

# World Journal of *Gastrointestinal Surgery*

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**REVIEW**

- 407 Exosomal noncoding RNAs in cholangiocarcinoma: Laboratory noise or hope?  
*Laschos K, Lampropoulou DI, Aravantinos G, Piperis M, Filippou D, Theodoropoulos G, Gazouli M*

**ORIGINAL ARTICLE****Retrospective Cohort Study**

- 425 Narrow pelvic inlet plane area and obesity as risk factors for anastomotic leakage after intersphincteric resection  
*Toyoshima A, Nishizawa T, Sunami E, Akai R, Amano T, Yamashita A, Sasaki S, Endo T, Moriya Y, Toyoshima O*

**CASE REPORT**

- 435 Gastric splenosis mimicking a gastrointestinal stromal tumor: A case report  
*Isopi C, Vitali G, Pieri F, Solaini L, Ercolani G*

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## Exosomal noncoding RNAs in cholangiocarcinoma: Laboratory noise or hope?

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### Abstract

Currently, extracellular vesicles and particularly exosomes have gained a lot of research interest due to their unique roles in several biological processes. Noncoding RNAs (microRNAs, long noncoding RNAs and circular RNAs) represent a class of functional RNA with distinct regulatory roles in tumorigenesis and cancer progression. Cholangiocarcinoma is a rare but highly aggressive type of malignancy that is very challenging to diagnose, especially in early stages; surgical resection still represents the sole potentially curative treatment option. Hence, there is an urgent need for the discovery of novel diagnostic and prognostic biomarkers. Hereby, we provide a comprehensive review of the most recent discoveries that focus on exosomal noncoding RNAs in cholangiocarcinoma with the aim to identify new molecular players that could be used as biomarkers and therapeutic targets.

**Key Words:** Cholangiocarcinoma; Long noncoding RNAs; MicroRNAs; Circular RNAs; Piwi-interacting RNAs; Exosomes; Extracellular vesicles

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**Core Tip:** Although there are currently several original research studies investigating the role of noncoding RNAs in cholangiocarcinoma, very few have focused specifically on exosomal noncoding RNA signatures. This is the first review to summarize and report current data regarding exosomal noncoding RNAs in cholangiocarcinoma and discuss their potential future clinical applications.

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## INTRODUCTION

Cholangiocarcinoma (CCA) is a type of highly heterogeneous group of epithelial malignancies that can originate from any division of the biliary tree. The classification of CCA is based on the anatomic location with regards to the liver and includes three subtypes: Intrahepatic, perihilar (Klatskin tumor) and distal extrahepatic<sup>[1]</sup>. The heterogeneous nature of this type of cancer also corresponds to several epidemiological, biological and clinicopathological features. Intrahepatic CCA is the second most common primary liver cancer after hepatocellular carcinoma, and its prognosis is very poor, mainly depending on the potential and extent of surgical resection<sup>[2,3]</sup>; the five-year survival rates drop to 2% for cases that are diagnosed with distant metastases<sup>[4]</sup>. Treatment options for inoperable and/or microscopically positive surgical resection margin (R1) cases, include chemoradiation therapy<sup>[5]</sup>. However, the response rates still remain very low, and the clinical management of the advanced CCA is lacking a “gold standard” chemotherapy regimen<sup>[6]</sup>.

Several risk factors have been associated with CCA pathogenesis including hepatobiliary disorders such as primary sclerosing cholangitis (PSC), the presence of choledochal cysts, hepatolithiasis, viral hepatitis B and C-induced chronic hepatitis and cirrhosis<sup>[7-9]</sup>. Moreover, parasite (*Opisthorchis viverrini* or *Clonorchis sinensis*)-induced infections<sup>[10]</sup> as well as genetic factors<sup>[11]</sup>, obesity, smoking and alcohol consumption have also been correlated with increased risk of CCA<sup>[9]</sup>.

CCA is a highly aggressive, rare type of malignancy that is very challenging to diagnose at an early, potentially curable stage. The confirmation of the diagnosis usually results from the combination of: (1) Imaging, such as computed tomography, magnetic resonance imaging, magnetic resonance cholangiopancreatography, endoscopic retrograde cholangiopancreatography and positron emission tomography; (2) Biochemical; and (3) Histological data<sup>[12]</sup>. Moreover, the assessment of cancer biomarkers, namely carbohydrate antigen 19-9 and carcinoembryonic antigen, is a common routine practice towards the diagnosis, but their usefulness remains controversial due to their low sensitivity and specificity to detect CCA in early stages<sup>[13]</sup>. Therefore, there is an urgent need for novel diagnostic and therapeutic approaches towards the prompt detection and management of CCA.

Exosomes represent a distinct type of extracellular vesicle (EV) that mediate intercellular communication and have recently emerged as promising biomarkers and therapeutic targets in the cancer field<sup>[14,15]</sup>. There is also evidence that circulating EVs play an important role in the pathogenesis of liver disease *via* the modulation of several cell signaling mechanisms<sup>[16]</sup>. Many studies have shown that there is a different expression of exosomal noncoding RNAs (ncRNAs) in cancer patients compared to healthy subjects and often mirrors the type of cancer. The mechanisms of EV packaging remain unclear; however, several factors that affect the composition of EVs have been described so far. These include: (1) The upregulation of a distinct RNA type in parental cells; and (2) The existence of sorting processes that may be biotype-specific<sup>[17]</sup>. The content of exosomes consists of a mosaic cargo including proteins, lipids and nucleic acids<sup>[18]</sup>. Tumor-derived (TD) exosomes can act as transporters of this load; the latest may include cancer-related signaling molecules that can be transferred to other recipient cells *via* exosome fusion with the target cell membrane.

The transported genetic information can subsequently regulate gene expression in the recipient cell, which in turn may trigger several tumor-related processes, such as tumor proliferation, epigenetic reprogramming, invasion and metastasis<sup>[19-22]</sup>.

Hereby, we review the current evidence regarding the roles of exosomal ncRNAs and discuss their diagnostic and therapeutic potential in CCA. For clarity, because the term “EVs” has been ambiguous in the literature and often coincides and/or is being confused with the term “exosomes,” in the present work we will try to include data that refer to primarily exosomal and secondarily EV-derived ncRNAs in CCA.

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## EXOSOMES IN CANCER: BIOGENESIS AND CHARACTERISTICS

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Compared to microvesicles (the second main type of extracellular vesicles), exosomes differ in size and biogenesis pathway. More specifically, exosomes are smaller than microvesicles ranging in diameter from 30 to 100 nm and originate from the endosomal network. Moreover, they are released to the extracellular space by multivesicular bodies (MVBs) following fusion with the cellular membrane. On the other hand, microvesicles are larger in size (50 to 1000 nm in diameter), and they are secreted to the extracellular environment through direct outward budding of the plasma membrane<sup>[15-19]</sup>.

The process of exosome biogenesis includes the following steps: (1) Early endosome creation from the plasma membrane; (2) MVB formation; and (3) Initial formation of exosomes as intraluminal vesicles (ILVs) in the MVBs. These components are either degraded by lysosomes or released as exosomes to the extracellular space after fusion with the plasma membrane. During this process, several biomolecules such as cytosolic proteins, lipids and nucleic acids are incorporated in the MVBs<sup>[20]</sup> (Figure 1).

Two main mechanisms orchestrate ILV formation and the packaging of bioactive exosomal cargo. The first one depends on the endosomal sorting complexes required for the transport system whereas the second depends on raft-based microdomains, such as tetraspanin-enriched microdomains, tetraspanins and lipids<sup>[21]</sup>.

Exosomes and their parental cell-specific cargos can be secreted by all eukaryotic cells, both healthy and tumor, into the extracellular environment. Then they can either enter neighboring recipient cells by endocytosis or travel through biological fluids such as blood, urine and saliva. Exosome uptake from the recipient cell takes place after cellular recognition and internalization. It has been reported that tumor cells can release ten times more exosomes than healthy cells and that TD exosomes exhibit pro-oncogenic properties, such as promoting cell proliferation, epithelial-to-mesenchymal transition (EMT), angiogenesis, metastasis and drug resistance<sup>[21,23]</sup> (Figure 2). With regard to CCA, bile EV concentrations were found to be significantly increased in patients with CCA suggesting that they could be used for diagnostic testing<sup>[24]</sup>. In conclusion, exosomes are considered to be crucial mediators of intercellular communication because they can transfer their content and alter biological responses in other cells.

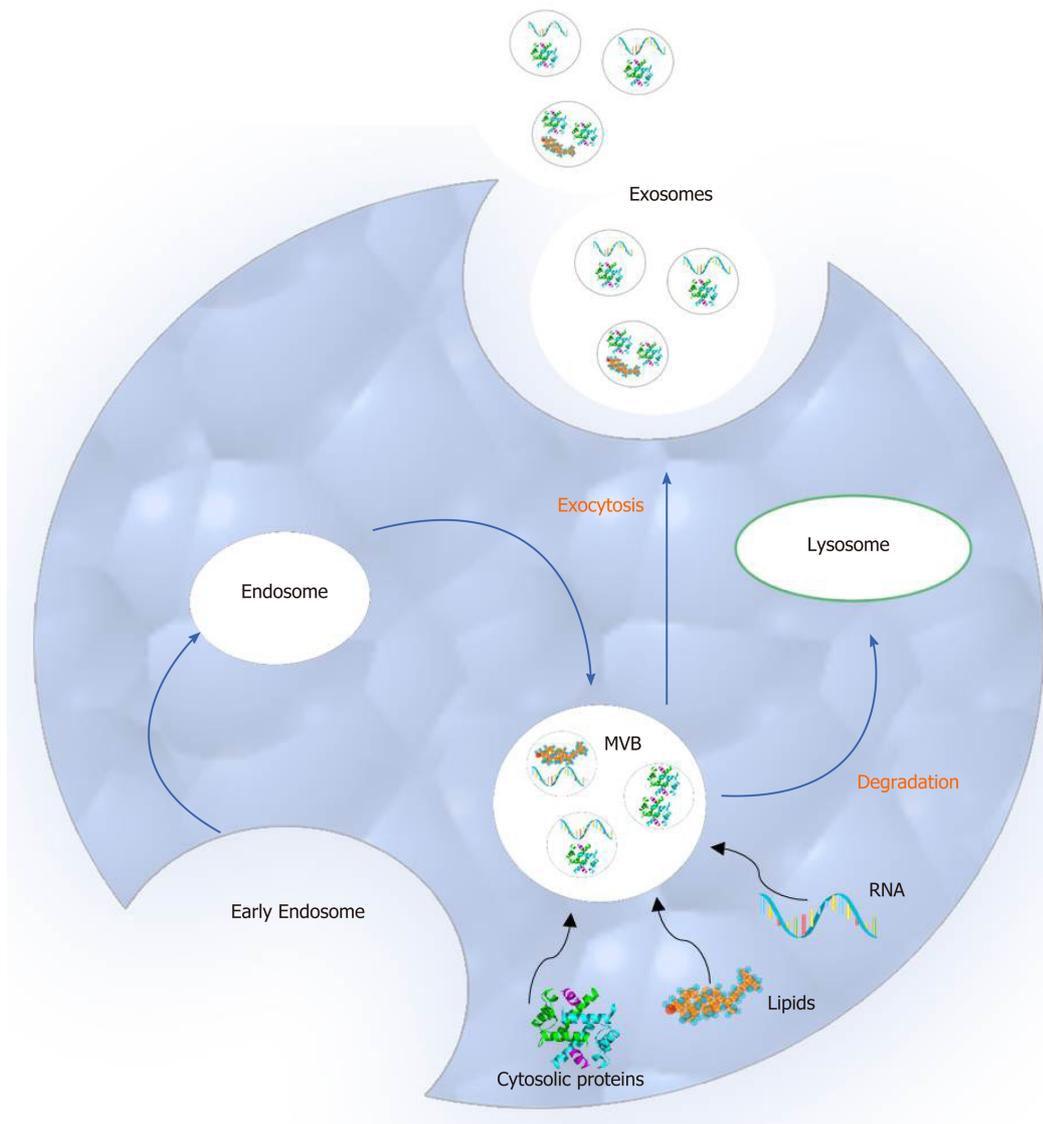
Accumulating evidence shows that exosomal components may play crucial roles in several cancer related processes such as angiogenesis and metastasis colonization. The “pre-metastatic niche” is the microenvironment created by TD exosomes and facilitates metastasis<sup>[25]</sup>. Recent work has also demonstrated that exosomes promote neoangiogenesis at the pre-metastatic niche by several mechanisms including: (1) Protein activation (*i.e.*, activation of transcription factor 2 and metastasis-associated protein 1)<sup>[26]</sup>; (2) Vascular permeability promotion *via* soluble E-cadherin<sup>[27]</sup>; and (3) The release of proangiogenic factors that promote neovascularization [microRNAs (miRNAs), vascular endothelial growth factor and cytokines]<sup>[28,29]</sup>. Moreover, several mechanisms implicating TD exosomal miRNAs, kinases and other factors affecting the process of metastasis have been identified in current literature<sup>[30,31]</sup>.

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## EXTRACELLULAR VESICLES IN CCA PATHOPHYSIOLOGY

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Over the last decade several studies have focused on the role of EVs and exosomes in biliary pathophysiology and CCA pathogenesis<sup>[16,32]</sup>. In 2010, Masyuk *et al.*<sup>[33]</sup> found that bile exosomes released by normal cholangiocytes directly interacted with primary cilia and inhibited cell proliferation *via* the ERK signaling pathway. During CCA development, EVs promote the myofibroblast-like transdifferentiation of bone marrow mesenchymal stem cells (MSCs) and thus favor the formation of tumor stroma. Moreover, they stimulated IL-6 production contributing further to CCA growth<sup>[34,35]</sup>.



**Figure 1 Biogenesis and release of exosomes.** Initial formation of the late endosome from early endosome. Subsequently, the late endosome transforms to multivesicular bodies. The latest can either get degraded by lysosomes or release exosomes to the extracellular environment following fusion with the cellular membrane. MVB: Multivesicular body.

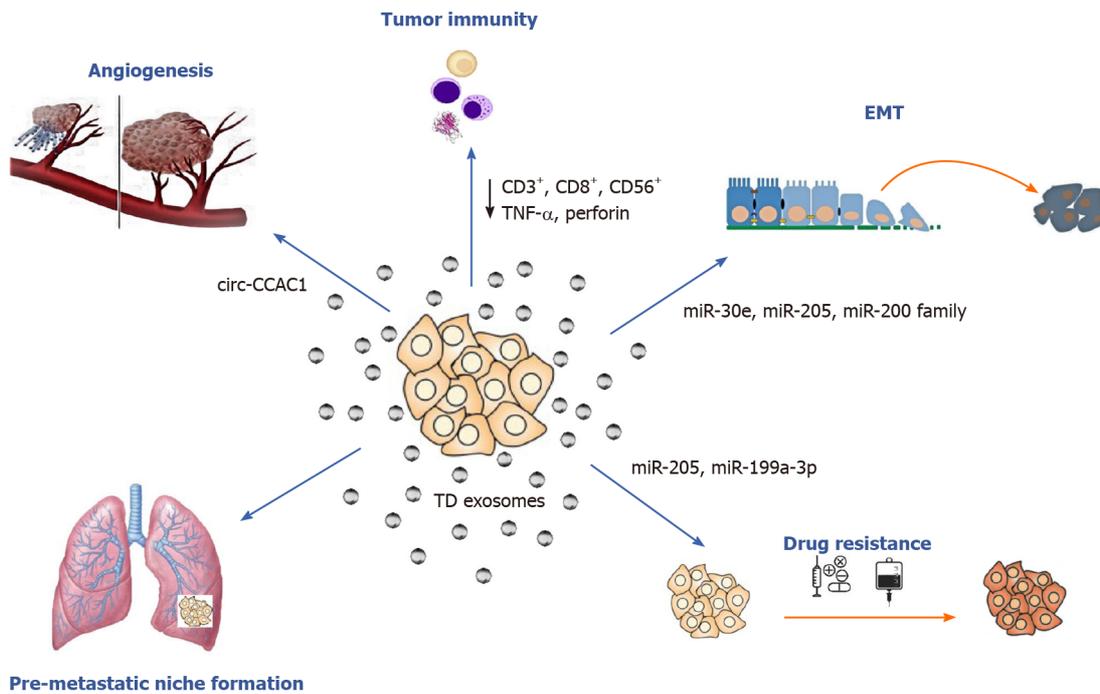
Chen *et al.*<sup>[36]</sup> reported that TD exosomes contributed to CCA escape from immune recognition by downregulating CD3<sup>+</sup>, CD8<sup>+</sup>, NK (CD56<sup>+</sup>) cells and by decreasing TNF- $\alpha$  and perforin production.

Additionally, CCA cell-derived EVs are loaded with a unique content that has been associated with tumorigenic effects. Proteomic analysis identified various oncogenic proteins such as epidermal growth factor receptor and integrin beta-4 in CCA *vs* healthy cholangiocyte-derived EVs<sup>[37]</sup>. Accordingly, Dutta *et al.*<sup>[38]</sup> reported that various cancer-related proteins (*i.e.*, large neutral amino acids transporter small subunit 1 (LAT1), 4F2 cell-surface antigen heavy chain, pyruvate kinase) were disclosed in CCA-derived exosomes compared to normal human cholangiocytes (H69), providing evidence for their direct intercellular transport by the exosomes. Recently, the phosphorylation level of exosomal heat shock protein 90 was also found to be significantly related with tumor malignancy in an *in vitro* model of isogenic CCA cells<sup>[39]</sup>.

## EXOSOMAL NCRNAs IN CCA: CURRENT EVIDENCE

### Exosomal miRNAs and CCA

Dysregulation of cellular miRNAs in several types of cancer has been a topic of



**Figure 2 Functional roles of exosomes in cholangiocarcinoma.** Tumor-derived exosomes are released to the tumor microenvironment and distant organs transferring bioactive molecules and thus regulating several cancer-related processes such as epithelial-mesenchymal transition, drug resistance, pre-metastatic niche formation, angiogenesis and tumor immunity. EMT: Epithelial-mesenchymal transition.

extensive investigation; currently there is emerging evidence that exosomal miRNA expression is also modified, suggesting that it may serve as a potential biomarker for cancer diagnosis and prognosis<sup>[40]</sup>. In 2013, Huang *et al*<sup>[41]</sup> first reported that amongst other exosomal RNA species, miRNAs were the most abundant in human plasma-derived exosomes. miRNAs are short ncRNAs consisting of 21–25 nucleotides that are critically involved in the regulation of gene expression<sup>[19]</sup>. The exosome-loading process starts after miRNA biogenesis and includes a complex of different components such as mature and pre-miRNAs, other RNA species, proteins and lipids. This cargo mirrors the content of the parent cell and is transferred to the recipient cell *via* fusion with the plasma membrane. Subsequently, transported miRNAs can play regulatory roles in the recipient cells<sup>[42]</sup>. Thus, CCA-derived exosomes can act as cancer migration and invasion mediators by transferring oncogenic miRNAs to normal cholangiocytes.

To date, several studies have investigated the role of miRNAs in the initiation and progression of CCA, but only a few have focused specifically on the exosomal miRNA profiling and role (Table 1). Reportedly, miR-205 can act as an oncogene or tumor suppressor<sup>[43]</sup>; miR-205 overexpression has been implicated in the development and progression of several cancers<sup>[44,45]</sup>. Okamoto *et al*<sup>[46]</sup> reported that miR-205 levels were associated with gemcitabine resistance in HuH28 cell lines and that miR-205 upregulation was related with restoring gemcitabine sensitivity. Interestingly, exosome-derived miR-205 from human CCA cell lines was found to be overexpressed, and knockdown of miR-205-5p expression repressed migration and invasion in CCA cell lines<sup>[47]</sup>. The same study also supported the role of exosomal miR-200 family members in CCA progression. Consistent with this observation, Shen *et al*<sup>[48]</sup> found that the miR-200 family was differentially expressed in peripheral blood-derived exosomes of CCA patients.

On the contrary, exosomal miR-199 family members and their clustered miRNA, hsa-miR-214-3p were found to be downregulated in human CCA cell line-derived exosomes, supporting their role in CCA carcinogenesis<sup>[47]</sup>. Furthermore, a panel of five miRNAs (miR-191, miR-486-3p, miR-1274b, miR-16 and miR-484) were upregulated in bile EVs from CCA patients *vs* a control group of patients suffering from PSC, biliary obstruction and bile leak. Of note, the study isolation protocol supported that the identified EVs were probably exosomes<sup>[49]</sup>.

It is well known that cancer invasion and metastasis have also been associated with EMT promotion. A recent study investigated the role of EV-miRNAs in regulating EMT process in CCA cells. The authors concluded that miR-30e expression was decreased by TGF- $\beta$ <sup>[50]</sup>; the latter has been previously identified as an EMT inducer<sup>[51]</sup>.

**Table 1 Potential clinical application of selected extracellular vesicle-derived microRNAs as biomarkers in cholangiocarcinoma**

microRNA	Expression	Type of EVs	EV source	Major finding	Potential application	Ref.
miR-205	↑	Exosomes	Human CCA cell lines	Downregulation of miR-205-5p decreased migration and invasion in CCA cell lines	Therapy monitoring/therapeutic target	[47]
Members of miR-200 family						
miR-200c-3p, miR-200b-3p, miR-200a-3p, miR-429 and miR-141-3p	↑	Exosomes	Human CCA cell lines	Supported the role of exosomal miR-200 family in CCA progression	Prognostic value	[47]
miR-200c-3p, miR-200a/c-3p	↑	Exosomes	Peripheral blood samples (36 patients)	(a) miR-200c-3p emerged as a potential diagnostic biomarker; and (b) miR-200a/c-3p emerged as a potential diagnostic and prognostic biomarker	Early diagnostic and prognostic value	[48]
miR-199 family	↓	Exosomes	Human CCA cell lines	Supported the role of miR-199 family in CCA carcinogenesis	-	[47]
miR-214	↓	Exosomes	Human CCA cell lines	Supported the role of miR-214 in CCA carcinogenesis	-	[47]
5 miR-based panel (miR-191, miR-486-3p, miR-1274b, miR-16 and miR-484)	↑	EVs	Bile samples (46 CCA <i>vs</i> 50 control patients with PSC, biliary obstruction and bile leak)	(a) The panel displayed a 67% sensitivity and 96% specificity for CCA diagnosis; and (b) tool for differential diagnosis between biliary obstruction of nonmalignant etiologies and CAA	Diagnostic value	[49]
miR-30e	↓	EVs	Nonmalignant human cell <i>vs</i> CCA cell lines	Encapsulation of miR-30e in EVs could suppress CCA cell invasion and migration by inhibiting EMT	EVs may be used as vehicles for delivery of therapeutic agents	[50]
miR-195	↓	EVs	Human liver stellate cell line	Coculture of CCA and stellate cell lines resulted in downregulation of miR-195. EV-mediated miR-195 transfer targeted tumor cells and inhibited proliferation in a rat model.	EVs may be used as vehicles for delivery of therapeutic agents	[52]
miR-604	↑	EVs	Serum	Displayed 0.944 diagnostic capacity for CCA	Diagnostic value	[53]
miR-551B	↑	EVs	Serum	Displayed 0.909 diagnostic capacity for CCA	Diagnostic value	[53]
miR-96-5p, miR-151a-5p, miR-191-5p and miR-4732-3p	↑	Exosomes	Blood	Stage II CCA patients displayed the highest levels	Diagnostic value in early CCA stages	[58]
miR-9-5p	↑	Exosomes	Human ICC samples	Significant association with malignancy promotion <i>via</i> ↑IL-6 expression in vCAFs	Prognostic value	[59]

EV: Extracellular vesicle; CCA: Cholangiocarcinoma; ICC: Intrahepatic cholangiocarcinoma; PSC: Primary sclerosing cholangitis; EMT: epithelial-mesenchymal transition; vCAF: Vascular cancer-associated fibroblast.

More importantly, this study demonstrated that miR-30e encapsulation in EVs could halt CCA cell invasion and migration by inhibiting EMT<sup>[50]</sup>. Similarly, miR-195 levels were downregulated in cholangiocarcinoma cells, and EV-incorporated miR-195 decreased cancer progression in a rat CCA model<sup>[52]</sup>.

Another recent study demonstrated that miR-551B and miR-604 were significantly upregulated in serum EVs, displaying an optimal diagnostic capacity for CCA<sup>[53]</sup>. Interestingly, Chang *et al.*<sup>[54]</sup> had previously reported that decreased miR-551b-3p levels were associated with poor overall survival of CCA patients. It is worth noting that

miR-551b-3p expression in cancer varies in the literature. For instance, miR-551b-3p upregulation has been reported in papillary thyroid carcinoma<sup>[55]</sup> and ovarian cancer<sup>[56]</sup>, whereas gastric cancer has been associated with miR-551b-3p downregulation<sup>[57]</sup>. Therefore, further research may be required in order to investigate the expression and functional roles of miR-551b-3p in CCA.

Recently, four miRNAs (miR-96-5p, miR-151a-5p, miR-191-5p and miR-4732-3p) were found to be significantly overexpressed in blood-derived exosomes of CCA patients<sup>[58]</sup>, and exosomal miR-9-5p was proposed as a potential prognostic biomarker for intrahepatic cholangiocarcinoma<sup>[59]</sup>. Mir-9-5p has been previously associated with angiogenesis promotion in cervical cancer<sup>[60]</sup>.

### **Exosomal long ncRNAs and CCA**

Long ncRNAs (lncRNAs) represent a subclass of ncRNAs (more than 200 nucleotides long) with distinct roles in several biological processes including cell proliferation, differentiation, invasion and metastasis. Several studies have found that lncRNAs can act as crucial mediators of cancer development, including CCA. Recent evidence supports their involvement in CCA progression *via* the competing endogenous RNA (ceRNA) network<sup>[61]</sup>. lncRNA levels in secreted exosomes have been suggested to be similar with those detected in plasma<sup>[62]</sup>. Although some lncRNA-specific loading mechanisms have been described in the literature<sup>[63]</sup>, the exact process that drives the exosomal loading with a specific biological cargo remains unclear.

Many studies have investigated the abnormal expression of specific lncRNAs and their association with CCA development and progression. However, little research has focused on the exosome and/or EV-lncRNA expression. Given that a very recent extensive review summarizes the roles of lncRNAs in CCA<sup>[64]</sup>, we will refer to and discuss current literature evidence based on studies investigating the functional roles of lncRNAs in EVs with an emphasis to exosomes (Table 2).

Ge *et al*<sup>[65]</sup>, reported that two lncRNAs (ENST00000588480.1 and ENST00000517758.1) were significantly upregulated in exosomes isolated from bile samples of CCA and benign biliary obstruction patients. This study demonstrated that both lncRNAs play crucial roles in CCA carcinogenesis and progression. Furthermore, a recent study identified the differential expression of several lncRNAs in urine and serum-isolated EVs from patients with CCA, PSC and healthy subjects. More specifically, the expression of three lncRNAs (MALAT-1, LOC643955 and LOC100190986) was significantly altered in serum EVs from CCA patients compared to patients with PSC<sup>[53]</sup>. Indeed, Tan *et al*<sup>[65]</sup> previously reported the oncogenic role of MALAT-1 in human hilar cholangiocarcinoma cell lines. The authors found that MALAT-1 was associated with prognosis and several clinicopathological parameters, such as stage, tumor size and perineural invasion. Furthermore, Shi *et al*<sup>[66]</sup> reported the aberrant expression of three lncRNAs (including MALAT-1) in plasma samples from hilar cholangiocarcinoma patients, suggesting that they may serve as candidate biomarkers for the early detection of hilar cholangiocarcinoma. Similarly, three lncRNAs (LOC100134868, HLA complex group 4 and LOC100134713) were differentially expressed in urine EVs from CCA patients compared to patients with PSC<sup>[53]</sup>. HLA complex group 4 was recently identified as one of the optimal feature coding genes participating in the ceRNA regulatory network and implicated in laryngeal cancer recurrence<sup>[67]</sup>.

In summary, despite the emerging role of exosomal lncRNAs as potential cancer biomarkers, to date very few researches have been focused on CCA. Most of the available data cannot determine direct associations between the exosomal lncRNAs and CCA development and progression. Moreover, to our knowledge no evidence exists regarding the sensitivity and specificity of lncRNAs in a clinical setting.

### **Circular RNAs and CCA**

Besides miRNAs and lncRNAs, circular RNAs (circRNAs) represent another subclass of bioactive ncRNAs. Originally, circRNAs were considered an RNA splicing byproduct with negligible functions. Recent findings suggested that exosomal circRNAs can serve as candidate cancer biomarkers due to their high stability in exosomes<sup>[68]</sup>.

CircRNA production originates from pre-mRNA back-splicing of exons, resulting in a single-stranded, closed, circular structure. Emerging evidence shows that they participate in several pathophysiological processes and that their expression is significantly altered during cancer development<sup>[69,70]</sup>. Conn *et al*<sup>[71]</sup> proposed that circRNAs are implicated in the EMT process and thus affect cell migration, invasion and tumor metastasis. Their regulatory role in the transcription process has also been reported<sup>[72]</sup>.

**Table 2 Potential clinical application of selected extracellular vesicle-derived long noncoding RNAs as biomarkers in cholangiocarcinoma**

lncRNA	Expression	Type of EVs	EV source	Major finding	Potential application	Ref.
ENST00000517758.1 and ENST00000588480.1	↑	Exosomes	Bile samples of CCA ( <i>n</i> = 35) and biliary obstruction patients ( <i>n</i> = 56)	(a) ENST00000588480.1 expression may contribute to tumorigenesis and CCA progression ( <i>via</i> p53 signaling pathway); and (b) Combined ENST00000588480.1 and ENST00000517758.1 exhibited higher sensitivity than CA19-9 (82.9% <i>vs</i> 74.3%)	Diagnostic value; Prognostic value/Therapeutic target	[63]
MALAT-1	↑	EVs	Serum	Upregulated in EVs from CCA <i>vs</i> PSC patients	Diagnostic value	[53]

lncRNA: Long noncoding RNA; EV: Extracellular vesicle; CCA: Cholangiocarcinoma; PSC: Primary sclerosing cholangitis.

Few studies have investigated the role of circRNAs in CCA tumorigenesis and progression. Cdr1as was found to be upregulated in cholangiocarcinoma tissues, and its expression level was positively correlated with clinicopathological parameters (tumor, node, metastasis stage, lymph node invasion and postsurgery recurrence). The authors also supported that high Cdr1as expression was associated with poor overall survival, highlighting the potential role of this circRNA as a prognostic biomarker<sup>[73]</sup>. Another study found that hsa\_circ\_0001649 was downregulated in CCA tissues, and it was associated with tumor size and grade. Moreover, it was suggested that upregulation of hsa\_circ\_0001649 resulted in tumor suppression both *in vivo* and *in vitro*<sup>[74]</sup>. Increased hsa\_circ\_0001649 expression was also negatively correlated with tumor progression in hepatocellular carcinoma<sup>[75]</sup> and in pancreatic ductal adenocarcinoma<sup>[76]</sup>. Its potential role as a tumor suppressor in gastrointestinal malignancies was further supported by a recent study; upregulation of hsa\_circ\_0001649 inhibited tumor growth and metastasis in gastric cancer cells<sup>[77]</sup>. Finally, Xu *et al*<sup>[78]</sup> proposed that circ\_0005230 inhibited cell apoptosis and promoted cell proliferation and metastasis in CCA cells.

To the best of our knowledge, only two studies have investigated the possible association of exosomal circRNA expression in cholangiocarcinoma so far. According to Wang *et al*<sup>[79]</sup>, circRNA 0000284 was found to be significantly upregulated in CCA cell lines, tumor tissues and plasma exosomes. Furthermore, exosome-mediated circ-0000284 transfer to neighboring normal cells resulted in tumorigenesis and CCA progression. Hence, circ-0000284 was reported to exhibit autocrine and paracrine actions through exosomal intercellular communication. Recently, circ-CCAC1 was found upregulated in circulating EVs exerting a potential role in CCA diagnosis and prognosis<sup>[80]</sup>.

### Piwi-interacting RNAs and CCA

Piwi-interacting RNAs (piRNAs) represent the largest class of ncRNAs; piRNAs are 26 to 31 nucleotides long and specifically interact with piwi-domain containing proteins<sup>[81]</sup>. More recent evidence suggests that piRNAs are involved in gene regulation at epigenetic and posttranscriptional levels, emerging as new mediators in the process of carcinogenesis<sup>[82,83]</sup>. In 2016, Yuan *et al*<sup>[84]</sup> noticed that piRNAs were differentially expressed in plasma-derived exosomes of cancer patients compared to healthy subjects. Currently, the majority of published studies have focused on the potential role of piRNAs as diagnostic and prognostic biomarkers in other types of malignancies, such as colorectal cancer and hepatocellular carcinoma<sup>[85,86]</sup>.

On the other hand, very little is known about the roles of piRNAs in CCA. Chen *et al*<sup>[87]</sup> reported that piwi-like protein 2 was significantly overexpressed in both hilar CCA tissues and the QBC939 cell line. Based on this observation, Gu *et al*<sup>[88]</sup> further investigated exosomal piRNA signatures and found that several piRNAs were differentially expressed in CCA patients compared to healthy individuals. More importantly, the authors suggested that piR-10506469 was significantly overexpressed in plasma-derived exosomes from CCA patients and that piR-10506469 and piR-20548188 were significantly downregulated after surgery, suggesting that these piRNAs may serve as potential diagnostic and prognostic biomarkers.

## DEREGULATION OF NCRNAs IN CCA: MOLECULAR MECHANISMS

In terms of epigenetic modifications, miRNA downregulation has often been associated with hypermethylation of their promoters. For instance, epigenetic silencing of tumor suppressor miR-370 in human CCA has been linked to hypermethylation of its promoter by IL-6-dependent overexpression of DNA methyltransferases<sup>[89]</sup>. Interestingly, An *et al*<sup>[90]</sup> suggested that miR-370 silencing in CCA follows Knudson's "two-hit hypothesis" mechanism *via* IL-6 mediated maternal to paternal epigenotype switch. Furthermore, CpG island hypermethylation of miR-373 resulted in miR-373 downregulation in hilar cholangiocarcinoma<sup>[91]</sup>. Accordingly, increased methylation of CpG sites upstream of miR-376c gene was found in intrahepatic CCA cell lines<sup>[92]</sup>. CCA tumorigenesis and progression has also been associated with Notch pathway activation<sup>[93]</sup>. Enhancer of zeste homolog 2 (EZH2) and DNA methylation-induced miR-34a silencing resulted in the promotion of CCA cell growth through activation of the Notch pathway<sup>[94]</sup>. Interferon regulatory factor-1 (IRF-1) has been suggested as a tumor suppressor in CCA<sup>[95]</sup>, and miR-383 has been recently found to directly target IRF-1<sup>[96]</sup>. These findings suggest that the targeting of IRF-1 by miR-383 may be the molecular basis for IRF-1 downregulation in CCA.

ncRNAs can regulate gene expression at several levels (epigenetic, transcriptional and posttranscriptional). On this basis, the regulatory scenario is enormous and remains under investigation. The exact underlying mechanisms of many ncRNA functions remain unclear. In the following section will focus on the molecular mechanisms of exosomal miRNAs in CCA.

The hypomethylated status of the miR-429 promoter has been correlated with increased miR-429 expression in CCA. Goepfert *et al*<sup>[97]</sup> demonstrated that epigenetically dysregulated miR-429 directly targeted cadherin-6 and promoted tumor growth. Exosomal miR-551b levels were upregulated in EVs isolated from CCA patients<sup>[53]</sup>. LncRNA SMARCC2 acts as a "sponge" RNA promoting the aberrant miR-551b-3p expression in gastric cancer<sup>[57]</sup>. On the other hand, Chang *et al*<sup>[54]</sup> demonstrated that miR-551b-3p directly targeted and decreased CCND1 expression, inhibiting CCA cell cycle progression and proliferation. miR-551b downregulation in breast cancer patients was associated with hypermethylation of its promoter<sup>[98]</sup>. Exosome-derived miR-205 and members of the miR-200 family were found to be overexpressed in CCA cell lines<sup>[47]</sup>. It has been previously suggested that these miRNAs cooperatively regulate EMT by targeting the transcriptional repressors ZEB1 and SIP1<sup>[99]</sup>. Moreover, miR-205 upregulation was associated with enhancing gemcitabine sensitivity in CCA cell lines; however, the authors could not identify possible target genes that could be associated with chemosensitivity<sup>[46]</sup>. Another study suggested that miR-205 promotes tumor invasion and metastasis in ovarian cancer *via* suppressing PTEN/SMAD4 expression<sup>[44]</sup>. MiR-199a-3p has also been implicated in increasing cisplatin sensitivity by inhibiting the mTOR pathway and by downregulating the MDR1 gene<sup>[100]</sup>. Recently, Zhang *et al*<sup>[101]</sup> reported that exosomes can act as carriers of miR-199a-3p in hepatocellular carcinoma. Interestingly, the authors concluded that intravenous injection of exo-miR-199a-3p increased resistance to cisplatin, offering a novel option for cisplatin refractory cancers. KEGG pathway analysis revealed that several CCA-associated, exosomal miRNA (miR-96-5p, miR-151a-5p, miR-191-5p and miR-4732-3p) target genes were enriched in the MAPK signaling pathway, suggesting their role in the process of neurogenesis<sup>[58]</sup>. miR-9-5p is another important miRNA that was upregulated in CCA-derived exosomes; miR-9-5p expression in intrahepatic cholangiocarcinoma was correlated with IL-6 upregulation in vascular cancer-associated fibroblasts *via* EZH2 overexpression<sup>[59]</sup>. Furthermore, Wei *et al*<sup>[60]</sup>, suggested that it could promote angiogenesis in cervical cancer by targeting suppressor of cytokine signaling 5.

Accumulating evidence has demonstrated that lncRNAs may interact with miRNAs as ceRNAs and regulate gene expression at the posttranscriptional level. Several lncRNAs, such as lncRNA TUG1, lncRNA HULC, lncRNA H19, PVT1 and LINC01296 have been identified as ceRNAs in CCA<sup>[64,102]</sup>. Furthermore, another oncogenic lncRNA, SPRY4-IT1, interacts with EZH2, lysine specific demethylase 1 and DNA methyltransferase 1 serving as a molecular scaffold<sup>[64]</sup>. In addition, some lncRNAs, such as SNHG1, directly interact with EZH2 and regulate gene transcription<sup>[103]</sup>.

Exosomal MALAT-1 was upregulated in serum isolated EVs from CCA patients. Mechanistically, MALAT-1 acted as a ceRNA *via* miR-204-dependent CXCR4 regulation<sup>[65]</sup>. Furthermore, Wang *et al*<sup>[104]</sup> supported that MALAT-1 exerted its oncogenic functions in CCA by activating PI3K/Akt pathway. Zinc fingers and homeoboxes 1 (ZHX1) functions as a transcription repressor binding DNA methyltransferase 3B; ZHX1 was associated with CCA development and

metastasis<sup>[105]</sup>. A recent study supported that suppression of the lncRNA MALAT1/miR-199a/ZHX1 axis inhibited glioblastoma progression<sup>[106]</sup>. Given that exosomal MALAT-1 and miR-199a roles in CCA have been previously described, future studies could investigate the potential role of MALAT1 in ZHX1 regulation by sponging miR-199a. The lncRNAs, ENST00000588480.1 and ENST00000517758.1 have been associated with promoting CCA development *via* the p53 signaling pathway<sup>[63]</sup>.

The functions and underlying molecular mechanisms of circRNAs in cancer have gained increasing scientific interest. Some of the proposed mechanisms include their ability to (a) regulate gene transcription by binding to RNA polymerase II; (b) alter protein activity; and c) act as ceRNAs<sup>[107]</sup>.

The oncogenic role of circ\_0005230 in CCA was first described by Xu *et al*<sup>[78]</sup>, who proposed its role as a ceRNA by sponging miR-1238 and miR-1299. Circ\_0005230 was previously associated with an unfavorable prognosis of breast cancer patients; reportedly, it can act as a miR-618 sponge and thus enhance CBX8 expression<sup>[108]</sup>. Increasing evidence supports the role of hsa\_circ\_0001649 as a tumor suppressor<sup>[74-77]</sup>. Its expression has been correlated with the ERK and Wnt/ $\beta$ -catenin pathway<sup>[77]</sup>. Matrix metalloproteinases play crucial roles in several cancer-related processes such as tumor neovascularization and metastasis<sup>[109]</sup>. Matrix metalloproteinase-9 was significantly regulated by hsa\_circ\_0001649 expression in CCA cells<sup>[74]</sup>.

Concerning exosomal circRNAs in CCA, circ-0000284 was identified as a ceRNA, directly binding to miRNA-637 and thus stimulating LY6E expression<sup>[79]</sup>. Finally, the newly identified circ-CCAC1 promoted CCA progression *via* YY1 upregulation by sponging miR-514a-5p. YY1 is a transcription factor and gene target of miR-514a-5p. The authors suggested positive correlations among circ-CCAC1, YY1 and CAMLG expression levels<sup>[80]</sup>.

## EXOSOMAL NCRNAs IN CCA: POTENTIAL DIAGNOSTIC AND THERAPEUTIC APPLICATIONS, FUTURE EXPECTATIONS

Exosomes and their diverse cargos represent a relatively novel and very promising field of investigation in cancer research. Their distinct properties have been valued by scholars, and they have currently become a research hotspot. CCA cells harbor specific ncRNA expression profiles transferred by EVs to neighboring or distant cells. Exosomal secretion into bodily fluids offers a novel, non-invasive liquid biopsy approach by detecting specific ncRNA profiles in serum or urine. Interestingly, although glomerular infiltration seems to affect the number of detectable ncRNAs in urine, some ncRNAs were found to be significantly upregulated in urine EVs isolated from patients with CCA<sup>[53]</sup>. These findings indicate a potential diagnostic value as noninvasive biomarkers for CCA. Moreover, we suggest that further research should focus on exosomal ncRNA signatures that have been associated with early stage CCA detection<sup>[47,48,58,66]</sup>, especially because late stage disease is not amenable to curative treatments.

Although EV isolation and characterization techniques have been improved in recent years, several concerns still arise regarding the accurate extraction and quantification of exosomal ncRNAs. Thus, caution in interpreting current research results is clearly warranted. With regard to CCA studies, several points should be taken into consideration to explain the conflicting results<sup>[49]</sup>: The relatively small number of published studies; the low patient sample size; and the different specimen collection, storage and processing procedures. This contradiction may also be attributed to the different ncRNA expression patterns among tissues and fluids of different origin. Moreover, despite exosomes being considered relatively stable<sup>[110]</sup>, there is still a need for developing universal methodologies in order to establish reproducible and reliable data.

Another topic of great importance in CCA management is associated with the role of systemic therapy. Despite insufficient response, chemotherapy remains the mainstay for patients with advanced CCA. Gemcitabine, cisplatin, 5-fluorouracil and oxaliplatin represent the chemotherapy agents that are still included in current therapeutic options<sup>[111]</sup>. Targeted and immunotherapy agents have also shown some potential in specific patient subpopulations. A comprehensive assessment of interpatient and intratumor heterogeneity is essential in order to understand the underlying drug resistance mechanisms and move towards a more personalized approach. In this context, exosomal ncRNAs may serve as biomarkers associated with response to several therapeutic regimens. There are currently only a few studies that have investigated the potential role of exosomal miRNAs as monitoring biomarkers in

cancer treatment. For instance, a recent study suggested that plasma exosomal miR-125b expression may act as a tool for the early detection of resistance to mFOLFOX6-based chemotherapy in advanced and recurrent colorectal cancer patients<sup>[112]</sup>. Similarly, Wei *et al.*<sup>[113]</sup> reported that exosomal miR-222-3p played an important role in gemcitabine resistance by targeting SOCS3 in non-small cell lung cancer patients. Another exosomal miRNA, miR-425-3p was also proposed as a potential prognostic marker in cisplatin-resistant non-small cell lung cancer patients<sup>[114]</sup>. Exosome-transferred miR-199a-3p mimics seemed to reverse cisplatin resistance in hepatocellular carcinoma cells<sup>[101]</sup>. However, to the best of our knowledge, no study has investigated exosome-derived ncRNA signatures in CCA drug resistance. Therefore, additional research in this field is needed in order to identify new biomarkers.

The high potential of exosomes in CCA diagnosis, prognosis and therapeutic approaches has gained considerable research interest. In line with the observation from Kitdumrongthum *et al.*<sup>[47]</sup>, one of the most promising therapeutic strategies for exosomes is the inhibition of oncogenic ncRNAs. Selectively loaded exosomes could serve as vehicles for the targeted delivery of molecules with antitumor properties (antioncogenic ncRNA agents). To expand this perspective, such molecules may also include ncRNAs that mimic endogenous, tumor suppressing ncRNAs. Indeed, Ota *et al.*<sup>[50]</sup> suggested that miR-30e encapsulated in EVs may suppress CCA metastasis by inhibiting EMT and thus could serve as a therapeutic agent. Similarly, EV-mediated miR-195 transfer was found to target tumor cells and inhibit CCA cell proliferation in a rat model<sup>[52]</sup>. An interesting procedure has already been described and includes the isolation and insertion of designated therapeutic biomolecules into exosomes. Then, the modified exosomes are reintroduced to the patient and regulated cellular functions that inhibited tumor growth<sup>[115]</sup>. From a safety perspective, autologous EV administration has been well tolerated, exhibiting mild inflammatory responses<sup>[116]</sup>.

Moreover, the design and efficacy of exosome-based therapeutic approaches demands comprehensive understanding of exosome pharmacokinetics. So far, the *in vivo* tracking of exosomes includes fluorescence labeling or radiolabeling methods<sup>[117]</sup>. The half-life of exosomes and EVs have been reported in the literature<sup>[118,119]</sup>. It is interesting to mention that exosomes isolated from patient fluids or tissues have longer circulation times due to low immunogenicity<sup>[120]</sup>. However, there are several factors that affect exosome concentration in systematic circulation and target tissues. Apart from the route of administration and dose, the pharmacokinetic parameters of exosomes depend on individual genetic variations, blood flow, organ volume and clearance<sup>[121,122]</sup>. Several bioengineering strategies, including polyethylene glycol-based formulations, have been proposed in order to improve the exosome half-life in circulation and target tissues<sup>[123]</sup>. Further research focusing on tissue-specific analytical methods towards dose individualization is needed due to variations in exosome absorption, distribution, metabolism and elimination.

Considering the above, an optimal drug delivery system should be able to escape immune defense mechanisms, transfer the incorporated cargo selectively to tumor sites and display minimum toxicity to normal tissues. Exosomes, as a natural body product, can avoid phagocytosis, enter target cells and escape degradation by lysosomes with limited immune response<sup>[124]</sup>. In general, EV tropism depends on the nature of the progenitor cell<sup>[125]</sup>. Thus, the appropriate exosome selection for engineering is crucial. Indeed, an increasing number of studies have proposed EV modification towards enhancing targeted drug delivery and anticancer efficacy. For instance, paclitaxel-loaded EVs from prostate cancer cells displayed improved efficacy, and the targeted delivery was partially attributed to surface proteins. Moreover, the authors suggested that the use of autologous cancer cell-derived EVs offer an advantage because they are taken up by both parent cells and other cells in the tumor microenvironment<sup>[126]</sup>. Accordingly, genetic modification of MSCs-derived and dendritic cell-derived exosomes has been a subject of extensive research in several tumor types<sup>[127-131]</sup>. Various methods for EV engineering have been described; electroporation for miRNA loading and incubation for loading chemotherapy drugs into exosomes are two of the most commonly used techniques<sup>[132]</sup>.

In conclusion, despite that numerous studies have suggested possible therapeutic applications for exosomes, very few clinical trials have been conducted until now. Indeed, our brief search in ClinicalTrials.gov did not reveal any clinical trial using exosomes for CCA treatment. On the other hand, there are very few interventional clinical trials (phase I-II) investigating the role of exosomes as drug-delivery microsystems in the treatment of other types of cancer<sup>[133-136]</sup>. This may be attributed to the fact that there are still several questions that need to be answered, such as the exact recipient cell uptake processes of exosomes *in vivo*. Nevertheless, we did identify one

prospective observational study aiming to characterize TD exosomal ncRNAs as potential biomarkers in CCA<sup>[137]</sup>. Finally, it is worth mentioning that one recently added interventional clinical trial aims to investigate exosomal PD-L1 and miRNA expression profiles in non-small cell lung cancer patients receiving immunotherapy<sup>[138]</sup>.

## CONCLUSION

Exosomal ncRNAs represent a fast growing and promising field of current cancer research. CCA-derived exosomes are loaded with unique ncRNA signatures that may serve as important tools for the early diagnosis and prognosis of cholangiocarcinoma. Further, targeted, large-scale research is needed in order to identify new diagnostic and prognostic biomarkers with acceptable sensitivity and specificity in CCA. Although the concept of exosomes as delivery “nanosystems” of antitumor biomolecules is very recent, it provides a very promising prospect in CCA treatment due to the limited treatment options for this type of cancer.

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