

MicroRNA *miR-1* is Up-regulated in Remote Myocardium in Patients with Myocardial Infarction

(infarcted tissue / remote myocardium / microRNA expression)

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Abstract. MicroRNAs are small regulatory RNA molecules that mediate regulation of gene expression, thus affecting a variety of physiological, developmental and pathological conditions. They are believed to be new promising therapeutic targets. In recent studies two muscle-specific microRNAs were discovered to contribute to heart diseases and development: *miR-1* and *miR-133*, but there is little data on their expression patterns in human myocardial infarction. We performed simultaneous expression analysis of *miR-1*, *miR-133a*, *miR-133b* in samples of infarcted tissue and remote myocardium from twenty-four patients with acute myocardial infarction. MicroRNA expression was analysed using quantitative real-time PCR and compared to the expression patterns in myocardium of eight healthy adults who died in accidents. We found ~3.8-fold *miR-1* up-regulation in remote myocardium when compared to infarcted tissue or healthy adult hearts. As *miR-1* has been shown in animal models and clinical studies to contribute to arrhythmogenesis by regulating pacemaker channel genes, our finding of *miR-1* up-regulation in patients with myocardial infarction indicates that it might be responsible for the higher risk for arrhythmias in these patients. In addition, *miR-133a/b* down-regulation in infarcted tissue and remote myo-

cardium was observed, indicating *miR-133a/b* involvement in the heart response to myocardial infarction. We conclude that *miR-1* and *miR-133* seem to be important regulators of heart adaptation after ischaemic stress.

Introduction

MicroRNAs (miRNAs) are non-coding single-stranded RNA molecules of 20–24 nucleotides in length. They are post-transcriptional regulators of gene expression. By targeting mRNAs, they consequently inhibit mRNA translation and protein synthesis or cause mRNA degradation. miRNAs have been found to regulate a variety of developmental and physiological functions (e.g. stem cell differentiation, neurogenesis, haematopoiesis, immune reactions, metabolism (Fazi and Nervi, 2008; Williams, 2008)). They are associated with various pathologic conditions, such as cancer, autoimmune diseases, inflammatory and neurodegenerative disorders (Williams, 2008). There is growing evidence suggesting that miRNAs are also involved in the regulation of development, physiologic and pathologic conditions of the heart (Scalbert and Bril, 2008).

Many miRNAs are expressed in a tissue-specific manner. Recent studies defined muscle-specific expression of certain miRNAs, their target genes and their expression in various diseases (Yang et al., 2008). Among mammalian miRNAs identified so far, *miR-1* and *miR-133* are believed to have a muscle-specific expression pattern (Chen et al., 2006). Both have been demonstrated to regulate heart development (Chen et al., 2006; Liu et al., 2007; Thum et al., 2008), and both have been demonstrated to be dysregulated in hypertrophic and failing hearts (Care et al., 2007; Sucharov et al., 2008; Thum et al., 2008), but there is little data on their expression patterns in human myocardial infarction (MI) (Yang et al., 2007; van Rooij et al., 2008; Yin et al., 2008; Roy et al., 2009).

We therefore performed expression analysis of *miR-1*, *miR-133a* and *miRNA-133b* in infarcted and remote myocardium of patients with acute MI, using a quantitative real-time polymerase-chain reaction (qRT-PCR) approach.

Received April 15, 2009. Accepted October 6, 2009.

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Abbreviations: CAD – coronary artery disease, Ct – cycle threshold, FFPE – formalin-fixed paraffin-embedded, *GJA1* – gene encoding protein connexin 43, *HCN2* – hyperpolarization-activated cyclic nucleotide-gated channel genes, *KCNJ2* – gene encoding K⁺ channel subunit Kir2.1, MI – myocardial infarction, *miR* – microRNA gene, miRNA – microRNA, NMEC – National Medical Ethics Committee, PCR – polymerase chain reaction, qRT-PCR – quantitative real-time PCR, RT – reverse transcription, RM – remote myocardium, SPSS – Statistical Package for the Social Sciences.

Material and Methods

Patients and tissue samples

Our study included autopsy heart samples from 24 patients with MI. MI was diagnosed clinically by symptoms and/or electrocardiographic changes, and confirmed by elevated plasma levels of markers of cardiac necrosis. Among 24 patients with MI, 14 were males and 8 females, aged 60–94 years. Diabetes mellitus and arterial hypertension were recorded in 9 and 12 patients, respectively. Four patients received reperfusion treatment, and 5 patients had documented ventricular fibrillation and/or ventricular tachycardia.

The duration of MI at the time of death was estimated on the basis of histological and clinical data. All cases of MI included in our study were up to 7 days old.

In all cases, samples from MI and the border zone were available; in 10 cases, samples from remote myocardium were also included. All autopsies were performed within 24 hours after death. Tissue samples were fixed in 10% buffered formalin and embedded in paraffin (FFPE, formalin-fixed paraffin-embedded). Five paired tissue samples (infarcted tissue and remote myocardium) were preserved in RNAlater (Applied Biosystems/Ambion, Austin, TX) for subsequent RNA isolation.

RNA isolation and analysis of extracted RNA

FFPE heart tissue samples: Tissue samples were cut at 25 μm from FFPE tissue blocks using a microtome. For the isolation procedure, three 25- μm sections were used. Total RNA isolation was performed using RecoverAll Total Nucleic Acid Isolation (Ambion) according to the manufacturer's protocol, with a few modifications. One ml of xylene (Merck, Whitehouse Station, NJ) was added for de-paraffinization, followed by brief vortexing and incubation at 50 °C for 15–30 min. After the ethanol-washing step, pellets were air-dried and digestion with Protease (Ambion) was performed overnight at 50 °C (until the tissue was completely digested). The RNA was eluted twice in 30 μl of nuclease-free water.

RNAlater preserved heart tissue samples: Autopsy samples preserved in RNAlater (Ambion) were used for RNA extraction with QIAzol Lysis Reagent (Qiagen, Hilden, Germany) and chloroform. After extraction, total RNA was cleaned with a miRNeasy Mini Kit (Qiagen), performing on-column DNase Digestion, using RNase-Free DNase Set (Qiagen). The RNA was eluted twice in 40 μl of nuclease-free water.

RNA quality and quantity: The concentration of RNA extracted was measured using NanoDrop-1000 (Thermo Scientific, Waltham, MA) and tested for UV/vis ratios. The A_{260}/A_{230} ratio needs to be above 1.0 and the A_{260}/A_{280} ratio needs to be above 1.8. The integrity and presence of small RNAs (< 200 nucleotides) was analysed in a Bioanalyzer 2100 (Agilent, Santa Clara, CA), which is a microfluidics-based platform. The Small RNA Assay and Bioanalyzer 2100 allow separating, verifying and optimizing miRNA after the extraction procedures. After applying 1 μl of the sample to the high-resolution

Small RNA Assay and using Small RNA Ladder as a reference, we obtained the detailed view of the RNA of 6–150 nt in range.

miRNA reverse transcription and TaqMan-based qRT-PCR analysis

All the reagents and instruments were from Applied Biosystems, except where otherwise indicated. As the internal control gene, *hsa-miR-26b* was used according to the Applied Biosystems Application Note (Wong et al., 2007). Looped primers for specific miRNA reverse transcription, including *hsa-miR-1*, *hsa-miR-133a*, *hsa-miR-133b* and *hsa-miR-26b* were utilized following the manufacturer's protocol. Briefly, a reverse transcription (RT) reaction was performed using 15- μl master mix with 10 ng of total RNA sample. Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out using the Applied Biosystems 7900 Real-Time PCR System in 20- μl PCR master mix containing 10 μl TaqMan 2 \times Universal PCR Master Mix, 1 μl TaqMan assay, and 9 μl RT products diluted 10-fold. All the qRT-PCR reactions were performed in triplicates and the signal was collected at the endpoint of every cycle.

To eliminate the possibility of miRNA degradation and potential false results (Bravo et al., 2007), the isolation procedure, reverse transcription and qRT-PCR reaction of samples were performed within two days, with the isolation procedure being done on the first day.

Statistical analysis

To present the relative gene expression, the $2^{-\Delta\Delta\text{Ct}}$ method was used, in which the fold change of the tested group (infarcted tissue, remote myocardium) was calculated relative to the calibrator group (healthy adult hearts) (Livak and Schmittgen, 2001). The calculated ΔCt in different groups (infarcted tissue, remote myocardium) were compared to the ΔCt of the control group (healthy adults) and tested for statistical significance using Mann-Whitney test for independent groups of samples (24 infarcted tissue or 10 remote myocardium versus eight healthy adult hearts) or Wilcoxon signed rank test for paired samples (infarcted tissue versus remote myocardium from the same patient) with a cut-off point at $P < 0.05$ (Yuan et al., 2006). The SPSS (Statistical Package for the Social Sciences) analytical software ver. 16 (SPSS Inc., Chicago, IL) was used. When the ΔCt of the tested groups was significantly higher or lower than the ΔCt of the control group, the expression change was considered to be statistically significant.

Results

RNA quality and quantity

The concentration of RNA extracted was measured using NanoDrop-1000 (Thermo Scientific) and tested for UV/vis ratios. The samples showing the A_{260}/A_{230} ratio above 1.0 and A_{260}/A_{280} ratio above 1.7 were subjected to analysis for the integrity and presence of small

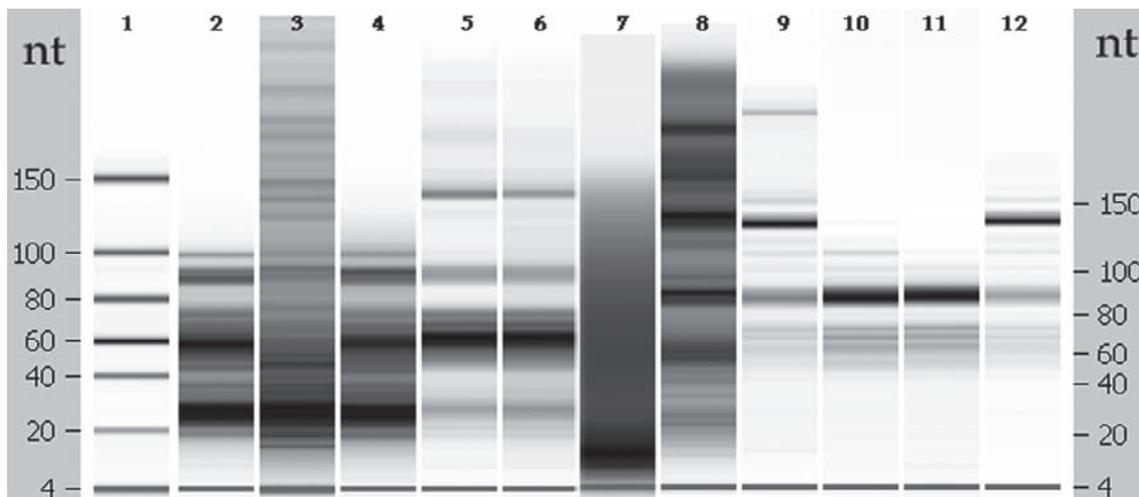


Fig. 1. Bioanalyzer 2100 (Agilent) analysis of the small RNAs (< 200 nt). The number above the lane represents: 1, Small RNA Ladder (6–150 nt); 2–6, small RNA extracted from RNAlater-preserved autopsy heart tissue; 7–8, small RNA extracted from FFPE autopsy heart tissue; 9–12, small RNA extracted from EDTA-preserved peripheral blood samples used as control samples.

RNAs by Bioanalyzer 2100 (Agilent). The detailed view of the RNA of 6–150 nt in range revealed the presence of ~22 nt long miRNA molecules as shown in Fig. 1.

TaqMan-based qRT-PCR results

Using qRT-PCR, we analysed the expression of muscle-specific miRNAs in infarcted tissue and remote myocardium compared to healthy adult hearts. The results are summarized in Figs. 2–4. All three miRNAs were not expressed in the aorta, which served as a negative control, and there was no correlation between the expression patterns of *miR-1* or *miR-133a/b* and the presence of diabetes, arterial hypertension or reperfusion treatment.

miR-1 expression in infarcted tissue and remote myocardium compared to healthy adult hearts

We found significant *miR-1* up-regulation (~3.8-fold) in ten remote myocardium samples when compared to the infarcted tissue (Wilcoxon signed rank test, $P = 0.03$) or healthy adult hearts (Mann-Whitney test, $P = 0.04$). In contrast, *miR-1* showed neither up- nor down-regulation in the infarcted tissue of MI patients when compared to healthy adult hearts. The results are summarized in Fig. 2.

miR-133a expression in infarcted tissue and remote myocardium compared to healthy adult hearts

There was no statistically significant difference in the expression of *miR-133a* between infarcted tissue and remote myocardium (Wilcoxon signed rank test, $P > 0.05$). However, we found lower expression of *miR-133a* in the infarcted tissue (~2.2-fold) and remote myocardium (~1.7-fold) of MI patients compared to healthy adult hearts, but the difference did not reach statistical signifi-

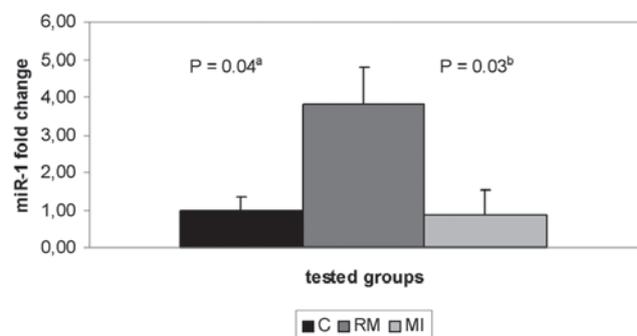


Fig. 2. *miR-1* expression fold change in human myocardial infarction compared to healthy adult hearts. MI (infarcted tissue), RM (remote myocardium), C (control – healthy adult hearts). ^a Mann-Whitney test, expression in remote myocardium compared to healthy adult hearts; ^b Wilcoxon signed rank test, expression in remote myocardium compared to infarcted tissue.

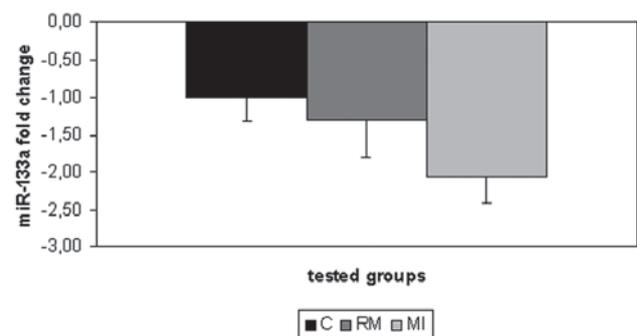


Fig. 3. *miR-133a* expression fold change in human myocardial infarction compared to healthy adult hearts. MI (infarcted tissue), RM (remote myocardium), C (control – healthy adult hearts).

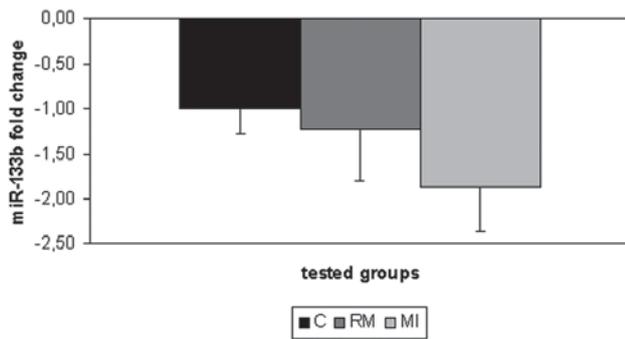


Fig. 4. *miR-133b* expression fold change in human myocardial infarction compared to healthy adult hearts. MI (infarcted tissue), RM (remote myocardium), C (control – healthy adult hearts).

cance (Mann-Whitney test, $P > 0.05$). The results are summarized in Fig. 3.

miR-133b expression in infarcted tissue and remote myocardium compared to healthy adult hearts

There was no statistically significant difference in the expression of *miR-133b* between infarcted tissue and remote myocardium (Wilcoxon signed rank test, $P > 0.05$). We found lower expression of *miR-133b* in the infarcted tissue and remote myocardium (~1.7-fold and ~1.8-fold, respectively) of patients with MI compared to healthy adult hearts, yet the differences were not statistically significant (Mann-Whitney test, $P > 0.05$). The results are summarized in Fig. 4.

Discussion

miR-1 and *miR-133* are clustered on the same chromosome loci and transcribed together in a tissue-specific manner. Using cell culture and animal model experiments, it has recently been shown that *miR-1* and *miR-133* have different roles in muscle development: *miR-1* promoting myoblast differentiation and *miR-133* myoblast proliferation (Chen et al., 2006; Liu et al., 2007) and apoptosis, showing a pro-apoptotic role of *miR-1* and anti-apoptotic role of *miR-133* (Xu et al., 2007). It has been suggested that *miR-1* and *miR-133* also play an important role in determining cardiac automaticity in the developing heart, possibly by down-regulating *GJA1* and *KCNJ2* (Yang et al., 2007), and by re-expression of the pacemaker channel genes *HCN2* (hyperpolarization-activated cyclic nucleotide-gated channel genes) (Luo et al., 2008). In adult life, *miR-1* and *miR-133* have been found to be dysregulated during physiologic and pathologic hypertrophy (Care et al., 2007) and heart failure (van Rooij et al., 2007; Thum et al., 2008), both in humans and experimental animals.

In this study, we analysed the expression of three muscle-specific miRNAs, *miR-1*, *miR-133a* and *miR-133b* in infarcted tissue and remote myocardium

from patients with MI, in comparison to healthy adult hearts. The most significant finding was ~3.8-fold *miR-1* up-regulation in remote myocardium from patients with MI, but not in the samples from infarcted tissue. This finding is consistent with previously reported *miR-1* up-regulation (~2.8-fold) in patients with coronary artery disease (CAD), but not in an animal model of MI, which showed ~2.6-fold up-regulation in the ischaemic zone of MI but not in the non-ischaemic zone (Yang et al., 2007). According to the animal model, over-expression of *miR-1* in the hearts of adult rats leads to the development of severe cardiac arrhythmias by targeting mRNAs of ion channel genes, *GJA1*, which encodes connexin 43, and *KCNJ2*, which encodes the K^+ channel subunit Kir2.1. Knockdown of endogenous *miR-1* can inhibit ischaemic arrhythmias (Yang et al., 2007). Consistently, five of ten patients included in our study with elevated *miR-1* expression in remote myocardium had documented arrhythmias (i.e. ventricular fibrillation and/or ventricular tachycardia). Our finding of *miR-1* up-regulation in patients with MI indicates that it might be responsible for the higher risk of arrhythmias in these patients. These findings raise hope that inhibition of *miR-1* might reduce the life-threatening arrhythmias, a frequent cause of death in patients with cardiovascular disease.

In addition, we also found down-regulation of *miR-133a* and *miR-133b* in infarcted tissue and remote myocardium in patients with MI when compared to healthy adult hearts, but this down-regulation did not reach statistical significance, possibly due to a small number of cases included in our study. Muscle-specific *miR-133* is believed to be a negative regulator of hypertrophic growth of heart muscle, since *miR-133* showed down-regulation in patients with hypertrophic cardiomyopathy and in mouse models of cardiac hypertrophy (Care et al., 2007). Our finding of *miR-133* down-regulation could be associated with cardiac hypertrophy, a common pathological response to a number of cardiovascular diseases including ischaemic heart disease and MI. By undergoing a remodelling process and hypertrophic growth, heart adapts to the impaired cardiac function. The remodelling process in the heart also includes electrical remodelling, which increases the risk of arrhythmogenesis by re-expression of the pacemaker channel gene *HCN2* (Luo et al., 2008). The *HCN2* gene has been demonstrated as a target of *miR-133*.

In conclusion, our findings of *miR-1* up-regulation and *miR-133a/b* down-regulation in remote myocardium in MI are consistent with the suggestion that these microRNAs may contribute to an increased risk of arrhythmias after MI by down-regulating *GJA1* and *KCNJ2* (targets for *miR-1*) and re-expression of *HCN2* (target for *miR-133*) (Yang et al., 2007; Luo et al., 2008). Our findings also support the suggested role of *miR-1* up-regulation and *miR-133a/b* down-regulation in remodelling processes after ischaemic stress (Care et al., 2007). However, these results should be confirmed in a larger series of patients.

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