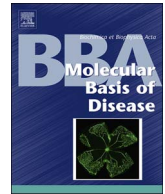




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BBA - Molecular Basis of Disease

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Review

MicroRNAs and extracellular vesicles in cholangiopathies[☆]P. Olaizola^{a,1}, P.Y. Lee-Law^{a,b,c,1}, A. Arbelaiz^a, A. Lapitz^a, M.J. Perugorria^{a,b,d}, L. Bujanda^{a,b,d}, J.M. Banales^{a,b,d,*}^a Department of Liver and Gastrointestinal Diseases, Biodonostia Health Research Institute – Donostia University Hospital, University of the Basque Country (UPV/EHU), San Sebastian, Spain^b National Institute for the Study of Liver and Gastrointestinal Diseases (CIBERehd, “Instituto de Salud Carlos III”), Spain^c Department of Gastroenterology and Hepatology, Radboud University Medical Centre, Nijmegen, Netherlands^d IKERBASQUE, Basque Foundation for Science, Bilbao, Spain

ARTICLE INFO

Keywords:

microRNAs
Extracellular vesicles
Cholangiopathies
Diagnosis
Pathogenesis
Therapy

ABSTRACT

Cholangiopathies encompass a heterogeneous group of disorders affecting biliary epithelial cells (i.e. cholangiocytes). Early diagnosis, prognosis and treatment still remain clinically challenging for most of these diseases and are critical for adequate patient care. In the past decade, extensive research has emphasized microRNAs (miRs) as potential non-invasive biomarkers and tools to accurately identify, predict and treat cholangiopathies. MiRs can be released extracellularly conjugated with lipoproteins or encapsulated in extracellular vesicles (EVs). Research on EVs is also gaining attention since they are present in multiple biological fluids and may represent a relevant source of novel non-invasive biomarkers and be vehicles for new therapeutic approaches. This review highlights the most promising candidate miRs and EV-related biomarkers in cholangiopathies, as well as their relevant roles in biliary pathophysiology. This article is part of a Special Issue entitled: Cholangiocytes in Health and Disease edited by Jesus Banales, Marco Marzoni, Nicholas LaRusso and Peter Jansen.

Research strategy: PubMed search (April 2017) was done with the following terms: “microRNA”, “miRNA”, “miR”, “extracellular vesicles”, “EV”, “exosomes”, “primary biliary cholangitis”, “primary biliary cholangitis”, “PBC”, “primary sclerosing cholangitis”, “PSC”, “cholangiocarcinoma”, “CCA”, “biliary atresia”, “BA”, “polycystic liver diseases”, “PLD”, “cholangiopathies”, “cholestatic liver disease”. Most significant articles in full-text English were selected. The reference lists of selected papers were also considered.

Abbreviations: 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; ADPKD, autosomal dominant polycystic kidney disease; ADPLD, autosomal dominant polycystic liver disease; AE2/SLC4A2, Cl[−]/HCO₃[−] anion exchanger 2; ALP, alkaline phosphatase; AMA, anti-mitochondrial antibody; ARPKD, autosomal recessive polycystic kidney disease; Ars2, arsenic resistance protein 2; ASGPR1, asialoglycoprotein receptor 1; AUC, area under the curve; BA, biliary atresia; CA19-9, cancer antigen 19-9; CCA, cholangiocarcinoma; CCL2, C-C motif chemokine ligand 2; Cdc25A, cell division cycle 25A; CDH6, cadherin-6; CDK6, cyclin-dependent kinase 6; CHEK2, checkpoint kinase 2; c-Myc, myc proto-oncogene protein; CXCL1, chemokine (C-X-C motif) ligand 1; eCCA, extrahepatic cholangiocarcinoma; EGFR, epidermal growth factor receptor; ELK1, ETS domain-containing protein Elk-1; EMT, epithelial mesenchymal transition; ERK, extracellular signal-regulated kinases; EV, extracellular vesicle; FDA, food and drug administration; FIBG, fibrinogen gamma chain; FOXA1, forkhead box protein A1; FOXO1, forkhead box protein O1; FXR, farnesoid X receptor; GSK3β, glycogen synthase kinase-3 beta; HCC, hepatocellular carcinoma; HDAC4, histone deacetylase 4; HSC, hepatic stellate cell; iCCA, intrahepatic cholangiocarcinoma; IGF1R, insulin-like growth factor-1 receptor; IL, interleukin; IL1β, interleukin-1 beta; InsP3R3, type III inositol 1,4,5-triphosphate receptor; ITGB4, integrin beta-4; MAPK, mitogen activated protein kinase; MAP3K8, mitogen-activated protein kinase kinase kinase 8; MBD2, methyl-CpG-binding domain protein 2; Mcl-1, induced myeloid leukemia cell differentiation protein Mcl-1; MDR1, multidrug resistant protein 1; miR, microRNA; MMP, matrix metalloproteinase; MSC, mesenchymal stem cell; mTOR, mechanistic target of rapamycin; MVB, multivesicular body; NCAM1, neural cell adhesion molecule 1; NDRG2, N-myc downstream-regulated gene 2; NUA1, (nua) family kinase 1; PBMC, peripheral blood mononuclear cells; PBC, primary biliary cholangitis; pCCA, perihilar cholangiocarcinoma; PDC-E2, pyruvate dehydrogenase complex-E2; PDCD4, programmed cell death protein 4; Per1, period circadian protein homolog 1; PLD, polycystic liver disease; PSC, primary sclerosing cholangitis; PSMD10, proteasome 26S subunit non-ATPase 10; PTEN, phosphatase and tensin homolog; PTPN14, tyrosine-protein phosphatase non-receptor type 14; RB, retinoblastoma protein; RECK, reversion-inducing cysteine-rich protein with Kazal motifs; RhoC, ras homolog family member C; ROC, receiver operating characteristic; Smad4, small mothers against decapentaplegic homolog 4; SULT2A1, sulphotransferase 2A1; TET1, ten-eleven translocation 1; TGFβR2, transforming growth factor beta receptor 2; TIMP3, metalloproteinase inhibitor 3; TNFα, tumor necrosis factor alpha; TRAIL, TNF-related apoptosis-inducing ligand; UDCA, ursodeoxycholic acid; VEGF, vascular endothelial growth factor; XIAP, X-linked inhibitor of apoptosis protein

[☆] This article is part of a Special Issue entitled: Cholangiocytes in Health and Disease edited by Jesus Banales, Marco Marzoni, Nicholas LaRusso and Peter Jansen.

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<http://dx.doi.org/10.1016/j.bbadis.2017.06.026>

Received 16 May 2017; Received in revised form 27 June 2017; Accepted 28 June 2017

Available online 13 July 2017

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1. Introduction

Bile duct epithelial cells (*i.e.* cholangiocytes) are important in health and disease. They represent a small proportion (3–5%) of the total liver cell population but, nonetheless, play essential roles for normal liver function including the alkalization and fluidization of the primary bile produced by hepatocytes. Biliary diseases, also termed as cholangiopathies, encompass a wide spectrum of etiologies comprising genetic, infectious, immune-mediated, drug-induced, vascular, neoplastic or idiopathic. Although their pathogenesis still remains obscure, chronic inflammation and cholestasis seem to be common events that exacerbate the wound-healing response leading to the development of liver fibrosis and cirrhosis. Most cholangiopathies lack valid diagnostic and/or prognostic biomarkers, as well as adequate targets for therapy, demanding the need for further research. In the last years, the discovery of microRNAs (miRs) has represented a revolution and a paradigm shift (Fig. 1), postulating them as promising biomarkers and targets/tools for therapy. These small (18–23 nucleotides) endogenous non-coding RNAs play significant roles in most physiological and pathological cellular events including proliferation, differentiation, migration, senescence and survival, by regulating post-transcriptional gene expression [1,2]. Cholangiopathies display aberrant miR signatures in cholangiocytes, immune cells, liver tissue and biological fluids among others, evidencing their potential value in diagnosis, prognosis and therapy [3–5]. MiRs can be released into the extracellular medium associated with lipoproteins or encapsulated in extracellular vesicles (EVs), thus participating in intercellular communication (Fig. 2) [6]. EVs are small lipid-enclosed spheres secreted by many cell types and found in multiple biological fluids including bile, blood and urine. There are different types of EVs according to their origin, size, molecular composition and biological function. Regarding their origin, EVs are classified into exosomes, plasma membrane-derived vesicles and apoptotic bodies. Besides miRs, they can also contain other nucleic acids, lipids and proteins. To date, the most studied EVs are exosomes, which are released extracellularly upon exocytic fusion of endosome-derived multivesicular bodies (MVBs) with the plasma membrane of the cells. Then,

the exosomal content can be further delivered into recipient cells where it can regulate gene expression and cellular functions. Changes in the transcriptomic and proteomic EV content have been reported in different cholangiopathies, pointing out their potential value as non-invasive biomarkers (Fig. 1) [7].

This review provides current knowledge on the role of miRs and EVs in the pathogenesis of biliary diseases, and their potential therapeutic value. Moreover, the most promising miRs and EV-related biomarkers as new non-invasive diagnostic and prognostic tools, and their therapeutic value will be discussed. Finally, future directions on basic and clinical investigations will be highlighted.

2. MicroRNAs in cholangiopathies

2.1. Fibro-inflammatory cholangiopathies

Primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC) and biliary atresia (BA) are cholangiopathies characterized by chronic inflammation, cholestasis and biliary fibrosis. Along the disease progression, cirrhosis, portal hypertension and, ultimately, liver failure may arise in these three disorders.

2.1.1. Primary biliary cholangitis (PBC)

PBC is a chronic cholestatic liver disease of unknown etiology linked to autoimmune processes targeting small and medium intrahepatic bile ducts. Without treatment, PBC may progress to liver fibrosis, cirrhosis and, ultimately, liver failure [8]. PBC mainly affects middle-aged women (~90%) and has been associated with environmental toxins, infectious agents and certain genetic factors [9]. Diagnosis is based on clinical and serological parameters such as elevated levels of alkaline phosphatase (ALP) and the presence of specific anti-mitochondrial antibodies (AMAs) against pyruvate dehydrogenase complex-E2 (PDC-E2) in up to 95% of patients [10,11]. Moreover, a high proportion of PBC patients also presents serum anti-nuclear antibodies (ANAs) [12]. However, individuals without these serological features require a liver biopsy to determine the diagnosis. The choleretic bile acid

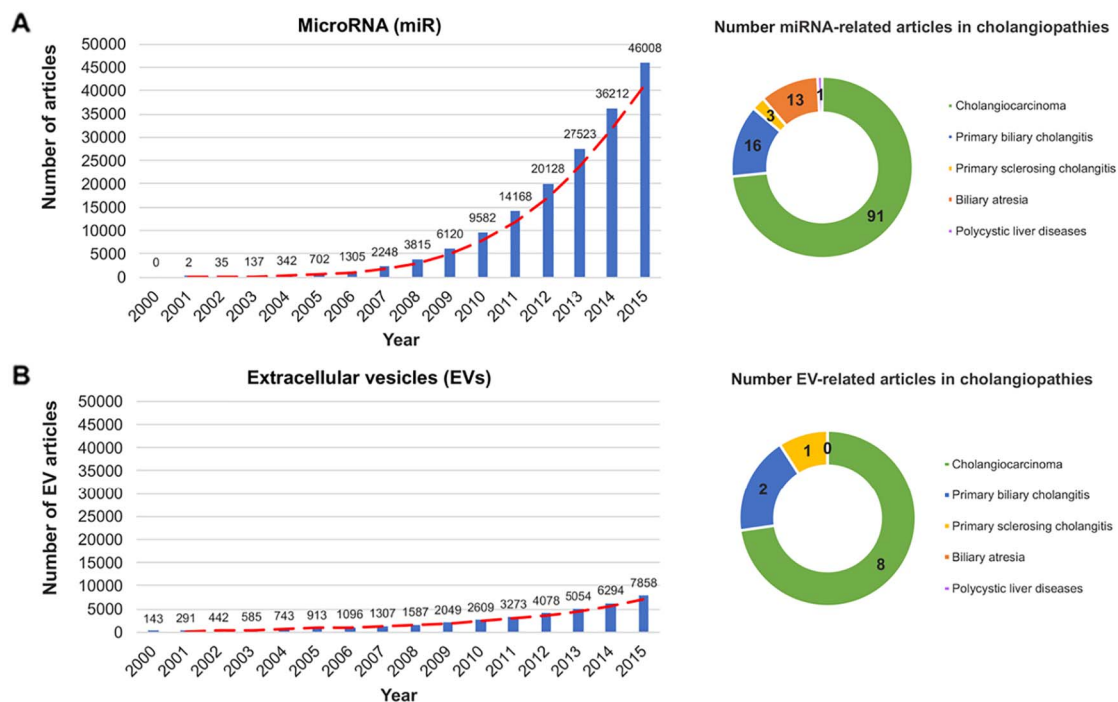


Fig. 1. Trends of miR- and EV-related articles since 2000. A) Exponential growth in the number of miR-related articles (*upper left panel*) and total number of articles related to each type of cholangiopathy (*upper right panel*). B) Exponential growth in the number of EV-related articles (*lower left panel*) and total number of articles related to each cholangiopathy (*lower right panel*). Red lines indicates the trends.

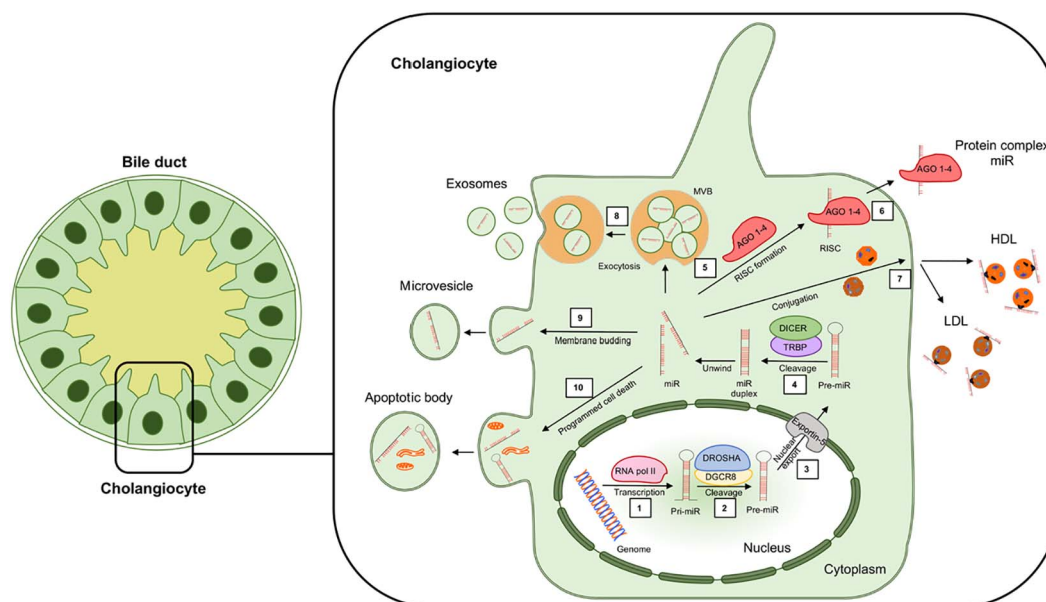


Fig. 2. Biogenesis and release of miRNAs. Primary miRNAs (Pri-miRNAs) are synthesized by RNA pol II or III (1). This initial RNA form, with 5' cap and poly-A tail, is then cleaved by the microprocessor complex formed by DROSHA/DGCR8 generating the hairpin-shaped Pre-miR (Pre-miRNAs) (2). The Pre-miRNAs are then exported to the cytoplasm via Exportin-5 (3), where they are processed by DICER/TRBP into a double-stranded mature miR (4). One strand of the miR duplex can enter the RISC assembling pathway (5) or be released by the cell, while the complementary strand is often degraded. In order to be released into the extracellular space, the mature miR strand can associate to AGO 1–4 proteins (6) or to lipoproteins (HDL, LDL) (7). On the other hand, miRNAs can be loaded into exosomes that are extracellularly released upon exocytic fusion of MVB with the plasma membrane (8) or into microvesicles formed by the blebbing of the cellular plasma membrane (9). Additionally, miRNAs can be discharged in apoptotic bodies along with other cellular-derived material (10).

RNA pol II: RNA polymerase II; Pre-miR: precursor miRNA; RISC: RNA-induced silencing complex; AGO: Argonaute protein; HDL: high density lipoprotein; LDL: low density lipoprotein; MVB: multivesicular body.

ursodeoxycholic acid (UDCA) is the mainstay treatment, which is administered daily and chronically. Nonetheless, depending on age, up to 50% of PBC patients lack an adequate response to UDCA treatment and have lower long-term survival than the general population [13,14]. For those UDCA non-responders, the farnesoid X receptor (FXR) agonist obeticholic acid (OCA) has recently been approved by the food and drug administration (FDA) as monotherapy or combination with UDCA. OCA improves some markers of cholestasis but also induces side effects like pruritus [15]. Therefore, it is fundamental to identify accurate biomarkers for specific and early diagnosis, prognosis and response to therapy, as well as new therapeutic targets for individualized patient care in PBC.

Several studies have investigated the expression of miRNAs in serum [16,17] and liver tissue [18], as well as in specific cell types including peripheral blood mononuclear cells (PBMCs) [19,20] and biliary epithelial cells [21] from PBC patients. The serum from PBC patients was characterized by an altered miR profile [17,22], with upregulation of both miR-122-5p and miR-141-3p, and downregulation of miR-26b-5p that conferred a higher diagnostic value than the serum levels of ALP and ANAs (Table 1) [16]. In addition, deep sequencing analysis of serum samples from PBC patients identified miR-139-5p to be downregulated in patients with advanced PBC vs healthy controls and tended to be lower in advanced PBC patients vs early PBC patients [23], suggesting its potential value to predict the disease progression. The role of miRNAs as biomarkers to predict the response to treatment in PBC was highlighted by the fact that overexpression of miR-299-5p in serum was associated with non-response to UDCA treatment compared to both PBC responders and healthy controls [20]. On the other hand, alterations in the miR expression profile were also reported in PBMCs from PBC patients [22]. In particular, downregulation of miR-181a, miR-181b, miR-374b, and miR-425 was found in CD4⁺ T cells from PBC patients compared to healthy controls. Of note, the downregulation of miR-425 in CD4⁺ T cells induced inflammatory cytokines production [24].

Cholestasis is one of the main hallmarks of PBC and is caused, partially, by downregulation of both the Cl⁻/HCO₃⁻ anion exchanger 2

(AE2/SLC4A2) and the type III inositol 1,4,5-triphosphate receptor (InsP3R3) in cholangiocytes [25–29]. Remarkably, the etiopathogenic role of AE2 in PBC was also highlighted by the fact that *Ae2*^{-/-} mice spontaneously developed different PBC-like features, including serum specific AMAs [30,31]. One of the triggering causes for both AE2 and InsP3R3 downregulation in PBC cholangiocytes is miR-506, which was found overexpressed in these cells and directly targeted both mRNAs leading to cholestasis [21,32]. Different pro-inflammatory cytokines found overexpressed in PBC livers such as interleukins (ILs) 8, 12, 17, 18 and tumor necrosis factor alpha (TNFα) enhanced the transcriptional activity of *miR-506* gene promoter in cholangiocytes, subsequently leading to altered expression of proteins involved in several biological processes, particularly in mitochondrial energy metabolism [33]. MiR-506 induced PBC-like features in cholangiocytes including cell dedifferentiation, stress, susceptibility to bile-salt induced apoptosis, dysregulation of mitochondrial metabolism and PDC-E2 overexpression, ultimately promoting the activation and proliferation of PBMCs from PBC patients when co-cultured [33]. These data point out the relevant role of miR-506 in the etiopathogenesis of PBC and its potential therapeutic regulatory value.

2.1.2. Primary sclerosing cholangitis (PSC)

PSC is a chronic cholestatic liver disease affecting both intra- and extrahepatic large bile ducts [34,35]. Most patients are middle-aged men (~60%) and up to 80% also present inflammatory bowel disease, most commonly ulcerative colitis [8,34,35]. Importantly, PSC patients have a 400-fold increased risk for developing cholangiocarcinoma (CCA) compared to the general population [36]. Similar to PBC, hereditary and environmental elements are associated with PSC, but the etiopathogenesis remains unclear. There is no effective medical treatment to alter the disease course and liver transplantation is the only curative option [34,35,37]. In PSC, studies on the pathogenesis and role of miRNAs as biomarkers or tools for therapy are still limited.

A report from 2016 pointed out the relevance of serum miRNAs as potential biomarkers for the diagnosis of PSC and CCA (Table 1) [38].

Table 1

MiRs as diagnostic biomarkers for cholangiopathies.

AUC, area under the curve; BA, biliary atresia; CA19-9, cancer antigen 19-9; CCA, cholangiocarcinoma; iCCA, intrahepatic cholangiocarcinoma; MiR, microRNA; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; SEN, sensitivity; SPE, specificity.

Disease	MiR	Expression	Source	Number of patients	SEN (%)	SPE (%)	AUC	Reference
PBC	122-5p + 141-3p + 26b-5p	Up (122-5p + 141-3p) Down (26-5p)	Serum	PBC (n = 82) vs healthy controls (n = 60)	80.5	88.3	0.905	[16]
PSC	200c	Down	Serum	PSC (n = 40) vs healthy controls (n = 40)	–	–	0.740	[38]
BA	200a	Up	Serum	BA (n = 24) vs other forms of neonatal hyperbilirubinemia (n = 24)	83.3	83.3	0.862	[44]
	200b	Up	Serum		79.2	79.2	0.807	
	429	Up	Serum		70.8	91.7	0.806	
	140-3p	Down	Plasma	BA (n = 44) vs cholestatic disease controls (n = 20) and healthy controls (n = 20)	66.7	79.1	0.750	[46]
	4429	Down	Serum	BA (n = 35) vs non-BA neonatal cholestasis (n = 20)	83.3	80.0	0.789	[45]
	4689	Up	Serum		66.7	80.0	0.722	
CCA	9	Up	Bile	CCA (n = 7) and gallbladder cancer (n = 2) vs choledocholithiasis patients (n = 9)	88.9	100.0	0.975	[67]
	145	Up	Bile		77.8	100.0	0.975	
	942	Up	Bile		77.8	100.0	0.765	
	302c	Up	Bile		88.9	100.0	–	
	199a-3p	Up	Bile		88.9	100.0	–	
	222	Up	Bile		88.9	100.0	–	
	105	Up	Bile		77.8	100.0	–	
	21	Up	Serum	iCCA (n = 74) vs healthy controls (n = 74)	87.8	90.5	0.908	[68]
	21	Up	Plasma	iCCA (n = 25) vs healthy controls (n = 7)	–	–	0.940	[71]
	26a ^a	Up	Serum	CCA (n = 66) vs healthy controls (n = 66)	84.8	81.8	0.899	[73]
	106a ^a	Down	Serum	CCA (n = 103) vs healthy control (n = 20)	81.6	85.0	0.890	[76]
	150	Up	Plasma	iCCA (n = 15) vs healthy controls (n = 15)	80.6	58.1	0.791	[75]
	192	Up	Serum	<i>O. viverrini</i> CCA (n = 10) vs healthy controls (n = 32)	74.0	72.0	0.809	[79]
	21 + 192	Up	Urine	<i>O. viverrini</i> CCA (n = 22) vs healthy controls (n = 21)	81.8	71.4	0.849	[80]
	483-5p + 194	Up	Serum	CCA (n = 40) vs healthy controls (n = 40)	–	–	0.810	[38]
	483-5p + 222	Up	Serum	CCA (n = 40) vs PSC (n = 40)	–	–	0.770	
	1281	Down	Serum	CCA (n = 31) vs PSC (n = 40)	55.0	90.0	0.830	[81]
	126	Down	Serum		68.0	93.0	0.870	
	26a	Down	Serum		52.0	93.0	0.780	
	30b	Down	Serum		52.0	88.0	0.780	
	122	Down	Serum		32.0	90.0	0.650	
	412	Up	Bile	PSC/CCA (n = 12) vs PSC (n = 52)	50.0	89.0	0.810	[81]
	640	Up	Bile		50.0	92.0	0.810	
	3189	Up	Bile		67.0	89.0	0.800	
	1537	Up	Bile		67.0	90.0	0.780	
	1537 + CA19-9	Up	Bile		73.0	93.0	0.910	

^a Also prognostic biomarker.

In a discovery phase, 21 miRs were found differentially expressed in PSC, 33 in CCA and 26 in both groups compared to healthy controls, as well as 24 miRs in PSC vs CCA with area under the receiver operating characteristic (ROC) curve (AUC) > 0.700 [38]. After a validation phase in a second cohort of patients, miR-200c was confirmed to be downregulated in PSC vs healthy controls, whereas increased levels of both miR-483-5p and miR-194 were found in CCA vs healthy controls as well as both miR-483-5p and miR-222 in CCA vs PSC [38]. Combination of these particular miRs further improved the specificity and accuracy of diagnosis.

In terms of liver pathophysiology, and particularly in cholestasis, pregnane X receptor (PXR) induced the expression of *sulphotransferase 2A1* (*SULT2A1*) to convert lithocholic acid into a less toxic form and, thus, prevented liver injury. However, PSC patients are characterized by disease-specific impairment of *SULT2A1* expression following PXR activation. In PSC, *SULT2A1* expression might be regulated by miR-378a-5p, which was found overexpressed in PSC vs PBC livers and was predicted by bioinformatics tools to target *SULT2A1* gene expression [39]. On the other hand, it was reported that miR-21 promotes biliary hyperplasia [40] and miR-7a enhances cholangiocyte proliferation [41] in animal models of cholestasis and sclerosing cholestasis, respectively, suggesting a potential role of these miRs in the pathogenesis of PSC. However, the role of these or other miRs in the PSC etiopathogenesis remains still unknown.

2.1.3. Biliary atresia (BA)

BA is a progressive and destructive cholangiopathy affecting the

intra- and extrahepatic bile ducts of neonates and that causes severe cholestasis [42]. The etiopathogenesis of this disease remains still obscure but several genetic and environmental factors have been postulated to participate in its development [43]. Without treatment, BA patients seldom survive more than 2 years [42]. To date, the only effective therapy is the Kasai portoenterostomy, which restores the bile flow. However, early diagnosis and intervention are crucial for the prognosis if this disease. BA is diagnosed by operative cholangiography and/or liver biopsy, which are invasive and time consuming, highlighting the urgent need for non-invasive alternatives.

Serum of BA patients may contain promising miR biomarkers with high sensitivity and specificity for the diagnosis (Table 1). Indeed, up-regulation of miR-200a, miR-200b and miR-429 (miR-200 cluster) was reported in BA patients compared to other forms of neonatal hyperbilirubinemia (NH) [44]. On the other hand, miR-4429 downregulation and miR-4689 upregulation were also reported in another study with potential diagnostic value [45]. Additionally, by using next-generation sequencing, miR-140-3p was found downregulated in plasma of BA patients compared to cholestatic disease patients and healthy controls, showing diagnostic potential [46]. Interestingly, the same study tried to identify previously reported dysregulated miRs (i.e. miR-200 family [44], miR-21 [47,48], miR-29a [48,49], miR-222 [47,50,51]) implicated in human and experimental BA, however only upregulation of miR-200 family was validated [46].

Different dysregulated miRs in BA are believed to be associated with pro-inflammatory and pro-fibrotic processes (Table 2) [22]. MiR-19b, which was found downregulated in liver tissue of BA patients [52],

Table 2

MiRs involved in non-tumor cholangiopathies.

AE2, Cl[−]/HCO₃[−] anion exchanger 2; BA, biliary atresia; Cdc25a, cell division cycle 25A; FOG2, friend of Gata 2; FOXA2, forkhead box protein A2; IGF1: insulin-like growth factor 1; IGF1R, insulin-like growth factor 1 receptor; IL6R, interleukin 6 receptor; InsP3R3, type III inositol 1,4,5-triphosphate receptor; Ngn-3, neurogenin-3; N-Ras, neuroblastoma RAS viral oncogene homolog; PBC, primary biliary cholangitis; PLD, polycystic liver disease; PPP2R2A, protein phosphatase 2 regulatory subunit B alpha; PSC, primary sclerosing cholangitis; PTEN, phosphatase and tensin homolog; Smad, small mothers against decapentaplegic; STAT3, signal transducer and activator of transcription 3; TGFβ, transforming growth factor beta; TNFα, tumor necrosis factor alpha.

Disease	MiR	Expression	Target	Function	Sample	Reference
PBC	506	Up	AE2, InsP3R3	Secretion	Cell lines, tissue	[21,33]
	139-5p	Down	c-FOS, TNF-α	Inflammation	Tissue, serum	[23]
	425	Down	N-Ras	Inflammation	Serum	[24]
PSC	7a	Up	Ngn-3	Proliferation	Animal model	[41]
	21	Up	Smad	Fibrosis, proliferation	Tissue, animal model	[40]
BA	21	Up	PTEN	Fibrosis	Tissue	[47]
	29a	Up	IGF-1, IGF-1R	Cell death, inflammation	Animal model	[49]
	200a, 200b, 200c	Up	FOXA2	Inflammation, proliferation	Tissue, animal model	[44]
	200b	Up	FOG2	Proliferation, migration, fibrosis	Tissue	[53]
	222	Up	PPP2R2A	Fibrosis	Tissue, animal model	[50,51]
	19b	Down	TGFβ	Fibrosis	Tissue	[52]
	124	Down	STAT3, IL-6R	Inflammation, proliferation	Tissue, animal model	[54]
PLD	15a	Down	Cdc25a	Proliferation	Cell lines, tissue	[59]

directly targeted transforming growth factor beta receptor 2 (TGFβR2) gene expression and is believed to indirectly induce downstream TGFβ signaling, which is involved in hepatic stellate cells (HSCs) activation [52]. Additionally, increased miR-222, miR-200b and miR-21 in liver tissue of BA patients may also stimulate HSCs activation promoting fibrosis [47,50,53]. On the other hand, the pro-inflammatory cytokine IL6, upregulated in liver of BA patients, has been reported to enhance cholangiocyte proliferation through miR-124 and miR-200 family [54].

2.2. Polycystic liver diseases (PLD)

PLD comprise a heterogeneous group of congenital cholangiopathies inherited in dominant [*i.e.* autosomal dominant polycystic liver disease (ADPLD) or autosomal dominant polycystic kidney disease (ADPKD)] or recessive form [*i.e.* autosomal recessive polycystic kidney disease (ARPKD)] and characterized by progressive development of multiple fluid-filled biliary cysts (> 10), which are the main cause of morbidity [55–57]. PLD can be found isolated (*e.g.* ADPLD) or associated with renal cystogenesis (*e.g.* ADPKD and ARPKD). Current surgical and/or pharmacological treatments do not improve the prognosis of these diseases, and liver transplantation remains the only curative option. Hepatic cystogenesis in PLD is characterized by functional alterations in bile duct epithelial cells [58] that include miRs dysregulation [59,60]. To date, there is only a previous report highlighting the important role of miRs in PLD pathophysiology [59,60]. In this study, an abnormal miR expression profile was found in cholangiocytes isolated from an animal model of ARPKD (*i.e.* the PCK rat), which has the same human orthologous gene mutated (*i.e.* *PKHD1*). Of note, most of the dysregulated miRs were found downregulated in PCK cholangiocytes [59]. Of particular interest is miR-15a, which was found highly downregulated in both rat and human cystic cholangiocytes [59]. MiR-15a directly targets cell division cycle 25A (Cdc25a) promoting cystic cholangiocyte cell proliferation [59]. Interestingly, experimental targeting of miR-15a with specific anti-sense oligonucleotides inhibited cystic cholangiocytes growth [59].

2.3. Cholangiocarcinoma (CCA)

CCA includes a heterogeneous group of malignancies with biliary differentiation features that may arise from different liver cell types including mature cholangiocytes. CCA is the second most frequent liver tumor accounting for 10–20% of all primary liver neoplasms [61,62]. Attending to their anatomical location, CCAs are classified as intrahepatic (iCCA), perihilar (pCCA) and extrahepatic (eCCA) [63]. Epidemiological studies indicate that CCA worldwide incidence has

been rising in the last decades [63,64] ranging from 0.30 to 8.75 per 100.000 individuals depending on the geographical area [61,63,64]. Several risk factors, including PSC, cirrhosis, viral hepatitis B and C, hepatolithiasis, congenital biliary malformations as well as the hepatobiliary flukes endemic in East Asia (*i.e.* *Opisthorchis viverrini* and *Clonorchis sinensis*), have been identified for CCA [64]. However, the etiology of most CCAs still remains unknown [63,64]. Since CCAs are often asymptomatic in early stages, diagnosis is usually conducted when the disease is advanced and widespread [63,64]. The current diagnostic strategy comprises a combination of imaging methods, non-specific tumor biomarkers in serum [*i.e.* carbohydrate antigen 19-9 (CA19-9)] and histological analyses of tumor biopsies. Late diagnosis compromises the potential curative options, which are mainly based on surgery, leading to poor prognosis [63,64]. Furthermore, the responsiveness of CCA to current chemotherapies is very limited [63,64]. Therefore, early detection of these tumors is crucial for those patients with risk factors and for those that present recurrence after surgery. During the last decade, a significant number of research articles have been published on the role of miRs in CCA (Fig. 1A) pointing out their relevant value as non-invasive biomarkers (Table 1) and potential targets for therapy.

2.3.1. MicroRNAs as biomarkers

To strengthen the diagnosis of CCA and monitor tumor progression, elevated CA19-9 levels in serum are commonly used as a complementary approach to imaging methods. However, the sensitivity and specificity of this non-invasive biomarker is modest, particularly in early stages of the disease [65,66].

Increasing evidence points out the relevance of miRs as biomarkers for CCA. Indeed, several dysregulated miRs have been described in serum, plasma, urine or bile from CCA patients, showing high AUC values for diagnosis (Table 1). High-throughput real-time PCR-based assays performed in human bile samples showed that miR-9, miR-105, miR-145, miR-199-3p, miR-222, miR-302c and miR-942 levels were higher in both CCA and gallbladder cancer compared to patients with choledocholithiasis [67] and acknowledged miR-9 as a reliable diagnostic indicator for biliary tract cancer [67]. In addition, different studies revealed miR-21 as a potential biomarker candidate for the diagnosis of iCCA [68,69], as it was found overexpressed in both serum and plasma from these patients compared to healthy individuals [70,71]. Clinical stage and tumor differentiation degree were reported to correlate with the levels of miR-21 in CCA tissue, and high miR-21 expression has been linked to poor overall survival, evidencing its promising role as a prognostic biomarker [70,72]. Similarly, levels of miR-26a were shown to be increased in CCA tissues, cell lines and

serum from CCA patients compared to healthy controls [73,74]. Furthermore, miR-150 levels were found upregulated in plasma from iCCA patients [75] and its combination with CA19-9 improved the individual diagnostic capacity of both biomarkers [75]. In contrast, the expression of serum circulating miR-106a was reported downregulated in CCA compared to healthy controls, but its diagnostic value was lower than CA19-9 [76]. As previously mentioned for PSC-CCA, serum miR-483-5p and miR-194 were found upregulated in CCA patients compared to control individuals and their combination improved the diagnostic potential of each miR [38]. Remarkably, increased serum levels of miR-222 and miR-483-5p were identified in CCA vs PSC patients [38]. A different study provided a panel of 5 serum miRs (miR-1281, miR-126, miR-26a, miR-30b and miR-122) able to discriminate between PSC and CCA patients [77]. Furthermore, a distinct miR expression pattern was identified in bile from PSC-CCA vs PSC patients (miR-412, miR-640, miR-1537 and miR-3189) [77]. Interestingly, combination of miR-1537 and CA19-9 levels displayed higher diagnostic capacity than CA19-9 alone [77].

MiRs have also been postulated as prognostic biomarkers for CCA. Downregulation of miR-106a in serum from CCA patients was associated with higher risk of lymph node metastasis and inversely correlated with overall survival [76]. Likewise, downregulation of miR-150-5p in plasma, bile and tumor tissue from CCA patients negatively correlated with CA19-9 levels and tumor grade [78]. On the other hand, increased serum levels of miR-192 were associated with metastasis and poor overall survival in liver fluke-associated CCA patients compared to healthy controls [79]. Of note, increased miR-192 and miR-21 levels were found in urine from liver fluke-associated CCA patients and their combination improved the diagnostic power of these two miRs alone [80].

The overall diagnostic capacity of miRs in CCA was emphasized in two independent meta-analysis, where the pooled of 11 miRs from 8 independent studies displayed AUC values of 0.900 and 0.880, respectively [81,82]. miRs in bile and serum showed higher diagnostic value (i.e. 0.957 and 0.957, respectively) than tissue (0.847) and urine (0.745) [81].

2.3.2. MicroRNAs in CCA pathology

Aberrantly expressed miRs in CCA tumor cells have been described to participate in pathological processes including cell proliferation, differentiation, survival, invasion/migration, epithelial mesenchymal transition (EMT), epigenetics and chemoresistance (Fig. 3) [22]. These miRs can function as oncogenes or tumor suppressors (Table 3).

2.3.3. Onco-microRNAs

Several upregulated miRs in CCA cells function as onco-miRs promoting tumor growth. For instance, miR-21 stands out as a pivotal regulator of several pathophysiological processes such as cell proliferation, survival, EMT, invasiveness/metastasis and chemoresistance via direct targeting of different tumor suppressors including programmed cell death protein 4 (PDCD4), metalloprotease inhibitor 3 (TIMP3), reversion-inducing cysteine-rich protein with Kazal motifs (RECK), tyrosine-protein phosphatase non-receptor type 14 (PTPN14), 15-hydroxyprostaglandin dehydrogenase (15-PGDH), phosphatase and tensin homolog (PTEN) and phosphatidylinositol 3-kinase (PI3K) [68,70,83–86], among others [87]. In addition, arsenic resistance protein 2 (Ars2), involved in miR biogenesis, was found overexpressed in CCA tissue and cell lines leading to increased miR-21 expression, which in turn inhibited its downstream targets further contributing to CCA cell proliferation and oncogenesis [69].

The mRNA of different genes involved in the blockade of cell cycle such as p15, p21 and Cyclin E1 has been reported to be directly targeted by the oncogenic miR-224, leading to enhanced cell cycle progression and tumor growth [88]. Another miR with a potential role in cell cycle progression is miR-34a, which was found to directly inhibit period circadian protein homolog 1 (Per1) expression and its interaction with

checkpoint kinase 2 (CHEK2), preventing cell cycle arrest [89]. Emerging data have demonstrated that miR-191 directly targets ten-eleven translocation 1 (TET1), which in turn demethylates and thereby represses p53 expression in CCA cells. Hence, upon miR-191 upregulation, the tumor suppressor activity of p53 is inhibited and leads to cell survival and proliferation [90]. Furthermore, miR-429 expression was reported to be increased in CCA, correlating with the hypomethylated status of its promoter [91]. Functional *in vitro* studies showed that miR-429 directly targeted the tumor suppressor cadherin-6 (CDH6) inducing tumor cell growth [91]. Likewise, increased cellular proliferation and apoptosis evasion were shown in iCCA upon miR-31 upregulation through RAS p21 GTPase activating protein 1 (RAS1) direct inhibition [92]. RAS1 suppression led to increased levels of the active RAS form (GTP-bound) as well as to increased ERK1/2 phosphorylation further activating the MAPK signaling pathway and ultimately enhancing tumor growth [92]. MiR-421 was identified as another onco-miR in CCA that promoted tumor cell proliferation, migration and colony forming *via* FXR inhibition [93]. CCA cell survival was associated with miR-25, which protected tumor cells against TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis *via* direct targeting of TRAIL Death Receptor-4 (DR4) [94].

The expression of the tumor suppressor N-myc downstream-regulated gene 2 (NDRG2) was found to be repressed by miR-181c, resulting in cell cycle progression, cell proliferation and chemoresistance [95]. Additionally, miR-181c correlated with the expression of mesenchymal markers N-cadherin and vimentin, and negatively correlated with the epithelial marker E-cadherin, therefore, promoting EMT [95]. Moreover, miR-221 was shown to target the tumor suppressor PTEN and promote migration and invasion in CCA cells through the β -catenin signaling pathway-mediated EMT, as it favored β -catenin translocation into the nucleus. Moreover, β -catenin could activate c-Jun, known to induce miR-221 expression. Thus, miR-221, β -catenin and c-Jun signaling pathways form a positive feedback loop through PTEN inhibition that enhances EMT [96]. Finally, miR-24 has been found to act as an onco-miR by partially repressing the tumor suppressor protein menin and, hence, stimulating proliferation, migration, invasiveness and angiogenesis in CCA tumors [97].

2.3.4. Tumor suppressor microRNAs

Different tumor suppressor miRs are downregulated in CCA cells leading to tumor growth. In particular, downregulation of miR-494 in CCA cells induced the expression of its direct target cyclin-dependent kinase 6 (CDK6) leading to cell cycle progression [98,99]. Similarly, miR-122 was able to inhibit the expression of genes involved in cell cycle progression including Cyclin G1 and insulin-like growth factor 1 receptor (IGF1R), and its baseline downregulation in CCA resulted in cell proliferation [100]. Regarding cell survival, decreased miR-29b levels in CCA cells promoted the expression of its target induced myeloid leukemia cell differentiation protein Mcl-1 (Mcl-1), an anti-apoptotic Bcl-2 family member that protects cells against TRAIL-mediated programmed cell death [101]. Similarly, decreased miR-410 expression in CCA cells resulted in the upregulation of its target, the X-linked inhibitor of apoptosis protein (XIAP), leading to tumor cell growth and invasiveness [102]. Likewise, reduced miR-212 levels in CCA cell lines led to the upregulation of its direct target forkhead box protein A1 (FOXA1), promoting increased tumor cell proliferation and invasion [103]. On the other hand, miR-145 was pointed out as another tumor suppressor found downregulated in CCA cells [104]. MiR-145 prevents the tumor growth, proliferation and invasion of iCCA cells by directly targeting novel (nua) family kinase 1 (NUAK1), which further negatively regulated Akt/Forkhead box protein O1 (FOXO1) pathway and matrix metalloproteinase (MMP) expression halting iCCA progression [104]. Moreover, decreased miR-150-5p levels in CCA cells promoted cell proliferation, migration and invasion *via* upregulation of its direct target the oncogenic ETS domain-containing protein Elk-1 (ELK1) [78].

Regarding EMT, migration and invasiveness, decreased miR-214

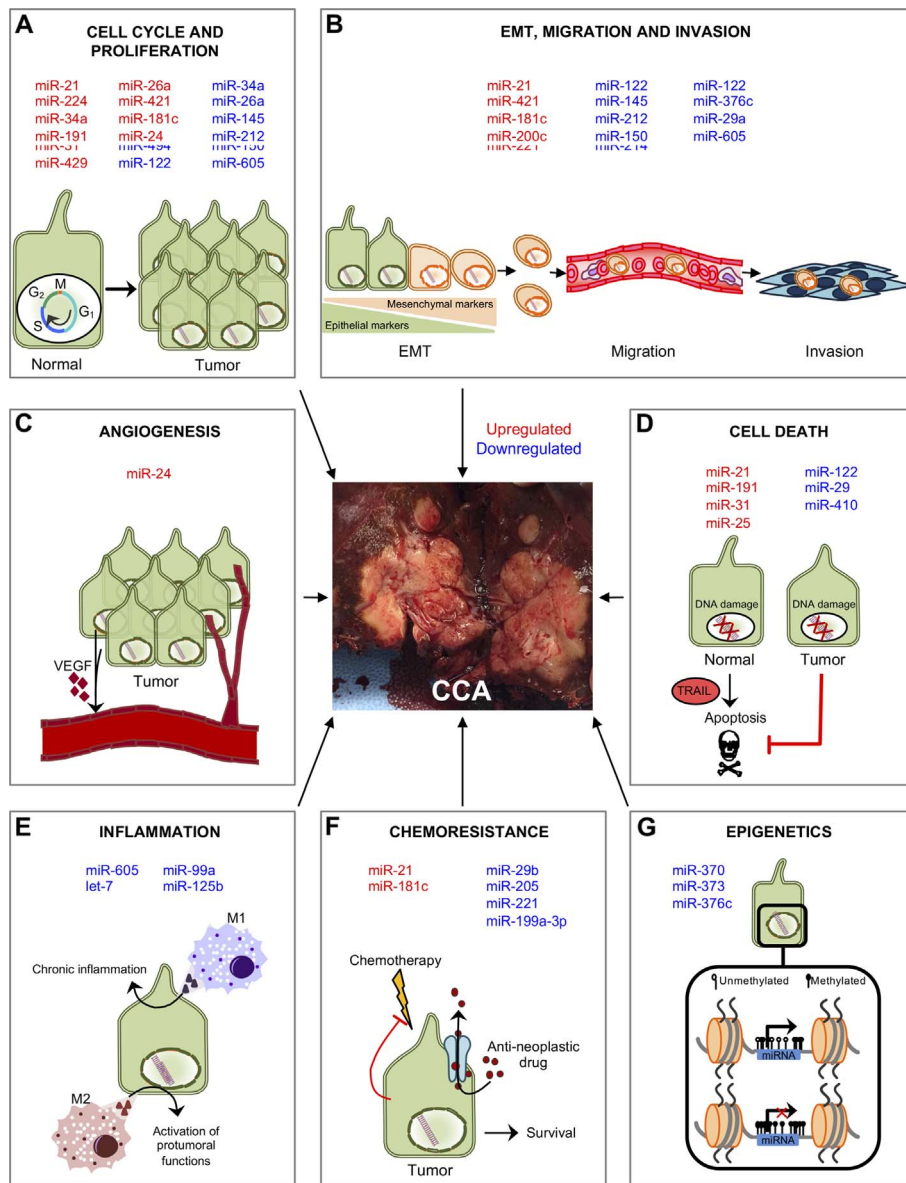


Fig. 3. Aberrant miR expression in the pathogenesis of CCA. Dysregulated miRs in tumorigenic cellular events: cell cycle dysregulation and proliferation (A); EMT, migration and invasion (B); angiogenesis (C); cell death (D); inflammation (E); chemoresistance (F) and epigenetics (G). The central image corresponds to a human liver with CCA. Upregulated miRs are shown in blue color and downregulated ones in red color.

CCA, cholangiocarcinoma; M1, macrophage type 1; M2, macrophage type 2; TRAIL, TNF-related apoptosis-inducing ligand; VEGF, vascular endothelial growth factor.

levels were reported in metastatic iCCA tissues compared to non-metastatic ones, leading to decreased expression of the epithelial marker E-cadherin and induced expression of the EMT transcription factor Twist [105]. In addition, downregulation of miR-122 in human CCA has been suggested to be involved in tumor cell migration and invasion through dysregulation of MMP2, MMP9, RECK, E-cadherin and N-cadherin expression [100]. The activation of the ERK/MMP2/MMP9 signaling pathway and the consequent proliferation, migration and invasion were induced upon miR-138 downregulation in CCA cells *via* Ras homolog gene family member C (RhoC) upregulation [106]. Similarly, miR-200c was reported to mediate EMT in CCA cells *via* direct targeting of neural cell adhesion molecule 1 (NCAM1) [107]. On the other hand, the epidermal growth factor (EGF)-dependent migration of iCCA cells was associated with downregulated miR-376c expression *via* upregulation of its direct target growth factor receptor-bound protein 2 (GRB2). Additionally, interleukin-1 beta (IL1 β) and MMP9 were suggested to be functioning downstream of GRB2 signaling [108]. Likewise, TGF β may be responsible of the reduced miR-29a levels in CCA cell lines and tissue, leading to the stimulation of tumor growth and metastasis *via* activation of its direct target histone deacetylase 4 (HDAC4) [109].

Inflammation has been established as a relevant component of

tumor progression. MiR-605 was found downregulated in iCCA tissue leading to overexpression of its direct target 26S proteasome ATPase regulatory subunit 10 (PSMD10), which prevented retinoblastoma protein (RB) from inhibiting interleukin 6 (IL6) [110]. Induction of the IL6/STAT3 pathway ultimately promoted cell proliferation and invasion *in vitro* and tumor growth *in vivo* through the upregulation of Cyclin D1, vascular endothelial growth factor (VEGF), MMP2 and MMP9 [110,111]. The IL6/STAT3 pathway has also been described as the target of the let-7/miR-99a/miR-125b cluster, which was found downregulated in CCA tissues [112]. Essentially, this miR cluster directly targeted central inflammatory elements including IL6, IL6R and IGF1R, which in turn activated STAT3 downstream signaling enhancing CCA progression [112]. Downregulation of miRs in CCA may be caused by hypermethylation of their promoters. Decreased miR-370 levels in CCA cells were linked to hypermethylation of its promoter *via* IL6-dependent activation of DNA methyltransferases. Consequently, the expression of miR-370 was reduced and the expression of its target the mitogen-activated protein kinase kinase kinase 8 (MAP3K8) was enhanced contributing to tumor growth [113]. In addition, the paternal allele of miR-370 was often found silenced through genomic imprinting in CCA patients [114]. Moreover, due to the overexpression of IL6 in

Table 3

miRs involved in biliary tumorigenesis. 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; CCA, cholangiocarcinoma; CDH6, cadherin-6; CDK6, cyclin-dependent kinase 6; c-Met, tyrosine-protein kinase Met; c-Myc, myc proto-oncogene protein; DR4, death receptor 4; ELK1, ETS domain-containing protein Elk-1; EMT, epithelial mesenchymal transition; FOXA1, forkhead box protein A1; FXR, farnesoid X receptor; GRB2, growth factor receptor-bound protein 2; GSK3B, glycogen synthase kinase 3 beta; HDAC4, histone deacetylase 4; ICCA, intrahepatic cholangiocarcinoma; IGF1R, insulin-like growth factor 1 receptor; IL6, interleukin 6; IL6R, interleukin 6 receptor; KRT19, keratin 19; MAP3K8, mitogen-activated protein kinase kinase 8; MBD2, methyl-CpG-binding domain protein 2; Mcl-1, induced myeloid leukemia cell differentiation protein Mcl-1; miR, microRNA; MMP2, matrix metalloproteinase 2; MMP9, matrix metalloproteinase 9; mTOR, mechanistic target of rapamycin; NCAM1, neural cell adhesion molecule 1; NDRG2, N-myc downstream-regulated gene 2; NUA1, NUA family kinase 1; PDCD4, programmed cell death protein 4; Per1, period circadian protein homolog 1; PIK3R, phosphoinositide-3-kinase regulatory subunit 1; PSMD1, proteasome 26S non-ATPase regulatory subunit 1; PTEN, phosphatase and tensin homolog; PTPN14, tyrosine-protein phosphatase non-receptor type 14; PTTG1, pituitary tumor-transforming gene 1 protein; RASA1, RAS p21 protein activator 1; RECK, reversion-inducing cysteine-rich protein with Kazal motifs; RhoC, ras homolog family member C; Smad4, small mothers against decapentaplegic homolog 4; TET1, ten-eleven translocation 1; TIMP3, metalloproteinase inhibitor 3; TOP2A, DNA topoisomerase 2-alpha; Twist, twist basic helix-loop-helix transcription factor; WNT10B, Protein Wnt-10B; XIAP, X-linked inhibitor of apoptosis protein.

ROLE IN TUMORIGENESIS	MIR	DISEASE	TARGET	FUNCTION	SAMPLE	REFERENCE
Onco-miR	21	ICCA	PTPN14, PTEN	Proliferation, tumor growth, apoptosis	Cell lines, tissue, serum	[69]
	21	O. viverrini ICCA	PDCD4	Proliferation, tumor growth, migration, oncogenesis,	Cell lines, tissue, animal model	[72]
	21	CCA	PDCD4, TIMP3, E-Cadherin, N-Cadherin, Vimentin, PTEN	Proliferation, apoptosis, EMT, migration, invasion, oncogenesis, chemoresistance	Cell lines, tissue	[68, 83-86]
	24	CCA	Menin	Proliferation, migration, invasion, angiogenesis	Cell lines, animal model	[97]
	25	CCA	DR4	Apoptosis	Cell lines, tissue	[94]
	26a	CCA	GSK-3b	Proliferation	Cell lines, tissue	[74]
	31	ICCA	RASA1	Proliferation, apoptosis	Cell lines, tissue	[92]
	34a	CCA	Per1	Proliferation, cell cycle progression, tumor growth, invasion	Cell lines, tissue	[89]
	181c	CCA	NDRG2	Proliferation, tumor growth, EMT, migration, invasion, chemoresistance, senescence	Cell lines, tissue	[95]
	191	ICCA	TET1	Proliferation, cell cycle progression, tumor growth, apoptosis, EMT, migration, invasion	Cell lines, tissue, animal model	[90]
	221	CCA	PTEN	EMT, migration, invasion	Cell lines, tissue	[96]
	224	CCA	p15, p21, Cyclin E1	Cell cycle progression, tumour growth	Cell lines, tissue	[88]
	421	CCA and oallbladder cancer	FXR	Proliferation, migration	Cell lines, tissue	[93]
	429	CCA	CDH6	Tumor growth	Cell lines	[91]

¹miR cluster.

CCA patients, the maternal allele may also become hypermethylated and suppress the expression of miR-370 from this allele too, further disrupting its tumor suppressor activity [114]. Similarly, miR-373, also found in a CpG island, was shown to be downregulated in pCCA. In consequence, its direct target the methyl-CpG-binding domain protein 2 (MBD2) became overexpressed [115]. Interestingly, MBD2 can also bind to miR-370 promoter, initiating a positive feedback loop by CpG island methylation, therefore, intensifying the repression of miR-370 [116,117]. Epigenetic modifications also regulated the expression of miR-376c in iCCA, as higher methylation levels of CpG sites upstream miR-376c gene were observed [108].

Downregulation of certain miRs is known to confer CCA a chemoresistant phenotype. Thus, decreased levels of miR-29b, miR-205 and miR-221 in CCA cells induced chemoprotection against gemcitabine [118]. Moreover, downregulation of miR-199a-3p in CCA cells decreased their sensitivity to cisplatin by inducing the expression of multidrug resistant protein 1 (MDR1) and by inhibiting the mechanistic target of rapamycin (mTOR) signaling pathway [119].

The role of some miRs in cholangiocarcinogenesis remains controversial as different studies have defined certain miRs to function as both onco-miRs and tumor suppressor miRs. For instance, decreased levels of miR-26a expression in CCA cells exhibited proliferative properties resulting in incremented tumor growth both *in vitro* and *in vivo*, via direct targeting of keratin type I cytoskeletal 19 (KRT19) [120]. In contrast, overexpression of miR-26a was also reported to enhance CCA cell proliferation and tumor growth in nude mice by directly targeting glycogen synthase kinase-3 beta (GSK3 β) and further activating the β -catenin signaling pathway and some of its downstream genes including myc proto-oncogene protein (c-Myc), cyclin D1, and peroxisome proliferator-activated receptor δ (PPAR δ) [74]. Likewise, miR-34a has been described as both onco-miR and tumor suppressor miR. As aforementioned, miR-34a overexpression halted cell cycle arrest by blocking Per1 expression and hampering its interaction with CHEK2 [89]. However, miR-34a has also been characterized as a tumor suppressor, and can be inhibited by TGF β , which is found upregulated during CCA development and progression. Consequently, TGF β -mediated downregulation of miR-34a resulted in the overexpression of other miR-34a downstream targets (*i.e.* CDK6, Cyclin D1, c-Myc and small mothers against decapentaplegic homolog 4 [SMAD4]) promoting CCA cell proliferation, as well as migration and EMT [121–123].

3. Extracellular vesicles in cholangiopathies

3.1. Extracellular vesicles (EVs)

EVs are nano- or micro-sized lipid bilayer spheres produced by diverse cell types and released into the extracellular media where they participate in intercellular communication both in physiological and pathological conditions [124–127]. Although EVs were first identified in 1946, the general interest of the scientific community on EVs did not appear until the last decade, where an increasing number of scientific reports, specific societies and journals emerged (Fig. 1B). According to their biogenesis, EVs are classified as exosomes, microvesicles and apoptotic bodies [128] (Fig. 4). Exosomes (~40–150 nm) are generated in the MVBs of cells, which derive from early endosomes that are formed by the endocytosis of the plasma membrane [125]. Microvesicles (also called as microparticles: ~40–1000 nm) are formed and released by direct budding of the plasma membrane of cells [129,130]. Finally, apoptotic bodies (~100–5000 nm) are produced by cells undergoing apoptosis and their size and morphology are more heterogeneous [129,130]. EVs are found in different biological fluids such as blood, urine, saliva, breast milk, ascitic fluid, seminal fluid and bile, among others [124]. Likewise, EVs contain a specific subset of proteins, RNA species, metabolites and lipids, which play important roles in intercellular communication and in the regulation of multiple physiological processes including immune response [124,131], homeostasis of

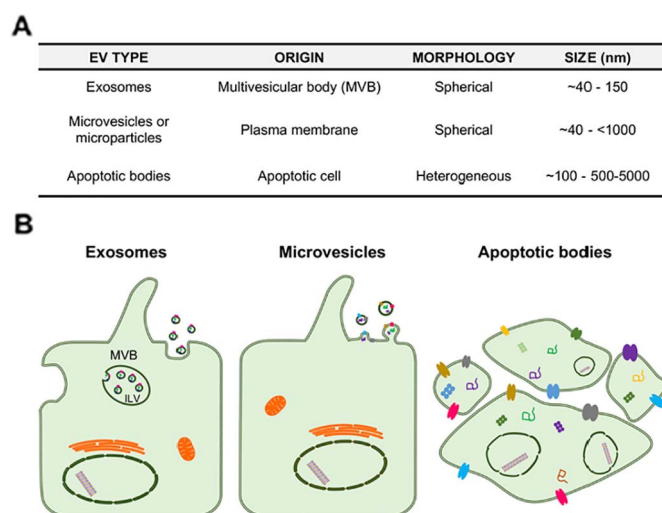


Fig. 4. EV classification according to biogenesis. A) General features of exosomes, microvesicles/microparticles and apoptotic bodies. B) Graphical representation of the different EV types.

MVB, multivesicular body; ILV, intraluminal vesicles.

the circulatory system [124], pregnancy process mediating the fetal-maternal communication, [124,132] embryonic development [124] and tissue repair [124,133,134]. Moreover, EVs participate in the pathogenesis of different diseases and are also considered as a source for biomarkers and as new therapeutic vehicles for drugs or external biomolecules delivery [124,126,135–137].

Several studies have pointed out the relevant role of EVs in the regulation of different aspects of liver function in health and disease [138–140]. Similarly, the role of EVs in biliary pathophysiology has also been investigated in the last years (Fig. 1B). The first evidences of the physiological role of EVs in biliary physiology were given by the identification of EVs in bile [141]. Bile EVs were partially secreted by cholangiocytes and directly bound to their primary cilium inhibiting cell proliferation in an ERK-dependent manner [141]. These data evidenced that EVs may contribute to the maintenance of the homeostasis of the biliary epithelia in normal conditions.

3.2. EVs as source of biomarkers for cholangiopathies

The value of EVs as source of biomarkers for cholangiopathies has been investigated in PSC and CCA. A panel of miRs (miR-191, miR-486-3p, miR-1274b) was reported upregulated in EVs isolated from bile of CCA patients compared to several biliary benign disorders (*i.e.* cholelithiasis, PSC, chronic pancreatitis and Sphincter of Oddi dysfunction) showing diagnostic value (Table 4) [6]. Besides miRs, specific proteome profiles were reported in serum EVs from PSC, CCA, hepatocellular carcinoma (HCC) and healthy individuals [7] with diagnostic value between groups (Table 4). Of note, pantetheinase (VNN1), C-reactive protein (CRP), fibrinogen gamma chain (FIBG), immunoglobulin heavy constant alpha 1 (IGHA1), alpha-1-acid glycoprotein 1 (A1AG1) and gamma-glutamyltranspeptidase 1 (GGT1) proteins were all found overexpressed in EVs from CCA patients compared to PSC, healthy control and HCC, showing differential diagnostic value [7]. In addition, different proteins such as ficolin-2 (FCN2), inter-alpha-trypsin inhibitor heavy chain H4 (ITI4), FIBG, plasma protease C1 inhibitor (IC1) or serum amyloid P-component (SAMP), among others, were found overexpressed in serum EVs from early stage CCAs (I-II) compared to PSC patients, showing higher diagnostic value than CA19-9 [7]. On the other hand, non-invasive differential diagnosis of iCCA vs HCC is sometimes difficult. Interestingly, different proteins were found concentrated in EVs from iCCA patients compared to HCC such as FIBG, A1AG1, vitamin D-binding protein (VTDB), CRP, presenting higher

Table 4

EVs associated biomarkers for cholangiopathies.

A1AG1, alpha-1-acid glycoprotein 1; AMPN, aminopeptidase B; AUC, area under the curve; CCA, cholangiocarcinoma; CRP, C-reactive protein; FIBG, fibrinogen gamma chain; IC1, plasma protease C1 inhibitor; IGHG4, immunoglobulin heavy constant gamma 4; ITGB4, integrin beta-4; miR, microRNA; EV, extracellular vesicles; FCN1, ficolin 1; HCC, hepatocellular carcinoma; IGHA1, immunoglobulin heavy constant alpha 1; NEP, nuclear export protein; PIGR, polymeric immunoglobulin receptor; PSC, primary sclerosing cholangitis; SAMP, serum amyloid P-component; SEN, sensitivity; SPE, specificity; VNN1, pantetheinase; VTDB, vitamin D binding native protein.

Biomarkers in EV	Name	Disease	EV source	Number of patients	SEN (%)	SPE (%)	AUC	Reference
miR	miR-191	CCA	Bile	CCA (n = 46) vs controls ^a (n = 50)	67.0	96.0	–	[6]
	miR-486-3p	CCA	Bile		67.0	96.0	–	
	miR-1274b	CCA	Bile		67.0	96.0	–	
Protein	AMPN	PSC	Serum	PSC (n = 30) vs controls (n = 32)	83.3	62.5	0.789	[7]
	FCN1	PSC	Serum		76.7	68.7	0.771	
	NEP	PSC	Serum		63.3	90.6	0.761	
	PIGR	PSC	Serum	CCA (n = 43) vs controls (n = 32)	76.7	71.8	0.742	[7]
	VNN1	PSC	Serum		76.7	59.3	0.741	
	AMPN	CCA	Serum		90.7	65.6	0.878	
	VNN1	CCA	Serum	2 independent cohorts	72.1	87.5	0.876	[7]
	PIGR	CCA	Serum		83.7	71.8	0.844	
	IGHA1	CCA	Serum		81.4	75.0	0.820	
	CRP	CCA	Serum	CCA (n = 43) vs PSC (n = 30)	79.1	68.7	0.808	[7]
	FIBG	CCA	Serum		88.4	63.3	0.796	
	A1AG1	CCA	Serum		76.7	70.0	0.794	
	S10A8	CCA	Serum	2 independent cohorts	69.8	66.6	0.759	[7]
	S10A9	CCA	Serum		74.4	60.0	0.740	
	SAMP	CCA	Serum		79.1	57.6	0.740	
	PIGR	CCA I-II	Serum	CCA I-II (n = 13) vs controls (n = 22)	75.0	95.4	0.905	[7]
	AMPN	CCA I-II	Serum		91.7	72.7	0.833	
	FIBG	CCA I-II	Serum		100.0	68.1	0.833	
	IGHG4	CCA I-II	Serum	CCA I-II (n = 13) vs PSC (n = 30)	66.7	96.4	0.818	[7]
	IGHA1	CCA I-II	Serum		91.7	59.0	0.814	
	FCN2	CCA I-II	Serum		100.0	80.9	0.956	
	ITIH4	CCA I-II	Serum	iCCA (n = 12) vs HCC (n = 29)	91.7	80.9	0.881	[7]
	FIBG	CCA I-II	Serum		91.7	80.9	0.881	
	IC1	CCA I-II	Serum		75.0	80.9	0.821	
	SAMP	CCA I-II	Serum	iCCA (n = 12) vs HCC (n = 29)	75.0	80.9	0.821	[7]
	FIBG	iCCA	Serum		83.3	89.6	0.894	
	A1AG1	iCCA	Serum		83.3	82.1	0.845	
	VTDB	iCCA	Serum	iCCA (n = 12) vs HCC (n = 29)	75.0	89.2	0.823	[7]
	CRP	iCCA	Serum		75.0	79.3	0.802	
	VNN1	iCCA	Serum		83.3	68.9	0.802	

^a Including PSC, biliary obstruction and bile leak.

diagnostic capacity than CA19-9 or alpha-fetoprotein (AFP), a non-specific tumor marker commonly used to support the diagnosis and monitoring of HCC [142]. Moreover, serum EVs from HCC patients showed increased levels of proteins such as galectin-3-binding protein (LG3BP) compared to healthy controls, with higher diagnostic value than AFP [7]. On the other hand, changes in the population of microvesicles (MVs) have been reported for both CCA and HCC. Increased amount of MVs positive for Annexin V, epithelial cell adhesion molecule (EpCAM) and asialoglycoprotein receptor 1 (ASGPR1) was found in serum from both HCC and CCA patients compared to cirrhotic patients. The amount of these particular MVs exhibited diagnostic value for the presence of liver tumors compared to cirrhotic livers and, interestingly, their amount decreased after tumor resection, supporting a correlation with the presence of the tumor [143].

Besides the potential of EVs as biomarkers, their value for therapeutic purposes has been explored for CCA. A recent study indicated that restoration of miR-195 levels in CCA cells by the injection of miR-195 laden EVs coming from stellate cells overexpressing miR-195 reduced their growth and improved survival in a CCA orthotopic mouse model [144].

3.3. EVs in biliary pathophysiology

Increasing evidence indicates that EVs participate in the pathophysiology of biliary diseases. In PBC, plasma EVs have been suggested to immunomodulate the peripheral blood-derived antigen presenting cells (monocytes and dendritic cells) regulating the expression of the co-stimulatory molecules CD86 and CD80 [145]. Thus, autologous

incubation of PBC plasma-derived EVs with PBMC induced the upregulation of CD86 in CD14⁺ monocytes, CD40 in CD11c⁺ dendritic cells and the downregulation of both CD80 and CD40 in the CD14⁺ monocyte populations. However, PBC plasma EVs did not show changes on monocyte cytokine production. Furthermore, PBC plasma-derived EVs exhibited increased amount of miR-451a and miR-642a-3p compared to healthy controls, which have been associated with several immune regulatory processes including dendritic cell cytokine profile regulation, T cell IL6 production or the toll-like receptor 4 translation to monocytes [145]. As PBC courses with selective destruction of the biliary epithelial cells [146], the immunomodulatory effects of the apoptotic bodies coming from the dying cholangiocytes could have an impact on the progression of PBC. With that idea, the protein content of the apoptotic bodies coming from normal intrahepatic cholangiocytes treated with the toxic glycochenodeoxycholic bile acid was explored by mass-spectrometry analysis. Of note, these apoptotic bodies presented several proteins related to immune reaction including those involved in NF-κB, ERK and Notch signaling pathways, and in the modulation of IL8 and its signaling receptor [147].

Regarding biliary tract malignancies, several reports highlighted the role of EVs in the pathogenesis of these tumors, particularly in intercellular communication and tumor microenvironment, where CCA-derived EVs may modulate stromal cells and inhibit the immune response favoring tumor development and progression. CCA cell-derived EVs could activate mesenchymal stem cells (MSC) resulting in the release of important pro-inflammatory cytokines such as IL6, chemokine (C-X-C motif) ligand 1 (CXCL1) and C-C motif chemokine ligand 2 (CCL2) that promote the migratory capacity of CCA cells. Therefore, CCA cell-

derived EVs may modulate the tumor stroma to favor tumor growth. Regarding immunomodulation, CCA cell-derived EVs reduced the presence of CD3 + CD8 + CD56 + natural killer cells and CD3 + CD56 + cytotoxic cell populations in PBMCs cultures and the production of TNF α and perforin [148]. The pro-tumorigenic effect of CCA cell-derived EVs may come from oncogenic molecules present in these vesicles. In this regard, the protein content of CCA-derived EVs *in vitro* was studied by mass spectrometry-based proteomics in liver fluke-associated CCA cells [142,149] and in two idiopathic CCAs cell lines [142,149]. Liver fluke associated CCA-derived EVs present an upregulation of several oncogenic proteins including galectin-3 binding protein (LGBP), large neutral amino acids transporter small subunit 1 (LAT1), 4F2 cell-surface antigen heavy chain (4F2hc), pyruvate kinase (KPYM) and EPCAM, among others. Other important proteins involved in cholangiocarcinogenesis that were also found upregulated in CCA-derived EVs [142,149] included the epidermal growth factor receptor (EGFR), integrin beta-4 (ITGB4) [142]. Liver flukes are the main risk factor for CCA development in several Southeast Asian countries [61]. In this regard, *O. viverrini* liver fluke-produced EVs promoted cholangiocyte proliferation [150].

4. Conclusion and future directions

Both miRs and EVs play significant roles in the pathophysiology of cholangiopathies and are promising non-invasive tools for diagnosis, prognosis and to predict treatment response. Several miRs and EV-related proteins present in serum, bile or urine showed high sensitivity and specificity for the diagnosis of different cholangiopathies. The use of miRs and EV-related biomarkers could be a promising future option as the first diagnostic step in early detection of cholangiopathies, specifically in individuals with known risk factors for CCA (*i.e.*, liver flukes, hepatitis B and C, or PSC). Furthermore, the majority of miRs found dysregulated in biological fluids were not validated in other studies, pointing out the need to standardize the isolation, purification and amplification methods. Moreover, special attention should be directed to bridge the gap between pre-clinical and clinical studies. Thus, future international collaborative investigations are urgently needed to validate the most promising biomarkers and to start their implementation in the general clinical practice. On the other hand, elucidating the role of the dysregulated miRs in the pathogenesis of cholangiopathies is key to uncover novel therapeutic targets. However, most of the pathophysiological studies were carried out using *in vitro* analysis, demanding the need of *in vivo* studies in order to test the potential impact of miR-based therapies for the treatment of biliary diseases.

Disclosures

Authors disclose no conflicts.

Grant support

Spanish Ministry of Economy and Competitiveness [J.M. Banales (FIS PI12/00380, FIS PI15/01132 and Miguel Servet Program CON14/00129), M.J. Perugorria (FIS PI14/00399 and Ramon y Cajal Programme RYC-2015-17755)] cofinanced by “Fondo Europeo de Desarrollo Regional” (FEDER); “*Instituto de Salud Carlos III*” [CIBERehd: J.M. Banales, and L. Bujanda], Spain; “Diputación Foral Gipuzkoa” (L. Bujanda: DFG14/007; J.M. Banales: DFG15/010, DFG16/004), Departments of Industry, Tourism, Trade and Health of the Basque Country (L. Bujanda: 2013111173; M.J. Perugorria: 2015111100) and BIOEF (Basque Foundation for Innovation and Health Research: EITB Maratoia BIO15/CA/016/BD to J.M. Banales); P. Olaizola is funded by the Basque Government (PRE_2016_1_0269) and P.Y. Lee-Law by the European Association for the Study of the Liver (EASL; Sheila Sherlock Award).

Transparency document

The <http://dx.doi.org/10.1016/j.bbadis.2017.06.026> associated with this article can be found, in online version.

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