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Using microRNA as Biomarkers of Drug-Induced Liver Injury

William F. Salminen*, Xi Yang, Qiang Shi and Donna L. Mendrick

Division of Systems Biology, National Center for Toxicol ogical Research, Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079, USA

Abstract

MicroRNAs (miRNA) are small non-coding RNAs that regulate gene expression post-transcriptionally. They bind to complementary sequences on target mRNA and typically down regulate expression or increase the rate of degradation; however, the roles of miRNA are still evolving and some miRNA have been shown to increase specific gene translation. miRNA holds a unique position among RNA for use as a biomarker due to its unique stability. Unlike mRNA, miRNA has been shown to be remarkably stable in a variety of tissues and body fluids. This greatly facilitates the use of miRNAs as clinical biomarkers of disease and injury since sample handling and processing is much less problematic when compared to mRNA. miRNA expression profiles have been extensively investigated for distinguishing cancerous vs. non-cancerous tissue. Taking this approach one step further, profiles of miRNA in cell-free body fluids have also been able to distinguish patients with different types of cancer and even provide prognostic information about disease outcome. The rationale behind this approach is that cancerous masses release miRNA into the systemic circulation and changes in the pattern and amount of miRNA can be used to detect the type of cancer. A recent extension of this approach is using miRNA in cell-free body fluids to detect organ injury. Several studies have shown increased serum levels of specific miRNA after myocardial or hepatocellular injury. Since some miRNA exhibit tissue specific expression, it is possible that miRNA profiles could be used to not only identify gross organ injury but also distinguish between different types of organ injury (e.g., heart vs. liver). This article will provide an overview of the role of miRNA in the cell, review the literature on using miRNA profiles to identify organ injury, and highlight the potential use of miRNA for assessing drug-induced liver injury. It should be noted that at the time of this writing, none of the profiles have been qualified for clinical use by the FDA.

Keywords: miRNA; Drug-induced liver injury; Biomarker

Introduction

MicroRNAs (miRNA) are small non-coding RNAs that regulate gene expression post-transcriptionally [1]. It has been estimated that >60% of human protein-coding genes are regulated by miRNAs [2]. They typically down regulate gene expression; however, the roles of miRNA are still evolving and some miRNA have been shown to increase specific gene translation [3]. miRNA have even been shown to be released from cells and regulate gene translation in distant cells, reminiscent of cell-to-cell communication by cytokines and other soluble factors that are actively released from cells [4,5]. To date, over 720, 408, and 1424 miRNAs have been identified in mice, rats, and humans, respectively (Sanger miRBase Release 17).

miRNA holds a unique position among RNA for use as a biomarker due to its unique stability. Unlike mRNA, miRNA has been shown to be remarkably stable in a variety of tissues and body fluids [6-8].This stability greatly facilitates its use as a clinical biomarker of disease and injury since sample handling and processing are much less problematic when compared to mRNA. miRNA-based biomarkers also have many advantages over protein-based biomarkers primarily due to the fact that miRNA is a relatively simple molecule that can be detected using standard, robust molecular biology techniques such as real time quantitative polymerase chain reaction (RT-qPCR).

miRNA expression profiles have been extensively investigated for distinguishing cancerous vs. non-cancerous tissue [1,9-12].Profiles of miRNA in cell-free body fluids have also been able to distinguish patients with different types of cancer and even provide prognostic information about disease outcome [13,14]. The hypothesis is that cancerous masses release miRNA into the systemic circulation and, therefore, changes in the pattern and amount of miRNA can be used to detect the type of cancer. Studies are showing promise in using miRNA in cell-free body fluids to detect organ injury. Since some miRNA exhibit tissue specific expression, it is possible that miRNA profiles could be used to not only assess tissue injury but also distinguish between different organs (e.g., heart vs. liver). The following sections provide an overview of the role of miRNA in the cell, review the relevant literature on using miRNA profiles to identify organ injury, and highlight the use of miRNA in assessing drug-induced liver injury (DILI).

miRNA transcription, processing, and function

miRNAs are a newly discovered class of non-coding RNA that regulate entire intracellular pathways primarily at a posttranscriptional level. miRNAs are approximately 21 - 24 nucleotides long and are highly conserved among species. Some miRNAs are derived from introns of protein-coding genes; whereas, others are derived from non-protein-coding RNA transcripts. Both of these miRNA derivation processes have provided insight into the unique role for these regions of DNA that were previously believed to have no relevant cellular function. Many miRNAs are located in clusters of 2-19 miRNA hairpins encoded in close proximity and can be derived from a single transcript. The processing of miRNA into the final mature form differs from mRNA and involves transcription from the relevant sequence of DNA and subsequent unique cleavage steps. The sequence of events is diagramed

***Corresponding author:** W. F.Salminen, Division of Systems Biology, National Center for Toxicological Research, Food and Drug Administration, 3900NCTR Road, Jefferson, AR 72079, USA, Fax: 1-870-543-7736; E-mail: William.Salminen@fda.hhs.gov

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in Figure 1 and more details can be found in various reviews of the synthesis, processing, and function of miRNA [15-19].

Similar to mRNA, most miRNAs are transcribed by RNA polymerase II; in this case, primary miRNAs (pri-miRNAs) are generated. Pri-miRNAs are produced in a wide array of lengths, often several thousand nucleotides long, and similar to mRNA are capped at the 5'end and polyadenylated at the 3' end [15,18]. Within the nucleus, the pri-miRNAs are processed by the ribonuclease III enzyme Drosha in cooperation with DGCR8 releasing small, approximately 70-nucleotide-long, stem-loop-structured molecules called precursor miRNAs (pre-miRNAs). The pre-miRNAs are exported from the nucleus into the cytoplasm by the protein Exporten 5 in a GTP-dependent process. Once in the cytoplasm, the pre-miRNAs are processed into approximately 22-nucleotide long mature RNA duplexes by the enzyme Dicer in cooperation with the double-stranded RNA-binding protein TRBP. The mature miRNA duplex consists of a guide strand and a passenger strand. The Dicer-TRBP complex, in cooperation with argonaute 2, unwinds the duplex [20]. The guide strand is then incorporated into the argonaute-containing RNA interference-induced silencing complex (RISC) while the passenger strand is degraded [21]. The exact mechanism of strand selection is unclear but the guide strand generally exhibits lower thermodynamic stability at the 5' end [22].

The miRNA/RISC complex is the ultimate effector of miRNA-mediated gene repression. Most reactions involve binding of the miRNA/RISC complex to the 3' untranslated region of the target mRNA; however, the mechanisms of miRNA regulation of gene expression are still evolving and there are likely other mechanisms that result in gene regulation such as binding to the 5' untranslated region of the target mRNA [9]. If the miRNA exhibits perfect complementation to the target mRNA sequence, the mRNA is targeted for degradation. If the miRNA has less than perfect complementation, mRNA translation is repressed. In mammalian cells, the seed region of miRNAs (2-8 nucleotides) is the primary determinant of target recognition within the 3'-untranslated region of the target mRNA [23]. The majority of reactions requires perfect complementation between the seed region and the target mRNA; whereas, pairing outside the seed region is less stringent. Since the seed region is small and it is the primary target recognition determinant, a single miRNA can potentially regulate hundreds of target mRNAs. The concentration of a given miRNA can be regulated at all of the various processing steps, providing additional control over gene regulation [16].

Many studies have shown that miRNAs are deregulated under different pathological conditions, such as cancer and liver injury [10,15,17,24]. In the case of cancer, up-regulation of miRNA expression can induce or suppress tumor formation depending on

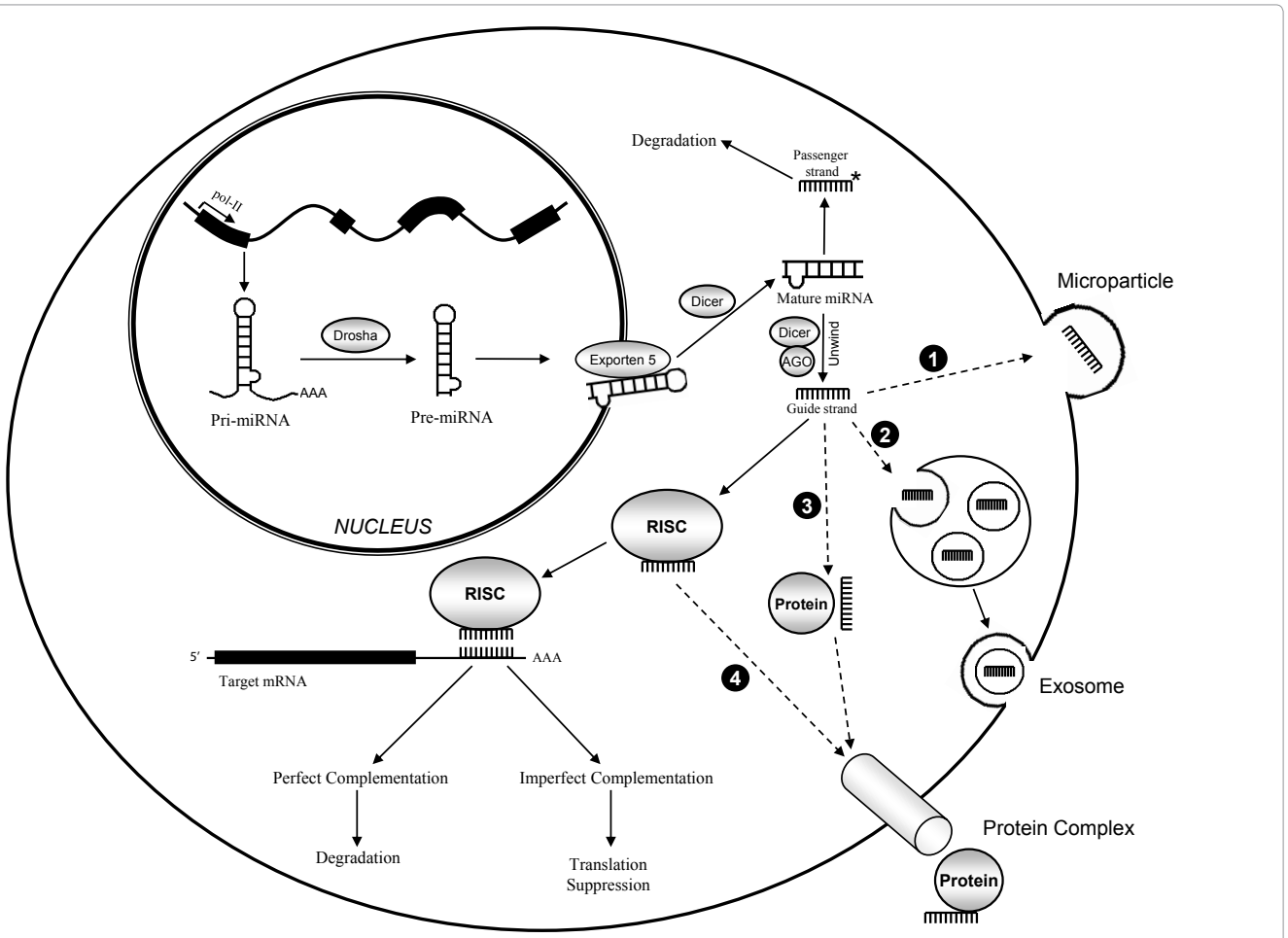

Figure 1: miRNA transcription, processing, gene targeting, and extracellular release. After processing and selection of the guide strand, miRNA may be released from the cell as 1) microparticles, 2) exosomes from microvesicular bodies, and 3) & 4) protein complexes. RISC is the RNA interference-induced silencing complex that contains argonaute proteins.

their normal role of inhibiting tumor suppressive or oncogenic target mRNAs, respectively. For example, miRNA-21 targets multiple tumor suppressor genes, including Bcl2, PTEN, and Fas ligand. Increased expression of miRNA-21, as found in some cancers, results in the downregulation of multiple mRNA targets and subsequent increased proliferation and/or decreased apoptosis [16]. In general, miRNAs and their targets have reciprocal expression patterns and miRNA regulation of gene expression appears to act like a rheostat to fine tune gene expression of many genes as opposed to gross regulation of a single gene [25]. In addition, there is redundancy built into miRNA regulation of gene expression with many miRNAs being part of families with related sequences that are potentially involved in the same pathways [15]. Overall, miRNAs play a pivotal role in regulating gene expression and changes in miRNA expression are likely to either alter biological pathways, such as with cancer induction, or represent the response to changes in cellular homeostasis, such as during DILI. From a biomarker perspective, both of these factors are important and changes in miRNA expression are likely to serve both as biomarkers of disease/injury and provide novel insights into the biological pathways that are altered during disease/injury.

Overview of DILI and the need for new biomarkers

DILI is one of the leading causes of drug attrition during drug development and post-marketing drug withdrawal [26-29]. DILI is also a common cause of patient morbidity. Current biomarkers can detect liver injury but there are many inadequacies that make them less than ideal. For example, serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are the primary biomarkers of hepatocellular injury. Unfortunately, limitations exist with the use of both enzymes since 1) elevations can reflect nonhepatic injury, particularly skeletal muscle injury, 2) elevations can occur after a critical therapeutic window has passed, such as with acetaminophen overdose, 3) elevations can occur in the absence of histological evidence of injury, such as with statins, 4) concentrations do not discriminate between different etiologies of liver injury, and 5) levels do not provide any insight into disease prognosis. The histopathological analysis of liver biopsies is another marker for liver disease; however, the biopsies are invasive and not routinely performed. Therefore, there is a need for new biomarkers that are not just sensitive but are also specific and prognostic.

The liver is anatomically and physiologically complex and liver injury can affect one or more of these factors. Therefore, DILI presents as diverse biochemical, histologic, and clinical abnormalities and can mimic all forms of liver injury ranging from asymptomatic elevations of hepatic enzymes to fulminant hepatic failure [30]. With the advent of various "omics" technologies (i.e., genomics, proteomics, and metabolomics), the spectrum of hepatotoxic effects caused by different drugs has widened exponentially such that it is unlikely that any two drugs will produce the exact same spectrum of effects using these exquisitely sensitive techniques that involve thousands of different endpoints [31]. However, even though the spectra are unlikely to match exactly, there are likely to be sufficient similarities to group drugs into broad categories [32].

Clinically, DILI is classified in terms of the clinical liver disease; the three most common types of injury are hepatocellular, cholestatic, or mixed hepatocellular/cholestatic. Hepatocellular often involves direct damage of the hepatocytes such as the centrilobular necrosis caused by acetaminophen. This type of damage is often associated with elevated serum ALT and AST levels due to leakage from damaged hepatocytes. Cholestatic injury often involves damage to some part of the bile excretion apparatus resulting in impaired bile excretion. This type of injury is often associated with elevated serum levels of biomarkers reflective of bile duct injury such as gamma glutamyl peptidase, alkaline phosphatase, and bilirubin. Mixed injury presents with a mixture of both types of effects. In addition to these three clinical types of injury, each one may be associated with a systemic syndrome. For example, in addition to the liver injury, some drugs may cause hypersensitivity reactions in other organ systems (e.g., Stevens-Johnson syndrome, renal dysfunction, myocarditis) suggesting an immunoallergic component to the injury [33,34]. Unfortunately, drugs rarely produce a single clear clinical picture making the diagnosis of DILI difficult. For example, amoxicillin/clavulanic acid usually causes cholestatic injury but can also produce acute hepatocellular injury or a mixed type injury [30,35]. There are also other types of DILI such as microvesicular steatosis, non-alcoholic steatohepatitis, chronic hepatitis, cirrhosis, and veno-occlusive disease; however, these are much less common.

Despite the broad classification of drugs as causing hepatocellular, cholestatic, or mixed injury, they all are likely to share the common trait of having a mechanistic basis to the injury. For a compound like acetaminophen, the biochemical basis has been studied for years and has explained the clinical picture of injury [36]. However, even for this extensively studied drug, research continues to fine tune our understanding. The mechanism of injury for most compounds with a very confusing clinical picture (e.g., mixed injury with systemic hypersensitivity reaction) is unknown but it is likely that there is a biochemical basis behind the injury. The adaptation seen to the DILI caused by some drugs provides clues about the molecular processes that might play a role in DILI. For most patients on a given drug, no adverse effects are noted; however, some patients may experience transient, asymptomatic increases in serum ALT. The majority of these patients adapt to the drug as indicated by a return of serum ALT to baseline levels despite continued treatment. A small percentage of these patients will continue to experience increased ALT levels evolving into other clinical signs of liver injury and possibly leading to liver failure. Although the clinical outcome is much different for the three groups of patients (non-responders, adapters, and sensitive), the basic biochemical events are likely to be similar. The difference separating the three groups of people could be due to a multitude of reasons and include both genetic and environmental factors [37]. Since the cellular response to the drug involves a common biochemical basis, it is likely that "omics" analyses, such as miRNA analysis, will reveal the affected pathways and can be used to assess not only the type of liver injury but the mechanisms behind the injury.

Using miRNA profiles to detect organ injury

Starting in the late 1990's, a wide array of studies have shown that miRNA levels are differentially expressed in the target tissue during disease or injury. It is important to note that patterns found to date have not been subjected to rigorous qualification so remain unproven. Cancer has been the focus of much study and it has been clearly shown that miRNA expression patterns differ between cancerous and non-cancerous tissue [10,38-43]. More recently, miRNA expression profiles after tissue injury have been able to distinguish normal versus injured tissue (reviewed below). These studies have clearly shown that miRNA profiles are altered in the target tissue during disease or injury; however, their application to clinical diagnosis is limited since they require invasive sampling of the tissue.

The ideal DILI biomarker would be present in readily accessible body fluids such as blood or urine and would meet the criteria listed in Table 1. Many studies have shown that cell-free miRNA is present

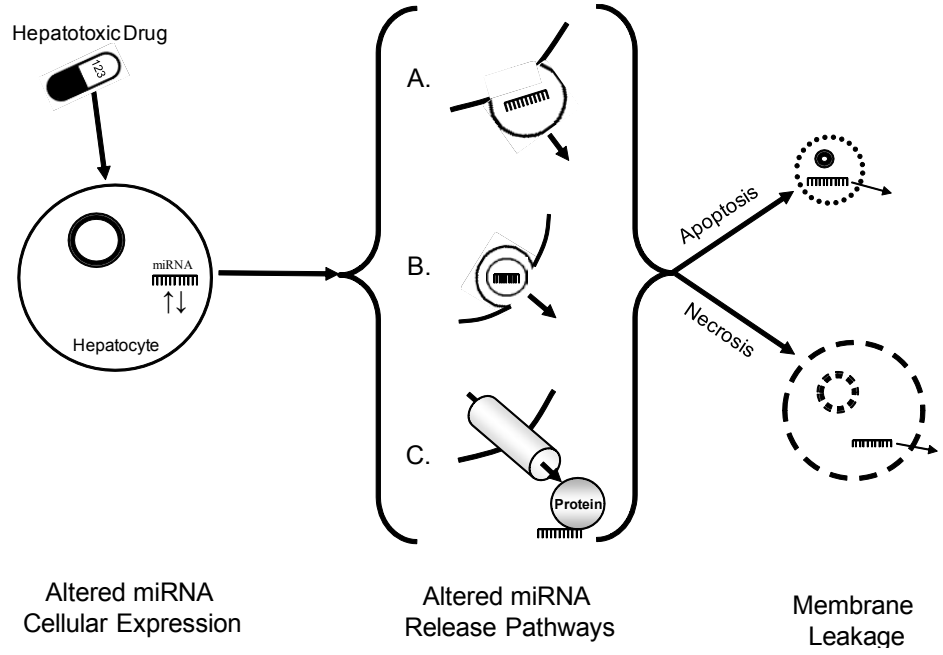

Figure 2: Pathways of miRNA release from hepatocytes after exposure to a hepatotoxic drug. The cellular level of a given miRNA may be increased or decreased leading to corresponding changes in the amount of miRNA released from the cell. miRNA release processes may be altered such as A) microparticles, B) exosomes, and C) protein complexes. Apoptotic or necrotic cell death compromises the cell membrane causing leakage of miRNA.

in readily accessible body fluids and meet many of the ideal biomarker criteria (reviewed below). Studies have shown that the level of plasma or serum miRNA is altered in patients with various types of cancer [14,44-51]. In some diseases, miRNA levels are increased and in others they are decreased, but they share the common feature of being able to distinguish normal patients from those with cancer. For example, four serum miRNAs were differentially expressed in patients with hepatocellular carcinoma and the level of miRNA-221 correlated with tumor size, cirrhosis, and tumor stage [52].

Although miRNA detection is theoretically simpler and subject to fewer confounders than developing protein based assays, there are some technical issues associated with miRNA measurement. There are several different platforms for miRNA detection including Northern blotting, RT-qPCR, microarray, and Next-Generation sequencing. Unfortunately, at this time, there appears to be limited correlation between the different platforms, most likely due to the strong influence of different primer designs on the measurements for RT-qPCR and microarrays, the two most commonly used detection methods [53]. Many different protocols for sample preparation are used making comparisons between studies difficult. In contrast to total RNA, assessment of miRNA quantity and quality is less than ideal. For sample analysis, some studies use an enriched small RNA fraction whereas others use total RNA; however, the influence of the fractionation procedure has not been systematically investigated. Finally, and especially for cell-free miRNA analysis, a variety of different normalization processes have been used to control for technical and biological variability but none of them are ideal [14,39,53,54].

miRNA levels in the plasma or serum have been used to distinguish patients suffering from different types of organ injury, such as myocardial damage. A multitude of studies have shown that plasma/serum miRNA levels can distinguish normal patients or animals from those experiencing myocardial injury [55-59]. For example, miR-1 was significantly elevated in the plasma from patients suffering acute myocardial infarction compared to controls [60] and, in a separate study, a panel of 20 miRNAs was shown to predict acute myocardial infarction in patients with high specificity, sensitivity, and accuracy although these have not been put forward for biological qualification [61]. Studies have also shown that the plasma/serum miRNAs are altered for a variety of other types of organ injury or disease such as cerebral infarction, skeletal muscle damage, muscular dystrophy, systemic lupus erythematosus, traumatic brain injury, and diabetes [62-67]. Several recent studies have shown that the plasma/serum level of various miRNAs are altered after various types of liver injury and disease such as hepatitis B [68] and C [69] infection, chronic hepatitis [70], non-alcoholic fatty liver disease [70], and hepatic injury and rejection after transplantation [71]. These studies clearly show that miRNAs are differentially released during organ injury and can be used to identify affected patients. Figure 2 diagrams the likely progression of miRNA release into the blood that accompanies organ injury, in this case, hepatocellular injury. miRNAs present within circulating blood cells are also being explored to detect organ injury. A recent study showed that miR-1291 and miR-663b in the peripheral total blood are highly specific and sensitive biomarkers to determine acute myocardial infarction [61]. In rat models of brain injury, miR-298, miR-155, and miR-362-3p in the blood have been proposed as candidate biomarkers to predict such damages [72]. In kidney disease, blood miR-142-5p, -155, and -223 have been shown be to up-regulated and correlated well with the human renal allograft status [73]. These studies indicate that circulating blood cells and their miRNA levels may act as sentinels of disease in other organs.

In normal cells, miRNA are released into extracellular fluid, such as blood, via several different processes as outlined in Figures 1 and 2; however, it is important to keep in mind that this is an evolving area of research and there are likely additional mechanisms for

extracellular miRNA release that have yet to be discovered [74]. The first two mechanisms involve release of miRNA in cell-derived vesicles. Microparticles are vesicles that are derived from outward budding of the cell membrane and contain various cellular constituents, including miRNA. They are typically 100-1000 nm in size. Exosomes are internally derived vesicles that are contained within multivesicular bodies, the cellular organelles that integrate both endocytic and secretory pathways. Some multivesicular bodies fuse with the plasma membrane and release their contents (i.e., the exosomes) into the extracellular fluid. Exosomes are typically 30-100 nm in size thus being smaller than microparticles. The third mechanism involves release of miRNA bound to cellular proteins, such as argounate-2 and HDL [75,76]. Various studies have shown that these extracellular miRNAs can alter biological processes in distant cells and may play a role in cell-to-cell communication [5,77-80].

In cells undergoing stress, homeostasis is altered leading to altered blood miRNA levels via a variety of different mechanisms (Figure 2). Cells undergoing stress may alter the cellular levels of miRNAs and this in turn leads to altered blood levels without any change in the normal processes of miRNA release from the cell. Disturbances in the release processes due to cell stress would directly influence how much miRNA is released into the blood. If cell injury is severe and apoptotic or necrotic death ensues, it is likely that in addition to changes in the normal synthesis and release pathways, miRNAs are also released through the compromised cellular membrane. The interplay of these factors will ultimately determine how the concentration of a given miRNA in the blood changes after tissue injury and in what form the miRNA is released (e.g., microparticle, exosome, or protein complex).

The alteration of cell-free miRNAs is not restricted to the blood. Altered levels of miRNAs in a wide array of body fluids such as urine, sputum, feces, bile, cerebrospinal fluid, and saliva have been detected in patients suffering various diseases or organ injury [8,77,81-86]. Similar to blood, miRNA in these body fluids appear to be much more stable than exogenously added RNA. It is clear that cell-free miRNAs are present in a wide array of body fluids and they hold great promise in the diagnosis of organ injury.

miRNA as biomarkers of DILI

As mentioned previously, new biomarkers of DILI are needed since the current biomarkers have various flaws such as induction by other types of organ injury or lack of prognostic value. As shown for a variety of different types of diseases and organ injury, miRNA shows great promise of meeting many of the criteria of an ideal DILI biomarker (Table 1). For the liver, a variety of different diseases and injury such as acetaminophen- and carbon tetrachloride-induced hepatotoxicity, nonalcoholic steatohepatitis, FAS-induced acute liver injury, ischemia/reperfusion, and fibrosis have been shown to alter hepatic miRNA levels [87-93]. In a seminal study by Wang et al. [94] that used an acetaminophen-induced mouse model of hepatotoxicity, the level of many plasma miRNAs inversely correlated with the level of hepatic miRNAs, indicating that for these miRNAs, hepatic injury caused the release of miRNAs into the circulation. For example, miRNA-122 and miRNA-192 exhibited high levels in the liver and upon injury were released into the blood with concurrent decreases in the liver. The changes in these miRNAs not only preceded changes in serum ALT levels but also exhibited much less variability in animal to animal responses. Similar clinical results were recently obtained in patients with acetaminophen poisoning [95]. The serum level of two relatively liver-specific miRNAs, miR-122 and miR-192, were substantially higher in patients suffering from acetaminophen-induced acute liver injury compared to healthy controls. The serum level of a heart-enriched miRNA, miR-1, was not altered in these patients and the serum level of a brain-enriched miRNA, miR-218, was slightly higher in the acetaminophen patients. Serum miR-122 levels correlated with peak ALT but not prothrombin time or total bilirubin concentrations. In a smaller cohort of patients suffering from non-acetaminophen-induced acute liver injury, serum miR-122 was also increased. Other studies have also shown that plasma or serum miRNAs are altered after other types of liver injury and disease [69-71,96-98].

We have recently shown that urinary miRNA levels can distinguish control from acetaminophen-treated rats [99]. Similar to the plasma miRNA results from Wang et al. [94], urinary miRNA levels were both more sensitive and more consistent biomarkers of acetaminophen exposure when compared to serum ALT and AST. For example, urinary miRNAs were increased at a low acetaminophen dose that failed to alter serum ALT and AST. In addition, at the high acetaminophen dose, only 2 of 7 animals exhibited increased ALT/AST levels; whereas, every animal had elevated urinary miRNA concentrations for at least several miRNAs that were screened. These results show that urinary miRNAs may be suitable biomarkers for liver injury; however, additional work is need to determine the specificity of the findings and if they are translatable to the clinic.

Qualification of cell-Free miRNA as biomarkers of DILI and data needs

The available published data show that the level of various miRNAs in serum, plasma, and/or urine may serve as new biomarkers of DILI. However, more studies and data are required before the various miRNAs are qualified as true biomarkers of DILI that can supplement and/or replace existing biomarkers. The FDA and ICH have issued guidance on biomarker qualification and the content of data submissions [100-102]. Although specific testing and qualification plans are not provided in the guidance documents, they do highlight the fact that robust data are required to qualify a new biomarker. As part of the qualification process, it will be important to determine how cell-free miRNAs meet the various specifications of an ideal biomarker (Table 1). Although cell-free miRNAs do not have to exceed every criterion, it will be necessary to determine how they compare to existing biomarkers of DILI. It will also be important to determine if individual or batteries

Specific
• Preferentially (or exclusively) produced in target tissue
• Able to differentiate pathologies
Sensitive
• Early indication of disease before clinical symptoms develop
• Present at low concentrations in controls and exhibits significant increase after injury
• Long half-life
Diagnostic
• Proportional to degree of severity of pathology
Prognostic
• Can predict disease progression
Robust
• Not confounded by unrelated conditions
• Rapid, simple, accurate, and inexpensive detection
Translatable
• Data can be used to bridge pre-clinical and clinical results
Non-invasive
• Released from tissue into accessible fluid sample

Table 1: Characteristics of an ideal DILI biomarker.

of cell-free miRNAs are associated with specific drugs, diseases, or injuries or if they are just a generic indicator of gross liver injury, regardless of the insult. Inter-individual differences and variability in responses will need to be determined. Most marketed drugs that cause DILI fall into the rare "idiosyncratic" category and adversely affect only a limited number of patients. Ideally, cell-free miRNA will be shown to be superior to existing biomarkers in identifying idiosyncratic DILI earlier in the course of the disease so that the drug can be withdrawn before serious injury occurs. Finally, no existing biomarker of DILI provides prognostic information and it will be important to determine if cell-free miRNA fills this data gap.

Conclusion

Over the past decade and especially in the past few years, a lot of research has been conducted looking at the ability of miRNAs to serve as biomarkers of disease or injury. miRNAs hold great promise as biomarkers in extracellular fluids since they are stable, have a simple chemistry, are potentially organ specific, and are easily detected using readily available and sensitive methods. The use of cell-free miRNAs as biomarkers of DILI is in its infancy but several studies have shown promising results and cell-free miRNAs have been successfully used to detect other types of organ injury. Future studies will help determine the role cell-free miRNAs play in detecting DILI and whether they will simply be adjuncts to existing biomarkers or will show superior performance and supplant existing biomarkers.

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