

Targeting cancer stem cells in cholangiocarcinoma (Review)

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Abstract. The incidence of cholangiocarcinoma has been increasing steadily over the past 50 years, but the survival rates remained low due to the disease being highly resistant to non-surgical treatment interventions. Cancer stem cell markers are expressed in cholangiocarcinoma, suggesting that they serve a significant role in the physiology of the disease. Cancer stem cells are frequently implicated in tumor relapse and acquired resistance to a number of therapeutic strategies, including chemotherapy, radiation and immune checkpoint inhibitors. Novel targeted therapies to eradicate cancer stem cells may assist in overcoming treatment resistance in cholangiocarcinoma and reduce the rates of relapse and recurrence. Several signaling pathways have been previously documented to regulate the development and survival of cancer stem cells, including Notch, janus kinase/STAT, Hippo/yes-associated protein 1 (YAP1), Wnt and Hedgehog signaling. Although pharmacological agents have been developed to target these pathways, only modest effects were reported in clinical trials. The Hippo/YAP1 signaling pathway has come to the forefront in the field of cancer stem cell research due to its reported involvement in epithelium-mesenchymal transition, cell adhesion, organogenesis and tumorigenesis. In the present article, recent findings in terms of cancer stem cell research in cholangiocarcinoma were reviewed, where the potential therapeutic targeting of cancer stem cells in this disease was discussed.

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1. Introduction

Cholangiocarcinoma (CCA) is a diverse and collective malignancy that is derived from the biliary epithelium (1). Broadly recognized risk factors for CCA include liver fluke infection, primary sclerosing cholangitis, cirrhosis, viral hepatitis, congenital anomalies of the biliary tree and hepatolithiasis (1). In addition, inflammatory bowel disease, obesity and genetic predisposition have been reported to be associated with a higher risk of developing CCA (Fig. 1) (1). Although the incidence of CCA in the United States remains relatively low, at 1.26 cases per 100,000 people as of 2015, its prevalence has been steadily increasing over the past 50 years (2). According to analysis by race, CCA has the highest overall incidence in Asian Americans at 1.87 cases per 100,000 people, followed by Caucasian Americans at 1.23 cases per 100,000 people and lastly African Americans, which is 1.17 per 100,000 people (2). However, the mortality rate of CCA in African Americans has increased dramatically over the past decade, increasing 45% compared to ~20% in Asian Americans and Caucasian Americans (3).

The current 5-year survival rate in patients with early stage CCA who undergo curative-intent surgery is 30% (4). Patients with advanced disease at diagnosis have limited treatment options and poor prognosis. In a previous surveillance program of 825 patients with CCA, regardless of treatment modality or tumor pathology, the median overall survival (OS) was found to be 7 months, whilst the 5-year survival rate was revealed to be 5.7% (5). Due to aggressive tumor growth and the presence of metastases at diagnosis, ~86% of patients with CCA patients are ineligible for either curative-intent surgery or palliative resection (5). Patients ineligible for curative surgery are provided a combinatorial chemotherapy of gemcitabine and cisplatin either as adjuvant to surgery or as the first line of care. However, combination chemotherapy confers minimal survival benefit, where it modestly prolongs the median OS to 11.7 months and progression free survival to 8 months compared with a historical OS of 2.5-4.5 months with supportive care (6,7). This highlights the urgent need for the development of novel effective treatment options for patients who are ineligible for surgery.

Newly developed therapies have demonstrated promising clinical efficacy against chemotherapy-refractory

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CCA. In a phase II study of patients with fibroblast growth factor receptor (FGFR)-altered advanced biliary tract cancer (BTC), a selective pan-FGFR kinase inhibitor exerted impressive anti-tumor activity with a disease-control rate (DCR) of 82% (8). Additionally, in another phase I study involving patients with isocitrate dehydrogenase 1 (IDH)-positive CCA, which represents ~25% of all cases of CCA, the IDH1 inhibitor ivosidenib exhibited a well-tolerated safety profile with an overall response rate (ORR) of 5% and an OS of 13.8 months (9). However, these aforementioned promising targeted therapies were only viable for a relatively small percentage of patients with CCA with the specific IDH1 and FGFR mutations aforementioned (9).

Within the last decade, immunotherapy has become a major pillar of cancer therapy. Immune checkpoint inhibitors (ICIs) act by targeting dysregulated immune checkpoints, including programmed death protein 1 (PD-1) and programmed death ligand (PD-L1), which affect anti-tumor immunity in several types of cancers (10). Accumulating evidence have demonstrated encouraging results in applications with ICIs alone for treating hepatocellular carcinoma (HCC), with response rates reaching ~15-20% (11,12). However, ICIs have demonstrated minimal efficacy for the treatment of CCA (13,14). In a recent study of advanced BTCs that were treated with a combination of an ICI and microwave ablation, the ORR was found to be 12.5% (15). Novel therapeutic strategies to combat CCA progression are therefore urgently sought. One emerging target in this research field is cancer stem cells (CSCs), also referred to as tumor-initiating or -propagating cells.

2. Defining cancer stem cells

There is increasing consensus supporting the existence of a distinct cellular hierarchy within a tumor, where CSCs are unique originators of all tumor cells and are responsible for tumor growth (16-18). CSCs have been implicated in CCA along with a variety of other solid tumors, including breast, brain, colorectal (CRC), pancreatic, liver, melanoma, ovarian and prostate cancer (Tables I and II). It is hypothesized that CSCs survive following the initial stages of cancer therapy and thereby facilitate relapse and metastasis, where they are responsible for acquired resistance to conventional cancer treatment regimens, including radiation therapy and the more recently discovered immunotherapy (18-20). CSCs may be able to evade the immune system by altering their immunogenicity, enabling the avoidance of rejection mediated by the immune system *in vivo* (21). CSCs are defined by their enriched capacity for self-renewal and differentiation into explicit malignant progenies. Tumors with CSC-enriched phenotypes are considerably more plastic than originally anticipated, which are in turn heavily influenced by the tumor microenvironment, rendering the design of therapeutic methodology against them difficult (22). In addition, although previous reports suggested a frequency of <1 CSC per 1,000 cancer cells, the proportion of CSCs with tumorigenic capacity could be much higher (23,24). CSCs can be uniquely characterized by their cell-surface markers, where several markers have been used to identify CSCs in various types of cancers such as CCA (Tables I and II).

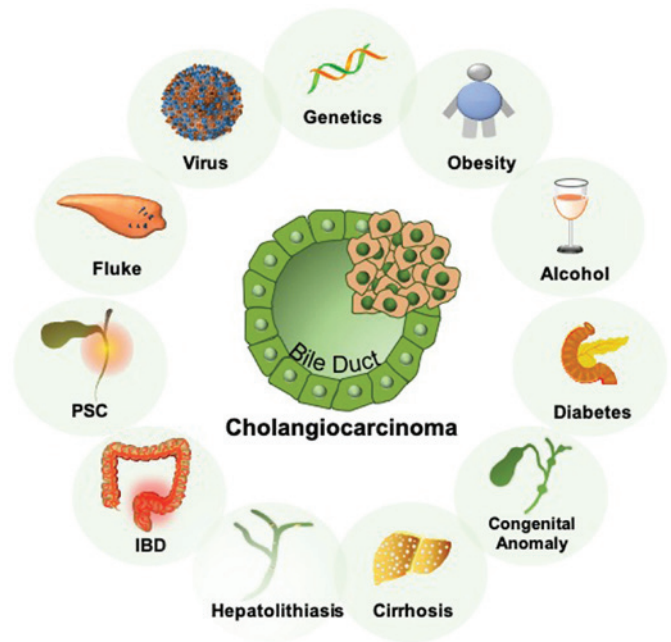


Figure 1. Established risk factors of cholangiocarcinoma. PSC, primary sclerosing cholangitis; IBD, inflammatory bowel disease.

CD133. CD133, also known as prominin-1, is a pentaspan trans-membrane glycoprotein that appears to be an epithelial marker in tissues in addition to being a CSC marker (24). Although the precise function of CD133 remains unclear, considerable evidence exists for the increased capabilities for tumor initiation in CD133⁺ cell cultures and tumor xenografts (24-28). The presence of CD133 along with other suspected CSC markers has also been associated with poorer overall survival in patients with CCA (29). In a previous study of 29 patients with intrahepatic CCA who had undergone major hepatectomies, only 8% of CD133⁺ patients remained alive 5 years following surgery, compared with 57% among CD133⁻ patients (P=0.02) (29). Consistent with this notion, CD133⁺ liver cancer cell lines appear to exhibit significantly higher resistance to autophagy as a result of IFN- γ treatment compared with the corresponding CD133⁻ cell lines *in vitro* (28). This finding has been corroborated *in vivo*, where CD133⁻ tumors shrank in cirrhosis-associated HCC cells in mice treated with IFN- γ . By contrast, tumors of the CD133⁺ phenotype were resistant and instead became further enriched with CD133⁺ expression (28).

CD44 and CD24. CD44 is a cell-surface glycoprotein that is involved in cell-cell adhesion and migration (30,31). Functionally, it is involved in leukocyte homing and activation, wound healing and cell migration (30,31). CD44s, the conventional isoform of CD44, is expressed in normal epithelial cells and serves as an adhesion molecule in the extracellular matrix (30,31). In some carcinomas of epithelial origin, variant isoforms of CD44 have been implicated in tumor metastasis and invasion (32). Previous studies have suggested CD44 and its variant isoforms to be responsible for cellular stemness characteristics, associated with resistance to reactive oxygen species (ROS) in CSCs and implicated in the progression of malignancies in gastrointestinal system (30,31). However, it should be noted that the clinical relevance of different CD44 isoforms are

Table I. Cancer stem cell markers from various solid tumors.

Marker	Type of cancer	<i>In vitro</i> assay	<i>In vivo</i> study (Xenograft)
CD133	Brain	Sphere formation (94)	NOD/SCID mice (106), nude mice (94)
	Breast	Colony formation (95)	NOD/SCID mice (95)
	Colon	Clonosphere formation (96,97)	NOD/SCID mice (96,107)
	Kidney	Colony formation (98)	SCID mice (98)
	Hepatic	Colony formation (97,99)	NOD/SCID (97), SCID mice (99)
	Lung	Sphere formation (100)	SCID mice(100)
	Melanoma	Colony formation (101)	N/A
	Ovarian	Sphere formation (102,103)	NSG mice (102), NOG mice (103)
	Pancreatic	Colony formation (104)	N/A
	Prostate	Colony formation (105)	Nude mice (108)
CD90	Brain	Sphere formation (109)	NOD/SCID mice (103)
	Hepatic	Sphere formation (110)	NOD/SCID mice (110)
	Lung	Colony and sphere formation (111)	NOD/SCID mice (111)
	Ovarian	Colony formation (112)	NOD/SCID mice (112)
	Stomach	Sphere formation (113)	ALB/c nude mice (113)
ALDH1	Brain	Colony formation (114)	Nude mice (114)
	Breast	IHC staining (115)	BALB/c mice (120)
	Colon	Colony (116) and sphere formation (117)	Nude mice (117)
	Hepatic	Colony formation (99)	SCID mice (99)
	Lung	Colony formation (118)	N/A
	Melanoma	N/A	NOD/SCID mice (121)
	Ovarian	Sphere formation (102,103)	NSG TM mice (102), NOG [®] mice (103)
	Pancreatic	N/A	NMRI nu/nu mice (122)
	Prostate	Sphere formation (119)	NOD/SCID mice (119)
CD44	Brain	Colony (123) and sphere formation (124)	CD44 KO mice (123), nude mice (124)
	Breast	IHC staining (115)	N/A
	Colon	Colony formation (125)	NOD/SCID (131), BALB/c mice (125)
	Kidney	IHC staining (126)	N/A
	Hepatic	Colony formation (127)	NOD/SCID mice (127)
	Lung	Colony and sphere formation (111)	NOD/SCID (111), BALB/c mice (132)
	Ovarian	Sphere formation (128)	NOD/SCID mice (128)
	Pancreatic	N/A	NOD/SCID mice (133)
	Prostate	Colony (105) and sphere formation (129)	NOD/SCID mice (129)
	Stomach	IHC staining (130)	SCID mice (134)
EpCAM	Breast	Sphere formation (135)	NOD/SCID mice (135)
	Colon	Colony formation (136)	N/A
	Hepatic	Sphere formation (110)	NOD/SCID mice (110)
	Ovarian	Sphere formation (128)	N/A
	Pancreatic	N/A	NOD/SCID mice (133)
CD24	Breast	IHC staining (115)	BALB/c mice (120)
	Colon	Colony formation (137)	NOD/SCID mice (137)
	Hepatic	Colony formation (138)	Nude mice, SCID mice (138)
	Ovarian	Sphere formation (128)	NOD/SCID mice (128)
	Pancreatic	N/A	NOD/SCID mice (133)
SOX2	Brain	Colony formation (139)	NOD/SCID mice (139)
	Breast	Colony formation (140)	Nude mice, BALB/c mice (140)
	Lung	Colony formation (141)	Nude mice (141)
NANOG	Brain	Sphere formation (142)	Nude mice (142)
	Hepatic	Colony and sphere formation (138)	NOD/SCID, BALB/c mice (138)
	Prostate	Sphere formation (143)	NOD/SCID mice (143)
	Testis	IHC staining (144)	N/A

NOD, non-obese diabetic; SCID, severe combined immunodeficient; KO, knockout; ALDH1, aldehyde dehydrogenase 1; EpCAM, epithelial cell adhesion molecule; SOX2, SRY-box transcription factor 2.

Table II. Cancer stem cell markers in cholangiocarcinoma.

Marker	<i>In vitro</i> assay	<i>In vivo</i> study (xenograft)
CD133	Sphere formation (24)	BALB/c, NOD/SCID mice (24)
CD90	Sphere formation (24)	BALB/c mice, NOD/SCID mice (24)
ALDH1	Colony formation (46)	NOD/SCID mice (46)
CD44	Sphere formation (36)	NOD/SCID mice (45)
EpCAM	Sphere formation (24)	NOD/SCID mice (45)
CD24	Sphere formation (36)	NOD/SCID mice (45)
SOX2	Immunohistochemical human samples (50)	NOD/SCID mice (139)

ALDH1, aldehyde dehydrogenase 1; EpCAM, epithelial cell adhesion molecule; SOX2, SRY-box transcription factor 2; NOD, non-obese diabetic; SCID, severe combined immunodeficient.

highly dependent of the type of cancer. For example, CD44v6 appeared to be unrelated to the CCA progression, whilst CD44v9 appears to be clinically relevant to the disease (32-34). In a previous immunohistochemistry analysis of CCA tumors, CD44v9 was found to associate with the expression of inflammatory markers cyclooxygenase-2 (COX2) and S100 Calcium Binding Protein P, where CD44v9 expression was found to be higher in CCA associated with liver fluke infection (32).

There have been disparities in the findings regarding the use of CD24 as a CSC marker. The CD44^{high}/CD24^{low} cell phenotype has been repeatedly utilized as a signature of CSCs in breast tumors, where they were demonstrated to be chemoresistant following chemotherapy (35). Similar findings were also documented in a previous *in vitro* study of CCA (36). In CCA cell lines, a shift from CD44^{high}/CD24^{high} to CD44^{high}/CD24^{low} was observed in cells resistant to epidermal growth factor receptor inhibition (36). By contrast, pharmacological depletion of ROS scavengers resulted in increased sensitivity to radiotherapy and depleted clonogenicity in the CD24⁺CD90⁺-enriched cell population, suggesting that the CD24⁺CD90⁺ combination may be responsible for mediating resistance to radiation in CSCs (37). In patients with CCA who received chemotherapy and radiation, CD24 expression was previously found to be associated with a lower median survival time (38). To verify these findings, further research on the individual role of CD24 in CSCs and cancer progression is required.

Epithelial cell adhesion molecule (EpCAM). EpCAM is a downstream signaling target of the Wnt pathway (39,40). Wnt signaling was previously demonstrated to be simultaneously decreased in colon cancer cells following EpCAM knock-down (39). Furthermore, it was previously found in HCC that EpCAM expression is dependent on the nuclear accumulation of β -catenin (40). EpCAM has been applied as a prognostic marker for a number of epithelial cancers, including HCC and CCA (41-44). In accordance with *in vitro* studies of the individual tumorigenic potential of CSC markers, CD44⁺CD24⁺EpCAM⁺ cells isolated from extrahepatic CCA xenografts in immunocompromised mouse exhibited higher tumorigenicity compared with those of the CD44⁺CD24⁺EpCAM⁻ phenotype (45).

Aldehyde dehydrogenases (ALDH). ALDH belong to a family of intracellular enzymes that are involved in cellular

detoxification, differentiation, and drug resistance (46,47). Although ALDH1 has been most commonly applied as a CSC marker in breast cancer, it has also been previously implicated in CCA and HCC (46,47), where the expression level of ALDH1 was found to be correlated with poor prognosis in patients with CCA (46,47). In addition, ALDH1 expression has been demonstrated to potentiate mesenchymal properties in the CCA cell line TFK-1 (46). However, conflicting evidence exists with regards to the role of ALDH1 in CRC compared with that in CCA. In CRC, it was hypothesized that the expression of extracellular, rather than intracellular CSC markers, may serve as superior indicators of tumor stemness (23,24).

SRY-box transcription factor (SOX)2, NANOG, and octamer-binding transcription factor 4 (OCT4). SOX2, NANOG and OCT4 are all transcription factors essential for the maintenance of stemness in embryonic stem cells and have been previously used as markers for CSCs (48). They directly communicate with each other during embryonic development, where they suppress differentiation into progenitor cells (48). NANOG, OCT4, and SOX2 expression have all been previously revealed to be associated with poor prognosis in rectal cancer, glioma and CCA (49). In rectal cancer, expression of ≥ 2 in comparison to ≤ 1 of these markers was found to associate significantly with poorer OS. In particular, OCT4 was also demonstrated to be independently associated with poor tumor differentiation, higher N stage and larger tumor size in rectal cancer (48). Likewise in CCA, co-expression of OCT4 and Nanog was found to be associated with the most inferior of the clinical outcomes (49). Elevated SOX2 expression has also been previously associated with poorly differentiated tumors, metastasis and insignificantly, vascular invasion and tumor stage in CCA (50). Patients with CCA in which SOX2 was overexpressed, exhibited significantly lower OS with no difference in DFS (50).

Overall, based on the data available on CSC markers, it is important to characterize CSCs in tumors by using multiple markers instead of reliance on any single marker.

3. Signaling pathways involved in CSC stemness and potential targets

In addition to the expression of cell surface molecules that are typically associated with stem cells, CSCs exhibit classical

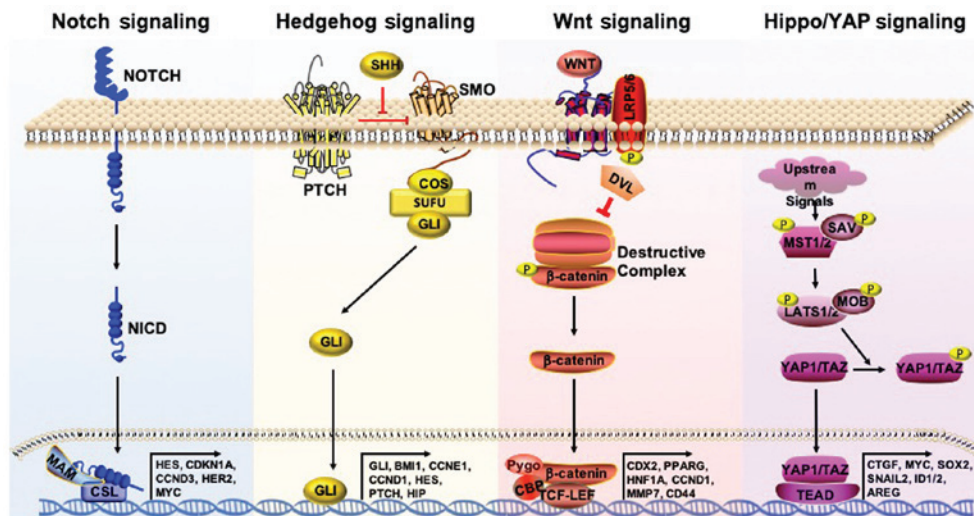


Figure 2. Pathways involved in CSC Development. Hippo/YAP1, Notch, Hedgehog, and Wnt signaling are all pathways implicated in the development of stemness features, including EMT, cell migration and proliferation, and tumorigenesis. The regulation of their target genes plays a vital role in CSC development. YAP1, yes-associated protein 1; EMT, epithelial-to-mesenchymal transition; CSC, cancer stem cells; MAM, mastermind; CSL, CBF-1, suppressor of hairless, lag-1; HES, hairy and enhancer of split; CDKN1A, cyclin dependent kinase inhibitor 1A; CCND3, cyclin D3; COS, costal; HER2, human epidermal growth factor receptor 2; PTCH, patched; SHH, sonic hedgehog; SMO, smoothened; SUFU, suppressor of fused homolog; CCND1, cyclin D1; HIP, hedgehog-interacting protein; LRP5/6, Low-density lipoprotein receptor-related protein 5/6; DVL, disheveled; Pygo, pygopus; TCF-LEF, T-cell factor/lymphoid enhancer factor; CBP, CREB binding protein; CDX2, caudal type homeobox 2; PPARG, Peroxisome proliferator activated receptor- γ ; HNF1A, hepatic nuclear factor 1 α ; MMP7, matrix metalloproteinase 1; MST1/2, macrophage stimulating 1; SAV, salvador family WW domain containing protein 1; MOB, phocein; LATS1/2, large tumor suppressor kinase 1/2; TAZ, tafazzin; TEAD, transcriptional enhancer factor; CTGF, connective tissue growth factor; SOX2, SRY-box transcription factor 2; ID1/2, inhibitor of differentiation 1/2, AREG, amphiregulin.

features of stem cells such as the ability to differentiate into explicit progenies (51). Signal transduction pathways that are highly active in embryonic and adult stem cells, including Notch, Hedgehog and Wnt, are also highly active in CSCs (Fig. 2) (19). In this section, the various signaling pathways involved in CSC maintenance and research that are focused on targeting these pathways will be outlined.

Hippo/Yes-associated Protein (YAP1). The Hippo/YAP1 is a signaling pathway that is highly conserved among the majority of mammalian species. It has recently garnered significant attention due to its reported role in regulating CSCs (52). Core kinases involved in this pathway include macrophage stimulating (MST) 1/2 and large tumor suppressor kinase (LATS) 1/2, which are under regulation by scaffolding proteins Salvador family WW domain containing protein 1 and phocein (MOB), respectively (Fig. 2). Activation of MST and LATS leads to the phosphorylation and subsequent inactivation of YAP1. This inhibits YAP1 from entering the nucleus, where it would normally bind to the transcriptional enhancer factor (TEAD) and SMAD families of transcription factors, leading to the transcription of a number of oncogenic genes, including SOX9, amphiregulin, MYC and Gli1 (53-55).

YAP1 overexpression is associated with tumorigenesis, epithelial-to-mesenchymal transition (EMT), in addition to tumor angiogenesis and invasion, physiological processes that have been previously demonstrated to lead to unfavorable prognoses in malignancies, including CCA, HCC, lung, brain, ovarian, breast, bladder and colon cancer (56-60). YAP1 expression was found to be an independent prognostic factor in both human and animal studies of CCA (57,59). Previous studies have demonstrated that liver-specific MOB1a/lb double knockout (61) and MST1/2 conditional knockout mice (62)

exhibited phenotypically mixed HCC/CCA inducible tumors. These findings suggest that the Hippo/YAP1 signaling pathway is critical for liver tumorigenesis. Intriguingly, the introduction of activated YAP1 and myristoylated AKT in the biliary tract, coupled with biliary ligation, triggered CCA formation in an IL-6-dependent manner within 6-8 weeks in >70% of the mice tested (63). This was performed through the ectopic expression of the constitutively active AKT and YAP1 using the Sleeping Beauty transposon transfection system. This study not only highlighted the potential role of inflammatory cytokines on CCA oncogenesis but also suggests YAP1 to be an important driver of CCA tumorigenesis.

A number of studies have indicated that the Hippo/YAP1 pathway serves an integral role in the maintenance of CSCs. YAP1 overexpression directly upregulates SOX9 in esophageal cancer, which in turn endows cancer cells with stem-like properties (64,65). In addition, YAP1 can induce the expression of the embryonic stem cell transcription factor SOX2 and co-operate with the pro-inflammatory COX2 pathway to expand the population of CSCs in urothelial cancer (66). Immunohistochemistry analysis of COX2 and YAP1 expression in bladder cancer samples previously demonstrated that these two proteins can mediate resistance to treatment, whilst the *in vitro* inhibition of these proteins significantly reduced tumor growth (66). Additionally, the expression of YAP1 was found to be upregulated in CSCs in non-small cell lung cancer (NSCLC) cell lines, which was believed to contribute to their capacity for self-renewal forming angiogenic tubules (67). This previous study also showed that knocking down YAP1 expression can significantly reduce the spheroid forming and proliferative capacity of NSCLC (67). Interestingly, the effects of YAP1 were revealed to be mediated through induction of SOX2, which then directly interacts with OCT4, in a manner

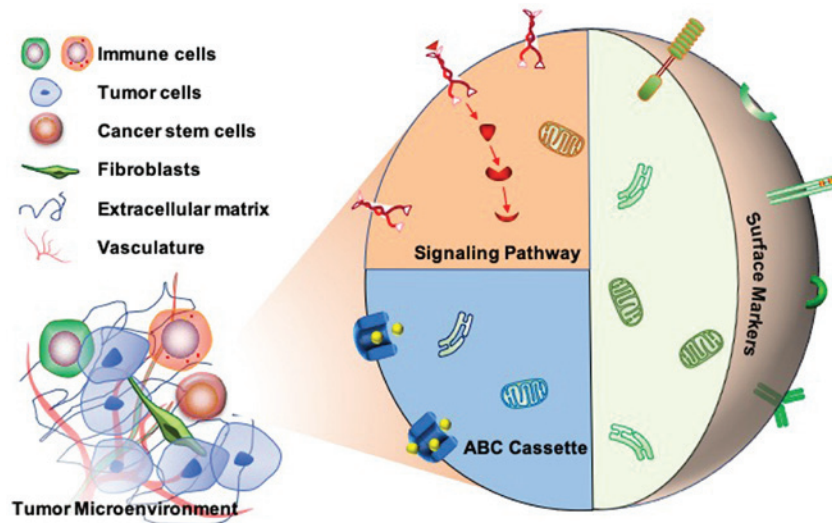


Figure 3. Strategies to target cancer stem cells. Many strategies aimed at eradicating CSCs have been developed and the main areas have been summarized, including targeting cell surface markers, signaling pathways and drug efflux transporters. ABC, ATP-binding cassette.

that was independent of TEAD2 (67). It was reported previously that long noncoding RNAs are highly expressed in CSCs of HCC, which are required for the self-maintenance of liver CSCs (68). Among those, lncBRM initiates YAP1 signaling activation to drive the self-renewal of liver CSCs (68). In conclusion, these findings suggest that YAP1 likely serve a pivotal role in the maintenance of stemness in CSCs.

Until recently, targeting the Hippo/YAP1 signaling pathway has proved challenging due to its complexity and substantial crosstalk with other pathways. Verteporfin, a photodynamic drug that was traditionally used for treating macular degeneration, has recently emerged as a YAP/tafazzin (TAZ) inhibitor, where it has demonstrated promising preclinical results in cancers such as CCA (57). The combination of verteporfin and rapamycin was previously found to inhibit intrahepatic CCA cell proliferation and tumor growth (57), where verteporfin activated mTOR whilst inhibiting STAT3 phosphorylation in CCA (57). However, to the best of our knowledge, no clinical studies on the effects of verteporfin on CCA or CSCs in other cancers have been performed.

In a recent study, LEE011 was found to inhibit cyclin-dependent kinase 6 (CDK6), whilst CA3 inhibited YAP1, using both *in vivo* and *in vitro* models of esophageal cancer (69). YAP1 and CDK6 expression were also revealed to associate positively with each other and with resistance to radiation (69). Combined treatment using both CA3 and LEE011 reduced tumor volume to a greater degree compared with either treatment alone. These findings suggest that YAP1 may synergize with CDK6 to induce cell proliferation and resistance to chemotherapy in cancer (69). However, to date, no clinical studies have been conducted using the novel Hippo/YAP1 inhibitors. Further research devoted to the effects of Hippo/YAP1 on CSC formation may prove to be beneficial for the treatment of refractory cancers such as CCA.

Sonic Hedgehog. Hedgehog (Hh) protein activation by Patched triggers a signaling cascade that regulates proliferation, metastasis and invasion in cancer cells (70). In addition, it has also been implicated in the stemness maintenance of CSCs (19). A

previous study has found that hypoxia induces Hh activation in CCA, which was demonstrated to be positively associated with the induction of EMT, and upregulation of the stemness markers, including NANOG, Oct4, SOX2, CD133 *in vitro* (27).

Application of the Hh inhibitor Saridegib, in combination with gemcitabine, did not exert notable therapeutic effects on metastatic pancreatic cancer in phase II trials (71). Additionally, whilst there were promising preclinical and phase I data for this trial, control patients exhibited longer OS compared with those treated with the Hh inhibitor (71,72). Glasdegib and Vismodegib are currently the two FDA-approved Hh signaling inhibitors that have been extensively studied in relation to malignancies such as acute myeloid leukemia (73). However, since research on their effects in other cancers have been mostly discontinued, they have not been studied clinically in CCA.

Notch. The Notch signaling pathway has been extensively studied in cancer, where it was suggested to be important for the regulation of cell survival and apoptosis (74). Notch is cleaved sequentially by tumor necrosis factor- α converting enzyme (TACE) and γ -secretase, resulting in the release of the intracellular domain of Notch (NICD). NICD then translocates into the nucleus where it mediates the transcription of target genes, including hairy and enhancer of split-1, NF- κ B, cyclin D1 and c-myc (75). The Notch1 receptor and the Notch ligand Jagged 1 was found to be overexpressed in four human CCA cell lines (76). In addition, overexpression of NICD in mouse livers has been previously found to induce cystic CCA tumor development (77). These observations suggest that Notch can serve as a potential target for controlling CSCs (78).

γ -secretase inhibitors are among the largest class of Notch inhibitors, with >12 different clinical trials having previously applied this class of drug (19). However, none have progressed to phase III trials due to inefficacy or intolerance and therefore development of drugs of this class has been discontinued. Antibodies specifically targeting Notch 1-3 have also been developed, but like γ -secretase inhibitors, research on this class of drug has been repeatedly discontinued (19). By

Table III. Active clinical trials targeting CSCs in solid tumors.

Trial identifier	Type of solid tumor	Experimental arm	Phase
NCT03949283	Recurrent ovarian carcinoma, platinum-resistant ovarian cancer	Chemo ID assay	III
NCT02232633	HCC, CCA	BBI503	II
NCT03632798	Recurrent ovarian cancer	Chemo ID assay	III
NCT03632135	Recurrent glioblastoma	Chemo ID assay	III
NCT02642094	Breast cancer	Rapamycin	II
NCT03548571	Glioblastoma	Dendritic cell immunization, adjuvant temozolomide	II, III
NCT03298763	Lung adenocarcinoma	MSCTRIAL	I, II
NCT02859415	Thoracic cancer	Mithramycin	I, II
NCT02279719	HCC	BBI608, BBI503, in combination with sorafenib	I, II
NCT03186937	Triple negative breast cancer	Hominex-2	II
NCT02370238	Metastatic breast cancer	Paclitaxel, reparixin	II
NCT03030287	Ovarian, peritoneal, fallopian cancer	OMP-305B83, paclitaxel	I
NCT03927573	NSCLC, breast, pancreatic, urogenital	GEM3PSCA	I
NCT03572283	Pancreatic cancer	Bethanechol	I
NCT02753127	Colorectal	Napabucasin + FOLFIRI	III
NCT02157051	Breast cancer	CD105/Yb-1/SOX2/CDH3/MDM2-polyepitope plasmid DNA vaccine	I
NCT03466450	Glioblastoma	Glasdegib and temozolomide	I, II
NCT03816163	Pancreatic cancer	Zolbetuximab + nab-paclitaxel + gemcitabine	II
NCT02432326	Solid tumors	BBI608, BBI503	I
NCT02483247	Solid tumors	BBI503 in combination: capecitabine doxorubicin nivolumab pembrolizumab paclitaxel or sunitinib	I, II
NCT02467361	Metastatic cancer	BBI608 in combination: pilimumab, nivolumab, or pembrolizumab	I, II
NCT02776917	Breast cancer	Cirmtuzumab + paclitaxel	I
NCT03851614	Mismatch repair proficient CRC, PAC, leiomyosarcoma	Durvalumab in combination with olaparib or cediranib	II
NCT02231723	PDAC	BBI608 in combination with four standard chemotherapies	I
NCT02024607	CRC, HCC, PDAC, CCA, EAC or gastric cancer	BBI608 in combination with 7 standard chemotherapies	I, II
NCT01781455	Solid tumors	BBI503	I, II
NCT02903771	Ovarian, peritoneal, fallopian cancer	Cantrixil	I
NCT01372579	Breast cancer	Eribulin mesylate, carboplatin	II

NSCLC, non-small cell lung cancer; CRC, colorectal cancer; HCC, hepatocellular carcinoma; PDAC, pancreatic ductal adenocarcinoma; PAC, pancreatic adenocarcinoma; CCA, cholangiocarcinoma; EAC, esophageal adenocarcinoma.

contrast, the anti-delta like canonical notch ligand 4 antibody, Demcizumab, has progressed to randomized phase II trials in lung and pancreatic cancer after exhibiting a manageable safety profile and an ORR of 51% in phase I NSCLC trials (19). No data on its use in CCA are currently available.

Wnt/β-catenin. The Wnt/β-catenin signaling pathway is one of widely studied pathways in cancer research. Notably, CRC is initiated by mutations in genes such as adenomatous polyposis coli (APC), which activates the Wnt/β-catenin pathway (79). In the canonical Wnt/β-catenin pathway, Wnt ligands bind to Frizzled and low-density lipoprotein receptor-related protein receptor complexes, initiating the recruitment of scaffold proteins and disruption of the

β-catenin destruction complex. Mutations in this complex, including that of APC, can lead to β-catenin accumulation in the cytosol. The accumulated β-catenin then translocates into the nucleus where it associates with the TCF family of transcription factors and a number of co-activators, including TAZ, to initiate the transcription of target genes. It has been previously reported that the canonical Wnt/β-catenin signaling pathway is activated in human CCA, where the inhibition of Wnt/β-catenin signaling reduced proliferation whilst inducing apoptosis *in vivo* (80). However, it remains unclear how the Wnt signaling pathway serves a role on the stemness of CSCs. The canonical Wnt/β-catenin pathway has been previously reported to be directly regulated by TAZ, an effector of the Hippo/YAP1 pathway (81).

LY2090314 is a glycogen synthase kinase 3 inhibitor, which induces the accumulation of β -catenin (Fig. 2). It has been shown that LY2090314 treatment in conjunction with nab-paclitaxel in a preclinical model of pancreatic cancer prolonged mice survival (82). However, regimens consisting of LY2090314 in combination with pemetrexed and carboplatin, demonstrated suboptimal safety profiles and minimal clinical efficacy in patients with advanced pancreatic cancer in a previous phase I clinical trial (83). BBI503 is a novel stemness kinase inhibitor that inhibits Nanog and serine/threonine kinase 17a, which induces β -catenin accumulation by stabilizing the β -catenin destruction complex (Fig. 2). Phase I data on BBI503 indicated prolonged OS and disease control in patients with CRC tumors with positive Nanog expression (84,85). Phase II trials in both patients with HCC and CCA in addition to those with other types of solid tumors are under way (84,85).

JAK/STAT. STATs are a family of cytoplasmic transcription factors that serve key roles in maintaining cancer stemness. They exert significant influence on cellular survival, proliferation, differentiation and apoptosis by mediating responses to cytokine, hormone and growth factor signaling. Genetic variations in the JAK/STAT signaling pathway appear to be associated with CRC (86). Notably, abnormalities in STAT3 have been revealed to be involved in the oncogenesis of a number of cancers, where it was demonstrated that STAT3 and both JAK1 and JAK2 are involved in CRC cell growth, survival, invasion and migration through the regulation of target gene expression, including Bcl-2, E-cadherin, vascular endothelial growth factor and matrix metalloproteinases (86).

Napabucasin (BBI608), a drug that targets the transcription factor STAT3 to reduce stemness characteristics, has reached phase III trials (87). In phase II trials in CRC, napabucasin, in combination with standard chemotherapy, exhibited an impressive DCR of 93% and an ORR of 33% (88). Napabucasin has also been studied preclinically in CCA, where treatment with this drug resulted in general cytotoxicity and inhibited cancer stemness (89). In addition, napabucasin has been shown to inhibit colony formation and significantly downregulate the expression of several stemness-related genes, including CSC markers ALDH1 and CD133 in CCA cells (89).

4. Challenges and ongoing research targeting CSCs

To the best of our knowledge, no single agent is currently available that can effectively target CSCs. One of the reasons is the substantial crosstalk among the pathways aforementioned. For example, phosphorylated YAP in the cytoplasm can associate with β -catenin to promote its degradation (90). Furthermore, nuclear β -catenin can associate with TAZ and SMAD to induce transcription of Wnt target genes. Recently, it was discovered that the loss of MST1/2 in hepatocytes, a core kinase of the Hippo pathway, led to the activation of Notch signaling (91). This resulted in severe liver enlargement and HCC formation due to a positive feedback loop with YAP/TAZ (91). Knockdown of β -catenin expression in the livers of MST1/2-null mice was found to increase tumorigenesis, revealing an inhibitory role of Wnt in relation to YAP (91). Additionally, Hh can interact with both the Wnt and Notch pathways, where increased Hh signaling can upregulate the

expression of the Notch ligand and Jagged 2 in neuronal stem cells, thereby maintaining stemness (92). It is possible that this phenomena is conserved in cancer. Secreted frizzled protein 1 is an integral protein upstream in the Wnt signaling cascade that was previously identified as a target of Hh signaling through the actions of Gli1/2 (93). All aforementioned crosstalk between the signaling pathways increase the complexity and challenge of targeting CSCs using a single agent.

Given the role of CSCs in cancer relapse and metastasis, there is an urgent need for the development of novel therapies that target CSCs to effectively eradicate cancer (Fig. 3). Even with the knowledge of the pathways involved in the development and maintenance of CSCs, designing pharmacological agents for targeting these pathways has proven to be exceedingly difficult. Although there are only a small number of clinical drugs currently available that are hypothesized to target CSCs exclusively, a number of clinical trials targeting CSCs in different types of cancer, including CCA, are currently ongoing (Table III).

5. Conclusions

CSCs have the ability to hide from the immune system and resist therapies designed to kill cancer cells. YAP1 is a downstream effector of the Hippo/YAP1 pathway, where the dysregulation of this pathway leads to oncogenesis and the enhancement of cell stemness. A number of drugs have attempted to targeted cancer stemness with modest effects in clinical trials. The Hippo/YAP1 pathway has become an attractive target for novel CSC inhibition. Although novel therapeutic agents targeting CSCs have demonstrated promise in preclinical research, none have demonstrated satisfactory outcomes in clinical settings. The complexity of crosstalk between signaling pathways therefore warrants further research. Given the resistance of CCA to non-surgical treatment, patients with CCA may benefit substantially from research on this topic.

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Availability of data and materials

The datasets generated during the current study are not publicly available because they consist of publicly accessible information but are available on reasonable request.

Authors' contributions

NAM, JF, and CX collected information and wrote the manuscript. SZG collected information and edited the manuscript. All authors read and approved the final version of this manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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