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CURRENT OPINION

Circulating MicroRNAs in Drug Safety Assessment for Hepatic and Cardiovascular Toxicity: The Latest Biomarker Frontier?

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Abstract Drug-induced liver and cardiovascular injuries are important aspects of safety evaluations of numerous drugs in development. Therefore, reliable and predictive biomarkers to allow detection of early signs of drug-induced liver and cardiovascular injuries are required in clinical and preclinical pharmaceutical evaluation. MicroRNAs (miRNAs) are reported to be present in body fluids (blood, urine, etc.), and these 'circulating miRNAs' have been proposed as toxicological biomarkers of drug-induced tissue injury in preclinical and clinical practice. To be used as biomarkers of drug toxicity, such miRNAs need to show rapid and injured-tissue–specific upregulation in body fluids after injury, be more sensitive than existing protein markers such as alanine aminotransferase (ALT) and troponins, and be able to identify the toxicants responsible, if possible. In this article, we focus on the current knowledge of circulating miRNAs, which have potential for use in assessment of drug-induced liver and cardiovascular injuries. In addition, we discuss an important question regarding normalization of the expression levels of certain circulating miRNAs in body fluids.

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1 Introduction

MicroRNAs (miRNAs) are endogenous non-coding RNAs of ~22 bp in length, which suppress gene expression in a sequence-specific manner and play important roles in a wide range of physiological and pathological processes [1, 2]. miRNA was first identified in *Caenorhabditis elegans* as RNA molecules that were complementary to the 3′ untranslated regions of the target transcript, such as the lin-4 and let-7 genes [3, 4]. On the basis of miRBase release 20.0, more than 1,800 human miRNAs have been registered [5], with a large number being evolutionarily conserved [6, 7]. It has been reported that miRNAs are expressed in all animal cells and have fundamental roles in cellular activities such as development, cellular differentiation, proliferation, cell-cycle control, apoptosis, metabolism, and cancer [7]. miRNAs can downregulate gene expression by affecting mRNA stability and influencing protein synthesis in a sequence-specific manner [8]. Similarly to mRNA, some miRNAs are produced in a cell- or tissue-specific manner [9, 10]. Those specific miRNAs would be released to ouside of these cells followed by cell injury. Thus, it is strongly suggested that injured cells or tissues could be identified by detecting such miRNAs circulating in body fluids.

Recent studies have suggested that miRNAs are not only localized within the cell (cellular miRNA) but are also present in extracellular spaces such as body fluids [11–16]. Interestingly, these circulating miRNAs are resistant against RNase- and repetitive freezing and thawing cycle-mediated degradation, owing to the formation of complexes with high-density lipoproteins [17] or Argonaute 2 [18], or envelopment in microvesicles [19, 20], exosomes [21, 22], or apoptotic bodies [23, 24]. miRNAs are detectable and highly stable in circulation [25, 26], making

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them attractive for biomarker discovery. On the basis of these findings, the profile of circulating miRNAs in a wide range of body fluids, including urine, blood, cerebrospinal fluid, bronchial lavage, and breast milk, has been exploited as a biomarker in various diseases, including cancer [27], drug-induced tissue injury [9], and inflammation [28]. In oncology, many studies have demonstrated that circulating miRNAs are a potential diagnostic biomarker of chronic lymphocytic leukemia [29], non-small-cell lung cancer [30], colorectal cancer [31], and many other cancers [12, 32]. These data consistently show that circulating miRNAs are potentially useful biomarkers for cancer diagnosis and prognosis. Recently, several studies have reported that the levels of specific circulating miRNAs have been used to detect and monitor tissue injury induced by drugs, although the number of such studies is relatively small.

In this article, we discuss recent findings regarding the potential of circulating miRNAs as biomarkers of liver and cardiovascular toxicity.

2 Use of Circulating miRNAs as Biomarkers of Liver Injury

Drug-induced liver injury is an adverse event that frequently leads to cessation of drug testing in clinical trials, restrictions on drug use, and removal of drugs from the market [33–35]. Assessment of liver injury induced by drugs is a major safety issue during drug development. Existing biomarkers of liver injury—for example, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin—provide reasonable indicators of damage or altered function [36]; however, serum ALT and AST activities are known to be increased in other organ injuries [37, 38]. A number of exploratory biomarkers are currently under investigation, including high-mobility group box 1, cytokeratin 18, glutamate dehydrogenase, sorbitol dehydrogenase, albumin mRNA, α-glutathione S-transferase, and F-protein [39, 40]. Although several of these exhibit a high degree of sensitivity for early liver injury and varying degrees of cell-type specificity, they do not discriminate between drug and non-drug etiologies. Therefore, reliable new biomarkers of liver injury are urgently required for clinical use and for preclinical pharmaceutical evaluation. In recent years, several studies have demonstrated the possibility of evaluating drug-induced liver injury by measuring circulating miRNA levels (see Table 1).

The first study of a circulating miRNA profile in liver injury was conducted by Wang et al. [9]. The authors reported that two liver-enriched miRNAs (miR-122 and miR-192) were observed to be reliable serum biomarkers of acetaminophen-induced acute liver injury in mouse plasma in a dose-dependent and exposure duration-dependent manner, which paralleled that of ALT and caused histopathological changes in the liver. Moreover, these miRNAs were detected earlier than ALT and at lower doses, indicating greater sensitivity, and this change was more specific for viral-, alcohol-, and chemical-induced liver injury than for other organ damage, and was a more stable and reliable biomarker [41]. ALT and AST have also been shown to be increased in extrahepatic injury such as muscle damage or cardiac injury [38, 42]. Both of these parameters were increased in plasma samples from rats that had been treated with 2,3,5,6-tetramethyl-*p*-phenylenediamine (a skeletal muscle toxicant), although no hepatocellular degeneration or necrosis was observed. In the same plasma samples, levels of miR-133a, but not miR-122, were significantly elevated (more than 500-fold) when compared with levels in plasma samples from control rats [43]. In contrast, an approximately 6,000-fold increase in miR-122 and only a minimal increase in miR-133a (approximately 10-fold) was observed in rats treated with the liver toxicant trichlorobromomethane, indicating that miR-122 and miR-133a in plasma could be injured-tissue–distinguishable markers and clearly relative to ALT and AST. Interestingly, the specificity of serum miR-122 for acetaminophen-induced acute liver injury in humans was also examined in a heterogeneous group of patients with liver injury relative to comparator cohorts of healthy volunteers and patients with chronic proteinuric kidney disease [44]. That study showed that miR-122 levels in serum were raised and correlated with an existing biomarker of drug-induced liver injury, indicating that circulating miR-122 could be a novel and valuable diagnostic biomarker of human liver injury. In addition to drug-induced liver injury, several studies have reported that serum levels of miR-122 were increased in HBV- or HCV-induced liver injury [41, 45–47]. These data suggest that elevated circulating levels of miR-122 reflect liver injury independent of the cause of the injury. Therefore, pathogen-specific biomarkers are needed to identify the cause of human liver injury.

Yamamura et al. [48] reported that plasma miRNA profiles exhibited different patterns depending on the differentiating pathogenesis of acute and chronic liver injury, as well as hepatocellular injury, cholestasis, steatosis, steatohepatitis, and fibrosis in rat models, thus suggesting that plasma miRNA profiling could be useful for distinguishing the different types of liver injury when compared with conventional biomarkers, such as ALT and alkaline phosphatase (ALP). Interestingly, the state of circulating miRNAs in serum/plasma differed depending on the type of liver injury. Circulating miRNAs including miR-122 and miR-155 were predominantly associated with the exosome-rich fraction in alcoholic liver disease, whereas these miRNAs were present in the protein-rich fraction in drug-

Table 1 Tissue injury-associated microRNAs (miRNAs) in blood or urine

Injured tissue	Toxicants/diseases	Upregulated miRNAs	Body fluid	Species	References
Liver	Acetaminophen	miR-122, -192	Plasma	Mouse	[9]
Liver	Acetaminophen	miR-122, 146a, -155	Plasma (protein-rich fraction)	Mouse	[49]
Liver	Cytidine-phosphate-guanosine, lipopolysaccharide	miR-122, 146a, -155	Plasma (exosome-rich fraction)	Mouse	[49]
Liver	Ethanol	miR-122, -155	Plasma (exosome-rich fraction)	Mouse	[49]
Liver	Trichlorobromomethane	miR-122	Plasma	Rat	[43]
Liver	Acetaminophen	miR-122	Plasma	Human	[44]
Liver	Acetaminophen	miR-20b-3p, -34c*, -185, -291a-5p, -296, 330*, -433, -434, -484, -664	Urine	Rat	[50]
Liver	Hepatitis B, hepatitis C	miR-122	Plasma	Human	[41, 45–47]
Heart	Isoproterenol	miR-208	Plasma	Rat	[55]
Heart	Ischemic preconditioning, ischemia/reperfusion	miR-1	Plasma	Rat	[56]
Heart	Acute myocardial infarction[a]	miR-1	Urine	Rat	[57]
Heart	Acute myocardial infarction	miR-133a/b	Plasma	Human	[58–61]
Heart	Acute myocardial infarction	miR-499	Plasma	Human	[59, 60, 62]
Heart	Acute coronary syndrome	miR-1/-21/-499[b]	Plasma	Human	[63]

[a] Acute myocardial infarction induced by left anterior descending coronary artery ligation

[b] Combination use of the three miRNAs

induced liver injury [49]. These data indicated that extracellular distribution of miRNAs is distinct under different pathological conditions—for example, severe and rapid damage or mild and slower damage—in the liver.

Use of urine samples to detect miRNA as a biomarker could also be useful. Yang et al. [50] reported that specific miRNA profiles in urine were identified in acetaminophen- or carbon tetrachloride-induced liver injury rats. Ten miRNAs (miR-20b-3p, -34c*, -185, -291a-5p, -296, -330*, -433, -434, -484, and -664) were commonly increased by treatment with both acetaminophen and carbon tetrachloride in this model. Interestingly, the alteration pattern in urine reflected that in the liver, indicating that patterns of urinary miRNA hold promise as a good biomarker of hepatotoxicant-induced liver injury.

3 Use of Circulating miRNAs as Biomarkers of Safety for Assessment of Cardiovascular Toxicity

Cardiac safety biomarkers that translate from drug discovery through preclinical to clinical development and into the market are also necessary. Like hepatotoxicity, cardiotoxicity is an important cause of compound attrition in preclinical and clinical development. Cardiovascular toxicity accounted for 9% of withdrawals of prescription drugs from the worldwide pharmaceutical market from 1960 to 1999 [51]. In the cardiac field, biomarkers such as cardiac myoglobin, creatine kinase-MB isoenzymes, and troponins are now routinely used for assessment of myocardial injury [52]. Unfortunately, many of these markers have reduced sensitivity or less specificity, or do not allow timely diagnosis. Therefore, a multiple biomarker strategy may circumvent these limitations by adding accuracy and predictive power.

A recent report has suggested that serum levels of cardiac-expressed miRNAs react to cardiac injury in a manner similar to that of cardiac enzymes (Table 1). Most previous studies have focused on four miRNAs (miR-1, miR-133a/b, miR-208, and miR-499) as biomarkers of cardiac injury. Of these, miR-208 is expressed exclusively in the heart; the other miRNAs are expressed in both heart and skeletal muscle in humans and rats [53]. The first study of circulating miRNA in plasma in myocardial injury was

conducted by Ji et al. [54], who reported that plasma levels of miR-208, which is produced exclusively in the heart, increased in isoproterenol-induced myocardial injury. Plasma levels of miR-208 show good correlation with plasma levels of cardiac troponin I, a classic and gold-standard marker of myocardial injury [55]. In addition to miR-208, other skeletal muscle-enriched miRNAs, such as miR-1, miR-133a/b, and miR-499, have been evaluated.

As far as we know, only the study by Ji et al. [54] has demonstrated a relationship between circulating miRNA levels and drug-induced cardiovascular injury. Thus, circulating miRNAs as a biomarker of acute myocardial infarction (AMI) are also described in this section to explore their potential as biomarkers of drug-induced cardiovascular toxicity. Cheng et al. [56] reported that serum levels of circulating miR-1 were significantly increased in patients with AMI and were positive correlated with serum creatine kinase-MB levels. Moreover, they reported that the levels of circulating miR-1 in urine were significantly elevated in patients with AMI and showed a positive correlation with serum troponin I levels [57]. In addition, a time-course study using rats showed an obvious delay in the increase of miR-1 levels in urine when compared with that in blood, but the increase of miR-1 levels in urine was sustained longer than that in blood. Consistently, serum levels of miR-133, which belongs to the same cluster and is cotranscribed with miR-1, have been found to be elevated in humans after AMI [58–61]. Several additional studies have shown that circulating levels of the myosin-related miR-499 are elevated in patients after AMI [59, 60, 62]. Despite these encouraging results, the number of samples in the aforementioned studies is insufficient to provide clear proof of the diagnostic power of miRNA signatures and their value for clinical testing of AMI patients. Oerlemans et al. [63] then examined the expression of several miRNAs in the serum of 106 acute coronary syndrome (ACS) patients and 226 patients who had chest pain but were not diagnosed as having ACS. The expression levels of the combined three miRNAs (miR-1, -21, and -499) were significantly higher in the ACS patients than in the non-ACS patients. Interestingly, the combination of these three miRNAs resulted in a significantly greater area under the receiver operating characteristic curve (AUC) of 0.94 than that of high-sensitivity troponin T (0.89). This suggests that these three circulating miRNAs in blood are strong biomarkers for detecting myocardial injury. However, almost all of the miRNAs described in this section, especially in the second half, may not necessarily relate to cardiovascular injury induced by drugs, because few miRNA studies have investigated drug-induced cardiovascular toxicity. Therefore, it is necessary to investigate circulating miRNAs as reliable biomarkers of cardiovascular injury induced by drugs in humans.

4 A Remaining Question in Methodology: How to Normalize Circulating miRNAs

Detection of miRNAs by real-time quantitative polymerase chain reaction (qPCR) has the advantages of being robust, relatively inexpensive, and sensitive to even small amounts, because of signal amplification [64]. Yet many factors dictate the quality of real-time qPCR results, such as differences in the quality of the starting materials and RNA extraction or transcript efficiencies [65]. Thus, a suitable normalizer is required to eliminate as much variation as possible to increase the accuracy of expression measurements. Although small RNA molecules such as 5S and U6 are frequently used as reference genes, they may change widely depending on pathological conditions [66], thus suggesting that these small RNA molecules are not suitable as internal controls in all studies. Synthetic spike-in miRNAs, mainly *C. elegans* miRNAs without homology to mammalian miRNAs, are also used for normalization, but they cannot correct sample-to-sample variations and are unstable in crude plasma [67]; thus, no housekeeping miRNA/small RNA or universal normalizer has yet been established. In addition, evidence reported in previous studies suggests that the transcript levels of some housekeeping genes vary considerably in response to changes in experimental conditions and/or patient conditions [68, 69].

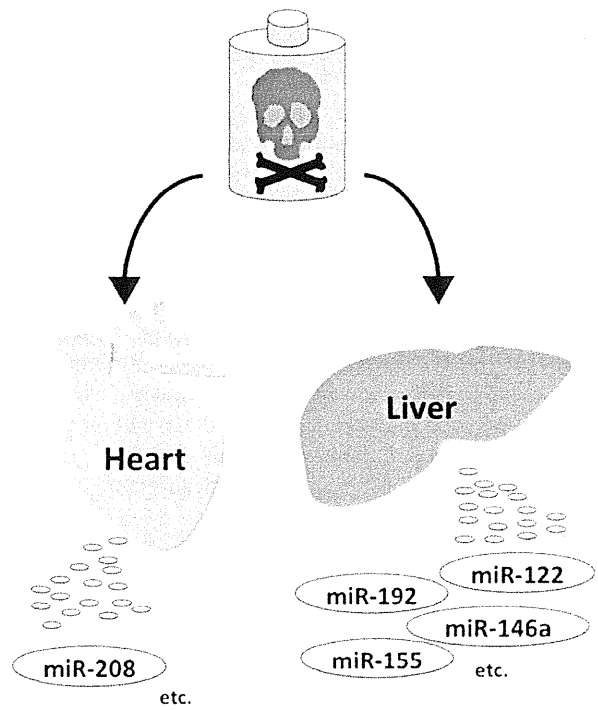

Fig. 1 Circulating microRNAs (miRNAs) as possible biomarkers of liver and heart injury induced by drugs

Wang et al. [70] reported that miR-103 was a suitable reference gene for plasma miRNA analysis in an acetaminophen-induced liver injury model in rats. This suggests that miR-103 is a suitable normalizer in miRNA analysis using plasma/serum, although it will be necessary to evaluate stability in various drug-induced injury models. According to this view, it may be necessary to perform a well-controlled analysis of circulating miRNAs in a large cohort of patients and healthy volunteers. These studies provide further evidence that miRNAs may be useful as serum biomarkers for clinical use.

5 Conclusion

In this article, we discuss recent findings regarding the possibility of circulating miRNA as a biomarker of liver and cardiovascular toxicity (Fig. 1). Although the field of miRNA-related toxicological studies is still in its infancy, novel, reliable, and sensitive miRNA biomarkers that can be used for assessment of tissue-specific toxicity will continue to be discovered in the future, and it is hoped that the disadvantages of circulating miRNAs as biomarkers will be conquered, especially for use in clinical applications. Circulating miRNAs in biological fluids have great potential to contribute to drug development and clinical therapy.

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