Overexpression of microRNA-133b reduces myocardial injuries in children with viral myocarditis by targeting Rab27B gene

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Abstract: The present study is to measure the expression of microRNA (miRNA or miR)-133b in circulating blood of children with viral myocarditis before and after drug treatment, and to investigate its relationship with the severity of myocardial lesions. A total of 36 children patients with viral myocarditis who received treatments at our hospital between June 2014 and June 2016 were enrolled in the present study, including 21 boys and 15 girls (age range, 9 months – 16 years). Quantitative real-time polymerase chain reaction was used to determine the expression of miR-133b in peripheral blood of patients and cardiomyocytes infected with CVB3. CCK-8 assay was used to test the proliferation of cardiomyocytes. ELISA was used to determine the levels of creatine kinase (CK-MB) and lactate dehydrogenase (LDH) in peripheral blood and cardiomyocyte culture supernatants. Western blotting and ELISA were performed to measure the levels of Rab27B protein in peripheral blood and cardiomyocyte culture supernatants. Bioinformatics was used to predict the target gene of miR-133b. Silencing of Rab27B gene was achieved by transfection with its small-interfering RNA. Dual luciferase reporter assay was carried out to test whether miR-133b directly targets Rab27B. Reduced expression of miR-133b in peripheral blood was possibly correlated with myocardial injuries in viral myocarditis.

Key words: miR-133b; Myocardial injury; Viral myocarditis; Rab27B.

Introduction

Viral myocarditis (VM) is a common infectious disease in pediatric clinical practice, which is caused by viral infection or autoimmune disorders (1). Viral myocarditis can develop into acute heart failure (2). It is shown that coxsackievirus B3 (CVB3) is one of the most common viruses causing viral myocarditis (3). According to clinical studies, the mortality rate of viral myocarditis in young people is as high as 21%, and sudden death caused by viral myocarditis itself or fatal ventricular arrhythmia caused by viral myocarditis accounts for about 20% in children (4, 5). Persistent viral myocarditis has a poor prognosis with a 5-year survival rate of approximately 50%. Persistent chronic inflammation can lead to myocardial cell necrosis, myocardial hypertrophy or cell apoptosis, or even develop into myocardial fibrosis, dilated cardiomyopathy (DCM) and heart failure (6). In recent years, the incidence of viral myocarditis has shown a rising trend, attracting the attention of researchers (7). However, there is a lack of specific treatment for viral myocarditis at present, and the treatment is still focused on the controlling of malignant cardiac arrhythmia and heart failure. Therefore, it is necessary to study the molecular mechanism of viral myocarditis.

MicroRNA (miRNA or miR) molecules are a class of conservative single-strand small molecules (18 – 22 nucleotides) that are encoded by endogenous genes (8). miRNA can bind to the 3’-untranslated region (UTR) of mRNA at the seeding region at the 5’-terminus (No. 2 – 8 nucleotides) (9). If miRNA is completely complementary to the target gene mRNA, it degrades the mRNA; if the binding is incompletely complementary, the mRNA is not degraded but its translation is inhibited (10). Therefore, miRNA is an important molecule in the posttranscriptional regulation of genes. Studies confirm that miRNA molecules participate in the regulation of nearly 90% human genes, affect biological processes such as cell proliferation, differentiation, metabolism and development, and play important roles in the occurrence and development of a variety of diseases, such as cancer, infection and neurodegenerative diseases (11-13). miRNA plays an important role in heart disease, and it is found that miRNA is involved in the processes of cardiac hypertrophy, arrhythmia, myocardial cell...
apoptosis and viral infection of myocardial cells (14, 15). For example, miR-1 is widely expressed in myocardial cells, and inhibits the expression of serum response factor (SRF) and histone deacetylase 4 (HDAC4), both of which participate in the regulation of myocardial hypertrophy, proliferation and apoptosis (16). More studies show that expression of miR-23a promotes myocardial hypertrophy (17, 18). In addition, miR-133b is abundantly expressed in cardiac myocytes (19), but its expression in peripheral blood of children with viral myocarditis and the underlying molecular mechanism have rarely been reported before. In the present study, we measure the expression of miR-133b in circulating blood of children with viral myocarditis before and after drug treatment, and to investigate its relationship with the severity of myocardial lesions.

Materials and Methods

Patients
A total of 36 children patients with viral myocarditis who received treatments at our hospital between June 2014 and June 2016 were enrolled in the present study, including 21 boys and 15 girls (age range, 9 months – 16 years). Clinical diagnosis was carried out according to the standards of viral myocarditis, which were set up by the cardiovascular group of pediatric branch of Chinese Medical Association in 2000. According to the left ventricular ejection fraction and cardiac troponin I measured by echocardiography, children with myocarditis were divided into mild VM group (n = 35; EF > 50%; cardiac troponin < 1 μg/L or normal) and severe VM group (n = 21; EF < 50%; cardiac troponin I > 0.2 μg/L or above normal). All patients received combined treatments such as large doses of vitamin C, protection of important organ functions, symptomatic treatment, increasing myocardial nutrition, and antiviral treatment. Children with severe VM received large doses of gamma globulin (2 g/kg body weight, immune globulin via intravenous dripping) and methylprednisolone (15mg/ kg body weight for 3 days). Venous blood samples were collected from children with viral myocarditis who were followed up regularly after treatment, and used as normal control. All procedures were approved by the Ethics Committee of Hubei University of Medicine. Written informed consents were obtained from all patients or their families.

Automatic biochemical analysis
Creatine kinase (CK-MB) in peripheral blood was determined using a CK-MB detection kit (DiaSys, Shanghai, China) according to the manufacturer’s manual. Briefly, 250 μl reagent I was incubated at 37 °C for 3 min before addition of 10 μl samples. Then, the mixture was incubated in water bath at 37 °C for 3 min before addition of 50 μl reagent II. Absorbance changes were monitored within 5 min, and CK-MB activity was calculated from the rising rate of average absorbance (per minute) in the linear section. The result was calculated by an automatic biochemical analyzer (AU5800; Beckman Coulter, Brea, CA, USA). Normal reference range was 0-24 U/L. For the determination of CK-MB in culture supernatant of cardiomyocytes, the cells in each group were cultured for 72 h before collecting the supernatant, followed by centrifugation at 10,000 rpm for 15 min. The determination was performed according to the steps described above. Each test was performed in triplicate.

Detection of lactate dehydrogenase (LDH)
The detection of LDH in peripheral blood or supernatant of cardiomyocytes was performed strictly using a LDH detection kit (Applygen Technologies, Inc., Beijing, China) according to the manufacturer’s manual. Each test was performed in triplicate.

Cells
Neonatal mouse cardiomyocytes (ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured with serum-free cardiomyocyte medium (ScienCell Research Laboratories, Carlsbad, CA, USA). These cells were seeded on 6-well plates, and divided into control group and CVB3 group. After being cultured at 37 °C and in 5% CO₂ for 24 h, the medium was discarded, and the cells were washed with Hank’s solution twice. In the infection group, 500 μl 100 TCID₅₀ CVB3 virus (Wuhan Virus Research Institute, Chinese Academy of Sciences, Wuhan, China) was added; in negative control (NC) group, 500 μl complete medium was added. The cells were cultured at 37 °C and in 5% CO₂ for 2 h before changing the medium to complete medium. After cultivation for another 72 h, the cells were used for the extraction of RNA and protein.

Cardiomyocytes were seeded onto 24-well plates containing complete medium supplemented with 10% fetal bovine serum, and divided into NC group, CVB3 group, and miR-133b+CVB3 group. When reaching 50 – 60% confluence, 1.25 μl miR-133b mimics (20 pmol/ μL; RiboBio, Guangzhou, China) and 2 μl Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) were added into two individual vials containing 50 μl OptiMeni medium, respectively. Five minutes later, the liquids in the two vials were mixed before standing still for another 15 min. Then, the mixture was added onto the cells for an incubation of 6 h. After changing DMEM medium supplemented with 10% fetal bovine serum, the cells were cultured under normal condition before use. HeLa cells were used to determine the tissue culture infective dose of CVB3 virus. In addition, 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum, and seeded for dual luciferase reporter assay when reaching 90% confluency.

Quantitative real-time polymerase chain reaction (qRT-PCR)
Peripheral blood (250 μl) or 10⁶ cells were mixed with 1 ml Trizol (Thermo Fisher Scientific, Waltham, MA, USA) for lysis. After lysis, total RNA was extracted using phenol chloroform method. The purity of RNA was determined by A260/A280 and A260/A230 spectrophotometry (Nanodrop ND2000, Thermo Scientific, Waltham, MA, USA). Then, cDNA was obtained using a reverse transcription using PrimeScript RT Reagent Kit (Takara, Dalian, China) at 1 μg RNA and stored at -20°C.

To test the expression of miRNA, SYBR® PrimeScript™ miRNA RT-PCR Kit (Takara, Dalian, China).
was chosen, using U6 as internal reference. The qRT-PCR reaction system (20 μl) contained 1μl cDNA, 10 μl qRT-PCR-Mix, 0.5μl upstream primer (5’-TGTG-GTCCCCCTTCAACCGCTA-3’), 0.5μl downstream universal primer (provided by the kit), and 8μl ddH2O. Each sample was tested in triplicate. The PCR protocol was: initial denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 60 s and annealing at 60°C for 30 s (iQ5; Bio-Rad, Hercules, CA, USA). The 2-ΔΔCt method was used to calculate the relative expression of miR-133b against internal reference.

Cell Counting Kit 8 (CCK-8) assay
Cells in each group were seeded into 96-well plates at a density of 5,000 cells per well in triplicate. For every 24 h, the cells were incubated with CCK-8 reagent (Beyotime, Shanghai, China) for 30 min. Absorbance at 490 nm was read on a microplate reader (168-1000; Model 680, Bio-Rad, Hercules, CA, USA) at 24 h, 48 h, and 72 h, and proliferation curves were plotted using absorbance values at each time point.

Enzyme-linked immunosorbent assay (ELISA)
Culture supernatants of cardiomyocytes were collected after cultivation for 72 h and centrifuged at 10,000 rpm for 15 min. The contents of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in the supernatants were determined using respective ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s manuals. Each test was performed in triplicate.

Western blotting
After being cultured for 72 h, the cells were washed with pre-cooled phosphate-buffered saline. Then, Radio-Immunoprecipitation Assay (RIPA) lysis buffer (600 μl; 50 mM Tris-base, 1 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% TritonX-100, and 1% sodium deoxycholate; Beyotime Institute of Biotechnology, Shanghai, China) and phenylmethylsulfonyl fluoride (1%) were added to the samples. After lysis for 15 min on ice, the mixture was centrifuged at 12,000 g/min and 4°C for 10 min to obtain supernatants. Protein samples (50 μg) were then mixed with equal volume of 2× sodium dodecyl sulfate loading buffer before denaturation in boiling water bath for 10 min. Afterwards, the samples (5 μl) were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100 V. The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (300 mA, 2 h) and blocked with 50 g/L skimmed milk at room temperature for 1 h. Then, the membranes were incubated with goat anti-mouse polyclonal Rab27B primary antibody (1:1,000; Abcam, Cambridge, UK) and mouse anti-mouse GAPDH primary antibody (1:5,000; Abcam, Cambridge, UK) at 4°C overnight. After extensive washing with phosphate-buffered saline with Tween 20 for 5 times of 5 min, the membranes were incubated, respectively, with goat anti-mouse (1:5,000; Abcam, Cambridge, UK) and rabbit anti-goat (1:5,000; Abcam, Cambridge, UK) horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature before washing with phosphate-buffered saline with Tween 20 for 5 times of 5 min. Then, the membrane was developed with enhanced chemiluminescence detection kit (Sigma-Aldrich, St. Louis, MO, USA) for imaging. Image lab v3.0 software (Bio-Rad, Hercules, CA, USA) was used to acquire and analyze imaging signals. The relative content of target protein was calculated against GAPDH.

Dual luciferase reporter assay
The potential target genes of miR-133b were predicted using bioinformatics. miR-133b was identified to be capable of binding with 3’-UTR of Rab27B mRNA. According to bioinformatics results, wild-type (WT) and mutant seed regions of miR-133b in the 3’-UTR of Rab27B gene were chemically synthesized in vitro, added with Spe-1 and HindIII restriction sites, and then cloned into pMIR-REPORT luciferase reporter plasmids. Plasmids (0.5 μg) with WT or mutant 3’-UTR DNA sequences were co-transfected with miR-133b mimics into 293T cells. After cultivation for 24 h, the cells were lysed using dual luciferase reporter assay kit (Promega, Fitchburg, WI, USA) according to the manufacturer’s manual, and fluorescence intensity was measured using GloMax 20/20 luminometer (Promega, Fitchburg, WI, USA). Using renilla fluorescence activity as internal reference, the fluorescence value of each group of cells was measured.

Statistical analysis
The results were analyzed using SPSS 17.0 statistical software (IBM, Armonk, NY, USA). All measurement data were expressed as means ± standard deviations. Intragroup comparison was performed using paired t-test. P < 0.05 was considered statistically significant.

Results
Reduced expression of miR-133b in peripheral blood is possibly correlated with myocardial injuries in viral myocarditis
To measure the expression of miR-133b in peripheral blood, qRT-PCR was performed. The data showed that
miR-133b alleviates viral myocarditis via Rab27B.

Expression of miR-133b is significantly reduced in cardiomyocytes infected with CVB3 virus

To determine the expression of miR-133b in cardiomyocytes infected with CVB3 virus, qRT-PCR was carried out. The data showed that the expression of miR-133b in cardiomyocytes infected with CVB3 virus was significantly lower than that in control group (P < 0.05) (Fig. 2A). The result indicates that expression of miR-133b is significantly reduced in cardiomyocytes infected with CVB3 virus.

Overexpression of miR-133b inhibits cardiomyocyte injuries caused by CVB3 virus infection

To test whether miR-133b participates in cardiomyocyte injuries caused by CVB3 virus infection, we transfected cardiomyocytes with miR-133b mimics. CCK-8 assay showed that the absorbance of cardiomyocytes infected with CVB3 virus was significantly lower than that in NC group at 48 h and 72 h (P < 0.05). Moreover, up-regulated expression of miR-133b significantly increased the absorbance of cardiomyocytes infected with CVB3 virus (P < 0.05) to levels similar with NC at 48 h and 72 h (Fig. 2B). The contents of CK-MB and LDH in culture supernatants from cardiomyocytes infected with CVB3 were significantly higher than control group (P < 0.05), but co-transfection with miR-133b mimics significantly reduced CK-MB and LDH levels in cardiomyocytes infected with CVB3 virus (P < 0.05) (Fig. 2C and D). The results suggest that overexpression of miR-133b inhibits cardiomyocyte injuries caused by CVB3 virus infection.

Overexpression of miR-133b inhibits the enhanced production and release of cytokines TNF-α and IL-6 by cardiomyocytes infected with CVB3 virus

To measure the production and secretion of cytokines TNF-α and IL-6 by cardiomyocytes, Western blotting and ELISA were employed. Western blotting showed that TNF-α and IL-6 expression in cardiomyocytes infected with CVB3 was significantly higher than that in NC (P < 0.05). By contrast, co-transfection with miR-133b mimics significantly reduced the expression of TNF-α and IL-6 in cardiomyocytes infected with CVB3 virus (P < 0.05) (Fig. 3A and B). ELISA showed that the contents of TNF-α and IL-6 in supernatants of cardiomyocytes infected with CVB3 virus were significantly higher than those of NC (P < 0.05), but co-transfection with miR-133b significantly decreased the release of TNF-α and IL-6 by cardiomyocytes infected with CVB3 virus (P < 0.05) (Fig. 3C and D). The results indicate that overexpression of miR-133b inhibits the enhanced production and release of cytokines TNF-α and IL-6 by cardiomyocytes infected with CVB3 virus.

Rab27B promotes injuries of cardiomyocytes induced by CVB3 infection

The potential target genes of miR-133b were predicted using bioinformatics. miR-133b was identified to be capable of binding with 3'-UTR of Rab27B mRNA. Western blotting showed that Rab27B protein expression in cardiomyocytes infected with CVB3 virus was significantly lower than that in NC group (P < 0.05) (Fig. 4A). In addition, the level of miR-133b in peripheral blood from children with viral myocarditis was significantly lower than that in NC group (P < 0.05) (Fig. 1A). In addition, the level of miR-133b was moderately correlated with the contents of CK-MB and LDH in peripheral blood (R² = 0.747 and 0.680, respectively) (Fig. 1B and C). The results suggest that reduced expression of miR-133b in peripheral blood is possibly correlated with myocardial injuries in viral myocarditis.
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significantly higher than that in NC group (P < 0.05), while transfection with miR-133b mimics significantly reduced the elevated expression of Rab27B protein in cardiomyocytes infected with CVB3 virus (P < 0.05) (Fig. 4A). Silencing of Rab27B gene expression by siRNA decreased the expression of Rab27B protein (P < 0.05; Fig. 4B) and the proliferation of cardiomyocytes infected with CVB3 (P < 0.05; Fig. 4C). ELISA showed that down-regulated expression of Rab27B gene by siRNA significantly reduced the secretion of CK-MB and LDH by cardiomyocytes infected with CVB3 virus (P < 0.05; Fig. 4D and E). These results suggest that Rab27B promotes injuries of cardiomyocytes induced by CVB3 infection.

Rab27B facilitates the synthesis and release of cytokines TNF-α and IL-6 by cardiomyocytes

To test whether Rab27B affects the synthesis and release of TNF-α and IL-6 by cardiomyocytes, Western blotting and ELISA were performed, respectively. Western blotting showed that expression of TNF-α and IL-6 proteins in cardiomyocytes in CVB3+siR-Rab27B group was significantly reduced compared with that in CVB3+NC group (P < 0.05) (Fig. 5A and B). ELISA showed that release of TNF-α and IL-6 cytokines in supernatants of cardiomyocytes in CVB3+siR-Rab27B group was significantly lower than that in CVB3+NC group (P < 0.05) (Fig. 5C and D). The results indicate that Rab27B facilitates the synthesis and release of cytokines TNF-α and IL-6 by cardiomyocytes.

miR-133b binds to the 3'-UTR seed region of Rab27B mRNA

To identify the interaction between miR-133b and the 3'-UTR of Rab27B mRNA, dual luciferase reporter assay was performed. The fluorescence value of cells co-transfected with miR-133b mimics and pMIR-REPORT-WT luciferase reporter plasmids was significantly lower than that in NC group (P < 0.05). By contrast, the fluorescence value of cells co-transfected with miR-133b mimics and pMIR-REPORT-mutant luciferase reporter plasmids was not significantly different from that in NC group (P > 0.05) (Fig. 6). The result suggests that miR-133b binds to the 3'-UTR seed region of Rab27B mRNA.

Discussion

Viral myocarditis is a common disease in children. It is reported that the incidence of viral myocarditis in children has increased significantly in recent years. Because of the atypical clinical manifestations of viral myocarditis, untimely diagnosis and treatment will seriously affect the prognosis of the children (20). The main pathological changes in viral myocarditis are cardiomyocyte injuries, including direct cardiomyocyte damages caused by virus and immune injuries (21).
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Abundant production and release of cytokines IL-6 and TNF-α play important roles in myocardial injuries. Our data show that production and release of IL-6 and TNF-α by cardiomyocytes infected by CVB3 are higher than NC group, while overexpression of miR-133b has decreased the production and release of IL-6 and TNF-α by the infected cardiomyocytes, suggesting that miR-133b inhibits the synthesis and release of IL-6 and TNF-α. In addition, Targetscan (www.targetscan.org) shows that Rab27B is a target gene of miR-133b. It is reported that Rab27B protein is a member of Ras-related small GTP enzyme superfamily that has specific subcellular localization, playing important roles in the processes of cell secretion, endocytosis, signal transduction and development (28). Rab27B also plays important roles in tumor proliferation, invasion and metastasis (29), but its effect in myocardial injuries is rarely reported. Our data demonstrate that Rab27B expression is enhanced in cardiomyocytes infected by CVB3, and overexpression of miR-133b reduces the expression of Rab27B in the infected cardiomyocytes. Moreover, silenced expression of Rab27B promotes the proliferation of cardiomyocytes infected by CVB3, reduces the contents of CK-MB and LDH released by the cells, and decreases the levels of TNF-α and IL-6 in the cells and culture supernatants. Dual luciferase reporter assay discovers that miR-133b directly binds to the 3'-UTR of Rab27B, suggesting that Rab27B is a target gene of miR-133b. In the present study, we have identified Rab27B as a target gene of miR-133b. Actually, miRNAs can regulate multiple targets, so we will further investigate mechanism of miR-133b in the future. In conclusion, miR-133b expression in peripheral blood from children with viral myocarditis is reduced, and negatively correlated with the levels of CK-MB and LDH, the markers for myocardial injuries. miR-133b inhibits the proliferation of cardiomyocytes and the release of cytokines TNF-α and IL-6 by targeting Rab27B, and alleviates myocardial injuries. Therefore, miR-133b is a potential diagnostic and therapeutic target for viral myocarditis.

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Disclosures

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