Circulating MicroRNAs as Biomarkers and Potential Paracrine Mediators of Cardiovascular Disease

Shashi K. Gupta, MSc; Claudia Bang, MSc; Thomas Thum, MD, PhD

MicroRNAs (miRNAs) comprise a class of small, non-coding RNAs that control expression of complementary target mRNAs. Dysregulation of intracellular miRNA expression has been described in various diseases, including a number of cardiovascular conditions. Functional studies have shown a role for miRNAs in cardiac fibrosis, hypertrophy, angiogenesis, and heart failure.1–3 These findings suggest a new therapeutic entry point for cardiac disease and illustrate the broad therapeutic potential of miRNA modulation. Initial reports have detected circulating extracellular miRNAs in the serum/plasma of patients with cancer.4 Despite the existence of RNases, miRNAs remain stable in serum and other body fluids. One explanation is the inclusion of miRNAs into lipid or lipoprotein complexes such as exosomes5 or microvesicles.6 Subsequently, altered concentrations of miRNAs have been found in patients with various cardiovascular diseases. Here, we review the current knowledge about circulating miRNAs during coronary artery disease (CAD), myocardial infarction, and heart failure (Table). A further new and exciting function of circulating miRNAs in the cardiovascular system may be their potential to serve as paracrine signaling molecules.7

Secretion, Stability, and Potential Function of Circulating MiRNAs

The detection of circulating miRNAs in serum/plasma8,9 suggests that miRNAs may fulfill biological functions outside the cell and serve as potential biomarkers for diseases. Circulating miRNAs are protected from RNase-dependent degradation by several mechanisms, including their inclusion in microvesicles, exosomes, and apoptotic bodies as well as through the formation of protein-miRNA complexes resistant to degradation (Figure). In addition, circulating miRNAs are quite stable even after multiple freeze-thaw cycles.8 Recent studies have shown that miRNAs are actively secreted in microvesicles or exosomes from different cell types.6,10 Despite the current knowledge about the existence of circulating miRNAs and the intercellular transfer of miRNAs from donor cells to recipient cells, the underlying mechanisms of the cellular secretion of miRNAs are not fully understood. MiRNA loading into exosomes depends on the association of the RNA-induced silencing complex with multivesicular bodies.11,12 Neutral sphingomyelinase 2, which regulates the biosynthesis of ceramide, was identified to control, at least in part, the secretion of miRNAs.13 Cells that have been confirmed as secreting miRNAs through exosomes include mast cells5 and embryonic stem cells. For the latter, it has been shown that miRNA-enriched microvesicles can be transferred to mouse embryonic fibroblasts in vitro, thus influencing the expression of genes in adjacent cells.14 Further, miRNAs from Epstein-Barr virus-infected cells are transported to uninfected recipient cells through exosomes.7 Brain microvascular endothelial cells are able to take up glioblastoma-derived microvesicles enriched with miRNAs and miRNAs with subsequent alteration of the genetic endothelial program.10 Exosomes have been identified as an active component of conditioned medium from human embryonic stem cell-derived mesenchymal stem cells. Injection of such exosomes into a pig or murine model of cardiac ischemia/reperfusion injury has resulted in reduced cardiac damage and improved outcome.15,16 Cardiomyocyte progenitor cells release exosomes that stimulate the migration of endothelial cells.17 MiRNAs also can be released directly from damaged cells (eg, after myocardial infarction).18 Endothelial-derived apoptotic bodies enriched with miR-126 can be transported into atherosclerotic lesions and convey paracrine alarm signals to recipient vascular cells that trigger the recruitment of progenitor cells and alleviate atherosclerosis.17 As noted, miRNAs can be protected from degradation by the formation of specific protein-miRNA complexes. Mammalian cells release a significant number of RNA-binding proteins into the culture medium after serum deprivation.19 For instance, nucleophosmin 1 is able to bind miRNAs and might be involved in miRNA exportation, packaging, and protection of extracellular miRNAs from degradation.

How can intercellular communication be mediated by exosomes? Exosomes released from a specific cell type may act as signaling complexes through the binding of exosomal membrane proteins with a surface receptor on target cells, resulting in intracellular stimulation (Figure).20,21 Alternatively, exosomes may bind to surface receptors on target cells with subsequent endocytotic internalization by the recipient cells (Figure).22 After internalization, exosomes can fuse with the membranes of endosomes, leading to release of their contents into the cytosol of target cells. Exosomes can remain segregated within endosomes and may transfer their content to lyso-
somers or may be released within the cells following fusion with the plasma membrane.\textsuperscript{22} Finally, exosomes can fuse with the target cell membrane and release their genetic contents inside the recipient cells in a nonselective manner (Figure).\textsuperscript{22} Circulating extracellular miRNAs likely represent a novel mechanism of intercellular communication. Which of the proposed mechanisms is mainly involved in the release of miRNAs and paracrine intercellular communication during cardiac disease remains to be determined.

**Detection, Quantification, and Normalization of Circulating MiRNAs**

The use of circulating miRNAs as potential biomarkers in clinical scenarios depends on the sensitivity of methods used to detect them. Real-time quantitative reverse transcriptase-polymerase chain reaction is the most common and sensitive method used to date to quantify circulating miRNAs. Detailed descriptions of the methods for quantification of miRNAs in plasma or serum are available.\textsuperscript{23} Quantification of circulating miRNAs with high sensitivity is challenging because of (1) the very low amounts of RNA recovered from plasma or serum and (2) the lack of proper endogenous controls for normalization. However, because the RNA yield from plasma/serum is very low, accurate normalization procedures are important. The use of spiked-in control miRNAs is helpful in normalizing for isolation differences. Synthetic *Caenorhabditis elegans* miRNAs (such as miR-39, miR-54, and miR-238) were added after denaturation of plasma/serum during RNA isolation. Several other endogenous circulating miRNAs also can be used for normalization, such as miR-17-5p, U6,\textsuperscript{18} 5S rRNA,\textsuperscript{26} miR-454, and RNU6b,\textsuperscript{8} although it cannot be ruled out that there are changes of the concentration in different patient populations on medication or with cardiovascular risk factors.

<table>
<thead>
<tr>
<th>Disease Type</th>
<th>miRNAs Upregulated</th>
<th>miRNAs Downregulated</th>
<th>Time Point</th>
<th>No. Samples</th>
<th>Species</th>
<th>Correlation</th>
<th>Ref No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMI</td>
<td>miR-1, miR-133a, miR-133b, miR-499-5p</td>
<td>miR-122, miR-375</td>
<td>517±309 min</td>
<td>33 STEMI, 17 healthy</td>
<td>Human</td>
<td>Cardiac TnI, except for miR-499-5p</td>
<td>24</td>
</tr>
<tr>
<td>AMI</td>
<td>miR-208a, miR-1, miR-133a, miR-499</td>
<td></td>
<td>4.8±3.5 h</td>
<td>33 AMI and non-AMI, 30 healthy</td>
<td>Human</td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>AMI</td>
<td>miR-499</td>
<td></td>
<td>48 h</td>
<td>9 AMI, 5 UAP, 9 CHF, 10 healthy</td>
<td>Human</td>
<td></td>
<td>31</td>
</tr>
<tr>
<td>AMI</td>
<td>miR-1</td>
<td></td>
<td>8.5±3.82 h</td>
<td>31 AMI, 20 healthy</td>
<td>Human</td>
<td>CK-MB</td>
<td>30</td>
</tr>
<tr>
<td>AMI</td>
<td>miR-1</td>
<td></td>
<td>93 AMI, 66 healthy</td>
<td>Human</td>
<td>QRS widening</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>AMI</td>
<td>miR-208b, miR-499, miR-133a</td>
<td>miR-223</td>
<td>12 h</td>
<td>36 AMI, 36 healthy</td>
<td>Human</td>
<td>Cardiac TnI, CPK except for miR-223</td>
<td>33</td>
</tr>
<tr>
<td>VM</td>
<td>miR-208b, miR-499, miR-133a</td>
<td>miR-223</td>
<td>14 acute, 20 post-VM, 20 healthy</td>
<td>Human</td>
<td>With severity of VM</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>HF acute</td>
<td>miR-499, miR-122</td>
<td></td>
<td>33, 34 healthy</td>
<td>Human</td>
<td></td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>HF diastolic</td>
<td>miR-423-5p</td>
<td></td>
<td>20 hypertension, 39 diastolic dysfunction, 20 healthy</td>
<td>Human</td>
<td>miR-133a correlated to NT-proBNP</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>HF</td>
<td>miR-423-5p</td>
<td></td>
<td>30 HF, 20 non-HF, 39 healthy</td>
<td>Human</td>
<td>NT-proBNP, EF</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>CAD</td>
<td>miR-133a, miR-208a</td>
<td>miR-126, miR-17, miR-92a, miR-155, miR-145</td>
<td>822 DM</td>
<td>Human</td>
<td>Statin therapy</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td>miR-499, miR-126</td>
<td></td>
<td>18 h, 6 h, 18 h, 24 h, 3 h</td>
<td>4–5 Mice</td>
<td></td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>LAD ligation</td>
<td>miR-1, miR-133a, miR-133b, miR-499-5p, miR-208a</td>
<td>miR-126</td>
<td>67 CAD, 31 healthy</td>
<td>Human</td>
<td></td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>LAD ligation</td>
<td>miR-208a</td>
<td></td>
<td>3 h</td>
<td>6 Rat</td>
<td></td>
<td>Myocardial infarct size</td>
<td>30</td>
</tr>
<tr>
<td>LAD ligation</td>
<td>miR-1</td>
<td></td>
<td>6 h</td>
<td>12 Rat</td>
<td></td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Isoproterenol-induced myocardial injury</td>
<td>miR-208</td>
<td>8 h</td>
<td>8 Rat</td>
<td>Cardiac TnI</td>
<td>26</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AMI indicates acute myocardial infarction; CAD, coronary artery disease; CHF, congestive heart failure; CK, creatine kinase; DM, diabetes mellitus; EF, ejection fraction; HF, heart failure; LAD, left anterior descending coronary artery; NT-proBNP, N-terminal prohormone brain natriuretic peptide; STEMI, ST-segment elevated myocardial infarction; UAP, unstable angina pectoris; VM, viral myocarditis.
Circulating MiRNAs as Emerging Biomarkers of Cardiovascular Disease

Circulating miRNAs may have great potential for use as clinical biomarkers because easy, noninvasive detection in various medical conditions is possible. Circulating miRNAs have been shown as biomarkers in various diseases, including cancer where they also are of prognostic relevance. There is now growing evidence that circulating miRNAs also can be used as biomarkers in cardiovascular diseases (Table).

CAD and Cardiovascular Risk Factors

Fichtlscherer et al reported reduced levels of miR-126, members of the miR-17-92 cluster, inflammation-related miR-155, and smooth muscle-enriched miR-145 in patients with CAD compared with healthy controls. In contrast, cardiac muscle-enriched miRNAs (miR-133a, miR-208a) tended to be higher in patients with CAD. Correlation studies revealed that vascular and inflammation-linked miRNAs were altered by vasculoprotective therapies with inhibitors of the renin-angiotensin system, aspirin, and statins. There is additional evidence that cardiac risk factors affect circulating miRNA levels. It was shown that patients suffering from prevalent diabetes have significantly decreased levels of miR-20b, miR-21, miR-24, miR-15a, miR-126, miR-191, miR-197, miR-223, miR-320, and miR-486 but a modest increase of miR-28–3p. MiR-126 data were confirmed in 822 patients in univariate and multivariate analyses. In patients with diabetes, the reduction of miR-126 was confined to circulating vesicles in plasma.

Myocardial Infarction

In a cohort of 93 patients with acute myocardial infarction (AMI), the muscle-enriched miRNA miR-1 was significantly upregulated in the circulation compared to non-AMI controls. MiR-1 levels correlated with abnormal QRS widening in patients with AMI, whereas no correlation was found with ST-segment alterations or levels of cardiac troponin (Tn) I or creatine kinase (CK) MB. MiRNA-1 also may serve as a potential predictor of AMI. Other studies have shown miR-1 to be upregulated in AMI, but in contrast to Ai et al, these
studies demonstrated a positive correlation of miR-1 levels with CK-M levels, whereas D’Alessandra et al. showed that miR-1 upregulation in patients with AMI correlates with cardiac TnI levels. Additional miRNAs that were found to be upregulated in patients with AMI (ST-segment elevation myocardial infarction) include miR-133a, miR-133b, miR-208b, miR-499, and miR-499-5p, whereas miR-122, miR-223, and miR-375 were lower than in controls. Plasma levels of heart-specific miR-208a became detectable in patients with AMI with a detection sensitivity of 90.9% but were undetectable in healthy controls. A parallel analysis of miR-208a along with TnI measurements 4 hours after the onset of symptoms showed miR-208a to be detectable in all affected individuals, whereas TnI was seen only in 85% of patients. MiR-208b and miR-499 correlated with TnT levels in patients with AMI, suggesting that miR-208a, miR-208b, miR-499, and other miRNAs may be alternatives or even superior to conventional biomarkers for the early detection of AMI (see Limitations section).

Heart Failure and Viral Myocarditis
MiRNAs have been shown to play an important role in mediating the transcriptional changes observed during heart failure, so a change in profile of circulating miRNAs may be expected in this context. Tijsen et al. showed circulating miR-423-5p as a potential biomarker of heart failure. Patients were recruited from a dyspnea registry, and interestingly, the level of miR-423-5p distinguished between dyspnea due to heart failure and dyspnea without heart failure. Circulating miRNA-423-5p correlated with N-terminal prohormone brain natriuretic peptide levels and ejection fraction. Indeed, miR-423-5p was specifically enriched in the blood of heart failure cases, and receiver operator characteristic curve analysis showed miR423-5p to be a diagnostic predictor of heart failure. Another study found a significant increase in miR-499 levels in patients with acute heart failure, whereas no changes were found in diastolic heart failure. In patients with viral myocarditis, mild elevation of miR-208b and miR-499 was found.

Circulating miRNAs in Animal Models of Cardiovascular Disease
Circulating miRNAs also have been evaluated in different animal models of heart disease. Isoproterenol treatment in rats induced myocardial injury and resulted in increased circulating miR-208 levels. In contrast, miR-208 levels remain unaffected in renal infarction models, thoracotomy surgery, or cardiac hypertrophy, demonstrating use as a potential and specific biomarker for myocardial injury. Upregulation of miR-208 levels also was reported by Wang et al. and D’Alessandra et al. in a model of coronary artery ligation in rats and mice, respectively. MiR-208a was elevated after 1 hour of left anterior descending coronary artery occlusion and reached a peak at 3 hours. Circulating miR-1 also was increased after myocardial infarction in animal models, and a positive correlation between infarct size and serum miR-1 levels after ischemia/reperfusion has been reported. In contrast to cardiac injury, miR-1, miR-133a, and miR-133b levels in mice with acute hind-limb ischemia were transiently reduced. Finally, miR-499-5p is increased after coronary artery ligation in mice, and levels closely correlate with TnI.

Limitations and Future Directions
The potential of selected circulating miRNAs or miRNA combinations to be used as specific biomarkers of distinct cardiovascular diseases is great. However, with few exceptions, the number of patients in the individual studies (Table) to date has been extremely low. It will be difficult to determine appropriate suitable endogenous controls because the expression profile of circulating miRNAs may change depending on the patient’s cardiovascular disease state and medication. Finally, prognostic data for circulating miRNA levels in cardiovascular disease currently are lacking but may be available soon from several laboratories.

Conclusions
MiRNAs emerge as potentially interesting and powerful new biomarkers for cardiovascular disease. Their role as paracrine signaling molecules in cardiovascular diseases remains to be determined.

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Disclosures
Dr Thum has filed patent applications for the use of miRNAs as therapeutics and biomarkers in cardiovascular disease.

References


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