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Anti-Apoptotic Effect of MicroRNA-30b in Early Phase of Rat Myocardial Ischemia-Reperfusion Injury Model

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ABSTRACT

This study aimed to investigate the effect of microRNA-30b (miR-30b) in rat myocardial ischemic-reperfusion (I/R) injury model. We randomly divided Sprague–Dawley (SD) rats (n = 80) into five groups: 1) control group; 2) miR-30b group; 3) sham-operated group; 4) I/R group, and 5) I/R+miR-30b group. Real-time quantitative polymerase chain reaction, immunohistochemical staining and Western blot analysis were conducted. TUNEL assay was employed for testing cardiomyocyte apoptosis. Our results showed that miR-30b levels were down-regulated in I/R group and I/R + miR-30b group compared with sham-operated group (both $P < 0.05$). However, miR-30b level in I/R + miR-30b group was higher than I/R group ($P < 0.05$). Markedly, the apoptotic rate in I/R group showed highest in I/R group ($P < 0.05$). Additionally, the results illustrated that protein levels of Bcl-2, Bax, and caspase-3 were at higher levels in ischemic regions in I/R group, comparing to sham-operated group (all $P < 0.05$), while Bcl-2/Bax was reduced ($P < 0.05$). Bcl-2 level and Bcl-2/Bax were obviously increased in I/R + miR-30b group by comparison with I/R group, and expression levels of Bax and caspase-3 were down-regulated (all $P < 0.05$). We also found that in I/R + miR-30b group, KRAS level was apparently lower and p-AKT level was higher by comparing with I/R group (both $P < 0.05$). Our study indicated that miR-30b overexpression had anti-apoptotic effect on early phase of rat myocardial ischemia injury model through targeting *KRAS* and activating the Ras/Akt pathway. J. Cell. Biochem. 116: 2610–2619, 2015.

KEY WORDS: MICRORNA-30B; ANTI-APOPTOSIS; ISCHEMIC-REPERFUSION INJURY; KRAS; RAS/AKT PATHWAY; BCL-2; BAX; CASPASE-3

Coronary heart disease is a common cause of death and disability, accounting for 12.8% of deaths around the world, and is associated with myocardial ischemia-reperfusion (I/R) injury [Hausenloy and Yellon, 2013]. Therapeutic interventions for myocardial infarction, such as percutaneous coronary intervention, cardiac surgery, and thrombolytic therapy in fact resulting in the ischemic heart gaining blood perfusion and oxygen supply within a short time, leading to I/R injury that includes persistent ventricular systolic disorder, arrhythmia, and augmentation of the infarct size [Frohlich et al., 2013]. Endothelial injury and dysfunction also has a negative effect on I/R injury by impeding nitric oxide production that regulates platelet aggregation, radical scavenging, oxygen consumption, and leukocyte adhesion [Zweier and Talukder, 2006; Haliga et al., 2013]. Myocardial I/R rapidly increases mitochondrial membrane permeability, resulting in damage to mitochondrial membrane potential and release of pro-apoptotic factors [Ioroi et al., 2014]. Cardiomyocyte apoptosis is a key factor in the progression of I/R injury; therefore, current

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drug development focuses on inhibiting myocyte apoptosis and alleviating cardiac injury as a critical step in improving I/R injury [Liu et al., 2014a; Song et al., 2014].

MicroRNA (miRNAs) are small non-coding RNA molecules that are 18–25 nucleotides in length, found in introns or intergenic regions of plants, animals, and some viruses, and function in RNA silencing and post-transcriptional regulation of gene expression [Di et al., 2014]. It is estimated that more than 1000 miRNA genes exist in the human genome, and >500 miRNAs have been sequenced and cloned, and they collectively regulate the functions of hundreds of target genes [Berezikov et al., 2005]. The miRNAs have gained significant attention in recent years, and in particular relevance to cardiovascular system, miRNA pathways appear to have remarkable roles in cardiovascular physiology and disease progression [Horie et al., 2010; Ono et al., 2011]. MicroRNA-30b (miR-30b) is a member of the miR-30 family with key roles in regulating tumorigenesis by targeting V-Ki-ras2 Kirsten rat sarcoma viral oncogene (KRAS) [Tanic et al., 2012; Zhong et al., 2013]. Phosphatidylinositol 3-kinase (PI3K) via interaction with its downstream target, phosphorylated protein kinase B (p-AKT), activates signaling pathway to prevent cardiomyocyte apoptosis and protects the myocardium from I/R injury [Zhang et al., 2014b]. Previous studies identified that miRNAs negatively regulate PI3K/Akt pathway, and when inhibited, promote cell survival and confer protection against I/R injury [Keyes et al., 2010; Liao et al., 2014]. The mechanisms involved in miRNA-mediated protection against I/R injury appear to be related to the decreased apoptosis of myocardial cells [Qin et al., 2012]. Interestingly, miR-30b-mediated protection against I/R induced apoptosis implicates in targeting KRAS gene and augmenting Ras–PI3K–Akt activation, thus, over-expression of miR-30b can inhibit I/R induced cardiomyocyte apoptosis [Leite-Moreira et al., 2013; Liao et al., 2014; Li et al., 2015]. Our study investigated if the anti-apoptotic effects of miR-30b extended to the cardiovascular system and more importantly, if these functions had relevance to I/R injury, and therefore, we tested this hypothesis using the early phase of rat I/R injury model.

MATERIALS AND METHODS

ETHICS STATEMENT

The study was approved by the Institutional Ethics Committee of the Second Hospital of Jilin University. All procedures were in accordance with Regulations on Animal Management Committee of the Second Hospital of Jilin University. Every effort was made to minimize the suffering of rats.

ANIMALS

Male Sprague–Dawley (SD) rats (n = 80, Grade SPF, No. 2009-0004), weighing 220–250 g, were obtained from the Experimental Animal Center of the Second Hospital of Jilin University. Animals were maintained at room temperature with free access to water and food. All the experimental procedures were performed with the approval of the Experimental Research Institute of the Second Hospital of Jilin University.

MATERIALS

The materials used in the study were: plVTHM/miR-30b (Baozhu Biotechnology Co., Ltd, Shenzhen); Trizol reagent (Invitrogen, UK); TaqMan® MicroRNA Assay (Applied Biosystems); protein lysate (Beijing CellChip Biotechnology Co., Ltd, China); Bradford protein assay kit (Beyotime Institute of Biotechnology, China); miR-30b primer, U6, KRAS primer, β-actin (Guangzhou Funeng Genes Co., Ltd); anti-KRAS (catalog number, 3965), anti-p-AKT (catalog number, 8275), and anti-total-AKT (catalog number, 9916) antibodies (Cell Signaling Technology, Inc., China); antibodies against caspase-3 (catalog number, ab2302), Bcl-2 (catalog number, ab7973), Bax (catalog number, ab7977) (Abcam Inc., Cambridge, MA); horseradish-peroxidase (HRP)-labeled goat anti-rabbit secondary antibody (catalog number, PI1000) and horse anti-mouse secondary antibody (catalog number, PI2000) (Vector Laboratories, Burlingame).

ANIMAL TREATMENTS

SD rats (n = 80) were randomly divided into five groups: 1) control group, rats transfected with plVTHM as a blank control using coronary artery injection; 2) miR-30b group, rats transfected with plVTHM/miR-30b; 3) sham-operated group, rats undergoing thoracotomy without ligation of the coronary artery; 4) I/R group, rats transfected with plVTHM as a blank control and conducted with ischemia-reperfusion after 14 days and 5) I/R+miR-30b group, rats transfected with plVTHM/miR-30b and conducted with ischemia-reperfusion after 14 days.

MIRNA TRANSFECTION

Rats anesthetized with intraperitoneal injection of 10% chloral hydrate (350 mg/kg) were connected by the intubation with small animal ventilator, whose respiratory frequency was 70 times/min with 6.0 ml/kg tidal volume and respiratory ratio of 1: l. Subsequently, the heart was exposed after cutting skin of neck, the left side of the chest skin through the third and fourth ribs. By lentiviral vector-based transfection, the cardiomyocytes of all SD rats were transfected with 10 μl plVTHM/miR-30b and plVTHM using coronary artery injection, as shown in Figure 1. After 3 days, these cardiomyocytes were given daily intraperitoneal injection of 100 thousand units of penicillin to prevent infection.

ESTABLISHMENT OF THE RAT I/R INJURY MODEL

In brief, the heart was exposed through a left thoracotomy under sterile conditions. The left anterior descending coronary artery (LAD) was ligated with a suture beneath 1–2 mm of the junction of pulmonary conus and aorta. I/R injury was induced by LAD coronary artery occlusion for 45 min followed by 30 min reperfusion of LAD. After completion of reperfusion period, cardiac impulse of rats was recorded, and the skin was sutured layer by layer. In the sham-operated group, LAD coronary artery was sutured passing through the vessels, but without LAD coronary artery occlusion. Every rat was treated once daily with intraperitoneal injection of 100 thousand units of penicillin G for preventing from infection for a successive 3 days.

Fig. 1. The sequence and length of miR-30b in the recombinant plasmid pIVTHM/miR-30b.

INDWELLING SPECIMEN

Rats were injected with 10% chloral hydrate (350 mg/kg) with ligation on LAD coronary artery, and then were injected 4 ml 1% Evans blue dye into left ventricular. The hearts were washed using ice-cold physiological saline, and the ischemic area was identified as Evans blue unstained region. The ventricles of hearts were transversely cut into two parts. The tip of the heart was incubated by 4% paraformldehyde for 8 h, then preserving with sodium azide for morphologic studies. Tissues from ischemic area of the heart (bottom) were preserved in freezing tube by liquid nitrogen and then transferred into refrigerator under 80°C. At 14 days after transfection, rats in control group and miR-30b were sacrificed at once, and rats in sham-operated group, I/R group and I/R + miR-30b group were also sacrificed after 2 h reperfusion.

RNA EXTRACTION AND REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

The expression levels of miR-30b and KRAS were determined by reverse transcription (RT) of total RNA from ischemic areas followed by Real-time quantitative Polymerase Chain Reaction (PCR) procedure. Total RNA was isolated using the TRIzol reagent and the purity and concentration of RNA was tested by UV spectrophotometer and was observed by agarose gel electrophoresis. Using Taqman® microRNA RT kit (Applied Biosystems company), RT was performed on DEPC-treated 7 μl reaction volume of 10 ng total RNA, 1.5 μl 10 × RT buffer, 0.15 μl d NTP (100 nmol/L), 1 μl Multiscribe™ (50 U/μl), and 0.19 μl RNasin (20 U/μl), and 3 μl primer under following conditions: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min. The Real-time PCR system was performed in 10 μl Taqman® universal PCR master mix containing 1 μl 20 × Taqman® probe, 1.6 μl RT products and 7.4 μl DEPC water. The sequences of miR-30b and KRAS primers for amplification were showed in Table I. PCR programs were carried out as follows: 40 cycles for 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min. Specificity of PCR assay was confirmed by melting curve of PCR products and electrophoresis on 3.5% NuSieve. The experimental condition was made three times. PCR analysis was implemented by using Opticon Monitor 3.0 software (Bio-Rad, Hercules, CA). Threshold value was set at the rock bottom of each amplification curve with the parallel rising trend and then threshold cycle (Ct) was calculated. The $2^{-\Delta\Delta Ct}$ method was applied to analyze data.

IMMUNOHISTOCHEMICAL STAINING

Tissues from ischemic areas of the heart were sliced in each group, and were deparaffinized in citrate the antigen retrieval buffer (pH 6.0). Sections were kept in 3% hydrogen peroxide solution at 37°C for 10 min, followed by incubated in 3% sheep serum for 1 h and wash three times with phosphate buffer solution (PBS) for 5 min. Anti-Bcl-2, anti-Bax, and anti- caspases-3 rabbit polyclonal antibodies were diluted 1:200, 1:200, and 1:100, respectively, which were incubated with sections at 4°C overnight and washed by PBS three times (5 min). Sections were exposed to HRP-labeled goat anti-rabbit secondary antibody (1:400) solution at 37°C for 2 h. After three time washes by PBS, color was assessed using diaminobenzidine (Beijing Zhongshan Golden Bridge Biological Engineering Co., Ltd) for 5–10 min. The staining was brown and observed by an invert florescence microscope (Nikon), equipped with a digital camera. The areas of Bcl-2-, Bax-, and caspase-3-positive cells were counted with image analysis software (NIS-Elements BR 3.0).

WESTERN BLOT ANALYSIS

Tissues (0.25 g) from ischemic areas were prepared in Eppendorf tube (EP tube) adding protein lysate, purchased from Beijing Cellchip Biotechnology Co., Ltd. The products were centrifuged, and when separating the supernatant, the precipitate was saved. Protein concentrations were checked by Bio-Rad assay kit. Equal amounts of protein were separated by SDS–PAGE and then blotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked by nonfat dried milk, and subsequently incubated with primary antibodies for 1 h at room temperature (KRAS, 1:1000;

TABLE I. The Sequences of Forward Primers and Reverse Primers of MicroRNA-30b, U6, V-Ki-ras2 Kirsten Rat Sarcoma Viral Oncogene, and β-actin

Gene	Primer Sequence
miR-30b	F: 5′-CGCGCTGTAAACATCCTACAC-′3 R: 5′-GTGCAGGGTCCGAGGT-′3
U6	F: 5′-GCTTCGGCAGCACATATACTAAAAT-′3 R: 5′–CGCTTCACGAATTTGCGTGTCAT-′3
KRAS	F: 5′-CCCTCGAGGGTGAAAATACTTTTGCATT-′3 R: 5′-GCTCTAGAGCTTTTAGATCACTTCACAGCA-′3
β-actin	F: 5′-AGGCCCCTCTGAACCCTAAG-′3 R: 5′-CCAGAGGCATACAGGGACAAC-′3

KRAS, V-Ki-ras2 Kirsten rat sarcoma viral oncogene.

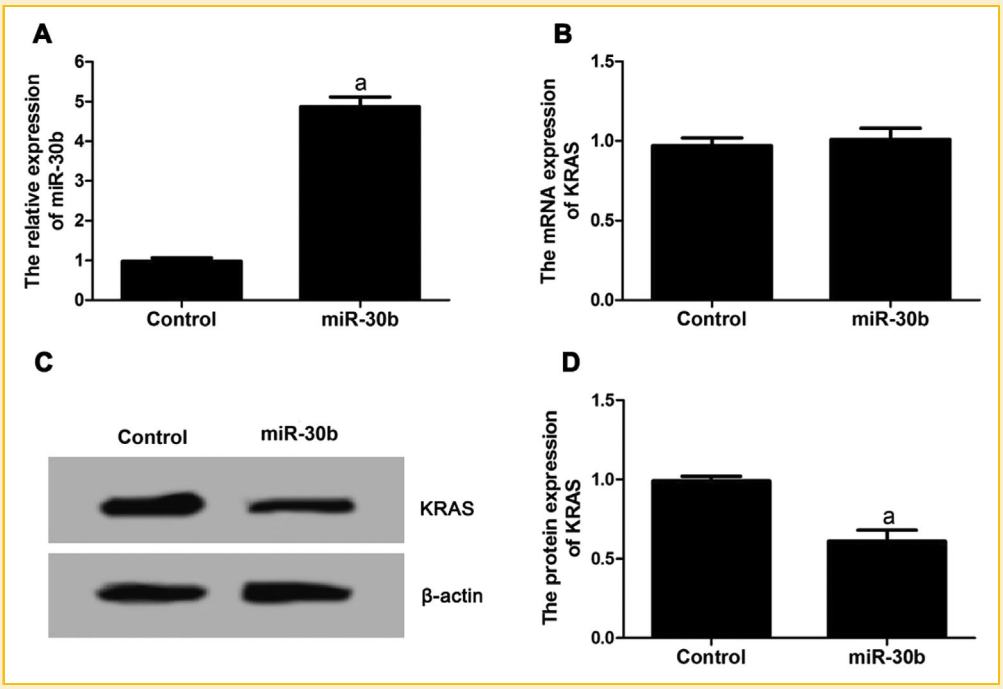

Fig. 2. Levels of miR-30b and KRAS of rats after transfection measured by Real-time quantitative Polymerase Chain Reaction and Western blot analysis. A and B: Real-time quantitative Polymerase Chain Reaction testing mRNA level of miR-30b and KRAS after transfection; C and D: Western blot analysis testing protein level of KRAS after transfection, a: compared with control group, $^{a}P < 0.001$.

Bcl-2, 1:1000; Bax, 1:2000 and caspase-3, 1:5000). After three washes, the blots were incubated with secondary antibodies (1:200) at 37°C for 1 h. Statistical analysis of scanned images was assessed quantitatively by software IPP6.0 (Media Cybernetics, Bethesda) through determining the Optical Density (OD) of positively stained substances. The experiment was repeated in triplicate, with results expressed mean ± standard deviation (SD).

STATISTICAL ANALYSIS

The SPSS 19.0 software was used for statistical analysis. The measurement data were presented as mean ± SD. LSD- *t* test was applied to compare differences. The comparison between groups was performed by applying One-way ANOVA. Differences were considered significant at $P < 0.05$.

RESULTS

MORTALITY

Two rats were died in control group owning to intraoperative cardiac arrest. In I/R group, five rats died owning to intraoperative ventricular fibrillation (n = 2), cardiac arrest (n = 2) and post-operative acute left heart failure (n = 1), and only one rat died in I/R + miR-30b group owning to intraoperative ventricular fibrillation.

CONSTRUCTION OF MIR-30B LENTIVIRAL VECTOR

The precursor and flanking sequences of miR-30b were amplified and cloned into the blunt-EcoRI site of the lentiviral vector pLVTHM. After electrophoresis in 3% agarose gel, that PCR product was about 510 bp (Fig. 1A), which was consistent with the expected product. The result indicated that the recombinant plasmid was successfully constructed (Fig. 1B).

EXPRESSION LEVELS OF MIR-30B AND KRAS AFTER TRANSFECTION

At 14 days after transfection, expression level of miR-30b was up-regulated in miR-30b group than that in control group (4.87 ± 0.25 vs. 0.98 ± 0.08, t = 58.89, $P < 0.001$), which was seen in Figure 2A, while there was no difference in KRAS mRNA level between the two groups (1.01 ± 0.07 vs. 0.97 ± 0.05, t = 1.776, $P = 0.087$) (Fig. 2B). Notably, miR-30b level was high compared to

TABLE II. Real-Time Quantitative Polymerase Chain Reaction Testing Expression Levels of MiR-30b and KRAS in Rat Myocardial Ischemic Areas After Transfection

Groups	Cases (n)	MiR-30b expression	KRAS mRNA expression
Sham-operated group	16	0.97 ± 0.05	0.97 ± 0.08
I/R group	11	0.57 ± 0.03[a]	1.51 ± 0.11[a]
I/R + miR-30b group	15	0.73 ± 0.07[a,b]	1.48 ± 0.09[a]
F		186.400	160
P		<0.001	<0.001

I/R, ischemic/reperfusion; a: compared with sham-operated group; b: compared with I/R group.
[a] $P < 0.05$.
[b] $P < 0.05$.

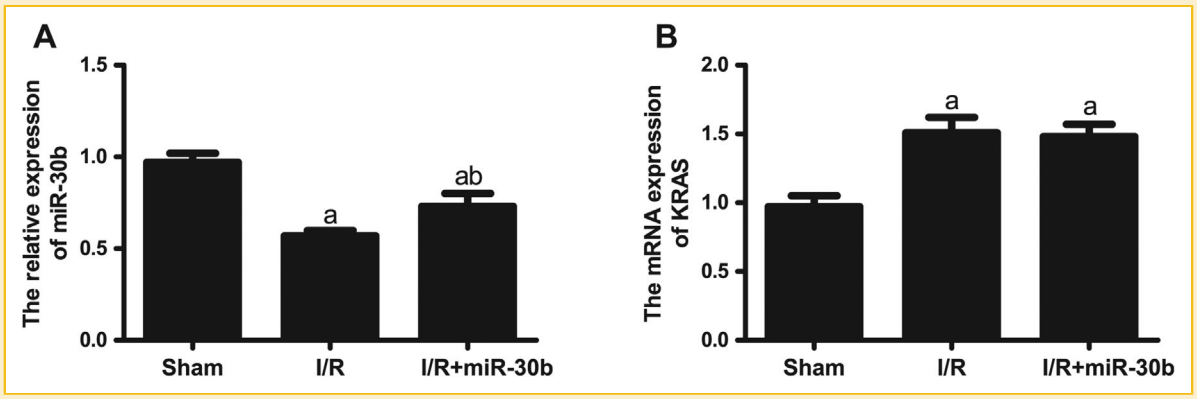

Fig. 3. Real-time quantitative Polymerase Chain Reaction testing expression level of miR-30b of rat myocardial ischemic areas in sham-operated group, I/R group and I/R + miR-30b group. I/R, ischemic/reperfusion; a: compared with sham-operated group, $^{a}P < 0.05$; b: compared with I/R group, $^{b}P < 0.05$.

control, and concomitantly, KRAS protein levels were significantly reduced (0.61 ± 0.07 vs. 0.99 ± 0.03, $t = 19.74$, $P < 0.001$) (Fig. 2C–D). The result demonstrated that miR-30b expression was negatively associated with KRAS protein level.

EXPRESSION LEVELS OF MIR-30B AND KRAS IN MYOCARDIAL ISCHEMIC REGIONS AFTER TRANSFECTION

The miR-30b and KRAS levels in myocardial ischemic regions were shown in Table II and Figure 3. When compared with sham-operated group, miR-30b expression levels were down-regulated in I/R group and I/R + miR-30b group (both $P < 0.05$). However, expression level of miR-30b in I/R + miR-30b group was higher than I/R group ($P < 0.05$). The KRAS mRNA levels were increased in I/R group and I/R + miR-30b group, which had no statistical difference (both $P > 0.05$).

TUNEL ASSAY TESTING I/R-INDUCED CARDIOMYOCYTE APOPTOSIS

The rate of cardiomyocyte apoptosis was significantly different in sham-operated group, I/R group and I/R + miR-30b group ($F = 731.6$, $P < 0.001$). Markedly, the apoptotic rate in I/R group and I/R + miR-30b group was higher than sham-operated group (both $P < 0.05$), and it showed highest rate in I/R group ($P < 0.05$) (Table III, Fig. 4). The result suggested that miR-30b could inhibit cardiomyocyte apoptosis.

TABLE III. TUNEL Assay Testing I/R-Induced Cardiomyocyte Apoptosis after 14 d of Transfection

Groups	Cases (n)	Number of cell apoptosis	Total cells	Apoptotic rate
Sham-operated group	16	2.00 ± 0.33	99.27 ± 12.57	1.46 ± 0.17
I/R group	11	28.34 ± 1.86	117.30 ± 10.24	16.45 ± 1.66^{a}
I/R + miR-30b group	15	16.77 ± 2.21	119.55 ± 10.25	$10.05 \pm 0.95^{a,b}$

I/R, ischemic/reperfusion; a: compared with sham-operated group; b: compared with I/R group.
$^{a}P < 0.05$.
$^{b}P < 0.05$.

EXPRESSION LEVELS OF IN BCL-2, BAX AND CASPASE-3 IN MYOCARDIAL ISCHEMIC REGIONS

As seen in Figure 5, the myocardium in I/R group showed striking structural changes and more inflammatory cell infiltration when compared to sham-operated group. The structure in I/R + miR-30b group had less changes and inflammatory cells. In I/R group, Bcl-2, Bax, and caspase-3 expression levels were significantly increased (all $P < 0.05$). Compared with I/R group, anti-apoptotic protein Bcl-2 was also elevated in I/R + miR-30b group, but pro-apoptotic proteins (Bax and caspase-3) were lower than I/R group (both $P < 0.05$) (Table IV). Western blot analysis showed that protein levels of Bcl-2, Bax, and caspase-3 were statistically different in sham-operated group, I/R group and I/R + miR-30b group (Bcl-2: $F = 302.7$, $P < 0.001$; Bax: $F = 1576$, $P < 0.001$; Caspase-3: $F = 444.7$, $P < 0.001$; Bcl-2/Bax: $F = 294.8$, $P < 0.001$). The protein levels of Bcl-2, Bax, and caspase-3 were at higher levels in ischemic regions in I/R group, comparing to sham-operated group (all $P < 0.05$), while Bcl-2/Bax was reduced ($P < 0.05$). As shown in Figure 6, Bcl-2 expression level and Bcl-2/Bax were obviously increased in I/R + miR-30b group by comparison with I/R group, and expression levels of Bax and caspase-3 were down-regulated (all $P < 0.05$). The results showed that overexpressed miR-30b could attenuate cardiomyocyte apoptosis by controlling Bcl-2/Bax values.

ASSESSMENT OF RAS/AKT SIGNALING PATHWAY IN I/R RAT MODEL AFTER TRANSFECTION

KRAS protein level in ischemic regions was higher in I/R group compared with sham-operated group, which showed statistical significance ($P < 0.05$). In I/R + miR-30b group, KRAS expression level was also significantly increased ($P < 0.05$). The result illustrated that miR-30b could suppress KRAS protein level in myocardial ischemic areas (Fig. 7). By comparing with sham-operated group, p-AKT level in ischemic regions was sharply decreased ($P < 0.05$), but activation level of p-AKT in I/R + miR-30b

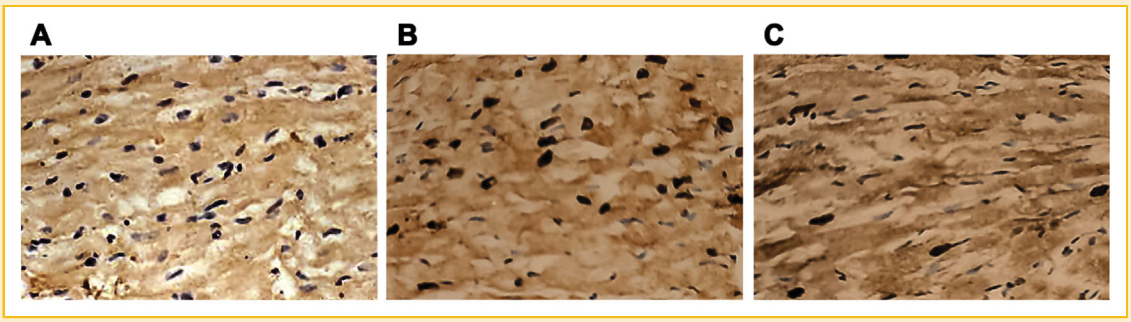

Fig. 4. TUNEL assay testing I/R-induced cardiomyocyte apoptosis after 14 d of transfection. A: sham-operated group; B: I/R group; C: I/R + miR-30b group; →, apoptotic cardiomyocytes.

group was up-regulated relative to I/R group ($P < 0.05$). The total AKT level was not statistically different in sham-operated group, I/R group and I/R + miR-30b group (all $P > 0.05$). The results indicated that overexpressed miR-30b could attenuate cardiomyocyte apoptosis by targeting KRAS and activating p-AKT.

DISCUSSION

Our results indicate that overexpression of miR-30b decreased V-Ki-ras2 Kirsten rat sarcoma viral oncogene (KRAS) protein level, suggesting that KRAS may be the target gene of miR30-b. The miR-30 family comprises miR-30a, -30b, -30c, -30d, -30e, and -384-5p, functioning as critical tumor suppressors [Liu et al., 2014b]. Emerging studies have reveal that miR-30b has an effect on tumor development involving in cell proliferation and apoptosis, and miR-30b is reduced in human colorectal carcinoma (CRC) [Calin and Croce, 2006; Slattery et al., 2011]. KRAS is recognized as a direct and functional target of miR-30b by modulating the 3′ untranslated region (3′ UTR) of mRNAs and repressing its translation [Bodemann and White, 2008]. Therefore, miR-30b may influence the protein level of KRAS. The Ras/ERK and Ras/Akt pathways are major

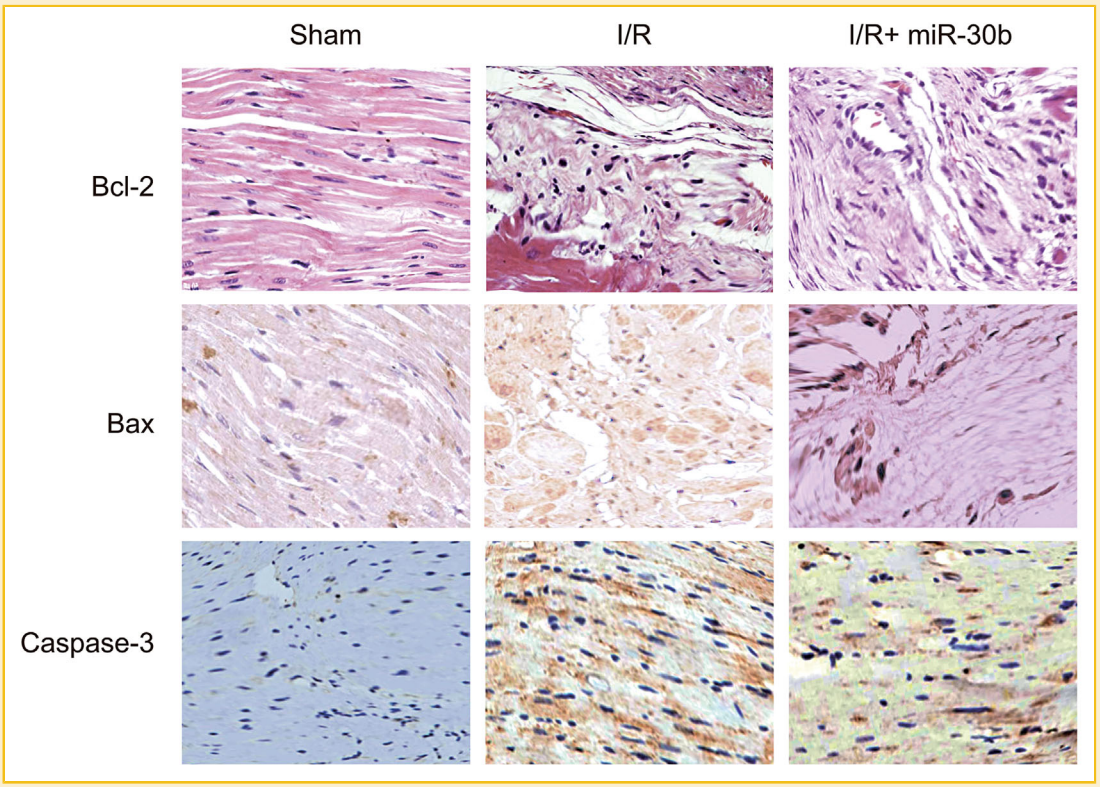

Fig. 5. Immunohistochemical staining testing expression levels of Bcl-2, Bax, and caspase-3 of rat myocardial ischemic areas after 14 d of transfection.

TABLE IV. Immunohistochemical Staining Testing Areas of Bcl-2-, Bax-, and Caspase-3-Positive Cells of Rat Myocardial Ischemic Regions in Sham-Operated Group, I/R Group and I/R + miR-30b Group

Groups	Cases (n)	Bcl-2	Bax	Caspase-3
Sham-operated group	16	9.87 ± 1.01	8.55 ± 1.27	6.04 ± 1.02
I/R group	11	13.89 ± 1.98^{a}	12.86 ± 2.44^{a}	16.58 ± 2.13^{a}
I/R + miR-30b group	15	$17.76 \pm 2.77^{a,b}$	$10.54 \pm 1.53^{a,b}$	$10.84 \pm 1.75^{a,b}$
F		58.08	30.52	136.4
P		<0.001	<0.001	<0.001

I/R, ischemic/reperfusion; a: compared with sham-operated group; b: compared with I/R group.
[a] $P < 0.05$.
[b] $P < 0.05$.

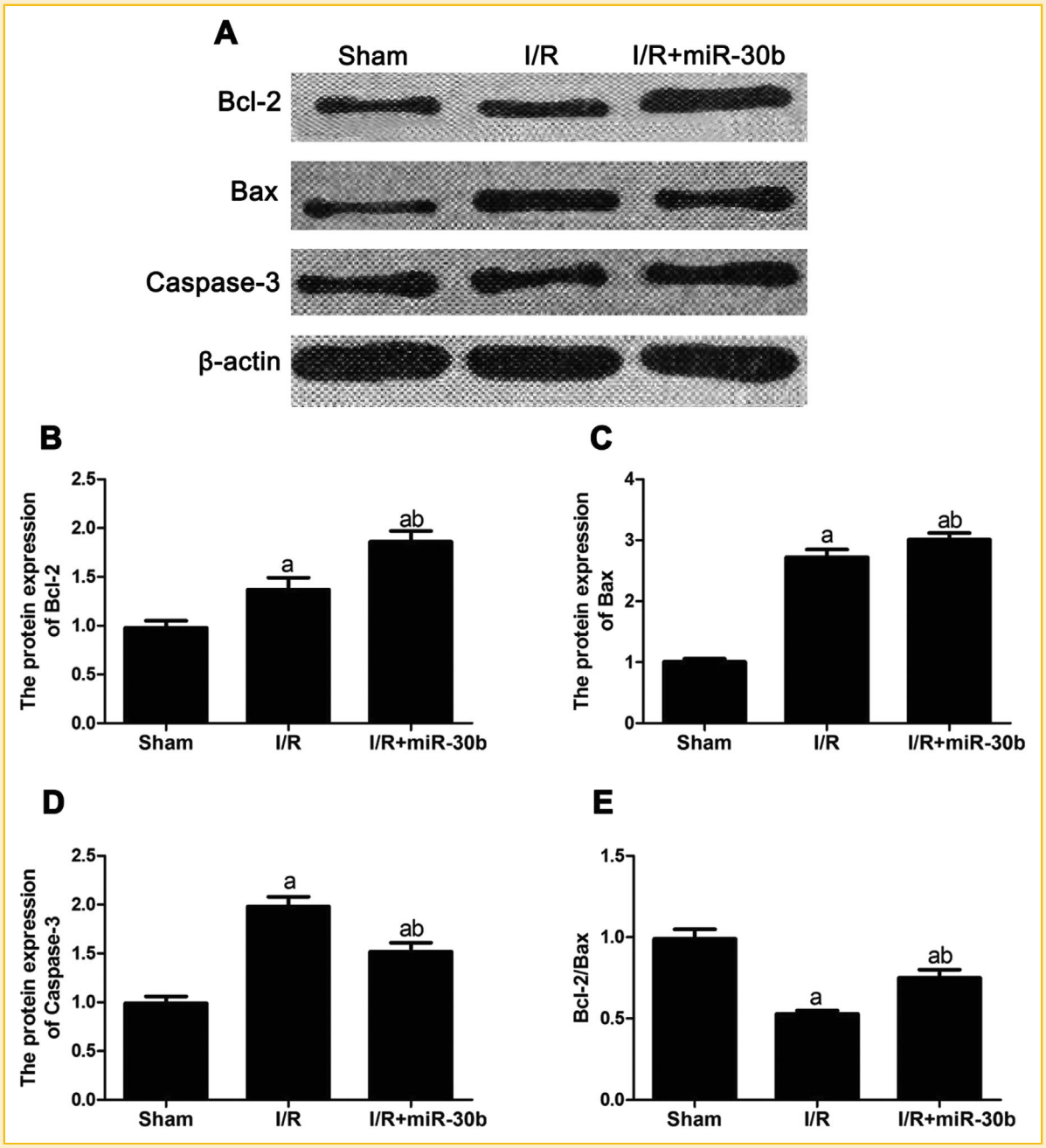

Fig. 6. Western blot analysis testing protein levels of Bcl-2, Bax, and caspase-3 of rat myocardial ischemic areas in sham-operated group, I/R group, and I/R + miR-30b group. a: compared with sham-operated group, [a] $P < 0.05$; b: compared with I/R group, [b] $P < 0.05$.

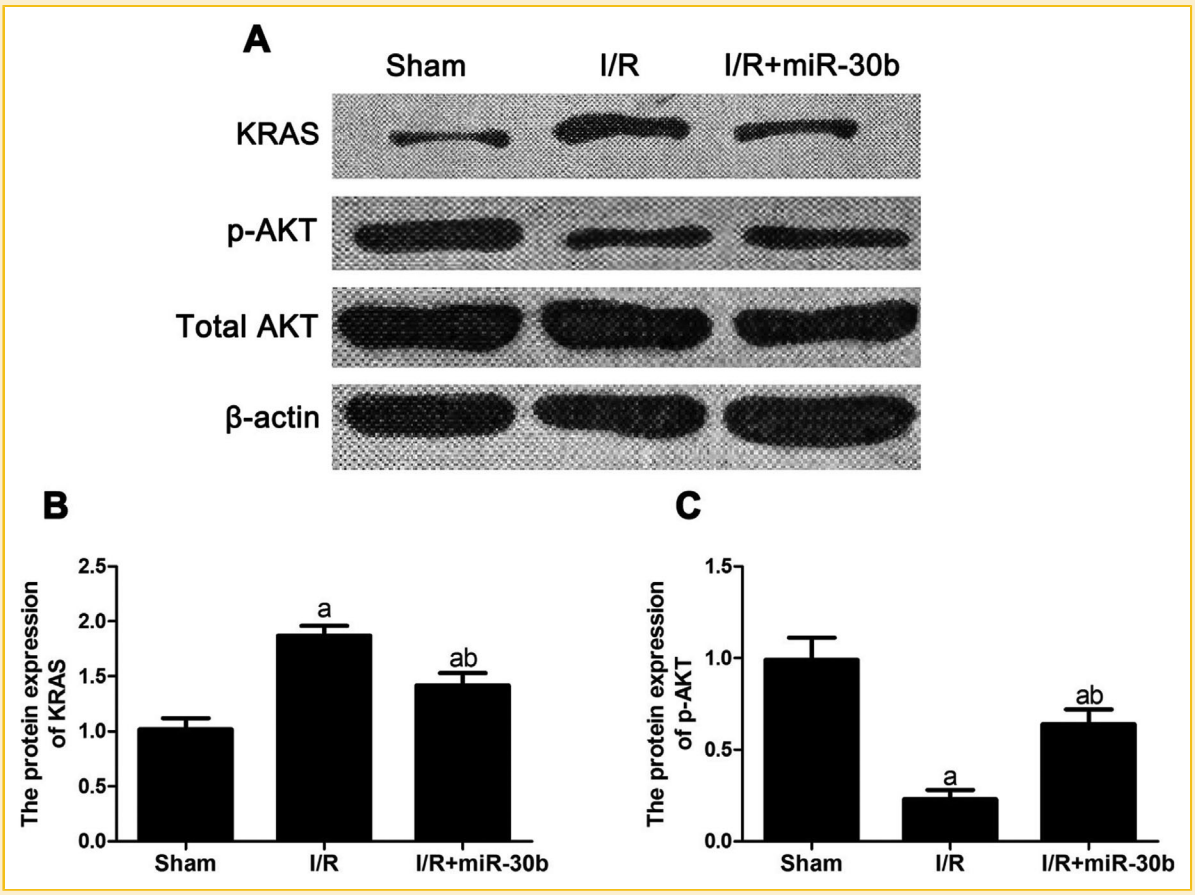

Fig. 7. Western blot analysis testing expression levels of KRAS and p-AKT of rat myocardial. a: compared with sham-operated group, $^{a}P < 0.05$; b: compared with I/R group, $^{b}P < 0.05$.

downstream pathways of activated KRAS, and the effects of miR-30b on activating Ras/Akt pathway in controlling of proliferation and apoptosis are achieved through targeting cell cycle regulators and apoptosis effectors including Bcl-2, Caspases, Bad. and Bim [Steelman et al., 2011a; Liao et al., 2014].

The Ras/PI3K/Akt pathway is highly conserved and Ras activates the PI3K/Akt pathway and promotes multistep and sequential interactions among several proteins [Hemmings and Restuccia, 2012]. Phosphorylation of Akt (p-AKT) is additionally activated by mammalian target of rapamycin (mTOR) [Sarbassov et al., 2005]. Full activation of Akt plays a remarkable role in regulating additional substrate-specific phosphorylation events in cytoplasm and nucleus, leading to inhibition of phosphorylation of pro-apoptotic proteins and activation of other mediators involved in diverse cellular activities such as metabolism, angiogenesis, survival, growth, proliferation, transcription, and protein synthesis [Guertin et al., 2006; Bozulic and Hemmings, 2009]. Ras activates multiple effector pathways, especially PI3K/Akt signaling pathway, which alters the activation, proteins stability, and cellular localization of downstream molecules such as Bcl-2, Bax, and Caspases [Steelman et al., 2011b; Yadav and Denning, 2011]. In the present study, we demonstrated that overexpressed miR-30b could attenuate cardiomyocyte apoptosis by controlling Bcl-2/Bax values. The activation/inhibition of Bcl-2, Bax, and caspases-3 determines pro-apoptotic or anti-apoptotic responses, suggesting the importance of Ras/PI3K/Akt signaling pathway in regulation of cell apoptosis [van der Weyden and Adams, 2007; Zhang et al., 2011].

Overexpression of miR-30b may inhibit cardiomyocyte apoptosis and attenuate I/R injury via targeting KRAS and activating Ras/Akt signaling pathway to modulate Bcl-2, Bax, and Caspases levels and exert its anti-apoptotic effect. Previous studies have shown that miR-30b expression during disease processes may have key influence on regulating cell cycle, including cell proliferation and apoptosis, through targeting multiple pathways [Balderman et al., 2012; Zhang et al., 2014a]. For example, miR-30b overexpression in CRC patients significantly suppressed phosphoinositide 3-kinase catalytic subunit delta (PIK3CD) expression, a vital component of PI3K/Akt signaling related with tumorigenesis involved in cell activities, suggesting that under certain pathological states, miR-30b could regulate cell migration and invasion via the PI3K/Akt signaling pathway [Zhang et al., 2014a]. The Bcl-2 protein is a principal member of anti-apoptotic regulators that plays a major role in inhibition of cell apoptosis through maintaining mitochondrial membrane integrity and regulating apoptotic signaling [Parsons and

Green, 2010]. The Akt activation mediated by miR-30b is significant for anti-inflammatory and anti-apoptotic response via suppression of Bax and caspase-3 expression and up-regulation of Bcl-2 expression [Bharti et al., 2012; Ouyang and Giffard, 2014]. The Ras/Akt is the key pathway of protecting myocardial cells from apoptosis induced by ischemia through targeting downstream molecules like Bcl-2, Bax, and caspase-3 [Chen et al., 2011]. Therefore, our study illustrated that miR-30b exhibited strong anti-apoptotic effect in early phase of rat myocardial ischemia injury model through targeting the initial steps of the Ras/Akt pathway.

In summary, our study provided the first evidence of anti-apoptotic effect of miR-30b in early phase of rat myocardial I/R injury model via the Ras/Akt pathway. We demonstrated KRAS as a target involved in miR-30b mediated anti-apoptotic effect, and miR-30b overexpression may inhibit cardiomyocyte apoptosis by inhibiting KRAS expression and enhancing p-AKT activation. Importantly, the findings may further help the treatment of myocardial I/R injury by targeting miR-30b level to prevent cardiomyocyte apoptosis.

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