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Cholangiocarcinoma tumor microenvironment highlighting fibrosis and matrix components

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Abstract

Cholangiocarcinoma (CCA) is an extremely aggressive malignancy characterized by a very limited prognosis and scarce treatment options. The majority of patients are diagnosed at an advanced stage and do not qualify for potentially curative surgical treatments, making CCA an increasingly prevalent global challenge. CCA is characterized by a highly reactive desmoplastic stroma, with complex mechanisms underlying the mutual interactions between tumor cells and stromal compartment. This review focuses on the recent studies examining CCA's biological features, with particular reference to the tumor reactive stroma (TRS) and its role in CCA progression, including matrix remodeling, angiogenesis and lymphangiogenesis, metastasis, and immune evasion. After giving a panoramic view of the relationship between the tumoral and stromal compartment (cancer-associated fibroblast, CAFs and tumor-associated macrophages, TAMs), this review also discusses the current therapeutic approaches to counteract CAFs and TAMs effects on CCA progression.

Keywords: Tumor microenvironments, CAFs, TAMs, CCA, tumor-reactive stroma, therapeutic target

INTRODUCTION

Cholangiocarcinoma

Cholangiocarcinoma (CCA) includes a heterogeneous group of malignancies originating in the biliary tract. According to the anatomical localization along the biliary tree, it is classified into intrahepatic (iCCA), accounting for about 10%, perihilar (pCCA), accounting for 50%-60% and distal (dCCA), rating 20%-30% of total CCAs[1]. Although it is considered a rare disease, CCA is the second most common primary liver cancer, and its incidence has been increasing worldwide in the last decades, even though with geographic dissimilarities[2,3]. Due to the fast rise of drug resistance in CCA cells, late diagnosis and lack of effective immunotherapies, CCA is often lethal; surgical resection, which represents the first-choice treatment, is not applicable to iCCA patients with advanced disease and, even when performed, the 5-year survival is low (less than 25%)[4]. On the other hand, available chemotherapeutics, commonly used for other tumors, showed poor efficacy in CCA, contributing to its high aggressiveness. Although the etiology of the different subtypes of CCA has not been fully understood, epidemiological studies conducted in recent years show that CCA heterogeneity can be ascribed to the interaction between genetic determinants and risk factors. Regarding genome aberrations, a bias to chromosomal instability and a high frequency of breakpoints, losses and gains have been observed in CCA, even if a limited number of studies are available, most of which are conducted in Asiatic patients[5]. In particular, according to comparative genomic hybridization studies, the most frequent alterations include losses at 6q, 3p, 9p, 14q, and 13q, gains at 1q, 7q, 7p, and 8q, and joined defeats at 6q and 3p[5,6]. Several gene mutations/rearrangements have also been reported, such as mutations in the proto-oncogene B-Raf, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (PIK3CA), PTEN, and rearrangements in reactive oxygen species proto-oncogene 1 (ROS1). Regarding iCCA, the most commonly observed aberrations include: mutations in isocitrate dehydrogenase 1/2 (IDH1/IDH2), mutations/rearrangements in FGF receptor 2 (FGFR2), mutations in cyclin-dependent kinase inhibitor 2A (CDKN2A) and mesenchymal–epithelial transition tyrosine-protein kinase Met (MET), mutations/amplifications of EGFR and mutations in Kirsten rat sarcoma virus (KRAS) and in tumor protein P53 (TP53), the last two also frequently found in perihilar and distal CCAs. Mutations in AT-rich interactive domain-containing protein 1A (ARID1A) and small mother against decapentaplegic 4 (SMAD4), rearrangements in protein kinase CAMP-activated catalytic subunit α/β (PRKACA/PRKACB) and amplifications of human EGFR 2 (HER2) are more common in eCCA[7-9]. Moreover, epigenetic modifications that cause genomic instability, such as methylations, histone modifications and changes in the expression of non-coding RNAs, can contribute to the biological features of CCA cells[10]. Besides other conditions (such as alcohol abuse), several pathologies have been identified as risk factors for CCA, including hepatic parasite infections (*Clonorchis Sinensis* and *Opistorchis Viverrini*), viral hepatitis (HCV and HBV), primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH), indicating that, similarly to other tumors, chronic liver inflammation and a fibrotic setting can predispose to CCA[2,3].

CCA AND TUMOR-REACTIVE STROMA

CCA, similarly to other solid tumors, is characterized by a highly reactive and fibrous tumor microenvironment, referred to as desmoplastic stroma, consisting of a cellular and a non-cellular compartment, which constitutes a dynamic and favorable environment for CCA development[11]. The tumor reactive stroma (TRS) plays a part in any aspect of tumorigenesis, including tumor growth, angiogenesis and invasiveness, as well as chemoresistance; indeed, the abundant connective tissue, acting as a physical barrier to prevent drugs from reaching cancer cells, can contribute to a lack of responsiveness of CCA to common chemotherapies[11,12].

As mentioned above, TRS consists of cellular and non-cellular components: the cell compartment is represented by immune (as lymphocytes, neutrophils, macrophages) and non-immune cells (such as fibroblast and endothelial cells), whereas the non-cellular part includes soluble mediators, exosomes and

extracellular matrix (ECM) molecules. All these elements interact with each other and with cancer cells, giving rise to an intricate network that influences every aspect of CCA cell biology, hence participating in any phase of tumor progression[11,12]. Among these factors, the ECM has been recently demonstrated to play a major role in cancer progression, as an aberrant ECM characterizes several solid tumors, such as pancreatic and breast cancer, including CCA[13]. For this reason, apart from the scarce sensitivity of tumor cholangiocytes to chemotherapeutics, targeting tumor microenvironment and particularly ECM or ECM-secreting cells could represent a good option for CCA treatment[14]. Moreover, once identified, specific altered ECM components could serve as diagnostic/prognostic markers in CCA patients.

This review summarizes the role of ECM remodeling in the development and progression of CCA, and also focuses on the functions of the main ECM-producing cell types, namely cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs)[15].

Role of extracellular matrix in CCA

ECM: general aspects

The ECM consists of a wide variety of fibrous and non-fibrous molecules that can be classified into collagens, glycoproteins (such as fibronectin, laminins, tenascins, and elastin) and proteoglycans (such as heparan sulfates, versican, and hyaluronan). Once secreted, these molecules arrange and organize themselves to form two main structures, differing in composition, localization and functions: basement membrane and interstitial matrix. Basement membrane, mainly composed of non-fibrillar collagen (collagen type IV, VI), laminins, nidogen and proteoglycans (mainly heparan sulfate), is a quite stable and dense matrix essential to keep cells on a separate compartment, thus maintaining cell polarity and tissue homeostasis[16]. The interstitial matrix, which consists of fibrous collagens (mainly type I, III, V), fibronectin and elastin, is a dynamic structure that can be remodeled in response to different stimuli and ensure tissue integrity, maintaining cell connections into the stroma and with the basement membrane[16]. Biochemical and physical properties of ECM are the result of ECM deposition, organization, post-translational changes (such as cross-linking), and degradation. These dynamic and finely regulated processes are essential to preserve tissue homeostasis in physiologic conditions. Conversely, dysregulation of these mechanisms leads to the altered ECM remodeling typical of chronic inflammation, fibrosis and cancer[17,18].

Cells can sense ECM changes through specific surface receptors (integrins, discoidin domain receptors (DDRs), and syndecans) that bind ECM molecules and can operate independently or in concert with other partners, such as protein kinase receptors[13]. Once activated, they induce a cascade of intracellular events leading to cytoskeleton reorganization and activation of signaling pathways, such as Rho family small GTPases, extracellular signal-regulated kinases (ERKs), c-Jun NH2-terminal protein kinases (JNKs), p38 mitogen-activated protein kinase (p38 MAPK) and phosphoinositide 3-kinase (PI3K), able to modulate a variety of biological activities[19,20]. It is therefore obvious that alterations of ECM composition or architecture can give rise to and support an environment favorable to cancer progression.

CCA-associated ECM

Desmoplastic tumors, such as CCA, are characterized by high amounts of ECM, with abnormal biochemical and mechanical properties, that impact tumor development, progression, and metastatic dissemination[13,21]. Indeed, an aberrant ECM influences both tumor and stromal cell behavior, inducing phenotypical changes that further sustain the abnormal ECM remodeling. On the other hand, phenotypical modifications occurring in cancer cells as a consequence of genetic/epigenetic alterations could possibly affect TME composition and function, as shown in other tumors. For example, the oncogenic KRAS-CXCR2 axis modulates CAF functions in Pancreatic ductal adenocarcinoma and contributes to CAFs heterogeneity[22].

Indeed, in iCCA, mutant KRAS regulates glucose metabolism in CCA cells, probably affecting TME organization[23].

As mentioned above, any aspect of ECM remodeling can contribute to tumorigenesis. Excessive deposition of fibrous molecules, such as interstitial collagens and fibronectin, their abnormal organization, along with degradation of normal ECM are crucial steps for tumor development[21]. Derangement of normal basement membrane, due to excessive degradation or reduced deposition, is responsible for cells to lose polarity, acquiring a motile phenotype and invading the parenchyma, and represents a prerequisite for tumor malignancy[13,21,24]. A concurrent rearrangement of the interstitial matrix is also essential. Increasing amounts of fibrous molecules, mainly collagen type I and fibronectin, organized in linearized structures, perpendicular to the tumor edge, are deposited, thus promoting cell migration. In particular, whereas fibronectin is a primary inducer of tumor cell motility, interstitial collagen mainly acts on fibroblasts and immune cells, other than cancer cells[21]. Among ECM components, CCA desmoplastic stroma is also enriched in laminin, organized in cross-shaped molecules that highly contribute to CCA invasiveness and aggressive phenotype[25-27]. Increased expression of laminin γ2 in CCA cells and tissue samples and its correlation with metastasis and poor outcome in CCA patients has been shown[28-31]. Moreover, upregulation of laminin β1 was observed in sarcomatoid CCA, although its role was not defined[32]. Accordingly, enhanced levels of laminin-binding integrin subunits (α2, α3, α6, β1,β4) were found in CCA cell lines and tissues, often correlating with tumor invasiveness[33,26]. In particular, the upregulation of integrin β4 was predictive of lymph node metastasis and low survival[27]. Of note, high expression of β4 and β6 integrin subunits has been exclusively detected in CCA, allowing to discriminate this tumor by other hepatic malignancies[33,34]. Similar findings were reported for the non-integrin receptor laminin receptor (LAMR), which was also found to be upregulated in CCA and correlated with adhesion, invasiveness and metastatic properties[35,36]. Interestingly, 67 kDa LAMR positively modulates lysyl oxidase-like-2 (LOXL2) expression in CCA, thus contributing to matrix stiffness (see below)[36]. Similarly, heparan sulfate and heparan sulfate proteoglycans have been shown to be augmented in TRS of diverse solid tumors, including CCA, and positively modulate cell proliferation, adhesion and motility[37-40]. These molecules can function as co-receptors of growth factor receptors, such as fibroblast growth factor receptor, FGFR, amplifying their signals and sustaining cancer growth[38].

Parallel to aberrant matrix deposition/organization, gradual degradation of ECM by specific proteolytic enzymes (mainly metalloproteinases, MMPs, and membrane type-metalloproteinases, MT-MMPs) is a critical process during tumor progression. These proteases: (a) allow the replacement of normal matrix with an aberrant tumor-derived ECM; (b) facilitate cancer and stromal cell motility by degrading the dense matrix that surrounds the cells. Furthermore, during the degradation of the ECM are released soluble mediators and other signaling molecules, otherwise linked to the ECM for spatial and temporal control of their activity, such as transforming growth factor β, TGFβ, fibroblast growth factor-2, FGF-2, and vascular endothelial growth factor-A, VEGF-A[41-43]. Finally, proteolytic degradation produces short ECM fragments that interact with ECM molecules or growth factor receptors and mediate pro- or anti-tumorigenic properties, as well as small proteins, called matrikines, that, acting as cytokines or chemokines, influence tumor and stromal cell behavior. Indeed, matrikines can modulate proliferation, migration, angiogenesis and protein synthesis and secretion, further contributing to a tumor-supporting microenvironment[44,45]. Along these lines, it is not surprising that the levels of ECM-degrading enzymes are often elevated in association with tumor progression. In CCA, overexpression of MMP-2, MMP-9, MMP-11 and MMP-14 has been detected, with differences among tumor subtypes, and correlates with poor outcome[46-48]. In a hamster model of CCA, high levels of MMP-9, together with iNOS and Rac1 expression, were associated with fibroblast accumulation, peribiliary fibrosis, tumor invasiveness, and short survival time[49]. In addition,

a study by Pak *et al.* demonstrated that CCA cells exposed to *Clonorchis Sinensis* excretory-secretory products showed increased migratory and invasive abilities, in association with enhanced expression and activity of MMP-2, mediated by ERK1/2 and NF-κB induction[50].

During cancer progression, increasing amounts of cross-linked collagen fibers are deposited, resulting in a stiff milieu that further sustains stromal cell activation and tumor cell migration and entry into the vascular system. A key role in this phase of ECM reorganization is played by lysyl oxidases (LOXs). LOXs are copper-dependent secreted enzymes involved in cross-linking ECM molecules that maintain the structural integrity of tissues and improve tensile strength. These proteins, which include lysyl oxidase (LOX) and lysyl oxidase-like (LOXL) 1-4, highly contribute to TME changes that drive tumor growth, invasion, epithelial-to-mesenchymal transition (EMT) and immunomodulatory properties, as well as angiogenesis and have been shown to be upregulated in metastatic cancers and correlated with low survival[51]. Concerning CCA, elevated expression of LOXL2 has been found in the stroma of tissue samples of CCA patients and correlated with bad prognosis[52], suggesting a potential role for LOXL2 in promoting tumor progression[53]. Accordingly, a study by Xu *et al.* reported that LOXL2 expression in CCA tissues was associated with lymph node invasion, differentiation, and poor outcome. By contrast, LOXL2 knockdown in CCA cells resulted in reduced *in vitro* invasiveness and lower ability to induce liver metastasis *in vivo*[53]. Moreover, high levels of LOXL2 in CCA specimens, in association with the interacting factor GATA6, correlated with increased expression of VEGF-A and microvessel density and were predictive of poor patient outcomes[54].

As mentioned, a stiff ECM is also responsible for altered angiogenic process, which boosts further changes in ECM and tumor dissemination[55]. If, on the one hand, the traction strength of a stiffened matrix affects vascular integrity, the abundant amounts of tenascin, interstitial collagens and fibronectin stimulate vascular outgrowth, resulting in the release of angiogenic factors and making endothelial cells more responsive to soluble mediators[36].

Besides structural molecules, non-structural glycoproteins (such as osteopontin, periostin, tenascin-C, thrombospondin-1, mesothelin and others) participate in ECM reshaping during tumorigenesis. These matricellular proteins, which contribute to cell-matrix interactions and cell signaling, deeply influence several aspects of CCA progression, such as cell proliferation, invasion, metastasis, EMT, and immune escape (see below)[56].

Finally, ECM reshaping is also essential for metastasis establishment. Once circulating tumor cells extravasate, their fate will depend on the features of the microenvironment in the distal organ. In this site, an ECM favorable to tumor growth can be already present or an ECM remodeling can be directly stimulated by tumor cells or by exosomes released by tumor and tumor-associated stromal cells. In the metastatic site, ECM modifications can be crucial for the awakening of disseminated dormant tumor cells[13,21]. In this regard, the key role of matrikines has been recently reported in iCCA[57].

Cancer-associated fibroblasts

Markers, origin and sub-classification

Concerning the cellular components of the TRS, the main cell population is represented by the so-called cancer-associated fibroblasts (CAFs), a population of activated fibroblasts that assume a relevant role as potential therapeutic targets in iCCA for promoting tumor progression[58]. CAFs are characterized by the expression of several specific markers, including α-smooth muscle actin (α-SMA)[58], fibroblast specific protein 1 (FSP-1)[59], the mucin-like transmembrane glycoprotein podoplanin, the cell surface

metalloprotease cluster of differentiation 10 (CD10)[60] and platelet-derived growth factor receptor β (PDGFRβ)[61].

Other markers used to identify CAFs include vimentin, alpha 1 collagen type I (COL1α1) and fibroblast activation protein alpha (FAP)[62,63]. Among these markers, FSP-1 has shown the highest percentage of expression in CAFs (up to 84.5%) and has been associated with an immature phenotype and lymph node metastasis, suggesting that CAFs phenotype is crucial for tumor progression[64].

Of note, high expression levels of α-SMA in CAFs have been associated with larger tumor size and decreased overall survival (OS) in CCA patients. In addition, CAF with high α-SMA positivity had a greater effect on the proliferation of tumor and non-tumor biliary cells than normal hepatic fibroblasts expressing lower levels of α-SMA[65,66].

CAFs can originate from quiescent hepatic stellate cells (HSCs), portal fibroblasts, and to a lesser extent, circulating mesenchymal cells derived from bone marrow[67]. Furthermore, CAFs may even be derived from pericytes and adipocytes[68,69]. Concerning iCCA, single-cell RNA sequencing technique has shown that HSCs represent the major source of CAFs in iCCA, since as much as 85%-95% of Col1a1-GFP$^+$ CAFs and 85%-93% of α-SMA$^+$ CAFs came from HSCs. Notably, HSC-derived CAFs are the main TRS cell population crosstalking with tumor cells[70].

Recently, Zhang *et al.*, analyzing human iCCA specimens and adjacent tissues by single-cell RNA sequencing, revealed that CAFs can be classified into six subclasses. The most abundant one consisted of CD146-positive cells expressing microvasculature signatures and high levels of inflammatory chemokines (IL-6, CCL8) and was, for this reason, renamed vascular cancer-associated fibroblasts (vCAFs). IL-6 secreted by vCAFs was responsible for the upregulation of enhancer of zeste homolog 2 (EZH2) in tumor cells, which exacerbated their malignancy. The second ones are matrix CAFs (mCAFs) expressing high levels of ECM proteins, such as collagen and periostin, and low levels of α-SMA. The third subclass is represented by the inflammatory CAFs (iCAFs) related to inflammatory response regulation and complement activation. The other subclusters consisted of antigen-presenting CAFs (apCAFs), EMT CAFs (eCAFs) expressing epithelium-specific marker genes, and lipofibroblast-c5-FABP1 involved in lipid metabolism[71], respectively.

Similar analyses were conducted by Affo *et al.* that uncovered five different subclasses of CAFs: inflammatory and growth factor-enriched CAFs (iCAFs), myofibroblastic CAFs (myCAFs), mesothelial CAFs (mesCAFs), multi-CAFs consisting of multiple subpopulations and other CAFs that were not included in any category. In particular, myCAF-expressing hyaluronan synthase 2 but not type I collagen, and iCAF-expressing hepatocyte growth factor (HGF) were reported to originate from HSCs and promoted tumor growth[70]. The markers of the different subclasses are represented in Table 1.

These two different classifications highlight the different functions and phenotypes of CAFs, providing evidence of inter-tumor heterogeneity in iCCA.

Of interest, it has been observed that CAFs are not only localized in CCA primary tumors, but can also be present in metastatic areas, as shown by Itou *et al.*, that detected α-SMA positive CAFs in metastatic lymph nodes of iCCA patients[72].

Table 1. Human CAF subtypes and markers

CAFs	Markers	References
All CAFs	α-SMA, COL1a2, and PDGFRβ	[71]
Vascular CAFs	CD146 (MCAM), MYH11, GJA4, RGS5 and IL-6 and CCL8	[71]
Matrix CAFs	COL5A1, COL5A2, and COL6A3, periostin (POSTN), FN1, LUM, DCN, and VCAN	[71]
Inflammatory CAFs	FBLN1, IGFI, CXCL1, IGFBP6, SLPI, SAA1, and complement genes (C3 and C7)	[71]
Antigen-presenting CAFs	CD74, HLADRA, and HLA-DRB1	[71]
EMT-like CAFs	KRT19, KRT8, and SAA1	[71]
Lipofibroblast–c5–FABP1	APOA2, FABP1, FABP4, and FRZB	[70]
Inflammatory CAFs	ADAMTS4 AGT APOE ARHGDIB CCL19 CCL21 COLEC11 CPE GEM GJA4 GPX3 HIGD1B IL6 ISYNA1 LHFP MAP1B MT1A NDUFA4L2 PDK4 RGS5	[70]
Myofibroblastic CAFs	APOD CCL11 COL1A1 COL1A2 COL3A1 COL5A1 COL6A3 CTGF CTHRC1 CYP1B1 FN1 INHBA ISLR LUM MMP14 POSTN PTGDS SERPINF1 SFRP2 SPON2 VCAN	[70]
Mesothelial CAFs	ANXA1 ANXA2 BDKRB1 C19orf33 C3 CALB2 CCDC80 CFB CRABP2 CXCL1 CXCL6 EFEMP1 EGFL6 EMP3 EZR HMOX1 HP HSPA6 IFI27 IGFBP6 ITLN1 KRT18 KRT19 KRT8 LINC01133 LOX MT1E MT1G MT1X MXRA5 PDPN PLA2G2A PRG4 PRSS23 PTGIS RP11-572C15.6 S100A10 S100A16 S100A6 SAA1 SAA2 SERPINE2 SH3BGRL3 SLC12A8 SLPI TM4SF1	[70]
Pan-CAFs	COL1A1 COL1A2 COL3A1 C1S ACTA2 C1R SERPINF1 PDGFRB	[70]

IL-33 produced by both cancer cells and CAFs inhibits CCA cell migration and can be considered a good CCA prognostic biomarker[73]. Moreover, high levels of interleukin-33 in cancer cells and cancer-associated fibroblasts correlate with good prognosis and suppressed migration in cholangiocarcinoma[73].

Activation and CAFs/CCA cells interaction

CAFs are present in the TRS in higher amounts with respect to normal stroma fibroblasts and exist in a permanent state of activation, resulting in a wide release of molecules that interact with cancer and immune cells, including TGF-β1, connective tissue growth factor (CTGF), epidermal growth factor (EGF), HGF and stromal cell-derived factor-1 (SDF-1) or CXCL12. Some of these mediators, such as TGF-β1 and tumor necrosis factor alpha (TNFα), are associated with poor outcomes in iCCA patients[67]. Recently, it has also been shown that CXCL12 levels correlated with metastasis and poor prognosis in iCCA[74]. This chemokine is mainly produced by CAFs and poorly expressed in normal fibroblasts, indicating a possible role of CXCL12 in the recruitment of these cells to the TRS[69].

CAFs also secrete extracellular matrix (ECM) factors, such as tenascin-C, periostin (POSTN), osteopontin, fibronectin, collagen type I, as well as different matrix metalloproteases such as MMP1, MMP2, MMP3 and MMP9, which create a favorable microenvironment for tumor progression[67].

In addition, CCA cells secrete cytokines to promote the activation and recruitment of CAFs, with TGF-β1 and platelet-derived growth factor D (PDGF-D) being the most relevant ones[67,75]. In particular, hypoxic CCA cells overexpress and release PDGF-D, which in turn binds to its related receptor PDGFRβ on CAFs, resulting in the release of VEGF A/C. This event sustains tumor lymphangiogenesis and stimulates CCA cell intravasation, leading to early spread to lymph nodes. Moreover, the use of imatinib to block PDGFRβ leads to inhibition of the VEGFs secretion and, consequently, a decrease in PDGF-D-induced fibroblast migration and CCA cell intravasation[76,77].

On the other hand, CAFs also produce PDGF-B, another member of the PDGF family, which binds PDGFR β expressed by CCA cells and activates Hedgehog signaling, which, in turn, protects tumor cells from TNFα-related apoptosis-inducing ligand (TRAIL). Notably, in a CCA orthotopic rat model, Hedgehog inhibition

by cyclopamine induced cancer cell apoptosis with the subsequent decrease of tumor growth[78].

CAFs also release heparin-binding epidermal growth factor (HB-EGF), which binds the epidermal growth factor receptor (EGFR) expressed by CCA cells. HB-EGF/EGFR axis activates the β-catenin pathway, resulting in increased motility of CCA cells, and induces the production of TGF-β1 which, in turn, promotes CAF recruitment and further release of HB-EGF, thus creating a continuous loop of activation. In this connection, the inhibition of EGFR with gefitinib in immunocompromised mice co-transplanted with CCA cells and human liver myofibroblasts results in a reduction of tumor incidence, growth and metastasis, suggesting that EGFR signaling is important for tumor growth and dissemination[79].

HB-EGF has also been shown to induce IL-6 production in CCA cells. In addition, the presence of KRAS mutation stimulates IL-6 expression in CCA cells, indicating that both the activation of EGFR signaling and mutated KRAS represent mechanisms leading to IL-6 upregulation in CCA tissues[23]. Moreover, IL-6 expression was associated with the tumor volume in a CCA-engineered mouse model and the use of siltuximab, an anti-IL-6 monoclonal antibody, inhibited IL-6 signaling driving a reduction of CCA growth both in vitro and *in vivo*[80], suggesting a role of this cytokine in CCA carcinogenesis. In this connection, literature data reported that the zinc finger E-box binding homeobox 1 (ZEB1), a transcription factor overexpressed in different types of cancer, favors the induction of EMT, stemness genes and CAF activation in CCA and promotes the release of soluble factors (CTGF, HGF, and IL6), enhancing tumor progression[81]. Moreover, its overexpression has been associated with poor prognosis in iCCA[81].

Among the interactions that can occur between CCA cells and CAFs, microRNAs seem to have a role in mediating CCA carcinogenesis, acting as oncogenes or tumor suppressors. It has been observed that exosomes produced by CCA cells could induce CAF activation. In particular, the downregulation of miR-34c contained in the CCA cell exosomes may convert fibroblasts into CAFs through the expression of pro-inflammatory and fibroblast-associated proteins, favoring increased CAF migration[82].

Among the different factors produced by CAFs, CXCL12 is a chemokine that has been demonstrated to have a key role in cancer progression. CXCL12 binds to two different receptors, CXCR4 and CXCR7, both expressed on CCA cells. The binding of CXCL12, released by activated HSCs, to these receptors on CCA cells has been proposed to induce different biological responses implicated in tumor progression[83,84]. Of note, CCA cells overexpress CXCR4, an effect induced by TNF-α produced from TAMs[85] or by HGF released by CAFs[86].

Angiotensin II (AngII) expressed by cancer cells and its receptor AT-1, present on both stromal and tumor cells, may also play a role in CAF activation. In particular, AngII promoted the survival of iCCA cells and HSC activation, indicating a role for AngII/AT-1 axis in tumor progression exerted through both autocrine and paracrine mechanisms[87].

Role of CAFS in immunomodulation

It has been demonstrated that CAFs can modulate immune cell activities. A CAF subset enriched in fibroblast activation protein (FAP) can recruit myeloid-derived suppressor cells (MDSCs) through the release of the chemokine (C-C motif) ligand 2 (CCL2)[88]. In addition, CAFs can potentiate MDSCs stemness features through IL-6- and IL-33-dependent 5-lipoxygenase /LTB4-BLT2 signaling[89].

Recent evidence showed that Caveolin-1 (CAV1) expressed by CAFs plays an important role in cellular senescence[90]. CAV1 overexpression in CAFs was related to poor overall survival (OS) and recurrence-free

survival of iCCA patients. Moreover, $CAV1^+$ CAFs correlated with the infiltration of $Foxp3^+$ TILs, indicating that $CAV1^+$ CAFs might attract regulatory T lymphocytes (Treg) cells into the TRS[91].

Moreover, it has been demonstrated that CXCL5 is able to induce iCCA growth and metastasis by recruiting intratumoral neutrophils through the activation of PI3K-Akt and ERK1/2-MAPK signaling pathways. In particular, CXCL5 overexpression seems to be induced by IL-1β released by CAF, and in turn, IL-1β release is mediated by CCA cells through paracrine mechanisms[92,93]. Moreover, CXCL5 overexpression alone, or together with the presence of intratumoral neutrophils, turned out to be an independent prognostic factor for iCCA patients[92,93].

Role of CAFS in angiogenesis

CAFs, in collaboration with CCA cells, create a rich lymphatic vasculature responsible for the early metastatization of this cancer to the regional lymph nodes, precluding therapeutic surgery[94]. Furthermore, angiogenesis has been related to a high risk of recurrence after surgery[95].

As aforementioned, PDGF-D released by CCA cells promotes the release of VEGFs from CAFs, molecules responsible for the recruitment of lymphatic endothelial cells (LECs) and their organization in anastomosed structures. Indeed, LECs localize in close adjacency with either CCA cells or CAFs in the TRS, suggesting an important role of these latter in promoting angiogenesis[77]. Consistently, the release of PDGF -BB from the vascular endothelium stimulates the recruitment of PDGFR-β- expressing HSCs and their subsequent adhesion to the vessel wall. In turn, HSCs release angiogenic factors under hypoxic conditions and surround LECs to stabilize the vessels[96-98]. In the context of TRS, in addition to cytokines, the hypoxic microenvironment can also exert a role in CAF activation by enhancing the expression of placental growth factor (PlGF), a VEGF family member, in HSCs and cancer cells. High circulating levels of PlGF were observed in blood samples from iCCA patients and correlated with poor outcomes. Moreover, the inhibition of PlGF reduced iCCA cell invasion, desmoplasia and TRS stiffness and abolished CAFs activation, suggesting a possible role of PlGF in CAFs recruitment. Of note, PlGF inhibition also increased the effects of chemotherapy in orthotopic mouse models of iCCA[69,99].

PIGF has been shown to increase VEGF-mediated angiogenesis through binding to VEGFR1 and Nrp1 expressed by endothelial cells. PIGF, unlike VEGF, is only overexpressed in pathologic conditions, such as hypoxia, and could be considered a possible target for iCCA treatment without the risk of affecting physiological angiogenesis[99,100].

CAFs in ECM remodeling

During cholangiocarcinogenesis, the ECM gradually undergoes great changes in the deposition of structural and non-structural proteins, including collagen type I, fibronectin and matrix-modified enzymes, resulting in a desmoplastic matrix. This stiff and thick atypical ECM drives abnormal behavior of both tumor and stromal cells, thus influencing processes related to cancer biology, such as inflammation, fibrogenesis, and angiogenesis.

Several cell types actively contribute to remodeling ECM versus a desmoplastic matrix, including CAFs, macrophages and tumor cells that underwent epithelial-to-mesenchymal transition (EMT)[75].

Among the non-structural proteins released in the desmoplastic ECM, tenascin-C, periostin and osteopontin were reported to boost cancer aggressiveness and represent biomarkers of poor prognosis for CCA patients[101]. Tenascin-C is mainly expressed by CAFS, but cancer cells are also involved in its

production. A high amount of tenascin-C are localized at the invasive front and are associated with poor outcome in iCCA patients. It has been observed that tenascin-C affects cancer cell invasion and metastasis through c-MET- and EGFR-dependent mechanisms[102]. In addition, tenascin-C may also contribute to creating an immune suppressive microenvironment, thus further promoting tumor growth and dissemination[102].

Recent evidence shows that besides tenascin-C, desmoplastic ECM also contains tenascin-W, which, unlike tenascin-C, is not present in normal hepatobiliary tissue and therefore could be considered a possible novel marker of iCCA stroma[103].

In this connection, periostin, which is known to be produced only by CAFs, is an independent prognostic factor for the overall survival of CCA patients. By interacting with integrin α5 expressed by CCA cells, it induces tumor cell proliferation and invasion through PI3K/AKT signaling and CCA cell migration through ITGα5β1/TWIST-2 mediated EMT[104,105].

Finally, osteopontin is involved in immunomodulation since it represents a key factor for the development of NK cells and the survival of T cells[106,107]. In iCCA patients, high stromal levels of osteopontin within TRS represent an independent predictor of poor prognosis, which correlates with tumor size and the presence of lymph node or macrovascular invasion[108]. Osteopontin promotes iCCA progression through MAPK1 and Wnt/β-Catenin signaling and could represent a potential novel prognostic biomarker and a potential target for iCCA patient treatment[109]. Recently, it has been observed that HSCs promote the expression of nuclear receptor family 4 subgroup A member 2 (NR4A2) in iCCA cells, then inducing tumor proliferation and invasion. This is potentially relevant since NR4A2 can stimulate osteopontin expression by enhancing the activity of the Wnt/β-catenin pathway[110].

Tumor-associated macrophages

As previously reported, a major risk factor for CCA development is represented by the persistent biliary inflammatory condition detected in chronic cholestatic injury due to liver flukes, PBC and PSC, as well as chronic viral infections (HBV or HCV) and liver cirrhosis[111]. The peculiar CCA desmoplastic stroma includes CAF cells and, to a less extent, TAMs. TAMs represent the major immune cell population that infiltrates the CCA tumor microenvironment and high amounts of infiltrating TAMs correlate with poor prognosis of patients with CCA[112]. However, whereas CAFs are distributed across the entire tumor mass, TAMs are mostly located at the invasive tumor front, putatively recruited by neoplastic cells[113].

TAMs, which mostly display a M2 macrophage phenotype, play a key role in cancer progression and immune evasion by releasing immunosuppressive cytokines[113,114]. TAMs infiltrating CCA are not apparently derived from resident macrophages ($CD68^+$) or liver Kupffer cells but, more likely, from circulating monocytes expressing the clusters of differentiation 14 and 16 ($CD14^+/CD16^+$). Their recruitment is mediated by several chemokines, including CCL2 (also known as MCP-1), the colony-stimulating factor 1 (CSF1) and VEGF, which are primarily released by CAFs, but also by intrahepatic macrophages[75,101]. Similarly, the stromal cell-derived factor 1 (SDF-1, also known as C-X-C motif chemokine 12 or CXCL12), CCL3 and CCL4 chemokines[115], as well as cytokines such as IL-1β, IL-4, IL-10 and IL-13 can play a role in recruitment and attraction of monocytes and macrophages[116]. Moreover, macrophage recruitment supported by CAFs and CCA cells involves the activation of specific intracellular pathways, such as Notch, IL-6/STAT3, as well as PI-3K regulatory signaling[116].

Literature data described the crosstalk between CCA cells and TAMs and TAMs were suggested to play a role in accelerating tumor progression by modulating tumor growth, inflammation, metastases, angiogenesis, and immune escape[75,113,116].

TAMs, proliferation, Epithelial-to-Mesenchymal Transition and metastasis in CCA

Literature studies suggest that TAMs can exert a pro-neoplastic role in CCA, including contribution to tumor growth, acquisition of invasive phenotype, and promotion of metastasis. In this connection, TAMs can sustain the proliferation of CCA cells by activating the Wnt/β-catenin pathway through the release of Wnt ligands, including Wnt3a and Wnt7b[117-120]. Consistently with Wnt/β-catenin activation, cytoplasmic and nuclear β-catenin expression is higher in CCA tissue, whereas its expression on the plasma membrane is reduced. Moreover, the exposure of CCA cells to conditioned medium collected from LPS-activated TAMs led to increased proliferation of cancer cells[118].

As it is well established, EMT is a critical event in cancer progression through which epithelial cancer cells acquire a mesenchymal and invasive phenotype and then spread to distant sites and metastasize. In this context, TAMs, which are the primary immune cells infiltrating the tumor microenvironment, act as local and systemic signaling sources to enhance almost every step of the tumor invasion-metastasis cascade (including EMT, invasion of the surrounding extracellular matrix, intravasation, survival in the circulatory system, extravasation and colonization), through the secretion of multiple factors[121,122]. Techasen *et al.* have demonstrated that conditioned media collected from lipopolysaccharide (LPS)-activated macrophages were able to induce typical EMT markers in CCA cells, including decreased E-cadherin and CK-19 expression as well as increased mesenchymal markers such as S100A4 and MMP9[123]. These phenotypical EMT-related changes were due to the release of several cytokines and growth factors in culture medium, such as TGF-β1, IL4, IL6, IL10 and TNF-α, by LPS-activated macrophages.

Moreover, Thanee *et al.* have reported that conditioned medium collected from $CD163^+$-macrophages activated by IL-4 resulted in increased migration of CCA cells coupled with increased expression of the mesenchymal marker N-cadherin[124]. Of relevance, increased amounts of $CD163^+$-macrophages in the CCA tumor microenvironment were significantly associated with patients' metastatic condition[124]. In this connection, macrophages stimulated with LPS were able to induce EMT-related reprogramming of CCA cells through TNF-α-induced activation of Snail and ZEB[125-127].

TAMs, matrix remodeling and angiogenesis

In the peculiar desmoplastic TRS of CCA has been documented a strong interplay between angiogenesis, lymphangiogenesis and ECM remodeling processes, with VEGF (particularly VEGF-A and VEGF-C) representing a crucial factor promoting CCA progression and metastatic spread. Indeed, VEGF secreted by CAFs and CCAs is able to regulate tumor-associated lymphangiogenesis, with VEGF-A, together with SDF-1 and CCL2, promoting the recruitment of TAMs which, in turn, contribute to matrix remodeling by releasing MMPs[128]. Accordingly, Subimerb *et al.* reported that TAMs, particularly those at the invasive tumor front, are the primary source of MMP9 within the CCA and contribute to invasiveness and metastatic dissemination[112]. Moreover, the amounts of infiltrating $MMP\text{-}9^+$-TAMs correlate with the low survival rate of CCA patients. Still concerning the role of VEGF-A released by TAMs, the previously cited study by Subimerb *et al.* reported that more than half of the CCA human tumor samples exhibited strong macrophage infiltration[112]. In addition, Subimerb *et al.* also observed increased levels of circulating $CD14^+$/$CD16^+$ monocyte cells, which are precursor cells of tissue-resident macrophages in CCA patients[129]. Of interest, these monocytes expressed high levels of VEGF, CXCL3 and epiregulin, which, in turn, can predict the tissue invasive nature of iCCA.

In this connection, a study by Hasita *et al.* showed that the treatment of macrophages with conditioned medium obtained from HuCCT1 cholangiocarcinoma cells resulted in a STAT3-related M2 polarization of macrophages and consequent increased expression and release of VEGF-A, TGF-β, IL-10 and MMP-2, mediators able to promote angiogenesis and matrix remodeling[130].

TAMs and immune evasion

In the specific context of CCA, TAMs can exert immunosuppressive properties by acting on the recruitment of cells involved in immune evasion, such as Treg, tumor-associated neutrophils (TANs) and MDSCs[101]. Hasita *et al.* reported a positive correlation between the amount of $CD68^+$ and $CD163^+$ TAMs within CCA stroma and the amount of $CD4^+$ and $FOXP3^+$ Treg infiltration[130]. Moreover, TAMs within CCA stroma, by secreting different cytokines, such as CCL22, CCL2, CCL17, IL-10, IL-4 and IL-8, can promote the recruitment of MDSCs and TANs, favoring the generation of an immunosuppressive microenvironment[119]. Along these lines, iCCA patients with poor prognosis showed activation of the GM-CSF-bone marrow axis, resulting in high levels of circulating myeloid cells. The shutdown of the GM-CSF axis restored the "good" immunity by facilitating cytotoxic T cell influx and reducing TAMs infiltration[131]. TAMs can also exert immunosuppressive activity by modulating immune checkpoints, including programmed cell death ligand-1 (PD-L1)[119]. PD-L1 can be expressed on the membrane of cancer cells and of antigen-presenting cells, including resident macrophages and TAMs. It can bind with Programmed death-1 (PD-1) surface receptors on T cells, then causing inhibition of their cytotoxic activity. It should be noted that, concerning PD-L1 expression in CCA tissue, a strong discrepancy across studies can be observed, which is likely to depend on several factors including the variety of sample sizes, the cancer localization, the statistical thresholds and the different antibodies used. However, the general agreement is that $PD\text{-}L1^+$-TAMs in both CCA murine models and human CCA patients are more abundant compared to CCA cells[132] and that high levels of PD-L1 are correlated with a more aggressive tumor phenotype and worst patients prognosis[133,134].

Finally, the expression of PD-L1 in both CCA and stromal cells is usually associated with an increased percentage of infiltrating TANs and TAMs[134].

Tumor reactive stroma as a therapeutic target

Classically, cancer therapies aim to target neoplastic cells as the only critical cellular actors in the context of clinical management. However, the TME contains several types of cell populations that can contribute to cancer progression. This aspect is particularly relevant in CCA, where the tumor microenvironment consists of a strongly altered desmoplastic stroma in which each stromal, immune and extracellular component may indeed represent a potential therapeutic target. In particular, CAFs and TAMs are considered the major cell contributors underlying CCA malignancy. It should be noticed that stromal cells (i.e., CAFs and TAMs) are genetically stable, whereas CCA cells can undergo multiple genetic/epigenetic modifications, resulting in a highly heterogeneous population. Therefore, a strategy designed to counteract the pro-tumorigenic TRS might represent a suitable approach. A summary of actual therapeutic strategies targeting TRS is provided in this section.

ECM as potential therapeutic target

As described, an aberrant ECM is a main feature of CCA and has a deep impact on tumor progression. A recent study performed on a CCA cell line (KKU-213A), comparing 2D cultures with a 3D silk fibroin-gelatin/hyaluronic acid/heparan sulfate (SF-GHHs) scaffold model mimicking ECM, demonstrated that SF-GHHs promoted cell adhesion, proliferation and spheroid formation, induced the upregulation of EMT and stemness markers and served as a reservoir of cancer stem cells[132]. Thus, a better understanding of the

processes driving an altered ECM remodeling in CCA is crucial to counteract desmoplastic reactions and develop potential therapeutic strategies. Due to the role of LOX family members in the cross-linking of ECM molecules, matrix remodeling and promoting cancer progression, several molecules targeting these enzymes have been tested. Although no study is available on CCA, LOX/LOXL inhibitors are reported to be effective in HCC preclinical studies and clinical trials of other types of tumors[51]. Salidroside, a glycoside extracted from *Rhodiola rosea*, has been shown to reduce cancer cell invasion and tumor growth and metastatic sprouting in pancreatic cancer through the downregulation of mRNA levels of LOX family members[133]. Moreover, the use of Escin Ia, a component of *Aesculus chinensis* Bunge fruits, reduced LOXL2 levels and inhibited EMT in breast cancer. These effects were due to the increased expression of E-cadherin and the concomitant decrease of vimentin, α-SMA, Snail, Slug, Zeb1, Zeb2, and Twist, related to a reduction of metastatic properties[134]. Among recently synthesized LOXL2 inhibitors, the (2chloropyridin-4-yl) methanamine 20 has been shown to be the most potent one[135]. However, also miRNAs exhibit an inhibiting effect on LOXL2 expression. In particular, miR-26a-5p and miR-29a-3p are able to downregulate LOXL2 expression, counteracting the development of TRS and the establishment of a premetastatic niche in HCC[136]. Other miRNAs, such as miR-26a/b, miR-29a/b/c and miR-218, have been shown to suppress LOXL2 levels, resulting in reduced cancer cell proliferation, migration, invasion and metastatic abilities in different tumors[137,138].

CAFs, as potential therapeutic targets

CAFs are mainly considered pro-neoplastic cells for their role in matrix remodeling as well as their interaction with cancer and other stromal cells to promote cancer progression. Then for this simple reason, they can be considered as a potential target for CCA treatment.

It is important to point out that CAFs, even if classified recently in multiple subsets, resemble a genetically stable and a more homogeneous cell phenotype compared to cancer cells which can undergo different genetic/epigenetic alterations originating a highly heterogeneous compartment. Therefore, CAFs represent theoretically easier and more effective CCA targets than tumor cells[94].

Literature data showed that BH3 mimetic navitoclax (BH3-only protein mimetics) was able to selectively promote Bax-dependent apoptosis in α-SMA$^+$ CAFs but not in normal fibroblasts or CCA cells. In this connection, in an orthotopic rat model of CCA, navitoclax selectively removed CAFs from TRS, resulting in decreased tumor growth and metastasis and improved survival, pointing out that the depletion of CAFs represents a promising CCA therapy[139].

Interestingly, it has been reported that CAFs can stimulate stemness features in MDSCs by acting on BLT2 in iCCA cells through LTB4, a downstream metabolite of 5-LO. This is relevant since high expression of tumoral BLT2 was related to iCCA patients' poor survival. Moreover, in subcutaneous patient-derived xenografts in NSG mice, treatment with a BLT2 antagonist alone did not affect tumor growth, while the combination of this molecule with gemcitabine resulted in decreased tumor cell proliferation[89].

Speaking of tyrosine kinase inhibitors able to abolish CAFs activities, Nintedanib is observed to affect FGFR, VEGFR and PDGFR, resulting in a reduction of CAF proliferation, α-SMA levels and pro-neoplastic effects, as well as in inhibition of tumor growth and of α-SMA positive CAFs, in *in vivo* study. Combination therapy with Nintedanib and gemcitabine was much more effective in inhibiting tumor growth than the single treatments[140].

In this connection, Imatinib mesylate, a tyrosine kinase inhibitor that can block PDGF-D signaling, has shown discouraging preliminary results. Similar to imatinib mesylate, the use of EGFR inhibitor gefitinib in clinical trials has given poor results for CCA treatment[69].

In addition, CAFs-conditioned medium (CM) promoted IL-6 mediated motility of CCA cells and CM from Resveratrol-treated cells completely abolished this effect by increasing the expression of E-cadherin and decreasing N-cadherin levels. Therefore, a nutraceutical like resveratrol could inhibit the malignant phenotype of cancer cells by acting on CAFs-related secreted factors[141]. By contrast, the inhibition of the Hedgehog (HH) signaling, stimulated by PDGF-B secreted by CAFs, has displayed promising effects in inhibiting CCA growth and progression. Cyclopamine's severe side effects in mice limited this drug to preclinical studies, while Vismodegib, a second-generation cyclopamine derivative, has been unveiled to be an effective anticancer agent in cholangiocarcinoma[142]. Hedgehog-antagonizing agents have also been displayed to be helpful in combination with conventional chemotherapy to treat cholangiocarcinoma, suggesting the use of hedgehog antagonists as therapeutic agents for this type of cancer[143-145].

Moreover, since CCA patients with low stromal expression of IL-6 and active autophagy pathway in cancer cells showed a better prognosis and a more effective response to post-surgery chemotherapy, IL-6 can represent another therapeutic target for CCA. Along these lines, impaired IL-6 production in CAFs by siRNA showed that IL-6 augments the autophagy-linked apoptotic response to 5-FU in human CCA cells. Indeed, impaired autophagy due to stromal inflammation was demonstrated in an experimental animal model of cholangiocarcinoma[146].

Finally, a novel therapeutic approach reveals that light-activated nano hyperthermia can affect the tumor microenvironment. Indeed, the use of multifunctional iron oxide nanoflowers decorated with gold nanoparticles (GIONF) could deplete CAFs from TRS, then reducing stroma stiffness and cancer regression[147] [Figure 1 and 2].

Targeting TAMs in CCA treatment

Actually, TAMs-oriented therapeutic approaches should aim at: (1) restoring M1 phenotypical polarization; (2) depleting TAMs; (3) interfering with the crosstalk between TAMs and cancer cells[119,148]. Boulter *et al.* reported a positive effect of liposomal clodronate (Lipclod) in ablation of TAMs: in this study, the injection of Lipclod in a xenograft model of human CCA cells as well as in TAA-induced rat CCA model, resulted in: i) a significant decrease of macrophages infiltration and consequent reduction of Wnt7b and related pro-proliferative genes (CCNE, CCND2, and BIRC5); ii) an increased number of apoptotic events associated with increased expression of BAX1; iii) a reduction of tumor growth[120]. Similarly, in the same experimental models, the inhibition of Colony stimulating factor 1 receptor (CSF-1R) with W2580 or AZD7507 counteracted macrophage recruitment and activation, resulting in the inhibition of iCCA growth and a decreased tumor burden[120]. Along these lines, Dwyer *et al.* reported a reduction of $F4/80^+$ and $CD206^+$ macrophages in SNU-1079-generated human cells xenograft model treated with an anti-MCP1 antibody (2H5): these mice showed significantly smaller tumors compared to control mice[149]. A similar effect was observed in TAA-induced mice CCA model with knockdown of fibroblast growth factor-inducible 14 (Fn14). The knockout of Fn14 blocked the TWEAK/Fn14/NF-Kb pathway, resulting in reduced MCP-1 levels, decreased TAMs accumulation and reduced tumor growth[149]. Yang *et al.* also observed a reduction in TAMs infiltration and iCCA progression by blocking CCL5 with specific siRNA raised against atypical protein kinase C iota ($aPKC_\iota$-siRNA), a protein involved in the induction of EMT[150]. Finally, concerning TAMs recruitment, Durvalumab (monoclonal antibody anti-PD-L1) given in combination with a CSF-1R inhibitor (SNDX-6532) is currently under ongoing clinical trial in cohorts of iCCA patients

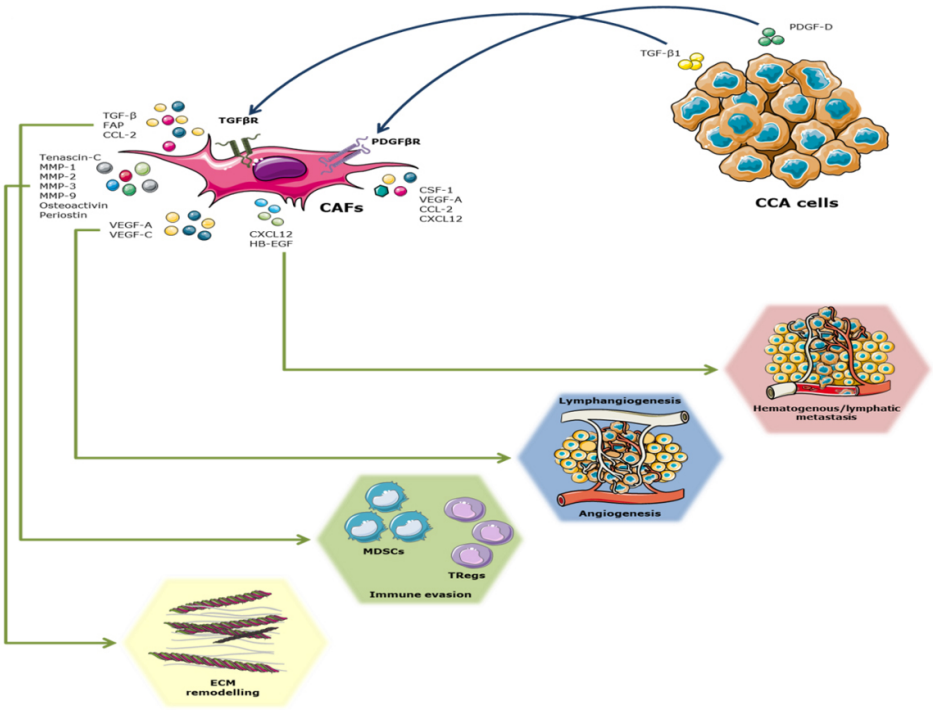

Figure 1. Role of CAFs in CCA progression. Functional crosstalk between CCA cancer cells and CAFs from a cancer mechanics perspective. As mentioned and described in the review text, CAFs affect the tumor microenvironment, modulating ECM remodeling, angiogenesis and lymphangiogenesis, metastasis and immune evasion, and then provide a microenvironment favorable to CCA progression.

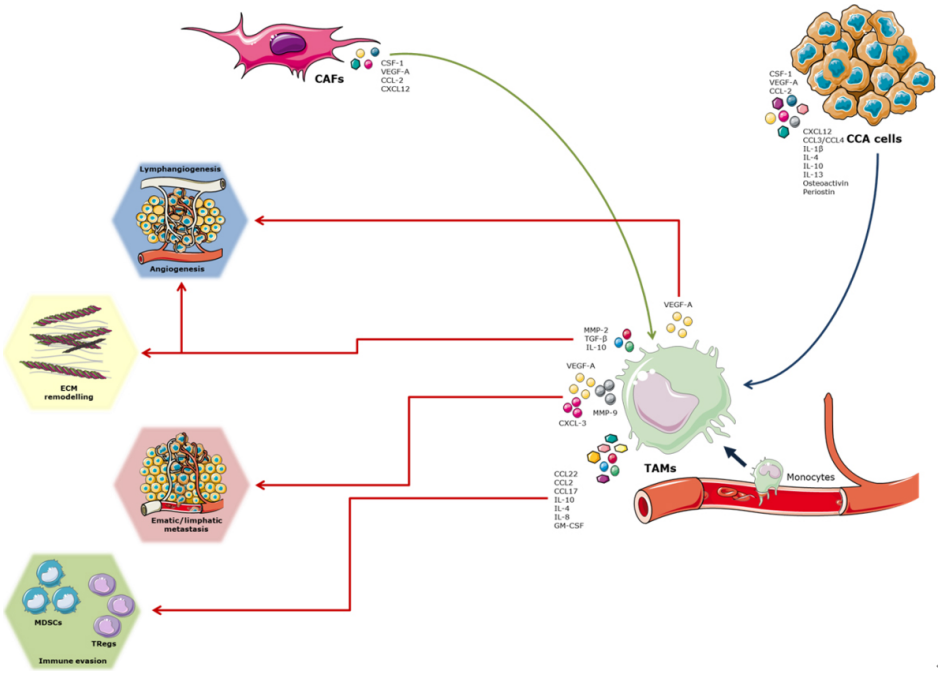

Figure 2. Role of TAMs in CCA progression. Graphic representation of the crosstalk between CAFs, TAMs and CCA cells during progression of cholangiocarcinoma. As described in the review text, TAMs can modulate different aspects of CCA progression, including ECM remodeling, angiogenesis and lymphangiogenesis, metastasis, and immune evasion.

(NCT04301778).

In a different approach, Diggs *et al.* have reported that the use of agonistic anti-CD40 antibody (clone FGK4.5) in combination with PD-1 therapy in different murine models (subcutaneous and intrahepatic tumor injection model, or AKT250 and YAP-driven mouse CCA model) can prevent the phenotypical switch from M1 to M2, resulting in reduced tumor growth, improved survival, increased infiltration of $CD4^+$, $CD8^+$ and NK immune cells and enhanced response to chemotherapy[151]. Actually, the drug GSK2636771, a PI3Kγ inhibitor, is under evaluation in the MATCH Screening Trial for solid tumor patients, including iCCA patients (NCT02465060), to verify its ability to revert TAMs polarization from the pro-neoplastic M2 to the anti-tumoral M1 phenotype. Finally, the use of antibodies against CD-47 (B6H12.2) in a transplenic intrahepatic metastasis mouse model led to enhanced macrophage phagocytosis of all macrophage subtypes (M1, M2, and TAMs-like monocyte-derived macrophage) and consequently suppressed CCA growth and metastasis[152].

CONCLUSION

CCA is an extremely aggressive and rare malignancy with limited treatment alternatives. The majority of patients are diagnosed at an advanced stage and do not qualify for potentially curative surgical treatments, making CCA an increasingly prevalent global challenge. CCA is characterized by a highly reactive desmoplastic stroma. As discussed above, a strict relation exists between the tumoral and stromal compartment, with complex mechanisms underlying the mutual interactions between CCA cells, CAFs, TAMs, and immune cells. Accumulating evidence demonstrates that each component of TRS (particularly CAFs, TAMs) has a critical role in affecting CCA progression, by directly or indirectly acting on ECM remodeling, angiogenesis and lymphangiogenesis, metastasis as well as by favoring immunosuppressive microenvironment [Figure 1]. Therefore, an improved understanding of cell interactions within the CCA microenvironment could enable the development of combinatorial therapies targeting both intrinsic (tumor cells-dependent) and extrinsic (stromal cells/TRS-derived) compartments and could have a concrete impact on clinical practice and patient outcomes.

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Authors' contributions

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