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MiR-155 and miR-148a reduce cardiac injury by inhibiting NF-κB pathway during acute viral myocarditis

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Abstract. – OBJECTIVES: Acute viral myocarditis (VM) is an important cause of sudden cardiac death and heart failure in healthy young person. Its pathogenesis is based on an adverse immune response evoked by infection of the cardiac muscle by cardiotropic viruses especially Coxackievirus B3 (CVB3). MicroRNAs (miRNAs) are short, noncoding RNA sequences that regulate gene expression at the posttranscriptional level. Recently, there are reports that disturbed miRNAs expression is associated with VM but the mechanism is not well understood.

MATERIALS AND METHODS: Herein, we screened 15 selected miRNAs in myocardial tissues of patients with acute CVB3 caused myocarditis and found the expression of miR-155 and miR-148a was up-regulated significantly.

RESULTS: Predicted by using bioinformatics tools and confirmed by dual-luciferase assay and western blot, we confirmed that RelA is a direct target gene of miR-155 and miR-148a. Subsequent in vitro functional study indicated that miR-155 function as immune response negative feedback factor that reduced cardiac myoblast cytokines expression during CVB3 infection. Further *in vivo* experiments indicated that miR-155 can improved mice survival rate when CVB3 infected.

CONCLUSIONS: So, our study indicated that miR-155 is a potential therapeutic target for viral myocarditis.

Key Words:
MicroRNA, NF-κB, Viral myocarditis, Coxackievirus, RelA.

Introduction

Myocarditis is defined as an inflammatory process of the myocardium which result in injury to the cardiac muscle cells and the manifestations range from subclinical to sudden death. Myocarditis may be induced by infectious, toxic or immunologic agents. Among the different infectious pathogens, viruses are the most common causes of myocarditis and serological studies, nucleic-acid hybridization and PCR-based studies of endomyocardial biopsy and autopsy specimens have shown that enteroviruses represent one of the most common groups of viruses detected in the myocardium[1]. Coxsackie virus B3 (CVB3) is believed to be the most common causative agent in human myocarditis. Viral myocarditis (VM) affects 5-20% of the human population which can be fatal in infants as well as children[2,3]. The pathogenesis of VM is based on an adverse immune response evoked by infection of the cardiac muscle by cardiotropic viruses, which leads to viral elimination as well as cardiac myocyte destruction, reparative fibrosis, and heart failure. The lack of effective therapies to treat myocarditis mandates a better understanding of the basic molecular mechanisms that govern the adequate and autodestructive inflammatory signaling pathways within the immune system[4].

MicroRNAs are short, noncoding RNA that repress gene expression at the posttranscriptional level by targeting the 3 -untranslated region of mRNA sequences. Bioinformatical study indicated that more than 60% of human genes may be regulated by miRNAs[5]. Gene expression studies revealed that miRNAs are differentially expressed in heart disease, and loss-of-function studies in mice firmly established that miRNAs control a variety of cellular processes essential to the heart[6,7]. At present, several miRNAs have been confirmed play crucial roles during VM pathogenesis, such as miR-155, miR-21, and miR-146a[7]. There are also reports indicated that miRNA may be used as new biomarkers for VM diagnosis, like miR-208b and miR-499-5p[8].

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In this study, we first detected the expression of 15 selected miRNAs which was reported related to myocarditis in myocardial tissues of patients with acute CVB3 caused myocarditis (n=6). We found miR-155 and miR-148a had a significant up-regulated expression compared with controls. Subsequently, we confirmed RelA is a direct target gene of miR-155 and miR-148a. *In vitro* functional study indicated that miR-155 function as immune response negative feedback factor that reduced cardiac myoblast cytokines expression during CVB3 infection. Further *in vivo* experiments indicated that miR-155 can improved mice survival rate when CVB3 infected. So miR-155 may be used as a potential therapeutic target for viral myocarditis.

Materials and Methods

Patient Material

All human materials were obtained from Zaozhuang Municipal Hospital, stored in -80°C immediately after collection and available for research purposes. The process of material collection was in accordance with the Declaration of Helsinki and the Ethical Committee of Zaozhuang Municipal Hospital. Total RNAs were extracted using right ventricular septal biopsies from patients with acute myocarditis (n=6) with a definite clinical history of myocarditis and confirmed virus presence in the cardiac biopsies. Controls (n=6) consisted of age-matched patients with unexplained ventricular tachy-arrhythmias but with a normal ejection fraction and the absence of systemic or cardiac inflammation or virus presence at the time of biopsy.

Cell Culture

HEK2148aT cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (FBS) Gibco, Gran Island, NY, USA), 100 IU/ml penicillin and 10 mg/mL streptomycin. All cells were maintained at 37°C under an atmosphere of 5% CO_2.

Cardiac myocytes from neonatal mice within 72 h of birth were prepared as previously reported[9]. Briefly, the hearts were minced finely and subjected to stepwise enzymatic digestion with 0.25% trypsin. The dissociated cells were washed with complete basal Eagle's medium and depleted of endothelial cells and fibroblasts by two sequential 1 h adsorptions to plastic flasks at 37°C. The nonadherent myocytes were removed, washed once, resuspended in complete basal medium, and dispensed into tissue culture wells. After a period of 48 h, the myocytes were attached firmly to the plastic. According to observations on the shape and beating activity of the cells obtained, more than 95% cells were identified as cardiac myocytes. The cells were used as described below.

Stem-loop qRT-PCR

Quantitative RT-PCR analysis was used to determine the relative expression of 15 candidate miRNAs. Total RNA was extracted from tissues samples, using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The expression levels of miR-1, miR-133a, miR-27a, miR-30a, miR-29b, miR-155, miR-146b, miR-148a, miR-199a, miR-208b, miR-223, miR-21, miR-499, miR-375 and miR-125a were detected by TaqMan miRNA RT-Real Time PCR. Single-stranded cDNA was synthesized by using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and then amplified by using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) together with miRNA-specific TaqMan MGB probes (Applied Biosystems, Foster City, CA, USA). The U6 snRNA was used for normalization. Each sample in each group was measured in triplicate and the experiment was repeated at least three times.

Dual Luciferase Assay

Full length of RelA 3'UTR were cloned into downstream of firefly luciferase coding region in pmirGLO vector (promega, Madison, WI, USA) to generate luciferase reporter vector. For luciferase reporter assays, HEK293T cells were seeded in 24-well plates. MiRNA mimics or miRNA inhibitors or their corresponding controls and luciferase reporter vectors were co-transfected by using lipofectamine 2000 (Invitrogen, Carlsbad, CA USA). Two days after transfection, cells were harvested and assayed with the Dual-Luciferase Assay (Promega, Madison, WI, USA). Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC).

Western Blotting

Protein extracts were boiled in SDS/β-mercaptoethanol sample buffer, and 20 g samples were loaded into each lane of 10% polyacrylamide gels. The proteins were separated by elec-

trophoresis, and the proteins in the gels were blotted onto polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech, St. Albans, Herts, UK) by electrophoretic transfer. The membrane was incubated with rabbit anti-RelA polyclonal antibody (Abcam, Cambridge, MA, USA) and mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 h at 37°C. The specific protein antibody complex was detected by using horseradish peroxidase conjugated anti-rabbit or anti-mouse antibody. Detection by the chemiluminescence reaction was carried using the ECL kit (Pierce, Appleton, WI, USA). The β-actin signal was used as a loading control.

Cytokine Assays

Levels of IL-1β, IL-6 of cell culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (eBioscience Inc., San Diego, CA, USA) following the manufacturers' instructions.

Mice

All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health. Ethical approval was obtained from Animal Ethics Committee of Zaozhuang Municipal Hospital before the study. Specific pathogen free male BALB/c mice (6 weeks old) were housed under pathogen-free conditions in an animal house and fed normal mouse chow, and given tap water *ad libitum*.

Six week old male mice were injected intraperitoneally with 10^4 plaque-forming units of CVB3 (nancy strain). Mice were observed every day for the development of clinical morbidity and mortality until day 9. The miRNA mimics 2'-O-methyl oligonucleotide and miRNA control were purchased from GenePharma Co. These mice received, every two days, either miR-155 mimics or miRNA control at a dose of 80 mg/kg body weight through tail vein injection.

Results

In this study, we selected 15 miRNAs including heart-associated (miR-1, -27b, -133a, -148a, -208b, and -499), immune-associated (miR-146b, 30a, -155, 199a, 125a, -375 and -223), and fibrosis associated (miR-21 and miR-29b) candidates. We detected the expression of this 15 miRNAs expression in biopsies of 6 VM patients and found the expression of miR-155 and miR-148a was significantly up-regulated (Figure 1). Since miRNAs regulate biological process by repressing target genes expression, we predicted the target genes of miR-155 and miR-148a by using online bioinformatics tools TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/). We found RelA, an important component of NF-κB signaling is a predicted target gene of miR-155 and miR-148a.

To investigate whether the expression of RelA is repressed by miR-155 and miR-148a, we first cloned the 3'UTR of RelA into pmir-GLO vector after firefly luciferase coding region (pGLO-RelA). HEK293T cells were transfected with miR-155 or-148a mimics with scramble sequence double strand short RNA molecule as control, or miR-155 or 148a inhibitor with scramble sequence single strand short RNA molecule as control. 48h after transfection, cells were lysed and luciferase activities were detected. As shown in Figure 2A, B, the relative firefly luciferase activity was significantly reduced when transfected with miR-155 and miR-148a mimics. On the contrary, firefly luciferase activity was up-regulated significantly when co-transfected with miRNAs antagonist. The results indicated that miR-155 and miR-148a can repress firefly luciferase activity by binding RelA 3'UTR. To further identify miR-155 and miR-148a target sites in RelA 3'UTR, two mutant RelA reporter vectors with 4 nucleotides variation were constructed. As shown in Figure 2A, B (right), the firefly luciferase activity was not repressed by miR-155 and miR-148a when 4 nucleotides replaced by adenine. The results indicated that miR-155 and miR-148a repress RelA expression by targeting 3'UTR.

Although RelA is a target gene of miR-155 and miR-148a, it is also unknown whether endogenous RelA is repressed by miR-155 and miR-148a. HEK293T cells were transfected with miR-155 or miR-148a, the expression of RelA was detected 48h after transfection. As shown in Figure 2C, the amount of RelA is significantly reduced by miR-155 and miR-148a. On the contrary, the protein level was up-regulated slightly by miR-155 and miR-148a antagonist.

To further unveil the biological function of disturbed miR-155 and miR-148a in the process of VM in cardiac cells, we constructed full length of mouse RelA 3'UTR into pmir-GLO vector. Results of dual-luciferase assay

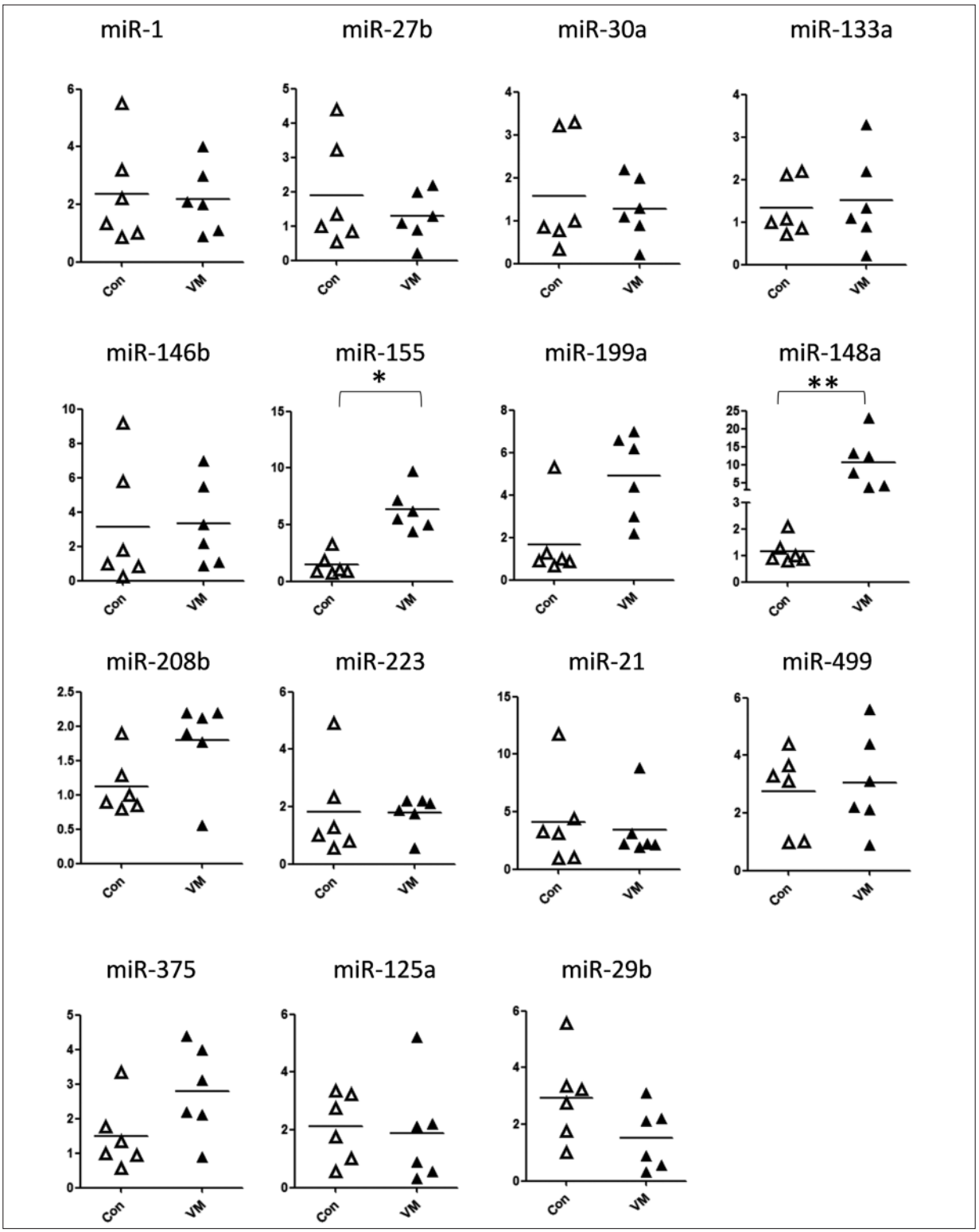

Figure 1. Expression of 15 selected miRNAs in cardiac biopsy from VM patients. Total RNAs were extracted using right ventricular septal biopsies from patients with acute myocarditis (n=6) with a definite clinical history of myocarditis and confirmed virus presence in the cardiac biopsies. Controls (n=6) consisted of age-matched patients with unexplained ventricular tachy-arrhythmias but with a normal ejection fraction and the absence of systemic or cardiac inflammation or virus presence at the time of biopsy. The expression of miRNAs was detected using stem-loop qRT-PCR, U6 was used as loading control. The results were analyzed using Student *t*-test and $p < 0.05$ was considered statistically significant. *$p < 0.05$, **$p < 0.01$.

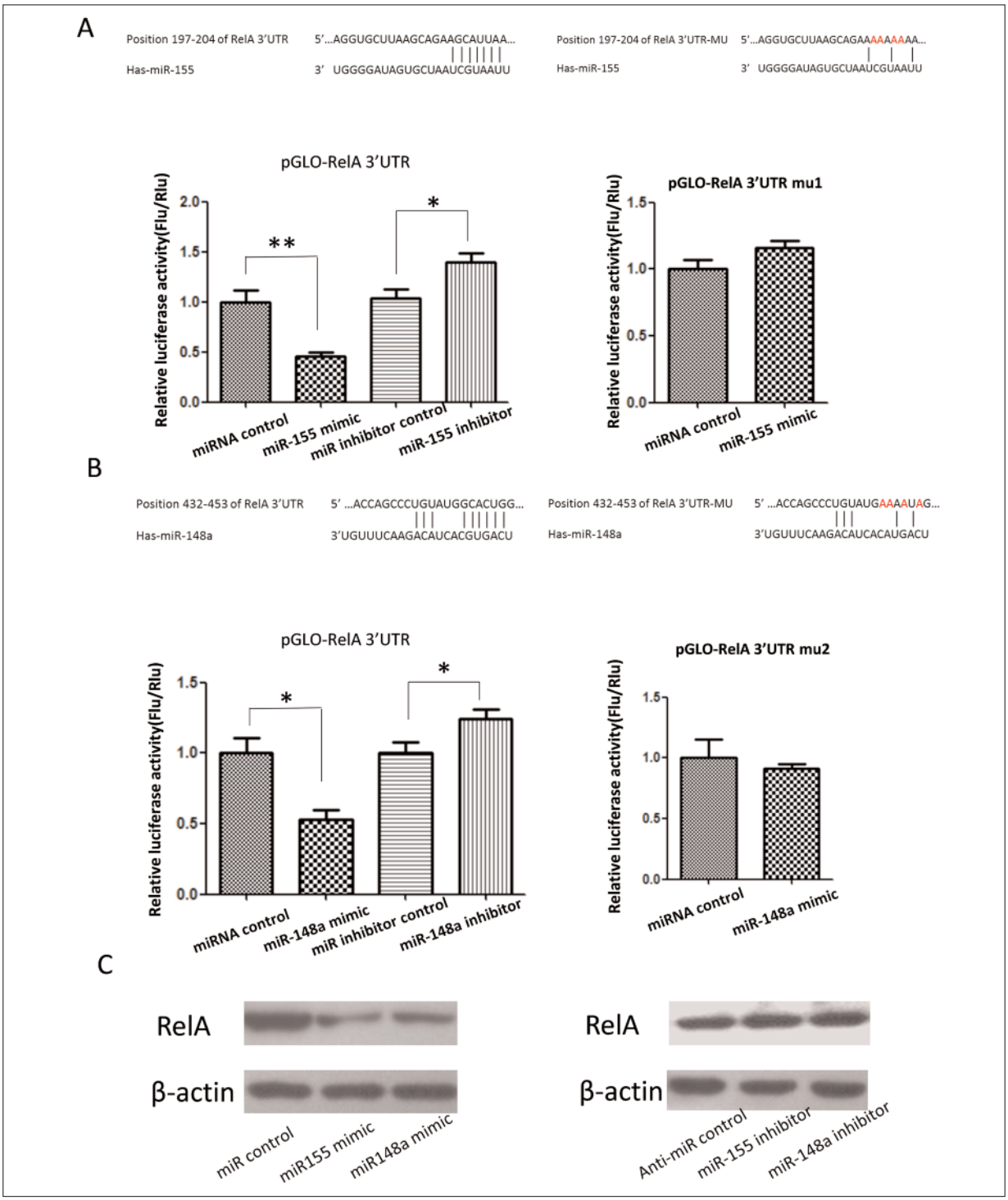

Figure 2. miR-155 and miR-148a repress RelA expression in HEK293T cells. ***A,*** Using TargetScan, we found miR-155 may target RelA by binding to the 3'UTR of RelA. HEK293T cells were co-transfected with pGLO-RelA, miR-155 mimic or inhibitor. The results demonstrated as firefly luciferase activity versus renilla luciferase activity. The mutant vector with 4 nucleotides variation was used to confirm the binding site of miR-155. ***B,*** Predicted using miRanda, we found miR-148a may repress RelA expression by binding to the 3'UTR of RelA directly. HEK293T cells were co-transfected with pGLO-RelA, miR-148a mimic or inhibitor. The results demonstrated as firefly luciferase activity versus renilla luciferase activity. The mutant vector with 4 nucleotides variation was used to confirm the binding site of miR-148a.The results were analyzed using *t*-test. p < 0.05 was considered statistically significant. **p* < 0.05, ***p* < 0.01. ***C,*** HEK293T cells were transfected with miR-155 or miR-148a or miRNA control. 48h after transfection, western blot was used to detect the expression of RelA.

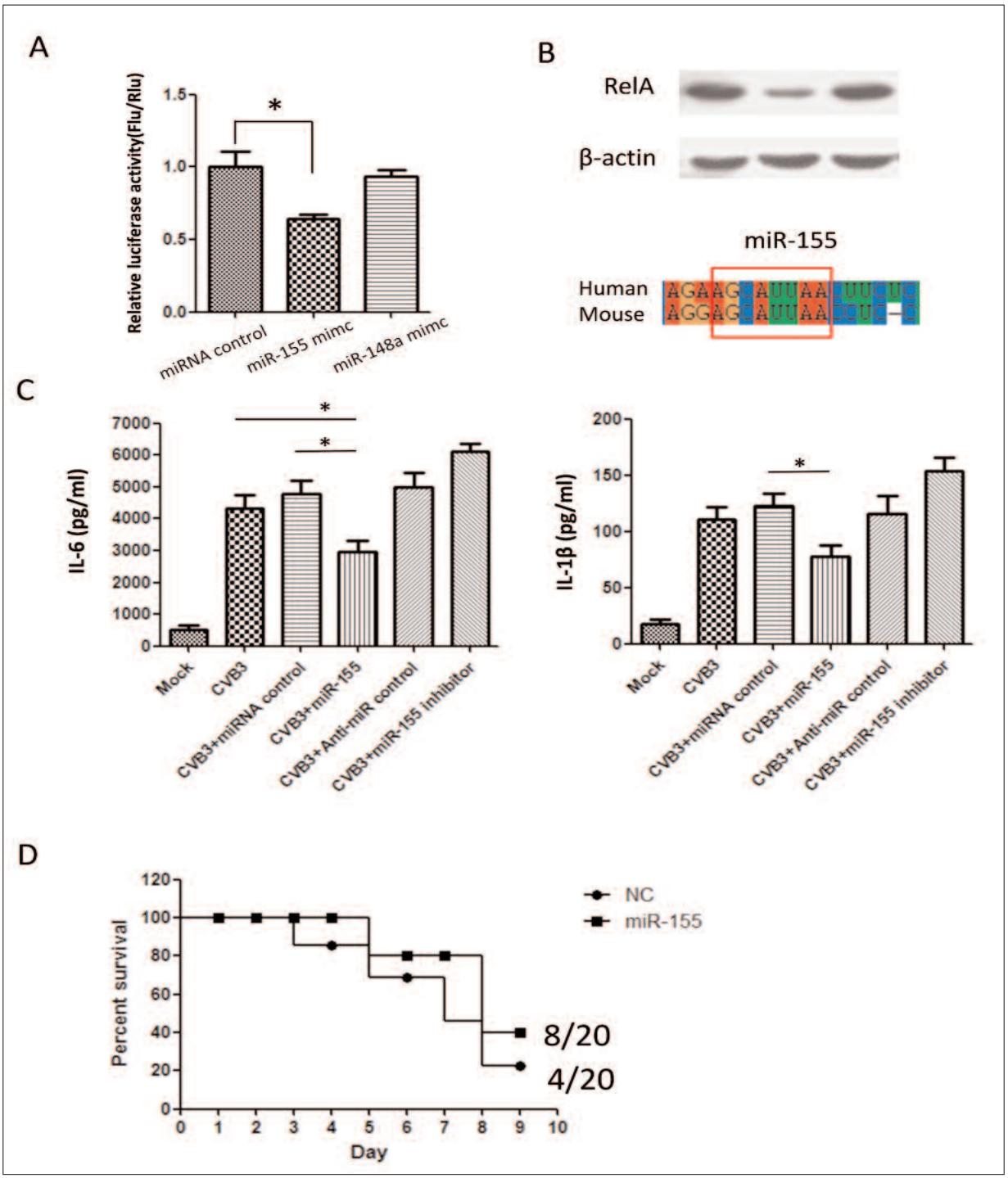

Figure 3. miR-155 not miR-148a improve mice survival rate by repress RelA expression. ***A,*** Mouse cardiac myocytes were co-transfected with mouse RelA 3'UTR reporter vestor and miR-155 or miR-148a. 48h after transfection, luciferase activity was detected. The results demonstrated as firefly luciferase activity versus renilla luciferase activity.The results were analyzed using *t*-test. $p < 0.05$ was considered statistically significant. *$p < 0.05$. ***B,*** Mouse cardiac myocytes were transfected with miR-155 or miR-148a, 48h after transfection, western blot was used to detect RelA expression. ***C,*** Mouse cardiac myocytes were transfected with miR-155, 48h after transfection, cells were infected with CVB3. Levels of IL-1β, IL-6 of cell culture supernatants were determined by enzyme-linked immunosorbent assay. The results were analyzed using *t*-test. $p < 0.05$ was considered statistically significant. *$p < 0.05$. ***D,*** Six week old male mice were injected intraperitoneally with 104 plaque-forming units of CVB3 (nancy strain). Mice were observed every day for the development of clinical morbidity and mortality until day 9. These mice received, every two days, either miR-155 mimics or miRNA control at a dose of 80 mg/kg body weight through tail vein injection.

indicated that mouse RelA is a target gene of miR-155 except miR-148a (Figure 3A). Subsequently, we isolated murine cardiac myocytes, transfected miR-155 and miR-148a mimics into this cell and then detected the endogenous RelA using western blot. We also compared the 3'UTR sequences of RelA between mouse and human. The result is in accordance with the results of dual luciferase assay-only miR-155 repressed RelA expression in murine cardiac myocytes and only the target site of miR-155 is conserved between human and mouse (Figure 3B).When infected by CVB3, the amount of IL-6 and IL-1β secreted from the cells transfected with miR-155 is significantly reduced indicated that NF-κB signaling was repressed.

To further assess the biological function of miR-155 *in vivo*, six week old male mice were injected intraperitoneally with 10^4 plaque-forming units of CVB3 and those mice received, every two days, either miR-155 mimics or miRNA control at a dose of 80 mg/kg body weight through tail vein injection. At day 9, 40% mice were survived in miR-155 group compared with 20% survival rate of the control group. The results suggested miR-155 may modulate immune response to CVB3 and protect the body from overdriven immune action.

Discussion

Acute viral myocarditis (VM) is an important cause of sudden cardiac death and heart failure in healthy young people and coxackievirus B3 is the most common pathogen. Meanwhile, CVB3 induces a high incidence of acute neonatal diseases and multiple organ injury[10]. MicroRNAs is a group of gene expression regulator and some of which have been confirmed associated with VM. In this study, we first screened 15 selected miRNAs in myocardial tissues of patients with acute CVB3 caused myocarditis and found the expression of miR-155 and miR-148a was up-regulated significantly. Predicted by using bioinformatics tools and confirmed by dual-luciferase assay and western blot, we confirmed that RelA is a direct target gene of miR-155 and miR-148a. The binding site of miR-155 is conserved between human and mouse, and we confirmed that the expression of RelA was also repressed in murine cardiac myoblast. Subsequent *in vitro* functional study indicated that miR-155 function as immune response negative feedback factor that reduced cardiac myoblast cytokines expression during CVB3 infection. Further *in vivo* experiments indicated that miR-155 and miR-148a can improved mice survival rate when CVB3 infected.

Our study indicates that miR-155 and miR-148a repress RelA expression in HEK293T cells, but only the binding site of miR-155 in RelA 3'UTR is conserved in human and mouse. And only miR-155 can also repress the expression of RelA in murine cardiac myocytes. Our results do not investigate the function of miR-148a in mice but miR-148a may have crucial roles during VM pathogenesis in human. So, more studies should be employed to unveil the function of miR-148a in human.

Conclusions

We identified that overexpressed miR-155 and miR-148a involved in the process of CVB3 caused VM. We first confirmed RelA is a direct target gene of miR-155 and miR-148a in human and the target site of miR-155 is conserved between human and mouse. miR-155 overexpression can modulate immune response to CVB3 by repress NF-κB pathway and improve the survival rate of mice infected with CVB3.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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