

# World Journal of *Gastrointestinal Surgery*

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

Konstantinos Laschos, Dimitra Ioanna Lampropoulou, Gerasimos Aravantinos, Maria Piperis, Dimitrios Filippou, George Theodoropoulos, Maria Gazouli

**ORCID number:** Konstantinos Laschos 0000-0001-5224-3192; Dimitra Ioanna Lampropoulou 0000-0003-3696-8550; Gerasimos Aravantinos 0000-0002-2106-1713; Maria Piperis 0000-0003-3892-9678; Dimitrios Filippou 0000-0001-5410-3046; George Theodoropoulos 0000-0003-4900-9711; Maria Gazouli 0000-0002-3295-6811.

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**Konstantinos Laschos, Dimitra Ioanna Lampropoulou, Gerasimos Aravantinos,** Second Department of Medical Oncology, General Oncology Hospital of Kifissia “Agiol Anargiroi”, Athens 14564, Greece

**Maria Piperis,** Radiation Therapy Department, Iatropolis, Athens 15231, Greece

**Dimitrios Filippou,** Department of Anatomy and Surgical Anatomy, Medical School, National and Kapodistrian University of Athens, Athens 11527, Greece

**George Theodoropoulos,** 1<sup>st</sup> Propaedeutic University Surgery Clinic, Hippocratio General Hospital, Medical School, National and Kapodistrian University of Athens, Athens 11527, Greece

**Maria Gazouli,** Department of Basic Medical Sciences, Laboratory of Biology, Medical School, National and Kapodistrian University of Athens, Athens 11527, Greece

**Corresponding author:** Maria Gazouli, PhD, Associate Professor, Department of Basic Medical Sciences, Laboratory of Biology, Medical School, National and Kapodistrian University of Athens, Michalakopoulou 176, Athens 11527, Greece. [mgazouli@med.uoa.gr](mailto:mgazouli@med.uoa.gr)

### 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.

## EXOSOMES IN CANCER: BIOGENESIS AND CHARACTERISTICS

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).

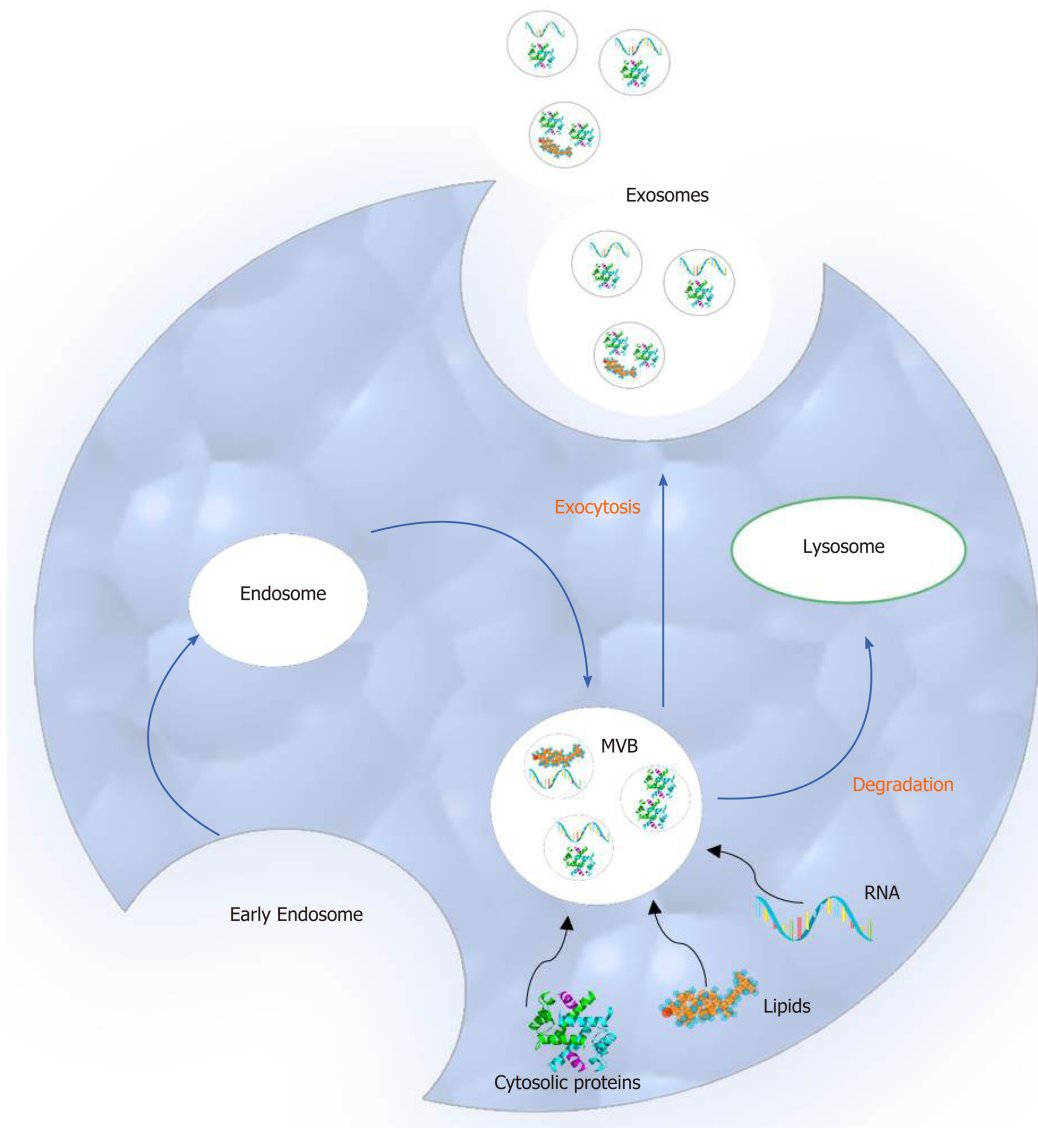
Two main mechanisms orchestrate ILV formation and the packaging of bioactive exosomal cargo. The first one depends on the endosomal sorting complexes required for the transport system whereas the second depends on raft-based microdomains, such as tetraspanin-enriched microdomains, tetraspanins and lipids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

## EXTRACELLULAR VESICLES IN CCA PATHOPHYSIOLOGY

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.

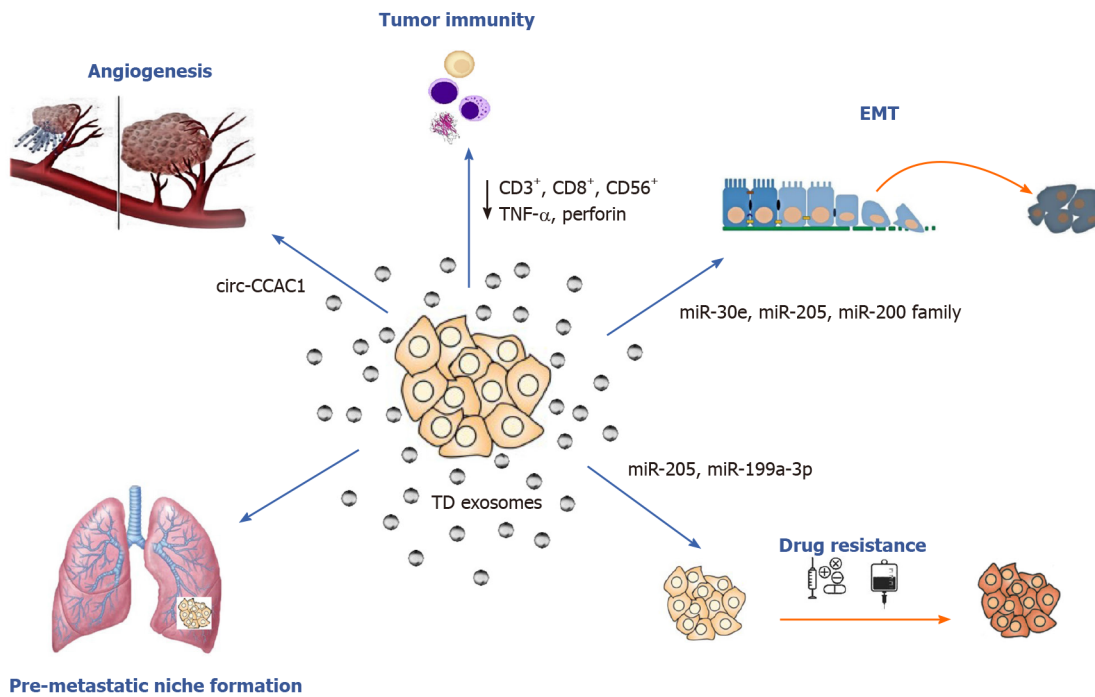
Chen *et al*<sup>[36]</sup> reported that TD exosomes contributed to CCA escape from immune recognition by downregulating CD3<sup>+</sup>, CD8<sup>+</sup>, NK (CD56<sup>+</sup>) cells and by decreasing TNF- $\alpha$  and perforin production.

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. Goeppert *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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## Retrospective Cohort Study

# Narrow pelvic inlet plane area and obesity as risk factors for anastomotic leakage after intersphincteric resection

Akira Toyoshima, Toshihiro Nishizawa, Eiji Sunami, Ryuji Akai, Takahiro Amano, Akiyoshi Yamashita, Shin Sasaki, Takeshi Endo, Yoshihiro Moriya, Osamu Toyoshima

**ORCID number:** Akira Toyoshima 0000-0002-5697-6251; Toshihiro Nishizawa 0000-0003-4876-3384; Eiji Sunami 0000-0002-3637-9732; Ryuji Akai 0000-0001-7241-6265; Takahiro Amano 0000-0002-0489-7519; Akiyoshi Yamashita 0000-0002-3519-084X; Shin Sasaki 0000-0003-3489-6821; Takeshi Endo 0000-0002-3394-0213; Yoshihiro Moriya 0000-0002-7865-8748; Osamu Toyoshima 0000-0002-6953-6079.

**Author contributions:** Toyoshima A is the lead investigator, performed operations, collected and analyzed the data, and wrote the manuscript; Nishizawa T performed the literature search and statistical analysis and wrote the manuscript; Sunami E performed the operations; Akai R and Amano T assisted the operations; Yamashita A drafted the conception; Sasaki S supervised the study and approved the final manuscript; Toyoshima O contributed to data management, interpretation, and revision.

### Institutional review board

**statement:** This retrospective study was approved by the ethics review board of the Japanese Red Cross Medical Center on July 31, 2019.

### Informed consent statement:

Patients were not required to give

**Akira Toyoshima, Ryuji Akai, Takahiro Amano, Shin Sasaki,** Department of Colorectal Surgery, Japanese Red Cross Medical Center, Tokyo 150-8935, Japan

**Toshihiro Nishizawa,** Department of Gastroenterology, International University of Health and Welfare, Narita Hospital, Narita 286-8520, Japan

**Toshihiro Nishizawa, Osamu Toyoshima,** Department of Gastroenterology, Toyoshima Endoscopy Clinic, Tokyo 157-0066, Japan

**Eiji Sunami,** Department of Surgery, The University of Kyorin, Tokyo 113-8655, Japan

**Akiyoshi Yamashita,** Department of Radiology, Japanese Red Cross Medical Center, Tokyo 150-8935, Japan

**Takeshi Endo,** Tokyo Midtown Clinic, Tokyo 107-6206, Japan

**Yoshihiro Moriya,** Miki Hospital, Iwate 029-4201, Japan

**Corresponding author:** Akira Toyoshima, MD, Doctor, Department of Colorectal Surgery, Japanese Red Cross Medical Center, 4-1-22 Hiroo, Shibuya-ku, Tokyo 150-8935, Japan. [toyosanaa@yahoo.co.jp](mailto:toyosanaa@yahoo.co.jp)

## Abstract

### BACKGROUND

Intersphincteric resection (ISR) has been increasingly used as the ultimate sphincter-preserving procedure in extremely low rectal cancer. The most critical complication of this technique is anastomotic leakage. The incidence rate of anastomotic leakage after ISR has been reported to range from 5.1% to 20%.

### AIM

To investigate risk factors for anastomotic leakage after ISR based on clinicopathological variables and pelvimetry.

### METHODS

This study was conducted at Department of Colorectal Surgery, Japanese Red Cross Medical Center, Tokyo, Japan, with a total of 117 patients. We enrolled 117 patients with extremely low rectal cancer who underwent laparotomic and laparoscopic ISRs at our hospital. We conducted retrospective univariate and

informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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multivariate regression analyses on 33 items to elucidate the risk factors for anastomotic leakage after ISR. Pelvic dimensions were measured using three-dimensional reconstruction of computed tomography images. The optimal cutoff value of the pelvic inlet plane area that predicts anastomotic leakage was determined using a receiver operating characteristic (ROC) curve.

## RESULTS

We observed anastomotic leakage in 10 (8.5%) of the 117 patients. In the multivariate analysis, we identified high body mass index (odds ratio 1.674; 95% confidence interval: 1.087-2.58;  $P = 0.019$ ) and smaller pelvic inlet plane area (odds ratio 0.998; 95% confidence interval: 0.997-0.999;  $P = 0.012$ ) as statistically significant risk factors for anastomotic leakage. According to the receiver operating characteristic curves, the optimal cutoff value of the pelvic inlet plane area was 10074 mm<sup>2</sup>. Narrow pelvic inlet plane area ( $\leq 10074$  mm<sup>2</sup>) predicted anastomotic leakage with a sensitivity of 90%, a specificity of 85.9%, and an accuracy of 86.3%.

## CONCLUSION

Narrow pelvic inlet and obesity were independent risk factors for anastomotic leakage after ISR. Anastomotic leakage after ISR may be predicted from a narrow pelvic inlet plane area ( $\leq 10074$  mm<sup>2</sup>).

**Key Words:** Intersphincteric resection; Anastomotic leakage; Pelvimetry; Pelvic dimensions; Pelvic inlet plane area; Rectal cancer

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**Core Tip:** Intersphincteric resection (ISR) is the ultimate sphincter-preserving procedure in extremely low rectal cancer. We investigated risk factors for anastomotic leakage after ISR based on clinicopathological variables and pelvimetry. Narrow pelvic inlet and obesity were independent risk factors for anastomotic leakage after ISR. Anastomotic leakage after ISR may be predicted from a narrow pelvic inlet plane area ( $\leq 10074$  mm<sup>2</sup>).

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## INTRODUCTION

Since Schiessel *et al*<sup>[1]</sup> first introduced intersphincteric resection (ISR) in 1994, the procedure has been increasingly accepted as the ultimate sphincter-preserving procedure in extremely low rectal cancer. ISR preserves the natural anus and avoids permanent colostomy. However, ISR is performed in the deep and funnel-shaped pelvic cavity, where access and visualization of the narrow pelvis is difficult<sup>[2,3]</sup>. Anastomotic leakage is the most critical complication that can cause reduced function or narrowing of the anal sphincter, possibly warranting a permanent colostomy<sup>[4-6]</sup>. According to a report from a committee (chaired by N Saito) sponsored by the Ministry of Health, Labour and Welfare of Japan, anastomotic leakage occurred in 23 (10.2%) of 225 patients after ISR<sup>[7]</sup>. The incidence rate of anastomotic leakage after ISR has been reported to range from 5.1% to 20%<sup>[8-10]</sup>.

Akasu *et al*<sup>[11]</sup> have reported on the risk factors for anastomotic leakage after ISR but have not analyzed pelvic size. Pelvic anatomy is a determinant factor of rectal dissection, as the narrow pelvic cavity and bony structures surrounding the rectum may hinder dissection maneuvers<sup>[12]</sup>. Pelvimetry is famous for its use in predicting

obstetric risk. In this study, we used pelvimetry to measure the inlet and outlet plane areas, particularly pelvic dimensions, to investigate risk factors for anastomotic leakage after ISR.

## MATERIALS AND METHODS

### Ethics

This retrospective study was approved by the ethics review board of the Japanese Red Cross Medical Center on July 31, 2019.

### Subjects

This study included subjects who underwent laparotomic and laparoscopic ISR as treatment for extremely low rectal adenocarcinoma with inferior margins less than 5 cm from the anal verge at the Japanese Red Cross Medical Center between 2005 and 2019. The spread of the rectal cancer was quantified in accordance with the clinical tumor-node-metastasis (TNM) classification (8<sup>th</sup> edition)<sup>[13]</sup>. Liver metastases were detected in all patients with stage IV cancer. The patients with peritoneal metastasis, multivisceral resection, gastrointestinal stromal tumor, and carcinoid were excluded.

Our indications for ISR included: (1) An inferior tumor margin less than 5 cm from the anal verge; (2) A moderate- to well-differentiated adenocarcinoma; (3) Local spread restricted to the internal sphincter without involvement of striated muscle; (4) The presence of some amount of T4 tumor (vaginal invasion); (5) Patients with resectable metastases to the liver or lungs; and (6) Normal sphincter function.

### Surgical procedure

The surgical procedure was performed in accordance with the method by Schiessel *et al*<sup>[1]</sup>. Laparotomy was performed with a small incision below the umbilicus, considering minimally invasive surgery. Under either laparotomy or laparoscopy, the inferior mesenteric artery (IMA) root was generally preserved, and the superior rectal artery was severed at the branching site of the left colic artery (LCA) or first branch of the sigmoid colic artery (SCA). When the IMA root was preserved, the lymph node around IMA root was separately resected to improve the curability. If tension was present at the anastomotic site or the color of the colon indicated an inadequate blood supply, the IMA was severed at the root and splenic flexure was mobilized. The rectum was divided transanally, removing a part or the entirety of the internal sphincter. Reconstruction was performed with a hand-sewn coloanal anastomosis from the anus. A closed suction drain was inserted in the left side of the pelvis and brought out at the lower angle of the wound. In some cases, an anal bougie (transanal drain) was inserted. Anastomotic leakage occurred in the second patient; thus, in all the subsequent patients, we constructed a covering stoma. The diverting stoma was closed 3-6 mo after surgery. Before the closure, we performed colonoscopy, contrast enema radiography, or computed tomography (CT). CT was performed each time there was a complication. The operations were performed by 3 staff surgeons (A, B, and C).

### Outcomes

Anastomotic leakage was defined as the presence of an anastomosis fistula during the first postoperative endoscopy or gastrografin enema. In addition, anastomotic leakages were classified into three categories according to the clinical management by Rahbari *et al*<sup>[14]</sup> as follows: Grade A requires no active therapeutic intervention; Grade B, an active therapeutic intervention without operation; and Grade C, re-laparotomy.

We conducted retrospective univariate and multivariate regression analyses on 33 items to elucidate the risk factors for anastomotic leakage after ISR. These items were categorized into three groups as follows: Surgery, tumor, and patient related. The surgery-related factors were operation type (laparotomy or laparoscopic surgery), circumferential resection margin (CRM +/-), site of IMA ligation (IMA/LCA or SCA), splenic flexure mobilization (with/without), construction of a diverting stoma (with/without), insertion of an anal bougie (with/without), blood loss, transfusion (with/without)<sup>[15]</sup>, surgeon (A, B, and C), surgical duration, and curability (A, B, or C). Tumor-related factors were tumor size, distance from the anal verge to the inferior margin of the tumor, patient categorization as clinical TNM classification, preoperative radiotherapy (with/without), and neoadjuvant chemotherapy (with/without). The patient related factors were age, sex, body mass index (BMI), American Society of

Anesthesiologists Physical Status (ASA-PS; I, II, or III), serum total protein level, hemoglobin level, prognostic nutritional index<sup>[7]</sup>, diabetes (hemoglobin A1c  $\geq$  5.8 g/dL), and nine pelvimetry measurements.

### Pelvimetry

We used the Synapse Vincent magnifying viewer (Fujifilm Medical Co., Ltd., Tokyo, Japan) to convert CT images into three-dimensional images. By using this imaging system, the anteroposterior (AP) diameter, transverse diameter, and total area were measured at the pelvic inlet and outlet. The length of the pubic symphysis and distance from the sacral promontory to the coccyx were measured to determine the anterior and posterior depths of the pelvis (Figure 1A-C). The lumbosacral (tortuosity) angle at the sacral promontory was also measured (Figure 1D).

### Statistical analyses

Differences between the leakage and non-leakage groups were detected using the Welch's *t*-test or Student *t*-test for continuous data. The  $\chi^2$  test was used for categorical secondary outcomes. The predictors found to be associated with anastomotic leakage in the univariate analysis ( $P < 0.05$ ) were analyzed by subsequent multiple logistic regression method to identify independent factors. If both diameter and area were statistically significant, the area was preferentially used in the multivariate analysis. The optimal cutoff value of the pelvic inlet plane area for predicting anastomotic leakage was determined using the receiver operating characteristic curve. *P* values of  $< 0.05$  were considered statistically significant in this study.

## RESULTS

We enrolled 117 patients [89 men; mean age, 61.3 years (range, 26-86 years)]. We observed anastomotic leakage in 10 (8.5%) of the 117 patients as follows: Grade A in 3 patients (30%), grade B in 5 (50%), and grade C in 2 (20%). In the 3 cases with grade A leakage, it was cured by conservative treatment such as antibiotic treatment. In the 5 patients with grade B leakage, the fistulae were closed using an alpha-cyanoacrylate monomer (Aron Alpha A Sankyo, Toagosei Co., Ltd., Tokyo, Japan), a tissue adhesive. Two patients with grade C leakage needed re-operation. One patient underwent permanent colostomy, and the other underwent nephrostomy for vesicorectal fistula. Closure of the diverting stoma was possible in 8 (80%) of the 10 patients. No operative deaths occurred.

Table 1 shows the correlation between the postoperative anastomotic leakage and clinicopathological variables. The univariate analysis revealed a statistically significant relationship between anastomotic leakage and higher BMI, smaller pelvic inlet area, shorter AP diameter of the inlet plane, shorter transverse diameter of the inlet plane, shorter AP diameter of the outlet plane, longer pubic symphysis, greater lumbosacral angle, and larger amount of bleeding.

In the multivariate analysis, we identified high BMI (odds ratio 1.674; 95% confidence interval: 1.087-2.58;  $P = 0.019$ ) and smaller pelvic inlet plane area (odds ratio 0.998; 95% confidence interval: 0.997-0.999;  $P = 0.012$ ) as statistically significant risk factors for anastomotic leakage (Table 2).

According to the receiver operating characteristic curves, the optimal cutoff value of the pelvic inlet plane area was 10074 mm<sup>2</sup> (Figure 2). Narrow pelvic inlet area ( $\leq 10074$  mm<sup>2</sup>) predicted anastomotic leakage with a sensitivity of 90%, a specificity of 85.9%, and an accuracy of 86.3%. The positive predictive value was 37.5%, and the negative predictive value was 98.9%.

## DISCUSSION

Narrow pelvic inlet and obesity were independent risk factors for anastomotic leakage after ISR. This is the first report of increased anastomotic leakage due to narrow pelvic inlet. Narrow pelvic inlet hinders the surgical procedures with the approach from the abdominal cavity, and the difficulty might lead to anastomotic leakage.

Rullier *et al*<sup>[16]</sup> demonstrated that male sex and obesity were independent risk factors for anastomotic leakage after low anterior resection (LAR). The male pelvis is narrower than the female pelvis; thus, male sex as a risk factor for anastomotic leakage might be due to the narrower pelvis in males.

**Table 1 Univariate analyses of clinicopathological variables related to anastomotic leakage after intersphincteric resection**

	No leakage	Leakage	P value
Patients number	107	10	
Age in yr	61.1 ± 12.7	64.3 ± 9.9	0.436
Male sex, <i>n</i> (%)	80 (74.8)	9 (90)	0.489
Body mass index	22.4 ± 3.3	25.6 ± 2.7	0.004
Anesthesiologists physical status			0.569
1	29	3	
2	75	7	
3	3	0	
Serum total protein level, g/dL	6.9 ± 0.5	7.1 ± 0.5	0.351
Blood hemoglobin level, g/dL	13.5 ± 1.8	14.6 ± 1.4	0.055
Prognostic nutritional index	49.7 ± 6.3	52.4 ± 4.9	0.205
Diabetes mellitus			0.326
No	93	7	
Yes	14	3	
Inlet plane: Antero-posterior diameter, mm	110.3 ± 10.8	100 ± 7.9	0.002
Inlet plane: Transverse diameter, mm	156.3 ± 35.4	136.4 ± 17.7	0.008
Area of the inlet plane, mm <sup>2</sup>	11330 ± 1279	9428 ± 636.1	< 0.001
Outlet plane: Antero-posterior diameter, mm	97.8 ± 8.9	90.6 ± 10.4	0.018
Outlet plane: Transverse diameter, mm	103.4 ± 10.1	102.8 ± 4.4	0.739
Area of the outlet plane, mm <sup>2</sup>	9572 ± 1501	9323 ± 999	0.487
Length of the pubic symphysis, mm	41.9 ± 5.6	45.7 ± 5.2	0.044
Distance from the sacral promontory to the coccyx, mm	125.5 ± 13.3	131.3 ± 14.8	0.193
Tortuosity angle	131.7 ± 8.7	140.4 ± 11.4	0.004
Tumors			
Tumor size, mm	41.3 ± 25.2	33.3 ± 19.7	0.335
Distance from the anal verge, mm	16.8 ± 19.2	20.4 ± 14.6	0.563
TNM stage			0.056
0	3	1	
I	35	6	
II	23	1	
III	34	1	
IV	12	1	
Preoperative radiotherapy			0.972
No	90	9	
Yes	17	1	
Neoadjuvant chemotherapy			0.341
No	89	10	
Yes	18	0	
Surgery			0.341
Laparotomy	89	10	
Laparoscopic surgery	18	0	

Circumferential resection margin			0.809
No	100	9	
Yes	7	1	
Ligated site of the artery			0.583
Inferior mesenteric artery	5	0	
Left colonic artery	41	3	
Sigmoid colon artery	61	7	
Mobilization of the splenic flexure			0.738
No	99	9	
Yes	8	1	
Diverting stoma			0.401
No	1	1	
Yes	106	9	
Anal bougie			0.341
No	89	10	
Yes	18	0	
Bleeding amount, mL	645 ± 709	1380 ± 1166	0.004
Blood transfusion, mL	8 ± 60	292 ± 621	0.181
Operator			0.418
X	87	9	
T	9	1	
S	11	0	
Operation time in min	367 ± 156	387 ± 167	0.698
Curativity			0.54
A	86	9	
B	8	0	
C	13	1	

TNM: Tumor-node-metastasis.

**Table 2 Multivariate analyses of clinicopathological variables related to anastomotic leakage after intersphincteric resection**

Variables	Multivariate analysis		
	Odds ratio	95%CI	P value
Body mass index	1.674	1.087-2.58	0.019
Area of the inlet plane	0.998	0.997-0.999	0.012
Outlet plane: Antero-posterior diameter	0.905	0.811-1.008	0.07
Length of the pubic symphysis	1.125	0.883-1.435	0.341
Tortuosity angle	1.049	0.941-1.17	0.39
Bleeding amount	1.001	0.999-1.003	0.144

CI: Confidence interval.



Zhou *et al*<sup>[17]</sup> evaluated the technical difficulties in LAR or abdominoperineal resection using three-dimensional reconstruction of CT images. Their multivariate analyses revealed that the AP diameter of the pelvic inlet, AP diameter of the pelvic outlet, and height of the pubic symphysis were factors that affect operative time. These results indicate that narrower and deeper pelvises could increase operating times.

Zur Hausen *et al*<sup>[18]</sup> also evaluated the clinical outcomes in LAR or abdominoperineal resection using CT pelvimetry. A shorter AP pelvic inlet diameter was associated with a higher rate of incomplete mesorectal excision. However, the number of cases were relatively small ( $n = 74$ ), and the study failed to show the association between pelvic diameters and incidence of anastomotic leakage. The authors concluded that preoperative pelvimetry may help identify difficult pelvic dissections preoperatively.

The present study shows that narrow pelvic inlet significantly increased the incidence of anastomotic leakage after ISR. Furthermore, narrow pelvic inlet area ( $\leq 10074 \text{ mm}^2$ ) predicted anastomotic leakage with a sensitivity of 90%, a specificity of 85.9%, and an accuracy of 86.3%. Identification of patients at high risk for anastomotic leakage may allow for the selective use of the indocyanine green fluorescence method or more-advanced access methods such as transanal total mesorectal excision or robotic-assisted laparoscopic surgery<sup>[19]</sup> for improving surgical outcomes.

In our study, obesity was also an independent risk factor for anastomotic leakage after ISR. Surgery for obese patients is generally more difficult because obesity contributes to inadequate exposure of the surgical field, which results in accidental injury. Yang *et al*<sup>[20]</sup> and Yamamoto *et al*<sup>[21]</sup> reported that obesity was a risk factor for anastomotic leakage after LAR. Rullier *et al*<sup>[16]</sup> reported that obesity was a risk factor for anastomotic leakage after ISR. A protective stoma is, therefore, suitable after ISR, particularly in obese patients. The first limitation of our study is that it was conducted within a single specialized institution. Second, this study was consecutive but retrospective. Third, the number of laparoscopic cases was small, and the effect of laparoscopic surgery was not determined. A follow-up study should be performed to confirm and clarify the characteristics of anastomotic leakage after ISR including laparoscopic surgery.

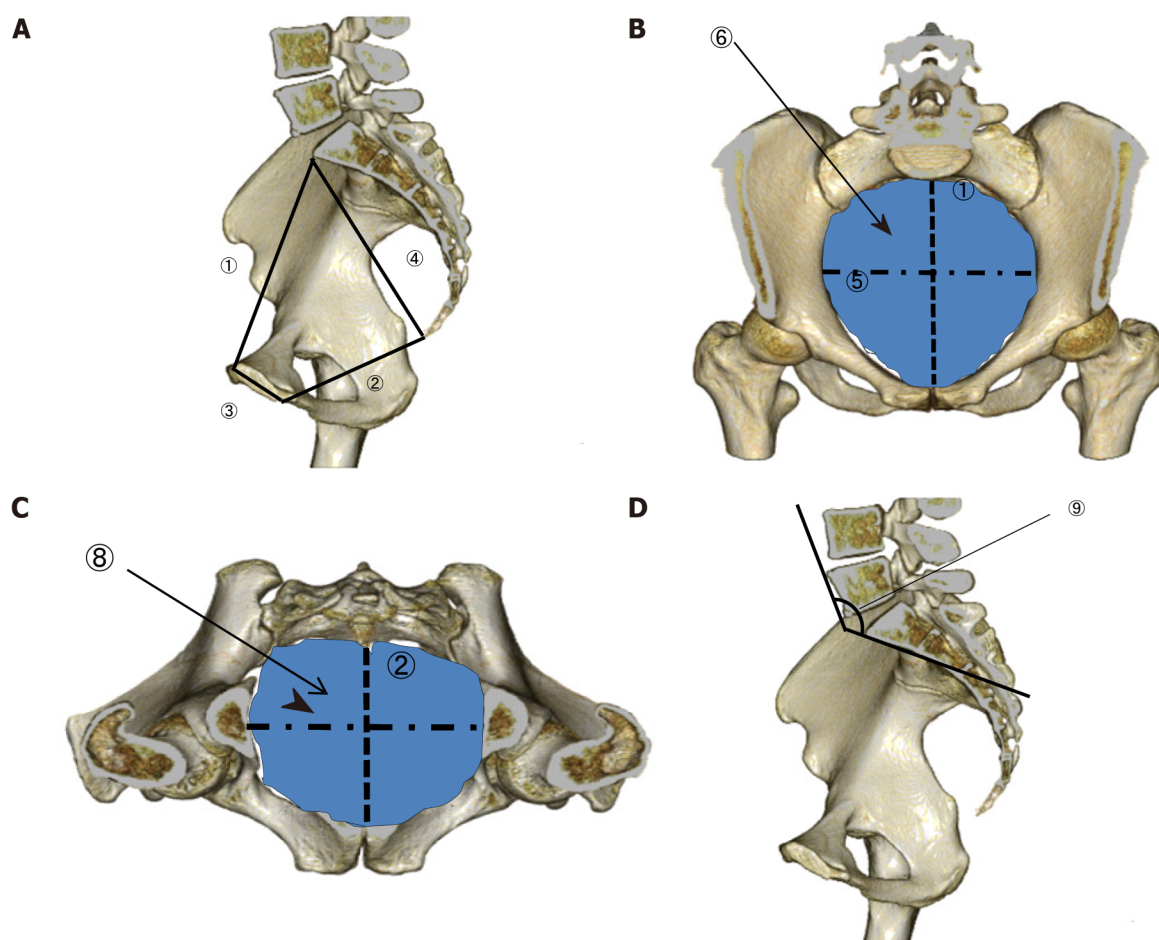
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## CONCLUSION

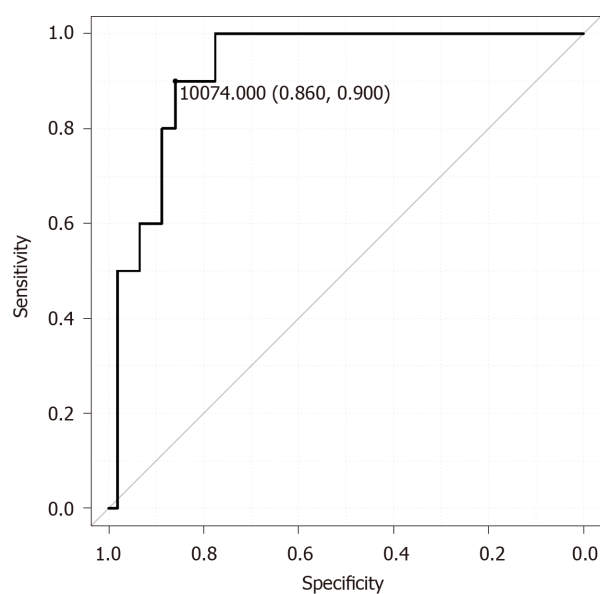
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In conclusion, narrow pelvic inlet and high BMI were associated with anastomotic leakage after ISR. Anastomotic leakage after ISR may be predicted from a narrow pelvic inlet plane area ( $\leq 10074 \text{ mm}^2$ ).





**Figure 1 Variables identified with three-dimensional pelvimetry.** A: Lateral view; B: pelvic inlet plane; C: Pelvic outlet plane; D: Lateral view. 1: Anteroposterior pelvic inlet diameter; 2: Anteroposterior pelvic outlet diameter; 3: Length of the pubic symphysis; 4: Distance from the sacral promontory to the coccyx; 5: Transverse pelvic inlet diameter; 6: Pelvic inlet area; 7: Transverse pelvic outlet diameter; 8: Pelvic outlet area; 9: Lumbosacral (tortuosity) angle at the sacral promontory.



**Figure 2 Receiver operating characteristic curve for predicting anastomotic leakage based on the pelvic inlet plane area.**

## ARTICLE HIGHLIGHTS

**Research background**

Intersphincteric resection (ISR) has been increasingly used as the ultimate sphincter-preserving procedure in extremely low rectal cancer.

**Research motivation**

Anastomotic leakage is the most critical complication that can cause reduced function or narrowing of the anal sphincter, possibly warranting a permanent colostomy.

**Research objectives**

This study investigated risk factors for anastomotic leakage after ISR based on clinicopathological variables and pelvimetry.

**Research methods**

We enrolled 117 patients with extremely low rectal cancer who underwent laparotomic and laparoscopic ISRs. Risk factors for anastomotic leakage after ISR that were analyzed using a multivariate analysis. Pelvic dimensions were measured using three-dimensional reconstruction of computed tomography images. The optimal cutoff value of the pelvic inlet plane area that predicts anastomotic leakage was determined using a receiver operating characteristic curve.

**Research results**

Higher body mass index and small pelvic inlet plane area were independently associated with anastomotic leakage after ISR. According to the receiver operating characteristic curves, the optimal cutoff value of the pelvic inlet plane area was 10074 mm<sup>2</sup>. Narrow pelvic inlet plane area ( $\leq 10074$  mm<sup>2</sup>) predicted anastomotic leakage with a sensitivity of 90%, a specificity of 85.9%, and an accuracy of 86.3%.

**Research conclusions**

Narrow pelvic inlet and obesity were independent risk factors for anastomotic leakage after ISR.

**Research perspectives**

A follow-up study should be performed to confirm and clarify the characteristics of anastomotic leakage after ISR including laparoscopic surgery.

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## Gastric splenosis mimicking a gastrointestinal stromal tumor: A case report

Claudio Isopi, Giulia Vitali, Federica Pieri, Leonardo Solaini, Giorgio Ercolani

**ORCID number:** Claudio Isopi 0000-0002-4186-8952; Giulia Vitali 0000-0002-4829-5544; Federica Pieri 0000-0002-2622-2985; Leonardo Solaini 0000-0002-5031-9285; Giorgio Ercolani 0000-0003-4334-5167.

**Author contributions:** Isopi C and Vitali G reviewed the literature and drafted the manuscript in consultation with Solaini L and Ercolani G; Isopi C and Vitali G are joint first authors; Pieri F performed the final pathology; Pieri F, Solaini L and Ercolani G critically revised the manuscript; All authors issued final approval for the version to be submitted.

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Claudio Isopi, Giulia Vitali, Leonardo Solaini, Giorgio Ercolani, Department of Surgery, Morgagni-Pierantoni Hospital, Forli 47121, Italy

Federica Pieri, Pathology Unit, Morgagni-Pierantoni Hospital, Forli 47121, Italy

Leonardo Solaini, Giorgio Ercolani, Department of Medical and Surgical Sciences, University of Bologna, Bologna 47100, Italy

**Corresponding author:** Leonardo Solaini, MD, Assistant Professor, Department of Surgery, Morgagni-Pierantoni Hospital, Via Carlo Forlanini, 34, Forli 47121, Italy. [leonardo.solaini2@unibo.it](mailto:leonardo.solaini2@unibo.it)

### Abstract

#### BACKGROUND

Mass lesions located in the wall of the stomach (and also of the bowel) are referred to as "intramural." The differential diagnosis of such lesions can be challenging in some cases. As such, it may occur that an inconclusive fine needle aspiration (FNA) result give way to an unexpected diagnosis upon final surgical pathology. Herein, we present a case of an intramural gastric nodule mimicking a gastric gastrointestinal stromal tumor (GIST).

#### CASE SUMMARY

A 47-year-old Caucasian woman, who had undergone splenectomy for trauma at the age of 16, underwent gastroscopy for long-lasting epigastric pain and dyspepsia. It revealed a 15 mm submucosal nodule bulging into the gastric lumen with smooth margins and normal overlying mucosa. A thoraco-abdominal computed tomography scan showed in the gastric fundus a rounded mass (30 mm in diameter) with an exophytic growth and intense enhancement after administration of intravenous contrast. Endoscopic ultrasound scan showed a hypoechoic nodule, and fine needle FNA was inconclusive. Gastric GIST was considered the most probable diagnosis, and surgical resection was proposed due to symptoms. A laparoscopic gastric wedge resection was performed. The postoperative course was uneventful, and the patient was discharged on the seventh postoperative day. The final pathology report described a rounded encapsulated accumulation of lymphoid tissue of about 4 cm in diameter consistent with spleen parenchyma implanted during the previous splenectomy.

#### CONCLUSION

Splenosis is a rare condition that should always be considered as a possible

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diagnosis in splenectomized patients who present with an intramural gastric nodule.

**Key Words:** Splenosis; Intramural gastric mass; Gastric nodule; Laparoscopic gastric surgery; Gastrointestinal stromal tumor; Case report

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**Core Tip:** Intramural gastric nodules are rare, but all differential diagnoses must always be considered. If feasible, a preoperative fine needle aspiration can help the surgeon in selecting the best treatment option. Splenosis is uncommon in the general population, but it must be considered in each patient with a history of splenectomy (especially after trauma). In this specific cluster it is reasonable to insist on ruling out splenosis even making a second histologic sampling after a first failure.

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**DOI:** <https://dx.doi.org/10.4240/wjgs.v12.i10.435>

## INTRODUCTION

The masses arising from the wall of the stomach are referred to as “intramural”. In these cases the endoscopic and radiologic features may lead to several differential diagnoses because several overlapping characteristics have been shown to exist among the various gastric masses. Intramural lesions can be benign or malignant, and the most common diagnosis is gastrointestinal stromal tumors (GISTs).

Only a preoperative sampling allows planning the best therapeutic approach, but when the nature of the nodule cannot be preoperatively determined, an assessment about size, possible diagnoses, patient’s characteristics and clinical symptoms should be done before considering an upfront surgical approach.

Herein, we present a case of an intramural gastric nodule mimicking gastric gastrointestinal stromal tumor, whose nature could be defined only after surgery.

## CASE PRESENTATION

### Chief complaints

A 47-year-old Caucasian woman was referred to our unit for an intragastric nodule detected during a gastroscopy.

### History of present illness

The gastroscopy was performed for long lasting epigastric pain and dyspepsia.

### History of past illness

Patient’s past medical history included: Asthma, hypothyroidism, migraine and a splenectomy for trauma.

### Personal and family history

No family histories were identified.

### Physical examination

The patient was in good general condition and slightly overweight (body mass index: 25.6). There were no abdominal mass and no pain on palpation.

### Laboratory examinations

Routine laboratory tests revealed no abnormalities.



### Imaging examinations

Endoscopy showed a 15 mm submucosal nodule bulging into the gastric lumen with smooth margins and macroscopically normal overlying mucosa. Biopsies were negative for malignancy and showed superficial chronic gastritis.

Consequently, a thoraco-abdominal computed tomography scan (Figure 1A and 1B) and an endoscopic ultrasound with a fine needle aspiration were planned. Those investigations found a roundish formation on the gastric fundus of about 30 mm in diameter with an exophytic development. The mass was in close contiguity with the left adrenal gland and the left pillar of the diaphragm with no signs of infiltration. The ultrasound appearance was of a solid mass with well-defined margins with a homogeneous and well vascularized internal texture in the absence of calcified or necrotic areas. The fine needle aspiration (FNA) was performed without complications, but the result was nondiagnostic due to inadequate tissue yield.

## FINAL DIAGNOSIS

Our main diagnostic suspect remained a gastric GIST and the symptoms could be related to the location of the mass. After a careful evaluation of the risks and benefits and according to the European Society for Medical Oncology guidelines<sup>[1]</sup>, the surgical excision was planned.

## TREATMENT

The laparoscopic resection was performed with a three trocars technique (10 mm supraumbilical and right hypochondrium and 5 mm left hypochondrium). After a careful lysis of the adhesions related to the previous splenectomy, the exophytic mass of the fundus was identified. The perigastric vessels were dissected in order to expose the nodule; the resection was performed with a linear stapler.

## OUTCOME AND FOLLOW-UP

The postoperative course was uneventful, and the patient was discharged on the seventh postoperative day. The final pathology of the specimen did not confirm our hypothesis but reported a rounded encapsulated accumulation of lymphoid tissue of 4 cm in diameter consistent with spleen parenchyma probably implanted during the previous splenectomy (Figure 2).

At the 6 mo follow-up the patient was symptom free.

## DISCUSSION

Ectopic splenic tissue can be found in the body as accessory spleens and splenosis<sup>[2]</sup>. The former is congenital and receives blood supply from the splenic artery. The latter is a benign condition caused by the spillage upon the peritoneal surface of cells from the spleen after splenic trauma or surgical procedures.

Splenosis is usually considered to be a rare phenomenon, but its real prevalence is difficult to define. Pearson *et al*<sup>[3]</sup> showed that recurrent splenic activity after urgent splenectomy is frequent, and according to Sikov *et al*<sup>[4]</sup>, its incidence could be as high as 76% in patients who had undergone splenectomy for trauma.

Splenosis is a benign condition, usually found incidentally and unless symptomatic surgery is not indicated<sup>[5]</sup>. In some cases the implantation could be responsible for serious conditions like gastrointestinal hemorrhage, pain from compression of the abdominal structures and bowel obstruction<sup>[6]</sup>. Splenosis may resemble several abdominal malignancies. As such several studies reported cases of splenosis mimicking a pancreatic mass<sup>[7]</sup>, lymphomas<sup>[8]</sup>, neuroendocrine tumors<sup>[9]</sup>, intramural colonic masses<sup>[10]</sup>, liver masses<sup>[11,12]</sup> and GISTs<sup>[13-16]</sup>. For this variability, the diagnosis of splenosis may be challenging. On a peripheral smear the absence of Howell-Jolly and Heinz bodies and siderocytes despite a history of splenectomy could mildly suggest the presence of a splenosis<sup>[17]</sup>. Imaging may not be accurate in defining this condition<sup>[18]</sup>. Differential diagnoses between benign<sup>[19-26]</sup> and malignant<sup>[27-32]</sup> forms and

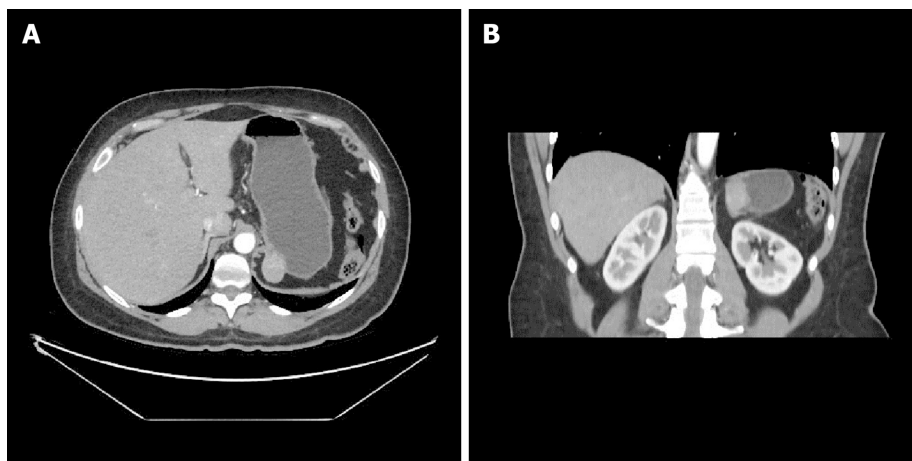


Figure 1 Preoperative abdominal computed tomography scan with intravenous contrast administration: A: Transverse; B: Coronal.

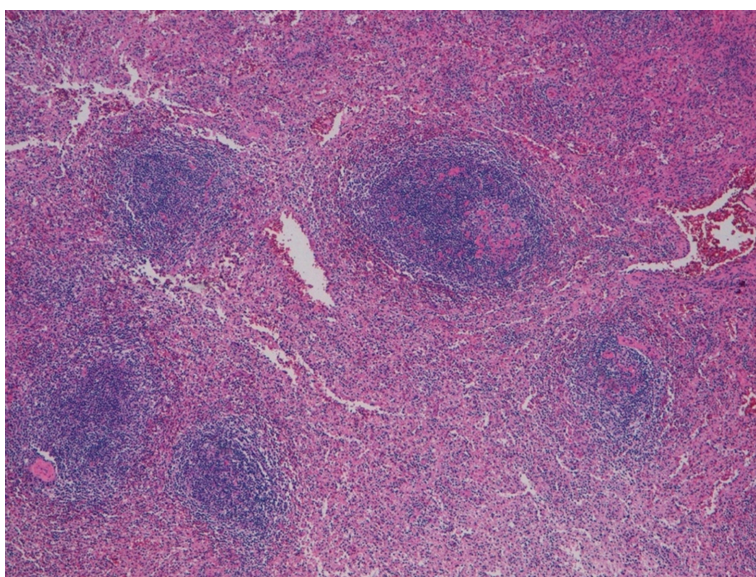


Figure 2 Lymphoid tissue found in the gastric nodule (hematoxylin and eosin staining,  $\times 4$ ).

the radiologic features of intramural gastric masses<sup>[33,34]</sup> are presented in the [Table 1](#).

Nowadays, there is a general consensus that the mainstay for the diagnosis of splenosis is the noninvasive scintigraphy using technetium-99m-labeled heat damaged red blood cell or indium 111-labeled platelets<sup>[35]</sup>. However, it must be highlighted that the real critical point in diagnosing splenosis is thinking about it in a suggestive past medical history.

During the assessment of a gastric intramural nodule, mass biopsy may help solving the diagnostic dilemma. However, in our case preoperative diagnosis was not possible, and the patient was submitted to surgery according to her symptoms and the most probable diagnosis.

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## CONCLUSION

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Splenosis is a rare condition that should always be considered as a possible diagnosis in patients who had undergone splenectomy. If feasible, a preoperative FNA may be the best preoperative investigation to rule out other diagnoses and to plan the most appropriate treatment.

**Table 1 Characteristics of intramural gastric masses**

	Location in the stomach	CT special features	Special features
<b>Benign lesions</b>			
Lipoma <sup>[19]</sup>	Antrum	Attenuation values -70 HU to -120 HU	Solitary, fibrous capsulated, soft (change in size and shape with peristalsis), no vessels
Leiomyoma <sup>[24]</sup>	Cardia	Low attenuation, endoluminal growth pattern	Negative for c-kit, positive for desmin and smooth muscle actin
Schwannoma <sup>[21]</sup>	Body	Minimal enhancement on the arterial phase	Absence of calcification, hemorrhage, necrosis; not encapsulated; positive for S-100
Glomus tumor <sup>[20]</sup>	Antrum	Strong enhancement on early-phase	Highly vascular; positive for calponin and smooth muscle actin
Inflammatory fibroid polyp <sup>[22]</sup>	Antrum	Enhancement on arterial phase	Positive for CD34 and vimentin
Hemangioma <sup>[25]</sup>	-	Strong enhancement on early-phase	Phleboliths are pathognomonic
Plexiform fibromyxoma <sup>[23]</sup>	Antrum	Myxoid tissue interspersed with vessels	Unique to the stomach, size from 2 cm to 15 cm
Ectopic pancreas <sup>[26]</sup>	Greater curvature	Similar to normal pancreas	-
Splenosis <sup>[5]</sup>	-	Enhancement on arterial phase	Splenectomized patients
<b>Malignant lesions</b>			
GIST <sup>[18]</sup>	Body	Smoothly circumscribed, bullseye sign	Positive for c-kit or dog-1; 50% greater than 2 cm
Non-GIST sarcoma (liposarcoma, leiomyosarcoma, unclassified sarcoma) <sup>[27]</sup>	-	Usually large, heterogeneous enhancement	Positive for desmin and smooth muscle actin, negative for c-kit
Lymphoma <sup>[33]</sup>	-	Wall thickening	Distant (more than close) and large adenopathy
Carcinoid <sup>[29]</sup>	-	Multiple small lesions	Reactive to synaptophysin and chromogranin A, hypergastrinemia related symptoms
Inflammatory myofibroblastic tumor <sup>[28]</sup>	-	Heterogeneously enhancing tumor (malignant appearance)	Borderline tumor, more frequent in young adults and children; reactivity for ALK
Metastasis <sup>[30-32]</sup>	-	-	"Homomorphic" endoscopic features; dyschromic lesions

CT: Computed tomography; GIST: Gastrointestinal stromal tumor; ALK: Anaplastic lymphoma kinase.

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