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Role of circulating microRNAs in liver diseases

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Abstract

MicroRNAs (miRNAs) are small RNAs regulate gene expression by inhibiting the turnover of their target mRNAs. In the last years, it became apparent that miRNAs are released into the circulation and circulating miRNAs emerged as a new class of biomarkers for

various diseases. In this review we summarize available data on the role of circulating miRNAs in the context of acute and chronic liver diseases including hepatocellular and cholangiocellular carcinoma. Data from animal models are compared to human data and current challenges in the field of miRNAs research are discussed.

Key words: Liver disease; Acute liver failure; MicroRNA; Liver fibrosis; Hepatocellular carcinoma; Autoimmune hepatitis; Cholangiocarcinoma

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Core tip: In this article, we aim to review the role of circulating microRNAs (miRNAs), a class of small non-coding RNAs involved in various pathological processes, in the context of liver disease. The focus is on current and future applications of miRNAs as potential diagnostic and prognostic biomarkers in the field of acute liver failure, liver fibrosis and cirrhosis, autoimmune liver disease as well as liver cancer.

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INTRODUCTION

MicroRNAs (miRNAs) are small RNAs that do not encode for proteins, but regulate gene expression^[1]. MiRNAs are transcribed by the RNA polymerase II or RNA polymerase II^[2-4]. The resulting 500-3000 nucleotides long transcripts (pri-miRNAs) are cleaved in a second step by the "microprocessor complex" into approximately 70 nucleotides long precursor miRNAs (pre-miRNA), which are actively exported from the nucleus into the cytoplasm. Finally, pre-miRNAs are processed by the

RNase III endonuclease “Dicer” into approximately 22 nucleotides long double stranded miRNAs, which bind to the Argonaute protein and are integrated into the “RNA-induced silencing complex”. Within this complex, miRNAs bind the 3′ or 5′ untranslated region of the target mRNAs, leading to a transcriptional or translational repression of the target mRNA^[2,4-6]. Alterations in miRNA expression profiles were described in organ development, aging, and cell death^[7], as well as in the pathophysiology of complex diseases such as inflammation, fibrosis and cancer^[8-13].

Besides their role in the regulation of gene expression, miRNAs have been described in body fluids, where they might serve as biomarkers^[14-17]. Based on their extraordinary stability, their less complex chemical structure and their lack of post-processing modifications, circulating miRNAs were suggested as “optimal” serum based biomarkers^[18]. Circulating miRNAs can be either bound to serum proteins and lipoproteins or be encircled in extracellular vesicles including exosomes, microvesicles or apoptotic bodies^[17,19]. As exosomes can be released by various hepatic cells (*e.g.*, hepatocytes and Kupffer cells) and can be transferred to other recipient cells to regulate expression profiles in these cells, they were suggested to play an important role in hepatic cell-cell-communication and in the pathophysiology of different liver diseases. Findings that miRNAs encircled in these vesicles are well protected from degradation furthermore highlight the potential of exosomal miRNAs to serve as potent biomarkers^[20-22]. With respect to the concept of “liquid biopsy” which has recently been suggested as a novel detection tool for malignant diseases^[23,24], miRNA might thus function as a potential “liquid biopsy” not only for malignant but also benign liver disease.

In this review, we evaluated studies indexed in Medline between 2006 and 2016. The terms “microRNA”, “liver”, “liver failure”, “fibrosis”, “cirrhosis”, “hepatocellular carcinoma”, “cholangiocarcinoma”, “autoimmune hepatitis”, “primary sclerosing cholangitis”, “primary biliary cholangitis”, “biomarker”, “diagnostic”, “prognostic” and combinations of these terms were used.

ACUTE LIVER FAILURE

Acute liver failure (ALF) is characterized by a massive loss of liver cell function based on various etiologies (*e.g.*, drug intoxication, viral or autoimmune hepatitis (AIH), Wilson’s disease or Budd-Chiari syndrome) without preexisting liver disease^[25,26]. Despite significant improvements regarding therapeutic options (*e.g.*, liver transplantation), ALF has remained a challenging clinical condition with mortality rates of about 50%^[27]. In this context, biomarkers allowing early diagnosis or estimation of patients’ fate might be helpful for the guidance of therapy^[28,29]. However, routinely used serum biomarkers for liver injury such as AST and ALT are not liver specific and only have a limited prognostic value^[30-32]. Therefore, new biomarkers are urgently needed to further improve patients’ individual treatment options and overall survival

in the context of acute liver injury.

In a pilot study on the potential of miRNAs as ALF biomarkers, Wang *et al.*^[18] demonstrated that liver specific miR-122 and miR-192 were elevated in sera of mice after acute Acetaminophen (APAP) intoxication compared to controls. Of note, miR-122 and miR-192 serum levels were increased in a dose- and exposure duration-dependent manner and were detectable significantly earlier than the classic serum aminotransferases^[18]. Consistently, circulating miR-122 and miR-192 levels were elevated in patients with APAP-induced ALF compared to healthy controls^[33]. Moreover, miR-122 serum levels returned earlier to normal when compared to ALT, indicating that circulating miR-122 might have a shorter half-life in comparison to ALT^[33]. High throughput sequencing of miRNAs in sera of patients with APAP overdose revealed 36 miRNAs to be elevated compared to healthy controls. Besides the already described miR-122 and miR-192, miR-483, miR-194 and miR-210 were additionally found to be increased in the sera of these patients^[32]. Antoine *et al.*^[29] demonstrated in a large cohort of patients with APAP-induced ALF that increased miR-122 serum levels are detectable very early after liver intoxication when serum ALT levels are still unaffected. Furthermore, levels of circulating miR-122 enabled the prediction of liver injury development with a high accuracy^[29].

An increasing number of studies have investigated circulating miRNAs regarding their prognostic potential for acute liver injury. Just recently, Russo *et al.*^[34] applied a microarray based expression analysis using a panel of 1733 miRNAs and 1658 pre-miRNAs in sera of 78 drug-induced liver injury (DILI) patients. These patients showed elevated serum levels of miR-122, miR-1246, -4270, -4433, -4463, -4484, -4532 and pre-miR-4767 as well as decreased serum levels of miR-455-3p, -1281 and pre-miR-4274 compared to healthy controls. Out of these, miR-122, miR-4463 and miR-4270 had a prognostic value as decreased serum levels correlated with the decrease of DILI patients within 6 mo. In this study, low albumin (less than 2.8 g/L) and low miR-122 serum levels (less than 7.89 relative fluorescent units) had a sensitivity of 100% and a specificity of 57% for the prediction of death in DILI patients^[34]. The prognostic value of miRNA profiles were further investigated in a retrospective study on patients with ALF caused by viral hepatitis, toxic liver injury, Budd-Chiari syndrome, Wilson’s disease, AIH or indeterminate etiology^[26]. In this study, serum levels of miR-122, miR-21 and miR-221 were found to be significantly increased in patients that showed a spontaneous recovery from ALF compared to non-recovered patients^[26]. Increased levels of circulating miR-122, miR-21 and miR-221 in patients with a spontaneous recovery from ALF were further associated with increased hepatocyte proliferation and liver tissue regeneration due to decreased expression of the respective miRNAs target genes in the liver like heme-oxygenase-1 (miR-122), programmed cell death 4 (miR-21), p27 and p57 (miR-221)^[26].

Table 1 Summary of circulating miRNAs as diagnostic biomarkers in various liver diseases

Medical condition	miRNA	Serum levels	# of patients	Method for determination	Ref.	
Acute liver failure (drug induced)	miR-122	↑	53	qPCR	Starkey Lewis <i>et al</i> ^[33]	
		↑	6	RNA sequencing, qPCR	Krauskopf <i>et al</i> ^[32]	
		↑	129	qPCR	Antoine <i>et al</i> ^[29]	
		↑	78	miRNA microarray	Russo <i>et al</i> ^[34]	
	miR-192	↑	53	PCR	Starkey Lewis <i>et al</i> ^[33]	
		↑	6	RNA sequencing, qPCR	Krauskopf <i>et al</i> ^[32]	
		↑	6	RNA sequencing, qPCR	Krauskopf <i>et al</i> ^[32]	
	miR-483	↑	6	RNA sequencing, qPCR	Krauskopf <i>et al</i> ^[32]	
	miR-194	↑	6	RNA sequencing, qPCR	Krauskopf <i>et al</i> ^[32]	
	miR-210	↑	6	RNA sequencing, qPCR	Krauskopf <i>et al</i> ^[32]	
	miR-4532	↑	78	miRNA microarray	Russo <i>et al</i> ^[34]	
	miR-455-3p	↓	78	miRNA microarray	Russo <i>et al</i> ^[34]	
	miR-1281	↓	78	miRNA microarray	Russo <i>et al</i> ^[34]	
	Liver fibrosis (CHC)	miR-122	↑	53	qPCR	Cermelli <i>et al</i> ^[37]
miR-34a		↑	53	qPCR	Cermelli <i>et al</i> ^[37]	
Liver fibrosis (NAFLD)	miR-122	↑	34	qPCR	Cermelli <i>et al</i> ^[37]	
		↑	28	qPCR	Salvoza <i>et al</i> ^[38]	
	miR-34a	↑	34	qPCR	Cermelli <i>et al</i> ^[37]	
		↑	28	qPCR	Salvoza <i>et al</i> ^[38]	
Liver cirrhosis	miR-513-3p	↑	67	miRNA microarray, qPCR	Roderburg <i>et al</i> ^[40]	
	miR-571	↑	67	miRNA microarray, qPCR	Roderburg <i>et al</i> ^[40]	
	miR-29	↓	67	miRNA microarray, qPCR	Roderburg <i>et al</i> ^[41]	
AIH	miR-21	↑	46	miRNA microarray, qPCR	Migita <i>et al</i> ^[47]	
	miR-122	↑	46	miRNA microarray, qPCR	Migita <i>et al</i> ^[47]	
PSC	miR-1281	↑	40	miRNA microarray, qPCR	Voigtländer <i>et al</i> ^[48]	
	miR-126	↑	40	miRNA microarray, qPCR	Voigtländer <i>et al</i> ^[48]	
	miR-200c	↓	30	miRNA microarray, qPCR	Bernuzzi <i>et al</i> ^[49]	
PBC	miR-505-3p	↓	10	RNA sequencing, qPCR	Ninomiya <i>et al</i> ^[50]	
	miR-197-3p	↓	10	RNA sequencing, qPCR	Ninomiya <i>et al</i> ^[50]	
	miR-122	↑	207	RNA sequencing, qPCR	Tan <i>et al</i> ^[51]	
		miR-141	↑	207	RNA sequencing, qPCR	Tan <i>et al</i> ^[51]
	miR-26b	↑	207	RNA sequencing, qPCR	Tan <i>et al</i> ^[51]	
		miR-21	↑	101	qPCR	Xu <i>et al</i> ^[58]
	↑		90	qPCR	Ge <i>et al</i> ^[62]	
		↑	121	qPCR	Tomimaru <i>et al</i> ^[64]	
		↑	457	miRNA microarray, qPCR	Zhou <i>et al</i> ^[68]	
	miR-121	↑	101	qPCR	Xu <i>et al</i> ^[58]	
		miR-223	↑	101	qPCR	Xu <i>et al</i> ^[58]
	↑		457	miRNA microarray, qPCR	Zhou <i>et al</i> ^[68]	
	miR-16	↓	90	qPCR	Ge <i>et al</i> ^[62]	
		↓	40	qPCR	El-Abd <i>et al</i> ^[63]	
	miR-122	↑	457	miRNA microarray, qPCR	Zhou <i>et al</i> ^[68]	
		miR-26a	↑	457	miRNA microarray, qPCR	Zhou <i>et al</i> ^[68]
	miR-192	↑	457	miRNA microarray, qPCR	Zhou <i>et al</i> ^[68]	
		CCA	miR-21	↑	25	RNA sequencing, qPCR
	↑		94	qPCR	Kishimoto <i>et al</i> ^[75]	
		miR-221	↑	25	RNA sequencing, qPCR	Correa-Gallego <i>et al</i> ^[74]
		miR-150	↑	15	qPCR	Wang <i>et al</i> ^[77]
		miR-224	↑	30	qPCR	Huang <i>et al</i> ^[76]
↑			30	qPCR	Huang <i>et al</i> ^[76]	

miRNA: MicroRNA; CHC: Chronic hepatitis C; NAFLD: Non-alcoholic fatty liver disease; qPCR: Quantitative RT-PCR; AIH: Autoimmune hepatitis; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cholangitis; HCC: Hepatocellular carcinoma; CCA: Cholangiocarcinoma; ↑: High circulating levels; ↓: Low circulating levels.

In summary, measurement of circulating miRNAs might represent important serum biomarkers for ALF and help to improve the prediction of patients' prognosis even at an early time point after liver injury. Table 1 summarizes potential diagnostic biomarker for ALF.

LIVER FIBROSIS AND CIRRHOSIS

Liver fibrosis and liver cirrhosis represent the most common end-points of chronic liver diseases such as alcoholic steatohepatitis, non-alcoholic steatohepatitis,

and viral hepatitis, which are all associated with a high morbidity and mortality. Currently, histology is considered the gold standard for the diagnosis and staging of liver fibrosis and/or cirrhosis. However, this procedure is related to a number of problems, including the risks for serious complications during liver biopsy, sampling errors and biases, variabilities in histopathologic interpretation and significant financial costs. Thus, alternative non-invasive strategies for the evaluation of liver fibrosis/cirrhosis are of increasing interest. In this context, besides other markers, circulating miRNAs have been

considered by many authors as promising serum based biomarkers with a potential for being used in clinical routine.

In the past, an overwhelming amount of data supporting a role for miRNAs in the development and progression of chronic liver diseases into liver cirrhosis and finally hepatocellular carcinoma (HCC) was presented (reviewed e.g., in^[35]). Based on these data, the group of El-Ahwany analyzed serum levels of different miRNAs with an established role in the activation of hepatic stellate cells (HSC) in sera of 66 subjects with early stage liver fibrosis and 65 subjects with late-stage fibrosis^[36]. Forty healthy subjects served as normal controls. In line to their role in the activation of HSC, serum concentrations of miR-138, miR-140, miR-143, miR-325, miR-328 and miR-349 were significantly elevated in patients with fibrosis compared to healthy controls. ROC analysis revealed a sensitivity and specificity of miR-138 of 89.3% and 71.43% for prediction of early stage fibrosis and of 89.3% and 93.02% for prediction of late stage fibrosis, respectively, demonstrating that analyses of circulating miRNAs might be helpful to detect even early stages of liver fibrosis. Besides these miRNAs, several groups demonstrated that levels of miR-34a, one of the best investigated miRNAs in the context of chronic liver diseases, are elevated in patients with liver fibrosis^[37-39]. In a large cohort of patients, Cermelli *et al.*^[37] described elevated levels of miR-34a in patients with both hepatitis C (CHC)- and NAFLD-dependent liver fibrosis. Interestingly, levels of miR-34 were independent of the viral load but reflected the stage of disease in both disease entities. In this study, miR-34a correlated with AST/ALT levels, stage of fibrotic disease, inflammatory activity and serum lipids in NAFLD patients, highlighting that levels of circulating miRNAs might reflect specific aspects in the pathophysiology of chronic liver diseases^[37]. In line with this assumption, we described elevated levels of miR-513-3p and miR-571 in patients with alcohol- or hepatitis C-induced liver cirrhosis. However, only serum level of miR-571 reflected the disease severity in liver cirrhosis, while miR-513-3p was independent on the stage of fibrosis or inflammatory activity in these patients^[40]. Besides these up-regulated miRNAs, a down-regulation of circulating miR-29 was found in patients with chronic liver injury and liver fibrosis. Levels of miR-29 correlated with the stage of liver fibrosis, MELD score and disease entity^[41]. In the context of alcohol induced liver injury, microarray based screening of exosomal miRNAs revealed an up-regulation of miRNA-192, miRNA-122, miRNA-30a, miRNA-744, miRNA-1246, miRNA 30b and miRNA-130a in blood sera of chronic alcohol-fed mice compared to healthy controls^[42]. Moreover, ROC curve analyses indicated a diagnostic potential of miRNA-192, miRNA-122, and miRNA-30a for the identification of alcohol-induced liver injury^[42].

Recently, the group of Matsuura *et al.*^[43] attempted to determine whether circulating miRNAs might be used to estimate disease progression in chronic hepatitis C

patients. One hundred and thirty CHC patients were prospectively followed. In this study, reduced plasma levels of the let7-family reflected a more advanced fibrosis stage whereas elevated concentrations of miR-122-5p were indicative for an increased inflammatory activity, but not for the degree of liver fibrosis^[43]. In another large cohort of CHC-patients, Trebicka *et al.*^[44] demonstrated that circulating miR-122 levels positively correlated with an enhanced inflammatory activity but negatively with liver fibrosis, which was most probably due to the loss of liver cells (as the major source of miR-122) during chronic liver injury. Interestingly, miR-122 serum levels were associated with the survival of CHC-cirrhosis patients independent of the MELD score, sex and age^[45], underscoring the potential of this liver specific miRNA in the diagnosis of liver fibrosis and cirrhosis.

In summary, circulating miRNAs might represent diagnostic and prognostic biomarkers in patients with liver fibrosis or cirrhosis.

AUTOIMMUNE LIVER DISEASE

Although autoimmune liver diseases have gained rising importance in the field of hepatology due to its increasing incidence over the last decades^[46], only very few studies have evaluated the involvement of circulating miRNAs in AIH, primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC).

To our knowledge only one study investigating circulating miRNAs in patients with AIH exists to date. In this study, serum samples of 46 type-1 AIH patients were screened for 2555 miRNAs using a microarray system and compared to patients with chronic hepatitis C and healthy controls. Circulating levels of miR-21 and miR-122 were significantly higher in AIH patients compared to both control groups. Interestingly, the authors observed a strong decrease of miR-21 and miR-122 levels after treatment with glucocorticoids, indicating a potential role of these miRNA not only as a diagnostic marker but also as a marker to assess treatment response^[47].

In PSC patients, serum levels of miR-1281 and miR-126 were shown to be significantly increased compared to healthy controls. Importantly, the elevation of these miRNAs in PSC patients was also significantly higher compared to CCA patients, arguing that miR-1281 and miR-126 might reflect disease-specific processes of PSC that do not or to a lesser extend occur during malignant transformation of bile duct cells into CCA^[48]. Moreover, Bernuzzi *et al.*^[49] described miR-200c as significantly down-regulated in patients with PSC in large screening approach including 667 miRNAs.

In PBC patients, a deep sequencing approach revealed circulating levels of miR-505-3p and miR-197-3p as significantly decreased when compared to healthy controls^[50]. However, this study was performed in a very small cohort of patients ($n = 10$) and needs further validation. In another study, Tan *et al.*^[51] establish a diagnostic serum miRNA panel in a cohort of 207 PBC

patients using a stepwise logistic regression model. The panel, consisting of miR-122, miR-141 and miR-26b, had an AUC of 0.905 for the discrimination between PBC patients and healthy control, which was superior to established biomarkers for PBC such as AP and ANA.

In summary, the role of circulating miRNA in autoimmune liver disease has so far only been analyzed in a very limited number of studies with comparatively small cohort sizes. Thus, further studies are needed to make a clear statement on the potential role of serum miRNAs as a biomarker for AIH, PSC and PBC.

LIVER CANCER

Circulating miRNAs have also become of increasing interest as biomarkers for hepatic and hepatobiliary malignancies. The following section reviews the emerging role of circulating miRNAs in the field of HCC and cholangiocarcinoma (CCA).

HCC

HCC represents the most common primary tumor of the liver and shows a steadily increasing incidence rate in most areas of the world^[52,53]. Despite being the sixth most common type of cancer worldwide, HCC is the second leading cause of cancer related death among men worldwide, corroborating the dismal prognosis of this disease^[54]. Even in medically developed countries such as the United States, HCC patients face a 1-year and 5-year survival rate of less than 50% and 10%, respectively^[55]. Since early detection of HCC is essential to provide patients with a potentially curative therapeutic approach and established tumor markers such as AFP feature a limited diagnostic potential especially at an early stage of disease, circulating miRNAs as biomarkers for HCC might help to improve the disease's poor prognosis.

As the most abundantly expressed miRNA in human liver tissue^[56], miRNA-122 was found to be up-regulated in serum samples of HCC patients, showing a sensitivity and specificity of 81.6% and 83.3%, respectively when compared to healthy controls^[57,58]. Nevertheless, as shown before, circulating levels of miR-122 were also described for different non-malignant hepatic diseases^[59], arguing for a rather unspecific characteristic of this miRNA. Interestingly, expression levels of miR-122 were decreased in HCC tissue samples^[60], suggesting a potential mechanism of miRNA secretion from HCC cells into the bloodstream.

Moreover, serum levels of exosomal miR-18a, miR-221, miR-222 and miR-224 were significantly higher whereas exosomal miR-101, miR-106b, miR-122 and miR-195 were significantly lower in patients with HCC compared to patients with chronic hepatitis B or liver cirrhosis^[61]. Furthermore, circulating levels of miR-16 were shown to be down-regulated in patients with HCC, correlating with tumor size and were further able to discriminate HCC from chronic HCV patients^[62,63]. In contrast non-malignant liver conditions such as NAFLD and chronic hepatitis C showed increased miR-16 serum levels^[37], making the

down-regulation of serum miR-16 levels in HCC a fairly specific marker for liver cancer. Serum levels of miR-21 represent a further promising tool for the diagnosis of HCC. Tomimaru *et al.*^[64] showed that circulating levels of miR-21 can reliably distinguish between HCC patients and healthy controls as well as patients with chronic hepatitis and are superior to the diagnostic potential of AFP. They also found a decrease of miR-21 serum levels after tumor resection, underlining the potential specificity of this miRNA for HCC^[64]. However, elevated levels of circulating miR-21 were also described in patients with different other types of gastrointestinal cancer^[65,66]. A recently published meta-analysis on circulating levels of miR-21 described a pooled sensitivity of 81.2% with a specificity of 84.8% for the diagnosis of HCC^[67].

Given the fact that the diagnostic power of a single miRNA is limited, various panels consisting of more than one circulating miRNAs have been evaluated as well. Using a microarray to screen 723 miRNAs, Zhou *et al.*^[68] found a panel of seven miRNAs (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801) that distinguished between HCC and healthy controls (AUC = 0.941), chronic hepatitis B (AUC = 0.842) and liver cirrhosis (AUC = 0.884) even at an early stage of disease (BCLC 0) in three independent cohorts of 934 participants. In comparison to AFP, another miRNA panel of seven miRNAs (miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505) was shown to have a superior AUC regarding the diagnosis of small-size (AUC = 0.833 vs AUC = 0.727) and early-stage (AUC = 0.824 vs AUC 0.754) HCCs. This panel did also have the ability to detect AFP-negative HCC patients^[69]. Similar results were obtained for a panel consisting of miR-15b and miR-130 that showed a sensitivity of 98.2% with a specificity of 91.5% for the diagnosis of HCC and had detection sensitivity of 96.7% for patients with low AFP serum levels (< 20 ng/mL)^[70].

Circulating miRNAs might also help to assess patients' outcome and their likelihood to benefit from different treatment options (surgery, systemic treatment, locally ablative treatment) in order to find a personalized therapy for individual patients. For instance, serum levels of miR-221 correlated with tumor size and tumor stage and patients with high levels of circulating miR-221 showed a significantly reduced overall survival compared to patients with lower miR-221 levels^[71]. Likewise, high serum levels of miR-122 were found to independently predict a poor overall survival in a cohort of 122 HCC patients^[72].

CCA

Although CCA represents a rare type of cancer in most parts of the world, it shares a very unfavorable prognosis with a 5-year survival rate of less than 5% for advanced disease stage^[73]. Again, early detection of CCA is necessary to offer patients a surgical tumor resection, which is the only potentially curative treatment option, but the established tumor markers CA19-9 and CEA have a

restricted diagnostic power. Besides the available data on their potential role as a biomarker for HCC, miRNAs have also been evaluated in few studies as a diagnostic tool for CCA.

Based on a CCA tissue expression analysis, which revealed 262 regulated miRNAs in tumor samples, circulating levels of miR-21 and miR-221 were found to be significantly elevated in patients with intrahepatic CCA, showing a high discrimination ability of miR-21 between patients and healthy controls (AUC = 0.94)^[74]. Nevertheless, these results are limited due to a small number of analyzed patients ($n = 25$). MiR-21 was further evaluated in a cohort of 94 patients with biliary tract cancer (BTC) and showed an AUC of 0.93 and 0.83 for the differentiation between BTC and healthy controls and BTC and non-malignant bile duct disease, respectively^[75]. Interestingly, serum levels of miR-21 decreased after surgical tumor resection^[75]. In another rather small study including a total of 30 CCA patients, Bernuzzi *et al.*^[49] identified circulating miR-483-5p and miR-194 as dysregulated in CCA patients. Furthermore, serum levels of miR-483-5p and miR-222 were able to discriminate between PSC and CCA patients. Other circulating miRNAs that were shown to be dysregulated in CCA patients are miR-224^[76] and miR-150^[77].

Some studies have also evaluated a potential use of miRNAs as a prognostic tool for CCA. Analyzing 103 patients with CCA, Cheng *et al.*^[78] described decreased serum levels of miR-106 as a predictor for poor survival. Moreover, elevated levels of circulating miR-26a correlated with disease stage and were reported to be an independent prognostic marker for CCA patients.

In summary, circulating miRNAs are of increasing interest for the diagnosis and prognosis of liver cancer. Although reliable data on serum/plasma miRNAs in the field of CCA are limited, circulating miRNAs are likely to play a decisive role for an early detection and the prediction of survival for both analyzed types of liver cancer in future. However, as the diagnostic and prognostic power of a single miRNA is limited, panels of different miRNA are needed to exceed the established biomarkers for liver cancer. In this context, larger studies will help to further evaluate and verify potential miRNAs for these purposes.

CONCLUSION

Circulating miRNAs represent a promising new tool for the diagnosis and prediction of prognosis for various acute and chronic liver diseases. Despite their obvious potential as biomarkers, there are several problems that prevent the use of circulating miRNAs as diagnostic tools in clinical routine. Most importantly, despite years of intensive research no consensus on optimal protocols for standardization of sample collection, data normalization and analysis was reached until now. As qPCR and microarray based measurements naturally depend on the design of miRNA specific primers or microarray probes, similarities between different miRNAs might result in

further difficulties regarding the comparison between studies. Moreover, data normalization issues mainly arise from the lack of a valid intrinsic RNA housekeeping gene for human serum samples and high inter-platform differences in miRNA quantification efficacy contribute to a poor comparability between studies. Finally, most studies are carried out as single center study including only a small number of patients. Therefore, next generation sequencing might have an important impact on the validation of miRNA profiles, as it allows mostly sequence independent, parallel measurement and detection of overall numbers of a broad spectrum of different miRNAs (reviewed *e.g.*, in^[79]). Thus, only if these present limitations can be overcome, circulating miRNAs might take the next step to be finally implemented in diagnostic algorithms or be used to estimate the clinical fate of patients with acute or chronic liver diseases.

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