



# Impact of microenvironment and stem-like plasticity in cholangiocarcinoma: Molecular networks and biological concepts

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## Summary

Clinical complexity, anatomic diversity and molecular heterogeneity of cholangiocarcinoma (CCA) represent a major challenge in the assessment of effective targeted therapies. Molecular and cellular mechanisms underlying the diversity of CCA growth patterns remain a key issue of clinical concern. Crucial questions comprise the nature of the CCA-origin, the initial target for cellular transformation as well as the relationship with the cancer stem cells (CSC) concept. Additionally, since CCA often develops in the context of an inflammatory milieu (cirrhosis and cholangitis), the stromal compartment or tumour microenvironment (TME) likely promotes initiation and progression of this malignancy, contributing to its heterogeneity.

This review will emphasize the dynamic interplay between stem-like intrinsic and TME-extrinsic pathways, which may represent novel options for multi-targeted therapies in CCA.

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## Introduction

Cholangiocarcinoma (CCA) is a highly malignant and heterogeneous adenocarcinoma of biliary-epithelial cells. CCA arises in the biliary epithelium [1] and together with hepatocellular carcinoma (HCC), represents the major primary liver cancer in adults. Over the past two decades, the incidence and mortality rate of CCA has increased worldwide, accounting for approximately 10% of primary liver cancer. For 70% of patients, tumours are

unresectable, advanced and metastatic. The 5-year survival rate for these patients is 0% to 10% [2].

CCA comprises several pathological entities that differ in gross clinical appearance and tumour localization (ILCA guidelines 2013 [3]): intrahepatic CCA (iCCA) develops in small intrahepatic bile ducts, whereas extrahepatic ductules give rise to both tumours in the bifurcation of the common bile duct (perihilar, pCCA) and in the distal biliary tree (dCCA) [2] (reviewed in [4]). These subtypes of CCA show partly different epidemiological behaviour and are associated to some degree with different risk factors, diverse origins and dissimilar backgrounds [5–7]. These malignancies are very heterogeneous in terms of cellular morphology, genomic alterations and response to drug-therapy. Thus, the CCA molecular classification (reviewed in [8,9]) is still intensely debated and currently not adapted to clinical trial design as well as decision-making. Several recent molecular classifications were proposed, enriching patient groups based on receptor tyrosine kinase (EGFR and HER2), MET, JAK/STAT, RAS/MAPK PI3K/AKT/mTOR, and inflammation (e.g., COX2) [10,11].

This review will focus on the current understanding of the pathobiological and molecular aspects of CCA initiation and progression, taking into consideration acquisition of stem-like properties and the cell-of-origin concept. Furthermore, we aim to emphasize the key role of CCA-associated inflammation and the biliary tumour microenvironment (TME) in the onset of this malignancy that may represent a potential therapeutic target.

### Key Points

- Cholangiocarcinoma is characterized by complex molecular heterogeneity, stressing the difficulty of optimal clinical management
- Therapeutic progress in liver cancer is hampered by genetic diversity and a lack of clear oncogene addiction. However, deep sequencing has identified several recurrent somatic mutation
- This malignancy often develops in the context of an inflammatory milieu e.g., cirrhosis and primary biliary or sclerosing cholangitis
- The tumour microenvironment or stromal compartment may represent a novel target option (e.g., JAK/STAT pathway, COX2 and NF-κB)

Keywords: Cholangiocarcinoma; Cancer stem cells; Tumour microenvironment; Tumour associated macrophages.

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Abbreviations: CCA, cholangiocarcinoma; CSC, cancer stem cell; HPC, hepatic progenitor cell; TME, tumour microenvironment; TAM, tumour-associated macrophages; iCCA, intrahepatic CCA; PSC, primary sclerosing cholangitis.



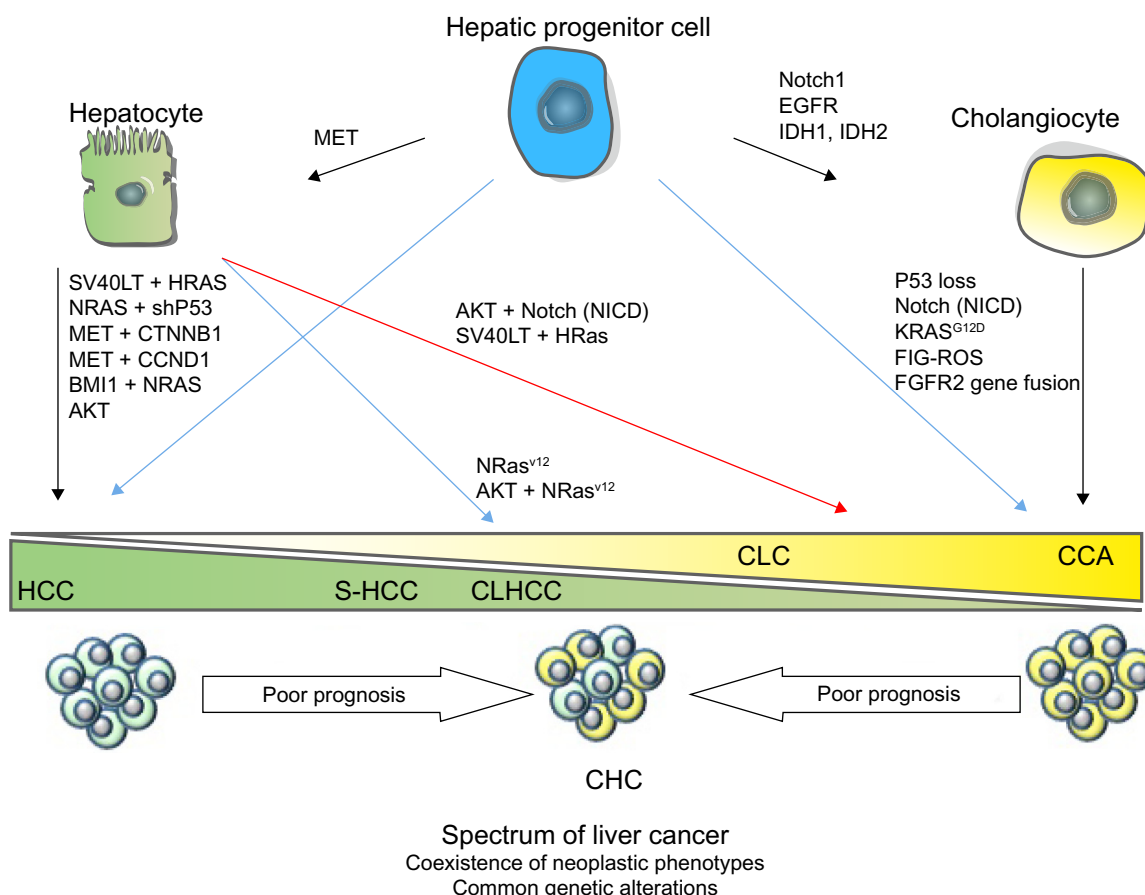
**Multiple cell origin of CCA**

The cellular origin of CCA has been intensely debated in the last years. In this category of tumours, molecular similarity is clinically important for therapeutic decisions and efficacy. Detailed analysis of the CCA spectrum has demonstrated the existence of rare mixed tumour types, such as CCA-HCC (CHC), with intermediate characteristics between HCC and iCCA [12]. This suggests that at least a subset of liver cancers could share a common hepatic stem/progenitor cell origin [13–18].

Hepatic progenitors cells (HPCs) reside in the smallest branches of the intrahepatic biliary tree: ductules and canals of Hering. HPCs act as a reserve cell compartment and are activated when hepatocytes and/or cholangiocytes are damaged (e.g., cholestasis) or their replication inhibited [19,20].

HPCs, situated in the canal of Hering, are bipotential; consequently, they can differentiate into hepatocytes or cholangiocytes. Interestingly, Kitade *et al.* recently described the role of the EGFR/NOTCH1 positive feedback-loop for HPC commitment towards the biliary epithelial cell lineage, while hepatocyte differentiation is driven by MET signalling [21]. After differentiation into malignant cells, bipotential HPCs undergo maturation

arrest and transformation, giving rise to a complete spectrum of tumour phenotypes with diverse hepatocellular and cholangiocellular differentiation characteristics e.g., cholangiocellular carcinoma (CLC) and CHC collision tumours. CLC is a subtype of CCA, in which more than 90% of the tumour is composed of small monotonous and/or anastomosing glands [20]. Besides its CCA-cellular region, these tumours also contain an HCC-like trabecular area, demonstrating the coexistence of multiple neoplastic phenotypes, which complicate CCA biology and ultimately treatment strategies. Since CLC tumours present both hepatocytic and cholangiocytic phenotypes, Komuta *et al.* proposed that CLCs might originate from HPCs [13,19,20]. In addition, CHC tumour cells simultaneously express hepatocytic (AFP) and cholangiocytic (CEA or CK19) markers along with the haematopoietic stem cell marker c-Kit [22], which leads to the conclusion that the primary liver intermediate carcinoma may be a distinct subtype that arises from bipotential HPCs. Thus, consistent with a progenitor cell origin, a whole range of phenotypic traits, unique to hepatocytes and cholangiocytes as well as progenitor cells, can be seen in CHC. Recently, a novel HCC subtype with molecular similarity to CCA (CCA-like HCC, CLHCC) was characterized by genomic analysis and stratified as an HCC-type



**Fig. 1. Multiple cells-of-origin in cholangiocarcinoma.** Cholangiocarcinoma (CCA) may originate by deregulation of oncogenic programs in a range of liver lineages. Alternatively tumours dedifferentiate and acquire progenitor features during carcinogenesis (dedifferentiation theory, red arrow). CCA heterogeneity may derive from different cells-of-origin as well as from diverse genetic mutations responsible for the dynamic differentiation/de-differentiation processes among different tumour subtypes. (HCC, hepatocellular carcinoma, CLC, cholangiocellular carcinoma; CHC, mixed CCA-HCC tumour types; CLHCC, CCA-like HCC; S-HCC, scirrhous HCC, a variant of HCC with abundant fibrous stroma).

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tumour that expresses CCA-like genes [23]. The authors found that CLHCC co-expressed characteristics of embryonic stem cells and hepatoblast-like genomic traits, suggesting its derivation from bipotential HPCs. Furthermore, this study provided insights into the heterogeneous progression of liver cancer, which imply a common cellular origin from different developmental stages. This paradigm was recently supported in a meta-analysis, which suggested that both iCCA and HCC share common genetic alterations, such as copy number variations (CNVs), including chromosomal gains (1q, 8q, and 17q) and losses (4q, 8p, 13q, and 17p), with high-level amplifications of 11q-13 [8]. This suggests that iCCA and HCC may be closely related at the molecular level. Indeed, a close genomic similarity between iCCA and a subset of HCCs with progenitor cell characteristics was shown in several recent studies [9–11]. Moreover, genomic [24,25] and genetic [26–28] analyses of CHC show closely related molecular alterations, suggesting that acquisition of CCA-like characteristics play a critical role in the heterogeneity of liver tumours with poor outcome and limited therapeutic potential.

The phenotypic complexity and presence of progenitor cell features in CCA can potentially be explained in two ways: either the cell-of-origin is a progenitor cell and/or alternatively tumours dedifferentiate to acquire progenitor features during transformation (dedifferentiation theory (reviewed in [29–31])). To this end, Fan *et al.* [32] recently suggested that iCCAs grow by lineage conversion, a process thought to occur during malignant transformation of hepatocytes through the simultaneous activation of e.g., NOTCH1 and AKT signalling. This study showed that dedifferentiation of transformed hepatocytes can form the basis for a malignant conversion into a cholangiocellular differentiation path, potentially contributing to the acquisition of stem/progenitor cell characteristics, which have been proposed to drive the growth of iCCA. Intriguingly, Holczbauer *et al.* [33] recently provided direct evidence that any cell in the hepatic lineage has the potential to be the designated cell-of-origin, and can give rise to the complete spectrum of liver cancers. In this study, the authors were able to perform a side-by-side comparison of liver tumourigenesis after transducing mouse primary HPCs, lineage-committed hepatoblasts and differentiated adult hepatocytes with transgenes encoding the oncogenes H-Ras and SV40LT. In support of the hypothesis that multiple cell types can be transformed and

converted to iCCA, Sekiya and Suzuki used the thioacetamide mouse model, and showed that in the early stages of tumour formation, Notch-mediated conversion of hepatocytes into biliary lineage cells could give rise to iCCA [34]. However, recently in the context of chronic biliary inflammation and p53 loss, Guest *et al.* emphasized the role of the biliary epithelium as the target of transformation and origin of iCCA formation [35]. This study further supports the evidence of Notch as a driver of biliary oncogenesis, demonstrating active signalling within the ductular tumour epithelium.

Although a marked diversity/plasticity of the underlying cell-of-origin is emerging from recent studies, current evidence suggests that most iCCA tumours are derived from undifferentiated cells with a stem-like capability (Fig. 1).

### Stemness features of CCA

Currently, the concept of stemness-driven carcinogenesis has added a new level of complexity in understanding CCA heterogeneity and drug resistance. Cancer stem cells (CSCs) represent a therapeutic challenging subpopulation, responsible for tumour initiation, progression and relapse. Based on similar properties of normal stem cells, CSCs are capable to self-renew, produce heterogeneous progeny, and divide unlimited (reviewed in [36]). It is equally plausible that CSCs may be derived from a restricted progenitor cell as well as from a more differentiated cell type. Notably, the cell-of-origin represents a stem-like cell that has acquired a cancer-promoting genetic or epigenetic alteration, and is not necessarily associated with the CSC-concept. Although, it has already been shown that HCC progression is driven by CSCs [37–40] very few studies have indicated that CCAs harbour phenotypic features of stem/progenitor cells (reviewed in [41]). Consistently, CSC-surface markers were recently proposed in CCA and include the expression of CD133 [42], CD24 [43], EpCAM [44], CD44 [45], and CD117 [46].

In order to develop novel CCA target strategies, it is important to shed light on the pathobiological and clinical aspects of putative stem-like features in this malignancy. Despite the molecular and phenotypic heterogeneity of CCA, in the next section we aim to highlight key regulatory pathways involved in promoting and maintaining CCA stem-like traits (Table 1).

**Table 1. List of stemness-related pathways in CCA.**

Pathways	Actionable targets	Role	References
Notch	Akt, c-myc	Maintenance of stem cell including hepatic progenitor cells	[32, 41, 47-52]
Wnt/ $\beta$ -catenin	c-myc, cyclin D1, survivin, CD133, EpCAM	Embryogenesis, maintenance of self-renewal	[41, 53, 54]
Hedgehog	Sonic-Hedgehog, PDGF-BB, Jagged-1, Notch	Embryonic development, cell differentiation, regeneration, stem cell biology	[41, 53, 55-57]
Hippo	YAP, WW45	Regulating expansion of liver progenitor cells (oval cells)	[58-70]
PI3K/AKT/PTEN	AKT/PTEN	iCCA pathogenesis	[1, 32, 41, 54, 71]
TGF- $\beta$ /IL-6	JAK/STAT	Self-renewal, EMT	[9, 54, 71-76]
RAS/RAF/MEK/ERK	MAPK	HPCs proliferation	[10, 41, 77, 78]

## Regulatory pathways involved in CCA-associated stemness: Emerging therapeutic targets

### Notch pathway

The Notch canonical signalling pathway is a highly conserved pathway controlling cell differentiation, proliferation and apoptosis, as well as maintenance of stem cells (including hepatic progenitor cells) ([41,47], reviewed in [48]) and morphogenesis of bile ducts (reviewed in [49]). In mammals, Notch receptors (Notch 1-4) are activated by five ligands (Jagged1, Jagged2, and Delta-like ligands 1, 3, and 4), responsible for the switch-on of downstream target genes, such as *Hes1* and *Hey1*. The expression of Notch receptors 1 and 3 correlates with cancer progression and poor survival in CCA [41], whereas the overexpression of Notch receptors 1 and 4 in HCC may exert tumorigenic effects [50]. Recently Fan *et al.* demonstrated that activated Notch e.g., the Notch intracellular domain (NICD), and Akt deregulation determined an oncogenic phenotypic switch from adult hepatocytes into precursors of iCCA, which express cholangiocyte markers, such as CK19 and SOX9 [32]. Since Notch signalling can contribute to either CCA or HCC, Villanueva *et al.* suggested that Notch could be deregulated in bipotential HPCs that are able to differentiate into either hepatocytes or cholangiocytes, depending on the supportive microenvironment [47]. Interestingly, Zender *et al.* convincingly showed that overexpression of the NICD in mouse livers caused the onset and development of iCCA [51]. Importantly, these tumours retained plasticity and features of bipotential HPCs; at 7 months, small epithelial/gland-like lesions in the livers stained positive for CK7, CK8, CK17, CK18, CK19, and CD34. The surrounding liver tissue showed a desmoplastic reaction similar to what is often observed in human iCCA (reviewed in [52]). Also, NICD, overexpressing bipotential HPCs, subcutaneously transplanted gave rise to tumours with CCA characteristics, whereas progenitor cells, overexpressing c-MYC/AKT, gave rise to HCC/hepatoblastoma-like tumours.

### Wnt/ $\beta$ -catenin signalling pathway

The Wnt/ $\beta$ -catenin pathway plays a crucial role in embryogenesis and its activation is essential for the maintenance of self-renewal (reviewed in [53]). Wnt ligands bind to Frizzled receptors, promote  $\beta$ -catenin translocation, TCF/LEF1 co-activation and induction of key liver cancer genes such as c-Myc, cyclin D1, Survivin. Notably,  $\beta$ -catenin is expressed in 58% of CCAs. In 8% of cases,  $\beta$ -catenin is mutated, which likely promotes the HPC compartment and is considered an early determinant in CCA-progression [41]. Additionally, Wnt expression is stimulated by commonly used progenitor markers, such as CD133 and EpCAM [54].

### Hedgehog signalling pathway

The Hedgehog (Hh) signalling pathway is associated with embryonic development, cell differentiation and stem cell biology. Activation of this pathway promotes CCA proliferation and survival, in addition to HCC carcinogenesis. Indeed, in 60% of HCCs there is an overexpression of Sonic Hedgehog (SHH), the predominant ligand of the Hh pathway in the liver. Importantly, in the context of stemness, Hh promotes HPC proliferation [41,53].

Further, SHH expression was shown to regulate both PDGF-BB [55] and Jagged-1 [56] signalling. As such, in a model of bile duct

ligation, Xie *et al.* demonstrated that impaired Hh signalling, inhibited the Notch pathway, and that Hh and Notch cooperate to control cell fate in adult liver repair [57].

### Hippo signalling pathway

The Hippo-signalling cascade is an evolutionarily conserved pathway implicated in multiple events during embryonic development and tumour onset [58–60]. Strong evidence also points to a significant role of Hippo signalling in stem cell regulation e.g. in HPCs (also termed oval-like cells) [61–63].

Recently Lu *et al.* [61] demonstrated that differentially regulated transcripts, affected by reduced Hippo-signalling, are in hepatocytes mostly involved in cell movement and immune response. This finding is consistent with an inflammatory or oval cell response, both of which occur following liver injury in situations where hepatocyte proliferation is attenuated [64–66].

Moreover, Lee *et al.* demonstrated that livers obtained from WW45 Liv-cKO mice were markedly larger than those of control animals, showing a notable increase in the number of immature progenitor cells (or oval-like cells) in the liver [63]. Importantly, these mice developed tumours with a mixed CHC-like phenotype, a tumour type thought to originate from transformed oval cells [67–69]. Indeed, liver/CHC tumours obtained from WW45 Liv-cKO mice were enriched in transformed oval cells. Consistently, Kim *et al.* provided clinical and pathological evidence that YAP1 (Yes-associated protein 1, a primary effector of the Hippo cascade) is frequently expressed in HCC and CHC mixed tumour types, which retain stemness-related markers, such as EpCAM and K19 [62].

Furthermore, recently it was proposed that the constitutive activation of YAP in the bile duct and in association with AKT is essential in inducing iCCA in a murine biliary injury model through a IL-33/ILC2/IL-13 circuit [70].

### PI3K/PTEN/AKT network

AKT signalling can be activated downstream of tyrosine kinase receptors, PI3K constitutive activation and loss of the phosphatase and tensin homolog (PTEN) [1]. *PTEN* is a tumour suppressor gene that antagonizes PI3K activity. *PTEN* deletion results in proliferation of CD133<sup>+</sup> cells and treatment of this cell population, using an AKT inhibitor, enhances the efficacy of radiation- and chemotherapy [41,54]. Fan *et al.* showed that the co-activation of AKT and N-Ras oncogenes caused the development of CHC-like liver tumours [32]. It was speculated that this malignant transformation could be the result of an expansion of HPCs or even through malignant conversion of hepatocytes into progenitor-like cells [32]. As such, the PI3K/AKT/PTEN network holds great promise as a therapeutic target in CCA and currently several clinical trials are ongoing (reviewed in [71]).

### TGF- $\beta$ /IL-6 and JAK/STAT signalling

The transforming growth factor- $\beta$  (TGF $\beta$ ) pathway plays a key role in the self-renewal and maintenance of the undifferentiated stem-cell state (reviewed in [72,73]). Disruption of the TGF $\beta$  pathway impairs stem cell differentiation and causes deregulated proliferation of HPCs, resulting in CCA development [54]. Early in tumour initiation, TGF- $\beta$  acts as a tumour suppressor, whereas at late stages it promotes tumour growth, metastasis and

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epithelial-mesenchymal transition (EMT) [74]. Among the processes involved in reprogramming, EMT likely plays an important role in the maintenance of stem-cell features. Sato *et al.* have demonstrated that TGF- $\beta$ 1/Snail activation induces EMT in CCAs *in vitro* and *in vivo* in resected CCAs, showing a strong correlation with an aggressive phenotype of CCA [74]. As such, inhibition of the TGF- $\beta$ /IL-6 pathway in HPCs may represent a novel therapeutic target in the clinical management of CCA [54].

Isomoto *et al.* previously demonstrated that IL-6-mediated STAT3 signalling is sustained in human CCA cells [75]. Binding of IL-6 to the gp130 receptor phosphorylates and activates the JAK/STAT pathway. STAT3 induces the transcription of target genes essential for cellular growth, differentiation and proliferation (reviewed in [9,71]). Furthermore, Zheng *et al.* showed that Gankyrin is an essential regulator of CCA tumour growth and metastasis [76]. This was achieved by activation of the IL-6/STAT3 signalling axis.

### RAS/RAF/MEK/ERK signalling pathway

The RAS/RAF/MEK/ERK cascade is a highly conserved signal transduction axis, whose activation results in a number of different physiological outcomes, including HPCs proliferation [41]. A global genomic and mutational profiling by Andersen *et al.* revealed a poor outcome of patients with mutated *KRAS*, characterized by deregulation of oncogenic signalling pathways together with an enrichment for CCA stem cell-like signatures [10]. Therefore, targeting the RAS/RAF/MAPK cascade using a MEK1/2 inhibitor e.g., selumetinib, originally used against metastatic biliary cancers [77], may represent an attractive therapeutic alternative in some iCCA cases.

Importantly, *KRAS* mutations have been detected in 30% of bile, taken from patients with primary sclerosing cholangitis (PSC), suggesting that it might be a potential event contributing to the malignant transformation of cholangiocytes [78].

### CCA-associated inflammation

Dissection of the molecular mechanisms underlying iCCA development has revealed a strong link between inflammation and tumorigenesis. During the course of chronic inflammation and cholestasis the biliary microenvironment releases endotoxins (e.g., lipopolysaccharides (LPS), pro-inflammatory cytokines (tumour necrosis factor- $\alpha$ , TNF- $\alpha$ ) and various interleukins (IL-1 $\beta$ , IL6) [79], which render the hepatic microenvironment favourable and permissive for cancer initiation, progression and resistance to anticancer therapeutics. In addition, recent studies have shown that cholangiocytes produce and release cytokines, such as IL-6, IL-8, TGF- $\beta$ , TNF- $\alpha$ , and the platelet-derived growth factor B chain (PDGF-B), all of which interact with the biliary epithelium in an autocrine/paracrine manner, thus regulating biliary cell homeostasis (reviewed in [80,81]). It is clear that this interplay between inflammatory signals and bile duct homeostasis plays an important role in biliary carcinogenesis [82]. An explanation of the phenomenon may be that intracellular cholangiocyte signalling, which elicits the development and growth of biliary tract cancers is altered during the course of malignancy in response to cytokine and growth factor stimuli.

Canonical IKK $\beta$ -dependent NF- $\kappa$ B signalling, a regulator of cell survival, immunity and inflammation, is a key pathway during

liver injury and inflammation (reviewed in [83]). Recent studies have shown that NF- $\kappa$ B and STAT3 signal transduction is engaged in extensive crosstalks in liver injury, inflammation and cancer formation [82]. Moreover, Anson *et al.* showed that by activating pro-inflammatory (e.g., IFN- $\gamma$ ) and anti-inflammatory (e.g., IL-4) mediators,  $\beta$ -catenin signalling produces an inflammatory milieu, responsible for aggressive liver tumour growth [84]. Further, altered cytokine profiles (CCL2, CCL5, CXCL10) may elicit a response not only in tumour cells but also in the surrounding liver parenchyma. As such, in a recent gene profile Andersen *et al.* laser micro-dissected a subset of tumours from iCCA patients and identified a stromal signature associated with poor prognosis [10]. This signature was characterized by upregulation of *IL6* and *TGF $\beta$ 3* gene expression and deregulation of chemokine receptors and ligands (*CXCR4*, *CCR7*, *CCL2*, *CCL5*, *CCL19*, *CCL21*), cytokine receptors (*IL3RA*, *IL7R*, *IL10RA*, *IL18RAP*) and interleukins (*IL6*, *IL16*, *IL33*). In addition, Sia *et al.* stratified iCCAs into two prognostic patient subsets with "inflammation" or "proliferation" phenotypic characteristics and enriched for immune-related signalling and STAT3 activation or oncogenic addiction (RAS, MAPK, and MET activation), respectively [11].

Furthermore, it is known that patients affected by biliary atresia retain high serum level of IL33. Consistently, IL33 is present in murine bile ducts with experimental biliary atresia. Li *et al.* recently demonstrated that administration of IL-33 to mice markedly increased the growth of cholangiocytes [70]. Notably, induction of the IL-33/ILC2/IL-13 circuit, in association with a constitutive activation of AKT and YAP in bile ducts, promotes iCCA in a murine model of biliary injury [70].

Although our current understanding of the immune-component in CCA is still limited, recent evidence strongly suggests a potential role of the inflammatory-controlled microenvironment in CCA onset, progression and clinical severity.

### Biliary tumour microenvironment

Molecular changes that regulate cell proliferation; survival and/or differentiation are well-known 'initiators' of tumour development. These events occur in the specific context of the tumour stroma. The tumour microenvironment (TME) represents a dynamic and actively causal component that supports aggressive tumour growth. It augments tumour heterogeneity and as such contributes to tumour progression, invasion and drug resistance.

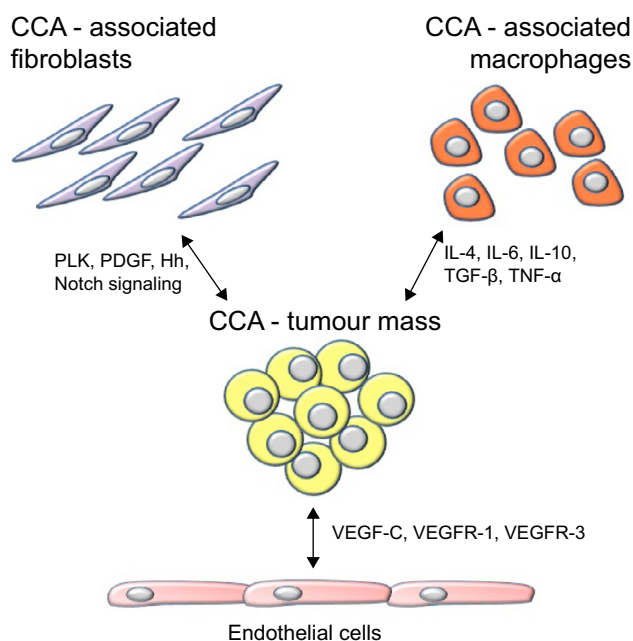
All subtypes of CCAs are associated with rapid proliferation of tumour-associated stromal cells, which contribute to the desmoplastic nature of this cancer. Cancer-associated fibroblasts (CAF) are key players in CCA invasiveness and in the generation of a desmoplastic reaction in CCA. Stromal cells, isolated from surgically resected CCAs, were recently characterized, and showed vimentin/ $\alpha$ -SMA-positivity and CK7/CK19-negative staining [85]. Primary cultures of human bile duct epithelial and stromal cells from CCA surgical specimens are in development, and these could represent powerful tools to investigate CCA tumour epithelial/stromal interactions [85]. Tumour spread requires that tightly adherent epithelial cells convert in to a more motile phenotype, expressing several mesenchymal features. During this process, typical mesenchymal programs are stimulated, including activation of specific molecules such as S100A4, a member of the S100 family of small calcium-binding proteins. S100A4 is commonly expressed by mesenchymal cells, macrophages as well

as epithelial cells during EMT [86]. Recently, Fabris *et al.* showed that after surgical resection nuclear S100A4 could be used as a prognostic marker for a subset of CCA patients [87]. Nuclear expression of S100A4 promotes invasiveness and metastasis of CCA cells, indicating that S100A4 is a potential therapeutic target. The role of CAFs or myofibroblasts (MFs) has recently demanded more attention since a crosstalk between MFs and the tumour epithelium itself may be promoting tumour growth (reviewed in [52]), and even correlate with CCA survival [88,89]. In the liver, MFs are derived from e.g., activated hepatic stellate cells (HSC), whose transition is stimulated by Hh signalling [90]. The MF-to-CCA crosstalk furthermore involves several signalling pathways, such as PLK [91], PDGF [92,93], Hh [56], and Notch [56,57]. In particular, Cadamuro *et al.* indicated that CCA cells recruit CAFs by secreting PDGF-D, which stimulates fibroblast migration through PDGFRB, Rho GTPase and JNK activation [94]. Recently, a key role of PLK2 was identified that links PLK and Hedgehog signalling in TRAIL-induced cell death, by demonstrating the ability of Hh to regulate PLK2 expression [91]. This event stabilizes Mcl-1, and thus confers resistance to TRAIL. Fingas *et al.* concluded that targeting either Hh or PLK signalling might restore CCA cell susceptibility to TRAIL-induced apoptosis [91,92]. Besides the involvement of several signalling pathways with potential therapeutic aim, which impact CAFs' role in tumour progression, CAFs themselves have been highlighted as a target. In two recent studies, BH3 mimetics were demonstrated in animal models to reduce the tumour burden by effectively targeting the stroma [95,96].

In addition to CCA-associated fibroblasts, the TME is enriched in a wide spectrum of immune cells that may exert a dual role in tumour development and progression. Indeed, immune cells can directly eliminate tumour cells or participate in the induction of an antitumoural immune response (reviewed in [97]). However, immune cells can also be recruited and appropriately programmed by tumour cells to favour growth and progression (reviewed in [97]). Tumour-associated macrophages (TAMs) are characterized by a distinct phenotypic polarization referred as "M1 and M2". M1-polarized macrophages manifest high levels of pro-inflammatory cytokines (IL-1, TNF- $\alpha$ , IL-6, and IL-23), high production of reactive nitrogen (NO<sup>-</sup>) and oxygen (ROS) intermediates that contribute to their tumouricidal activity and antitumour immunity. On the other hands, M2 macrophages serve as the main players, facilitating parasite containment, tissue remodelling and immune tolerance, which may be linked to tumour progression ([98–100], reviewed in [101]). Although many studies have shown the contribution of TAMs to tumour development and poor prognosis, the significance of TAM infiltration in human CCA is still unclear. However, Hasita *et al.* recently described an association between the ratio of CD68<sup>+</sup>/CD163<sup>+</sup> macrophages, regulatory T cells (Tregs) and vessel number in iCCA [102]. This study showed that the degree of microvascularization and tumour-infiltrating Treg cells was more intimately correlated with the number of CD163<sup>+</sup> M2 macrophages than with CD68<sup>+</sup> macrophages. Strikingly, patients with elevated levels of CD163<sup>+</sup> macrophages had a shorter disease-free survival compared to patients with CD68<sup>+</sup> [102]. Ohira *et al.* found that exposure of human macrophages to tumour cell-conditioned medium, derived from three different iCCA cell lines, resulted in a significant upregulation of CD163<sup>+</sup> macrophages as well as STAT3 expression and activation [103,104]. Supernatant, derived from HuCCT-1 cells, strongly induced STAT3-activation and

macrophage-polarization towards the M2-class with an increased expression of M2-type cytokines, such as IL-10, VEGF-A, TGF- $\beta$ , and MMP-2 [102]. These results suggest that in iCCA, macrophage differentiation into the M2-phenotype together with the contribution to angiogenesis and immunosuppression are dependent on STAT3-signalling. Further, the study determined that TNF- $\alpha$ , released by TAMs *in vivo*, could act on iCCA cells to increase the expression of CXCR4, which in turn was associated with an increased migration and invasion potential [103,104]. Techasen *et al.* suggested that various cytokines, secreted by activated macrophages, such as IL-4, IL-6, IL-10, TGF- $\beta$ , and TNF- $\alpha$ , consistent with a M2-phenotype, could induce EMT in CCA by enhancement of Snail nuclear translocation and reduction of E-cadherin expression. Indeed, addition of macrophage-conditioned medium to CCA cells reduced E-cadherin and CK19 expression, whereas it induced expression of the mesenchymal markers S100A4 and MMP-9 [86,105].

Bile duct tumours are surrounded by a rich vascular network, which provides an adequate support of oxygen and metabolites to malignant cholangiocytes in order to enhance tumour growth. This angiogenic potential is favoured by overexpression of the vascular endothelial growth factor C (VEGF-C), a protein that, stimulated by TGF- $\beta$  and  $\beta$ -catenin, is expressed by the surrounding mesenchyme as well as the malignant cells (reviewed in [106]). This suggests the existence of an autocrine/paracrine mechanism in the production of VEGF by malignant cholangiocytes, and further indicates that TAMs play an important role in regulating angiogenesis through VEGF. Interestingly, in HCC Zhuang *et al.* showed that increased peritumoural expression of VEGF-C in association with VEGFR-1 and VEGFR-3 correlated with an enhanced peritumoural distribution of macrophages, poorer overall survival and earlier tumour recurrence [107]. Moreover, it was suggested that VEGF autocrine and paracrine



**Fig. 2. Cholangiocarcinoma-associated microenvironment.** Tumour microenvironment related to cholangiocarcinoma includes cancer-associated fibroblasts (CAFs) and macrophage component. Crosstalk among different microenvironmental components is involved in the activation of CCA oncogenic signalling.

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effects support the expansion of the HPC-niche by stimulating HPCs as well as endothelial cell proliferation. This aspect could have important implication in pro-fibrotic processes and carcinogenesis [108] (Fig. 2).

### Future directions: Regulators of the CSC-niche

Corresponding to normal stem cells, CSC-features (e.g., self-renewal and differentiation) are regulated by the “CSC-niche” through complex undefined interactions (cellular components, soluble factors, cytokines and growth factors) [109,110]. The CSC-niche represents a unique microenvironment that supports self-renewal, regulates stemness and enhances drug resistance. The microenvironment surrounding CSCs has multiple important functions, including a mechanical anchorage and crosstalk interaction, mediated by direct contact and/or indirect extracellular factors. As such, the extracellular matrix may be an essential non-cellular component of the adult stem cell niche [111]. In HCC, increasing matrix stiffness promotes proliferation and resistance to chemotherapy, whereas surviving cells from “soft supports” have a significantly enhanced clonogenic capacity and increased expression of CSC-related markers, including CD44, CD133, KIT, CXCR4, OCT4, and NANOG [112]. Recently, Raggi *et al.* [39] used a 3D culture system to investigate the impact of epigenetics on the local microenvironment and reprogramming of hepatic CSCs. A combination of 2D cell density with a transient DNMT1-depletion, using the demethylating agent zebularine [37,113], altered the functional stem-like properties of HCC cells. This suggests that the cellular context is a critical determinant in the response to epigenetic alteration, which results in a long-term malignant reprogramming with enhanced antitumour effect.

Recently, experimental evidence across diverse tumour models has shown that CSCs constitute their own local microenvironment by recruiting and activating specific cell types, including immune components. Work by Jinushi *et al.* identified the milk fat globule-EGF factor 8 (*MFG8*) as a macrophage-derived factor, which potently increases tumour-initiating properties of murine colon and lung carcinoma cell lines [114]. This gene mainly activates STAT3 and SHH pathways in CSCs and further amplifies their drug resistance in cooperation with IL-6. These pathways (JAK/STAT and Hh) are major contributors in triggering tumorigenicity as well as resistance to therapy. These data suggest that TAMs play a key role in CSC maintenance and/or expansion. A recent study by Mitchem *et al.* showed that blockade of CCR2 or CSF1R in established tumours not only resulted in a significant decrease in tumour infiltration by macrophages, but also reduced the frequency of CD44<sup>+</sup>/ALDH1<sup>+</sup> pancreatic CSCs [115].

Therefore, since chronic liver inflammation (primary biliary cirrhosis, primary sclerosing cholangitis) and biliary tumour microenvironment have a key role in CCA-onset and clinical severity, a role of the TME unique to e.g., liver damage or background inflammatory state could be hypothesized. Indeed, it has been demonstrated that stem cell activators such as Wnt/ $\beta$ -catenin, TGF $\beta$ , Notch and Hh signalling pathways may also advance liver cancer [116]. For these reasons, the interplay between the CSC-niche and the inflammatory component need to be elucidated in CCA and might open new venues in CCA-combination therapy.

### Concluding remarks and clinical implications

The prognostic consequence for patients diagnosed with CCA is very dismal. The incidence of iCCA has increased alarmingly in the past decade, and we still only have a limited understanding of the complex molecular pathogenesis that causes drug resistance in this disease. Experiment evidence suggests that CCA may originate from multiple cells-of-origin, and that these contribute to the tumour heterogeneity and its complexity. Independent of origin, all cell stages of the hepatic lineage, including bipotential HPCs, dedifferentiation of non-CSCs and even spontaneous malignant conversion of adult hepatocytes may give rise to CCA. The regulatory signals that mediate this biological hierarchy may present novel opportunities in diagnosis and therapy. Promising targets include for example members of the Wnt/ $\beta$ -catenin, Notch and Hh pathways [117,118]. However, as more molecular studies are published, a multitude of promising therapeutic candidates will complicate traditional clinical management, underscoring the need for more individualized approaches. These pathways may involve targeting the microenvironment e.g., stromal compartment of the tumour, which includes inhibition of signals from CAFs, MFs, and TAMs as well as targeting the CSC-niche. For example, the HPC population is elevated in the setting of chronic liver disease, a condition of long-term inflammation and continued liver regeneration, which promotes CCA. This is furthermore likely supporting the CSC-niche. These observations emphasize the preventive nature and benefit of treating the underlying chronic liver inflammation by targeting e.g., NF- $\kappa$ B or COX2. The interaction between inflammatory, profibrotic signals and tumourigenesis mediated in part by interaction of the epithelial compartment with MFs and TAMs may present promising treatment options. Looking forward, innovative multi-targeted strategies, focused towards CCA-intrinsic pathways and TME-extrinsic mediators, will likely enhance the therapeutic efficacy and improve the potential with beneficial impact on the disease.

Insight into the environmental risk factors, molecular alterations, tumour plasticity and the epithelial-to-stromal crosstalk have shed light on potential new approaches for early detection and therapy, but require further translational and preclinical evaluation. Novel candidates for targeted therapy currently include the MET, FGFR2, JAK/STAT, RAS/RAF/MAPK, PI3K/AKT/mTOR pathways and isocitrate dehydrogenase (*IDH*) mutations. Besides, recent sequencing studies have highlighted *FGFR2* gene fusions in the progression of iCCA [119–122]. Of significance, *FGFR2* expression results in activation of the MAPK pathway, emphasizing potential therapeutic opportunities. Interestingly, epigenetic-driven therapy, affecting both DNA methylation and aberrantly expressed micro-RNAs, hold great promise in the treatment of liver diseases. *IDH* variants have been identified in up to 35% of CCA cases, making this one of the most prevalent hotspot mutations. In a recent study, Saha *et al.* showed that mutations in *IDH*, through hepatocyte nuclear factor 4- $\alpha$  (HNF4a), block HPCs from differentiating into the hepatocytic lineage [123]. Understanding the contribution of the TME as a driver in the pathogenesis of CCA is in its infancy but represents a promising option for “broad spectrum” treatment schemes. As such, future treatment strategies may likely be aimed towards the inflammatory and stromal tumour compartment.

**Conflict of interest**

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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