Potential Role of microRNAs in Cardiovascular Disease: Are They up to Their Hype?

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Abstract: Purpose of Review: Cardiovascular diseases remain the foremost cause of mortality globally. As molecular medicine unravels the alterations in genomic expression and regulation of the underlying atherosclerotic process, it opens new vistas for discovering novel diagnostic biomarkers and therapeutics for limiting the disease process. miRNAs have emerged as powerful regulators of protein translation by regulating gene expression at the post-transcriptional level.

Recent Findings: Overexpression and under-expression of specific miRNAs are being evaluated as a novel approach to diagnosis and treatment of cardiovascular disease. This review sheds light on the current knowledge of the miRNA evaluated in cardiovascular disease.

Conclusion: In this review we summarize the data, including the more recent data, regarding miRNAs in cardiovascular disease and their potential role in future in diagnostic and therapeutic strategies.

Keywords: Cardiovascular disease, diagnostic biomarkers, miRNAs.

INTRODUCTION

Cardiovascular disease remains the most prevalent cause of morbidity and mortality in the developed and developing countries [1]. Hence, concerted scientific efforts have been made to gain insight into pathogenesis of atherosclerosis and its sequelae, and discover diagnostic and predictive biomarkers as well as therapeutic strategies to combat these diseases. Progress in molecular medicine has revealed a complex post-transcriptional regulation in humans which may help us further understand the pathogenesis of this disease process.

miRNAs are single stranded, phylogenetically conserved, non-coding RNA molecules of ~22 nucleotides which inhibit mRNA translation to protein by interacting with its 3’ untranslated region (UTR) [2]. This binding results in mRNA degradation or translational inhibition and is termed post-transcriptional gene regulation, which contributes to tissue specificity of mRNA expression in human tissues. Furthermore, one miRNA can regulate hundreds of genes and one gene can be regulated by a number of miRNAs. Dysregulated expression patterns both cause and result from disease states [3].

As miRNAs can easily be detected by methods such as real-time PCR and microarrays, a number of groups have reported their use as biomarkers in cardiovascular diseases such as myocardial infarction, hypertension and heart failure. Although many of these studies require replication in independent study populations, the picture emerges that some miRNAs are quite specific for cardiovascular pathologies and are crucially involved in many aspects of cardiac development, homeostasis and pathobiology [4].

miRNA IN ATHEROSCLEROSIS

The influence of miRNAs in the regulation and control of crucial cellular processes, including signaling, proliferation, apoptosis, motility and angiogenesis has implicated aberrant miRNA expression in the development and progression of atherosclerosis. Nuclear factor κB (NF-κB) activation contributes to EC activation and dysfunction, which play critical roles during the development of atherosclerosis.

NUCLEAR FACTOR (NF-κB)

NF-κB is a pleiotropic signaling pathway. The mammalian NF-κB family consists of 5 transcription factors retained in the cytosol by a family of inhibitory proteins known as inhibitors of NF-κB (IκB). In the canonical NF-κB signaling pathway, the IκB kinase (IKK) complex is activated in response to a variety of stimuli. This leads to the release of
NF-κB heterodimers that translocate to the nucleus, where they bind to NF-κB elements that drive a wide range of gene expressions. NF-κB signaling is tightly and precisely regulated by different mechanisms at multiple levels, and failure to control this pathway contributes to the development of many chronic diseases, such as atherosclerosis [5]. In the arterial endothelium, NF-κB signaling is activated by many risk factors for atherosclerosis, diabetes, oxidized LDL, angiotensin II, and hemodynamic forces. The resulting NF-κB signaling leads to the expression of pro-inflammatory genes, including cytokines, adhesion molecules, and chemokines. Numerous studies convincingly demonstrate that inhibition of the endothelial NF-κB signaling pathway or its target gene expression ameliorates atherosclerosis.

**ENDOTHELIAL DYSFUNCTION**

Endothelial dysfunction and injury at sites with disturbed flow dynamics are critical events in the pathogenesis of atherosclerosis. Endothelial Cells (ECs) are primarily exposed to laminar flow (~15 dynes/cm²) in the arterial system, and shear stress in this range has an antithrombotic effect on endothelial cells. In contrast, reduction in shear stress, caused by oscillatory flow below a certain threshold, increases the turnover of ECs due to enhanced apoptosis and proliferation and induces a pro-inflammatory phenotype characterized by enhanced adhesiveness to leukocytes. This disturbed flow is characteristically found in athero-prone regions of the arterial tree such as branching points or the outer cover of the aortic arch. A subset of miRNAs has been identified that are induced by high shear stress mediating an atheroprotective role, including miR-10a, miR-19a, miR-23b, miR-101, miR-126 and miR-143/145 [6]. In contrast, low shear stress induced expression of miR-21a, miR-92a, and miR-663 resulting in an atheroprotective endothelial cell phenotype (Table 1). mRNA microarray analysis of the miR-10a knockdown in cultured human aortic ECs identified IκBα /NF-κB-mediated inflammation as the major pathway which was up-regulated after RNAi against miR-10a. Fang and Davies reported that in miR-92a knock-down mice, Kruppel Like Factor 4 (KLF4) and KLF2 levels were increased [7]. These are critical transcriptional regulators of endothelial homeostasis with an anti-inflammatory effect, and the control mice had an increased tumor necrosis factor (TNFα) induced vascular cell adhesion molecule-1 (VCAM-1) and E-selectin expression and blunted endothelial Nitric oxide synthase (eNOS) expression [8]. The study further showed that oxidized low-density lipoprotein (oxLDL) increases miR-92a expression primarily in low shear-stress areas, while therapeutic inhibition of miR-92a attenuates endothelial NF-κB activation and limits the development of atherosclerotic lesions in mice.

OxLDL contributes to atherosclerotic plaque formation and progression by several mechanisms, including the induction of endothelial cell activation and dysfunction, macrophage foam cell formation, and smooth muscle cell migration and proliferation. Vascular wall cells express on their surface several scavenger receptors that mediate the cellular effects of oxLDL. The lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is the main oxLDL receptor of ECs.

miRNA let-7g targets inhibit LOX-1 gene expression and under basal conditions LOX-1 expression is very low. Several stimuli, including oxLDL inhibit miRNA-let 7g expression by inducing transcription factor Oct-1 and up-regulate LOX-1 expression, which leads to the induction of endothelial dysfunction by several pathological stimuli (Fig. 1).

**MACROPHAGES**

Macrophages are the principal effector cells in atherosclerosis [4, 9]. NF-κB-dependent signaling pathways are again of crucial importance in the pro-inflammatory activation of macrophages. TNF-α and hyperlipidemia induce the expression of miR-103, which promotes the expression and release of endothelial chemokines CXCL1, CX3CL1 and

Table 1. microRNAs involved in atherosclerosis.

| Endothelial cells (stimulus) | miRNA     | Targets and function                                                                 | Reference |
|-----------------------------|-----------|-------------------------------------------------------------------------------------|-----------|
| High shear stress           | miR-10a   | Inhibits HomeoboxA1 (HOXA1) which facilitates phosphorylation of IkBαa prerequisite for NF-κB-mediated inflammation | [32]      |
|                             | miR-21    | Down-regulates phosphatase and tensin homologue (PTEN) pathway, increased eNOS phosphorylation and NO production | [33]      |
|                             | miR-126   | Mediates up-regulation of CXCL12 Suppresses VCAM-1 expression and leucocyte adhesion by endothelial cells | [6]       |
| Low oscillatory shear stress (OSS) | miR-92a | Overexpression Inhibits KLF2 mRNA and inhibits expression of eNOS | [7]       |
|                             | miR-663   | Mediates OSS-induced monocyte adhesion to ECs                                       | [8]       |
| Oxidized LDL                | miRNA-let7g| Binds and inhibits LOX-1 mRNA                                                       | [32]      |
|                             | miR-155   | Inhibits Bcl6 which inhibits NF-κB signaling                                         | [34]      |
|                             | miR-125   | Inhibits Endothelin-1 (ET-1) which leads to plaque stabilization                     | [35]      |
atherogenic monocyte adhesion (Table CCL2, by translational repression of KLF4, and increase NF-κB signaling and adhesion of monocytes to ECs [8, 10]. Notably, deficiency of KLF2 in macrophages accelerates atherosclerosis in hyper-cholesterolemic mice by increasing EC-apoptosis and reduced the leukocyte influx in the vascular endothelium [11, 12].

CCL2, by translational repression of KLF4, and increase atherogenic monocyte adhesion (Table 1; Fig. 2). Endothelial miR-92a also targets both KLF4 and KLF2, activating NF-κB signaling and adhesion of monocytes to ECs [8, 10]. Notably, deficiency of KLF2 in macrophages accelerates atherosclerosis in hyper-cholesterolemic mice by increasing monocyte adhesion and macrophage infiltration into atherosclerotic lesions. Also CCL2 secreted by ECs as well as smooth muscle cells promotes trans-endothelial migration of CCR2-expressing monocytes which migrate into the sub-endothelial space where they differentiate into macrophages. Over-expression of miR-181b inhibited an enriched set of NF-κB-responsive genes such as VCAM-1 and E-selectin, and reduced the leukocyte influx in the vascular endothelium [11, 12].

Inside the intima, the monocytes and VSMCs are loaded with modified LDLs through scavenger receptors, which internalize oxidized LDL, giving rise to foam cells. This scavenger-receptor mediated uptake is important for clearing oxidized LDL, but unregulated uptake of oxidized LDL leads to production of lipid laden foam cells which depend on cholesterol and lipid efflux pathways. This lipid uptake is regulated by miR-155 or miR-125a-5p, wherein these two miRNAs significantly reduce lipid uptake by inhibiting nuclear translocation of NF-κB that downregulates scavenger receptors (LOX-1, CD36, CD-68). miR-33a and miR-33b are intronic miRNAs located within the two SREBP (sterol regulatory element-binding proteins) genes, and regulate cholesterol and fatty acid metabolism. miR-33 inhibits expression of ABCA-1 (ATP binding cassette transporter-subfamilyA,
and ABCG1 and antagomiR to miR-33 has been shown to induce ABCA1 and cholesterol removal [13-15].

CHOLESTEROL HOMEOSTASIS

The hepatic low-density lipoprotein receptor (LDLR) pathway is essential for clearing circulating LDL cholesterol (LDL-C). In mice, inhibition of miR-148a increased hepatic LDLR expression and decreased plasma LDL-C. microRNA expression quantitative trait loci (miR-eQTL) studies using liver tissue from 424 morbidly obese individuals revealed an association of miR-128-1 and miR-148a expression with single nucleotide polymorphisms (SNPs) linked to abnormal human blood lipid levels, suggesting the relevance of these miRNAs, identified by genome wide association studies (GWAS), to human cardio-metabolic disorders. miR-122 is the most abundant miRNA in the liver and its inhibition results in a significant reduction of plasma cholesterol levels12 [16-18].

PLAQUE RUPTURE

Neo-angiogenesis and intra-plaque hemorrhage, drive blood components to atherosclerotic lesion, promoting plaque vulnerability due to enrichment in pro-oxidant, pro-inflammatory and proteolytic factors. miR-126 regulates endothelial response to angiopeiotin1 (Ang-1) which promotes angiogenesis followed by stabilization for maturation of the new vessel. miR-126 null mice were found to be partially viable but their vessels were fragile and leaky. Studies using human umbilical vein endothelial cells (HUVECs) demonstrated that miR-23-27-24 clusters exist in mammals and are highly expressed in vascularized tissues and miR-29 favors plaque instability due to reduction of collagen content [6].

CIRCULATING miRNA AND CARDIOVASCULAR DISEASE

Recently numerous studies have shown that plasma levels of many miRNAs are significantly altered in cardiovascular diseases. All cellular components involved in plaque and thrombus formation (e.g. endothelial cells, macrophages, smooth muscle cells) may potentially release miRNA or simply secrete less miRNAs in the circulation, and this deviation in miRNA profile, may serve as biomarkers for early atherosclerosis. Table 2 summarizes the miRNAs that are altered by myocardial ischemia/reperfusion injury.

miR-1 and miR-133a represent the most abundantly expressed cardiac miRs and derive from two bicistronic miR clusters, miR-1-1/miR-133a-2 and miR-1-2/miR-133a-1. The latter cluster lies in [19] intron 12 of mindbombE3 ubiquitin protein ligase 1, a protein involved in apoptosis. miR-1-1 and miR-1-2, as well as miR-133a-1 and miR-133a-2, are identical in humans. miR-133b, specifically expressed in the skeletal muscle, differs from miR-133a in the two terminal nucleotides at the 3'end. miR-1 and miR-133 are essential for skeletal and cardiac muscle cell proliferation and differentiation.

ACUTE MYOCARDIAL INFARCTION (AMI)

Several groups have investigated the fact that heart-specific miRNAs leak into the circulation after myocardial

Table 2. miRNAs in ischemic-reperfusion injury.

| miR    | Target                                      | Response to I/R injury                                                                 | Potential Role                                                                 | Reference |
|--------|---------------------------------------------|---------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|-----------|
| miR-1  | Heat shock protein-60 (Hsp-60) in adult cardiomyocytes & skeletal muscle | Down-regulation of multiple anti-apoptotic genes, provoke stress-induced apoptosis, arrhythmogenesis in mice | Diagnostic Biomarker Lower cardiac miR-1 levels may attenuate I/R injury | [36]      |
| miR-133| CASP-9, in adult cardiomyocytes & skeletal muscle | Anti-apoptotic role of miR-133, Protective effect against H2O2 triggered cell death , reduced fibrosis post-TAC | Diagnostic biomarker, Increased miR-133 may attenuate ischemic injury | [37]      |
| miR-21 | Rho-B in endothelial cells Inhibits PTEN in cardiac fibroblasts | Rho-B silencing impairs endothelial migration and tubulogenesis. PTEN upregulates matrix metalloproteinase-2 expression. Promoting fibrosis | Inhibition of miR-21 will promote angiogenesis, post-infarct fibrosis (latter role controversial) | [38]      |
| miR-29 | Mcl-1, an anti-apoptotic protein, of Bcl-2 family in cardiac fibroblasts/ endothelial cells | Down-regulation of miR-29 with antago miRs causes increased expression of collagen, down-regulated after MI in border zones, may contribute to post-infarct remodeling | Immediate down-regulation may be protective but long term inhibition may be detrimental | [39]      |
| miR-210, | Caspase-8, Ephrin A3 | Cyto-protection affected by ischemic preconditioning, pro-angiogenic effect, and influences process of leucocyte infiltration | Cardio-protective role at multiple levels | [40]      |
| miR-499 | Pdcd4, Pacs2, Dyrk2 | Knockdown of miR-499 associated with deletrious cardiac re-modeling | Inhibits mitochondrial apoptosis pathway and protects H2O2 injury in cardiomyocytes | [19]      |
infarction. Four cardiac miRNAs (miR-208a, miR-499, miR-1 and miR-133) are found to be consistently elevated in plasma of AMI patients within hours of myocardial infarction [19]. miR-208a and miR-499 were found to be rapidly induced in rodent models of MI and disappeared at 24hrs. In another study, serum miR-1 level was rapidly increased 200 fold after AMI in a rat model of AMI induced by coronary artery ligation with a peak at 6hrs and returned to baseline at 3 days [20].

Wang et al. confirmed that miR-208 was detectable in all patients of AMI within 4hrs, while troponin-T (cTnT) was detected only in 85% of patients, confirming superior sensitivity of miR-208 at early time points. Plasma miR-208b and miR-499 levels were elevated (>10^3-fold, p<0.001) in AMI patients compared to healthy controls. Receiver operating characteristics (ROC) curve analysis showed that the value of area under the curve was 0.947 for miR-499, and significantly correlated with cTnT, suggesting that this could emerge as an ideal biomarker of AMI [21].

In a study (n=444) miR-1, miR-133a and miR-208b levels were significantly increased in Non-ST elevation myocardial infarction or AMI compared with unstable angina, whereas miR-133b, miR-208a levels were not elevated in AMI compared with unstable angina (UA). However, considerable overlap existed in the plasma levels of these miRNAs, in unstable angina and AMI, precluding their use for further subdivision of acute coronary syndromes. More important their levels also correlated with Major Adverse cardiac events (MACE), risk of death and surrogate endpoints of infarct size and depressed ventricular function and could be used for further prognostication of patients [22].

Takatsubo cardiomyopathy remains clinically indistinguishable from AMI and no acute biomarkers exist to distinguish these two conditions. In a recent study, miR-16, miR-26a were elevated in this condition compared to AMI patients/healthy controls while miR-1 and 133a were elevated in the AMI group [23].

STABLE CORONARY ARTERY DISEASE

Circulating miR-133a, miR-208a levels are up-regulated while miR-126, miR-17, miR-92a, miR-155 levels are significantly down-regulated in patients with stable coronary artery disease compared to healthy subjects. Circulating miR-135a is increased by five-fold in circulating peripheral blood mononuclear cells (PBMCs) while miR-147 level is decreased by four-fold, and the ratio of miR135a/miR147 is increased by 19 fold suggesting the increased sensitivity of using the ratio as a biomarker for stable CAD. Furthermore Hoekstra et al. could find three miRNAs (miR-134, miR-198, miR-370) expressed differentially in CAD patients of unstable angina and patients with chronic stable angina [24]. Fichtlscherer et al., showed that in patients miR-145 expression (amongst other miRNAs) was significantly reduced in CAD patients compared with healthy controls [24].

miRNA IN ESSENTIAL HYPERTENSION

Vascular smooth Muscle Cells (VSMCs) rarely proliferate in adult tissues, but undergo major phenotypic changes from the contractile to the synthetic in response to environmental cues, a phenomenon known as switching or phenotypic modulation. This transition of VSMCs from a differentiated state to a dedifferentiated state plays a critical role in pathogenesis of cardiovascular diseases such as hypertension, vascular injury and arteriosclerosis. Several studies have focused on the role of miR-21, miR-221, miR-222, miR-143 and miR-145 in VSMC differentiation (Table 3). Three independent groups generated mouse models of miR-143/145 KO mice and showed that the cluster was necessary for VSMCs to acquire the contractile phenotype. Functional analysis of the vascular tone revealed that miR-143/145 KO mice had statistically significant arterial hypotension in comparison with wild-type under steady state conditions. Similarly the former exhibited reduced systolic blood pressure in response to angiotensin II stimulation. Expression levels of miRNA-143, miRNA-145 and miR-133 were significantly lower and miR-1and miR-21 levels were higher in hypertensive patients compared to controls [25, 26].

Shuqiang et al. demonstrated a link between human cytomegalovirus (HCMV) infection and essential hypertension and isolated an HCMV-encoded miRNA, hcmv-miR-UL112 from hypertensive subjects [27].

PLASMA miRNA IN HEART FAILURE (HF)

To explore whether circulating miRNAs can be used as biomarkers in patients with HF, Tijsen et al. performed miRNA arrays on plasma of 12 healthy controls and 12 HF patients [28]. Subjects with recent cardiac ische-

| miRNA  | Target function                                                                 | Reference |
|--------|----------------------------------------------------------------------------------|-----------|
| 221    | Promotes vascular smooth muscle cell (VSMC) proliferation through repression of cyclin-dependent kinase inhibitor p27Kip1 | [41]      |
| 222    | Knockdown of miR-221/222 elevates p27Kip1, c-kit and p57Kip2 and results in reduced VSMC proliferation and intimal thickening in response to injury. | [42]      |
| 143/145| Targets multiple targets, including KLF4, KLF5, ELK1, several actin remodeling proteins which are antagonistic to VSMC differentiation | [43]      |
| 146 a  | Expression of miR-146a is elevated in proliferative VSMCs                          | [44]      |
| 21     | Targets programmed cell death protein 4, required for differentiation of VSMC      | [41]      |
mIA/infarction were excluded, so results were less likely to be influenced by cardiac cell death and thus no increase in miR-208a, miR-499, miR-1, miR-208b was found in plasma of these HF patients. From these arrays, 16 candidate miRNAs were selected and validated in a second group of patients, consisting of 50 case subjects with complaints of dyspnea, 30 of whom were later diagnosed as having HF and 20 due to non HF. Seven miRNAs were validated in the plasma of patients with HF (miR-423-5p, miR-18b, miR-129-5p, miR-1254, miR-675, HS 202.1 and miR-622), among which miR-423-5p was most strongly related to the clinical diagnosis of HF, with an ROC showing an AUC of 0.91 (95% confidence interval 0.84-0.98). The observation that miR-423-5p is up-regulated in human failing myocardium suggests that the increased plasma levels are derived from the myocardium. Interestingly the abundance of some miRNAs was related to disease severity because it was found that miR-423-5p and miR-18b were higher in subjects with the poorest ejection fraction and NYHA classification.

miR-126 was negatively correlated with age, BNP levels and NYHA class in HF patients.

Sarcoplasmic/endoplasmic reticulum calcium ATPase 2a (SERCA2a) in cardiomyocytes, facilitates calcium uptake from the cytosol into the sarcoplasmic reticulum during excitation-contraction coupling, and decreased expression of SERCA2a is a hallmark of HF. To search for miRNAs that down-regulate SERCA2a expression Alexander et al. carried out a high-throughput screen of a whole-human-genome collection of miRNA using a ‘target-sensor’ construct consisting of the SERCA2a mRNA3’ UTR fused to enhanced green fluorescent protein (eGFP). A number of miRNAs were identified that were both evolutionarily conserved and had previously been reported to be up-regulated in patients with heart failure. Of these, miR-25 showed the most potent effect in in vitro experiments: it strongly affected calcium influx in a cardiomyocyte cell line, eliciting a physiological effect comparable to that of small interfering RNA (siRNA) against SERCA2a. Further in vivo experiments in TAC mice showed that overexpression of miR-25 using an adeno viral vector led to a reduction in myocardial contractile function.

PLASMA miRNA IN DIABETES MELLITUS (DM)

Zampetaki et al. did an extensive network analysis and revealed a unique plasma miRNA signature for DM, which included reduced levels of miR-132, miR-15a, miR-29b, miR-223 and elevated levels of miR-28-3p [29]. Among these miRNAs the endothelial derived miR-126 was most consistently related with DM, which is interesting because miR-126 was also one of the identified down-regulated miRNAs in CAD [30]. It is shown to play an important role in maintaining endothelial cell homeostasis and vascular integrity. These findings suggest that this unique plasma miRNA signature may become a valuable tool to predict microvascular and macrovascular complications.

miRNA AS THERAPEUTIC TARGETS IN CARDIO-VASCULAR DISEASE

miRNA are becoming an intriguing pharmacological target in the treatment of cardiovascular disease. The development of antisense oligonucleotide-mediated (anti-miR) knockdown and miRNA-mimic-mediated overexpression techniques might soon allow the regulation of any given miRNA in cardiovascular disease. Anti-miRs with 2'-o-methoxyethyl phosphorothioate substitutions have proven useful for the inhibition of miR-122 in the liver. miRNA mimics are synthetic RNA duplexes in which 1 strand is identical to the mature miRNA sequence (guide strand) and is designed to mimic the function of the endogenous miRNA. The other strand, “passenger strand” is often only partially complementary to the guide strand and typically linked to cholesterol to enhance cellular uptake. These miRNAs enhance the expression of miRNAs that are down-regulated in cardiovascular disease. Still there are several hurdles before these become a viable modality. First miRNAs often have several hundreds even thousands of predicted mRNA targets, but at physiological levels, miRNA most likely targets only a small fraction of them [31]. Forced overexpression of a miRNA, might result in regulation of physiologically irrelevant targets.

CONCLUSION

At the moment independent studies are identifying unique miRNA signatures for different cardiovascular disorders. This data would require validation in large-scale multicenter trials. miRNAs because of their tissue specificity and ease of identification in the plasma/blood are likely to launch an opportunity for better diagnostic biomarkers. Moreover the potential to block certain miRNAs, with dysregulated patterns in disease states would provide an exciting opportunity for therapeutic.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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