demonstrated that CAFs promote tumor growth by gathering myeloid-derived suppressor cells (MDSCs) via activation of leukotriene B4 receptor type 2 (BLT2). BLT2 activation was revealed to enhance cancer stemness and aggressiveness, and its blockade was sufficient to increase chemotherapeutic efficacy in iCCA patient-derived xenograft models[137].

The impact of Placental growth factor (PlGF) inhibition on the desmoplastic stroma and iCCA cell viability and invasion has been investigated[138]. PlGF belongs to the VEGF family and is highly expressed by CAFs in iCCA, contributing to invasive tumor growth through CAF/iCCA cell interactions. Genetic and/or pharmacologic PlGF inhibition in mice bearing orthotopic iCCA tumor resulted in an improvement due to antiangiogenic/antivascular effects and minimized inflammation provoking a decrease in desmoplasia and tissue stiffness, thereby also reducing hypoxia. Interestingly, a combination of anti-PlGF with GemCis versus single therapy elicited tumor growth delay and increased overall survival. However, in a previous ABC-03 trial, a randomized phase 2 trial of GemCis with or without cediranib (an oral VEGFR inhibitor) was negative, showing no efficacy in the treatment of patients with advanced biliary tract cancer[139,140].

Role of immunotherapy

Immunotherapy and, specifically, the modulation of immune checkpoint-mediated signaling pathways have emerged as a prominent therapeutic approach for several cancers, including iCCA. Although it is acknowledged that antitumor therapeutic approaches are hampered by the immunosuppressive TME, targeting not only stromal cells but also immune checkpoints, as well as finding a way to boost the immune system, has become a potential anticancer strategy in the last decade. However, data regarding the use of immunotherapy in iCCA are still scanty in terms of its efficacy and safety, therefore requiring more detailed studies. In this context, numerous clinical trials are investigating the antitumor efficacy of different immune checkpoint inhibitors (ICI), specifically PD-1, its ligand PD-L1 and CTLA-4, alone or in combination with other treatments for CCA. The presence of these immune checkpoints dampens the immune response provided by the activated T-cells supporting tumor cell survival. These immune checkpoint inhibitors, altering the PD-1, PD-L1, and CTLA-4 pathways, can boost antitumor immunity resulting in stronger clinical responses[141,142]. Cai *et al.* detected high levels of PD-1 and CTLA-4 in CCA, as well as in several other tumors, and their expression correlated with the immune subtypes in which IFN-γ and TGF-β were the main players[143]. Therefore, regarding cholangiocarcinoma, PD-L1 levels infer a potential response to ICI. In particular, it was also observed that PD-L1 positive tumors display a more protracted response to ICIs, due to a deficiency in mismatch repair mechanisms. This prompts microsatellite instability which in turn results in increased tumor mutational burden with concomitantly higher levels of tumor antigens[144,145]. Moreover, PD-L1 positive tumors induce the expression of several biomarkers, such as breast cancer gene 2 (BRCA2), TP53, BRAF, ring finger protein 43 (RNF43), transcription-associated topoisomerase 2a (TOP2A) mutations[146].

Spizzo *et al.*[147], using a next-generation sequencing (NGS) approach, characterized BRCA mutations in advanced biliary tract cancer (BTC) and identified a molecular subgroup as a potential candidate for the DNA-damage repair pathway (e.g., PARP inhibitors) treatment as monotherapy or in combination with immune checkpoint inhibitors. Indeed, in terms of personalized medicine, new trials are focusing on DNA damage repair (DDR) and, specifically, the emerging role of BRCA 1/2 in association with immune checkpoint inhibitors (ICIs)[148].

To increase the clinical data available, currently, numerous trials are actively aiming to investigate anti-CTLA-4 (such as ipilimumab or tremelimumab), anti-PD-1 (such as pembrolizumab or nivolumab), and anti-PD-L1 (such as durvalumab)[149]. Durvalumab showed promising antitumor activity and further investigation may provide more insights focused on the identification of biomarkers of response and emerging predictors, such as microsatellite instability (MSI), mismatch repair (MMS) and tumor mutational burden (TMB), associated with PD-L1 expression[150]. However, despite the promising data reported by the KEYNOTE-028 basket trial (NCT02054806)[151], the KEYNOTE-158 basket trial (NCT02628067) failed to reproduce these results concerning safety and efficacy in a larger cohort of patients with biliary tract cancer[152]. Presumably, the lack of specific criteria on the selected cohort revealed that the tumor genomic landscape might be in part responsible or have had a negative influence on the response to ICIs.

Up to the present day, combination therapies with ICIs and traditional chemotherapy or targeted drugs appear to be the right choice to prolong the survival of CCA patients. Recently, we have witnessed a changed approach to oncological patients and how the management of CCA is no longer shaped by the use of palliative chemotherapy or precision therapies alone. Instead, the new management aims to harness the potential synergies arising from concomitant administration of immunotherapy, chemotherapy, and targeted therapies[153]. Recent results from the TOPAZ-1 phase III trial (NCT03875235) showed that in patients with advanced biliary tract cancer, the combination of durvalumab plus GemCis improved overall survival (OS) and progression-free survival (PFS) when compared to placebo plus GemCis, with

manageable safety probably representing a new first-line standard of care regimen[154]. Besides the positive data from phase III TOPAZ-1 trial, recently (ending January 2023), another breakthrough in the treatment of patients with BTC is reported by the phase 3 KEYNOTE-966 trial (NCT04003636) of the PD-1 inhibitor pembrolizumab in combination with GemCis. The addition of pembrolizumab to GemCis elicited a significant improvement in OS *vs.* GemCis alone[155-157]. In another phase II trial (BilT-01) (NCT03101566), nivolumab plus GemCis or ipilimumab was not sufficient in improving the 6-month PFS. However, the high OS rate at 2 years in Arm A (nivolumab plus GemCis) suggested a potential benefit in a small cohort of patients[158]. A promising combination strategy involves the potential synergy of ICIs and anti-VEGF agents. VEGF, expressed by both CCA cells and TAMs, has an immune detrimental effect on tumor microenvironment, as it suppresses T cell response through inhibition of dendritic cell maturation[159]. Currently, IMbrave 151 (NCT04677504) represents the first study to assess the validity of combined PD-L1/VEGF blockade on a GemCis regimen in BTC. The aim of the trial is to consider the efficacy and safety of atezolizumab with bevacizumab in combination with GemCis *vs* atezolizumab, in combination with CisGem, in participants who have not received prior systemic therapy[160]. The combinatorial approach between anticancer drugs is used not only for the combination of ICI and classic chemotherapy but has also been used to evaluate the effect of chemotherapy and inhibitors of specific signaling pathways. For example, binimetinib (or MEK162), a specific inhibitor of Mitogen-activated protein kinase 1/2 (MEK1/2), was used in a phase I/II study (NCT01828034) as a first-line treatment of advanced BTCs together with GemCis with unsatisfactory results[161]. The treatment failure was likely due to the fact that all patients were treated with binimetinib regardless of whether they had MEK1/2 pathway deregulation. More promising results were reported by a phase Ib study (NCT02773459), in which binimetinib was administered in combination with capecitabine, an antimetabolite already in use for the treatment of gastric and breast cancers, in selected patients with BTCs pretreated with gemcitabine. These patients, all harboring mutations in the RAS/RAF/MEK/ERK pathway, showed acceptable drug tolerance and improvements in both OS and PFS[162].

To conclude, Viscardi *et al.*[163] published a very interesting meta-analysis to evaluate the existence of excess mortality in the ICI arm compared to the standard of care. Different patterns of progression upon ICI administration have been used to measure the raised mortality. Early death (ED), defined as death due to disease progression within 0-3 months, was used to quantify the excess mortality. The results of this study highlighted an increased ED risk, mainly in gastric and urothelial cancer patients, possibly due to the presence of a small subgroup sensitive to ICI and the absence of predictive biomarkers for ICI efficacy.

CONCLUSION AND FUTURE PERSPECTIVES

The diagnostic delay and the limited efficacy of the currently available therapies is a major challenge to disease management. iCCA, like other malignancies, is characterized by high heterogeneity, caused not only by the genomic, epigenetic, and molecular aberrations but also by the multifaceted interactions between cancer cells, CSCs, and the TME. Indeed, the relationship between TME and tumor cells is not yet fully understood. The crosstalk between tumor cells and TME, in particular, plays a decisive role in mediating the biological responses of iCCA, modulating the proliferation and chemoresistance typical of this tumor. The use of drugs capable of targeting the mediators of these interactions represents a promising approach for the treatment of iCCA, but the poor knowledge of the neoplasm-microenvironment interactions limits its applicability.

The most common cellular subpopulation in TME is represented by CAFs, which can originate from different types of fibroblasts and can differentiate in several sub-populations, contributing to enhancing iCCA features such as chemoresistance, inflammatory environment, invasion and immunosurveillance. A more in-depth understanding of this complex biological landscape will likely lead to improvements in the

clinical practice enabling a better stratification of the patients according to their clinicopathological features, genomic backgrounds and variations in the tumor microenvironment and immune response. This will help to maximize the safety and effectiveness of the therapeutic selection available. In this context, several compounds capable of selectively targeting CAFs or related signaling pathways are in an advanced phase of study and could lead to new therapeutic approaches for the treatment of patients with iCCA. At the same time, immunotherapy is receiving even more attention because data obtained from clinical studies suggest greater efficacy and fewer side effects than conventional therapies. Compared to monotherapy, combining ICIs with cytotoxic chemotherapy or targeted therapies is now actively investigated. Some available results validated the safety and efficacy of ICIs with GemCis, confirming that a combination strategy is a promising direction to pursue. Based on the data presented, we strongly believe that a combined approach aimed at blocking tumor growth, elimination of neoplastic cells and modifying the TME of iCCA are essential for the treatment of this deadly disease. Indeed, TME is fundamental in supporting and stimulating the characteristics of malignancy and chemoresistance typical of iCCA. Thus, we believe that further efforts are needed to better understand the tumor-TME crosstalk and to delineate clinical trials based on co-treatment with anticancer drugs and molecules capable of profoundly modifying the composition of both the cellular and matrix components of TME.

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Authors' contributions

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