Regulatory Noncoding RNAs in Cardiovascular Disease: Shedding Light on ‘Dark Matter’

Charan Reddy Kudumula, MD*

Abstract
Cardiovascular disease (CVD) remains a major cause of morbidity and mortality worldwide accounting for more deaths than any other cause. CVD is regulated by stage-specific gene expression which, in turn, is controlled by myriad of regulatory noncoding RNAs (ncRNAs or miRNAs). Rapid advancement in genome mining technologies has identified different miRNAs in various tissues and body fluids. It is now well accepted that miRNAs represent critical regulators of cardiovascular function. The present review focuses primarily on recent updates on the basic miRNA biology; CVD-miRNA based research progress and functional significance of circulating noncoding RNAs. We also discuss their potential use as biomarkers and/or therapeutic targets in CVD.

Keywords — Cardiovascular disease, myocardial infarction, microRNA, biomarker


I. INTRODUCTION

MicroRNAs (miRNAs)
For last half of the century, the concept of the term ‘RNA’ was largely restricted as an intermediate between the ‘gene’ and ‘protein’. Similar to the first RNA revolution in the 1980s when the enzymatic activity of RNA was reported,1 the discovery of miRNA represents the second RNA revolution.2 The recent outburst of large-scale genome sequencing, in silico interpretation of genomic data and their functional validation have revealed the genome with unprecedented resolution and challenged the central dogma that ‘DNA makes RNA makes protein’. Deep mining of genome has unraveled that the vast majority of intergenic regions in mammalian genome is transcribed extensively as non-protein-coding RNAs (ncRNAs).

While in the past thirty years, the analysis of coding RNA molecules, mostly messenger RNAs (mRNAs) were in the focus of research, the importance of non-coding RNAs has not been realized. Only a decade ago, miRNAs were discovered in mammals as a large class of evolutionarily conserved, key transcriptional and posttranscriptional inhibitors of gene expression thought to “fine tune” the translational output of target miRNAs3. In 1993 the first known single-stranded noncoding RNAs now defined as microRNAs (miRNAs) lin-4 was discovered as a critical modulator of temporal development in Caenorhabditis elegans.2 However, miRNAs were not recognized as a distinct class of post-transcriptional biological regulators until the early 2000s. Since then, explosive advancements in the field of miRNA biology have elucidated the basic mechanism of miRNA biogenesis and gene-regulatory function.

MicroRNAs (miRNAs; miR) have emerged as a novel class of endogenous, single stranded non coding RNAs (ncRNA’s) consisting of 15-22 nucleotides, which are not used as template for translation.4 Also, this major portion of the eukaryotic genome except protein-coding gene region was traditionally considered as inactive ‘trash’ left behind as evolutionary relics with no function. However, large-scale analyses of genome-wide activity unbiased of annotations with high-throughput sequencing technologies have demonstrated that these small RNAs have major role in control of gene expression.5 Acting as genetic switches or fine-tuners, miRNAs are key regulators of diverse biological and pathological processes including growth, development, organogenesis, apoptosis, cell proliferation and differentiation, myocardial infarcts (MI), ischemia/reperfusion (I/R) injury and heart failure (HF), providing glimpses of undiscovered regulatory mechanisms and potential therapeutic targets for the treatment of CVD.6,7 The present mini review focuses primarily on recent updates on the basic miRNA biology, CVD-miRNA based research progress and functional significance of circulating noncoding RNAs. We also discuss their potential use as biomarkers and/or therapeutic targets in heart disease.
MiRNA-biogenesis and function

Recently, the discovery of miRNAs, has opened new opportunities in cardiac disease biology. The heart is one of the first organs to function in a developing embryo. Currently, although our understanding of miRNA function in embryogenesis is rudimentary, the emerging role of the biogenesis and activity of miRNAs as key regulatory mechanisms in controlling developmental timing, tissue differentiation and maintenance of tissue identity during embryogenesis has been revealed.\(^8\)

MiRNAs regulate biological processes by binding to mRNA 3’-untranslated region (UTR) sequences to attenuate protein synthesis or mRNA stability.\(^9\) According to Sanger institute current databases report miR sequences for human’s miRNA (http://www.mirbase.org), for the time being, more than 1600 precursors and 2042 mature miRNAs have been discovered in humans and deposited. These miRNA genes have been catalogued in the miRBase online database (http://microrna.sanger.ac.uk).\(^10\) Most of the human miRNAs have been cloned and bioinformatic predictions indicate that mammalian miRNAs can regulate approximately 60% of all protein-coding genes.\(^12\) The expression of many miRNAs is specific to a tissue or developmental stage, and their expression pattern is altered during the development of various non-communicable diseases like HF and cancer.\(^14\)

For gene regulation via miRNAs, mainly three different mechanisms are known till date, which include translation repression, direct mRNA degradation and miRNA-mediated mRNA decay. Mostly, miRNAs bind with imperfect complementarity to their targeted mRNAs and thereby guide mRNA translation repression. They interact with targeted mRNAs primarily through the so-called seed, a 6–8 nt long region at their 5’-end. This seed is known to be highly conserved in miRNA families across different species.\(^15\) The algorithms to predict miRNAs from RNA-sequencing data are based on the assumptions that the precursors fold into a stable stem-loop structure, mature miRNAs are found on one arm of the stem, and these sequences are evolutionarily conserved.\(^16\)

The prediction of target mRNA from miRNA sequence consider stability of miRNA-mRNA duplex, secondary structure, nucleotide content within and neighboring to the tentative target sites, and position of seed complementary sites within the mRNA.

Figure 1 depicts the transcription regulation mechanisms exhibited by different types of well-characterized ncRNAs. The biogenesis of miRNAs is a complex multi-step process. It starts with RNA polymerase II-mediated transcription from the genome, leading to the formation of large RNA pre-cursor, pri-miRNA (primary miRNA transcript) with a typical hairpin morphology of variable length depending on the locus.\(^17\) A nuclear endonuclease, called DROSHA, then crops the distal stem portion of priRNA obtaining shorter chains (pre-miRNA).\(^19\) Pri-miRNA is transported to the cytoplasm by the nuclear receptor, exportin-5 and processed by DICER, an RNase III, to short double-stranded RNA containing the miRNA and the ‘star strand’ (miRNA*).\(^20\) miRNA* is degraded after stripping the miRNA strand to obtain mature miRNA.\(^21\) Mature miRNA interact with proteins like Argonaute endonuclease (Arg 2), in order to form the RNA-induced silencing complex (RISC), which directs mature miRNA towards the targeted mRNA and bind on their 3’ untranslated region (UTR).\(^19\) This miRNA/RISC complex is responsible for miRNA function. A single miRNA may modulate hundreds of miRNAs, and one miRNA has multiple predicted binding sites for miRNAs in their 3’UTR. After cleavage of a target mRNA, miRNAs are not destroyed; so they may recognize and modulate other miRNAs.\(^22\)

**Figure 1: Schematic representation of miRNA**

**Biogenesis and function**

MiRNA biogenesis is initiated with the processing of primary miRNA transcripts in the nucleus by the Drosha/Dgcr8, to generate a 70 nt Pre-miRNA, which are further processed into a miRNA duplex by Dicer, which is transported from nucleus to cytoplasm by exportin-5. In the cytoplasm, the Pre-miRNA is further trimmed to 21-25 nt double stranded RNA by Dicer into an miRNA:miRNA* duplex. Assembled into the RISC, one of the single stands negatively regulates gene expression by either translational repression or mRNA degradation.

**MiRNA’s and Cardiovascular Disease**

Globally, over 17.5 million people die annually due to cardiovascular diseases (CVDs), and this figure is predicted to increase to about 25 million by the year 2025. Thus, significant efforts have been made to determine the molecular and pathophysiological characteristics of the diseased heart and vasculature with the goal of developing novel diagnostic and therapeutic strategies for the early prediction of CVD. Although miRNAs are highly expressed in the heart, their roles in heart diseases are currently unclear. The search for specific
miRNAs as biomarkers for early prediction of cardiac complications and their functional characterization has been started extensively and a considerable number of studies have pointed out specific roles for several miRNA molecules during CVD. Table 1 summarizes the different classes of ncRNAs and their role as bio-markers for various cardiac complications.

TABLE-1: Circulating miRNAs as diagnostic markers in various CVDs. Results from several relevant reports were combined to give an overview

<table>
<thead>
<tr>
<th>Disease</th>
<th>miRNA biomarker</th>
<th>Study subjects</th>
<th>Tissue / Source and References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMI (IHD / Stroke)</td>
<td>miR-1, -30c, -133, -133a, -133b, -145, 208a, 208b, 499, 499-5p, -663b, -1291, -124</td>
<td>miR -126, -197, -223, -210</td>
<td>264 AMI &amp; 221 Controls (including 33 non-AMI with chest pain and 36 non-AMI with AP)</td>
</tr>
<tr>
<td>HF</td>
<td>miR -29b, -122, -142-3P, -423-5P</td>
<td>miR -107, 125b, -126, -139, -142-5P, -497</td>
<td>58 HF &amp; 76 Controls</td>
</tr>
<tr>
<td>CAD</td>
<td>miR-21, -27b, -13a, -134, -135, 135a, -198, -210, -370, 133, 133a, 133b, -280a, 34a, -146a, 146-5p, -100, -127, -145</td>
<td>miR-1, -17, -92a, -126, -145, -155, 181a, -221, -222, -1, -6, -147 hsa-miR-21, hsa-miR-17, hsa-miR-20a, and hsa-miR-92a</td>
<td>129 CAD &amp; 63 Controls</td>
</tr>
</tbody>
</table>

A. Myocardial infarction

Myocardial infarction (MI) is the world’s leading cause of morbidity and mortality. The pathological process of the MI is associated with dysregulated expression profile of genes that are important for cardiac function. MI induced by coronary artery occlusion is accompanied by cardiac remodeling at the site of infarction injury. This remodeling process involves fibrous tissue formation and extracellular matrix deposition are mediated by cardiac fibroblasts. Current biomarkers such as creatinekinase-MB (CK-MB) isoenzymes, uric acid (UA), myoglobin, adiponectin cathepsins, cellular cytokine (CD40 ligand), copeptin and cardiac troponin-I and T (cTnI and cTnT) are being used for the early prediction of MI. The incremental costs combined with high false positivity rate, questions the use of cTnI and cTnT as reliable methods for the early prognosis of MI. Therefore, multiple biomarker strategy may circumvent these limitations by adding accuracy and predictive power.

The miRNA function in cardiac complication was first studied in animal models, which showed that stress-induced cardiac hypertrophy mediated by miRNAs eventually led to HF. Recently, Da Costa Martins and De Windt gave an update on the role of miRNAs and their respective targets in cardiac growth and outlined the delicate balance of protagonist and antagonist miRNAs within the context of cardiac hypertrophy. Later, these studies lead to the identification of several miRNAs, which play potential roles in post-MI-induced cardiac remodeling. Bao et al have demonstrated that miRNAs, Let-7b and Let-7i can act as biomarkers of AMI and dilated cardiomyopathy (DCM) respectively. Let-7c expression alterations were strikingly similar in failing human and fetal hearts. A recent report suggests that elevated expression of cardiac-related miRNAs (miR-208a, miR-133, miR-133a, miR-133b, miR-145, miR-499, miR-499-5p and miR-1) in a manner similar to that of cardiac troponin iso-enzymes. The use of some of these miRNAs, particularly miR-208a as a biomarker for MI remains controversial. A single study described a significant increase in circulating miR-208a in AMI patients and showed high sensitivity and specificity of this miRNA for diagnosing AMI. However, others could not detect or could only find very low levels of miR-208a. These differences might relate to the timing of sampling, since miR-208a peaks 3 hrs after AMI and is restored to baseline after 24 hrs. These studies still require further validation.
B. Stroke

Strokes are mostly caused by obstructions in brain blood vessels (ischemic stroke: ~ 85% of cases) and, less often, by a disruption (hemorrhagic stroke) of a brain blood vessel. While 10% of the subjects die after ischemic stroke, in general, the prognosis for hemorrhagic stroke is even worse; with 38% of the cases resulting in death within 30 days. At present, stroke is diagnosed based on the patient examination by a clinician, complemented by brain imaging. However, clinical assessment of stroke is not always straightforward, since computerized tomography images are often not conclusive and do not detect mild ischemic strokes. Furthermore, specific biological markers to distinguish between different types of stroke are not yet available and need to be explored.

Coronary Heart Disease

Coronary Heart Disease (CHD)/Coronary artery disease (CAD) is a major problem worldwide including Asian countries. Atherosclerosis and thrombosis involves multiple cell types, and is regarded as an inflammatory disease of the vessel wall. CAD is characterized by endothelial activation, lipid accumulation and macrophage infiltration resulting in plaque formation, narrowing of the arterial lumen and in hardening and thickening of the arterial walls. Therefore, innovative and reliable biomarkers for atherosclerosis and plaque stability are much needed. A recent study has shown that circulating levels of vascular and inflammation-associated miRNAs are significantly down-regulated in patients with CAD. So far, 3 studies have addressed the potential of circulating miRNAs as biomarkers for CAD. Fichtlscherer et al. were the first to investigate the levels of plasma miRNAs in stable atherosclerotic disease in humans. Using miRNA arrays, circulating miRNA signatures were studied in plasma of 8 stable CAD patients versus 8 healthy volunteers and this resulted in the identification of 46 downregulated miRNAs and 20 significantly upregulated miRNAs in plasma of these patients. In another study, Hoekstra et al. have demonstrated that in stable and unstable CAD patients, 157 miRNAs were measured from PBMCs and differential expression was found. Most of the identified and validated downregulated miRNAs (miR-126, miR-92a, miR-155 and miR-17) were abundantly expressed in the endothelial cells of vessel wall. MiR-155 is mainly released by inflammatory cells, and since atherosclerosis is closely related to inflammation, one would expect increased levels of circulating miR-155 in CAD patients. Furthermore, patients with unstable AP could be discerned from stable patients, due to increased levels of miR-134, miR-198 and miR-370, further exemplifying the use of specific miRNA signatures in the identification of patients at risk for CAD.

Ren et al. have identified a distinct plasma miRNA expression pattern in vulnerable CAD patients. They found upregulation miR-106b/25 cluster, miR-17/92a cluster, miR-21/590-5p family, miR-126 and miR-451, in unstable CAD patients compared to patients with non-cardiac chest pain. Further, these authors found vascular and inflammation-associated miRNAs (hsa-miR-21, hsa-miR-17, hsa-miR-20a, and hsa-miR-92a) that were previously reported to be down-regulated in stable CAD patients were found to be upregulated. Circulating miR-126, which is enriched in apoptotic bodies, has been shown to mediate cardio-protective effects of endothelial apoptotic bodies. This miRNA decreased with increase in low density lipid cholesterol (LDL-c) levels in patients with stable CAD. Based on the reported changes in miR-126 level in association with LDL cholesterol, the circulating miRNA levels may reflect a compensatory response to inflammation under hyper-lipidemic background. It remains to be seen whether downregulation of miR-126 in these CAD patients is directly involved in inflammation or a compensatory response to this process. Further studies are necessary to explore the underlying mechanisms potentially linking LDL-c and circulating miR-126 levels in patients with CAD.

The decrease in the level of circulating endothelial miR-126 in the plasma of patients with stable CAD is a rather surprising observation, because the development of atherosclerotic lesions is known to be associated with endothelial activation. Fukushima et al. showed that plasma concentrations of miR-126 were negatively correlated with age. The underlying mechanism for the loss of miR-126 in the circulation with age is currently unknown but may relate to deteriorating perfusion, decreased renewal of endothelial cells, or cellular aging, which may lead to a reduction in the release of miR-126. In addition, both the vascular smooth muscle cell–enriched miR-145 and the inflammatory cell–related miR-155 were also found to be significantly down-regulated in patients with stable CAD. Among the above miRNAs, previous studies have indicated that miR-155, a typical multi-functional miRNA, plays a crucial role in immunity and inflammation. This observation was also not expected, because it is evident that atherosclerosis is associated with inflammation of the vessel wall, and inflammatory cells are the major source of miR-155.

It has been reported that a small numbers of miRNAs have shown their presence in platelets, and peripheral blood mononuclear cells (PBMCs). Freedman et al. have demonstrated that PBMCs and platelets expressed 19 miRNAs. However, it is not known why the non-nucleated cells like platelets would express miRNAs? Hoekstra et al. also studied the miRNA signature in the PBMCs of CAD patients. In a study group of 20 control subjects, 25 subjects with stable CAD, and 25 subjects with unstable CAD, real-time PCR analysis of 157 different miRNAs revealed that in PBMCs of both patient groups, circulating miR-135a was increased 5-fold, whereas miR-147 was decreased 4-fold compared with healthy control subjects. Interestingly, miR-147 previously has been reported to be associated with changes in the inflammatory capacity of immune cells by repressing tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). This suggests that PBMCs of CAD...
patients had an altered miRNA repertoire, possibly shifting to a more pro-inflammatory phenotype.

**Circulating miRNAs as Biomarkers**

Biomarkers are biological molecules found in blood and are used as markers of physiologic or pathologic processes taking place in the body. Most of the studies have evaluated patients presenting to emergency departments, underlining the need for an ideal biomarker for rapid recognition of type of CVDs. The recent discovery that miRNAs circulate in many body fluids (e.g., plasma, blood, placental fluid), suggests that circulating miRNAs can serve as new generation of biomarkers for CVD.\(^5,6,48,49\) In that respect, they are stable; their sequences are evolutionarily conserved, and are often tissue or disease specific, can be detected by highly sensitive and specific qPCR method as compared to immune-histochemical and biochemical approaches. Given the practical limitations of collecting tissue samples and the increasing feasibility and reliability of genomics-based diagnostics, there is a growing demand for non-invasive, specific and sensitive serum biomarkers is the need of the hour as before in early diagnosis of CVD. Tissue based molecular biomarkers can be in the form of histological and biochemical associated with pathologic changes, according to the stage of the disease. Serum biomarkers are even more appealing given the easy accessibility and have proteins shed, secreted, released from the tissues which can be detected in the circulation.

Current circulating biomarkers for CVD are based on specific proteins, such as cardiac troponins, CK-MB, uric acid (UA), Pregnancy-associated plasma protein-A (PAPP-A), Ischemia-modified albumin (IsMA) and Heart-type fatty acid binding protein (HFABP) and natriuretic peptides etc. Validation of some of these markers is still under investigation for their possible usefulness in an emergency department setting for early diagnosis of CVD.\(^29\)

Circulating miRNAs are resistant to RNAase digestion and remarkably stable in the RNAase-rich environment, and even after exposure to severe conditions such as extreme pH, high temperatures, repeated freeze-thawing, prolonged storage and other harsh conditions.\(^50\) Actually circulating miRNAs are heterogeneous as they exist both in non-vesicle form and in vesicle-associated form.\(^51\) Some circulating miRNAs are packaged into apoptotic bodies or microvesicles, while others are solely complexed with argonaute-2 (Ago2) protein, nucleophosmin 1 (NPM1) and high-density lipoproteins (HDL).\(^52\) Microvesicles, also called exosomes or microparticles, contain more than 100 miRNAs and can be delivered from one cell to another. Thus, circulating miRNAs might function as intercellular or inter-organ communication mediators, delivered to recipient cells and thereafter regulate the translation of their target genes.\(^52\)

In the past three years, several studies have reported the use of miRNAs as circulating biomarkers for diagnosis/prognosis of CVD such as ischemic heart disease (IHD), stroke and HF. Initial studies investigated the use of miR-124, a brain-specific miRNA, as a circulating marker of cerebral ischemia in rats where ischemic conditions were induced by middle cerebral artery occlusion (MCAO). MiR-124 levels were greatly increased in plasma after MCAO and peaked at 24 hrs (up to 150-fold compared to sham-operated animals), indicating its ability to serve as a systemic biomarker of cerebral ischemia and opening the possibility to probe for brain-specific miRNAs as biomarkers of tissue injury. With this initial idea that miRNAs would be released into the circulation from the injured heart, cardiac and skeletal muscle-specific miRNAs, a recent study examined four cardiac miRNAs (miR-208a, miR-499, miR-1, and miR-133a/b) and found to be consistently elevated in plasma of AMI patients within hours after the onset of infarction.\(^49\) Of these 4 miRNAs, miR-208a, is to the best of our knowledge the only heart-specific miRNA.\(^53\) The other 3 miRNAs (miR-499, miR-1, and miR-133), besides being abundantly expressed in the heart in response to hypoxic and ischaemic stress in cardiomyocytes, are also expressed in skeletal muscle.\(^53\) In contrast, Tijsen et al\(^24\) did not find increased miR-1, -208 or -499 in the plasma of AMI patients. Though, these authors identified a new miRNA, miR-423-5p as a biomarker specific for heart failure (HF), but it is not known what cell type (s) secrete miR-423-5p? Do increased levels of plasma miR-423-5p reflect the existence of a specific miRNA secretory pathway or does the miRNA release result from cell death and subsequent emptying of cellular contents into the extracellular space. It is clear from the studies discussed above that, blood based tests for screening purposes or disease monitoring would be more suitable as they are minimally invasive.

**Therapeutic potential of miRNAs**

At present, no reliable serum based biomarkers are available, which can be used to accurately predict patients who are at risk of developing CVD. However, the emergence of miRNAs has opened new opportunities for an early diagnosis of several cardiac diseases. Recently it has been reported that MiRNAs are dysregulated in cardiac and skeletal muscle disease, and have emerged as promising therapeutic targets in muscle wasting conditions where regenerative capacity is compromised.\(^54\) Further, it has been shown that selective miRNA-based strategy can as a therapeutic approach to inhibit vascular restenosis while preserving endothelial cell function.\(^56\) Recently, several investigators have demonstrated a functional role for microRNAs in the pathophysiology of cardiac arrhythmia.\(^57\) There are several promising data supporting the potential value of miRNAs as biomarkers, and these miRNAs can be robustly detected by commonly applied qRT-PCR, bioinformatics and microarray, and they have the potential to become a new class of clinical biomarkers.
Myocardial regeneration provides one of the most appealing therapeutic options to restore myocardial function to an injured or diseased heart, and there is some evidence that miRNAs could facilitate the process. Recently, it has been demonstrated that miR-15 family identified as a novel regulator of cardiac hypertrophy and fibrosis by inhibiting the TGFβ-pathway. 58 Sardu et al reported that miRNAs mediate the modulation of cardiac angiogenesis, apoptosis, fibrosis and membrane ionic currents. 59 β-Oxidative stress is a causal factor and key promoter of a variety of cardiovascular diseases associated with apoptotic cell death by causing deregulation of related genes. It has been demonstrated that, carvedilol, a β-adrenergic blocker protect cardiomyocytes by increasing miR-133 expression and suppress caspase-9 and subsequent apoptotic pathways. 60 Circulating miRNA-1 and -208a are novel, independent biomarkers for the diagnosis of AMI. Most significant successes so far with respect to the envisioned clinical transfer of delivery, off isolated RNA, hence development of novel serum-based RNA processing and miRNA quantification which makes it virtually impossible to measure the concentration and quality of the isolated RNA, hence development of novel serum-based biomarkers is often rather cumbersome. 64 Furthermore, modes of delivery, off-target effects, potential toxicity, reversibility and regulation of miRNA modulators also pose challenges in this field. Besides these, available arrays are constrained by incomplete miRNA coverage and issues in discriminating between closely related miRNAs. In advance of therapeutic targeting of specific miRNAs, better understandings on gene targets, functions and distribution of some of the miRNAs are needed to determine whether they can serve as potential therapeutic targets or not. Only a handful of targets for miRNAs have by now been discovered. It is critical to identify their gene targets and signaling pathways responsible for their cardiovascular effects in future studies, particularly how the expressions of miRNAs are regulated in CVD is currently unclear.

Given that miRNA research is still in infancy, more miRNA expression studies with myocardial specimens from patients with different cardiac diseases are required. The outcomes of such studies are awaited with interest. In conclusion, what we have learned about miRNAs to date is just the tip of the iceberg. If reported observations stand the test of time, the causes and early prognosis of miRNA based CVD may be suggested and further investigated.

References


van Rooij E, Quiat D, Johnson BA, Sutherland LB, Qi X, JA, Kelm, Jr RJ and Olson EN. A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance. *Dev Cell* 2009; 17 (5): 662-73.


