Relationship Between the Temporal Profile of Plasma microRNA and Left Ventricular Remodeling in Patients After Myocardial Infarction

Michael R. Zile, MD; Shannon M. Mehurg, MD; Jazmine E. Arroyo, BS; Robert E. Stroud, MS; Stacia M. DeSantis, PhD; Francis G. Spinale, MD, PhD

Background—microRNAs (miRs) are small noncoding RNAs that recognize and bind to mRNAs and inhibit protein translation or degrade mRNA. Studies in animal models have suggested that miRs play a translational or posttranslational regulatory role in myocardial growth, fibrosis, viability, and remodeling. However, whether specific temporal changes in miRs occur in patients during the left ventricular (LV) remodeling process that follows a myocardial infarction (post-MI) remains unknown. The current pilot study tested the hypotheses that plasma miRs could be reliably measured in post-MI patients and that there is a relationship between temporal changes in specific miRs and post-MI LV structural remodeling.

Methods and Results—LV end-diastolic volume (echocardiography) and plasma miR were measured in age-matched referent controls (CTLs, n=12) and post-MI patients (n=12) from day 2 through day 90 post-MI. Selected miRs (miR-1, miR-21, miR-29a, miR-133a, and miR-208) were measured using quantitative reverse transcription–polymerase chain reaction and normalized for endogenous small nuclear RNA U6. After MI, LV end-diastolic volume increased progressively compared with CTL; this was accompanied by time-dependent changes in specific miRs. For example, miR-21 initially decreased 2 days post-MI (0.3±0.1-fold versus CTL; P<0.05), increased 5 days post-MI (2±1-fold versus CTL; P<0.05), and returned to CTL values at later post-MI time points. In contrast, miR-29a increased 5 days post-MI (4±1-fold versus CTL; P<0.05) and then decreased to CTL at later time points. miR-208 increased 5 days post-MI (3±1-fold versus CTL; P<0.05) and remained elevated up to 90 days post-MI.

Conclusions—A time-dependent change in miRs occurred in post-MI patients, including an early and robust increase in miRs that has affected myocardial growth, fibrosis, and viability. Thus, serially profiling miRs in the plasma of post-MI patients may hold both mechanistic and prognostic significance.

Key Words: myocardial infarction ■ remodeling ■ microRNA

Clinical Perspective on p 619

Whether specific temporal changes in miRs occur in patients during the LV remodeling process that follows a myocardial infarction (post-MI) remains unknown. It is impractical to assess serial changes in miRs in post-MI patients using repetitive LV myocardial tissue biopsy specimens. However, because miRs are actively exported into the interstitial space and enter the plasma in a form protected from degradation, it may be possible to assess serial myocardial changes in miRs by using plasma sampling. Accordingly, the goals of the current study were as follows: (1) to develop a sensitive and reliable method to measure miRs in plasma in referent control subjects and post-MI patients and (2) to measure serial changes in specific miRs after an MI to determine whether there is a relationship between...
temporal changes in specific miRs and LV structural remodeling in post-MI patients.

**Methods**

**Subjects**

Twelve patients with a confirmed MI and 12 referent age-matched control subjects were enrolled in this pilot study after providing informed consent. All of the studies described herein were reviewed and approved by the Medical University of South Carolina Institutional Review Board. An ECG and/or a positive cardiac enzyme panel confirmed the MI. Patients were excluded from enrollment if there was a history of an MI, previous coronary revascularization surgery within the past 24 months, a history of an active malignancy, significant renal or hepatic dysfunction, or active rheumatological disease. The MI patients were treated according to American Heart Association (AHA)/American College of Cardiology (ACC) guidelines. The referent control group consisted of subjects with no evidence of cardiovascular disease. Cardiovascular disease was excluded by performing a complete medical history, a comprehensive physical examination, an ECG, and an echocardiogram.

**Protocol**

For the MI patients, studies were performed beginning at enrollment (post-MI day 1). Plasma from a peripheral vein blood sample was used to measure miR profiles at post-MI days 2, 5, 28, and 90. At post-MI days 1, 5, 28, and 90, an echocardiogram was obtained. For both referent controls and post-MI subjects, all plasma samples were drawn at the same time of the day, between 8 and 10 AM. Therefore, potential diurnal variations that may be present were avoided. For the referent control subjects, an echocardiogram and plasma sample were obtained once, at enrollment. All subjects abstained overnight before each study but took their morning medications as prescribed.

**Echocardiographic Data**

Transesophageal echocardiography was performed using a Sonos 5500 system with an S-4 MHz transducer (Agilent Technologies). Measurements were made with American Society of Echocardiography criteria. The 2D echocardiographic studies were performed using standard short- and parasternal long-axis views to obtain measurements of LV volumes. Images were coded and read in a blinded fashion, and this analysis remained unlinked to the miR levels until completion of the study.

**Plasma miR Measurements**

Small RNAs from plasma were isolated using the mirVana PARIS Kit (AM1556; Ambion), which is based on a denaturing/phenol chloroform extraction approach. Briefly, 400 μL of plasma was added to an equal amount of denaturing solution and incubated on ice for 5 minutes. Afterwards, 800 μL of an acid-phenol chloroform solution was added to the samples to inactivate RNAses and to create an aqueous RNA phase. This aqueous phase was removed and passed through glass-fiber filters (AM1556; Ambion), which is based on a denaturing/phenol chloroform extraction approach. Finally, the preamplification product was subjected to real-time polymerase chain reaction (CFX96 Real-Time System; BioRad). The relative cycle threshold (Ct) values for U6 snRNA were used as endogenous controls for normalizing the respective miR Ct values and were calculated as ΔCt (ΔCt=miR Ct-snRNA U6 Ct). Changes in miRNA ΔCt were reported as a fold change from the mean ΔCt of the referent controls, calculated as FC=2^ΔCt post-MI/ΔCt mean referent control, where “ΔCt post-MI” was calculated for each individual patient at each individual time point and each of these individual values was then subtracted from the mean value of all the referent controls. By using this method, a mean±SEM could be calculated for each miR. These normalized Ct values were computed for each sample, and these measurements were performed in duplicate. Referent control values were set at 1.0. Therefore, in post-MI patients, fold change values <1.0 represented a decrease in miR expression and fold change values >1.0 represented an increase in miR expression compared with referent control. In initial assays performed in triplicate using referent control samples, the coefficient of variation for individual miR values was <10% (Table 2).

Six miRs and 1 endogenous control were chosen for this study. A representative miR was chosen to target a translational or a posttranslational molecular regulatory role in each aspect of post-MI remodeling, including augmentation or inhibition of hypertrophy, extracellular matrix changes, apoptosis, and progressive dilation. miR-1 may blunt LV hypertrophy, augment apoptosis, and facilitate to progressive dilation; miR-208 augments hypertrophy and increases changes in the extracellular matrix; miR-21 and miR-133a inhibit apoptosis; and miR-29a inhibits changes in the extracellular matrix.

To validate our extraction efficiency, 8 human plasma samples were spiked with 10 ng of a foreign Caenorhabditis elegans miR-39 sequence (cel-miR-39; Integrated DNA Technologies) before extraction. The extraction efficiency was calculated as 76.8±0.6%. Also, the coefficient of variation between the spiked samples was determined as 2.2%. Negative controls were also run to verify the absence of genomic DNA contamination (reverse transcription control) and the absence of overall DNA contamination in the polymerase chain reaction system and working environment (template control). No genomic DNA contamination was detected.

<table>
<thead>
<tr>
<th>Table 1. Applied Biosystems’ miR Primers</th>
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<tbody>
<tr>
<td>miR</td>
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</tr>
<tr>
<td>miR-1</td>
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<tr>
<td>miR-21</td>
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<tr>
<td>miR-29a</td>
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<tr>
<td>miR-125b-3p</td>
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<tr>
<td>miR-133a</td>
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<tr>
<td>miR-208a</td>
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<tr>
<td>U6 snRNA</td>
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<p>| Table 2. Ct Values and Coefficient of Variation for 12 Referent Controls |
|--------------------------------|-------------------|------------------|</p>
<table>
<thead>
<tr>
<th>miR</th>
<th>Ct Values, Mean±SEM</th>
<th>Coefficient of Variation, %</th>
</tr>
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<tbody>
<tr>
<td>miR-1</td>
<td>28.55±0.69</td>
<td>8.40</td>
</tr>
<tr>
<td>miR-21</td>
<td>20.29±0.75</td>
<td>12.80</td>
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<tr>
<td>miR-29a</td>
<td>23.03±0.77</td>
<td>11.83</td>
</tr>
<tr>
<td>miR-133a</td>
<td>27.54±0.94</td>
<td>11.83</td>
</tr>
<tr>
<td>miR-208a</td>
<td>37.75±1.44</td>
<td>10.76</td>
</tr>
<tr>
<td>U6 snRNA</td>
<td>27.46±1.06</td>
<td>13.43</td>
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Table 3. Demographic Characteristics for Referent Control Subjects and Patients With MI

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Referent Controls (n=12)</th>
<th>Patients With MI (n=12)</th>
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<tbody>
<tr>
<td>Age, y</td>
<td>61±2</td>
<td>58±3</td>
</tr>
<tr>
<td>Male sex, No. (%)</td>
<td>5 (42)</td>
<td>9 (75)</td>
</tr>
<tr>
<td>Body surface area, m²</td>
<td>1.88±0.06</td>
<td>1.99±0.04</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>66±2</td>
<td>68±2</td>
</tr>
<tr>
<td>Arterial pressure, mm Hg</td>
<td>124±3</td>
<td>119±4</td>
</tr>
<tr>
<td>Systolic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic</td>
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There were time-dependent changes in the 5 measured miRs in the post-MI patients compared with the referent control subjects (Figure 2 and Figure 3). miR-1 and miR-21 decreased at day 2 post-MI; miR-29a, miR-133a, and miR-208 were unchanged at day 2 post-MI. miR-125 was not detectable at any time point in the post-MI patients. The value of snRNA U6 was unchanged from referent control at each point examined in the post-MI patients.

**Results**

Demographics

By experimental design, there were no differences in age between referent controls and post-MI patients (Table 3). The ratio of men/women was higher in the post-MI group. Heart rate and blood pressure were comparable between groups. Differences in medications reflect expected ACC/AHA guideline-based protocols for post-MI patients. In the referent control subjects, β-adrenergic blockers, angiotensin-converting enzyme inhibitors, and angiotensin receptor antagonists were used to treat mild increases in systolic pressure. Aspirin or anti-inflammatory agents were used for management of arthritic pain.

LV Structure

LV end-diastolic volume (EDV) increased in a time-dependent manner in the post-MI group, as shown in Figure 1. LV EDV was already increased compared with referent controls on day 1 post-MI. LV EDVs further increased from post-MI day 1 values at post-MI days 28 and 90.

miR Data

There were time-dependent changes in the 5 measured miRs in the post-MI patients compared with the referent control subjects (Figure 2 and Figure 3). miR-1 and miR-21 decreased at day 2 post-MI; miR-29a, miR-133a, and miR-208 were unchanged at day 2 post-MI. miR-1, miR-133a, and miR-208 increased at day 5 and remained elevated through day 90 post-MI. miR-21 and miR-29a were increased at day 5 but returned to normal by day 90 post-MI. miR-125 was not detectable at any time point in the post-MI patients. The value of snRNA U6 was unchanged from referent control at each point examined in the post-MI patients.
Plasma miRs
Given the fact that blood contains ribonucleases, it might be expected that neither serum nor plasma should contain any intact RNA. However, several recent studies\(^{18,19}\) have demonstrated the presence of miRs in healthy subjects and in patients with cancer and other disease processes. These facts lead to studies that addressed the question of whether quantitative reverse transcription–polymerase chain reaction products found in plasma samples were the result of contamination by degraded products of large-molecular-weight RNA, tRNA, or genomic DNA. Studies\(^{18,19}\) indicate that there is a stable reproducible population of miRs that exist in a form that is resistant to endogenous ribonuclease, possibly because it is packaged inside an exosome or is associated with other molecules. For example, some of the total RNA isolated from human plasma was degraded by treatment with exogenous ribonuclease; however, miRs were not degraded. The miRs were not degraded by treatment with DNase, multiple freeze-thaw cycles, prolonged incubation, or a large pH range.\(^{18}\) However, when miRs, not homologous to human miR, were added to human plasma, these miRs were degraded. In patients with known cancer in whom tissue samples demonstrate an increase in specific miRs, the plasma has also increased miRs.\(^{18}\)

Changes in miRs After MI
To our knowledge, there have been no clinical studies in which serial changes in a targeted group of miRs have been measured in the plasma of patients after MI. Previous clinical studies\(^{5,6,8–11,20,21}\) have been performed in patients with stable coronary artery disease, in patients with ischemia-induced cardiomyopathy, and at a single point early after an MI. Although significantly different in design and end points, these previous studies have important implications for the current study. Fichtlscherer and colleagues\(^5\) measured plasma miRs in patients with stable coronary artery disease. In their study, relative levels of miR-133a and miR-208a were marginally increased in patients with stable coronary artery disease and no evidence of myocardial dysfunction.\(^9\) This study examined patients potentially at risk of
developing an MI. Two more recent studies\textsuperscript{10,11} examined changes in miRs in patients early after an MI. In these studies,\textsuperscript{10,11} plasma miR-1 and miR-133a were increased, and these miRs were postulated to be novel biomarkers with diagnostic and prognostic value. Data from the current study markedly advanced these previous clinical studies by measuring plasma miRs both early and for several months after an MI; comparing data from MI patients with those from age-matched control subjects; examining a larger group of miRs chosen because they have affected myocardial growth, fibrosis, and viability; and relating changes in miRs to post-MI alterations in structure and function. For example, in the current study, plasma levels of certain miRs, such as miR-21 and miR-29a, increase early post-MI, whereas other miRs, such as miR-1, miR-133a, and miR-208, remained persistently elevated up to 3 months post-MI. Furthermore, higher levels of miR-29a early post-MI were related to greater remodeling late post-MI.

Downstream biologically relevant determinants of collagen homeostasis, such as matrix metalloproteinases (MMPs) and their endogenous tissue inhibitors, were examined in post-MI patients.\textsuperscript{22} In addition, collagen telopeptides have also been examined in post-MI patients.\textsuperscript{23,24} In each case, after an MI, determinants of collagen homeostasis favor degradation, and evidence of degraded collagen was present early after an MI, but favored fibrosis late after an MI. Data from these previous studies and the current study suggest the hypothesis that changes in miRs may precede, determine, and predict downstream collagen homeostatic events. However, proving a direct causal relationship between miRs and downstream collagen events must await further study.

However, the regional distribution of changes in miRs and the functional and mechanistic significance of the findings could not be determined from clinical studies using plasma samples. For these reasons, inferences from animal models of MI must be made and the serial changes after an MI that occur in these animal models must be compared with the current study.\textsuperscript{5,8,11–13} In rodent studies of myocardial tissue miR profiling post-MI, van Rooij et al.\textsuperscript{11} and other investigators\textsuperscript{11–13} demonstrated significant temporal and regional changes in miRs in the early (3–14 day) post-MI period. Indeed, these studies demonstrated early and dynamic changes in miR myocardial levels, such as miR-1, miR-21, miR-29a, and miR-133a, which were similar to the early plasma miR profile obtained in the present study. However, they also demonstrated regional heterogeneity in the changes in myocardial miR profiles that differed within the remote, border, and MI regions in these rodent post-MI models.\textsuperscript{8,12} Taken together, the findings from previous and present studies underscore several considerations in terms of profiling plasma miRs after an acute cardiovascular event, such as an MI. First, single-point measurements of plasma miRs may be insufficient to identify the relative magnitude and time-dependent nature of the dynamic changes in miRs after an acute event. Second, plasma measurements of miRs will be a summation of release from local tissue compartments and, therefore, will not be sensitive to regional heterogeneity in miR expression.

Although the present study demonstrated an association between certain plasma miR profiles and LV remodeling/dilation post-MI, the functional and mechanistic significance of the findings from the present study must be extrapolated primarily from rodent studies.\textsuperscript{5,6,8,13–15} For example, in a transgenic mouse model overexpressing miR-133a, reduced myocardial fibrosis was reported, which was accompanied by abnormalities in myocardial repolarization.\textsuperscript{15} In the present study, miR-133a was persistently elevated in patients after an MI, but whether the magnitude of this increase was sufficient to alter myocardial extracellular matrix remodeling remains speculative. One observation from previous rodent studies was that predicted posttranscriptional regulatory targets for miR-133, based on in silico mapping, were not necessarily predictive of in vivo findings.\textsuperscript{15} In the present study, the rationale for the selection of miRs for plasma profiling was based on previous in vivo observations in animal models, rather than on in silico mapping algorithms. For example, miR-21 myocardial levels were increased early post-MI in rodents to regulate myocardial fibroblast MMP-2 levels.\textsuperscript{13} Previous studies from this laboratory have demonstrated that dynamic changes in plasma MMP-2 levels occur in patients post-MI.\textsuperscript{22} Moreover, the temporal pattern of changes in plasma MMP-2 levels in this post-MI period were similar to those obtained in the present study with respect to miR-21. However, a recent study\textsuperscript{14} in transgenic mice suggests that ablation of miR-21 is insufficient to significantly alter adverse myocardial matrix remodeling. Thus, the mechanistic significance of the temporal changes in miR-21 in post-MI patients, which were observed in the present study, remains unclear.

One of the miRs that appears to hold biological relevance to extracellular matrix remodeling and, in particular, adverse post-MI remodeling is the miR-29 family.\textsuperscript{5,6,8} In the present study, a rapid surge in plasma miR-29a was observed early post-MI, which rapidly decreased at longer post-MI points. An interaction between fibrillar collagen expression and profibrotic signaling pathways and changes in the expression of the miR-29 family has been demonstrated in several animal model and human tissue studies.\textsuperscript{8} Data from the current and previous studies may suggest that early after a myocardial infarction, increased miR-29a expression may act to limit (or inhibit) a profibrotic ECM response, allow more ECM degradation, and result in more extensive LV remodeling (particularly LV dilation) in both the short- and long-term after an MI. However, these postulated mechanisms remain to be proved in future clinical and animal studies.

Conclusions

miRs can be reproducibly measured in the plasma of patients after an MI using a sensitive and reliable method. A time-dependent change in miR plasma levels occurred after an MI, particularly in those miRs that are associated with myocardial growth, fibrosis, and viability. Data from the current study suggest that serially profiling miRs in the plasma of post-MI patients may hold both mechanistic and prognostic significance.

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We had full access to the data and take full responsibility for the integrity of the data. We have read and agree to the article as written.

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Disclosures
None.

References
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