Unraveling the actin cytoskeleton in the malignant transformation of cholangiocyte biology

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ARTICLE INFO

Keywords:
Cholangiocarcinoma (CCA)
Actin cytoskeleton
Polarization
Cell motility
Pathobiology

ABSTRACT

Correct actin cytoskeleton organization is vital in the liver organ homeostasis and disease control. Rearrangements of the actin cytoskeleton may play a vital role in the bile duct cells cholangiocytes. An abnormal actin network leads to aberrant cell morphology, deregulated signaling networks and ultimately triggering the development of cholangiocarcinoma (CCA) and paving the route for cancer cell dissemination (metastasis). In this review, we will outline alterations of the actin cytoskeleton and the potential role of this dynamic network in initiating CCA, as well as regulating the course of this malignancy. Actin rearrangements not only occur because of signaling pathways, but also regulate and modify cellular signaling. This emphasizes the importance of the actin cytoskeleton itself as cause for aberrant signaling and in promoting tumorigenic phenotypes. We will highlight the impact of aberrant signaling networks on the actin cytoskeleton and its rearrangement as potential cause for CCA. Often, these exact mechanisms in CCA are limited understood and still must be elucidated. Indeed, focusing future research on how actin affects and regulates other signaling pathways may provide more insights into the mechanisms of CCA development, progression, and metastasis. Moreover, manipulation of the actin cytoskeleton organization highlights the potential for a novel therapeutic area.

Introduction

Cholangiocarcinoma (CCA) is a dismal disease caused by the malignant transformation of cholangiocytes, the epithelial cell layer lining the bile ducts [1,2]. The function of cholangiocytes is to transport and actively sense bile, a process that is highly dependent on the organization of intracellular actin filament networks. The actin cytoskeleton plays a major role in establishing and maintaining key cellular processes in cholangiocytes, such as membrane tension, structure and cell shape, polarity of membrane proteins, regulation of transporter and ion channels, and vesicular trafficking [3]. An abnormal actin network in cholangiocytes leads to aberrant cell morphology, deregulated signaling networks and can ultimately trigger the malignant development of CCA, paving the route for cancer cell dissemination (metastasis) [4]. Numerous cancer studies have described the impact of signaling pathways on the actin cytoskeleton, including CCA [5]. However, recent studies have highlighted that actin rearrangements not only occur because of the altered signaling pathways, but also regulate and modify cellular signaling [6–8]. This emphasizes the importance of the actin cytoskeleton as a direct cause for aberrant signaling and in promoting tumorigenic phenotypes.

In this review, we will outline alterations of the actin cytoskeleton and the potential role of this dynamic network in initiating and regulating the development of CCA. We will describe the changes of actin function in (a) sensing the extracellular milieu by the primary cilium, (b) the interface of extracellular matrix and cell membrane, (c) cell plasticity and motility and (d) nuclear localization and chromatin integrity. In the second part of the review, we will explain how actin can impair signaling pathways during CCA development, specifically its role in (a) biliary inflammation and (b) increased liver stiffness during liver fibrosis-to-cirrhosis. Finally, we will highlight therapeutic opportunities for targeting the actin cytoskeleton in CCA treatment.

Rearrangement of the actin cytoskeleton in CCA

The actin cytoskeleton is organized in a hierarchical structure based on the expression of different actin isoforms [4] (Fig. 1). Actin can function either in its monomeric form, as globular actin proteins (G-actin), or polymerize into longer actin filaments (F-actin). This polymerization process is tightly regulated by actin-binding proteins.

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https://doi.org/10.1016/j.tranon.2022.101531
Received 16 July 2022; Received in revised form 31 August 2022; Accepted 2 September 2022
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(ABPs). Different ABPs regulate the assembly/disassembly of F-actin through a complex network formation by actin branching or cross-linking of bundle filaments into actin stress fibers. If actin filaments interact with motor proteins, such as myosin, the actin cytoskeleton can mediate mechanical strength and contractility to the cell, which is essential for example in cellular migration and invasion. These specific processes are further regulated by Rho GTPases changing the activity status of ABPs and actin by post-translational phosphorylation. A panel of inhibitors is known to manipulate the actin organization. The most important inhibitors are summarized in Table 1.

In CCA, the actin cytoskeleton network is impaired at several different levels (Table 2). Aberrant signaling in CCA leads to deregulation of the actin cytoskeleton, cell morphology changes and subsequently to altered phenotypes such as, increased invasion [9,10], aberrant transcription, and loss of the primary cilium [11,12]. In the following section, we will discuss the role(s) and mechanism(s) of actin itself, and actin cytoskeleton components in oncogenic phenotypes by focusing on the actin cytoskeleton’s function in ciliogenesis, adhesion, epithelial-to-mesenchymal transition (EMT), cell motility, and chromatin integrity.

Sensing of the extracellular milieu: the primary cilium

The primary cilium of cholangiocytes is an essential signaling hub to transmit extracellular osmotic, chemical, and mechanical changes inside the cell [13]. Although, the primary structure of the cilium is formed by microtubules protruding out of the centrosome, the actin network plays an essential role in cilium formation, length, and receptor localization. We will particularly focus on the chemosensory pathway (Hedgehog (Hh) signaling) and the mechano-sensitive receptors (polycystin (PC-1 and PC-2), and their impact on the actin cytoskeleton in CCA (Fig. 2). Hedgehog (Hh) signaling is tightly regulated by the localization of the receptor smoothered (SMO), a G-protein-coupled receptor (GPCR)-

Table 1

| Inhibitor group                  | Effect on actin organization | REF       |
|---------------------------------|------------------------------|-----------|
| Cytochalasins (e.g., A and D)   | binds to actin filaments     | [125, 126]|
|                                 | prevents G-actin polymerization on barbed end |         |
|                                 | blocks assembly and disassembly of actin filaments |      |
| Latrunculin (e.g., A and B)     | prevents actin polymerization by binding to G-actin | [126] |
|                                 | enhances actin depolymerization |          |
| Jasplakinolide                  | induces actin polymerization and F-actin formation by actin nucleation | [127, 128] |
|                                 | actin stabilization, competitive binding to Phalloidin |   |
| Phalloidin                      | actin filament stabilization  | [128]     |
|                                 | competitive binding with Jasplakinolide |          |
|                                 | actin polymerization and bundling |        |
| Chondramide (e.g., A and C)     | binds to barbed end of monomeric G-actin and polymerized actin | [130] |
| Swinholide (e.g., A)            | induces actin nucleation and polymerization | [131]   |
| SMIFH2                          | inhibits formin-mediated F-actin formation, but not de novo F-actin formation | [132] |
|                                 | inhibition of non-myosin IIA |         |
| Fasudil                         | inhibitor of myosin light chain (MLC) phosphorylation | [56] |
| Blebbistatin                    | inhibits ATPase activity of myosin II | [133]   |

G-actin: globular actin; F-actin: filamentous actin; REF: references.

Fig. 1. Overview of basic actin cytoskeleton components and their impact on the formation of actin networks. Actin microfilament assembly is based on the treadmilling of globular actin (G-actin) to filamentous actin (F-actin). Actin nucleation and branching is regulated by actin-related protein 2/3 (ARP2/3) complex, formins and Wiskott-Aldrich syndrome proteins (WASPs). F-actin can be stabilized or bundled by fascin, filamin, actinin or tropomyosins and anchored to the membrane by L-plastin. Cellular contractions are mediated by myosin light chains (MLC) in the actomyosin network and post-translational phosphorylation by Rho GTPases (RHOA, RAC1, CDC42). Proteins promoting capping and disassembly of F-actin are CapZ, gelsolin and cofilin.
### Table 2

List of altered genes in actin cytoskeleton regulation during cholangiocarcinogenesis generated based on the KEGG signaling pathway “Regulation of actin cytoskeleton” (hsa04810). If not indicated otherwise, expression levels are in relation to benign/normal.

| Gene name | Gene/RNA/protein | Origin | Expression level | Stage in cholangiocarcinogenesis | Effect, regulation mechanism and clinical impact | REF |
|-----------|------------------|--------|------------------|---------------------------------|-----------------------------------------------|-----|
| ACTB gene | tissue up | CCA | Genomic amplification | [120] |
| ACTN1 protein | tissue up | iCCA | – | [134] |
| ACTN4 RNA (circular) | tissue up | iCCA | – | [136] |
| APC gene | tissue mutation | iCCA | – | [137] |
| ARAF gene | tissue mutation | iCCA | Constitutive active mutation N217I Increased viability | [138] |
| ARP3 protein | plasma up | liver fluke | – | [139] |
| BAIA2P2 protein | EVs (Patients, cell models) up | combined HCC/CCA | – | [140] |
| BRAF gene | tissue mutation | iCCA | Characterized in proliferation subclass | [141] |
| CDC42 protein | tissue down | congenital biliary atresia | Disruption of cell junctions and polarity | [142] |
| CXCL12 RNA | tissue, cell model up | liver metastasis in CCA | Increased migration/invasion | [145] |
| CXCR4 protein | cell models up | CCA | Increased migration/invasion | [10] |
| EGFR gene | tissue mutation | eCCA | Genomic amplification | [66] |
| ERK1 protein | tissue up | CCA | Poor survival | [143] |
| ERK2 protein | tissue up | CCA | – | [147] |
| EZR protein | tissue, cell models down | CCA | Increased migration | [148] |
| F2 RNA | tissue down | CCA | Poor overall survival | [146] |
| FAK protein | tissue up | iCCA | Activates Akt signaling | [151] |
| FGFR1 protein | tissue (mouse) up | IPNB | Induction of cholangiocarcinogenesis | [152] |
| FGFR2 gene | tissue (mouse) up | CCA | Constitutive active gene fusions | [153] |
| FGFR4 protein | tissue, cell models up | CCA | Glycosylated form leads to increased migration | [154] |
| FNAL protein | tissue up | pCCA | – | [155] |
| ITGA6 RNA | tissue, cell models up | PSC-derived CCA | – | [156] |
| ITGB1 RNA, RNA | tissue, cell models up | CCA | Increase migration, invasion, proliferation | [44] |
| ITGB1 protein | RNA, protein | cell models up | CCA | Increase migration, invasion, proliferation | [44] |
| ITGB3 RNA | cell models up | CCA | Increase migration, invasion, proliferation | [44] |
| ITGB4 protein | tissue up | iCCA | Inhibition by reduced miR-29–3p family | [44] |
| ITGB6 protein | tissue up | iCCA | Increase proliferation, migration, invasion | [45] |
| MEK1 protein | tissue up | hilar CCA | Increased proliferation, migration, invasion | [43] |
| KNG1 protein | bile up | CCA | Increased adhesion and proliferation, lymph node metastasis | [47] |
| MEK2 protein | cell models up | CCA | Increased adhesion and proliferation, inhibition by lovastatin | [47] |
| MYL9 RNA | tissue up | CCA | – | [157] |
| NRAS gene | tissue mutation | iCCA | Uregulation of fucosylated protein form | [158] |
| PDGFA RNA | tissue up | CCA | Compared to benign/ PSC | – | [159] |
| PDGFA protein | cell models up | CCA | Compared to PSC | – | [160] |
| PDGFA protein | cell models up | CCA | Compared to PSC | – | [161] |
| PDGFA protein | cell models up | CCA | Compared to PSC | – | [162] |
| PDGFA protein | cell models up | CCA | Compared to PSC | – | [163] |

(continued on next page)
Smo background induces a stronger chemotaxis phenotype than observed in lacking the ciliary localization domain (CLD) (embryonic fibroblasts (MEF) with two different

Hh signaling [11,15]. The stimulation of human and rat CCA cells by chemosensitive to SMO agonist purmorphamine, indicating a still active

transcription of Hh target genes. Although, the primary cilium is lost in CCA

like receptor, affecting chemotaxis, proliferation and apoptosis in CCA

in vivo [16,17]. These domains are both essential for proper ciliary trans

and known to affect the actin cytoskeleton (section 1C).

Mechanobiological changes in the bile flow are sensed by the ciliary protein complex PC-1/PC-2, containing the transmembrane receptor protein PC-1 and the calcium channel PC-2 [19]. Increasing intracellular calcium levels caused by changes in the bile flow inhibit adenylyl cyclase (AC)/cAMP, PI3K/Akt, and RhoA/ROCK signaling [18]. These pathways are all deregulated in CCA and known to affect the actin cytoskeleton (see section 1C).

Table 2 (continued)

| Gene name | Gene/ RNA/protein | Origin | Expression level | Stage in cholangiocarcinogenesis | Effect, regulation mechanism and clinical impact | REF |
|-----------|-------------------|--------|-----------------|----------------------------------|-----------------------------------------------|-----|
| RNA, protein | tissue | up | biliary atresia | Biliary defects and hedgehog pathway activation in zebrafish larvae, DNA hypomethylation | [164] |
| PDGFβ protein | tissue, cell models | up | CCA | – | [165] |
| PDGFc protein | tissue | up | CCA | – | [165] |
| PDGFd protein | tissue, cell models | up | eCCA | Invasive tumors present higher expression | [166] |
| PDGfra protein | tissue | up | CCA | – | [165] |
| PDGfrb protein | tissue | up | CCA | – | [165] |
| PI3CA gene | tissue | mutation | BTC | Mediates drug resistance | [169] |
| PI3CB protein | plasma | up | CCA (non-/liver-fluke) | – | [168] |
| PI3R1 RNA | cell models | up | CCA | – | [169] |
| PPP1CB RNA | cells (rat) | down | cholestasis | Inhibition by miR-218–5p in rat | [170] |
| PPP1R12A RNA | tissue | up | CCA | Increased proliferation and decreased apoptosis | [171] |
| PXN protein | cell models | up | iCCA | Clonorchis sinensis excretory-secretory products stimulate PXN expression and invasion | [172] |
| RNA, protein | cell models, xenograft | down | CCA | Clonorchis sinensis excretory-secretory products stimulate PXN expression and invasion | [173] |
| RAC1 protein | cell models | up | CCA | Induced by mechanical stretching | [56] |
| RAF1 gene | tissue | up | CCA | – | [55] |
| RhoA protein | cell models | up | CCA | – | [174] |
| ROCK2 RNA | cell models | up | CCA | – | [175] |
| SOS1 RNA | tissue | down | CCA | Inhibition by repressed miR-200h/c | [143] |
| SRC protein | cell models | mutation | cholangiocyes with mutated CFTR ΔF508 | Rearrangement of F-actin and increased inflammation | [176] |
| TIAM1 RNA, protein | tissue, cell models | up | CCA | – | [177] |
| VAV1 RNA protein | tissue | up | CCA | Positive correlation with CXCR4 expression | [178] |
| VCL protein | cell models | up | iCCA | Clonorchis sinensis excretory-secretory products stimulate VCL expression and invasion | [172] |
| RNA, protein | cell models | up | iCCA | VCL in combination with inactive LKB1 induces decreased adhesion, increased migration and metastasis | [179] |
| WAVE3 RNA | tissue, cell models | up | iCCA | Worsse overall survival | [180] |

iCCA: intrahepatic CCA; pCCA: perihilar CCA; eCCA: extrahepatic CCA; dCCA: distal CCA; IPNB: intraductal papillary neoplasm of the bile duct; BTC: biliary tract cancer; PSC: primary sclerosing cholangitis; EVs: extracellular vesicles; EMT: epithelial mesenchymal transition; REF: references.

iCCA: intrahepatic CCA; pCCA: perihilar CCA; eCCA: extrahepatic CCA; dCCA: distal CCA; IPNB: intraductal papillary neoplasm of the bile duct; BTC: biliary tract cancer; PSC: primary sclerosing cholangitis; EVs: extracellular vesicles; EMT: epithelial mesenchymal transition; REF: references.

like receptor, affecting chemotaxis, proliferation and apoptosis in CCA [11,14]. The canonical Hh signaling pathway gets activated by binding of the agonist Sonic hedgehog (Shh) to the ciliary located protein Patched 1 (PTCH1). PTCH1 is degraded in the lysosome, released, and enables the translocation of SMO into the cytoplasm. The translocation of SMO to the ciliary tip increases intracellular G-protein signaling and enables the translocation of SMO into the cilium. The translocation of

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pathways involved in autophagy, extracellular matrix (ECM) receptor interaction, focal adhesion and genes involved in actin cytoskeleton regulation [21]. Indeed, mutations in PC-1 in renal cells cause major rearrangements of the actin cytoskeleton, as shown by phalloidin staining of F-actin [22]. Organized F-actin on the basal membrane of normal cells is redistributed into disorganized, short and thick F-actin bundles in the cellular cortex of cystic cells [22]. This rearrangement is caused by two indirect regulatory mechanisms of actin mediated by PC-1. First, PC-1 induces F-actin polymerization by activation of nucleation factors (neural Wiskott-Aldrich syndrome protein (N-WASP) and actin-related protein 2/3 (ARP2/3)) through protein-protein interaction with the C-terminus of Pacsin-2 [23]. Second, PC-1 regulates actin-myosin interaction through Rho GTPase Activating Protein 35 (ARHGAP35). Wildtype PC-1 recruits ARHGAP-35 to the centrosome by
These studies were performed in renal cells, showing the localization of cilia formation, ciliary proteins and the centrosome are transported to inhibiting the activation of ROCK kinase signaling. PC-1 mutations 

Among ECM proteins, periostin (PN), laminin gamma 2 demonstrated to cooperate to carcinogenesis and tumor progression. The role of ECM in CCA has been experimentally performed in cholangiocytes. It is only recent that studies have identified actin and ABPs within the primary cilium and started to elucidate their function within renal cells. Using techniques like cryo-electron tomography, filamentous actin structures have been visualized in the primary cilium of MDCK-II cells. This is further supported by proximity-based proteomics of the cilary protein 5-hydroxytryptamine receptor 6 (HTR6) which revealed the localization of actin and ABPs at the ciliary base and within the cilium. Proteomics has helped to identify high abundance of actin proteins (ACTA1, ACTB, ACTG1), F-actin fragmentation proteins (GSN) and ABPs (ACTN1, ACTN4, TPM) within the cilium. A potential function of actin and ABPs in the cilium can be the correct organization of receptors, such as GPCR so-matostatin receptor type 3 (SSTR3) and the establishment of membrane polarity. The clustering of receptors in ‘receptor corals’ or free diffusion is essential in fine-tuning the downstream signaling. These studies were performed in renal cells, showing the localization of actin and ABPs in the primary cilium. We are still lacking similar studies to be performed in cholangiocytes.

**Cellular and extracellular matrix interface**

Besides the cilium, cholangiocytes sense their cellular environment and the ECM by plasma membrane proteins. Accumulation of ECM is at the origin of a desmoplastic reaction that typically characterizes CCA tumors. The role of ECM in CCA has been experimentally demonstrated to cooperate to carcinogenesis and tumor progression. Among ECM proteins, peristin (PN), laminin gamma 2 (LAMC2), osteopontin (SPP-1), secreted protein acidic and rich in cysteine (SPARC), thrombospondin-1 (THBS1), collagen type-1, 3 and 4 (COL1, COL3, COL4), are correlated with poor prognosis in human CCA. The expression of ITGB1 and ITGB3 in CCA cell lines (RBE and Huh-28) decreases the expression of mesenchymal markers favoring EMT and contribute to CCA cell invasion. As such, PN binding to ITGB1 induces the expression of mesenchymal markers favoring EMT and migratory properties of CCA cells via an AKT-dependent signaling pathway. A potential treatment option to target integrin overexpression in CCA is Lovastatin, a 3-hydroxy-3-methylglutaryl-coenzyme-CoA (HMG-CoA) reductase inhibitor, which was shown to inhibit the expression of ITGB1 and ITGB3 in CCA cell lines (RBE and Huh-28). Lovastatin treatment efficiently decreases the proliferation and migration of CCA cells by inhibition of stress-fiber formation and cellular adhesion. The above discussed research on integrin signaling solely focuses on their intracellular function to integrate external signals (so called outside-in signaling), but does not describe the integrin-mediated inside-out signaling. It still remains to be elucidated if and how intracellular actin rearrangements may affect ECM repositioning potentially enhancing desmoplastic phenotypes in CCA.

Interaction between ECM proteins and malignant cells drives the phenotypic cellular changes, acquiring metastatic features. If cultured on ECM gel (3-D culture model), CCA cells display a drastically modified actin cytoskeleton, with an increase expression of key ABPs such as L-plastin, ezrin (villin 2), fascin and cofilin-1. L-plastin localizes to actin-rich membrane structures in vitro, and its inhibition reduces CCA cell invasion. In human CCA samples, L-plastin is mainly localized in the cell nuclei, in which it may play a role in the regulation of nuclear actin and transcription (see section 1D). In tumor cells that display a more mesenchymal-like phenotype and can invade the basement membrane (metastasize), L-plastin is found in the cytoplasm. Uniquely, CCA (ITGB6) is correlated with clinicopathological features in iCCA including lymph node and distal metastases. In vitro, ITGB6 increases RAC1 activity in human CCA cell lines, resulting in remodeling of the actin cytoskeleton with an increase of F-actin polymerization and metalloproteinase-9 (MMP9) expression. These events both result in ECM degradation and cell migration. In hilar CCA, upregulation of ITGB6 is associated with poor prognosis. Other integrins are upregulated in CCA, including ITGA6, ITGB1, ITGB4 and ITGB6. ITGB1 is a prognostic factor for CCA, and both ITGB1 and ITGB6 are negatively regulated by the miR-29-3p family and contribute to CCA cell invasion. As such, PN binding to ITGB1 induces the expression of mesenchymal markers favoring EMT and migratory properties of CCA cells via an AKT-dependent signaling pathway. A potential treatment option to target integrin overexpression in CCA is Lovastatin, a 3-hydroxy-3-methylglutaryl-coenzyme-CoA (HMG-CoA) reductase inhibitor, which was shown to inhibit the expression of ITGB1 and ITGB3 in CCA cell lines (RBE and Huh-28). Lovastatin treatment sufficiently decreases the proliferation and migration of CCA cells by inhibition of stress-fiber formation and cellular adhesion. The above discussed research on integrin signaling solely focuses on their intracellular function to integrate external signals (so called outside-in signaling), but does not describe the integrin-mediated inside-out signaling. It still remains to be elucidated if and how intracellular actin rearrangements may affect ECM repositioning potentially enhancing desmoplastic phenotypes in CCA.

![Cellular and extracellular matrix interface](image-url)
cells cultured in 3-D release L-plastin into the extracellular milieu, a phenotypic trait not seen when culturing mixed hepatocellular-cholangiocarcinoma (CHC) cells [49]. Thus, L-plastin has been proposed to serve as a diagnostic biomarker that can differentiate tumor types (CCA and CHC).

**Cell plasticity and motility**

CCA is characterized by an early and high metastatic burden [2]. One prerequisite for metastasis formation is the tumor cell’s ability to migrate and invade into the tumor-adjacent tissue (liver parenchyma). Migration and invasion are two malignant phenotypes caused by several signaling pathways that lead to a rearrangement of the actin cytoskeleton. Tyrosine kinase receptors (RTKs) upon ligand binding trigger cell features, which allow for proliferation and migration of cancer cells. Several RTKs, including the fibroblast growth factor receptor (FGFR) [50] or epidermal growth factor receptor (EGFR) families [51,52] are major inducers of actin cytoskeleton remodeling (Fig. 4). Upon FGFR activation, CCA cells exhibit higher levels of actin polymerization in the cell periphery, with the formation of pseudopodia (for example, filopodia and lamellipodia), an effect that can be abrogated by using a MEK inhibitor [50]. Similarly, the chemokine CXCL12 (or SDF-1) induces pseudopodia and CCA cell invasion through its receptor CXCR4, MEK1/2, and PI3K pathways [10]. Also, downstream PI3K, the mTOR pathway appears critical for the formation of migratory protrusion structures. Everolimus, a potent inhibitor of mTOR signaling, alters the pathway affecting Rho GTPases in CCA triggering the migration and invasion of CCA cells [53]. In contrast to pro-invasive stimuli, Δ⁶-tetrahydrocannabinol (THC), the principal active component of cannabinoids, has an opposing effect, decreasing actin polymerization in CCA cells, and inhibiting migratory and invasive features [54]. These signaling pathways often affect Rho GTPases (RHOA, CDC42 and RAC1), which are key regulators of actin nucleation, polymerization, and contractility. Rho GTPases directly affect ARPs (as the nucleation factor N-WASP) or indirectly by activating the kinase ROCK. Subsequently ROCK phosphorylates myosin light chain phosphatases and actin-regulating LIM kinases. Interestingly, Rho GTPases themselves are deregulated in CCA. RAC1 is differentially expressed between 2 CCA subgroups in a subclass of CCA patients with better prognosis [55]. Mechanical stress caused by increased tissue stiffness stimulates the expression of RHOA and RAC1 (in both CCA and HCC cells) inducing migratory and invasive phenotypes [56], which can be abolished by pamidronate treatment, a drug used for treatment of osteoporosis [57].

**Nuclear actin and chromatin integrity**

Nuclear actin is the ‘cytoskeleton’ in the nuclear matrix, which participates in DNA repair, genome integrity, chromatin remodeling, and transcriptional regulation. The nuclear polymerization of actin has long been debated as it in its polymerized form cannot be stained by phalloidin [58]. In fact, the polymerized form of actin in the nucleus differs from the cytoplasmic conformation and accounts only for a small proportion of the primarily monomeric actin in the nucleus. Monomorphic actin and ARPs take part in modifying and remodeling chromatin. A key chromatin-remodeling complex is the ATPase-dependent Switch/Sucrose Non-Fermentable (SWI/SNF) complex. This complex is responsible for opening the chromatin by removing or repositioning histone octamers, allowing for active DNA repair, regulating transcription, and controlling genome stability by ATP hydrolysis [59,60]. In mammalian, three SWI/SNF complexes exist: (1) BAF (BRG1-associated factor complexes containing ARID1A), (2) PBAF (polybromo BRG1-associated factor containing PBRM1), and (3) ncBAF (noncanonical BAF). Using cryo-electron microscopy of S. cerevisiae BAF and human PBAF has shown that both complexes contain an actin-related subunit, which is located between the ATPase domain and the core complex, including Swi1 (ARID1) or PBRM1 [59,60] (Fig. 5). The actin-related subunit is essential for the structural complex integrity, binding of actin filaments for nucleosome recruitment and regulation of the ATPase activity of BRG1 [61]. The structure consists of one monomeric actin molecule and the oncogene ARP4 (or ACTL6A/BAF35) [59,60,62,63]. In hepatocellular carcinoma (HCC), overexpression of ARP4 is linked to poor prognosis and involved in activating Notch signaling thereby increasing cell migration, invasion and EMT in vitro.

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**Fig. 4.** Overview of receptor tyrosine kinase (RTK) and cytokine receptor signaling affecting Rho GTPases in CCA triggering the migration and invasion of CCA cells. RTKs (fibroblast growth factor receptor FGFR, epidermal growth factor receptor EGFR) and cytokine signaling (CXCL12, CXCR4) activate a kinase signaling cascade, Rho GTPases and mTOR signaling and subsequently trigger N-WASP and ROCK activation. The resulting elevated levels of F-actin induces the formation of lamellipodia and filopodia and actin-myxin mediated contraction, increased migration, and invasion (cellular phenotypes are highlighted in capital letters). The RTK signaling can be inhibited on several levels in the signaling cascade by MEK inhibitors, Pamidronate, Everolimus and THC (highlighted in red).

**Fig. 5.** Role and function of intracellular actin in chromatin remodeling Switch/Sucrose Non-Fermentable (SWI/SNF) complex in CCA. The chromatin remodeling complex SWI/SNF consists of the ATPase BRG1, the proteins PBRM1/ARID1A and is stabilized and linked to nuclear actin by the actin-related protein (ARP) module consisting of ARP4 and monomorphic actin. Loss-of-function mutations of SWI/SNF components or activation of Notch signaling by ARP4 overexpression are linked to worse survival in CCA. Loss of ARID1A function by stretch-induced nuclear F-actin induces YAP/TEAD4 signaling, expression of stem-like genes and increased sensitivity to the PI3K inhibitor MK-2206 (highlighted in red).
and tumor growth and metastasis in vivo [63]. In CCA, the actin-related SWI/SNF component remains unstudied. However, loss of function mutations in ARID1A (11–16%), ARID1B (5%), ARID2 (4–6%) and PBRM1 (21%) [64–67] are among the most recurrent mutations in CCA patients [68], which also are associated to worse prognosis [69,70]. Deletion of ARID1A in CCA (in vitro studies in HuCCT1 and RBE cells) decreases migration and invasion [70]. In patients with ARID1A mutations [71], CCAs are often characterized as the infiltrating mass-forming type, which may suggest a role of the actin cytoskeleton. In fact, ARID1A may be linked to the regulation of actin through Yes-associated protein 1 (YAP) and PI3K/Akt signaling. ARID1A knockout mouse under DDC (3, 5-diethoxybenzoyl-1,4-di-hydrocolloïdine) diet develop CCA-like lesions with increased YAP expression [72], emphasizing why upregulation of stem-like genes are observed in ARID1A knockout CCA cells [70]. Mechanistically, ARID1A binds to YAP, inhibiting the YAP/TEAD4 complex and activating transcription, a process that is regulated by mechanical stress and nuclear F-actin. Increased stretching of Hep293T cells causes actin at the nuclear border to start forming filaments and binding to ARID1A that prevents ARID1A-YAP complex formation [72]. Additionally, CCA cells expressing low levels of ARID1A are more sensitive to PI3K/Akt signaling inhibitor MK-2206 [73]. Interestingly, the PI3K substrate (PIP2) is required for SWI/SNF complex stability and binding of BRG1 to actin filaments [74]. PIP2 activates the complex N-WASP-ARP2/3 inducing actin filament nucleation and inhibiting the binding of the actin-severing protein coflin resulting in the formation of stable actin filaments.

**Actin remodeling and signaling—what comes first?**

Recent studies elucidated the important function of the actin cytoskeleton in liver homeostasis and early tumorigenesis. This highlights that changes in the actin cytoskeleton might not solely be a consequence of aberrant signaling, but can cause altered signaling in CCA [6]. Therefore, the second part of the review will provide arguments to study the actin cytoskeleton during early stages of CCA development, which could subsequently lead to alterations in signaling and CCA progression. We will describe actin cytoskeleton rearrangements in the context of CCA risk factors particularly during infection and liver cirrhosis.

**Role of actin cytoskeleton in biliary inflammation**

Chronic inflammation of the biliary tree is a key risk factor for CCA, and mainly caused by either cholestasis or viral, parasite and bacterial infections. The primary mechanism of infection is through bile secretory function in hepatocytes and cholangiocytes and is tightly regulated by transporters localized at the apical domain. Integrity of the actin cytoskeleton and the linker proteins are critical for the functional regulation of the apical transporters. In hepatocytes, the bile canalicular lumen comprises a pericanalicular actin cortex linked to integral plasma membrane proteins by radixin, a member of the ERM (ezrin-radixin-moesin) family, which acts as a cross-linker. A similar structural architecture is present in the apical domain of cholangiocytes, with expression of the ERM cross-linker protein ezrin, a protein expressed exclusively in the biliary lineage [75]. Genetically-engineered murine knockdown models either of radixin [76] or ezrin [77] have demonstrated the role of these ERM proteins in the liver epithelium. In radixin knockout mice, a loss of microvilli, a structure characterizing the canaliculi membrane of hepatocytes, along with an apical loss of multidrug resistance-associated protein 2 (MRP2) [76] cause hyperbilirubinemia in mice, a phenotype equivalent to Dubin-Johnson syndrome in humans. Ezrin-deficient mice develop a severe intrahepatic cholestasis following a dysregulation of the bile fluidity into the bile duct epithelium [77]. Ezrin deficiency in cholangiocytes results in loss of apical expression of several key transporters (such as cystic fibrosis transmembrane conductance regulator (CFTR), anion exchange protein (AE-2), and aquaporin-1 (AQP1)) and of ERM-binding phosphoprotein of 50 kDa (EBP50/NHERF-1), a PDZ-scaffold protein highly expressed in bile duct cells [77,78]. Ezrin-deficient mice have no prominent alterations in the cholangiocytes neither of microvilli nor their primary cilium structure. However, mutation in the above transporters may have consequences on the actin cytoskeleton and associated proteins. CFTR is a chloride transporter anchored at the apical plasma membrane to the actin cytoskeleton via EBP50 and ezrin [79]. In CFTR-defective cholangiocytes, the F-actin cytoskeleton is defective and EBP50 is mis-localized into the cytosol, resulting in destabilization of apical membrane organization and function [80]. The consequence of CFTR disorganization is an activation of proinflammatory signaling, simulated through a Src-dependent kinase feedback loop and subsequent activation of Toll-like receptor 4 (TLR4) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) upon endotoxin exposure.

Other sources of chronic inflammation include parasite and bacterial infections. In these cases, inflammation is caused by secretion of excretory-secretory products and internalization of liver fluke and bacteria into cholangiocytes and hepatocytes. The role of actin cytoskeleton rearrangement in parasite internalization is well-established [81,82]. Cryptosporidium parvum infection in cholangiocytes requires host cell actin remodeling at the attachment site. At the host-parasite interface, c-Src is activated and phosphorylates ABP (82), along with activation of the Cdc42 pathway and the nucleation machinery of ARP2/3 complex proteins. This process causes branching of actin filaments, facilitating the membrane protrusion and the parasite entry [83]. In addition, it may be further facilitated by water influx through the translocation of AQP1 and Na+/glucose cotransporter 1 (SGLT1) mediated by the actin myosin network regulator myosin IIIB [84].

Similarly, bacterial infection of epithelial cells is associated with an actin cytoskeleton remodeling and an inflammatory response. Actin polymerization is required for infection and internalization of Helicobacter pylori (H. pylori) and Shigella flexneri in CCA cells [85]. Pilus-like structures of H. pylori interact with integrin α5β1 on the surface of the host cell both in CCA [85] and HCC [86], and induces actin polymerization to allow internalization of the bacterium [85]. Once internalized, the bacterium stimulates expression of Nucleotide-binding oligomerization domain containing 1 (NOD1), TLR4, and TLR5, as well as activates the NF-κB pathway and interleukin 8 (IL-8) production in CCA cells [85]. In gastric cells, at the bacterium-host cell interface, H. pylori activates paxillin by phosphorylation at focal adhesion and regulates cytoskeletal reorganization to form actin stress-fibers, which favors cancer cell mobility and inflammation. Activation of paxillin by H. pylori depends on EGFR, FAK-Src, and PI3K/Akt signaling pathways [87]. Exposure of CCA cells in culture to H. pylori results in phenotypic changes, including the loss of cell-cell contact, filopodia protrusion, and induction of EMT-inducing transcription factors and cancer stem cell marker CD44. These features all favor malignant transformation of the biliary epithelium and progression of CCA [88]. H. pylori plays a role in altering stemness via its cytotoxin-associated gene CagA by inducing the expression of reprogramming factors (Splat-like transcription factor 4 (SALL4) and Kruppel-like factor 5 (KLF5)) as well as nuclear accumulation of β-catenin. In addition, these cells also display higher expression of epithelial splicing regulatory protein (ESRP1), which is involved in upregulating alternative splicing of CD44 (CD44<sup>alt</sup>) generating the stemness marker CD44 variant 9 [89].

**Impact of liver stiffness on the function of actin in mechanotransduction**

Cirrhosis is one of the key risk factors for CCA [2]. During the change from a normal to a cirrhotic liver, the liver tissue stiffness is increasing and is used as a diagnostic measure (fibrosis F0–3; cirrhosis (F4)) [90]. Increased liver stiffness negatively affects the function of the hepatocytes and overall liver function. In fact, mimicking cirrhosis by transferring hepatocytes onto a stiff matrix leads to nuclear deformation and reduces the hepatocyte function observed by decreased mRNA
expression of albumin and hepatocyte nuclear factor 4α (HNF4α) [90]. Expression of these genes can be restored by disruption of the cytoskeleton-nuclear interaction administering actin (Cytchalasin A) and microtubule inhibitor (nocodazole), highlighting the importance of the cytoskeleton in mechano-transduction. Besides the cytoskeleton and polarity protein complexes, YAP and transcriptional coactivator with PDZ-binding motif (TAZ) are key intracellular mechano-sensing effector proteins. In healthy hepatocytes and cholangiocytes YAP/TAZ signaling is inactive and contact-inhibition by neighboring cells prevents proliferation. The Hippo signaling pathway is a major inhibitor of YAP signaling, which is orchestrated through phosphorylation of YAP by the kinases LATS1/2 [91–93]. Phosphorylation of YAP excludes it from the nucleus and prevents its function as a transcription factor. Piezo-1 (PIEZO1) is a mechano-sensitive ion channel protein overexpressed in CCA cells, which induces migration and invasion in vitro as well as lung metastasis in vivo [91]. Activation of PIEZO1 by its agonist Yoda-1 induces EMT in CCA cells through the activation of YAP and reduced LATS1/2 phosphorylation. It is unknown, if PIEZO1 is regulating the phosphorylation of LATS1/2 directly or indirectly (direct activation of YAP). In this process, the actin cytoskeleton takes solely the role of a downstream effector of the Hippo pathway. Several lines of evidence argue for a LATS1/2-independent mechanism regulating YAP activity [94]. Neither knockdown of LATS1/2 nor YAP/TAZ mutants (insensitive to LATS1/2 phosphorylation in normal cells) can rescue YAP transcriptional activity, which indicate an additional inhibitory mechanism [94]. F-actin bundles can directly inhibit YAP signaling in cells in a stress-free surrounding. Knockdown or chemical inhibition of proteins preventing the formation of strong F-actin fibers (such as cofilin, CAPZ, gelsolin and formins) cause YAP activation, whereas inhibition of ARP2/3 has only minor effects on the YAP activity [94–96]. These studies in mammary epithelial and fibrotic breast cells have been reproduced in hepatocytes. Inhibitors of F-actin bundling (Cytchalasin D, latrunculin A) and the formin inhibitor (SMIFH) all induce the activation of YAP in hepatocytes in vitro [97]. Overexpression of the actin bundling protein (Fascin-1) in vivo in the murine liver induces YAP-dependent proliferation and dedifferentiation of ductular cells into atypical cells positive for cytokeratin 19 (CK19), counteracting the effects of the F-actin-capping protein subunit beta (CAPZB) and ARP2/3 [96]. Similar phenotypes can be observed in mice with liver specific Capzb knockout [7]. Additionally, these mice have impaired hepatocyte zonation and metabolism with improved glucose tolerance and decreased expression of gluconeogenic genes, which can be rescued by YAP inactivation [7].

Single-cell RNA-sequencing has shown that during liver injury elevated mechanical tension leads to YAP activation in biliary epithelial cells (BEC) [98]. Besides, the above-mentioned F-actin bundles, actin-myosin fibers play an essential role in this process. Mechanical stress following bile duct ligation results in expansion of the apical surface of hepatocytes, increased levels of F-actin and phosphorylation of actin-myxosin [97]. Stretching of CCA and HCC cells in vitro increases the expression of Rho GTPases and mediates the phosphorylation of myosin light chains (P-MLC) [56]. Since fasudil (inhibitor of MLC phosphorylation) can inhibit this process, both the expansion and bile canaliculi contractility are dependent on actin-myosin. Depending on the apical actin integrity, YAP is localized to the apical F-actin layer, but with increasing mechanical stress and contraction YAP is released and translocates to the nucleus. Disruption of F-actin by Capzb knockout impairs Notch activity and MLC phosphorylation. Further inducing YAP activity and resulting in a positive feedback loop [94]. Transcriptomic analyses of YAP targets in CCA and HCC have identified a positive feedback loop on the actin network via the AMPK kinase NUAk Family Kinase 2 (NUAK2) [8]. By combining analyses of (1) TEAD4-ChIP seq from liver of TetO-YAP mice, (2) YAP-ChIP-seq in HuCCT1 CCA cells, (3) RNA-seq of YAP/TAZ silenced mouse liver and (4) RNA-seq from TetO-YAP mice, Yuan et al. [8] have identified 14 YAP-regulated gene-targets in liver cancer. Among the identified genes are two well-known YAP-target genes (angiomotin (AMOT) and NUAK2). Depletion or chemical inhibition of NUAK-2 by HTH-02–006 partially rescued the YAP-dependent tumorigenesis in HCC and CCA in vitro and in vivo [8]. Mechanistically, NUAK-2 phosphorylates S445 in the myosin phosphatase target subunit (MYPT1), thereby inhibiting the MLC phosphatase (MLCP). Increased levels of MLCP phosphorylation and loss of actin fibers induce liver stiffness and YAP signaling, which subsequently triggers more actin fibers and actomyosin contraction [94].

In summary, increased cellular tension activates YAP and is favored by the formation of F-actin bundles and the actin-myosin network. In contrast, in healthy tissues with low contraction and space limitation, YAP activity is inhibited by ABPs, which promotes F-actin disassembly and branching. It remains unclear how the cell senses the content of F-actin. Further, it is controversial if F-actin regulation is through direct or indirect Hippo signaling or both or alternatively a secondary effect of cellular tension.

Therapeutic opportunities for targeting actin cytoskeleton in CCA

We have highlighted actin cytoskeleton deregulation through different signaling pathways and cellular components in CCA including the primary cilium, cell-ECM interaction, intracellular receptor signaling, and intranuclear functions. These pathways encompass therapeutic options for targeting either directly or indirectly to modify the deregulation of the actin cytoskeleton in CCA.

Major proteins and signaling pathways, such as Hh and HDAC6 signaling, are linked to the primary cilium, and have shown to affect the actin cytoskeleton in CCA. Among pharmacological inhibitors, SMO antagonizes Hh signaling, some of SMO inhibitors being already approved by the US Food and Drug Administration (FDA) in other cancer types. Cyclopamine is a natural alkaloid SMO inhibitor that effectively counteracts the development digestive tract cancer and CCA [99,100]. The continuous advancement in the development of cyclopamine derivatives has resulted in the second-generation compound vismodegib (GDC-0449), which are approved by the FDA for treatment in advanced basal cell carcinoma [101]. Interestingly, vismodegib inhibits the invasion of tumor cells in vivo in a rat model of CCA [11], a compound deserving to be investigated in the framework of CCA.

Two HDAC6 inhibitors (ACY-1215 and tubastatin A) have both shown favorable results in CCA. ACY-1215 inhibits the proliferation of cystic cholangiocytes in vitro and in vivo [26] and has shown promising results in a multi-institutional phase Ib/II study in relapsed lymphoma [102]. Tubastatin A represses CCA growth and restores the formation of the primary cilium [26], but due to complications in the delivery method of this compound, it has not yet moved into clinical trials [103]. Lovastatin is an inhibitor of cellular-ECM interaction, which is mediated by integrins [47]. As such, lovastatin has been used as a breast cancer prevention in patients with abnormal breast ductal cytology, but in a phase II setting it showed no significant change, questioning a beneficial application in CCA [104].

Intracellular signaling inducing actin rearrangements as well as cellular motility and plasticity are often mediated by RTKs such as EGFR, FGFR, and CXCR4, to which many tyrosine kinase inhibitors (TKI) have been developed. Within the TKI category are included inhibitors against the constitutively active FGFR2 fusion protein and prevalent FGFR mutations as seen in a subset of iCCA patients. This category includes the FDA approved pemigatinib [105], in fingatinib (phase 2) [106], and futibatinib (phase 1) [107]. Similar results have been achieved by the simultaneous inhibition of BRAF and MEK signaling combining dabrafenib and trametinib, showing a benefit in 20 out of 43 BRAFV600E-mutated CCA patients [108]. Also, an antagonist against CXCR4 (AMD3100) approved by the FDA had minor effects on solid tumors and showed significant adverse side effects [109]. Although, AMD3100 has been developed into the structurally similar compound (BPRX8070), which in the preclinical setting prevents tumor growth,
The actin cytoskeleton is significantly affected in CCA and contributes to the malignant transformation of cholangiocytes. Focusing future research on how actin affects and regulates other signaling pathways may provide more insights into the mechanisms of CCA cancer development, progression, and metastasis. Impact of the basic actin cytoskeleton components, such as actin isoforms (ARPs), ABPs, and Rho GTPases, remains largely unknown in cholangiocarcinogenesis. In contrast, direct inhibition of the actin cytoskeleton (Table 1) might help in the prevention of CCA development in individuals associated with risk factors like biliary inflammation and liver cirrhosis as emphasized in the second part of the review. Manipulation of the actin cytoskeleton and its organization highlights a potential for a novel therapeutic area often missed. Targeted disruption of the actin network in tumor cells by magnetic field-responsive supramolecular assemblies increases tumor cell death and thus, reduces tumor growth in vivo. Other promising drugs include chondramide B, miur-aenamide A (derived from myxobacteria strains), and jasplakinolide that induce actin stabilization and polymerization, and reduces tumor growth and invasion (Table 1). 

Conclusion and future perspectives

The actin cytoskeleton is a central pathway in the development of cholangiocarcinoma and tumor maintenance.

Funding

LD was awarded a PhD project grant from the Danish Cancer Research Foundation. JFLB was awarded an individual fellowship (EpICC) from the European Union Marie Skłodowska-Curie postdoctoral program. The laboratory of JBA is supported by the Novo Nordisk Foundation (0,058,419; 0,074,956), Danish Cancer Society (R167-A10784, R278-A16638), and the Danish Medical Research Council (1030-00070B). This project was supported by the European Union’s Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement no. 801,481 (IMED). LF belongs to a team supported by the Fondation pour la Recherche Médicale (Equipe FRM 2020 n° EQU202003010517), by Inca HRHG-MP-22-039 and ITMO Cancer of Aviesian within the framework of the 2021-2030 Cancer Control Strategy, on funds administered by Inserm.

Declaration of Competing Interest

JBA declares consultancy roles for Flagship Pioneering, SEALD and QED therapeutics.

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