Review Article

# MicroRNAs in Drug-induced Liver Injury

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## Abstract

Drug-induced liver injury (DILI) is a leading cause of acute liver failure, and a major reason for the recall of marketed drugs. Detection of potential liver injury is a challenge for clinical management and preclinical drug safety studies, as well as a great obstacle to the development of new, effective and safe drugs. Currently, serum levels of alanine and aspartate aminotransferases are the gold standard for evaluating liver injury. However, these levels are assessed by nonspecific, insensitive, and non-predictive tests, and often result in false-positive results. Therefore, there is an urgent need for better DILI biomarkers to guide risk assessment and patient management. The discovery of microRNAs (miRNAs) as a new class of gene expression regulators has triggered an explosion of research, particularly on the measurement of miRNAs in various body fluids as biomarkers for many human diseases. The properties of miRNA-based biomarkers, such as tissue specificity and high stability and sensitivity, suggest they could be used as novel, minimally invasive and stable DILI biomarkers. In the current review, we summarize recent progress concerning the role of miRNAs in diagnosing and monitoring both clinical and preclinical DILI, and discuss the main advantages and challenges of miRNAs as novel DILI biomarkers.


## Introduction

Drug-induced liver injury (DILI) is a growing challenge because of the increasing number of drugs used in medical care, and the increasing number of individuals who take them.[1,2] A database (www.livertox.nih.gov) has been established to provide up-to-date, comprehensive clinical information on DILI for both the general physician and the specialist. A recent epidemiologic study by Bjornsson and co-workers suggested that approximately 20 new cases of DILI per 100,000 persons occur each year.[2] DILI is also one of the most frequent causes for the termination of drug development or withdrawal of approved drugs, and therefore, has an enormous economic impact on health care expenditures.[3–6] Moreover, it is the leading cause of acute liver failure.[7] Of the estimated 10000 documented drugs developed for humans, more than 1000 have been associated with DILI.[8] The main causes of DILI in the United States are antibiotics, agents for the central nervous system, health foods and dietary supplements.[4,9,10] In China, traditional Chinese medicine and antituberculosis drugs are the major causes of DILI.[4,11,12] Children, women, and the elderly are more vulnerable to DILI, and the susceptibility is related to genetic and environmental factors.[13–15] Although there are many consistent features of DILI, early diagnosis is still challenging due to the lack of specific and sensitive clinical features.[8,16]

## Classification of DILI

DILI is usually categorized as non-idiosyncratic (predictable) and idiosyncratic (unpredictable).[17–19] The most common example of non-idiosyncratic DILI is from acetaminophen, which is one of the most commonly used medicines with a very high safety profile when used properly.[20,21] However, if misused, either intentionally or accidentally, significant liver injury can occur,[22] which has a short latency period, and is dose-related. This hepatotoxicity can also be studied in animal models.[23–26] Although idiosyncratic DILI has a longer/variable latency and is less common, it comprises the majority of clinical DILI cases.[27–29] Idiosyncratic DILI generally cannot be recapitulated in traditional animal models or in clinical trials.[28,30–32] Examples of this kind of DILI include those related to amoxicillin/clavulanate, non-steroidal anti-inflammatory drugs, and isoniazid.[13,33,34]

## Pathogenesis and treatment of DILI

The bulk of drug metabolism occurs in the liver, which is also vulnerable to damage from drug metabolites. The more common idiosyncratic type of DILI has no significant relationship with drug or dose,[27–29] and the mechanism can be divided into allergic[35] and metabolic idiosyncratic.[36] The allergic form is closely related to the high variability of the human leukocyte antigen system on chromosome 6,[37–39] whereas the metabolic form is closely associated with the genetic polymorphisms of individual drug-metabolizing enzymes, such as cytochrome P450 enzymes,[40] uridine diphosphate glucuronosyltransferase,[41] and N-acetyltransferase.[42] In addition, oxidative stress and the host inflammatory response also play important roles in the development of idiosyncratic DILI. There is mixed evidence to support the role of host factors such as age, sex, obesity,[43,44] and chronic liver disease in the development of DILI, and genetic predisposition appears to be a risk factor for injury from specific drugs.[38]

Treatment for most forms of DILI is focused on supportive care and requires longitudinal monitoring of the patient and

**Keywords:** Biomarker; Diagnosis; Drug-induced liver injury (DILI); MicroRNA.
**Abbreviations:** ALT, alanine aminotransferase; AST, aspartate aminotransferase; DILI, Drug-induced liver injury; miRNAs, microRNAs; ULN, upper limit of normal.
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laboratory work. Suspected cases of idiosyncratic DILI can be categorized as hepatitic, cholestatic, or mixed on the basis of the degree/ratio of abnormalities in the alanine aminotransferase (ALT) and alkaline phosphatase.[45] However, a careful evaluation for other causes of liver disease should be performed, and sometimes, a liver biopsy is needed. The mainstay of treatment for DILI is the immediate discontinuation of the offending drugs, and avoidance of drugs with similar chemical structures or pharmacologic effects. The timing of discontinuation of hepatotoxic drugs is controversial. Some investigators believe that the elevation of liver enzymes, including aspartate aminotransferase (AST) or ALT $> 3\times$ upper limit of normal (ULN) or alkaline phosphatase $> 1.5\times$ ULN, accompanied by elevation of bilirubin levels ($> 3\times$ ULN), indicate serious liver damage, and should trigger immediate discontinuation of suspected causative drugs.[46–49] The Federal Drug Administration has proposed the following criteria for immediate cessation of the drug in question: ALT $> 8\times$ ULN, ALT $> 5\times$ ULN for two weeks, ALT $> 3\times$ ULN accompanied by serum bilirubin $> 2\times$ ULN, pro-thrombin time-international normalized ratio $> 1.5\times$ ULN, or the appearance of liver damage symptoms. For oral agents, the severity of many cases of DILI can be decreased by reducing the residual drug in the gastrointestinal tract by means of gastric lavage, catharsis, and adsorption, or the use of diuresis, hemodialysis and other methods, within six hours of ingestion.[50,51]

## Diagnosis of DILI

It is very important to have a reliable and predictable biomarker for DILI, and considerable effort has been made to identify markers specifically for early detection. The Federal Drug Administration recently endorsed four standard serum biomarkers, including ALT, AST, total bilirubin and alkaline phosphatase, to help identify severe DILI events in clinical trials.[21,49,51] However, limitations of these current blood-based biomarkers include an unacceptable frequency of false positives/negatives, poor sensitivity, and lack of tissue specificity. For example, serum ALT activity is also associated with kidney damage and muscle necrosis.[52–54] Liver damage is only considered when serum ALT levels reach two to four times that of the control group, thus the best treatment period can easily be missed.[55] Fenofibrate can induce elevated serum transaminases, but does not cause significant liver damage, resulting in false positives.[56,57] In addition, the markers may become elevated only after substantial and sometimes irreversible tissue damage.[58] All of these characteristics decrease confidence in the utility of aminotransferases as biomarkers for DILI. Ideally, more sensitive and predictive biomarkers that respond very early before irreversible injury has occurred would offer improved outcomes.

## MicroRNAs

MicroRNAs (miRNAs) are a recently discovered class of small, endogenous, non-coding, single-stranded RNAs that are 19–24 nucleotides in length.[59] They are highly conserved, and regulate approximately 30% of all gene expression at a post-transcriptional level.[59] miRNAs were first reported in 1993 in a study by Lee *et al.* who showed that Lin-4 controlled the timing of sexual development in *Caenorhabditis elegans*.[60] To date, there are 24521 entries representing hairpin precursor miRNAs, which correspond to 30432 mature miRNAs expressed in 206 species (miRBase Release 20; http://www.mirbase.org). The major function of miRNA is to modulate gene expression either by translational repression or mRNA degradation. Binding of a miRNA to the 3′-untranslated region of mRNA with partial complementarity, as occurs in animals,[59,60] will inhibit translation, whereas perfect complementarity, such as occurs in plants,[59,61] will specifically direct mRNA cleavage resulting in target gene degradation.

Similar to other molecules involved in regulating gene expression, the expression levels of miRNAs differ significantly in various tissues and at distinct developmental stages.[59,61–63] The diversity of miRNA sequences, structures, abundance and expression make them powerful regulators of mRNA that are involved in development, proliferation, differentiation, apoptosis, energy metabolism and other physiologic processes.[63] Therefore, alterations in miRNA expression may reflect a change in the physiologic and pathologic states. Fig. 1 shows the number of retrievable manuscripts over the past 12 years in PubMed using "microRNA" and "biomarkers" as the keywords. An impressive and increasing number of related studies were observed, showing that the use of miRNA as a biomarker is currently one of the most important topics in the field.

Recent studies have shown that there are a large number of circulating miRNAs, and that altered miRNA expression profiles are closely related to disease. The discovery of miRNAs in circulating blood was first reported by Lawrie and co-workers in 2008.[64] Subsequently, the presence of stable miRNAs in plasma and serum was reported by other researchers.[65,66] Gilad and co-workers found that miRNA can be detected in serum, urine, saliva, and amniotic and other fluids, and placenta-associated serum miRNAs are significantly elevated during pregnancy.[67] These studies demonstrated that miRNAs are present in bodily fluid of humans and other animals, such as mice, rats, bovine fetuses, calves and horses, which laid the foundation for the use of miRNAs as noninvasive biomarkers, and their application in cancer prevention and disease diagnosis.[68–71]

### *MicroRNA and pathogenesis of DILI*

Responses to xenobiotics can be regulated or modulated by miRNAs in liver. For example, the exposure of rats to liver hepatotoxins, such as acetaminophen or carbon tetrachloride, results in altered expression of various miRNAs, including a decrease in miR-298 and miR-370, which are thought to regulate an oxidative stress-related gene.[72] An increase in levels of several oncogenic miRNAs, such as the 17–92 cluster, miR-106a, and miR-34, was detected in rat livers following exposure to tamoxifen, a potent hepatocarcinogen.[73] Another research group demonstrated that dioxins, which are ubiquitously present in the environment and tend to accumulate in humans and wildlife, alter the expression of miR-101a and its target, cyclooxygenase-2, which plays a significant role in liver damage.[74] Using the same method, Endo *et al.* found that in patients with suppressed expression of miR-106b, the hepatotoxic drug halothane caused upregulation of signal transducer and activator of transcription 3, which was involved with the resultant severe liver injury.[75] Furthermore, there are many studies reporting that drug-metabolizing enzymes, such as cytochrome P450 1B1, which is highly expressed in human liver, are targeted by certain miRNAs.[76–78] Taken together, these studies demonstrate that miRNAs play a significant role

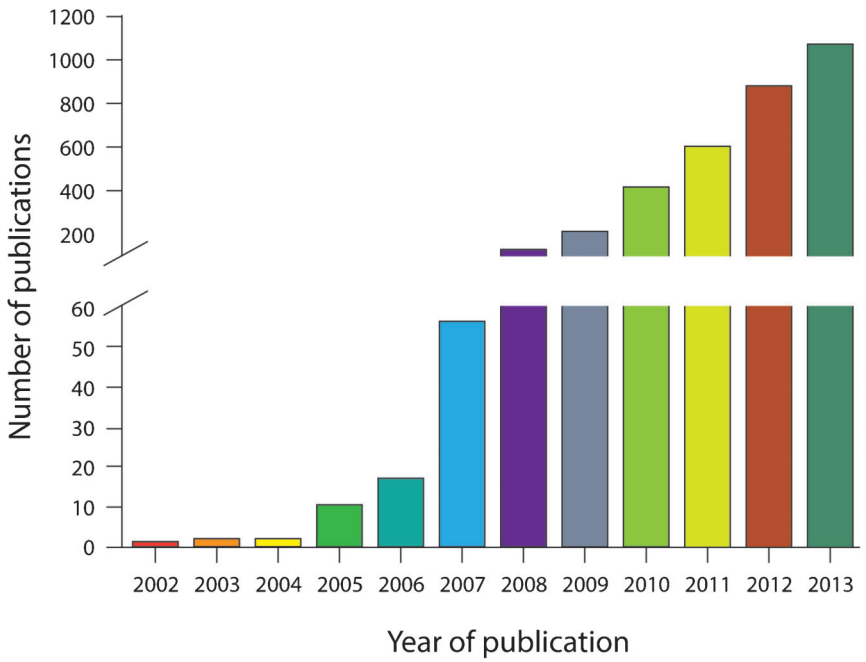


**Fig. 1. The number of articles listed on Pubmed concerning miRNAs as biomarkers published from 2002 to 2013**

in the pharmacologic and toxicologic progression of hepatotoxicity. As shown in Fig. 2, miRNAs may be involved in multiple DILI developmental processes via regulation of their target genes.

### *Circulating miRNAs as potential biomarkers of DILI*

Although studies of miRNAs as biomarkers are primarily concentrated in cancer research,[69,70] their potential as toxicologic biomarkers have also been recently explored.[79] Over the past several years, many animal and clinical studies have been published showing that miRNAs have an advantage over the conventional biomarkers for DILI. They are exceptionally stable, can be highly liver-specific and remarkably altered in pathologic states, are readily detectable in easily accessible bodily fluids, and are strictly conserved among species. Table 1 summarizes the published reports using circulating miRNAs in the blood and urine as new DILI biomarkers.

### *Serum or plasma miRNAs as biomarkers in experimental DILI*

A pioneer study by Wang *et al.* examined miRNAs as novel biomarkers in a well-established mouse model of acetaminophen-induced liver injury.[80] The authors demonstrated that the liver-enriched miR-122 and miR-192 were the top two miRNAs elevated in blood in a dose-and exposure time-dependent manner. The levels of these miRNAs preceded and paralleled serum ALT and AST levels and corresponding liver histopathology.

Several independent groups have provided additional data supporting the use of miRNAs as DILI biomarkers. In 2010, one group confirmed the time-dependent increase of plasma miR-122 levels in mice that correlated with liver histopathology induced by D-galactosamine and alcohol.[81] They also confirmed that the changes of miR-122 were larger than and preceded the changes in ALT. Importantly, significant increases in miR-122 were detected before obvious histopathologic changes in the liver, suggesting that miR-122 could be used for diagnosing and monitoring disease at early stages. Bala and co-workers discovered that serum plasma miR-122 and miR-155 were predominantly associated with the exosome-rich fraction in alcoholic and inflammatory liver injuries, whereas in acetaminophen-induced liver injury, these miRNAs were present mainly in the soluble protein-rich fraction.[82] These results suggest that circulating miRNAs may serve as biomarkers to differentiate between hepatocyte injury and inflammation, and the exosome- or protein-associated miRNAs may provide further specific mechanisms of liver pathology. The same group also compared 40 plasma miRNAs that were dysregulated with lethal or sub-lethal doses of acetaminophen, and found that miR-574-5p, miR-135a, miR-466g, miR-1196, miR-466f-3p and miR-877 were upregulated in the setting of lethal hepatotoxicity, while miR-342-3p, miR-195, miR-375, miR-29c, miR-148a and miR-652 were markedly downregulated.[83] Thus, differential expression of miRNAs in human plasma may be useful to distinguish lethal and sub-lethal hepatotoxicity.

Compared with mice, the susceptibility to acetaminophen-induced liver injury is lower in rats.[84] Using rat models created by the administration of chemical or special diets, Yamaura *et al.* examined the levels of the plasma miRNAs in acute liver injury (hepatocellular injury or cholestasis) and chronic liver injury (steatosis, steatohepatitis, and fibrosis).[85] Their results showed that miR-122 levels increased more quickly and dramatically than levels of aminotransferases, reflecting the extent of hepatocellular injury. Importantly, their study also demonstrated that the expression profiles of plasma miRNAs differed according to the type of liver injury,

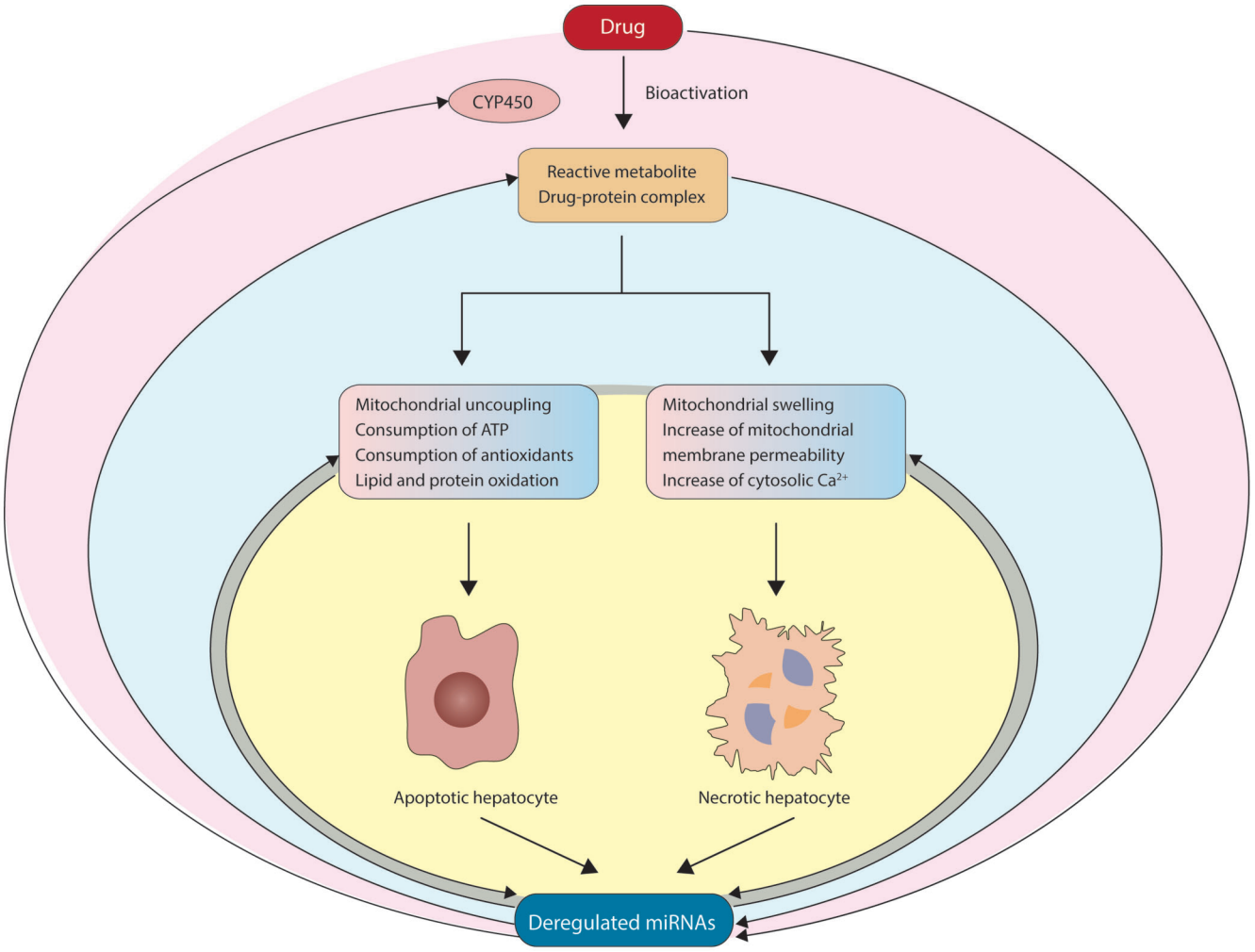


**Fig. 2. Pathways by which miRNAs may be involved in the development of drug-induced liver injury (DILI).** Drugs induce hepatocyte injury through their toxic metabolites generated via CYP450. The toxic metabolites can induce the consumption of ATP, production of reactive oxygen species and formation of drug-protein/DNA complexes, which result in mitochondrial uncoupling and hepatocellular apoptosis. When there is no ATP consumption, the reactive metabolites increase the mitochondrial permeability, causing an increase in cytosolic $Ca^{2+}$ and cell necrosis. All of these processes can affect the expression of miRNAs, and the deregulated miRNAs can, in turn, regulate the development of DILI through their target genes.

suggesting that these miRNAs could be specific and sensitive biomarkers for various types of liver injury. Su *et al.* also reported that miR-122, miR-192 and miR-193 have the potential to serve as sensitive, specific and noninvasive biomarkers for the diagnosis of herb-induced liver damage.[86] Furthermore, Starckx and co-workers demonstrated that the levels of miR-122 in rat plasma were significantly increased following administration of four well-characterized compounds associated with different types and mechanisms of liver toxicity: acetaminophen, allyl alcohol, alpha-naphthyl isothiocyanate, and phenobarbital.[87] The response of miR-122 secretion by mouse liver cells paralleled that of other markers, and was consistent with liver injury as indicated by ALT/AST and histopathologic evaluation. The changes of plasma miR-122 were also detected significantly earlier than other conventional biomarkers, and exhibited a wide dynamic range. Taken together, all the above discoveries demonstrate that miR-122 has great potential to be used as a biomarker of liver injury and may provide added value for assessing liver toxicity in both preclinical studies and the development of new drugs.

### *Urinary miRNAs as noninvasive biomarkers in experimental DILI*

The discovery of circulating miRNAs in the urine and other bodily fluids has created a new approach for the discovery of noninvasive biomarkers of organ injury.[88] For example, urine-derived miRNAs could become useful biomarkers for kidney[89] and bladder diseases,[90] or even for DILI. In a rat DILI model, urinary levels of some miRNAs were elevated, likely released from the liver after injury.[91] High-dose acetaminophen-treated rats showed elevations of serum ALT/AST, histologic signs of liver injury, and significant increases in urinary miRNAs levels. Although low-dose acetaminophen-treated rats did not show histologic signs of liver injury or changes in serum ALT/AST, urinary levels of nine miRNAs were substantially increased. Similarly, carbon

**Table 1. Circulating miRNAs in drug-induced liver injury (DILI)**

| Drug or chemical | Species | Sample type | Quantification method | miRNA expression in DILI | Clinical relevance | Reference |
|---|---|---|---|---|---|---|
| Acetaminophen, CCl4 | Rat | Liver | miRNA array, qPCR | miR-153↑, miR-302b↑, miR-337↑, miR-363↑, miR-409-5p↑, miR-542-3p↑, miR-29c↓, miR-298↓, miR-327↓, miR-342↓, miR-370↓, miR-376c↓, miR-494↓, miR-503↓ | Early diagnosis | [72] |
| Acetaminophen | Mouse | Plasma | miRNA array, qPCR | miR-122↑, miR-192↑, miR-193↑, miR-710↓, miR-711↓, miR-483↓ | Early diagnosis | [80] |
| D-GalN/LPS, alcohol | Mouse | Plasma | qPCR | miR-122↑ | Diagnosis and monitoring | [81] |
| Acetaminophen, unidentified drugs | Human | Plasma | qPCR | miR-122↑, miR-192↑ | Diagnosis and pharmaceutical evaluation | [92] |
| Alcohol, CpG/LPS, acetaminophen | Mouse | Plasma, Serum | qPCR | miR-122↑, miR-155↑, miR-146a↑ | Differentiate hepatocyte injury and inflammation | [82] |
| Acetaminophen, ANIT, allyl alcohol, phenobarbital, doxorubicin | Rat | Plasma | qPCR | miR-122↑ | Diagnosis and monitoring | [87] |
| Paraquat | Human | Serum | qPCR | miR-122↑, miR-192↓, miR-483↓, miR-711↓ | Diagnosis | [96] |
| Acetaminophen | Human | Whole Blood | miRNA array, qPCR | miR-29c↑, miR-19a↑, miR-19b↑, miR-802↑, miR-374a↓, miR-505↓ | Diagnosis | [93] |
| Acetaminophen, herb drug | Rat | Serum | miRNA array, qPCR | miR-122↑, miR-192↑, miR-193↑, miR-200a↑, miR-101a↑, miR-323↓, miR-322↓, miR-327↓, miR-380↓, miR-214↓, miR-342-3p↓ | Diagnosis | [86] |
| Acetaminophen | Mouse | Plasma | miRNA array, qPCR | miR-574-5p↑, miR-135a*↑, miR-466g↑, miR-1196↑, miR-466f-3p↑, miR-877↑, miR-342-3p↓, miR-195↓, miR-375↓, miR-29c↓, miR-148a↓, miR-652↓ | Medication guidance | [83] |
| Acetaminophen, ANIT, methapyrilene, CCl4 | Rat | Plasma | miRNA array, qPCR | miR-200a*↑, let-7c-1*↑, miR-503↑, miR-337-3p↑, miR-10b↑, miR-190↓, miR-743b↓, miR-449c↓, miR-410↓, miR-10b*↓ | Diagnosis of different types of liver injury | [85] |
| Acetaminophen, CCl4, penicillin | Rat | Urine | miRNA array, qPCR | miR-185↑, miR-296↑, miR-484↑, miR-434↑, miR-20b-3p↑, miR-330*↑, miR-433↑, miR-664↑, miR-291a-5p↑, miR34c*↑ | Diagnosis of different types of liver injury | [91] |

Abbreviations: ANIT, alpha-naphthyl isothiocyanate; CpG, cytidine-phosphate-guanosine; CCl4, carbon tetrachloride; D-GalN, d-galactosamine; LPS, lipopolysaccharide; qPCR, quantitative real-time polymerase chain reaction.

tetrachloride also led to an increase in urinary levels of 28 miRNAs, among which ten overlapped with the 44 identified in the acetaminophen-induced model. Therefore, urinary miRNAs appear to be another form of noninvasive DILI biomarker and may be useful for the classification of hepatotoxins. However, further studies are needed to examine their organ-specificity in detecting DILI.

### *Circulating miRNAs as biomarkers in human DILI*

A study examining human subjects who suffered DILI showed that the plasma levels of both miR-122 and miR-192 were substantially higher in patients who suffered acetaminophen-induced liver injury than in those who did not.[92] Moreover, levels of the liver-enriched miR-122, but not miR-192, correlated with serum ALT levels, consistent with results from a previous study in a mouse model.[80] Their findings also showed that the level of circulating miR-122 decreased to baseline much earlier than serum ALT did, suggesting that miR-122 has a shorter circulatory half-life. This work provided the first convincing evidence that circulating miRNAs could be used as a human DILI biomarker. Jetten *et al.* also demonstrated that miR-19b and miR-29c were upregulated in human blood cells after treatment with low-dose acetaminophen.[93] However, the level of ALT was not altered. These results suggest that the expression profile of circulating miRNAs may be altered at a very early stage when liver damage is undetectable using conventional markers. Indeed, another study showed that miR-122, along with high mobility group box-1 and full-length and caspase-cleaved keratin-18, were more sensitive than ALT at identifying acetaminophen-induced acute liver injury.[94] Thulin and colleagues also showed that serum keratin-18 and miR-122 levels were significantly increased at an earlier time point and to a greater extent than ALT in patients with DILI.[95]

Paraquat is one of the most common toxic herbicides, and is widely used around the world. By comparing paraquat-exposed human subjects with healthy donors, Dingand and co-workers found that the serum levels of miR-122 were strongly increased and correlated well with the status of liver function.[96] This pattern was similar to that was seen in the mouse acetaminophen-induced liver injury model.[80] However, miR-192, also identified in the mouse study, was unexpectedly decreased two-to-eight-fold in human hepatic samples.

## Conclusions

The prediction, diagnosis and management of DILI are very complicated issues. The current biomarkers or approaches to assess DILI include biochemical markers such as aminotransferases, total bile acids, histopathology or ultrastructural pathology, active metabolites and immune-related markers. However, due to the poor sensitivity, stability or specificity, these conventional toxicologic biomarkers cannot provide accurate information for early evaluation of liver hepatotoxicity. The development of novel, reliable and informative biomarkers remains an important issue in clinical and preclinical settings. Liver-specific miR-122 has been shown to be involved in various processes of liver development, differentiation, metabolism and stress responses.[97–99] Compared with the conventional hepatotoxic markers, circulating miR-122 can effectively and consistently distinguish intrahepatic from extrahepatic damage with higher sensitivity and specificity.[92–96] Thus, miR-122 is expected to be a preclinical and clinical biomarker of DILI.

Although the use of circulating miRNA levels for early diagnosis of DILI shows promise, further study is required before it is used in clinical applications. The major issues to be addressed include: 1) lack of association between miRNAs and mechanistic information on DILI; 2) lack of drug-specific miRNAs to diagnose various types of DILI; 3) lack of larger sample sizes validating the assay; and 4) lack of conventional detection methods that can be carried out in routine clinical laboratories, including standardized detection systems, standard reference materials, and quality control materials.[100,101] More studies are required to demonstrate the mechanisms underlying the phenotypes of DILI, including research and investigation on the alterations of miRNAs in liver tissues after treatments with various drugs representing a broad range of DILI types, combining miRNAs with other biomarkers, and multicenter collaborations.

In conclusion, the use of miRNAs, especially miR-122, as noninvasive biomarkers for DILI shows great potential, but is still in its infancy. More research on circulating miRNAs is needed to validate the use of miRNAs as novel biomarkers of DILI.

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## Conflict of interest

None

## Author contributions

Analyzing the data (LML, DW), drafting the manuscript (LML, KZ).

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