Experimental Study

MicroRNA-214 Inhibits Left Ventricular Remodeling in an Acute Myocardial Infarction Rat Model by Suppressing Cellular Apoptosis via the Phosphatase and Tensin Homolog (PTEN)

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SUMMARY

The aims of the present study were to determine the role of miR-214 on left ventricular remodeling of rat heart with acute myocardial infarction (AMI) and to further investigate the underlying mechanism of miR-214-mediated myocardial protection. AMI was induced in which adenovirus-expressing miR-214 (Ad-miR-214), anti-miR-214, or Ad-GFP had been delivered into rats hearts 4 days prior, while a phosphatase and tensin homolog (PTEN) inhibitor was administered via intra-peritoneal injection 30 minutes prior to AMI. Changes in hemodynamic parameters were detected and recorded. Left ventricular (LV) dimensions and LV/BW were measured. Quantitative RT-PCR was used to determine the miR-214 expression levels of the myocytes in the infarcted, border, and non-infarcted areas of the LV. Myocardial infarct size was also measured. Flow cytometry analysis was performed to examine cellular apoptosis. Western blot analysis was performed to examine PTEN expression. The results showed that miR-214 was upregulated in both border and infarcted areas. Myocardial cell apoptosis was decreased in the Ad-miR-214 group, but was increased in the anti-miR-214 group, while there were no differences among the Ad-GFP-group, PTEN-ad-miR-214 group, or PTEN-anti-miR-214 group. Myocardial infarct size, LV dimensions, heart rate (HR), and LV end-diastolic pressure (LVEDP) were decreased while the maximal rates of rise or decline in blood pressure in the ventricular chamber (± dp/dt) and LV systolic pressure (LVSP) were increased in the Ad-miR-214 group, all of which exhibited opposite changes in the anti-miR-214 group. PTEN was downregulated in the Ad-miR-214 group and upregulated in the anti-miR-214 group. PTEN was decreased in both the border and infarcted areas compared with non-infarcted areas. The study results suggest that Ad-miR-214 improves LV remodeling and decreases the apoptosis of myocardial cells through PTEN, suggesting a possible mechanism by which Ad-miR-214 functions in protecting against AMI injury. (Int Heart J 2016; 57: 247-250)

Key words: Adenovirus expressing transfer, QRT-PCR analysis, Western blot analysis, Rat acute myocardial model

Cardiovascular diseases constitute the major leading cause of death globally, and current estimates indicate that as many as 1 in 6 deaths per year can be attributed to coronary disease and associated myocardial ischemia in the United States.1) Cardiac remodeling after acute myocardial infarction (AMI) results in poor cardiac performance, which often leads to heart failure.2) Myocardial fibrosis results in mechanical stiffness, which contributes to ventricular contractile dysfunction.3)

MicroRNAs (miRNAs, miRs) are a class of endogenous, small (~22 nt) non-coding single-stranded RNAs, which have highly conserved sequences among species.4) Approximately 1,400 miRNAs have been identified thus far in humans, and this number is growing.5) An individual miRNA is as important as a transcription factor due to its ability to regulate the expression of multiple target genes.5) The majority of cellular functions, including apoptosis and necrosis, which are two key cellular events in cardiac remodeling after AMI, involve regulation by miRNAs.

Research has shown that miR-21, -24, -133, -210, -494, and -499 provide protection to myocytes against I/R-induced apoptosis, while miR-1, -29, -199a, and -320 promote apoptosis.6) Our previous studies have shown that miR-21 plays an important role in the protection of heart function and is a possible mechanism in ischemic disease.7) By analyzing conserved miRNAs that were upregulated in a rat AMI model, we identified miR-214 as a sensitive marker of ischemic injury.8) In the current study, we designed a rat AMI model with the aim of investigating whether miR-214 had a protective effect on heart function. Our findings suggest that miR-214 may pro-

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vide a potential novel therapeutic approach for the treatment of ischemic heart disease.

**METHODS**

**Rat AMI model:** AMI was induced by ligation of the left anterior-descending (LAD) artery as described previously. In brief, rats were anesthetized with chloral hydrate (10% 3 mL/kg, ip). Animals were intubated with a 19G stump needle and ventilated with room air using a MiniVent mouse ventilator. Under sterile conditions, the heart was exposed through a left thoracotomy in the fourth intercostal space. The LAD artery was then ligated approximately 2 mm distal from its origin with a 6.0 polypropylene suture. Proximal LAD artery ligation creates a reproducibly large lateral wall infarction in rats. The sham operation involved an identical procedure, except the suture was passed around the vessel without LAD occlusion. All protocols were approved by the Animal Ethics Committee of Hebei Medical University.

**Construction of adenovirus expressing miR-214, anti-miR-214, GFP, and PTEN inhibitor:** The adenovirus expressing miR-214, anti-miR-214, or the GFP control (Ad-GFP) was produced using the ViraPower™ Adenoviral Gateway™ Expression Kit (Invitrogen) according to the manufacturer’s protocol. The PTEN inhibitor (VO-OHpic) was obtained from Sigma.

**Adenovirus-mediated Ad-miR-214, anti-miR-214, or Ad-GFP gene transfer in vivo, and PTEN inhibitor injection:** As previously described, Ad-miR-214, anti-miR-214, or Ad-GFP was delivered into rat hearts 4 days prior to AMI. Briefly, rats were anesthetized with chloral hydrate (10% 3 mL/kg, ip). The pericardium was opened via the fourth intercostal space. The aorta and pulmonary artery were identified. A 23 G catheter containing 200 μL of adenovirus (expressing miR-214, anti-miR-214, or Ad-GFP) was advanced from the apex of the left ventricle to the aortic root. The aorta and pulmonary arteries were clamped distal to the site of the catheter and the solution was injected. The clamp was maintained for 10 seconds as the heart is volumetrically pumped against a closed system. The procedure allowed the solution that contained the adenovirus to circulate down the coronary arteries and perfuse the heart. After 10 seconds, the clamps on the aorta and pulmonary artery were released and the chest was closed. The PTEN inhibitor VO was administered by intra-peritoneal injection at a single dosage of 10 μg/kg 30 minutes prior to AMI in rats that had received Ad-miR-214, anti-miR-214, or Ad-GFP 4 days before.

**Measurement of infarct size and determination of infarcted, border, and non-infarcted areas:** At 24 hours after LAD ligation, the rats were anesthetized and 4 mL of 1% Evans blue dye was injected into the vena cava to delineate the non-infarcted portion of the heart. The myocardial ischemic area at risk was identified as the region lacking blue staining. The ventricles of the hearts were sliced transversely into 2-mm thick slices. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) at 37°C for 30 minutes to identify the non-infarcted and infarcted areas. Infarct size was expressed as the percentage of the ischemic area at risk. In myocardial slices, the non-infarcted area was defined as the Evans blue-stained area, the infarcted area was displayed as the TTC-unstained area, and the border area was identified as the Evans blue-unstained and TTC-stained area.

**Measurement of the dimensions of the left ventricles (LV) and LV relative weight:** Eight weeks following AMI, anesthetized rat hearts were arrested in diastole by an intravenous (iv) injection of saturated potassium chloride solution via the left jugular vein. The heart was excised and the LV was separated and weighed. The dimensions of the LV were measured in transverse slices at the level of the papillary muscle as previously described. The LV relative weight was the ratio of LV/BW.

**Hemodynamic examination:** Hemodynamic examination was performed as previously described. Hemodynamic parameters, including the heart rate (HR), left ventricular end-diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), maximal rate of rise in blood pressure in the ventricular chamber (+dp/dt max), and maximal rate of decline in blood pressure in the ventricular chamber (-dp/dt max) were measured and recorded by an 8-channel polygraph system (RM-6000).

**RNA extraction and qRT-PCR analysis:** Total RNA was obtained separately from the infarcted, border, and non-infarcted areas of the LV at 24 hours after LAD ligation, as well as from the sham group at the same time. RNA isolation was accomplished using Trizol (Invitrogen) reagent according to the manufacturer’s protocol. The total RNA concentration was measured at 260 nm using a NanoDrop 2000c spectrophotometer (Thermo Fisher). Quantitative miRNA analysis was performed using an All-in-One miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia). Approximately 300 ng of total RNA from each tissue was reverse-transcribed into cDNA using poly A polymerase primers (37°C for 60 minutes; 85°C for 5 minutes). Quantitative PCR was performed using an All-in-One miRNA qRT-PCR kit and probes (GeneCopoeia) with the following temperature profile: 95°C for 10 minutes followed by 40 cycles of 95°C for 10 seconds, 60°C for 27 seconds, and 72°C for 27 seconds. U6 expression level was used as an internal normalization control for each tissue. The primers used were provided by GeneCopoeia Inc. as follows: miRNA 214 forward, Cat#: RmiRQP1208; U6 forward, Cat#: RmiRQP9003; Reverse, Cat#:Po1011A. The Ct values of the target were normalized by subtraction of the U6 Ct values, thereby providing the ΔCt values. The relative expression level between treatments was then calculated using the following equation: relative gene expression = 2^-(ΔCt, sample-ΔCt, control).

**Western blot analysis:** Western blotting was performed according to standard protocols. Total protein extracts were isolated from adult hearts with 250 mM sucrose buffer. Antibodies against PTEN (EPIT MICS, 1:600) and GAPDH (EPIT MICS, 1:600) were used. Quantification of Western blots was performed using Image J v1.46 (NIH).

**Detection of apoptosis:** Cardiac myocyte apoptosis was measured by flow cytometry as described previously. Ad-miR-214, anti-miR-214, or Ad-GFP was transferred into the rat hearts 4 days before AMI, and cellular apoptosis was evaluated 24 hours after AMI in both border and infarcted areas using an Annexin V FITC/PI kit (BD Biosciences).

**Statistical analysis:** Statistical analyses were performed using SPSS 10.0 software. Data are presented as the mean ± standard deviation (SD). The difference between two groups was evaluated by the unpaired t test. The differences among the various groups were determined using the Kruskal-Wallis test. A probability P value of < 0.05 was considered statistically significant.
RESULTS

miR-214 expression signatures in different areas of infarcted rat hearts in the early phase of AMI: To determine the change in expression level of miR-214 in the different areas of infarcted hearts in the early phase of AMI, miRNAs were isolated from each region. As shown in Supplemental Figure 1, miR-214 expression was upregulated in the border and infarcted areas at 24 hours after AMI. In the infarcted area, miR-214 expression at 24 hours after AMI was 1.98-fold greater than that of non-infarcted areas; and in the border area, miR-214 expression at 24 hours after AMI was 2.28-fold greater than in the non-infarcted areas. As shown in Supplemental Figure 2, 4 days after injecting 2 × 10^10 pfu/rat adenovirus using our delivery method, Ad-miR-214 increased miR-214 expression while anti-miR-214 decreased miR-214 expression in the heart tissue. As shown in Supplemental Figure 3, the Ad-miR-214 could further increase the miR-214 in the infarcted and the border areas compared to that in the infarcted and border areas of the heart from control rats.

Hemodynamic examination: The hemodynamic index was determined and recorded 8 weeks after AMI. As shown in Supplemental Figure 4, Ad-miR-214 significantly increased LVSP, LV +dp/dt max, and LV -dp/dt min, and decreased HR and LVEDP when compared to the Ad-GFP group, while the effect of anti-miR-214 exhibited opposite effects in these parameters.

Effect of adenovirus-mediated miR-214 gene transfer on LV/BW: As shown in Supplemental Figure 5, 8 weeks after AMI, Ad-miR-214 significantly decreased the LV/BW ratio compared to that of the Ad-GFP group, while anti-miR-214 increased the ratio of LV/BW. These observations suggested that Ad-miR-214 improved the heart function in rats with AMI.

Effect of adenovirus-mediated miR-214 gene transfer on myocardial apoptosis: As shown in Supplemental Figure 6B, compared with the Ad-GFP group, the cellular apoptotic rate significantly decreased in the Ad-miR-214 group both in the border and infarcted areas. In contrast, the cellular apoptotic rate significantly increased in these same areas in the anti-miR-214 group. Compared with the Ad-GFP group, apoptosis in the infarcted area decreased 13.86% in the Ad-miR-214 group and increased 8.14% in the anti-miR-214 group; apoptosis in the border area decreased 7.95% in the Ad-miR-214 group and increased 9.14% in the anti-miR-214 group.

Effect of adenovirus-mediated miR-214 gene transfer on myocardial infarct size and LV dimensions: As shown in Supplemental Figure 7A, compared to control adenovirus (Ad-GFP)-treated rats, Ad-miR-214 reduced myocardial infarct size by 15%, while anti-miR-214 increased myocardial infarct size by 8% at 24 hours post-AMI. Representative TTC-stained hearts slices from rats treated with Ad-miR-214, anti-miR-214, and Ad-GFP are shown in Supplemental Figure 7B. It was expected that the reduced myocardial size and heart cell apoptosis should have functional results on LV remodeling such as a change in LV dimensions. To directly confirm this, the infarcted hearts from Ad-GFP, Ad-miR-214, and anti-miR-214 pre-treated rats were isolated at 8 weeks after AMI. As shown in Supplemental Figure 8B, the LV internal diastolic diameter in Ad-GFP-treated animals (10.01+/−0.21 mm) was significantly bigger than in the Ad-miR-214-treated animals (8.31+/−0.14 mm), and smaller than in the anti-miR-214-treated animals (12.31+/−0.14 mm). Myocardial infarct size was decreased by miR-214 24 hours after AMI and LV dimensions were decreased at 8 weeks after AMI.

PTEN is a miR-214 target gene that is involved in the Ad-miR-214-mediated apoptotic effects of cardiac cells: As shown in Supplemental Figure 9, compared with the Ad-GFP group, PTEN was downregulated at 4 days in the Ad-miR-214 group and upregulated at 4 days in the t-anti-miR-214 group. In addition, PTEN expression was decreased both in the border and infarcted areas at 24 hours after AMI, compared with the non-infarcted areas. Administration of PTEN inhibitor 30 minutes before AMI had no differential effect on the cellular apoptosis in either the infarcted area or border area in the Ad-GFP, PTEN-Ad-miR-214, and PTEN-anti-miR-214 groups at 24 hours after AMI. These results suggest that PTEN is a miR-214 target gene.

DISCUSSION

It is becoming increasingly clear that miRNAs fulfill multiple and specific functions in the post-infarct remodeling of reperfused myocardium. MiRNAs are capable of regulating cardiomyocyte hypertrophy, influencing myocardial contractile function, and promoting or protecting myocytes against I/R-induced apoptosis. The present study demonstrates that overexpression of miR-214 significantly improves hemodynamic, left ventricular function, and LV remodeling in a rat AMI model, and further suggests a possible miR-214-mediated mechanism related to the repression of myocyte apoptosis through the suppression of PTEN. The ability of miR-214 to reduce apoptosis may potentially lead to a novel therapeutic strategy.

Cardiovascular function is often evaluated by changes in various hemodynamic indices. The changes in HR, LVSP, LVEDP, and +/- dp/dt can be used to measure heart metergasis. It has been well established that elevated HR increases myocardial oxygen consumption in normal myocardium. HR reduction has also been demonstrated to ameliorate atherosclerosis and vascular inflammation, with an improvement in cardiovascular outcome. Furthermore, HR was also significantly associated with Killip class and LVEF on admission. The increase in LVEDP directly represents an increase in cardiac preload and also indirectly reflects the impairment of cardiac diastolic function. Our results showed that Ad-miR-214 improved LVSP, decreased HR and LVEDP, improved +/- dp/dt, and decreased left ventricular relative mass in the AMI rat model, which improved the systolic and diastolic function of the heart.

Apoptosis, which is programmed cell death, is the culmination of a series of changes in gene expression and DNA strand breaks. Apoptosis may be responsible for a significant amount of cardiomyocyte death during the acute ischemic stage of MI as well as for the progressive loss of surviving cells during the subacute and chronic stages. Furthermore, myocardial apoptosis is strongly associated with a major determinant of unfavorable LV remodeling and early symptomatic post-infarction HF. In the present study, the cardiomyocyte apoptotic rate decreased and myocardial infarction size was reduced in the miR-214 group compared with the control group at 24 hours post AMI. These results suggest that the attenuation of MI with miR-214 might be related to a reduction of cellular apoptosis.
Phosphatase and tensin homolog (PTEN) is a dual protein–lipid phosphatase that dephosphorylates the secondary messenger produced by PI3K and interrupts the downstream activation of AKT. PTEN has been implicated in ischemic heart disease. The inhibition of PTEN has been proposed to be an important strategy to improve myocardial survival following an ischemic episode. Additionally, a previous study showed that miR-21 activates AKT through suppression of PTEN. In the present study, we determined that miR-214 and PTEN have opposing expression and effects in an AMI rat model; compared with the control group, overexpression of miR-214 inhibits PTEN expression while knockdown of miR-214 upregulates PTEN expression. Upon pharmacological inhibition of PTEN activity, there was no difference in the cellular apoptosis in either the infarcted area or border area compared with the Ad-GFP control group. This result suggests that miR-214 protects the heart by repressing cardiomyocyte apoptosis through inhibition of PTEN. Our previous study has shown that PTEN is regulated by miR-214 and serves as an important target of miR-214 in cardiac myocytes. A similar study showed that miR-214 protects the heart against I/R injury by blunting Ca\(^{2+}\) overload and cell death in response to injury through its repression of NCK1, CaMKII\(\delta\), CypD, and BIM.

Based on early intervention-transfer of Ad-miR-214 before AMI, all analyses showed that overexpression of miR-214 following AMI decreased the size of the infarcted area, improved heart function and hemodynamic status, and inhibited left ventricular remodeling. The miR-214-mediated protective mechanism is related to a repression of cardiomyocyte apoptosis through PTEN inhibition. The cardio-protection rendered by miR-214 potentially represents a novel therapeutic approach for the treatment of ischemic heart disease.

**Disclosure**

Conflict of interest: The authors have declared that no competing interests exist.

**References**


**Supplemental Files**

Supplemental Figure 1, 2, 3, 4, 5, 6, 7, 8, 9
Please find supplemental files; https://www.jstage.jst.co.jp/article/ihj/57/2/57_15-293/_article/supplement