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Altered exosomal miR-181d and miR-30a related to the pathogenesis of CVB3 induced myocarditis by targeting SOCS3

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Abstract. – OBJECTIVE: MicroRNAs are a group of gene expression regulators and some of which have been confirmed to be associated with acute viral myocarditis (VM). This study aims to find new biomarkers for VM diagnosis and explore the roles of miRNAs during the pathogenesis of VM.

PATIENTS AND METHODS: 23 patients with acute myocarditis and 12 controls were included in this research. The expression of 10 candidate miRNAs in the serum exosome was examined by qRT-PCR. The direct targets were predicted using bioinformatics tools and then confirmed by dual luciferase assay and immunoblotting. Levels IL-6 of cell culture supernatants were determined by enzyme-linked immunosorbent assay. Six weeks old male mice were injected intraperitoneally with Coxsackievirus B3 (CVB3) and then treated by miRNA inhibitors through tail vein injection.

RESULTS: Five miRNAs were found to have disturbed expression in the exosome and may have the potential to be used as biomarker for VM diagnosis. Meanwhile, the expression of miR-30a and -181d was also altered in the cells after CVB3 infection. We identified SOCS3 as a direct target of miR-30a and -181d. Furthermore, during CVB3 infection, up-regulated miR-30a and -181d are related to enhanced IL-6 level via modulating SOCS3 expression. miRNA inhibitors injection increased mice survival rate after CVB3 infection.

CONCLUSIONS: miR-30a and -181d contribute to the over-activated inflammatory response to viral infection of the heart during coxsackievirus infection.

Key Words:
Viral myocarditis, microRNA, Coxsackievirus, SOCS3.

Introduction

Myocarditis is defined as an inflammatory process of the myocardium, which results in injury to the cardiac muscle cells, and the manifestations range from subclinical to sudden death. It is defined as an inflammation of the heart muscle, identified by clinical or histopathologic criteria[1,2]. Virus infection is a major cause of myocarditis and Coxsackievirus B3 (CVB3), a kind of enterovirus, which is believed to be the most common causative agent in human viral myocarditis. Viral myocarditis (VM) affects 5-20% of the human population, which can be fatal in infants as well as children[3,4]. 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 auto-destructive inflammatory signaling pathways within the immune system[5]. MicroRNAs (miRNAs) are short, noncoding RNAs that regulate 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[6]. Loss-of-function studies in mice firmly established that miRNAs control a variety of cellular processes essential to the heart[7,8]. MiRNAs play very important roles in maintaining

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normal human body physiology conditions, and abnormal miRNA expressions have been found related to many human diseases spanning from psychiatric disorders to malignant cancers[9-11]. Exosomes ranging in size from 40-100 nm in diameter, secreted by cells are proposed to be mechanism through which secreted cells pass signals to targeted cells. Recent studies indicate that the exosomal miRNAs were changed after virus infection, and some of which can be detected in the serum[12,13]. In this work, we detected 10 candidate miRNAs in the serum exosome samples from patients with myocarditis, which was induced by CVB3. Two miRNAs were found to be up-regulated. Furthermore, we showed these two miRNAs can modulate immune response of the cells after CVB3 infection by targeting SOCS3.

Patients and Methods

Participants

All human materials were obtained from Shandong University of Traditional Chinese Medicine, stored in -80°C immediately after collection and available for research purposes in accordance with the Declaration of Helsinki and the Ethical Committee of Shandong University of Traditional Chinese Medicine. Peripheral blood was obtained from patients with acute myocarditis (n=23) with a definite clinical history of myocarditis and confirmed virus presence in the cardiac biopsies. Controls (n=12) 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.

Exosome Isolation

Exosomes from serum samples were isolated using serial centrifugation method as described[14]. Patient serum samples were diluted with phosphate-buffered saline (PBS) in a 1:1 ratio[14]. Serum samples were then pre-treated with anti-IgG antibody (1:500 dilution) coupled to A/G sepharose beads (25 µl) to precipitate excessive non-specific immunoglobulins. After an overnight incubation at 4°C, the pre-treated samples were centrifuged at 5000 g for 15 min and the pre-cleared serum was used for exosomes isolation using a standard ultracentrifugation method. Briefly, the pre-cleared serum samples went through a series of centrifugation steps (2000 g for 20 min and 20,000 g for 45 min) and then transferred onto 30% sucrose solution, followed by ultra-centrifugation at 100,000 g for 2 h. Isolated exosomes were recovered from the sucrose solution and stored at -80°C until further analyses.

Cell Culture

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), 100 IU/ml penicillin and 100 µg/mL streptomycin. All cells were maintained at 37°C under an atmosphere of 5% CO_2.

Preparation of Viruses

CVB3, strain Nancy, was propagated in mycoplasma free HeLa cell cultures. 8 hours after CVB3 infection with 1 multiplicity of infection (MOI), the culture medium was removed and the cells were disrupted by three freezing (-20°C) and thawing cycles. After centrifugation at 1500 g, the cell debris was removed and virus particles were purified by chloroform extraction. Aliquots of purified virus were stored at -70°C.

Cytokine Assay

Levels IL-6 of cell culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (Abcam, Cambridge, MA, USA) following the manufacturers' instructions.

Real-time RT-PCR

Quantitative RT-PCR analysis was used to determine the relative expression of miRNAs. Total RNA was extracted from clinical or cell samples, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The expression levels of miR-10a-5p and miR-10a-3p were detected using 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 miR-16 was used for serum miRNA normalization and U6 snRNA was used for cell samples. Each sample in each group was measured in triplicate and the experiment was repeated at least three times.

Dual Luciferase Assay

Full length of SOCS3 3'UTR was cloned into downstream of firefly luciferase coding region

in pmirGLO vector (Promega, Madison, WI, USA) to generate luciferase reporter vector. For luciferase reporter assays, Hela cells were seeded in 48-well plates. MiRNA mimics or miRNA antagonists or their corresponding controls and luciferase reporter vector 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 luciferase activity/Renilla luciferase activity).

Immunoblotting

Protein extracts were boiled in SDS/β-mercaptoethanol sample buffer, and 30 μg samples were loaded into each lane of 10% polyacrylamide gels. The proteins were separated by electrophoresis, and the proteins in the gels were blotted onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, St. Albans, Herts, UK) by electrophoretic transfer. The membrane was incubated with rabbit anti-SOCS3 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 (HRP) 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.

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 the Shandong University of Traditional Chinese Medicine and Intensive Care Unit (ICU), Yantai Yuhuangding Hospital Affiliated to Qingdao University. Animal Ethics Committee was granted before the start of the study. Specific pathogen free male BALB/c mice (6 weeks old) were housed under pathogen-free conditions in an animal house and fed with 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 inhibitors 2'-O-methyl oligonucleotide and inhibitor control were purchased from GenePharma Co (Shanghai, China). These mice received, every two days, either miRNA inhibitors or inhibitor control at a dose of 80 mg/kg body weight through tail vein injection.

Statistical Analysis

Data were analyzed by using SPSS Statistical Package version 16. Independent two group's analyses used *t*-test. The overall survival curves were plotted according to the Kaplan-Meier method, and the generalized log-rank test was applied to compare the survival curves. $p < 0.05$ was considered statistically significant.

Results

To investigate the exosomal miRNA variation and identify biomarkers for myocarditis we selected 10 candidate miRNAs. Among them, miR-1, -27b, -148a and -133a are heart-associated; miR-146b, 30a, -155, -181d and -125a are immune-associated; miR-21 is fibrosis associated. The serum exosomes were separated by ultracentrifuge, and total RNAs were extracted using TRIzol Reagent followed by qRT-PCR detection. As shown in Figure 1A, the level of miR-30a, -181d and -125a was up-regulated and the level of miR-155 and -21 was down-regulated. Subsequently, we detected the exosomal and cellular level of these five miRNAs from CVB3 infected or non-infected HeLa cells, and found that only miR-30a and miR-181d were up-regulated significantly after CVB3 infection (Figure 1B).

To further investigate the function of disturbed miR-30a and -181d during the pathogenesis during CVB3 infection, we predicted the direct target genes using online bioinformatics tool: TargetScan (http://www.targetscan.org/vert_71). A total of 587 potential targets of both miR-181d and miR-30a were subjected to DAVID Bioinformatics Resources 6.8 for gene ontology analysis (https://david.ncifcrf.gov/). We found 6 genes are relates to T cell activation including SOCS3, which is a negative regulator of cytokine signaling. As shown in Figure 2A, there are predicted interaction between SOCS3 3'UTR and miR-30a and -181d. Subsequently, we constructed the reporter vector inserting the full length 3'UTR of SOCS3 into the 3'UTR region of firefly luciferase gene in the pmirGLO vector. HeLa cells were transfected with SOCS3

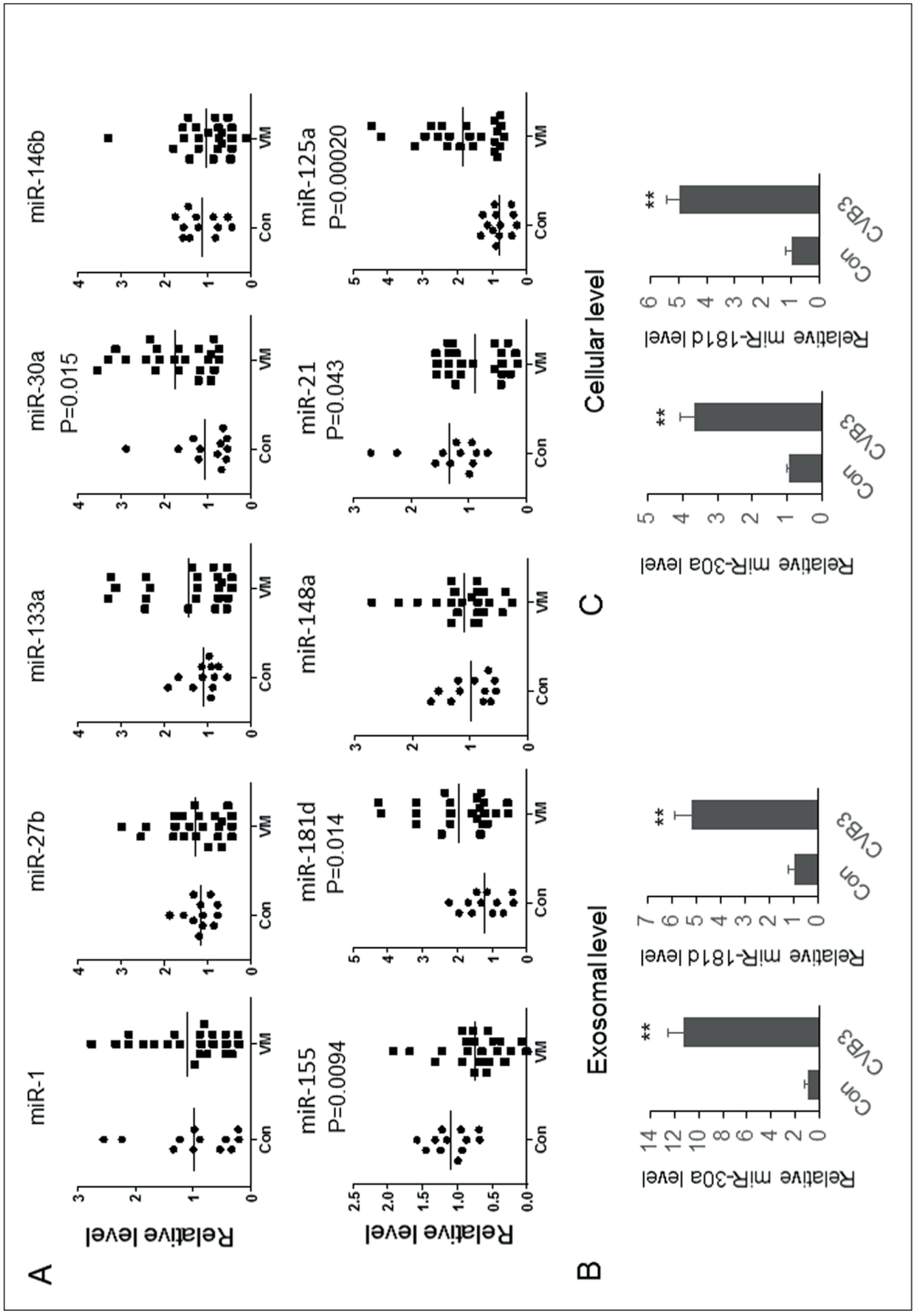

Figure 1. The expression of miRNAs was altered during CVB3 infection. ***(A)*** The level of ten selected miRNAs in the serum exosome was detected by qRT-PCR. The results were analyzed by Student's *t*-test and $p < 0.05$ was considered statistically significant. ***(B)*** HeLa cells were infected by CVB3. 48 hours after infection, exosomes were collected from the medium by ultracentrifuge. Total RNAs were extracted from the exosomes and cells. The expression of miR-30a and -181d was detected by qRT-PCR. The results were analyzed by Student's *t*-test and $p < 0.05$ was considered statistically significant. $*p < 0.05$, $**p < 0.01$.

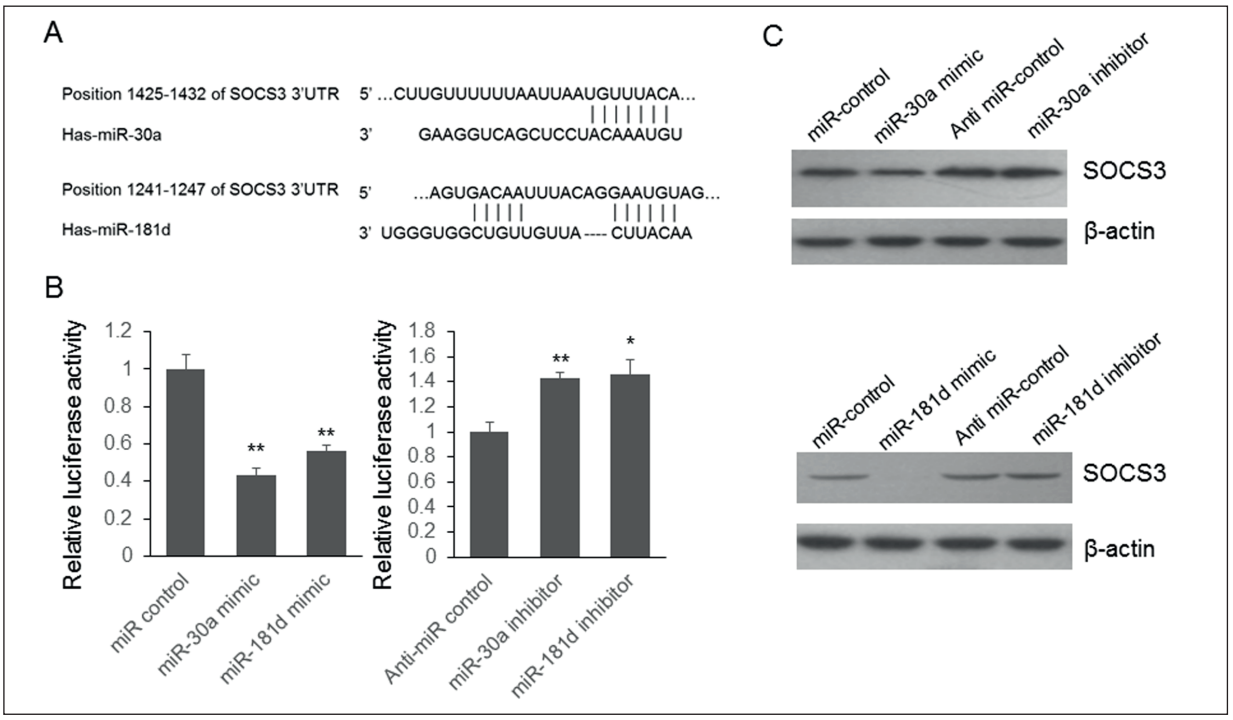

Figure 2. SOCS3 is a direct target of miR-30a and -181d. ***(A)*** Schematic diagram of the direct interaction between SOCS3 3'UTR and miR-30a and -181d. ***(B)*** Dual luciferase assay. HeLa cells were seeded in 48-well plates. MiRNA mimics or miRNA antagonists or their corresponding controls and luciferase reporter vector were co-transfected by using lipofectamine 2000. 48 hours after transfection, cells were harvested and assayed with the Dual-Luciferase Assay. Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity. ***(C)*** Immunoblotting.

reporter vector, and the mimic or inhibitor of miR-30a or -181d. 48 hours after transfection, cells were lysed and the luciferase activity was detected. As shown in Figure 2B, the relative luciferase activities were repressed by the mimic of miR-30a and -181d. Meanwhile, the luciferase activities were up-regulated by the inhibitors of miR-30a and -181d. These results indicated that miR-30a and -181d repress the expression of firefly luciferase activity via targeting the 3'UTR of SOCS3.

To further investigate whether endogenous SOCS3 expression is modulated by miR-30a and -181a, HeLa cells were transfected with miR-30a or -181d mimic. Cells were lysed 48 hours post transfection and the protein level of SOCS3 was detected by immunoblotting. As shown in Figure 2C, the expression of SOCS3 was repressed by the mimic of miR-30a or -181d, and up-regulated by the inhibitor of miR-30a or -181d, indicating that SOCS3 is a direct target of miR-30a and -181d.

To further unveil the biological function of disturbed miR-30a and miR-181d in the process of VM in cardiac cells, we isolated murine cardiac myocytes, transfected miR-30a and miR-181d mimics into this cell and then infected by CVB3. The amount of IL-6 secreted from the cells transfected with miR-30a or -181d is significantly up-regulated, and the cellular level of SOCS3 is down regulated (Figure 3A, B). Meanwhile, when transfected with miR-30a or -181d inhibitor, the level of IL-6 in the medium was reduced and the cellular SOCS3 was overexpressed (Figure 4A, B). These results indicated that the immune response to CVB3 in the cardiac cells is modulated by miR-30a and -181d via modulating SOCS3. To further assess the value of miR-30a and -181d inhibitor in clinical treatment, 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-30a and -181d inhibitors, or inhibitor control at a dose

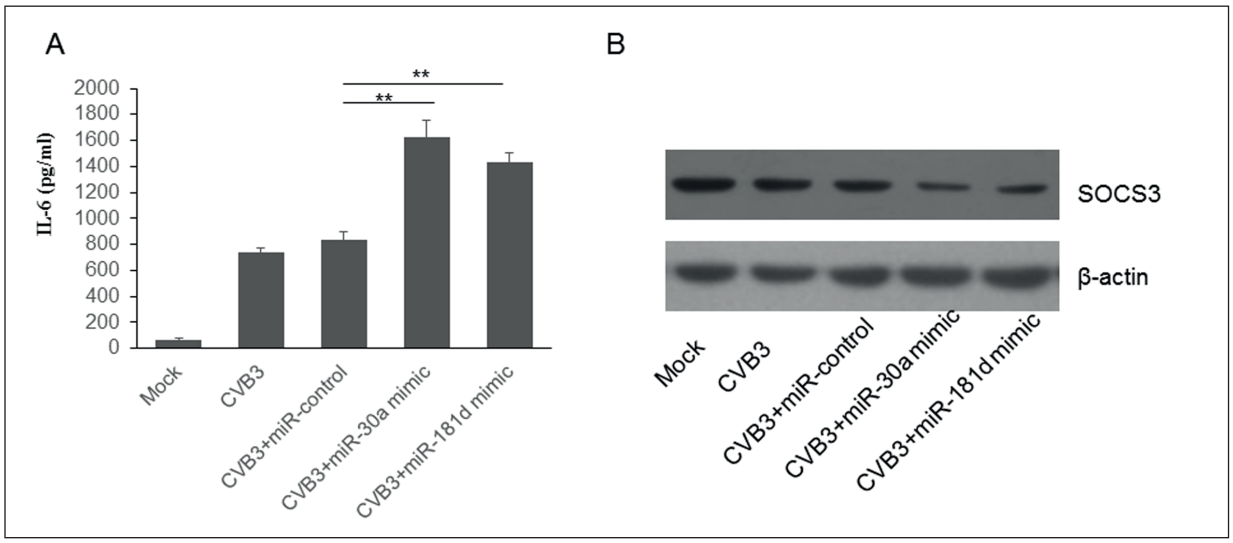

Figure 3. IL-6 level is modulated by miR-30a and -181d. Primary murine cardiac myocytes were transfected with miR-30a and miR-181d mimics and then infected by CVB3. The level of IL-6 in cell culture supernatants was determined by enzyme-linked immunosorbent assay ***(A)***, and the level of SOCS3 in cells was detected by immunoblotting ***(B)***.

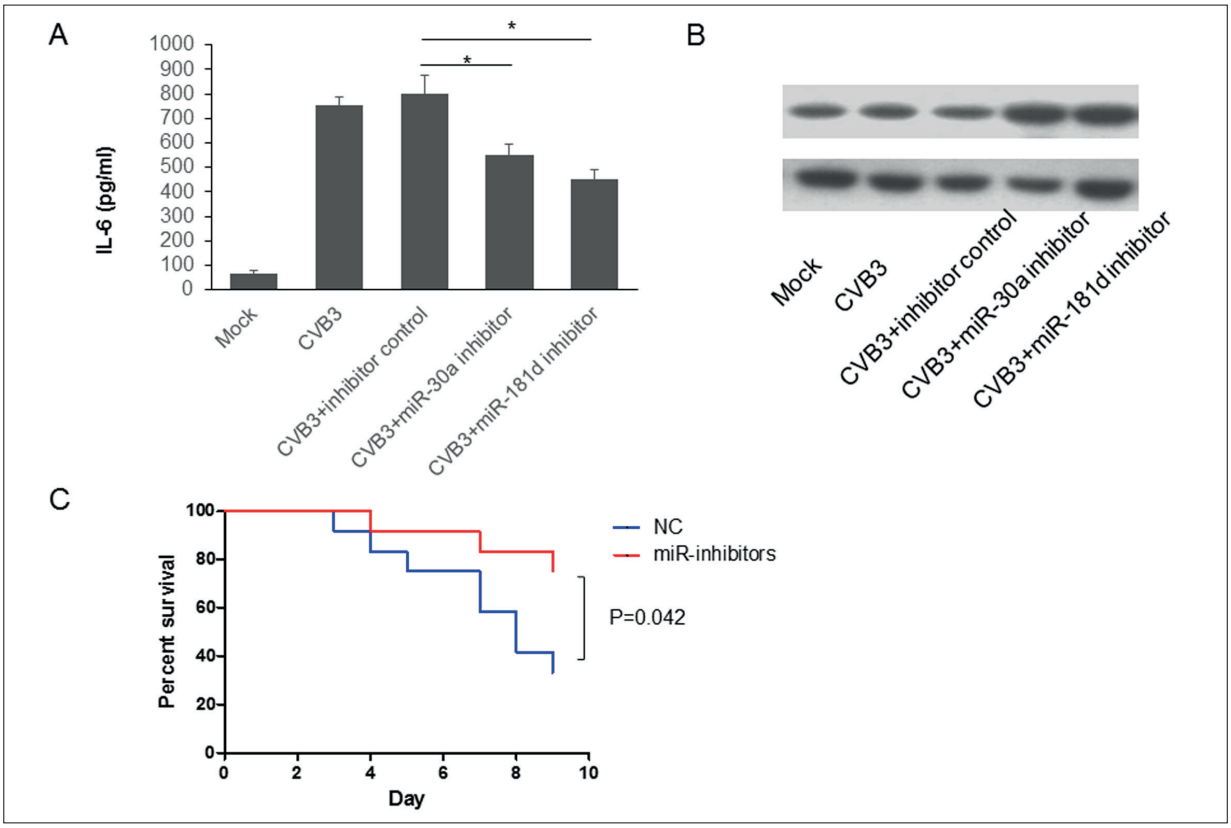

Figure 4. miR-30a and -181d inhibitors improve mice survival rate. Primary murine cardiac myocytes were transfected with miR-30a and miR-181d mimics and then infected by CVB3. The level of IL-6 in cell culture supernatants were determined by enzyme-linked immunosorbent assay ***(A)***, and the level of SOCS3 was detected by immunoblotting ***(B)*** and ***(C)*** 24 mice were separate into two groups randomly. Mice were injected intraperitoneally with 10^4 plaque-forming units of CVB3. Mice were observed every day for the development of clinical morbidity and mortality until day 9. These mice received, every two days, either miRNA inhibitors or inhibitor control at a dose of 80-mg/kg body weight through tail vein injection. The overall survival curves were plotted according to the Kaplan-Meier method, and the generalized log-rank test was applied to compare the survival curves.

of 80 mg/kg body weight through tail vein injection. At day 9, 75% mice were survived in miR-inhibitor group compared with 33% survival rate of the control group.

Discussion

Acute viral myocarditis (VM) is an important cause of sudden cardiac death and heart failure in healthy young people and coxsackievirus B3 is the most common pathogen. MicroRNAs are a group of gene expression regulator, some of which have been confirmed associated with VM. In this study, we first detected the expression of 10 candidate miRNAs in the serum exosome in 23 patients with VM and 12 controls. 5 miRNAs were found to have disturbed expression in the exosome and may have the potential to be used as biomarker for VM diagnosis. Meanwhile, 2 of them were also altered in the cells after CVB3 infection. Predicted using bioinformatics tools and confirmed by dual luciferase assay and Western blot, we identified SOCS3 as a direct target of miR-30a and -181d. Furthermore, during CVB3 infection, up-regulated miR-30a and -181d are related to enhanced IL-6 level via modulating SOCS3 expression. Thus, our data demonstrated that miR-30a and -181d contribute to the overactivated inflammatory response to viral infection of the heart during coxsackievirus infection.

Conclusions

We identified that overexpressed miR-30a and -181d involved in the process of CVB3 caused VM. We first confirmed SOCS3 is a direct target gene of miR-30a and miR-181d. miR-30a and -181d overexpression can modulate immune response to CVB3 by repressing SOCS3. The up-regulation of serum exosomal miR-30a and -181d may have the potential to be used as biomarkers for VM diagnosis.

Conflict of Interest

We confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgements

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