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REVIEW

miRNAs in non-alcoholic fatty liver disease

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Abstract **Non-alcoholic fatty liver disease (NAFLD) is a major cause of liver cirrhosis and hepatocellular carcinoma and is a considerable threat to public health. miRNAs are important post-transcriptional regulators of gene expression, and the dysregulation of miRNAs is involved in various biological processes in the liver, including lipid homeostasis, inflammation, apoptosis, and cell proliferation. Recently, a number of studies have described the association between miRNAs and NAFLD progression and have shown that circulating miRNAs reflect histological changes in the liver. Therefore, circulating miRNAs have potential use for the evaluation of NAFLD severity. In this review, we discuss the involvement of miRNAs in NAFLD pathogenesis and the key role of miRNAs in the screening, diagnosis, and staging of NAFLD.**

Keywords nonalcoholic fatty liver disease; nonalcoholic steatohepatitis; hepatocellular carcinoma; miRNA

Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined as the presence of hepatic steatosis (>5%–10% of hepatocytes are fatty) in people without history of excessive alcohol consumption (>21 drinks/week in men and >14 drinks/week in women) and other disease etiologies that result in fatty liver [1]. NAFLD is one of the most common liver disorders in the world, and its global prevalence in the general population is 6.3% to 33%, with a median prevalence of 20% [2]. Furthermore, the prevalence of non-alcoholic steatohepatitis (NASH) is 2%–3%, and up to 15% of NASH subjects progress to cirrhosis [3]. Although the initial histological change of NAFLD is simple steatosis, NAFLD is a spectrum disease that develops from NASH and fibrosis to cirrhosis and hepatocellular carcinoma (HCC) [4]. Therefore, NAFLD is a considerable threat to human health worldwide, and research on its pathogenesis, early diagnosis, and treatment is necessary. Similar to other complex diseases, multiple factors are involved in NAFLD development, including lipotoxicity, insulin resistance, endoplasmic reticulum stress, adipose tissue, gut microbiota, and genetics [5]. Our understanding of the pathogenesis of this disease remains limited because of its broad range and complexity. Furthermore, although histological assessment remains the gold standard for diagnosing NAFLD, this approach cannot be applied to the general population and has limited use for the early detection of NAFLD in high-risk patients. Therefore, further elucidation of the pathogenesis and natural history of NAFLD is necessary.

MicroRNAs are small (21–23 nucleotides), non-coding, highly conserved endogenous RNAs that regulate gene expression at the post-transcriptional level. Mature miRNA synthesis involves a series of steps. Pri-microRNAs are transcribed from microRNA genes by RNA polymerase II and then cleaved by a microprocessor complex in the nucleus, thus resulting in pre-microRNAs. The pre-microRNAs are then transported from the nucleus to the cytoplasm by Exportin 5 and then cleaved into double-stranded miRNAs via Dicer. The mature guide strand is installed into the RNA-induced silencing complex, which interacts with the target mRNAs while the passenger strand is degraded. Approximately 30% of human genes are regulated by miRNAs. The wide modulation of human genes by miRNAs indicates the key role of miRNAs in multiple physiological processes and diseases, including NAFLD [6].

Dysregulation of miRNA expression is a key pathogenic factor in many liver diseases, including viral hepatitis, alcoholic fatty liver disease, and hepatocellular cancer. Furthermore, evidence has demonstrated that miRNA expression changes with NAFLD progression [7].

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Circulating miRNAs are extremely stable and resistant to plasma RNase-mediated degradation in body fluids. Many studies have shown that an altered pattern of circulating miRNAs reflects histological changes and molecular events in the liver and responses to different NAFLD stages, thus suggesting that serum miRNAs may serve as constructive biomarkers of NAFLD [8]. Therefore, describing the altered expression of miRNAs in NAFLD is important to determine the exact mechanism of NAFLD and provide the early diagnosis and severity evaluation of NAFLD. The objectives of this review are to provide descriptions of altered miRNA profiles in NAFLD, assess the underlying mechanisms of the most-studied miRNAs in NAFLD progression, and explore the role of circulating miRNAs as biomarkers for the early diagnosis and evaluation of NAFLD to support the therapeutic strategies and monitoring of NAFLD treatment.

miRNAs involved in NAFLD pathogenesis

Evidence indicates that miRNAs are the key regulators of energy homeostasis and liver functions; therefore, the altered expression of miRNA profiles needs to be further studied to provide a foundation for exploring the particular mechanisms of miRNAs with respect to NAFLD (Fig. 1). An early study evaluated 4 upregulated miRNAs (miR-103, miR-31, miR-107, and miR-126-3p) and 2 downregulated miRNAs (miR-100 and miR-29c) in the liver tissue of ob/ob mice and streptozotocin-induced diabetic mice [9]. A microarray analysis of NASH patients also identified 46 differentially expressed miRNAs, among which miR-126 and miR-122 were downregulated and miR-21, miR-100, and miR-34a were upregulated [10]. Similarly, a study explored the expression profiles of miRNAs in NASH-related fibrosis mice induced by a methionine–choline deficient (MCD) diet. A total of 9 upregulated and 18 downregulated miRNAs were identified in this study. Among these miRNAs, miR-146a-5p was significantly downregulated and was shown to play a role in the activation of hepatic stellate cells (HSCs), as mediated by Wnt1 and Wnt5a [11].

Furthermore, a longitudinal study discovered that miRNAs are associated with dynamic progression from hepatic steatosis to HCC. This study also explored the dynamic expression levels of miRNAs during NAFLD progression in mice treated with a long-term high-fat diet (HFD). Four miRNAs (miR-340-5p, miR-484, miR-574-3p, and miR-720) were discovered to be involved in liver damage and tumorigenesis, and two miRNAs (miR-125a-5p and miR-182) were found to be dysregulated in early-stage NAFLD. Accordingly, miRNA profiles were differentially expressed in different NAFLD stages, thus suggesting that different pathological changes involve diverse miRNAs [12].

miR-122

miR-122 accounts for 70% of all miRNAs in the liver and is involved in a variety of biological processes. Therefore, miR-122 has been the focus of previous studies. Mice fed

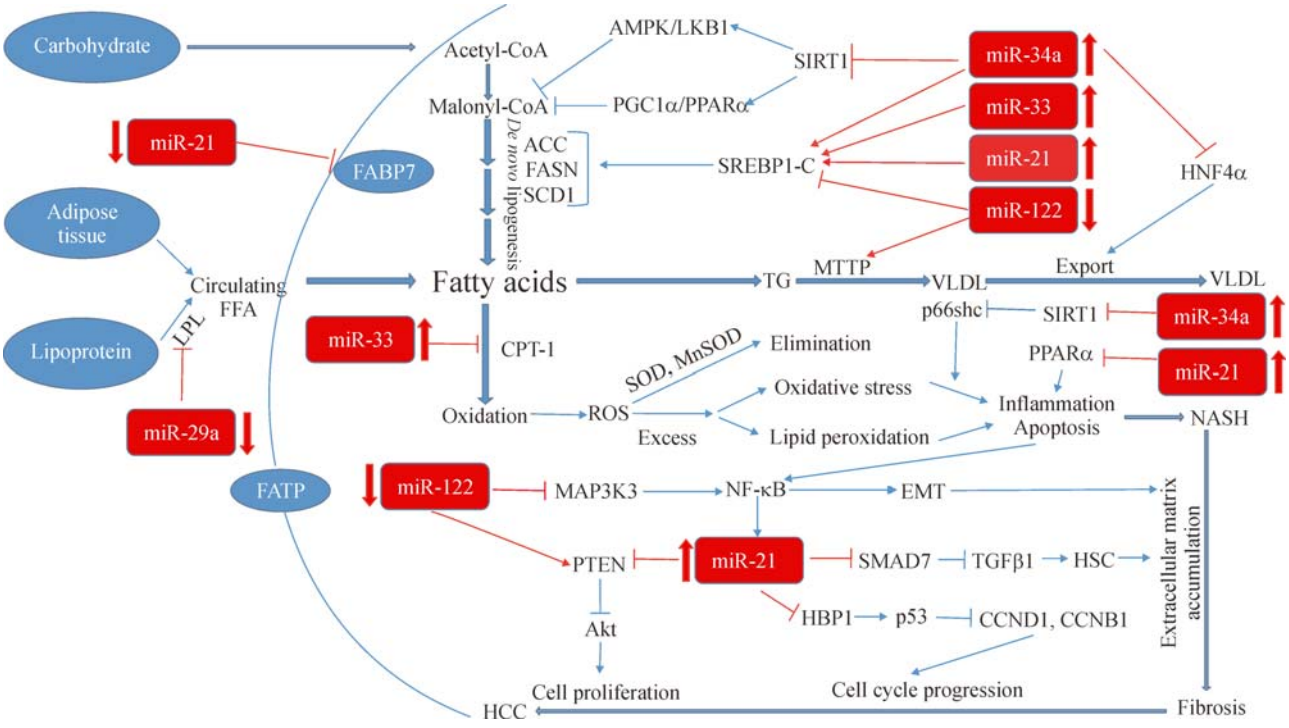

Fig. 1 miRNAs involved in the pathogenesis of NAFLD.

with a methyl-deficient diet had significantly decreased miR-122 levels [10], which were observed in NASH patients but not in simple steatosis patients or normal subjects [8]. Furthermore, mice carrying *Mir122a* deletion developed steatosis, which progressed to NASH, fibrosis, and HCC over the long term, thus suggesting the key role of miR-122 in the initiation and progression of NAFLD [13].

miR-122 modulates fatty acids, triglycerides, and cholesterol metabolism. Mice with *Mir122a* deletion exhibited lower serum cholesterol and triglyceride levels than WT mice and presented increased cholesterol and triglyceride contents in the liver. The distinct effect of miR-122 on cholesterol and triglyceride levels in the serum and liver may be attributed to the decreased expression of microsomal triglyceride transfer protein, which is related to very low-density lipoprotein (VLDL) assembly and secretion [13]. Furthermore, silencing miR-122 in cultured cells induced the overexpression of key genes involved in the regulation of lipid metabolism, including sterol regulatory element binding protein-1c (SREBP1c), sterol regulatory element binding protein-2 (SREBP2), fatty acid synthase (FASN), and HMG CoA reductase (HMGCR), whereas miR-122 overexpression yielded the opposite results [14]. However, another study found that antagonizing miR-122 with antisense oligonucleotide in HFD and normal-diet mice reduced the expression of lipogenic genes and increased hepatic fatty acid oxidation, as mediated by the activation of adenosine 5'-monophosphate-activated protein kinase (AMPK), thus resulting in improvement of hepatocyte steatosis [15]. This conflict has been observed in different studies, and the cause remains unclear because of distinct models and inhibition patterns. Therefore, additional direct targets involved in the effects of miR-122 must be identified to provide information on its underlying mechanism of action.

In addition to the involvement of miR-122 in lipid homeostasis, miR-122 also participates in the signaling pathways of NAFLD-related fibrosis and HCC. A relative deficiency of miR-122 concomitantly activated three targets, including mitogen activated protein kinase kinase kinase 3, vimentin, and hypoxia inducible factor-1α (HIF-1α). All three targets are related to epithelial–mesenchymal transition, which plays a key role in chronic inflammation, various fibrosis diseases, and cancer metastasis [16]. Moreover, Tsai *et al.* [13] found that mice lacking *Mir122a* had reduced phosphatase and tensin homolog (PTEN) expression and elevated Akt signaling activation, which is significantly associated with cell proliferation and cell invasion in cancer.

In general, the involvement of miR-122 in different stages of NAFLD demonstrates that miR-122 may serve as an indicator of NAFLD severity and provide additional insight on the diagnosis and staging of NAFLD in clinical practice.

miR-33a/b

SREBPs are transcriptional regulators of lipid homeostasis and play a vital role in the metabolism of cholesterol, fatty acids, and triglycerides, which are involved in the occurrence of NAFLD [17,18]. miR-33a and miR-33b are separately located in the intronic regions of host genes *srebp2* and *srebp1*, thus suggesting the co-transcription of miR-33 and SREBPs [19]. miR-33 contributes to the modulation of fatty acid metabolism and insulin signaling pathways, as well as the dysregulation of cholesterol synthesis and high-density lipoprotein (HDL) levels.

In fact, miR-33 overexpression inhibited the expression of genes involved in fatty acid oxidation (carnitine O-octaniltransferase, carnitine palmitoyltransferase 1A, and hydroxyacyl-CoA-dehydrogenase), whereas miR-33 inhibition enhanced this pathway in hepatocytes [20]. Another study demonstrated similar effects in a non-human primate model, and also found that silencing miR-33 reduced the expression of genes associated with fatty acid synthesis (SREBP1, FASN, ATP citrate lyase, and acetyl-CoA carboxylase α) and concomitantly elevated plasma HDL levels [21]. Interestingly, the chronic inhibition of miR-33 facilitated lipid accumulation and increased plasma triglyceride levels in mice; this condition may have been caused by the enhanced expression of nuclear transcription Y subunit γ, which is a co-activator required for SREBP-responsive genes [22]. For cholesterol metabolism, adenosine triphosphate binding cassette (ABC) transporters ABCA1 and ABCG1, which are the target genes of miR-33, were downregulated in NASH patients and animals and played key roles in reverse cholesterol transport and HDL formation [23].

Furthermore, miR-33 is associated with cell proliferation and G_1–S transition. Anti-miR-33 treatment in a mouse model promoted liver regeneration and increased the expression of cyclin-dependent kinase 6, a G_1/S-specific cyclin-D1 and a direct target of miR-33. However, few studies have explored the specific cell proliferative mechanism of miR-33 in NAFLD [24].

miR-33a/b is primarily responsible for lipid accumulation, which is the first event in NAFLD, and subsequently contributes to liver damage via lipotoxicity and oxidation stress. The involvement of miR-33 in cell proliferation provides additional information on the role of miR-33 in NAFLD pathogenesis.

miR-34a

miR-34a engages in multiple physiological processes, such as fatty acid oxidation and synthesis, triglyceride and cholesterol metabolism, and hepatocyte apoptosis. Several studies have shown that miR-34a is significantly elevated in ob/ob mice and NAFLD patients.

SIRT1 is a direct target of miR-34a and a key mediator of energy metabolism via multiple signaling pathways. miR-34a inhibition was found to activate fatty acid oxidation and attenuate steatosis as a result of the increased expression of hepatic silent mating type information regulation 2 homolog 1 (SIRT1), peroxisome proliferator-activated receptor-α (PPARα), and AMPK [25]. Another study defined the signaling pathways associated with miR-34a in NAFLD and observed that reduced miR-34a levels in the liver of a mouse model potentiated two pathways, namely, the SIRT1/peroxisome proliferator-activated receptor-γ co-activator 1α/PPARα pathway and the SIRT1/liver kinase B1/AMPK pathway, and decreased the expression of SREBP1c and its downstream targets FASN, stearoyl-CoAdesaturase1, and acetyl-CoA carboxylase. The first two pathways together contributed to decreased malonyl-CoA (mCoA) because of the elevated level of mCoA decarboxylase and led to CPT1 activation, which is associated with fatty acid oxidation [26]. The miR-34a/SIRT1/AMPK pathway also promotes the expression of HMGCR, an enzyme involved in cholesterol synthesis. Xu *et al.* [27] demonstrated that miR-34a inhibited VLDL secretion from the liver, which ultimately promoted liver steatosis and hypolipidaemia through interactions with hepatocyte nuclear factor 4α (HNF4α) in NASH patients and HFD-fed mice. In fact, HNF4α controls the expression of genes associated with lipid and glucose metabolism, and loss of HNF4 function caused fatty liver and decreased plasma lipid levels in mice [28,29]. Therefore, the miR-34a-HNF4α pathway is also involved in NAFLD.

Additionally, miR-34a/SIRT1 is associated with oxidative stress and apoptosis in NAFLD. The overexpression of miR-34a in the cultured hepatocytes of mouse models exacerbated FFA-induced apoptosis, whereas the inhibition of miR-34a via carnosic acid activates SIRT1 expression with a concomitant decrease in p66shc expression, ultimately attenuates lipid accumulation, and causes an anti-apoptotic effect in hepatocytes. p66Shc is a key redox enzyme that is involved in mitochondrial oxidation and apoptosis. Reduced malondialdehyde and increased superoxide dismutase (SOD) and manganese SOD were observed after miR-34a inhibition, thus indicating the significance of the miR-34a/SIRT1/p66shc pathway for NAFLD. Furthermore, another study of NAFLD patients demonstrated that miR-34a positively increased in the liver with disease severity and was accompanied by repressed SIRT1 activation and increased p53 acetylation [30].

Accordingly, miR-34a participates in both the steatosis and apoptosis of hepatocytes, thus suggesting that miR-34a level is linked to NAFLD severity and that miR-34a may serve as a novel biomarker for NAFLD progression.

miR-21

miR-21 is implicated in NAFLD pathogenesis and progression to HCC because of the involvement of miR-21 in multiple physiological processes.

miR-21 is involved in lipid homeostasis. A study demonstrated that miR-21 is downregulated in response to palmitic acid/oleic acid-induced hepatocyte steatosis and indicated that miR-21 mimic decreases serum triglyceride, free cholesterol, and total cholesterol levels by targeting the 3′-UTR of *hmgcr* [31]. Fatty acid binding protein 7 (FABP7) is a fatty acid binding protein that promotes fatty acid uptake and is a direct target of miR-21. Ahn *et al.* [32] found that miR-21 decreases in the livers of HFD mice and that lycopene treatment attenuates HFD-induced lipid accumulation by restoring miR-21 expression and inhibiting its downstream FABP7 levels.

miR-21 is overexpressed in NASH patients and low-density lipoprotein receptor-deficient ($Ldlr^{-/-}$) HFD-fed mice. miR-21 suppression reduces inflammation, liver injury, and fibrosis by activating PPARα expression but does not have an effect on lipid accumulation. The relationship between activated PPARα and unchanged lipid levels is inconsistent with the observation that PPARα induces fatty acid β-oxidation and improves hepatic steatosis. In fact, PPARα was shown to inhibit the proinflammatory signaling pathway and reduce liver injury and fibrosis regardless of its effect on lipid accumulation [33].

Additionally, inflammation and oxidative stress in NASH likely contribute to NASH-related fibrosis [34]. Transforming growth factor (TGF-β) signaling participates in fibrogenesis via HSC activation, which is responsible for the excess accumulation of extracellular matrix proteins [35]. A previous study demonstrated that induction of oxidative stress promoted the translocation of nuclear factor κB (NF-κB), which was observed to bind to the miR-21 promoter during NASH in mice and humans; miR-21 upregulation repressed the expression of Mothers against decapentaplegic homolog 7 (SMAD7), increased the expression of TGF-β, and ultimately caused fibrogenesis [36].

Moreover, Wu *et al.* [37] described the elevation of miR-21 expression in human hepatoma HepG2 cells with oleic acid treatment, as well as the reduction of lipid content and cellular proliferation with miR-21 inhibitor treatment. This effect is engaged in the dysregulation of high-mobility group box transcription factor 1 (HBP1)–p53 axis. Furthermore, the unsaturated fatty acid-induced overexpression of miR-21 via NF-κB diminishes PTEN expression, thereby triggering HCC development [38].

In general, miR-21 levels are distinct across different stages of NAFLD, thus indicating that certain miRNAs

may play different roles in the progression and extent of NAFLD-related liver injury.

miR-29

miR-29 is related to cell proliferation, extracellular matrix homeostasis, and gluconeogenesis. Silencing miR-29 contributes to multiple disease pathologies, such as fibrosis and rhabdomyosarcoma [39]. Therefore, the major effect of miR-29 in the liver is its involvement in fibrosis.

A number of studies have also focused on the function of miR-29 in lipid metabolism. miR-29 is downregulated in the livers of mice fed with a lipogenic methyl-deficient diet that causes NASH [40], and an miR-29a inhibitor promotes hepatic steatosis, thus demonstrating the importance of the miR-29 family for NAFLD. A recent report showed that plasma total and HDL cholesterol levels significantly decreased because of a global miRNA deficiency in Dicer1$^{fl/fl}$ mice. Notably, the altered lipid profile was mediated by the miR-29a-induced repression of lipoprotein lipase (LPL) in normal liver [41]. LPL plays a key role in breaking down circulating triglycerides and releasing their fatty acids for uptake by tissue, which use these fatty acids for β-oxidation or triglyceride synthesis. Antagonizing miR-29a facilitates the uptake of fatty acids in the liver and results in hepatic steatosis; this finding is consistent with those of previous studies wherein elevated levels of LPL were observed in NAFLD patients and HFD mice models [42]. An additional study performed by Kurtz *et al.* [43] observed decreased plasma cholesterol and triglyceride levels, fatty acid synthesis, and liver content as a result of locked nucleic acid (LNA) inhibitors of miR-29. They also showed that LNA29 silenced genes that are associated with the lipid synthesis pathway while activating transcriptional factors responsible for inhibiting lipid synthesis, such as Aryl hydrocarbon receptor, forkhead box O3, and SIRT1, thereby controlling lipid levels. These two studies described the involvement of miR-29 in different pathways at the molecular level, including the lipoprotein lipolysis pathway and the fatty acid and cholesterol synthesis pathway; however, the latter study did not show whether miR-29 causes hepatic steatosis.

Given that miR-29 is associated with fibrosis and hepatic lipid metabolism, the association between miR-29 and liver fibrosis may provide additional information for NAFLD staging and progression. Nevertheless, the underlying mechanism of miR-29 on NAFLD is unclear and additional studies should be conducted to demonstrate the correlation between miR-29 and NAFLD.

In addition to the miRNAs discussed here, various additional miRNAs are involved in NAFLD. The miR-212 expression was upregulated in the livers of HFD mice, and exercise attenuated hepatocyte steatosis via miR-212, which participates in the lipogenesis of NAFLD by interacting with fibroblast growth factor 21 (FGF21) [44]. Estrogen targeting miR-125b conferred protection against hepatic steatosis mediated by decreased FASN expression in the liver of a female mouse model [45]. Furthermore, miR-24 has been shown to promote lipid accumulation in the livers of HFD-treated mice and hepatocytes exposed to fatty acids, thus activating lipid synthesis via insulin-induced gene 1 downregulation and SREBP upregulation [46]. A microarray analysis revealed that miR-155 was upregulated by NF-κB, and tumor suppressor CCAAT/enhancer binding protein β (C/EBPβ) was downregulated in mice fed choline deficient and amino acid diets, which induces liver tumors [47].

Circulating miRNAs as biomarkers in NAFLD

A variety of imaging methods have been used to evaluate NAFLD in patients. Ultrasound and computed tomography scans, which are the most common methods for assessing hepatic triglyceride content, have limits in diagnosing mild hepatic steatosis and in reflecting the severity of inflammation. Although magnetic resonance spectroscopy provides highly accurate measurements of hepatic fat, it is time-consuming and requires complex procedures such as data acquisition and analysis, thus limiting its application in clinical practice [48]. The discovery of miRNAs in plasma indicates that miRNAs may be used as non-invasive biomarkers of disease.

Studies of hepatic miRNAs provide additional information for understanding the complex changes that occur during NAFLD progression. Furthermore, the release of extracellular vesicles containing miRNAs has been reported during the accumulation of lipotoxic lipids in hepatocytes, thus suggesting that circulating and liver miRNAs have a tight correlation and can be potentially used as biomarkers of liver damage [49]. Therefore, circulating miRNAs may be used as diagnostic or prognostic markers because they can be conveniently detected and correlated with histological changes. Without a doubt, a number of studies have focused on exploring changes in circulating miRNAs.

miR-122 has received considerable attention because of its wide involvement in the physiological processes of the liver. In a cross-sectional study of 443 subjects who attended health examinations in Japan, 5 serum miRNAs (miR-21, miR-34a, miR-122, miR-145, and miR-451) were elevated in NAFLD patients, and levels of serum miR-122 were correlated with the severity of liver steatosis [50]. Furthermore, a longitudinal study was conducted to explore the serum levels of miR-122 in HFD mice, in which miR-122 was upregulated prior to changes in alanine aminotransferase (ALT) during early-stage NAFLD, thus suggesting that miR-122 can be a potential

screening biomarker for NAFLD [51]. Additionally, in a mouse model of MCD-induced NASH, miR-122 serum levels increased and were correlated with ALT and aspertate aminotransferase (AST) levels, which are canonical biomarkers of liver damage [52]. Similarly, circulating miR-122 levels were upregulated in NASH patients compared with simple hepatocyte steatosis subjects and were associated with inflammatory activity and fibrosis stage [53], thus suggesting that monitoring miR-122 levels may be useful for assessing the development and severity of NAFLD-related liver injury. However, miR-122 may have low specificity in the diagnosis of NAFLD because increased serum levels of miR-122 are common in chronic liver diseases with different etiologies, including chronic hepatitis C and alcoholic fatty liver disease [54]. In addition to miR-122, Cermelli *et al.* [54] demonstrated that plasma miR-34a and miR-16 levels are high in NAFLD patients and miR-34a is associated with histological disease severity. Furthermore, circulating miR-181d and miR-99a are negatively associated with γ glutamyl transferase levels, and miR-197 and miR-10b are inversely correlated with inflammation in the liver [55]. Another research study found that 4 miRNAs (miR-122-5p, miR-1290, miR-27b-3p, and miR-192-5p) are differentially expressed in NAFLD patients compared with the control group. A 4-miRNA panel presented higher AUC and was shown to be a more sensitive and specific indicator of NAFLD than either miR-122, ALT, or fibrosis index 4 (FIB-4); this result indicates that a single miRNA is not powerful enough to reflect histological changes and act as a biomarker [56]. Therefore, a combination of multiple miRNAs and other biomarkers, such as cytokeratin 18, FGF21, ALT, and AST, may offer higher accuracy for assessing NAFLD.

Conclusions and perspectives

Several miRNAs are involved in the progression of NAFLD via various pathways, such as lipid metabolism, oxidative stress, fibrogenesis, and oncogenesis. A specific miRNA can be used to fine-tune specific processes by targeting different mRNAs, and miRNA levels vary during NAFLD progression. Therefore, a complex network exists for the regulation of related signaling pathways. Circulating miRNAs have recently emerged as biomarkers for the degree of NAFLD severity and can offer additional opportunities for the early diagnosis and staging of NAFLD. Although various studies have been performed to explore the role of miRNAs in NAFLD, multiple inconsistencies remain, and the relationship between miRNAs and histological changes in NAFLD is unclear. Therefore, further studies on the mechanism underlying the pathological progression of NAFLD are necessary. Moreover, a number of studies that focused on aberrant miRNAs in plasma were cross-sectional and cannot address the causal effects of miRNAs on NAFLD; therefore, additional long-term prospective studies are needed to clarify the predictive value of miRNAs for NAFLD. Furthermore, several miRNAs are dysregulated in multiple liver diseases and are not specific to NAFLD patients; therefore, additional studies focusing on the sensitivity and specificity of these miRNAs are required because biomarkers used in clinical practice should be able to effectively evaluate the presence and severity of disease. Given that miRNA profiles are associated with NAFLD staging, whether the joint effect of miRNAs or a combination of miRNAs and other biomarkers offer highly accurate and reliable information for NAFLD diagnosis, staging, and therapy monitoring is still unclear.

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Compliance with ethics guidelines

Zhen He, Cheng Hu, and Weiping Jia declare that they have no conflict of interest. This manuscript is a review article and does not involve a research protocol that would require approval by the relevant institutional review board or ethics committee.

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