

# MicroRNAs in Liver Disease: Bench to Bedside

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**MicroRNAs (miRs) are small non-coding RNAs that negatively regulate gene expression by pairing with partially complementary target sequences in the 3'UTRs of mRNAs to promote degradation and/or block translation. Aberrant miR expression is associated with development of multiple diseases including hepatic diseases. The role of miRs in the regulation of gene expression and rapid progress in the field of microRNA research are resulting in momentum toward development of diagnostic markers and novel therapeutic strategies for human liver diseases. Recent studies provide clear evidence that miRs are abundant in the liver and modulate a diverse spectrum of biological functions, thereby supporting an association between alterations of miR homeostasis and pathological liver diseases. Here we review the role of miRs in liver as their physiological and pathological importance has been demonstrated in metabolism, immunity, viral hepatitis, oncogenesis, fatty liver diseases (alcoholic and non-alcoholic), drug-induced liver injury, fibrosis as well as acute liver failure. (J CLIN EXP HEPATOL 2013;3:231–242)**

MicroRNAs (miRs) are a class of highly conserved, chemically stable, short non-coding endogenous RNAs of about 22 nucleotides (nt). They have recently emerged as important regulators of gene expression at the post-transcriptional level. MiRs were first discovered in 1993 in *Caenorhabditis elegans*.<sup>1</sup> There are approximately 17,000 miRs with more than 1900 in humans<sup>2</sup> which are thought to regulate about 60% of all human genes.<sup>3–5</sup> miRs have important regulatory roles modulating cell activities including differentiation and development, tissue remodeling, metabolism, intracellular signaling, immunity, proliferation and apoptosis.<sup>6,7</sup> There is functional redundancy in miR-mediated gene regulation since a single miR may bind to a number of mRNA transcripts and conversely, one mRNA could be targeted by several miR species. Due to their regulatory role in many cellular functions, alteration and/or abnormalities in miRs should continue to have increasing importance in human diseases. Recent studies have demonstrated that miR expression patterns are disease and tissue specific.<sup>8,9</sup> MiRs are abundant in

the liver and modulate a diverse spectrum of cellular processes associated with liver injury such as inflammation, apoptosis, and hepatocyte regeneration and deregulation of miR expression may be a key pathogenic factor in many liver diseases including viral hepatitis, hepatocellular carcinoma (HCC), metabolic and acute liver diseases; miR expression profiles are altered in many hepatic diseases compared to healthy subjects.<sup>10–14</sup> Many miRs are abundantly expressed in the liver; however miR-122 is liver specific, and is estimated to make up 70% of the total hepatic miR complement and is expressed at high levels (around 66,000 copies per cell).<sup>10–12</sup>

Studies suggest that miRs are not only localized within the cell but are also present in circulation. Extracellular circulating miRs can be found in lipid or lipoprotein complexes, apoptotic bodies, or actively secreted into microvesicles or exosomes, providing a mechanistic association between plasma miR level and specific organ dysfunction.<sup>15</sup> This suggests that circulating miRs may represent a new class of prognostic and diagnostic disease biomarkers.<sup>16,17</sup> Exosome secretion by hepatocytes has been characterized and the liver has been shown to release miRs into circulation in both normal physiological and pathological conditions.<sup>18</sup> The liver is a major contributor of circulating miRs and plasma miR levels partially reflect the dynamics of miRs in hepatocytes.<sup>19</sup> Hepatocyte apoptosis and necrosis also release cellular miRs directly into the circulation, explaining the reason for the superior sensitivity of serum miRs when compared to ALT or AST levels in diagnosing liver damage.<sup>20</sup> Bala et al have shown that miR-122 and miR-155 were predominantly associated in protein aggregates in acetaminophen-induced liver necrosis, whereas in alcoholic liver disease associated inflammatory damage, these two miRs were mainly found in the

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**Abbreviations:** miRs/miRNA: microRNA; HBV: hepatitis B virus; HCV: hepatitis C virus; HCC: hepatocellular carcinoma; DILI: drug-induced liver injury; IFN: interferon; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; ALD: alcoholic liver disease; ALF: acute liver failure; UTR: untranslated region; HSC: hepatic stellate cell; PPAR  $\gamma$ : peroxisome proliferator-activated receptor  $\gamma$ ; TNF: tumor necrosis factor; TGF: transforming growth factor

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exosome-rich vesicular fraction, suggesting an injury-specific distribution of miRs in the circulation.<sup>21</sup> Circulating miRs have tremendous stability in extreme conditions including a low pH environment, they are also resistant to the high endogenous RNase activity present in serum due to protein aggregation and vesicle enclosure and are stable in serum incubated at room temperature for at least 24 h or subjected to up to at least eight freeze-thaw cycles.<sup>17,22,23</sup> For these reasons circulating miRs are promising, non-invasive diagnostic biomarkers for liver disease.

## MICRORNA BIOGENESIS

MicroRNA genes can be intergenic or exist within introns or even in exons of long non-coding genes.<sup>24</sup> They are transcribed in the nucleus as a double stranded primary transcript (pri-miR) by RNA polymerase II which is several hundred nucleotides and includes an ~80 nt hairpin loop structure.<sup>25</sup> The pri-miR is post-transcriptionally capped at the 5' end and polyadenylated. A single pri-miRNA may contain up to six miRNA precursors. Subsequently, nuclear enzymes Droscha and Pasha then cleave the pri-miR converting it to a double stranded precursor miR (pre-miR) within the nucleus.<sup>26</sup> The pre-miR is then transported into the cytoplasm by the protein Exportin 5 where the RNase III enzyme Dicer cleaves the hairpin resulting in a 22 nt miRNA: miRNA\* duplex containing a leading and passenger strand (denoted by an asterisk).<sup>27,28</sup> Although either strand of the duplex can act as a functional miRNA usually the leading strand is incorporated into the RNA-induced silencing complex (RISC) via Argonaute protein where the miR binds to 3'UTR or 5'UTR of its target mRNA and the passenger strand is subsequently degraded.<sup>29,30</sup> Depending on the degree of complementarity between the miRNA and target mRNA, the target is either cleaved and degradation when perfect complementarity exists or translation of the target mRNA is blocked if only imperfect base pairing is achieved.<sup>31,32</sup> The sequence between the 2nd and the 8th nt from the 5' end is essential for target recognition.<sup>4</sup> Alteration in any of the above steps could contribute to development of diseases.<sup>33</sup>

## HEPATIC MICRORNAS AND LIVER DISEASE

MiR-122 is strongly up-regulated during embryonic development of the liver.<sup>34</sup> Quantitative analyses of miRs have demonstrated higher and dynamic expression in various developmental stages of liver during the fetal period.<sup>35</sup> MiR-30 is crucial for liver development and it is up-regulated during later developmental stages, influencing known regulators of hepatic function, including epidermal growth factor receptor.<sup>36</sup> Despite our limited understanding of the role of miRs in liver development, they are likely to be important regulators of cell lineage differentiation.

## MicroRNA-122

MiR-122 is a liver-specific, multifunctional RNA that controls liver homeostasis and interacts with many targets involved in lipid and cholesterol bio-synthesis, bilirubin and iron metabolism and oxidative stress-response pathways, among others.<sup>37-39</sup> It is highly expressed in hepatocytes due to its liver specific transcriptional regulation by hepatic nuclear transcription factors<sup>34,40</sup> and is elevated in most hepatic diseases including hepatitis C virus (HCV) and hepatitis B virus (HBV) infections as well as alcohol and drug-induced liver injury, HCC and NAFLD.<sup>13,14,37-45</sup> There are four miR-122 binding sites in the HCV genome<sup>42</sup> and miR-122 may promote viral replication by direct interaction with seed-sequence-binding to two target sites, S1 and S2, in the 5'-UTR of the HCV genome resulting in HCV-RNA genome stabilization and enhanced viral RNA abundance.<sup>42,43</sup> Dedifferentiation of hepatocytes during hepatocellular carcinogenesis is associated with the loss of miR-122.<sup>40,44,45</sup> Serum/plasma levels of miR-122 correlate with hepatic necro-inflammation, liver damage, cell death and increased aminotransferase levels in acute and chronic liver diseases.<sup>44,46-49</sup> Interestingly, hepatic and circulating miR-122 levels do not correlate in NAFLD<sup>14,39,50-55</sup> or viral hepatitis<sup>41,47,49,56</sup> although both have been statistically associated with various measures of disease severity in these studies. Together these studies show that miR-122 may play a role in most liver diseases.

## VIRAL HEPATITIS

Emerging evidence indicates that viruses use their own miRs to manipulate both cellular and viral gene expression.<sup>57</sup> Furthermore, viral infection can exert a profound impact on the host cellular miR expression profile, and several RNA viruses have been reported to interact directly with cellular miRs and/or to use these miRs to augment their replication potential.<sup>58</sup> MiRs are likely to have a role in viral hepatitis as cellular miRs play an important role in viral pathogenesis.<sup>59</sup> The level of plasma miR-122 exhibits an excellent correlation with the necro-inflammatory activity of HBV<sup>41</sup> and HCV<sup>47</sup> infections. Altered miR expressions during virus infection include host miRs that target virus sequences and host genes to modulate and influence viral replication.<sup>60,61</sup>

## Hepatitis B

HBV infection results in chronic hepatitis B (CHB) that confers a high risk of developing cirrhosis and liver cancer.<sup>62</sup> Most common prognostic parameters for sustained response after treatment include female sex, HBV genotype, high baseline ALT levels, low baseline HBV DNA, and HBsAg dynamics during treatment.<sup>63,64</sup> Novel markers are needed to improve the estimation of

prognosis as successful therapy significantly diminishes the risk of cirrhosis, liver failure and hepatoma. The occurrence of miRs has been shown in sera of HBV infected patients and these circulating miRs correlate positively with the severity of HBV-induced liver disease.<sup>65</sup> Around 100 miRs including miR-122, miR-16, miR-223, miR-19b, miR-20a, miR-92a, miR-106a, let-7b and miR-194, were amplified from HBV patients using TaqMan qRT-PCR assays.<sup>65</sup> The level of serum miRs in HBV patients was significantly higher in hepatitis B 'e' antigen (HBeAg) positive patients compared to HBeAg negative patients, with miR-122 and miR-194 being the highest differentially expressed.<sup>65</sup> This suggests that miR expression may be associated with HBeAg production in CHB patients. As HBeAg seroconversion is considered to be an important end point in CHB treatment, miR expression may aid the development of anti-HBV therapeutics.<sup>66</sup> However, a relationship between HB surface antigen (HBsAg) positivity and miRs was not found.<sup>65</sup> There was also no significant correlation observed between miR expression and ALT or HBV DNA levels. Patient age negatively correlated with miR levels, with lower levels of miR-122 and miR-194 in older population. It has also been shown that expression of miR-122, miR-572, miR-575, miR-638 and miR-744 was deregulated in chronic HBV patients and the levels of these miRs were significantly higher than ALT or AST in HBV; miR-122, miR-638, miR-575 and miR-572 were higher while miR-744 was lower.<sup>20</sup> MiR-155 has been demonstrated to play a role in antiviral immunity against HBV infection in human hepatoma cells, HepG2.<sup>67</sup> Differentially expressed miRs in CHB were also found to be associated with HBV induced HCC. A recent report demonstrated the potential of a plasma miR panel (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801) to identify HBV related HCC,<sup>68</sup> while a spate study found that levels of miR-25, miR-375, and let-7f are increased in the circulation of patients with HBV associated HCC.<sup>56</sup> Together these studies highlight the diverse roles that miRs play in the cellular response to CHB.

Treatment of chronic HBV with IFN leads to a sustained virologic response (SVR) in a limited number of patients (19%) with considerable adverse effects, sub-optimal pharmacokinetics and still remains the therapy in some developing nations. Zhang et al showed that a miR profile comprising 11 miRs (hsa-let-7a, hsa-miR-30a, hsa-miR-1290, hsa-miR-106b, hsa-miR-198, hsa-miR-1224-5p, hsa-miR-1281, hsa-miR-22, hsa-miR-638 and hsa-let-7f) can predict an initial treatment response (independent association with early virologic response) of IFN in HBV patients.<sup>19</sup> This study showed that there is a potential role of selected miRs in HBV life cycle. MiRs that altered HBsAg/HBeAg secretion were considered to possess HBV modulatory activity. While a miR-1281 inhibitor showed the highest potency in inhibiting HBV replication, let-7f,

miR-939, and miR-638 were also shown to inhibit viral replication.<sup>19</sup> MiR-939 and miR-638 were also shown to inhibit HBV viral antigen expression in a dose-dependent manner.<sup>19</sup> In a separate study, HBV replication was inhibited by miR-199a-3p, as a consequence of direct interaction of the miR and the HBV coding region.<sup>69</sup> Let-7a, c and f have been reported to be down-regulated in HepG2.2.15 cells that harbor the complete HBV genome and replicate actively by HBx protein.<sup>70,71</sup> MiR-29c also appears to have antiviral effect against HBV.<sup>72</sup>

Unlike HCV, it is unclear if miR-122 plays a crucial role in HBV replication. MiR-122 inhibition with a miR-122 antagomir significantly increased HBsAg and HBeAg secretion in HuH7 cells and miR-122 overexpression in HepG2 cells resulted in a marked reduction of HBsAg and HBeAg expression. This was associated with suppression of the anti-oxidative and anti-inflammatory gene heme oxygenase 1 (HMOX-1) and decreased HBV replication suggesting that miR-122 is antiviral for HBV, but pro-viral for HCV.<sup>73</sup>

## Hepatitis C

Studies have reported specific miRs altering HCV infection and/or being altered by the presence of the virus. The mechanism of interaction of miR-122 with the HCV genome is only partially understood. Interaction of miR-122 with the 5' non-coding region of the HCV genome was found to be critical for viral RNA abundance.<sup>11</sup> Down-regulation of miR-122 *in vitro* and *in vivo* has led to significant inhibition of viral replication.<sup>11</sup> However, viral infection and replication does not affect the expression of miR-122.<sup>74</sup> Recently, Marquez et al analyzed the expression of miR-122 and miR-21 in liver biopsy samples of HCV-infected patients and uninfected controls and found miR-122 levels to be inversely correlated with fibrotic stage, ALT, AST, while miR-21 correlated positively.<sup>75</sup> It was speculated that the dysregulation of miR-21 and miR-122 rather than the expression levels, could be related to fibrosis.

Several miRNAs have been shown to be involved in HCV entry, replication and propagation including: miRs 24, 149, 638 and 1182.<sup>59</sup> Sustained HCV replication also showed a marked dependence on miR-141 induction and the miR-141-targeted down-regulation and depletion of tumor suppressor "deleted in liver cell-1" (DLC-1) protein.<sup>76</sup> This is an example of "oncomiR addiction".<sup>76</sup> Since miRs target multiple genes, oncomiRs can be useful as potential targets of antagomir-based HCV therapy. Virus replication in infected hepatocytes was also induced by artificially increasing intracellular miR-141. The mechanism of miR-141 expression following HCV infection has yet to be determined, but depletion of this miR inhibits viral replication. It is established that miR-491 induces apoptosis,<sup>77</sup> suggesting its involvement in the early steps

of HCV infection followed by a decrease in its expression, which might favor the development of a hepatoma. A stimulatory effect of miR-192 and -215 has been demonstrated on HCV replication.<sup>78</sup> MiR-192 and -194 are liver-specific miRs<sup>79,80</sup> and are associated with SVR in chronic hepatitis C patients.<sup>81</sup>

Binding sites for miR-199a-3p have been found in the 5'UTR internal ribosome entry site (IRES) of HCV RNA (genotypes 1b and 2a) suggesting a direct interaction between these molecules.<sup>82</sup> It was also shown that overexpression of miR-199a-3p has an inhibitory effect on virus replication, whereas viral replication and protein expression are promoted by the suppression of miR-199a-3p via direct binding to the 5'UTR IRES. Increased miR-199a-3p expression was associated with SVR in chronic hepatitis C patients when compared with non-SVR.<sup>81</sup>

MiR-196b has been reported to have a target site in the NS5A coding region of HCV RNA<sup>57</sup> and its expression was found to be modulated upon IFN- $\beta$  treatment of primary mouse hepatocytes.<sup>57</sup> In addition to the direct targeting of the HCV RNA, Bach1, a repressor of HMOX1, is also a direct target of miR-196b.<sup>83</sup> As HCV infection causes oxidative stress and miR-196b inversely modulates HCV replication and HMOX1 expression, overexpression of miR-196b appears as a potential strategy to protect against liver injury during chronic hepatitis.

The relationship between HCV infection and miR-29 expression in hepatocytes and hepatic stellate cells (HSC) has been reported to show that the activation of HSCs leads to the down-regulation of miR-29.<sup>84</sup> Over expression of miR-29 in infected cells resulted in a 70% decrease of HCV replication as well as inhibition of HSC proliferation and collagen production. MiR-29a, -29b and -29c levels were noted to be increased in livers of HCV with SVR when compared to non-SVR,<sup>81</sup> thus suggesting a role of these biomarkers to monitor the response to anti-HCV treatments.

HCV infection results in modulation of miR that control viral particle entry and propagation, thus playing an important role in host immune evasion.<sup>37</sup> A marked increase of miR-155 has been observed in HCV-infected patients.<sup>85</sup> MiR-155 is thought to be involved in the Wnt signaling pathway which could contribute to HCV-induced hepatocarcinogenesis. MiR-449a plays an important role in modulating expression of YKL40 (an inflammatory marker) through targeting the components of the NOTCH signaling pathway following HCV infection.<sup>86</sup> In HCV patients, miR-449a was down-regulated, thereby up-regulating its target—NOTCH1, which further leads to up-regulation of TNF $\alpha$  mediated YKL40 expression. Thus, HCV induced down-regulation of miR-449a in human livers can up-regulate transcriptional factors leading to increased inflammatory response, promoting cell proliferation that can result in HCC.

Miravirsen (SPC3649), an antagomir, is a locked nucleic acid-modified oligonucleotide, complementary targeting miR-122 seed sequences, which is subsequently sequestered leading to a long-lasting HCV viral suppression.<sup>87</sup> A weekly injection of Miravirsen in HCV genotype-1 patients for one month followed by standard therapy has shown a continuous and prolonged antiviral activity, high barrier to resistance, with minimal side effects.<sup>88</sup> Further, Miravirsen treatment in HCV genotype 1 patients resulted in serum interferon-gamma inducible protein (IP)-10 reduction prior to decline in HCV RNA, suggesting its role in inducing a decrease in endogenous interferon pathway activity and provides a rationale to explore the utility of Miravirsen lead-in strategy prior to IFN-containing triple regimens.<sup>89</sup> Miravirsen has also demonstrated broad antiviral activity against HCV replicons resistant to NS3, NS5A and NS5B inhibitors.<sup>90</sup> Thus, this anti-miR-122 agent is a promising anti-HCV therapeutic.

MiRs have been reported to play role in IFN-mediated antiviral defense. IFN-mediated inhibition of HCV replication involves the induction of miRs which have complementary sequences within HCV genomic RNA.<sup>57</sup> IFN treatment also down-regulated miR-122 expression, that is required for HCV RNA 5'UTR interaction to promote viral RNA accumulation.<sup>11</sup> However, no positive correlation was observed between intrahepatic miRs or IFN induced miRs and HCV RNA levels in tissue samples from HCV infected patients, suggesting a complex role of miRs in HCV and IFN mediated effects.<sup>91</sup> MiRs 1, 30, 128, 196 and 296 are differentially expressed in HCV infected patients and induced by IFN- $\alpha$  treatment.<sup>92</sup> IFN- $\beta$  has shown to induce several miRs like miR-296, miR-351, miR-431 and miR-448 with sequence complementarity to the HCV RNA genome resulting in inhibition of HCV RNA replication.<sup>57</sup> Interestingly, miR-122 was substantially down-regulated (80%) in IFN- $\beta$  treated Huh cells.<sup>57</sup> This is also supported by evidence of lower pre-treatment levels of miR-122 among non-responders as compared to patients achieving complete EVR with dual therapy using IFN and ribavirin.<sup>91</sup>

Modulation of one or more of these miRs, may represent a new anti-HCV therapy that could also be used in conjunction with standard treatments to increase their efficiency.

## ACUTE LIVER FAILURE (ALF)

ALF is a complex multisystemic disease marked by degeneration and necrosis of the liver which induces hepatosis, coagulopathy, jaundice and hepatic encephalopathy, in the absence of a history of chronic liver disease.<sup>93</sup> It is a life-threatening condition with high mortality rates and it is often difficult to diagnose, given the lack of specific symptoms. Early intervention is necessary for positive outcomes.

During both acute and chronic liver damage, miR-122 was markedly reduced in the injured liver and correlated inversely with hepatic damage and ALT levels, thus suggesting its involvement in ALF in humans.<sup>94</sup> Paraquat exposure in humans led to a marked elevation of miR-122 and concomitant down-regulation of miR-483 and miR-711,<sup>95</sup> similar to that seen in acetaminophen-overdosed mice.<sup>18</sup> Wang et al have shown up-regulation of serum miR-122 and miR-192, preceding the elevation of transaminases, particularly ALT, after acute hepatic intoxication by acetaminophen in mice, yet decreased levels of these miRs were observed in liver tissue.<sup>18,41</sup> As these miRs could be detected before marked cell death occurs in the liver, they have the potential to serve as better indicator of liver failure than liver enzymes.<sup>41,96</sup> A recent study showed that miR-155, miR-146a, miR-125a, miR-15b and miR-16 were up-regulated and miR-1187 was down-regulated significantly during ALF in mice.<sup>97</sup> Hepatic miR-1187 regulates hepatocyte apoptosis by targeting caspase-8, a key protease in the death receptor signaling pathways.<sup>97</sup> D-galactosamine (D-GalN) plus lipopolysaccharide (LPS) induced ALF in mice that resulted in down-regulation of miR-1187 and an up-regulation of caspase-8 (both mRNA and protein level) and was predicted to target the caspase-8 mRNA 3'UTR.<sup>97</sup> These and other studies demonstrate that miRs regulate death receptor-mediated hepatocytes apoptosis in ALF.<sup>98,99</sup> Serum miRs that regulate hepatic apoptotic pathways may be shed into the circulation and reflect the degree of hepatic cell death could serve as reporters for progression of ALF.

## DRUG INDUCED LIVER INJURY (DILI)

Acetaminophen induced ALF is a significant cause of DILI and mortality in the United States<sup>100</sup> and worldwide.<sup>101</sup> Acetaminophen-related DILI is characterized by massive and coordinated hepatocyte necrosis without inflammation.<sup>102</sup> There is emerging evidence that the amount of circulating miRs can be used as a diagnostic and prognostic biomarker in DILI.<sup>18</sup> Acetaminophen administration results in increased miR-122, miR-155, miR-125b and miR-146a levels and these signatures could potentially represent biomarkers of acetaminophen-induced liver injury.<sup>21</sup> MiR-122 and -192 are shown to exhibit dose and exposure dependent changes in plasma parallel to aminotransferases and hepatocellular damage.<sup>18</sup> They were not only found to be significantly higher but also detected earlier in patients with acetaminophen-induced liver injury as compared with healthy controls.<sup>46</sup> MiR-122 levels correlated with peak ALT levels but not with prothrombin time, serum bilirubin or creatinine.<sup>46</sup> This may be explained by circulatory kinetics of these markers and it is possible that miR-122 release is an earlier event than the cessation of hepatic-clotting factor

production or heme-metabolism alteration during DILI. However, miR-122 was significantly higher in non-acetaminophen DILI patients, compared to healthy controls. This confirms that the levels of miR-122, but not miR-192, are significantly higher in the circulation after multiple forms of liver injury, including hepatitis and malignancy, suggesting that miR-122 holds superior diagnostic potential over miR-192. MiR-122 returned to baseline before serum ALT activity in acetaminophen DILI patients both with and without liver transplantation, indicating that it has a shorter circulating half-life and consequently might reflect dynamic changes in the histopathology of liver more accurately than ALT. However, the circulatory half-life of miR-122 has not been ascertained in humans. The intracellular half-life of miR-122 has been estimated to be >24 h.<sup>103</sup>

The exact mechanism of miR release during DILI remains unclear. It is not yet known whether serum miR-122 originates from sporadic diffused damaged liver cells or from distinct zonal patterns of injured hepatocytes. The hepatocytes in an injured liver may release miR-122 through a protein carrier pathway.<sup>104</sup> MiR-122 release during DILI may be biphasic, with early energy-dependent export followed by massive necrotic leakage. The liver tissue-specific origin of serum miR-122 provides an opportunity to discriminate between hepatic and non-hepatic serum ALT elevations. However, miR-122 alone may not be an ideal DILI biomarker because it does not distinguish benign clinical ALT elevations from serious liver injury potential.<sup>105</sup> It is also not specific enough to uniquely identify acetaminophen toxicity.<sup>106</sup> Future challenges will be to determine whether miR-122 can provide clinical prognostic value over current biomarkers of DILI in prospective clinical trials and real-life clinical situations with other etiologies of liver disease.

Lethal dosing of acetaminophen in murine model studies shows differential expression of a unique pattern of circulating plasma miRs, which is unique from the plasma miR profile associated with sub-lethal dosing and may be useful in the future to distinguish lethal and sub-lethal acetaminophen toxicity in humans. The goal would be to eventually have a miR acetaminophen nomogram to be used for human patient care, similar to the previous effective Rumack-Matthew nomogram.<sup>107,108</sup> After administration of a lethal or sub-lethal dose of acetaminophen, more than 40 potential miRs were both, greater than 2-fold up- as well as down-regulated in the lethal (500 mg/kg) compared to sub-lethal (150 mg/kg) dosing.<sup>109</sup> MiRs 574-5p, 135a, 466g, 1196, 466f-3p, and 877, are up-regulated in the setting of lethal compared to sub-lethal APAP associated hepatotoxicity, whereas miRs 342-3p, 195, 375, 29c, 148a and 652 are markedly down-regulated, supported by elevated ALT levels and histologic evidence.

## ALCOHOLIC LIVER DISEASE (ALD)

ALD is a major global health problem. Chronic alcoholism results in a broad spectrum of clinical features including steatosis, inflammation, fibrosis and cirrhosis, leading to increased risk of HCC. Increased inflammation and fat accumulation are the hallmarks of ALD. The role of miRs in ALD is an active research area, and recent studies suggest that alcohol exposure can modulate miR expression in hepatocytes.<sup>110</sup> Aberrant expression of miRs has been observed after alcoholic liver injury.<sup>111,112</sup> Treatment of normal human hepatocytes and cholangiocytes with ethanol and LPS induced a significant increase of miR-34a expression.<sup>113</sup> Overexpression of miR-34a decreased ethanol induced apoptosis in both hepatocytes and cholangiocytes. It was also shown that miR-34a contributes to alcoholic liver injury and tissue repair by modulating cell proliferation, remodeling and migration by directly targeting caspase-2 and sirtuin1, genes which are known to regulate apoptosis. Thus, therapeutic strategies based on targeting miR-34a may be potentially beneficial in ALD.

MiRs mediate several ethanol pathologies, including disruption of neural stem cell proliferation and differentiation in the exposed fetus, gut leakiness that contributes to endotoxemia and alcoholic liver disease, and possibly cancer.<sup>113-117</sup> Ethanol has been shown to repress miRs 9, 21, 153 and 335 in neural stem cells and neural progenitor cells thus altering cellular development and maturation.<sup>113</sup> Alcohol has been found to increase miR-212 expression and its over-expression is correlated with hyperpermeability of monolayer barrier (tight junction) by down-regulating protein Zonula occludens 1 translation.<sup>114</sup> Long-term exposure to alcohol can also induce endothelin 1 (ET-1) and hypoxia-inducible factor 1 and inhibit expression of miR-199 in liver sinusoidal endothelial cells.<sup>115</sup> MiR-199 is involved in ET-1 expression in human endothelial cells and functions as a negative regulator of ET-1 transcription and thus vascular tone in ALD.<sup>115</sup> A profound increase was found in serum miR-122 levels in alcohol-fed mice with a linear correlation between serum ALT and serum miR-122 levels.<sup>21</sup> MiR-27b, miR-214, miR-199a-3p, miR-182, miR-183, miR-200a, and miR-322 were found to be down-regulated, whereas miR-705 and miR-1224 were increased after 4 weeks of alcohol feeding in mice.<sup>116</sup> Alcohol has been shown to down-regulate miR-199 in rat liver sinusoidal endothelial cells and human endothelial cells.<sup>115</sup> Bala et al, showed significant induction of miR-132, increase in miR-155 in the livers of alcohol fed mice, while expression on miR-125b was down-regulated in the hepatocytes of alcohol fed mice.<sup>117</sup> Thus, alcohol consumption alters miR expression in a variety of cell types.

MiRs have been implicated to be essential regulators of the immune response and activation of the innate immune system in alcoholic steatohepatitis.<sup>118,119</sup> It has been shown that alcohol-induced gut leakiness is a key factor

in ALD and it allows endotoxin to enter the circulation and initiate liver damage and that ethanol increases miR-122 expression.<sup>117</sup> Induction of miR-155 and -132 is shown to contribute to increased TNF $\alpha$  production by Kupffer cells (KCs) in response to LPS.<sup>117</sup> Endotoxemia and direct alcohol exposure modulate hepatic miRs 182, 183, 705, 1224 and 199a-3p.<sup>120</sup> Alcohol particularly targets and increases miR-155 in macrophages, regulating TNF $\alpha$  production and prolonged alcohol exposure induces miR-155 in RAW264.7 macrophage and KCs.<sup>112</sup> Therefore induction of miR-155 may be involved in both LPS and oxidative stress signaling pathways, and hence contributing to the progression of ALD.<sup>117</sup> The physiological significance of alcohol-induced miR-155 in hepatocytes needs further investigation.

## NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

NAFLD, one of the most common chronic liver disease worldwide, is a spectrum of disorders that range from simple hepatic steatosis without significant inflammation or fibrosis, to non-alcoholic steatohepatitis (NASH) with varying degrees of inflammation and fibrosis, which may lead to cirrhosis, liver failure and hepatocellular carcinoma (HCC) despite the absence of significant alcohol consumption. Although insulin resistance, intestinal microbiota and oxidative hepatocellular injury are implicated, there still remains uncertainty in the pathogenesis, molecular interactions and regulatory mechanisms of NAFLD.

Since the initial study by Cheung et al showing differential expression of 46 (23 up-regulated and 23 down-regulated) hepatic miRs in patients with NASH and metabolic syndrome compared to subjects with normal liver histology, a number of additional studies, but mostly in animal models of NAFLD, have been done.<sup>14</sup> Together these studies suggest that differentially expressed miRs in humans and animal models of NASH regulate genes with diverse functions involved in the pathogenesis of NAFLD, including metabolism of lipid and glucose, regulation of the unfolded protein response, endoplasmic reticulum stress, oxidative stress, cellular differentiation, inflammation and apoptosis.<sup>14,36</sup> Notably, miR-34a has been shown to be up-regulated in both human serum<sup>50,51</sup> and liver in humans and animal models of NAFLD.<sup>14,55,121,122</sup> Two recent studies suggest two differing mechanisms for the involvement of miR-34a in NASH pathogenesis through down-regulation of SIRT-1; a) leading to AMP kinase dephosphorylation and subsequent decreased phosphorylation of HMGCoA, ultimately leading to cholesterol accumulation<sup>121</sup>; b) increased acetylation of p53 causing activation of apoptosis.<sup>123</sup> MiR-122 is significantly down-regulated in humans and animal models of NAFLD patients.<sup>12,14</sup> The targeted deletion of miR-122a in mice results in NASH, fibrosis and HCC.<sup>54</sup>

In miR profile analysis of high fat fed rat liver, 14 miRs were up-regulated and six were down-regulated, that might represent a distinctive molecular signature in the transition of steatosis to steatohepatitis.<sup>53</sup> MiR-34a and miR-146b were shown to be significantly over-expressed (99% and 80%, respectively) in human NASH.<sup>14</sup> Nakanishi et al showed hepatic miR-335 was up-regulated and level was closely correlated with the expression of adipocyte differentiation markers, i.e., PPAR- $\alpha$  and FAS in adipocyte in obese mice.<sup>124</sup> MiR-10b also targets PPAR- $\alpha$ , which plays a major role in NAFLD pathogenesis, and may regulate steatosis in murine models of NAFLD.<sup>125</sup> Changes in miRs, particularly increased expression of miR-705 and -1224 and decreased expression of miR-182 and -199a have also been contributed to impaired hepatic lipid homeostasis and inflammatory cascade in alcoholic as well as non-alcoholic steatohepatitis.<sup>116</sup> Together these studies may provide novel diagnostic markers and therapeutic targets for NAFLD.

## CIRRHOSIS/LIVER FIBROSIS

Liver fibrosis is a wound-healing response to injury that occurs in most chronic liver diseases. During this process an accumulation of scar tissue forms in the liver parenchyma. The mechanisms of fibrogenesis are poorly understood and thus there is no effective therapy. HSCs are thought to be the main effector cell type of fibrogenesis.

Activation of this normally quiescent vitamin A storage cell results in production of TGF $\beta$  and deposition of extracellular matrix (ECM) proteins such as collagen into the surrounding parenchyma.<sup>126-129</sup> Left unchecked this process will result in cirrhosis which is associated with high morbidity and mortality. MiR-21 regulates TGF $\beta$ , TLAM1 and PTEN that are directly involved in epithelial-to-mesenchymal transition, which is thought to contribute to organ fibrogenesis.<sup>130</sup> MiR-21 can also contribute to fibrogenesis by inducing extracellular signal-regulated kinase pathways in fibroblasts, which also produce collagen and ECM.

Increasing evidence suggests that miRs are key regulators of hepatic fibrogenesis, in particular by regulating gene expression in HSCs. Expression of the miR-199 and -200 families has been correlated with progression of liver fibrosis.<sup>131</sup> Increased levels of miR-199 and miR-200 families were found in the fibrotic liver of patients and up-regulation of these miRs resulted in significantly increased expression of fibrosis-related genes in an HSC cell line.<sup>131</sup> MiR-150 and miR-194 levels were significantly reduced in a bile duct ligation (BDL) rat model of fibrosis compared to sham operated control rats.<sup>132</sup> Moreover, overexpression of miR-150 or miR-194 was shown to reverse the activated phenotype of stellate cells (i.e., expression of alpha smooth muscle actin and collagen genes) in a human stellate cell line, LX2 via inhibition of c-myc and rac1 expression.

Thus, miR-150 and miR-194 could be potential therapeutic targets for fibrosis.<sup>132</sup>

Significant miR-132 down-regulation in fibrotic livers influences HSC activation, as shown in both carbon tetrachloride (CCl<sub>4</sub>) and BDL models of fibrosis.<sup>133</sup> Down-regulation of overexpressed miR-27a and 27b allowed restoration of activated HSCs and decreased HSC proliferation.<sup>134</sup> However, silencing of miR-27a and 27b didn't affect cell apoptosis and other characteristics of activated HSCs. Guo et al demonstrated decreased expression of miR-15b and miR-16 during the activation of HSCs and restoration of these miRs in primary HSCs resulted in a significantly elevated rate of apoptosis through a caspase signaling pathway.<sup>135</sup> Transient overexpression of miR-181b could promote HSC cell proliferation and cell cycle progression.<sup>136</sup> In addition, miR-19b has been shown to inhibit TGF- $\beta$  signaling and decrease type I collagen expression. Thus, this novel regulator of TGF- $\beta$  signaling in HSCs suggests a potential therapeutic approach for liver fibrosis.<sup>137</sup> The expression of miR-146a was down-regulated in HSC in response to TGF- $\beta$ 1 stimulation in dose-dependent manner and overexpression of miR-146a suppressed TGF- $\beta$ -induced HSC proliferation, and increased HSC apoptosis.<sup>138</sup> These studies indicate that miR-mediated gene regulation in HSCs is a major mechanism for controlling fibrogenesis.

In a murine CCl<sub>4</sub> model of induced liver fibrosis, miR-29 members (miR-29a, -29b, -29c) were significantly down-regulated, while miR-34 family (miR-34a, miR-34b and miR-34c) was found to be the most up-regulated.<sup>139</sup> MiR-29b may be a novel regulator of type I collagen expression.<sup>140</sup> MiR-29 families are shown to be very important regulators of liver fibrosis and could serve as potential biomarker for advanced cirrhosis. It can act as novel anti-fibrogenic mediator by repressing collagen synthesis via direct binding to its 3'UTR and also by interfering with pro-fibrogenic cell communication via platelet-derived growth factor (PDGF)-C and insulin-like growth factor (IGF)-I.<sup>141</sup> TGF- $\beta$  stimulation led to decreased miR-29 levels, but to pronounced up-regulation of collagen synthesis; hepatocyte growth factor (HGF) stimulation led to elevated miR-29 expression, but to repression of collagen synthesis.<sup>141</sup> Low plasma levels of miR-29a correlate with advanced liver fibrosis in humans. Several fibrosis-associated genes are potential targets of miR-29, and numerous studies have demonstrated that this miR is involved in the formation and/or dissolution of fibrosis in several organs (<http://www.targetscan.org>). Thus, dysregulation of miR-29 may be a factor in hepatic fibrosis.

In cirrhosis, a low serum miR-122 level was associated with hepatic decompensation, ascites, hepatorenal syndrome and spontaneous bacterial peritonitis.<sup>142</sup> The possible explanation for this may be reduced release from hepatocytes or higher volume distribution in patients with ascites. It is also conceivable that in cirrhotic patients

who have less functional hepatocytes, the total release of miRs upon damage might be lower than in patients with higher amounts of healthy liver tissue. MiR-122 levels had significant correlation with MELD score and an inverse correlation with creatinine and INR. Thus it may be a new independent potential marker for mortality and prediction of survival of patients with cirrhosis, supplementing the MELD score. In addition, miR-571, miR 513-3p and miR-652 may be potential novel non-invasive markers for cirrhosis induced by alcoholic hepatitis or hepatitis C, since in patients with cirrhosis, serum levels of miR 513-3p and miR-571 are increased whereas miR-652 is reduced.<sup>143</sup>

In conclusion there is a great deal of interest to better understand the role of miRs in the regulation of fibrogenesis-related genes in HSCs and other cell types to ultimately determine if miRs could be exploited as novel biomarkers for liver fibrosis diagnosis and therapeutic targets for miR-based anti-fibrotic gene therapy.

## ROLE OF MICRORNAS IN LIVER TRANSPLANTATION

Currently, histological examination of the liver is the gold standard of care to assess hepatic function and disease recurrence in the post-liver transplant (LT) setting.<sup>144</sup> But its use is limited by its invasiveness, risk of complications and its sampling variability.<sup>145</sup> Thus, non-invasive tests of liver fibrosis such as the AST-to-platelet ratio index (APRI), fibroSURE, FibroSCAN etc are being used. However, none of them are validated for clinical use. Hence there is a need for novel non-invasive marker and miR signature may be able to predict severity of fibrosis post-transplant. Compared to non-transplant patients, hepatitis C infection in immunosuppressed liver transplant recipients is characterized by an accelerated fibrogenesis and faster decompensation of allograft cirrhosis<sup>146,147</sup> and liver transplant for HCV infection is associated with decreased patient and graft survival when compared to other indications,<sup>148,149</sup> the mechanisms for which remain largely unknown.

A set of 9 miR signatures associated with progression of fibrosis were differentially expressed and have been recognized to identify early post-LT stage patients at high risk to develop severe progression to fibrosis/cirrhosis associated with HCV recurrence.<sup>150</sup> Of these 9 miRs, three were up-regulated (miR-155, -34a and -222) and six were down-regulated (miR-23b, -361, -455, -30b, -30c, and -27b). MiR-21 has been shown to regulate pro- and anti-inflammatory cytokines and has been implicated in the development of fibrosis leading to chronic rejection following liver transplantation.<sup>75,151</sup> This has been also validated in an independent set of HCV+ LT recipients and demonstrated organ specificity (molecular pathways involved in liver injury, malignancy and hepatic disease), and relationship with immune response (T cell lineages

development).<sup>150</sup> Farid et al have shown that circulating miR-122, miR-148, miR-194 are sensitive biomarkers for hepatocyte injury and rejection after liver transplantation.<sup>152</sup> Disease specific miR signatures would be helpful to monitor post-LT transplant success and supersede the need for multiple invasive, dangerous liver biopsies.

## FUTURE DIRECTIONS

Despite the high potential of miRs as novel biomarkers for liver disease, some complex challenges remain before clinical utility. Multicenter studies are needed to cross-validate findings.<sup>153</sup> Another challenge to the use of miRs as markers is that their mRNA targets are not easily identified by computational methods.<sup>153</sup> Levels of miR in whole blood are much higher than plasma or serum miR levels.<sup>154</sup> Serum and plasma levels of specific miRs are not readily comparable.<sup>155</sup> Given the technical challenges, there are conflicting data regarding up-regulation or down-regulation of circulating miR in various pathologies.<sup>156-158</sup>

MicroRNAs increase the chance for early diagnosis due to their upstream positions in regulation cascades and are readily discovered by genomic tools such as oligonucleotide microarrays and deep sequencing.<sup>158,159</sup> They can be amplified and detected in body fluids in a clinical setting by real-time quantitative PCR. However, analyzing complex patterns of miR expression requires intensive bioinformatics analysis. Many microRNAs represent promising targets for therapeutic gene therapy approaches although many technical challenges remain. Potential delivery vehicles such as adeno-associated virus vectors can be designed to express precursors or small interfering inhibitors of miRs with relatively good specificity for hepatocyte delivery. Nanoparticle-mediated miR delivery is also gaining attention.

## CONCLUSION

Differential expression of miRs has been correlated with a number of clinically important liver diseases. MicroRNAs represent a novel class of biomarkers due to their specific disease profiles which are stable and easily detected in clinical samples. However, the application of miRs for clinical therapy is still in its infancy and further investigation is required to better understand the significance of miRs in disease and provide rational decision making for their utility as biomarkers for disease classification, detection, prognosis and therapy. The use of miR replacement therapy utilizing short RNA duplexes that mimic under expressed miRs and chemically modified, single stranded oligonucleotide miR inhibitors that antagonize overexpressed miRs in various diseases are promising novel miR-based therapeutic options. Still, numerous challenges remain because of the lack of sub-optimal delivery systems, insufficient cellular uptake, off-target effects and cellular toxicity.

More work is needed to evaluate their long-term *in vivo* efficacy, specificity, and systemic toxicity prior to the future use of these emerging strategies.

## CONFLICTS OF INTEREST

All authors have none to declare.

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