MicroRNA-486-5p down-regulation protects cardiomyocytes against hypoxia-induced cell injury by targeting IGF-1

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Abstract: Background: MicroRNA has been reported to play an important role in congenital heart disease (CHD) in children. Recently, microRNA-486-5p (miR-486-5p) has been found increased in patients with cyanotic heart disease compared with those without heart disease. The present study aimed to investigate the effect of miR-486-5p on hypoxia-induced cardiomyocyte injury to reveal the role of miR-486-5p in cyanotic congenital heart disease (CCHD). Methods: Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to determine the expression of miR-486-5p in hypoxia-induced H9C2 cells. Dual luciferase reporter gene assay was used to confirm IGF-1 was a direct target of miR-486-5p. miR-486-5p inhibitor and IGF-1-siRNA were transfected into H9C2 cells. The cell viability was detected by MTT. Cell apoptosis was detected using flow cytometry. The expression of IGF-1, Bcl-2, caspase-3, caspase-9, and Bax mRNA and protein were detected by RT-qPCR and western blotting, respectively. Results: miR-486-5p expression gradually increased with prolonged hypoxia time in H9C2 cells. Dual luciferase reporter gene results confirmed IGF-1 was a direct target of miR-486-5p. In addition, inhibition of miR-486-5p significantly increased the hypoxia-induced decrease in cell survival and attenuated hypoxia-induced apoptosis. Furthermore, inhibition of miR-486-5p significantly attenuated the hypoxia-induced decrease in the level of IGF-1 and Bcl-2 and the increase in pro-apoptotic proteins such as caspase-3, caspase-9 and Bax. These effects could be reversed by IGF-1-siRNA. Conclusion: The data demonstrated that inhibition of miR-486-5p increased cardiomyocyte growth and reduced cell apoptosis under hypoxic conditions by targeting IGF-1, indicating that miR-486-5p may be an effective target for the treatment of CCHD.

Keywords: Cyanotic congenital heart disease, miR-486-5p, IGF-1, cell viability, apoptosis

Introduction

Cyanotic congenital heart disease (CCHD) is the most common innate malformation in infants and children, in which chronic hypoxia is the basic pathophysiologic process [1]. Chronic hypoxia is a key pathologic change in patients with CCHD. Studies have reported that patients with CCHD are in a state of hypoxic perfusion. They can still survive a certain time, and rarely develop into heart failure [2], but the detailed molecular mechanism is still unknown. Therefore, studying the protective response mechanism of cardiomyocytes to chronic hypoxia may serve as an in-depth study of the treatment of myocardial injury in cardiac surgery.

MicroRNAs (miRNAs) are endogenous, small noncoding RNAs of 19-22 nucleotides that negatively regulate gene expression by interaction with 3’-untranslated (UTR) regions of target mRNAs at the posttranscriptional or translational level [3]. miRNAs have been demonstrated to participate in a broad range of cardiac pathophysiology and cardiovascular function [4, 5]. Previous study has reported that miR-486-5p is involved in many physiologic and pathologic events, including cell proliferation, migration, and differentiation [6, 7]. miR-486-5p has been reported increased in patients with cyanotic heart disease compared with those without heart disease. Insulin-like growth factor-1 (IGF-1) is an essential regulator of cardiomyocyte...
miR-486-5p and congenital heart disease

homeostasis, such as promoting cell growth, inhibiting apoptosis, and signaling [8]. miR-486-5p directly regulates IGF-1 expression in HCC tumor progression [9]. However, the possible effects of miR-486-5p on hypoxic adaptation in cardiomyocytes has not been well-studied.

Therefore, the aim of the present study was to investigate the role of miR-486-5p in cyanotic congenital heart disease and to reveal a possible mechanism.

Materials and methods

Cell culture and cell treatment

Embryonic rat heart-derived H9C2 cells were purchased from American Type Culture Collection (Manassas, VA, USA) and incubated in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco, USA).

After serum-starving overnight, cells were maintained in an atmosphere containing a gaseous mixture of 94% N₂, 5% CO₂, and 1% O₂ at 37°C for durations of 12, 24, 48, and 72 h respectively.

Cell transfection

miR-486-5p inhibitor, IGF-1-siRNA, and their negative controls were synthesized from Ribobio (Guangzhou, China). H9C2 cells were transfected with miR-486-5p inhibitor, inhibitor control, IGF-1-siRNA, control-siRNA, or miR-486-5p inhibitor+IGF-1-siRNA using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instruction. After 48 h of transfection, the efficiency of transfection was analyzed using qRT-PCR.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA from cells were extracted using TRizol (Invitrogen; Thermo Fisher Scientific Inc.) according to the manufacturer’s protocol. 1 µg of total RNA was reverse-transcribed into cDNA using TaqMan microRNA Reverse Transcription kit (Invitrogen), following the manufacturer’s protocol. PCR analysis was performed using SYBR Premix Ex Taq GC kit (Takara, Japan) on an ABI PRISM 7900 HT sequence-detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). miR-486-5p expression was detected using a mirVana™qRT-PCR microRNA detection kit (Life Technologies; Thermo Fisher Scientific, Inc.). The primer sequences used were as follows: miR-486-5p-F: 5’-ACACTCCAGCTGGTGCTACTGAGGTCGCCC-3’; miR-486-5p-R: 5’-TCAACTGGTGTCTAAGGAA-3’; IGF-1-F: 5’-AATTTTTTCCGTCGCTG-3’; IGF-1-R: 5’-TCTGTCTATCGTATGTTTAC-3’; Caspase-3-F: 5’-TACCTCTAATGGGTGTGTG-3’; Caspase-3-R: 5’-GTTAACACGAGGAGGATG-3’; Caspase-9-F: 5’-GCACATATACAACCCCAAT-3’; Caspase-9-R: 5’-GCTGGACACCCACCTTA-3’; Bax-F: 5’-GTGCCCAGTTAGTCAGGAC-3’; Bax-R: 5’-CCATGTTGGTGTTCCCAT-3’; Bcl-2-F: 5’-CCAGCGTGTTGTCGAGTCATAAT-3’; Bcl-2-R: 5’-ATGCTTCAATGAGGCAATCCACC-3’; GAPDH-F: 5’-GAGTTGCTGGTGTTTCCCTG-3’; GAPDH-R: 5’-GAGTTGCTGGTGTTTCCCTG-3’; Bax-F: 5’-GTGCCCAGTTAGTCAGGAC-3’; Bax-R: 5’-CCATGTTGGTGTTCCCAT-3’; Bcl-2-F: 5’-CCAGCGTGTTGTCGAGTCATAAT-3’; Bcl-2-R: 5’-ATGCTTCAATGAGGCAATCCACC-3’; GAPDH-F: 5’-GAGTTGCTGGTGTTTCCCTG-3’; GAPDH-R: 5’-GAGTTGCTGGTGTTTCCCTG-3’. U6-F: 5’-TGTTAGGTGACAATTCGCCACT-3’; U6-R: 5’-CGCTGAGGCTGACCCGATG-3’. GAPDH and U6 were used as the internal control for mRNA and miRNA expression, respectively. We used the 2⁻ΔΔCq method to quantify relative gene expression [10].

Western blot assay

Total protein from H9C2 cells was extracted using lysis buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) after 24 h of incubation. BCA assay (Thermo Fisher Scientific, Inc.) was used to measure the protein concentrations. Equal amounts of protein (25 µg/lane) were subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF; Roche). Then, the membranes were blocked with 5% skim milk for 1 h at room temperature, and subsequently incubated with primary antibodies: IGF-1 (1:1000, Cell Signaling Technology, USA), anti-caspase 3 (1:1000, Cell Signaling Technology, USA), Bcl-2 (1:500; Santa Cruz Biotechnology), Bax (1:500; Santa Cruz Biotechnology), caspase-9 (1:500; Santa Cruz Biotechnology), and GAPDH (1:200; Santa Cruz Biotechnology) overnight at 4°C. Then we incubated with an appropriate horseradish peroxidase-linked secondary antibody at room temperature for 1 h. Protein bands were visualized using an enhanced chemiluminescence system (Pierce) according to the manufacturer’s instructions and quantified using Quantity One Version 4.6 Image software (Bio-Rad).
miR-486-5p and congenital heart disease

MTT assay

Cell survival was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. H9C2 cells was seeded in 96-well plates and incubated in hypoxic conditions for 72 h. After the hypoxic treatment, 20 µl MTT (5 mg/ml, Sigma) was added into each well and further cultured for 4 h. Then, 150 µl dimethyl sulfoxide/well (DMSO, Sigma) was added and incubated for 30 min at 37°C. Absorbance at 490 nm was measured using a microplate spectrophotometer (BioRad Laboratories, CA; USA).

Luciferase reporter assay

Targetscan software was used to predict the potential target of miR-486-5p. To confirm the relation between miR-486-5p and IGF-1, the 3’UTR of IGF-1 mRNA containing the putative target site for miR-486-5p was amplified by PCR from genomic DNA and cloned into the psi-CHECKTM-2 vector (Promega). The 100 ng psi-CHECK-2 luciferase reporter plasmids containing either the wild-type or the mutated IGF-1 3’UTR and miR-486-5p mimic (100 nM) or mimic control (100 nM) were cotransfected into H9C2 cells for 48 h using Lipofectamine 2000 (Invitrogen). 48 h later, luciferase activity in these transfected cells was measured with the Dual Luciferase Assay System (Promega, Madison, WI, USA) according to manufacturer’s instructions. Firefly luciferase was used to normalize the Renilla luciferase.

Flow cytometry

Cell apoptosis was analyzed by flow cytometry. Briefly, after hypoxic induction for 72 h, H9C2 cells were harvested and re-suspended with binding buffer according to the manufacturer’s instructions. Annexin V-FITC (10 µl) and propidium iodide (PI, 5 µl) was added into H9C2 cells and stained for 10 min in the dark at room temperature. The samples were immediately analyzed by FacsCalliburflow cytometer (BD Biosciences, Franklin Lakes, NJ; USA).

Statistical analyses

All data are expressed as mean ± SD. Statistical analyses were performed using SPSS software version 18.0 (IBM Corp., Armonk, NY, USA). Comparisons among various groups were performed using one-way analysis of variance (ANOVA) or Student’s t-test. P < 0.05 was considered significant.

Results

miR-486-5p was up-regulated in the hypoxic cardiomyocytes

H9C2 cells were exposed to hypoxia for 0, 12, 24, 48 and 72 h. The expression levels of miR-486-5p were gradually increased in a time-dependent manner (Figure 1). These results indicated that miR-486-5p was up-regulated in the hypoxic cardiomyocytes.

IGF-1 was a target of miR-486-5p

TargetScan predicted IGF-1 was a possible target gene of miR-486-5p (Figure 2A). To confirm whether miR-486-5p can directly target the IGF-1 3’UTR, a luciferase reporter plasmid containing wild type or mutant IGF-1 3’UTR were co-transfected with the miR-486-5p inhibitor into HEK293T. As shown in Figure 2B, miR-486-5p inhibitor significantly reduced the luciferase reporter activity of wild type IGF-1 3’UTR, but not of the mutant IGF-1 3’UTR.

Down-regulation of miR-486-5p increased hypoxia-induced cardiomyocyte cell survival

To investigate the potential role of miR-486-5p in the hypoxic cardiomyocytes, H9C2 cells were transfected with miR-486-5p inhibitor, inhibitor control, IGF-1-siRNA, or siRNA control. Transfection efficiencies were detected by qRT-PCR. As
shown in Figure 3A, 3B, miR-486-5p inhibitor transfection caused a significant decrease of miR-486-5p expression (Figure 3A) and IGF-1-siRNA transfection caused a significant decrease of IGF-1 (Figure 3B and 3C). In addition, miR-486-5p inhibitor resulted in the up-regulation of mRNA and protein expression of IGF-1 in hypoxia-induced H9C2 cells, which was reversed by IGF-1 siRNA (Figure 3D and 3E).

MTT assay was used to detect cell viability. As shown in Figure 3F, after 72 h exposure to hypoxia, the cell viability was significantly decreased compared with the control group. Downregulation of miR-486-5p efficiently increased H9C2 cell viability in hypoxic conditions at 72 h, which was reversed by IGF-1-siRNA transfection.

**Down-regulation of miR-486-5p inhibited hypoxia-induced cardiomyocyte apoptosis**

To detect the effect of miR-486-5p on hypoxia-induced apoptosis, flow cytometry was used. As shown in Figure 4, the percentage of cell apoptosis at 72 h after the induction of hypoxia was significantly increased compared with the control group. Downregulation of miR-486-5p significantly decreased the apoptosis rate compared with the control group under hypoxic conditions, which was reversed by IGF-1-siRNA transfection. In addition, changes in apoptosis-related molecules were measured by western blot and qPCR. As shown in Figure 5, inhibition of miR-486-5p significantly increased the protein and mRNA expression levels of IGF-1 and Bcl-2, and decreased the protein and mRNA expression levels of caspase-3, caspase-9 and Bax under hypoxic conditions, which was reversed by IGF-1-siRNA transfection.

**Discussion**

Chronic hypoxia is a common pathophysiologic process in patients with CCHD [10, 12], which leads to high mortality and morbidity. In this study, miR-486-5p was found to be up-regulated in the hypoxia-induced H9C2 cells. In addition, downregulation of miR-486-5p significantly increased hypoxia-induced cardiomyocyte cell survival and inhibited hypoxia-induced cardiomyocyte apoptosis by targeting IGF-1. Therefore, inhibition of miR-486-5p might be a mechanism for cardioprotection against chronic hypoxia.

Abnormal miRNA expression has been reported to lead to CHD [13]. For example, higher levels of miR-138 and miR-23b expression were observed in CCHD patients compared with acyanotic CHD patients [14, 15]. A recent study has reported that miR-486-5p was increased in patients with cyanotic heart disease compared with those without heart disease [16]. Consistent with a previous report, the present study results revealed that higher levels of miR-486-5p were found in CCHD patients compared with the acyanotic CHD patients. miRNA mediates a variety of biologic processes, such as proliferation and apoptosis. miR-486-5p has been studied in a variety of cancer cells. A previous study has reported miR-486-5p inhibited colorectal cancer cell migration and invasion by targeting PIK3R1 [7], miR-486-5p inhibited proliferation and invasion of NSCLC cells by regulating GAB2 [17]. In this study, we found that inhibition of miR-486-5p significantly increased the cell proliferation and decreased apoptosis of H9C2 cells under hypoxic conditions. Therefore, miR-486-5p may be part of a mechanism resulting in the development of CCHD.

Caspase-3 has been recognized as an essential member of the caspase family involved in cellular degradation during apoptosis [18].
miR-486-5p and congenital heart disease

**Figure 3.** miR-486-5p down-regulation increased H9C2 cell viability which was decreased by hypoxic treatment. A: H9C2 cells were transfected with miR-486-5p inhibitor or inhibitor control for 48 h, then the level of miR-486-5p was detected using qRT-PCR; B and C: H9C2 cells were transfected with IGF-1-siRNA or control-siRNA for 48 h, then the mRNA and protein level of IGF-1 was detected using qRT-PCR and western blotting respectively; D and E: H9C2 cells were transfected with miR-486-5p inhibitor, inhibitor control, or miR-486-5p inhibitor+IGF-1-siRNA for 48 h; then the mRNA and protein levels of IGF-1 were detected using qRT-PCR and western blotting respectively; F: H9C2 cells were pre-transfected with/without miR-486-5p inhibitor, inhibitor control, or miR-486-5p inhibitor+IGF-1-siRNA for 6 h, then the cells were incubated for a further 72 h under/not-under hypoxic conditions. MTT assay was used to determine cell viability. Data are expressed as mean ± SD. **P < 0.01 vs. Control; ##P < 0.01 vs. Hypoxia; &P < 0.01 vs. inhibitor.

Bcl-2 family proteins are key regulators in cell apoptosis and include anti-apoptotic protein Bcl-2 and pro-apoptotic proteins Bax and Bad [19]. Marginal changes in Bcl-2/Bax ratios result in inhibition or promotion of cell death. In this study, miR-486-5p inhibition decreased the rate of cardiomyocyte apoptosis in vitro by increasing Bcl-2 expression and decreasing Bax, Caspase-3, and Caspase-9 levels.

IGF-1 is a critical cardiac survival factor that can increase protein metabolism and promote cell growth [20]. A previous study has reported that chronic hypoxemia has a direct or indirect effect to reduce serum IGF-1 concentrations in patients with CCHD [21]. IGF-1 can directly alleviate hypoxia-induced cardiomyocyte apoptosis [22, 23]. IGF-1 treatment has been reported to up-regulate Bcl-xl expression but down-regulates pro-apoptotic Bax protein in heart mitochondria to protect myocardium against ischemia-reperfusion injury [24]. The dual luciferase reporter gene assay results confirmed that miR-486-5p directly binds the 3’UTR of IGF-1, and decreases its expression level in H9C2 cells. Our study results suggested that inhibition of miR-486-5p in hypoxia-induced cardiomyocytes increased cell proliferation and decreased apoptosis by dysregulating expression of IGF-1 protein and mRNA.

In summary, our results showed that miR-486-5p was up-regulated in hypoxia-induced H9C2 cells. Down-regulation of miR-486-5p increased cell viability and reduced apoptosis of cardiomyocytes under hypoxic conditions by targeting IGF-1. These results of the present study indicated that miR-486-5p may be a critical target for the clinical treatment of patients with CCHD.

**Disclosure of conflict of interest**

None.

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miR-486-5p and congenital heart disease

**Figure 4.** miR-486-5p down-regulation decreased H9C2 cell apoptosis increased by hypoxic treatment. H9C2 cells were pre-transfected with/without miR-486-5p inhibitor, inhibitor control, or miR-486-5p inhibitor+IGF-1-siRNA for 6 h, then the cells were incubated for a further 72 h under/not-under hypoxic conditions. FCM was used to determine cell apoptosis. Data are expressed as mean ± SD. **P < 0.01 vs. Control; ##P < 0.01 vs. Hypoxia; &&P < 0.01 vs. inhibitor.
miR-486-5p and congenital heart disease

Figure 5. Effect of miR-486-5p down-regulation on apoptosis-related gene expression in H9C2 cells. H9C2 cells were pre-transfected with/without miR-486-5p inhibitor, inhibitor control, or miR-486-5p inhibitor+IGF-1-siRNA for 6 h, then the cells were incubated for a further 72 h under/not-under hypoxic conditions. Western blot and qRT-PCR were used to detect the protein and mRNA levels of IGF-1, Bcl-2, Bax, Caspase 3 and Caspase 9. Data are expressed as mean ± SD. **P < 0.01 vs. Control; ##P < 0.01 vs. Hypoxia; &&P < 0.01 vs. inhibitor.

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miR-486-5p and congenital heart disease


