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Differential expression and functions of microRNAs in liver transplantation and potential use as non-invasive biomarkers

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Abstract

MicroRNAs (miRNAs) are important regulators in many biologic processes and have been implicated in the control of genes relevant to acute rejection and liver functions. Here we review the miRNAs specifically expressed in allografts during acute rejection and discuss potential roles for these miRNAs in liver dysfunction. We focus on miRNAs dysregulated both in the liver and in peripheral blood mononuclear cells and include a discussion of the potential for these miRNAs as non-invasive biomarkers to reflect liver status posttransplant.

Keywords

MicroRNA; circulating miRNA; liver transplantation; acute rejection; liver dysfunction; noninvasive biomarker

Introduction

Liver transplantation remains the ultimate treatment for many end-stage liver diseases. Although the liver has a unique immunologic microenvironment that promotes tolerance rather than immunity [1], rejection frequently occurs following liver transplantation and lifelong immunosuppression is still the norm for most recipients of liver allografts [2, 3]. Moreover, recurrent hepatitis C virus (HCV), hepatitis B virus (HBV) and vascular complications after liver transplantation remain problems causing graft failure and death [4, 5, 6, 7]. There is considerable interest in developing biomarkers for early detection of liver rejection and complications posttransplant with the ultimate goal of improving long-term graft outcomes. MiRNAs are small noncoding RNA molecules of about 22 nucleotides that regulate the posttranscriptional expression of target genes [8]. MiRNAs are important regulators in many biologic processes including innate and adaptive immune responses; they

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may govern expression of genes relevant to allograft rejection [9]. MiRNAs are extremely stable in plasma or serum suggesting potential use of noninvasive biomarkers [10, 11]. Previous studies from our group and others have reported the miRNA profile during heart, kidney and small intestine rejection [11, 12, 13]. In this review, we examined and discussed the miRNAs in liver allografts with the goal of determining miRNAs that are liver-specific and those that are expressed in all organs during graft rejection. The potential functions of candidate miRNAs in liver diseases and immune responses from bioinformatics analysis and current publications are discussed.

Differential expression of miRNAs after liver transplantation

MiRNAs have emerged as important regulators of immune system and immune functions [14]; they regulate central elements of the adaptive immune responses [15, 16]. Detection of intragraft and circulating miRNA expression is the first step to identify important miRNAs in transplantation and explore potential miRNA biomarkers of allograft injury and function. Studies have shown that alterations in miRNA expression of renal allografts associate with acute rejection (AR), suggesting miRNA expression patterns are predictive of allograft status [12, 17]. Moreover, miRNA levels in urine samples provide a strategy for earlier diagnosis of renal allograft function. For example, miR-210 levels identify patients with acute rejection and predict long-term kidney function [18]. Differential expression of miR-142-3p, -204 and -211 was observed in urine samples of patients with chronic allograft dysfunction, supporting the potential use of miRNAs as noninvasive markers for monitoring graft function [19]. A study by Asaoka et al. found a positive correlation between the intragraft expression levels of miR-142-3p, -886-3p and -132 and 17 mRNAs including CTLA4 and GZMB during acute cellular rejection in patients following small intestinal transplantation [13]. We detected microRNA expression profiles in heart graft and graft infiltrating lymphocytes (GILs) between AR and non-rejection groups (NR) using mouse heart transplantation model [11]. Interestingly, expression of miR-143-3p also increased significantly in GILs from our study, suggesting that miR-142-3p may have an important role in the activation of infiltrating cells during AR. In order to identify differential expression of miRNAs during AR in liver transplantation, we profiled miRNA expression (our unpublished data) between allogeneic and syngeneic liver grafts on day 7 posttransplant by TaqMan array rodent miRNA A Card in rat orthotopic liver transplantation (OLT) model (n=3). This time point was chosen for analysis because our previous experiments demonstrated that severe rejection apparent at seven days in our model of allogeneic OLT [20]. Of the 226 rat mature miRNAs examined, 156 miRNAs with Ct < 35 in all graft samples were included for analysis. Unsupervised hierarchical clustering of miRNA expression patterns classified the allogeneic liver grafts distinctly from the syngeneic liver grafts (Figure 1A), suggesting that differential expression of miRNAs associates with liver allograft rejection. Forty-six miRNAs were significantly changed in allogeneic liver grafts as compared to syngeneic liver grafts (Figure 1B). Of these miRNAs, 28 miRNAs showed expression fold change > 2, including increased expression of miR-204, miR-210 and -142-3p, and decreased expression of let-7b, -122, -200a and -31. A recent study also examined intragraft miRNA levels between AR and NR groups using rat OLT model [21]. Of those significantly changed miRNAs, about 30% miRNAs overlapped in our

data, including 10 increased miRNAs (miR-17, -27a, -93, -130b, -142-3p, -210, -221, -222, -342 and -363) and 5 decreased miRNAs (miR-31, -101, -122, -192 and -194). The results demonstrate that miRNAs are differentially expressed in liver allografts during rejection, which may play important roles in liver function and immune response.

Functions of liver-specific miRNAs in liver allograft rejection

Since miRNAs are highly conserved in both structure and function across species [8, 22], we compared our data of liver miRNA expression to the profiles observed in heart, kidney and small bowel allografts in order to determine if the differentially expressed miRNAs are liver-specific (Table 1A). We chose these miRNA expression profiles for comparison because of the same miRNA microarray platform was used in the four studies, in order to minimize discrepancy caused by different composition of miRNA probes and detection methods. Interestingly, miR-142-3p was found increased in all solid organ grafts during allograft rejection (Table 1B). The miRNA miR-126-5p was decreased in liver, heart and kidney grafts. Of note, miR-204 expression was increased significantly in liver grafts during rejection, but it showed decreased expression in heart and kidney grafts. We next selected 13 miRNAs that were only differentially expressed in liver grafts, either increased or decreased with a fold change >2 during rejection for further analysis (Table 1B), including miR-122, miR-101 and let-7b, which are the most abundantly expressed miRNAs in liver [23]. These data suggests that some miRNA expression may be organ-specific in allograft rejection; the miRNAs differentially expressed only in liver graft may associate with changes in liver status posttransplant. We utilized IPA (Ingenuity Systems, www.ingenuity.com) to predict the potential functions of these differentially expressed miRNAs. The predicted targets of each miRNA were firstly obtained from TargetScan (www.targetscan.org), and then evaluated in IPA for a core analysis. The miRNA let-7b is predicted to be involved in liver fibrosis, hepatitis, hypoplasia, necrosis, and proliferation (Table 2), suggesting roles in liver functions posttransplant. The miRNA let-7b is a member of let-7 miRNA family which has a highly conserved genomic organization [24]. The let-7 family has reported roles in human liver development [25] and human hepatocellular carcinoma by inhibiting Bcl-xL expression [26]. Moreover, the let-7 miRNA family enhances heme oxygenase-1 (HO-1) by suppressing Bach1 and attenuates oxidant injury in human hepatocytes; overexpression of let-7 may protect hepatocytes from oxidant injury [27]. Another miRNA, miR-122, which constitutes 70% of the total miRNA pool in the liver, is involved in liver regeneration (Table 2). The sequence of the mature miR-122 miRNA is completely conserved across species and this miRNA is specific to the vertebrate lineage [28]. Moreover, it has been shown that mice lacking miR-122 possessed several key phenotypes of human liver diseases, including hepatic inflammation, fibrosis, and a high incidence of hepatocellular carcinoma [29, 30]. MiR-122 plays role in the HCV replication cycle [31] and a therapeutic targeting miR-122, Miravirsin (SPC3649) has been shown to provide continuous and prolonged anti-viral activity in patients with HCV. Our miRNA microarray data shows decreased expression of both let-7 and miR-122 in liver grafts during rejection (Table 1A) and suggests additional studies focused on increasing expression of these miRNAs could improve liver graft function. Our approach also suggested other miRNAs, including miR-101, -205 and -367c, that could alter liver functions (Table 2). However, elucidation of their roles in liver

dysfunction is limited to a few studies in hepatocellular carcinoma (HCC). For example, miR-101 inhibits human HCC cells migration and invasion, downregulation of miR-101 associates advanced tumor progression of human HCC [32]. Of note, two studies reported that miR-101 inhibits cell proliferation of human HCC cells [33, 34], which is consistent with our bioinformatics prediction of a potential role for miR-101 in proliferation. In summary, these liver graft-specific miRNAs may play important roles in liver functions posttransplant, holding promise for the development of miRNA-based therapeutics and potentially as biomarkers of liver status.

MiRNAs in immune response during allograft rejection

A major pathological feature of acute rejection after OLT is lymphocyte infiltration into the graft [35]. Thus, it is important to examine the contribution of lymphocyte miRNAs during graft rejection. We found that miR-142-3p was differentially expressed in all solid organ allografts during acute rejection (Table 1B), but was not predicted to be associated with liver dysfunction, suggesting that infiltrating lymphocytes may be the source of miR-142-3p expression. Indeed, miR-142-3p is highly expressed in monocytes, B and T lymphocytes as well as in several hematopoietic cell lines [36, 37]. MiR-142-3p is dominantly expressed in CD8 T cells and its expression is dynamically altered during the differentiation process [38]. Thus, increased expression of miR-142-3p in grafts during acute rejection is likely caused by higher numbers of infiltrating cells. Studies have shown that dysregulation of miR-142-3p is important in the innate immune response and some autoimmune diseases. For example, miR-142-3p expression is decreased in dendritic cells (DCs) after LPS stimulation; it regulates DC responses to LPS by targeting on IL-6 mRNA [39]. Reduced expression of miR-142-3p in the CD4 T cells of patients with systemic lupus erythematosus (SLE) results in aberrant T cell responses and B cell hyperresponsiveness [40]. Importantly, a recent study showed that expression of miR-142-3p was significantly higher in B cells from operationally tolerant renal transplant recipients [41]. Although the specific role of miR-142-3p in immune cells after transplantation remains to be studied, evidence suggests that regulation of miR-142-3p expression in lymphocytes may impact graft outcomes. The miRNA miR-126 was also found to be differentially expressed in solid organ grafts during rejection (Table 1A). Studies suggest a role for miR-126 in allergy, with inhibition of miR-126 resulting in diminished Th2 responses, inflammation, and airway hyperresponsiveness [42]. Another study demonstrated that miR-126 regulates DNA methylation in CD4 T cells by directly targeting Dnmt1, leading to T cell autoreactivity [43]. However, a functional role for miR-126 in alloimmune response has not been identified. In previous work we demonstrated that miR-17, -31 and -130b, which are expressed in immune cells [44], were increased in the lymphocytes infiltrating the heart during rejection [11], suggesting potential roles of these miRNAs in alloimmune responses during rejection. These miRNAs were similarly expressed in rejecting liver allografts (Table 1A). Studies demonstrated that miR-17 facilitates T cell expansion upon antigen challenge, promotes the *in vivo* Th1 response and effector T cell responses by targeting TGF β R2 and CREB1, and inhibits Treg differentiation [32, 33]. MiR-31, expressed uniquely in T cells among human immune cell subsets, plays a role in differentiation of naive T cells to T regulatory cells by negatively regulating FOXP3 expression [47, 48]. MiR-130b expression

is dramatically increased following T cell activation and able to inhibit CD69 expression [49]. Taken together with our findings, these miRNAs may play important roles, such as activation, proliferation and differentiation in lymphocytes during liver graft rejection.

MiRNAs as non-invasion biomarkers for acute rejection in liver transplantation and liver diseases

Circulating miRNAs, which are extremely stable and protected from RNAase mediated degradation in body fluids, have emerged as candidate biomarkers for many diseases [50, 51]. The use of miRNAs as noninvasive biomarkers is of particular interest in the transplantation field, because examination of tissue biopsies is costly, invasive and can be associated with complications. Here, we summarize the recent reports describing circulating miRNAs associated with liver transplantation and liver disease (Table 3), which hold promise to be noninvasive biomarkers for early detection of liver graft rejection and complications posttransplant. It was demonstrated that hepatocyte-derived miRNAs, miR-122, -148a and -194, correlated with hepatic injury and acute rejection after liver transplantation [52]. The serum levels of these miRNAs were increased in patients with liver injury and positively correlated with aminotransferase levels. Importantly, miRNA expression was elevated earlier than aminotransferase levels during acute rejection. In another recent study plasma miRNA expression was examined in the rat OLT model during acute rejection and the results indicated that plasma miR-122 and miR-192 were increased during liver injury, and plasma miR-146a levels associate with cellular rejection [21]. Several studies have quantitated circulating miRNAs as potential biomarkers for HBV and HCV infection. Four serum miRNAs, let-7c, miR-23b, miR-122, and miR-150, which differentially expressed in patients with HBV, shows a high level of accuracy in discriminating HBV cases from the non-infected controls [53]. The miRNAs, miR-155, -125b, -146 and -122 were increased in serum from HCV patients as compared to controls, the increased serum levels of miR-122 correlated with increased ALT and AST levels [54]. Increased serum miR-21 levels were found to be correlated with the histological activity index in the liver, and the ALT, AST, bilirubin, and γ -glutamyltransferase levels in patients with HCV infection [55]. Another study reported that serum levels of miR-122, -34a and -16 were significantly increased, with miR-21 unchanged in patients with HCV [56]. The conflicting results of serum miR-21 levels may be attributed to the endogenous control used for normalization. Thus, it is critical to choose proper endogenous controls for normalization in these studies. Currently, the spiked-in miRNAs that are not expressed in human or rodent, such as cel-miR-39 and cel-miR-54, are good controls to normalize sample-to-sample variation during RNA extraction [10, 11, 54, 57]. Two studies showed that serum miR-122 and miR-192 levels were substantially higher in acetaminophen-induced acute liver failure as well as in a mouse model of acetaminophen-induced acute liver injury [58, 59]. In patients with biliary atresia (BA), it was reported that miR-200b/429 cluster was significantly increased in the serum of patients with BA relative to infants with non-BA cholestatic disorders [57]. Taken together, these studies indicate that increased expression of miRNAs, including miR-122 can be detected in the serum or plasma from patients with many liver diseases. Circulating miRNAs possess many of the characteristics of an ideal biomarker for liver status since they are non-invasive, stable and easily detected in the

blood. Thus, it provides an inspiring potential to emerge as novel blood-based biomarker in liver transplantation.

In summary, we identified, predicted and discussed the potential functions of miRNAs after liver transplantation. Acute liver graft rejection results in altered expression of a subset of miRNAs in liver grafts, which may have important roles in liver dysfunction posttransplant. Decreased expression of miR-122 and let-7b in liver grafts is likely associated with liver status during rejection. Further, we suggest that, the miRNAs, such as miR-142-3p that are expressed in all organ grafts, associate with lymphocyte alloimmunity during rejection. Modulation of expression of these miRNAs may lead to improved liver function and reduced alloimmune response after liver transplantation. Moreover, circulating miRNAs may be promising biomarkers for reflecting liver graft status posttransplant.

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Highlights

- Differential expression of miRNAs associates with liver allograft rejection.
- Liver graft-specific miRNAs play important roles in liver functions posttransplant.
- MiRNAs play important roles, such as activation, proliferation and differentiation in lymphocytes during liver graft rejection.
- Potential use of miRNAs as noninvasive biomarkers to reflect liver status posttransplant.

Figure 1. Differential expression of miRNAs associates with liver allograft rejection

Each column corresponds to the expression profile of syngeneic or allogeneic liver grafts on day 7 posttransplant. The color of each cell reflects the fold-change of the expression level of each miRNA relative to the average expression level across all samples. Green represents a decrease in expression, whereas red represents an increase.

A. Unsupervised hierarchical clustering of overall miRNAs expression in liver grafts.

B. Unsupervised hierarchical clustering of the miRNAs with differential expression at $p < 0.05$ in liver grafts.

Table 1A

MiRNA profiles in liver, heart, kidney and small bowel allografts

Rodent				Human			
Liver		Heart		Kidney		Small bowel	
Allo (n=3) vs Syn (n=3)		Allo (n=9) vs Syn (n=9)		AR (n=3) vs NR (n=4)		AR (n=15) vs NR (n=11)	
<i>MicroRNA</i>	<i>Fold</i>	<i>MicroRNA</i>	<i>Fold</i>	<i>MicroRNA</i>	<i>Fold</i>	<i>MicroRNA</i>	<i>Fold</i>
miR-204	19.31	miR-147	13.48	miR-142-5p	18.48	miR-494	9.66
miR-196c	13.33	miR-451	13.26	miR-142-3p	16.61	miR-886-3p	8.3
miR-20b-3p	5.85	miR-96	12.05	miR-155	14.53	miR-19a	5.98
miR-222	5.40	miR-182	6.95	miR-650	11.31	miR-223	4.03
miR-363	5.24	miR-210	6.45	miR-223	9.2	miR-142-3p	3.26
miR-376c	4.74	miR-183	5.71	miR-146a	3.65	miR-16	3.26
miR-142-3p	3.73	miR-500	4.67	miR-21	3.45	miR-146b-5p	2.95
miR-127	3.66	miR-7	4.34	miR-146b	2.9	miR-132	2.55
miR-210	2.64	miR-142-3p	3.43	miR-342	2.9	miR-146a	2.35
miR-17-3p	2.39	miR-21	3.24	miR-425-5p	2.13	miR-26b	2.22
miR-500	2.35	miR-130b	3.10	let-7a	0.67	miR-150	2.2
miR-221	2.27	miR-25	2.71	miR-100	0.62	let-7e	2.13
miR-130b	2.05	miR-222	2.70	miR-186	0.62	miR-20b	2.07
miR-27a	2.00	miR-18a	2.63	miR-99a	0.57	miR-195	2.03
miR-342	1.97	miR-324-3p	2.56	miR-365	0.55	miR-26a	1.85
miR-339-3p	1.93	miR-15b	2.53	miR-141	0.52	miR-886-5p	1.79
miR-34c	1.91	miR-339	2.49	miR-296	0.52	miR-125a-5p	1.77
miR-674	1.88	miR-652	2.45	miR-497	0.51	miR-100	1.77
miR-17	1.80	miR-155	2.44	miR-152	0.51	miR-193b	1.76
miR-93	1.44	miR-362-3p	2.43	miR-200a	0.5	let-7d	1.68
miR-7b	1.35	miR-200c	2.40	miR-26a	0.49	miR-10a	1.65
miR-542-3p	1.29	miR-221	2.38	miR-26b	0.49	miR-126-3p	1.49
miR-92a	1.19	miR-324-5p	2.28	let-7c	0.48	miR-139-5p	1.31
miR-130a	0.74	miR-142-5p	2.28	miR-130a	0.48	miR-374b	1.17
miR-361	0.72	miR-10a	2.18	miR-126-3p	0.48	let-7g	1.17
miR-126-5p	0.70	miR-132	1.94	miR-195	0.48	miR-192	0.18
let-7d	0.66	miR-148b	1.82	miR-32	0.46	miR-194	0.1
miR-182	0.60	miR-744	1.74	miR-125b	0.45	miR-375	0.07
miR-190	0.58	miR-92a	1.73	miR-30a-5p	0.44		
miR-145	0.56	miR-361	1.72	miR-30d	0.44		
let-7c	0.51	miR-20a	1.70	miR-203	0.43		
let-7a	0.50	miR-532-3p	1.61	miR-204	0.43		
let-7b	0.48	miR-188	1.60	miR-30e-5p	0.43		
miR-101	0.48	miR-340-3p	1.56	miR-10b	0.42		
miR-194	0.48	miR-301a	1.37	miR-125a	0.41		
miR-139-5p	0.46	miR-23b	0.72	miR-30b	0.4		

Rodent				Human	
miR-31	0.46	miR-30a	0.70	miR-196b	0.4
miR-192	0.45	miR-26a	0.65	miR-199a	0.4
miR-200a	0.44	miR-30b	0.64	miR-660	0.4
miR-96	0.38	miR-365	0.63	miR-30e-3p	0.36
miR-802	0.37	miR-203	0.62	miR-429	0.36
miR-205	0.34	miR-34b-3p	0.57	miR-181c	0.36
miR-183	0.32	miR-195	0.57	miR-126-5p	0.35
miR-122	0.31	miR-139	0.56	miR-193a	0.35
miR-375	0.21	miR-145	0.55	miR-378	0.35
miR-499	0.03	miR-30e	0.55	miR-10a	0.34
		miR-335	0.54	miR-30a-3p	0.32
		miR-193b	0.54	miR-27b	0.32
		miR-424	0.52	miR-30c	0.31
		miR-30c	0.47	miR-143	0.26
		miR-126-5p	0.45	miR-489	0.26
		miR-208	0.42	miR-193b	0.24
		miR-1	0.38	miR-9	0.2
		miR-136	0.38		
		miR-126-3p	0.36		
		miR-133a	0.35		
		miR-224	0.34		
		miR-497	0.34		
		miR-455	0.32		
		miR-199*	0.31		
		miR-204	0.29		
		miR-676	0.27		
		miR-208b	0.24		
		miR-135b	0.18		
		miR-135a	0.13		
		miR-302b	0.11		

Rodent-only miRNAs were removed from heart and liver miRNA profiles

MiRNA profiles of heart, kidney and small bowel grafts were adapted from reference [11], [12] and [13]

Table 1B

Differential expression of miRNAs during graft rejection

In liver, heart, kidney and small bowel
miR-142-3p*In liver only (fold change > 2)*

miR-196c

miR-20b-3p

miR-363

miR-376c

miR-127

miR-17-3p

let-7b

miR-101

miR-31

miR-802

miR-205

miR-122

miR-499

Table 2

Potential functions of miRNAs in liver diseases

Diseases	MiRNAs	P values
Biliary Hyperplasia	miR-205	4.37E-04
Hepatocellular Carcinoma	miR-205	4.37E-04
Liver Damage	miR-122	1.20E-04
Liver Dysplasia	miR-205	6.20E-03
Liver Fibrosis	let-7b	2.66E-03
Liver Hepatitis	let-7b	3.92E-03
	miR-205	9.53E-03
Liver Hyperplasia/Hyperproliferation	miR-205	4.37E-04
Liver Hypoplasia	let-7b	1.27E-03
Liver Necrosis/Cell Death	let-7b	8.98E-06
	miR-101	6.77E-03
Liver Proliferation	let-7b	6.47E-06
	miR-101	4.91E-03
	miR-367c	3.13E-03
Liver Regeneration	miR-122	2.89E-02

MiRNAs predicted to be involved in liver diseases at P value < 0.01 were selected

Table 3

Circulating miRNA expression in liver transplantation and liver diseases

MicroRNA	Source	Disease	Species	miRNA Expression change	Correlation with ALT/AST activities	Detection method	Endogenous control	Groups	Ref
miR-122, -148a	serum	acute rejection after liver transplantation	human	increase	yes	Taqman microRNA assay	N/A	rejection, nonrejection, control	[52]
miR-122, -148a, -194	serum	peritransplant ischemic liver injury	human	increase	yes	Taqman microRNA assay	N/A	transplant recipients, control	
miR-146a	microvesicles from plasma	cellular rejection after liver transplantation	rat	increase	no	miRNA microarray, RT-qPCR	cel-miR-54	acute rejection, control	[21]
miR-122, -192	microvesicles from plasma	liver injury	rat	increase	miR-122 and -192 correlate with ALT	RT-qPCR	cel-miR-54	liver injury, control	
let-7c, miR-23b, -122, -150	serum	ocult hepatitis virus B infection (OBI)	human	increase	high levels in HBV	Taqman microRNA assay	spiked-in plant miR-168	OBI, HBV, control	[53]
miR-122, -125b, -146a, -155	serum	HCV	human	increase	miR-122 expression correlates with ALT/AST levels	Taqman microRNA assay	spiked-in cel-miR-39	cHCV treatment naive patients, sustained responders, healthy control	[54]
miR-21	serum	chronic hepatitis C virus infection (CHC) and CHC-associated HCC	human	increased in CHC, but no change between CHC and CHC-associated HCC	yes	Taqman microRNA assay	miR-16	CHC, CHC with HCC, healthy control	[55]
miR-122, -34a -16	serum	chronic hepatitis C infection (CHC) and non-alcoholic fatty-liver disease (NAFLD)	human	increase in CHC and NAFLD	miR-122 and miR-34a correlates with ALT/AST levels	Taqman microRNA assay	spiked-in cel-miR-238	CHC, NAFLD, healthy control	[56]
miR-122, -192	plasma	drug-induced liver injury	mouse	increase	yes	Array hybridization, SYBR-based qPCR	N/A	acetaminophen, PBS	[58]
miR-122, -192	serum	drug-induced liver injury	human	all increased in APAP ALI patients, miR-122 increased in non-APAP ALI patients	yes	Taqman microRNA assay	U6 snRNA	APAP-induced Acute Liver Injury Patients, Healthy Controls, APAP no ALI Patients, non-APAP ALI Patients	[59]
miR-200b/429 cluster	serum	biliary atresia (BA)	human	increase	N/A	TaqMan microRNA microarray, Taqman microRNA assay	spiked-in cel-miR-54, cel-miR-238	biliary atresia, cholestatic controls	[57]