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Cholangiocarcinoma: novel therapeutic targets

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Abstract

Introduction

Cholangiocarcinoma (CCA) is a liver cancer derived from the biliary tree with a less than 30% five-year survival rate. Early diagnosis of CCA is challenging and treatment options are limited. Some CCA patients have genetic mutations and several therapeutic drugs or antibodies have been introduced to target abnormally expressed proteins. However, CCA is heterogeneous and patients often present with drug resistance which is attributed to multiple mutations or other factors. Novel approaches and methodologies for CCA treatments are in demand.

Area covered

This review summarizes current approaches for CCA treatments leading to the development of novel therapeutic drugs or tools for human CCA patients. A literature search was conducted in PubMed utilizing the combination of the searched term “cholangiocarcinoma” with other keywords such as “miRNA”, “FGFR”, “immunotherapy” or “microenvironment”. Papers published within 2015-2019 were obtained for reading.

Expert opinion

Preclinical studies have demonstrated promising therapeutic approaches that target various cells or pathways. Recent studies have revealed that hepatic cells coordinate to promote CCA tumor progression in the tumor microenvironment, which may be a new therapeutic target. Although

further studies are required, novel therapeutic tools such as extracellular vesicles could be utilized to manage CCA and its microenvironment.

Keywords: Cholangiocarcinoma, immunotherapy, tumor microenvironment, microRNA, long non-coding RNA, extracellular vesicles

Article highlights

- Cholangiocarcinoma is a cancer that may emerge in any part of the biliary tree or cholangiocytes with a < 30% five-year survival rate
- Treatments of cholangiocarcinoma are limited, hence novel therapeutic approaches are required
- Numerous signaling pathways are involved in CCA development or progression, and small molecules targeting these pathways, such as antagonists against serotonin receptors, may have the potential as a novel therapeutic drug for CCA.
- Various targets have been unearthed based on genetic aberrations or the microenvironment
- Non-coding RNAs are associated with tumor progression and invasion in cholangiocarcinoma
- Targeting the tumor microenvironment and immunotherapies had promising results in recent studies.
- Extracellular vesicles could be utilized to manage tumor progression by delivering non-coding RNAs

Abbreviations

2-HG = D-2-hydroxyglutarate; AANAT = aralkylamine N-acetyltransferase; ABCA1 = ATP-binding cassette transporter A1; αSMA = alpha smooth muscle actin; ASMT = acetyl serotonin O-methyltransferase; Bcl-2 = B-cell leukemia 2; BCL9 = B-cell lymphoma 9; BICC1 = bicaudal C homolog 1; CAFs = cancer-associated fibroblasts; CCA = cholangiocarcinoma; CD47 = cluster of differentiation 47; ceRNAs = competing endogenous RNAs; COL6A3 = collagen type VI alpha 3; CTLA4 = cytotoxic T-lymphocyte associated protein 4; DCs = dendritic cells; DDC = dopa decarboxylase; DEN = dimethyl nitrosamine; ECM = extracellular matrix; EMT = epithelial-mesenchymal transition; EVs = extracellular vesicles; FAP = fibroblast activation protein; FGFR = fibroblast growth factor receptor; GITR = glucocorticoid-induced tumor necrosis factor receptor; HSCs = hepatic stellate cells; HSP90 = heat shock protein 90; IBD = inflammatory bowel disease; IDH = isocitrate dehydrogenase; IL = interleukin; IRF1 = interferon regulatory factor 1; KRAS = Kirsten rat sarcoma viral oncogene homolog; lncRNAs = long non-coding RNAs; MAOA = monoamine oxidase A; miRNAs = microRNAs; MORC2 = Microchidia family CW-type zinc finger 2; MSCs = mesenchymal stem cells; NDMA = *N*-nitroso dimethylamine; NK = natural killer; ROS = reactive oxygen species; PD-1 = programmed cell death protein 1; PD-L1 = programmed cell death ligand 1; PBMCs = peripheral blood mononuclear cells; PRKAR1A = protein kinase type I-alpha regulatory subunit; PPHLN1 = periphilin 1; PSC = primary sclerosing cholangitis; SHTN1 = shootin 1; SOX4 = SRY-box 4; TAA = thioacetamide; TACC3 = transforming acidic coiled-coil containing protein 3; TAMs = tumor-associated macrophages; TGF-β1 = transforming growth factor beta 1; TH =

tyrosine hydroxylase; Th17 = IL-17-expressing lymphocytes; Tregs = regulatory T lymphocytes; TP53 = tumor protein 53; TPH = tryptophan hydroxylase; Twist1 = twist-related protein 1.

1. Introduction

Cholangiocarcinoma (CCA) is a type of malignancy that emerges from the biliary tree. Based on statistics from cancer.net, the 5-year survival rate for patients with early-stage CCA is 30%. If the tumor has spread to the regional lymph nodes, the 5-year survival rate is 24%. If CCA has spread to a distant part of the body, the 5-year survival rate drops to 2%. CCA is heterogeneous and currently classified as intrahepatic, hilar or perihilar, or distal CCA (Figure 1) (1). An incidence of intrahepatic, perihilar, and distal CCA is 8%, 50%, and 42%, respectively, according to a previous report using 564 CCA patients at a single institution (2). CCA is the second most common primary hepatic malignancy after hepatocellular carcinoma (HCC) and accounts for 10-20% of total primary liver cancers (1, 3). Although it is recognized as a rare cancer since the estimated incidence in the US is 1.67 per 100,000 people (1), CCA is an aggressive malignancy characterized by difficulties in early diagnosis followed by limited treatment options, poor prognosis, and high mortality rates (4). As a result, 5-year survival rates are approximately 10% in the US (5). Curative surgical resection is the sole effective treatment but patients often have recurrences and metastases after surgery. Postoperative adjuvant chemotherapy can improve the survival and cure rates after surgery, but the effects of chemotherapy are limited, and the 5-year survival rate with potentially curative surgery is under 30% (6).

Various factors are associated with CCA development. Liver fluke infections caused by parasites such as *Opisthorchis viverrini* or *Clonorchis sinensis* are a common risk factor for CCA especially in Southeast Asia (4). Parasite infection often induces hepatolithiasis, which is the existence of gallstones in the bile ducts and increases the risk of CCA (7). Since CCA is a biliary tract cancer derived from the biliary tree, biliary disorders such as primary sclerosing cholangitis (PSC) are risk factors for CCA development (8). Inflammatory bowel disease (IBD) is closely associated with PSC and often co-exists which is referred to as PSC-IBD. PSC-IBD patients are at risk for CCA development, and the long duration of IBD is associated with the increased risk of CCA (9). Patients with choledochal cysts also have a high risk of CCA (4, 10). Chronic hepatitis and cirrhosis caused by a viral infection such as hepatitis B or C virus have been recognized as the major risk factor, especially for intrahepatic CCA (3, 10). Genetic traits that induce mutations, polymorphisms, or genetic aberrations may increase the risk of CCA development (11). Other reported factors include obesity, smoking, and alcohol drinking (10). Previous studies performed in the US and China have found that metabolic syndrome increases the risk of CCA (12, 13). The incidence of CCA has been increasing worldwide in recent years as evidenced by increased patients with obesity or metabolic syndrome, suggesting that CCA is a growing health concern (14).

Previous studies have identified various genes and pathways associated with the pathophysiology of CCA. Preclinical studies target these candidate pathways to establish novel treatments that are more effective and less invasive than current treatment options. This review summarizes current therapeutic targets and approaches for CCA, focusing on preclinical research studies that represent potentials for the development of novel CCA therapies. A literature search

was performed using PubMed. The keywords used were the combination of "cholangiocarcinoma" with other keywords such as "immunotherapy" or "miRNA" (e.g., "cholangiocarcinoma immunotherapy"). Papers published during 2015-2019 were obtained for reading and consideration for citing. Highly cited or important literatures were also considered regardless of publication dates.

2. Emerging therapeutic targets for CCA

2.1. Genetic aberrations

Genomic sequencing analyses for CCA patients have identified various genetic aberrations, such as mutations/polymorphisms, abnormal amplification, and chromosomal translocation or fusion (15). Previous studies have identified various candidate genes associated with CCA status including isocitrate dehydrogenase (IDH), fibroblast growth factor receptor (FGFR), tumor protein 53 (TP53), and Kirsten rat sarcoma viral oncogene homolog (KRAS) (11, 16). These genes are expressed abnormally because of genetic aberrations, or expressed proteins have different functions due to neomorphic mutations. One strategy is to target these proteins for the management of CCA. For example, mutations in IDH genes alter the function of IDH leading to the production and accumulation of an oncometabolite D-2-hydroxyglutarate (2-HG) that contributes to carcinogenesis (17). IDH inhibitors, AG-221 (enasidenib) and AG-120 (ivosidenib), have been approved by the FDA for various cancers, including CCA. Clinical trials are currently ongoing for AG-221, AG-120, and AG-881 (NCT02577406, NCT01915498, NCT02989857, NCT02989857, NCT02481154, NCT03343197, NCT02632708). DNA sequencing for CCA patients identified genetic alterations or aberrations in FGFR (18, 19). The

fusion of FGFR2 with another gene, such as bicaudal C homolog 1 (BICC1), shootin 1 (SHTN1), transforming acidic coiled-coil containing protein 3 (TACC3), or periphilin 1 (PPHLN1), is commonly found in intrahepatic CCA patients (19, 20). Since these fusion FGFR2-X proteins are associated with tumor growth and metastases, inhibition of FGFR2 is another therapeutic strategy for CCA. A number of drugs have been introduced and are under clinical trials that target FGFR. For example, a previous study has demonstrated that the recently developed FGFR inhibitor derazantinib has anti-cancer effects in human CCA cell lines HuCCT1 and CCLP cells. Fusion gene expression of FGFR2-PPHLN1 increases the sensitivity of CCA cell lines to derazantinib, suggesting that this drug can be effective against fusion FGFR in intrahepatic CCA (21). A clinical trial for derazantinib is currently ongoing (NCT03230318). BGJ398 and Debio1347 are drugs targeting FGFR, and clinical trials are ongoing for them (NCT02150967 and NCT03834220, respectively). However, some CCA patients have multiple FGFR mutations and develop resistance against these drugs due to secondary FGFR mutations. Goyal *et al*. have introduced TAS-120 as a pan-FGFR inhibitor and have demonstrated its anti-cancer effects in intrahepatic CCA patients who demonstrate resistance against BGJ398 or Debio1347 (22). Another study has shown that the fusion protein FGFR2-TACC3 is a client of heat shock protein 90 (HSP90), and the combination of BGJ398 and a HSP90 inhibitor, ganetespib, have a higher anti-cancer effect than the single use of these inhibitors (23). Treatments with BGJ398 plus ganetespib significantly decreased tumor size *in vivo* using a xenograft model with NIH3T3 cells expressing FGFR2-TACC3 fusion (23). These studies suggest that targeting genes that represent genetic aberrations or variations is a promising strategy developing therapeutic drugs leading to novel treatments or chemotherapies for CCA patients. For more information on targeted genes and clinical trials, see a previous schematic review (24).

2.2. The tumor microenvironment

2.2.1. Cells associated with the CCA microenvironment

CCA tumors are often accompanied by the dense stroma containing immune cells and extracellular matrix (ECM). This tumor microenvironment plays a vital role in the progression and metastases of CCA (25). Various hepatic cells orchestrate and contribute to the tumor microenvironment development (26, 27). ECM is predominantly secreted by activated hepatic stellate cells (HSCs) or myofibroblasts, and ECM-producing cells that contribute to the tumor microenvironment development are referred to as cancer-associated fibroblasts (CAFs). Tumor-associated macrophages (TAMs) secrete inflammatory and fibrogenic cytokines such as interleukin 6 (IL-6) and transforming growth factor-beta 1 (TGF-β1) leading to cancer cell proliferation and activation of HSCs and myofibroblasts. Immune cells infiltrate into the liver and accumulate in the tumor microenvironment. These cells include neutrophils, dendritic cells (DCs), and regulatory T lymphocytes (Tregs).

2.2.2. Cancer-associated fibroblasts as a therapeutic target

Since the tumor microenvironment promotes CCA progression and invasion, it is a strategy to target the microenvironment and associated cells for the management of CCA. For example, a previous study has demonstrated that activated myofibroblasts express elevated levels of B-cell leukemia 2 (Bcl-2) and become more sensitive to a Bcl-2 inhibitor, navitoclax, compared to quiescent myofibroblasts (28). Navitoclax treatment induced apoptosis in CAFs leading to

decreased ECM deposition and stroma area as well as tumor size *in vivo* using rat xenograft models transplanted with rat CCA cell line BDEneu cells (28). TGF-β1 is a cytokine that activates HSCs and myofibroblasts leading to fibrogenesis. Thioacetamide (TAA) causes hepatic fibrosis, and long-term TAA treatments induce CCA in rodents. Administration of a monoclonal antibody against TGF-β, 1D11, which neutralizes all three TGF-β isoforms, decreased TAA-induced liver fibrosis as well as CCA development in rats, indicating that targeting HSCs or fibroblasts by inhibition of fibrogenesis and ECM deposition may lead to the prevention or treatment of CCA (29). Resveratrol (3,4',5-trihydroxy-*trans*-stilbene) is a natural polyphenol compound contained in grapes, berries, and peanuts. Treatments of human CCA cell line KKU-213 and KKU-100 cells with culture media of CAFs isolated from CCA tumors induced IL-6 secretion in CCA cells promoting cell proliferation indicating a cross-talk between CCA cells and CAFs (30). Culture media of CAFs pre-treated with Resveratrol decreased IL-6 secretion as well as cell proliferation and migration in CCA cell lines *in vitro* (30). These studies suggest that the tumor microenvironment and associated cell types, such as CAFs, could be therapeutic targets to develop novel treatments for CCA.

2.2.3. Immune checkpoints of macrophages and T cells

The tumor microenvironment contains various infiltrated immune cells, such as TAMs, neutrophils, and T cells. A therapeutic approach targeting immune cells is referred to as immunotherapy, and previous studies have demonstrated promising effects. Macrophages have phagocytic activity and can destroy cancer cells by phagocytosis. However, CCA tumors express elevated levels of cluster of differentiation 47 (CD47) compared to HCC tumors for immune

escape (31). CCA tumor cells interact with TAMs via CD47, which mediates an anti-phagocytic signal, and high expression of CD47 in CCA cells helps them to escape phagocytosis by TAMs (32). Administration of an anti-CD47 monoclonal antibody B6H12.2 increased phagocytic ability as well as infiltration of TAMs and decreased colonization of injected KKU-213 cells into the liver *in vivo* using NOD Rag-2$^{-/-}$Jak3$^{-/-}$ mice (31). T cells are another type of lymphocytes that have the ability to kill cancer cells. Programmed cell death protein 1 (PD-1) is a receptor expressed in the membrane of immune cells. Cancer cells express the ligand for PD-1 (PD-L1) on the cell surface, resulting in cancer cell interaction with T cells. This PD-1-PD-L1 interaction inactivates T cells leading to the survival of cancer cells. Inhibition of this immune checkpoint is a strategy to increase T cell functions and inhibit cancer progression in various malignancies (33). In CCA, a previous study has analyzed tumor tissues from 192 intrahepatic CCA patients and has found that PD-L1 overexpression in CCA tumors is correlated with poor survival rates of patients (34). This study has also found that a higher PD-L1 expression is correlated with higher population of CD8$^+$ T cells in the tumor area (34). Another study has analyzed samples from 320 intrahepatic CCA patients and has found that CCA tissues have higher expression levels of PD-L1 as well as a higher population of PD-1$^+$ T cells compared with peritumor tissues (35). High expression levels of PD-1 and PD-L1 were negatively correlated with survival rates (35). Zhou *et al*. have isolated lymphocytes from resected tumor tissues of intrahepatic or perihilar CCA patients to analyze the population of lymphocyte subsets (36). CCA tumors contained a higher population of Tregs and lower population for natural killer (NK) cells, NK T cells, and CD3$^+$CD8$^+$ T cells compared with non-cancerous liver tissues (36). This study has demonstrated that lymphocytes in CCA tumors express elevated levels of co-stimulatory receptor glucocorticoid-induced tumor necrosis factor receptor (GITR) and co-inhibitory receptors PD-1

and cytotoxic T-lymphocyte associated protein 4 (CTLA4) compared with lymphocytes in tumor-free livers (36). Treatments with an agonist for GITR or antagonists for PD-1 or CTLA4 increased proliferation and functions of CCA-derived T cells (36). These studies suggest that targeting lymphocytes and blocking immune checkpoints, such as the PD-1-PD-L1 axis, may lead to the development of novel CCA treatments by restoring functions of macrophages or T cells and inducing CCA cell deaths. Clinical trials are currently ongoing for pembrolizumab, a monoclonal antibody against PD-1 (NCT02703714, NCT02628067, NCT03111732), and atezolizumab, a monoclonal antibody against PD-L1 (NCT03201458, NCT03818997).

2.2.4. T cell activation via dendritic cells

DCs are antigen-presenting cells that can activate T cells (37). It is another strategy for immunotherapies to target DCs promoting T cell activation and CCA cell deaths. A previous study isolated peripheral blood mononuclear cells (PBMCs) from healthy donors, stimulated them, and differentiated them into DCs (38). Generated DCs were incubated with protein lysate or total RNAs harvested from human CCA line KKU-100 cells. These CCA-pulsed DCs induced differentiation of PBMCs and increased the number of $CD3^{+}CD8^{+}$ T cells compared to control DCs (38). Lymphocytes activated by CCA-pulsed DCs demonstrated anti-cancer effects by inducing apoptosis in KKU-213 cells, indicating the therapeutic potential for the generation of DCs that activate lymphocytes as anti-CCA effector cells (38). Another study has demonstrated that cAMP-dependent protein kinase type I-alpha regulatory subunit (PRKAR1A) is highly expressed in CCA tissues compared to adjacent healthy liver tissues (39). Transduction of PBMC-derived DCs with lentivirus carrying PRKAR1A cDNA generated PRKAR1A-presenting

DCs, and isolated effector T cells activated by these presenting DCs induced elevated cell deaths in KKU-213 cells compared with T cells stimulated by control DCs (39). Honokiol is a bioactive compound produced in the plant *Magnolia*. Treatments of human CCA cell line KKU-213L5 cells with honokiol induced apoptosis, and PBMC-derived DCs pulsed with honokiol-treated KKU-213L5 cell lysate activated isolated T cells that inhibited proliferation of co-cultured KKU-213L5 cells (40). These studies suggest that targeting DCs is a promising approach of immunotherapy for CCA patients.

2.3. Melatonin and circadian rhythms

Reactive oxygen species (ROS) and ROS-induced oxidative stress is a hallmark of cancers (41). A previous study compared patients with CCA and pancreatic cancer and found that serum levels of reactive oxygen metabolites were significantly higher in CCA patients (42). Serum antioxidant capacity was lower in CCA patients compared with pancreatic cancer patients, indicating the imbalance of oxidative stress in CCA (42). Melatonin or *N*-acetyl-5-methoxytryptamine is a hormone produced from tryptophan in the pineal gland. Melatonin has strong effects as an antioxidant detoxicating ROS as well as reactive nitrogen species (43). Therefore, it could have anti-cancer effects and could be utilized for the treatments of various cancers (44). An *in vitro* study using human CCA cell line KKU-M055 and KKU-M214 has demonstrated that melatonin induces apoptosis and inhibits cell proliferation of CCA cells showing the anti-cancer effects of melatonin (45). As mentioned, infection with the liver fluke *O. viverrini* is a risk factor for CCA. A previous study performed *O. viverrini* infections in hamsters and administered melatonin orally for 30 days. *O. viverrini* infection induced oxidative DNA damage and melatonin administration decreased DNA and liver damage *in vivo* (46). Infection of

O. viverrini plus *N*-nitroso dimethylamine (NDMA) administration causes CCA development in hamsters. Melatonin administration significantly decreased tumor volumes and improved survival rates (47). Melatonin may also affect the tumor microenvironment and immune cells. Elevated numbers of neutrophils and IL-17^+ cells (Th17) in the tumor area are associated with poor survival rates of CCA patients (48), and infection of *C. sinensis* disrupts the balance of Treg/Th17 in mice, which may lead to the development of CCA (49). A previous study has demonstrated that melatonin administration decreases the number of infiltrating lymphocytes as well as Tregs and Th17 at the tumor area in CCA hamsters induced by *O. viverrini* and NDMA (50). Melatonin is synthesized from serotonin in a two-step process. Aralkylamine *N*-acetyltransferase (AANAT) converts serotonin into N-acetyl serotonin, and acetyl serotonin O-methyltransferase (ASMT) converts further into melatonin (51). A previous study has found that CCA tumor tissues, as well as CCA cell lines, express significantly lower levels of AANAT and ASMT compared to control non-malignant tissues or cells (52). Overexpression of AANAT decreased cell proliferation and increased apoptosis in Mz-ChA-1 cells (52). This study has also demonstrated that melatonin administration decreases tumor size *in vivo* using nude mice with Mz-ChA-1 xenograft (52). These studies suggest that melatonin administration or induction of melatonin synthesis may have anti-cancer effects against CCA.

Melatonin regulates sleep and circadian rhythms (53). Since melatonin represents anti-cancer effects, maintenance of circadian rhythms may also contribute to the prevention or inhibition of CCA. Dimethyl nitrosamine (DEN) is a carcinogen that develops liver tumors. DEN administration is utilized for rodents as the models of HCC or CCA depending on experimental conditions (54, 55). A previous study has demonstrated that DEN administration develops liver

tumors in mice, and the disruption of circadian rhythms by 8-hour advance light exposure every two days exacerbates liver damage and tumor development compared to mice with 12-hour light and 12-hour dark cycle (56). Mteyrek *et al*. generated double knockout mice that lack clock genes *Cry1* and *Cry2* (57). These *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice had more severe liver damage and primary liver cancers compared with wild-type mice during chronic exposure to DEN (57). This study has demonstrated that DEN administration induces liver cancers that are the mixture of HCC and CCA, but in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, tumors are predominantly CCA, which is determined by histopathological analyses (57). Another study has demonstrated that expression levels of clock gene *Per1* are downregulated in CCA tissues and CCA cell lines, and overexpression of *Per1* inhibits CCA cell line proliferation *in vitro* and decreases tumor volumes *in vivo* using xenograft models (58). These findings suggest that clock gene networks and their expression levels are associated with CCA development and progression, and circadian rhythms may be a therapeutic target for CCA.

2.4. Serotonin and dopamine

Melatonin is produced from serotonin. Serotonin is a neurotransmitter produced from L-tryptophan by tryptophan hydroxylase (TPH) (59). Although melatonin has anti-oxidative and anti-cancer effects, previous studies have reported the association of elevated serotonin secretion with tumor growth and metastases in various cancers (60). In CCA, elevated expression of TPH1, as well as serotonin secretion, was observed in CCA tissues and CCA cell lines (61). Administration of TPH1 inhibitor *p*-chlorophenylalanine decreased tumor size in the xenograft model using Mz-ChA-1 cells, indicating the potential of serotonin as a therapeutic target for CCA (61). Monoamine oxidase A (MAOA) is an enzyme that is involved in the degradation

process of monoamines, including serotonin and dopamine. A previous study has demonstrated that expression levels of MAOA are downregulated in CCA tissues, and low MAOA expression is associated with poor survival rates in patients with intrahepatic or hilar CCA (62). PSC is a bile duct disorder representing a high risk for CCA development as mentioned. A recent study has demonstrated that expression levels of MAOA are decreased in cholangiocytes of *Mdr2*$^{-/-}$ mice, which are a mouse model of PSC, and administration of antagonists against serotonin receptors 5HTR2A, 5HTR2B, or 5HTR2C improves liver conditions by inhibiting ductular reaction and liver fibrosis (63). These studies suggest that serotonin synthesis and degradation is associated with CCA, and serotonin receptor antagonists may be a therapeutic tool to regulate cholangiocyte functions and CCA development.

Dopamine is another monoamine neurotransmitter produced from tyrosine by tyrosine hydroxylase (TH) and dopa decarboxylase (DDC). As well as serotonin, dopamine can be degraded by MAOA. A previous study has demonstrated that expression levels of TH and DDC, as well as dopamine secretion, are upregulated in CCA cell lines and inhibition of dopamine synthesis using DDC inhibitor L-(-)-α-methyldopa decreases tumor volumes in a xenograft model *in vivo* (64). Since MAOA expression is significantly downregulated in CCA tumors (61), serotonin and dopamine levels are upregulated, and this may contribute to CCA progression and metastases. These findings suggest that antagonists targeting dopamine receptors may have anti-cancer effects against CCA. However, there are five types of dopamine receptors, and the functional roles of each receptor in CCA are undefined. Expression levels of the five dopamine receptors are significantly different and inconsistent between CCA cell lines (64). Further studies

are required to elucidate whether agonists or antagonists on specific dopamine receptors represent anti-cancer effects for CCA.

2.5. Non-coding RNAs

2.5.1. MicroRNAs

MicroRNAs (miRNAs) are small non-coding RNAs that typically consist of 19-24 nucleotides (65). Regulation of gene expression by miRNAs play an essential role in the pathophysiology of CCA and targeting miRNA-mediated gene expression may lead to the development of CCA therapies. A previous study has demonstrated that expression levels of miRNA miR-551b-3p are significantly downregulated in CCA tumor tissues compared to healthy bile duct tissues (66). Low miR-551b-3p levels were associated with poor survival rates of patients as well as CCA cell proliferation (66). This study has also demonstrated that miR-551b-3p targets cyclin D1 (CCND1), leading to suppressed CCND1 expression, which is associated with CCA cell growth arrest and apoptosis (66). A study using 60 CCA tissue samples has found that expression levels of miR-186 are downregulated, and patients with low miR-186 expression have poor survival rates compared to patients with high miR-186 expression (67). Overexpression of miR-186 inhibited cell proliferation of human CCA cell lines CCLP1 and SG231 cells as well as tumor growth in xenograft mouse models (67). This study has identified twist-related protein 1 (Twist1) as the target of miR-186 (67). Another study has also demonstrated that miR-186 is significantly downregulated in CCA tissues and found that Microchidia family CW-type zinc finger 2 (MORC2) is the target of miR-186 (68). Wan *et al*. have demonstrated that miR-383 is overexpressed in intrahepatic CCA tumors as well as human CCA cell lines compared to non-

malignant tissues or cholangiocytes (69). Patients with higher miR-383 expression have poorer survival rates than patients with low miR-383 expression, and miR-383 suppresses interferon regulatory factor 1 (IRF1) (69). These studies suggest that miRNAs play a vital role in the pathophysiology of CCA regulating gene expression associated with CCA progression. Numbers of previous studies have identified various candidate miRNAs and their targets associated with CCA. Table 1 represents selected miRNAs identified in CCA tumors or cell lines.

2.5.2. Long non-coding RNAs

Long non-coding RNAs (lncRNAs) are RNA molecules that consist of over 200 nucleotides and are not translated into proteins (65). While the major functions of miRNAs are to silence and regulate mRNAs of target genes, lncRNAs have various functions, including X-chromosome inactivation and telomere regulation (65). Recent studies have demonstrated that lncRNAs function like a sponge to anneal miRNAs and inhibit their functions, promoting gene expression, which is a target of sponged miRNAs. This function of lncRNA as competing endogenous RNAs (ceRNAs) may be essential for the pathophysiology of CCA, and ceRNA functions may be a novel therapeutic target to regulate CCA progression. For example, Sun *et al*. have demonstrated that lncRNA KCNQ1OT1 is significantly upregulated in 62 CCA tumor samples compared to healthy liver tissues, and high expression of KCNQ1OT1 is associated with poor survival rates (70). Human CCA cell lines CCLP1 and RBE cells expressed higher levels of KCNQ1OT1 than normal cholangiocytes, and inhibition of KCNQ1OT1 decreased cell proliferation and invasion of CCA cells inducing apoptosis (70). This study has also demonstrated that KCNQ1OT1 sponges miR-140, which targets SRY-box 4 (SOX4), leading to elevated expression of SOX4 in

CCA tissues (70). Inhibition of miR-140 induced CCA cell proliferation, but inhibition of SOX4 decreased proliferation, indicating the functional role of KCNQ1OT1 as ceRNAs regulating SOX4 expression and CCA proliferation (70). Another study analyzed 20 CCA tumors and adjacent non-cancerous liver tissues and found that expression levels of lncRNA LMCD1-AS1 were significantly elevated in CCA tumors (71). Expression levels of LMCD1-AS1 were associated with cell growth, invasion, and apoptosis in HuCCT1 and RBE cells *in vitro* (71). This study has demonstrated that LMCD1-AS1 functions as a miRNA sponge for miR-345 that targets and regulates collagen type VI alpha 3 (COL6A3), leading to elevated COL6A3 expression and CCA cell proliferation (71). A previous study using 64 CCA patients has found that high expression of lncRNA LOXL1-AS1 is associated with high TNM stages and poor survival rates (72). LOXL1-AS1 promoted CCA cell proliferation, migration, and invasion by sponging miR-324-3p inducing the expression of its target ATP-binding cassette transporter A1 (ABCA1) (72). Elevation of NNT-AS1 expression has been identified in 48 CCA tissues and associated with tumor size, TNM stage, and poor survival rates (73). NNT-AS1 acts as ceRNAs by sponging miR-485 and regulates B-cell lymphoma 9 (BCL9) expression indirectly (73). Inhibition of NNT-AS1 decreased tumor size *in vivo* using xenograft mouse models with CCLP1 cells (73). These studies suggest that targeting lncRNAs and their functions may be a promising approach for the management of CCA progression. Numbers of previous studies have identified various lncRNAs and their primary miRNA targets for sponging and secondary (indirect) targets for the regulation of expression. Table 2 includes selected lncRNAs identified in CCA.

2.6. Extracellular vesicles

Various types of cells secrete small membrane-bound vesicles. Exosomes are ~100 nm in diameter and produced in endosome networks and are released through multivesicular bodies (74). Microvesicles or microparticles are larger than exosomes (0.1-1 μm in diameter) and are formed directly from the plasma membrane by outward budding (74). Exosomes and microvesicles are types of extracellular vesicles (EVs), and recent studies have shown that EVs may play a key role in the pathogenesis of liver diseases, including CCA (75, 76). EVs released from donor cells contain various mediators inside, including proteins, miRNAs, and lncRNAs, and can regulate physiological cell events by delivering these cargo mediators into recipient cells leading to disease conditions (77, 78). In CCA, Haga *et al*. demonstrated that EVs secreted from human CCA cell lines, KMBC and HuCCT1, induced elevated expression of markers for the activation of myofibroblasts or CAFs, fibroblast activation protein (FAP), and alpha-smooth muscle actin (αSMA), in human bone marrow-derived mesenchymal stem cells (MSCs) (79). Since MSCs transdifferentiate into myofibroblasts or CAFs, they contribute to the development of the tumor microenvironment and CCA growth (80, 81). This study indicates that EV-mediated communication between CCA cells and MSCs may be essential for CCA development.

Tumor cells secrete higher numbers of EVs than healthy cells. A previous study collected bile and serum samples from patients with malignancies, including CCA, and found that EV concentrations in bile were significantly higher in patients with malignancies and bile EV concentrations could be utilized for diagnostic testing for malignancies (82). Tumor-derived EVs contain unique cargos inside that could be utilized as a biomarker. Arbelaiz *et al*. analyzed proteome profiles of EVs isolated from serum of patients with CCA or PSC and found that the levels of various proteins were different from serum EVs isolated from healthy individuals (83).

These findings suggest that CCA-derived EVs may be useful for diagnostic testing by analyzing concentrations or cargo contents.

EVs could also be utilized as therapeutic tools to regulate CCA cell functions by delivering cargo mediators. Ota *et al*. have found that expression levels of miR-30e are significantly downregulated in human CCA cell lines, and Snail, which is associated with epithelial-mesenchymal transition (EMT), is the target of miR-30e (84). This study has also demonstrated that the transfection of HuCCT1 cells with miR-30e mimic elevate miR-30e levels carried in HuCCT1-derived EVs, and these miR-30e-enriched EVs inhibited Snail expression and EMT in other HuCCT1 leading to decreased cell proliferation and invasion (84). There is cross-talk between CCA tumor cells and cells in the tumor microenvironment such as CAFs promoting CCA progression (26, 27). Li *et al*. have demonstrated that human HSC line LX2 cells cocultured with CCA cell lines express decreased levels of miR-195, and upregulation of miR-195 in LX2 cells inhibits cell proliferation and invasion of cocultured CCA cells (85). LX2-derived EVs that carry elevated levels of miR-195 inhibited tumor growth and improved survival rates in xenograft rat models (85). These studies suggest that EVs carrying designed cargos can be utilized as drug/mediator carriers to manage CCA tumor progression.

3. Conclusion

Current studies have identified a number of therapeutic targets for CCA. Classic approaches of CCA treatments include the administration of antibodies or inhibitors for proteins that are expressed abnormally or have mutations in CCA patients, such as IDH and FGFR2. Several inhibitors for these proteins have been under evaluation in clinical trials. Other studies have

demonstrated that various signaling pathways are involved in CCA development or progression, and small molecules targeting these pathways, such as antagonists against serotonin receptors, may have the potential as a novel therapeutic drug for CCA. Targeting the tumor microenvironment and immunotherapies had promising results in recent studies. Although further studies are required, EVs could be utilized as a miRNA carrier for the management of CCA by regulating CCA cells or microenvironment-associated cells such as CAFs. Figure 2 summarizes current approaches for CCA therapies.

4. Expert opinion

4.1. CCA heterogeneity

The development of inhibitors based on genetic mutations or aberrations is a promising therapeutic approach for CCA and several drugs/inhibitors are currently under clinical trials. However, this approach may not be universal because CCA is heterogeneous and can be caused by various factors (10). For example, the incidence of mutations in CCA cases is 9% for IDH1 and 3% for IDH2 (24). This means that the effects of IDH inhibitors are limited and cannot be utilized for all CCA patients. Some CCA patients have multiple mutations or fusion proteins and have resistance against drugs, such as FGFR inhibitors. Therefore, the efficacy of inhibitors may be limited and other approaches for novel therapies will be required. In addition, the location or the origin of CCA tumors should be considered. As mentioned earlier, CCA can be classified as intrahepatic, hilar, or distal (extrahepatic) CCA according to the tumor location. Specific gene mutations or aberrations may be observed only in specific classifications. For example, FGFR2 fusions were identified mainly in intrahepatic CCA, and mutations in IDH1 or IDH2 were rare or

not found in extrahepatic CCA (24). Although intrahepatic CCA has been classically recognized as adenocarcinoma with other rare variants, Nakanuma *et al*. have identified a number of histological variations in intrahepatic CCA tumors and have introduced subclasses for intrahepatic CCA: conventional type, bile ductular type, intraductal type, and other rare variants (86). Another study also identified variations in immunohistochemical profiles in intrahepatic CCA and introduced subclasses: mucin-producing or mixed intrahepatic CCA and cholangiolocellular carcinoma (87). These studies indicate the heterogeneity of intrahepatic CCA, which is a result of the heterogeneous origins of CCA tumor cells. For hilar and distal CCA, it is highly likely that the tumor emerges from the bile duct epithelia (i.e., cholangiocytes). For intrahepatic CCA, however, the tumor can emerge from not only cholangiocytes, which line the intrahepatic bile ducts, but also hepatic progenitor cells (HPCs), which are located in the Canal of Hering, the intermediate location between cholangiocytes and hepatocytes (88). CCA tumors that are derived from HPCs are specifically referred to as cholangiolocellular carcinoma (89, 90). Furthermore, previous studies have demonstrated that hepatocytes can transdifferentiate into cholangiocyte-like cancerous cells by expressing biliary markers such as CK8 and CK19 and become intrahepatic CCA tumors in mice (Figure 3) (91, 92). These findings suggest that the mechanism of the tumor development may differ depending on the origins of CCA tumors, and this is probably the reason why current CCA therapies are disappointing with limited efficacy. Future CCA treatments may need to be designed or modified depending on the location, origin, histological profiles, or genetic traits of CCA tumors for individual patients.

Although further studies are required to understand the detailed mechanisms of CCA development with various locations and origins, these studies will be challenging due to the

limited availability of CCA animal models. Various CCA animal models have been introduced, but there is still no gold-standard models to mimic intrahepatic CCA with different origins (55). The most common and widely utilized model is the xenograft model, which transplants human CCA cell lines into the liver of nude mice. Although this model is useful to analyze the effects of drugs against tumor growth, it cannot mimic the development of CCA tumors in different location such as extrahepatic bile ducts. Xenograft models are useful only for studies of tumor growth and progression, not carcinogenesis *in vivo*. Further studies are required to establish novel CCA models that mimic the CCA development in different locations with different origins.

4.2. The tumor microenvironment and immunotherapy

Targeting the tumor microenvironment is relatively a new concept for CCA treatments, and further studies are needed to establish a novel CCA therapy. Current studies in the field focus on the functional roles of hepatic cells, such as CAFs and TAMs, in the CCA microenvironment development and progression. Detailed mechanisms are still largely undefined and need to be elucidated to identify candidate cells or pathways leading to the development of novel drugs. HSCs/myofibroblasts/CAFs play a vital role in ECM secretion and fibrosis development in CCA. Previous studies have revealed that HSCs are activated by cholangiocytes during biliary damage indicating the communication between cholangiocytes and HSCs, which contributes to liver fibrosis (93, 94). Although communication between CCA tumor cells and CAFs is still unclear, targeting cholangiocytes may be an approach to regulate HSC activation as CAFs and inhibit fibrogenesis in CCA. Another study has demonstrated that miR-34a is upregulated in liver samples of patients with steatohepatitis due to heavy alcohol drinking, and miR-34a regulates

senescence, activation, and fibrogenesis in HSCs during alcoholic liver injury (95). miR-34a is also upregulated in human CCA cell lines including Mz-CHA-1 and TFK-1 cells (58). Although the roles of miR-34a in CCA cells or CAFs are undefined, these findings indicate that the approaches or candidate targets suggested in different animal models or liver diseases may be utilized for CAFs in CCA. Therapeutic drugs targeting HSCs in cholangiopathies or alcoholic steatohepatitis could be effective to regulate CAFs leading to decreased stroma tissues and CCA progression.

Immunotherapies target immune cells especially in the tumor microenvironment. Approaches of immunotherapies include inhibition of immune checkpoints, such as anti-PD-1 antibodies, or activation of T cells by drugs or other hepatic cells, such as DCs, to facilitate CCA cell deaths. Cytokine-induced killer cell-based immunotherapy is another approach to increase activated T lymphocyte subsets leading to tumor cell deaths, although current studies are limited for CCA treatments, and further studies are required (96). Since drugs targeting gene aberrations such as IDH inhibitors are effective only in limited numbers of patients as mentioned, novel CCA treatments that are effective in higher amount of the population of CCA patients have been demanded. Although some previous studies represent promising therapeutic effects of immunotherapies, such as PD-1 or PD-L1 inhibitors, there are issues in current PD-1 inhibitors: i) Only limited numbers of patients are responsive to those inhibitors; ii) Recurrences can happen; and iii) Patients often suffer side effects due to autoimmune responses (97). Immunotherapy is a relatively novel treatment for CCA and could be utilized for patients who do not respond to classic treatments such as chemotherapy. It is also expected that immunotherapy may increase therapeutic effects of chemotherapy or other treatments due to robust immune

functions and responses. Combination of immunotherapy with chemotherapy or radiotherapy may provide better effects than the single treatment although further studies are required. Current studies in immunotherapy for CCA focus mainly on PD-1 or PD-L1 inhibitors, but there are other immune checkpoint pathways such as CD47-SIRPα pathway and B7-CTLA4 pathway (98, 99). Drugs targeting other pathways may have better effects than the PD-1-PD-L1 pathway. Future studies may lead to the development of immunotherapy, which is universally effective to CCA patients.

4.3. Extracellular vesicles

EVs and EV-based therapies are relatively new concepts in liver diseases. Hepatic cells communicate with each other, and EVs play an important role in this cell-to-cell communication and the pathophysiology of liver diseases (75, 100). Primary liver cancers, HCC and CCA, secrete EVs that regulate the functions of other liver cells developing the tumor microenvironment and promoting tumor growth (79, 101). In other words, hepatic cells can be regulated by EVs to improve or manage diseased conditions. Previous studies have demonstrated that EVs secreted from stem cells have therapeutic effects and can be utilized to attenuate diseased conditions such as liver fibrosis (102, 103). EVs carrying specific cargo miRNAs can deliver these miRNAs into liver cells *in vivo* and regulate cell functions such as fibrogenesis (103). Transfection of cells with miRNA mimics allows production of EVs carrying elevated levels of target miRNAs (84), and electroporation will also allow modification of EV cargos for miRNA mimics or inhibitors (104, 105). These previous studies indicate that EVs with designed cargo mediators have potential for novel CCA therapies as drug/mediator carriers. A previous

study has introduced a novel CCA therapy utilizing polymer-based nanoparticles delivering miR-210 (106). However, artificial polymeric nanoparticles could induce immune responses or rejection in the human body while EVs are natural particles produced from cultured cells, and the possibility of rejection or side effects will be relatively low. Future studies will develop an efficient methodology to create EVs carrying target mediators and will represent their efficacy as drug/mediator carriers for the management of CCA.

4.4.Non-coding RNAs

Current studies suggest that upregulation or downregulation of miRNAs or lncRNAs is associated with CCA status. Targeting non-coding RNAs in CCA is a relatively new field and no drugs have proceeded to clinical trials to date. A large number of non-coding RNAs, especially lncRNAs, have been identified in CCA tumor cells, but many studies utilized only CCA cell lines *in vitro* or did not identify candidate target genes to be regulated. This review mainly introduces studies that have been published recently and have represented target genes clearly (Table 2 and Table 3). For more information of other non-coding RNAs in CCA, see recent review articles (107-110). Current studies mainly focus on the roles of non-coding RNAs in CCA development or progression and the methodology of novel therapies targeting non-coding RNAs has not been established to date. Possible approaches include RNA interference. Some non-coding RNAs are elevated in CCA tumors and RNA interference technology using shRNAs or siRNAs could be useful to inhibit CCA progression. For example, lentiviral plasmid-mediated miR-10a inhibition decreased tumor size in CCA xenograft models with CCLP1 cells *in vivo* (111). Some non-coding RNAs are downregulated in CCA, and overexpression or

supplementation of candidate non-coding RNAs may be effective in inhibiting CCA growth. For example, overexpression of miR-551b-3p decreased cell proliferation and induced apoptosis in HuCCT-1 cells *in vitro* as well as decreased tumor volumes in HuCCT-1 xenograft mice *in vivo* (66). Some lncRNAs function as ceRNAs by sponging miRNAs, and the interaction between lncRNAs and proteins is often required for their functions. Small molecules that disrupt this RNA-protein interaction could be utilized to inhibit ceRNA functions. For example, lncRNA MEG3 interacts with PTBP1 in order to function leading to cholestatic liver injury (112). Small molecules that interfere with the MEG3-PTBP1 interaction may have therapeutic effects for bile duct injury. The same concepts may be utilized for CCA treatment, although current studies are limited and further studies are required. As mentioned, EVs can carry mediators such as miRNAs, lncRNAs, and their inhibitors, and those cargo mediators can be modified by cell transfection or electroporation. A previous study has demonstrated that injected EVs via the tail vein can be delivered to HCC tumors more than to adjacent healthy liver tissues in mice (113). CCA tumors may have a higher ability to engulf injected EVs and EVs may be an efficient mediator carrier to deliver candidate RNAs selectively into CCA tumors and inhibit tumor growth. Although more experimental data is required, targeting non-coding RNAs has the potentials for the development of novel CCA treatments.

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Declaration of interest

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Figure 1. Classes of cholangiocarcinoma. Cholangiocarcinoma (CCA) can be classified as intrahepatic, hilar or perihilar, or distal CCA according to the location where the tumors emerge. *The incidence is from the previous study analyzing 564 CCA patients at a single institution (2).

Figure 2. Current strategies for the treatments of cholangiocarcinoma. Cholangiocarcinoma (CCA) patients often have genetic mutations or aberrations causing abnormal protein expression or function. Antibodies or inhibitors for these proteins can be utilized to inhibit CCA progression. CCA cells express receptors for various mediators such as melatonin or serotonin, and administration of these mediators could regulate CCA cell proliferation. CCA cells express elevated levels of proteins such as PD-L1 which bind to PD-1 of T cells to escape T cell-induced cell deaths. Antibodies targeting these immune checkpoint proteins inhibit PD-1-PD-L1 interaction promoting T cell function and killing CCA cells. T cells can be activated by dendritic cells, and stimulation of dendritic cells by pulsing with CCA cell lysates could lead to effective T cell activation leading to inhibition of CCA growth. Recent studies have demonstrated that extracellular vesicles (EVs) can be utilized as therapeutic tools to deliver cargo microRNAs (miRNAs) and regulate cell proliferation or function of CCA cells or cells associated with the tumor microenvironment such as hepatic stellate cells, myofibroblasts, or cancer-associated fibroblasts.

Figure 3. Origins of cholangiocarcinoma cells. Cholangiocarcinoma (CCA) is heterogeneous and can emerge from various origins. Hilar/perihilar and distal CCA are mainly derived from the bile duct epithelia (i.e., cholangiocytes). Intrahepatic CCA can be derived from cholangiocytes,

hepatic progenitor cells, or hepatocytes. Classical intrahepatic CCA classified as adenocarcinoma is derived from cholangiocytes that line intrahepatic bile ducts. Hepatic progenitor cells are located in the Canal of Hering and can contribute to CCA development as cancer stem cells. Some previous studies classified this CCA phenotype as cholangiolocellular carcinoma. Other studies have demonstrated that hepatocytes can transdifferentiate into cholangiocyte-like cancerous cells expressing biliary markers CK8 and CK19 in rodents. It is not fully defined whether this hepatocyte-derived CCA is identical to cholangiocyte-derived classical intrahepatic CCA or cholangiolocellular carcinoma or a different phenotype which has different characteristics and responses against therapies. This heterogeneity of the CCA origin may contribute to the poor efficacy of current CCA treatments.

Tables

Table 1. Selected candidate miRNAs identified in CCA

miRNA	**Sample source**	**Upregulated or downregulated**	**Target**	**Association**
miR-551b-3p (66)	15 CCA tissues	Downregulated	CCND1	Poor survival rates of patients
miR-186 (67)	60 CCA tissues	Downregulated	Twist1	Poor survival rates of patients

miR-186 (68)	44 CCA tissues	Downregulated	MORC2	CCA cell proliferation *in vitro*
miR-383 (69)	82 CCA tissues	Upregulated	IRF1	Poor survival rates of patients
miR-10a (111)	CCA cell lines	Upregulated	PTEN	Tumor volume in xenograft mice
miR-191 (114)	21 CCA tissues	Upregulated	FRP1	CCA cell proliferation *in vitro*
miR-490-3p (115)	51 CCA tissues	Downregulated	Akirin2	CCA cell proliferation *in vitro*
miR-329 (116)	CCA cell lines	Downregulated	PTTG1	Tumor volume in xenograft mice
miR-142 (117)	100 plasma samples from CCA patients	Upregulated	PTEN	Poor survival rates of patients
miR-124 (118)	20 serum samples from CCA patients	Downregulated	UHRF1	CCA cell proliferation *in vitro*

miR-876 (119)	35 CCA tissues	Downregulated	BCL-XL	Tumor volume in xenograft mice
miR-494 (120)	34 CCA tissues	Downregulated	WDHD1	Tumor volume in xenograft mice
miR-424 (121)	10 CCA tissues	Downregulated	ARK5	Poor survival rates of patients
miR-122 (122)	11 CCA tissues	Downregulated	ALDOA	Tumor volume in xenograft mice
miR-21 (123)	57 CCA tissues	Upregulated	PTEN, PTPN14	Poor survival rates of patients
miR-200b/c (124)	14 CCA tissues	Downregulated	SUZ12, ROCK2	Tumor volume in xenograft mice

Table 2. Selected lncRNAs that are associated with CCA progression and function as ceRNAs

lncRNA	**Sample source**	**Upregulated or downregulated**	**Primary target**	**Secondary target**	**Association**

KCNQ1OT1 (70)	62 CCA tissues	Upregulated	miR-140	SOX4	Poor survival rates of patients
LMCD1-AS1 (71)	20 CCA tissues	Upregulated	miR-345	COL6A3	CCA cell proliferation *in vitro*
LOXL1-AS1 (72)	64 CCA tissues	Upregulated	miR-324-3p	ABCA1	Poor survival rates of patients
NNT-AS1 (73)	48 CCA tissues	Upregulated	miR-485	BCL9	Poor survival rates of patients
SNHG1 (125)	CCA cell lines	Upregulated	miR-140	TLR4	Tumor volume in xenograft mice
UCA1 (126)	66 CCA tissues	Upregulated	miR-122	Undefined	Poor survival rates of patients
Lnc-ATB (127)	30 CCA tissues	Upregulated	miR-200c	CCND1/CDK2	Tumor size and TNM stage of patients
MEG3 (128)	20 CCA tissues	Downregulated	miR-361	TRAF3	Cell viability *in vitro*
SPRY4-IT1 (129)	70 CCA tissues	Upregulated	miR-101-3p	EZH2	Poor survival rates of patients
ZFAS1 (130)	64 CCA tissues	Upregulated	miR-296	USF1	Poor survival rates of patients

Table 3. Comparison of current therapeutic strategies for CCA.

Strategy	Example	Pros	Cons
Antibodies or inhibitors for proteins with aberrations	Enasidenib for IDH, derazantinib for FGFR	Promising effects, clinical trials ongoing, easier administration (oral tablet)	Effective only for the limited percentage of patients
Targeting cells in the tumor microenvironment	Navitoclax (Bcl-2 inhibitor), 1D11 (anti-TGF-β antibody)	May effective for higher population of patients	Only suggested in animal models or *in vitro* studies
Immunotherapy	Pembrolizumab (anti-PD-1), atezolizumab (anti-PD-L1)	Promising effects, clinical trials ongoing, may improve effects of other therapies	Effective only for the limited percentage of patients, recurrence, side effects
Messengers and their signaling pathways	Melatonin, serotonin receptor inhibitors	May effective for higher population of patients	Only suggested in animal models or *in vitro* studies
Non-coding RNAs	Overexpression of miR-186, NNT-AS1 inhibitors	Novel approaches, may be utilized for other cancers	Only suggested in animal models or *in vitro* studies

Extracellular vesicles	miR-30e-enriched EVs, miR-195-enriched EVs	Novel approaches, may have the low possibility of rejection	Only suggested in animal models or *in vitro* studies

Figure 1

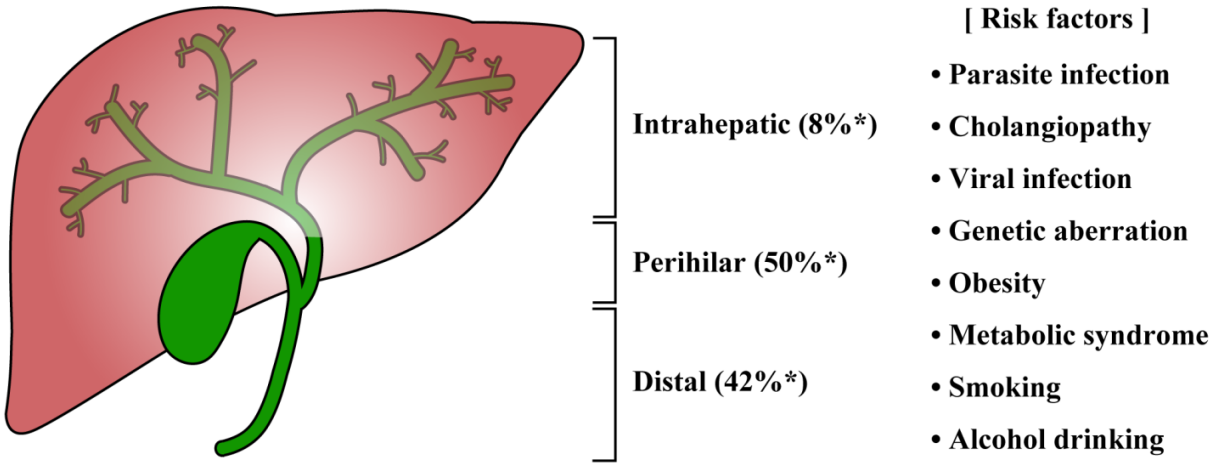

Figure 2

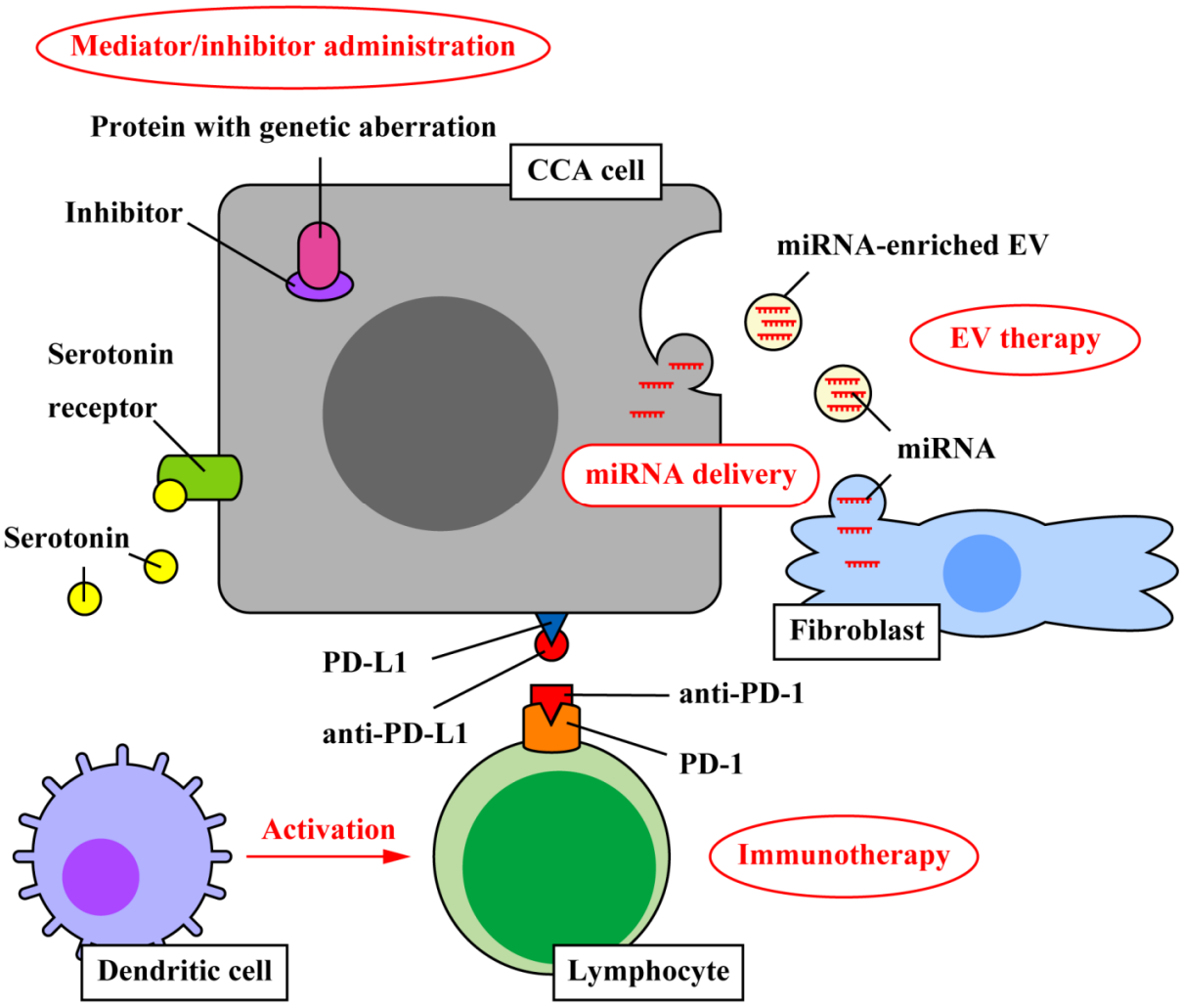

Figure 3