Original Article

MiR-423-5p inhibits human cardiomyoblast proliferation and induces cell apoptosis by targeting Gab 1

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Abstract: Background: Dilated cardiomyopathy (DCM) is a heart disease characterized by ventricular dilatation, systolic dysfunction, and progressive heart failure. MicroRNAs are important regulators of gene expression, influencing the progression of DCM. This study aimed to reveal the role of microRNA-423-5p (miR-423-5p) in human cardiomyoblast cell (HCM) and its potential mechanisms. Materials and methods: Serum samples were obtained from DCM patients and used to analyze miRNAs levels compared to healthy control. After transfected with miR-423-5p mimics in HCM cells or miR-423-5p mimics+pcDNA3.1+HA-Gab 1, the functions of proliferation and apoptosis were investigated. Quantitative polymerase chain reaction analysis and western blot were performed to detect the expression of related proteins in HCM cells. The target gene of miR-423-5p was determined by luciferase assay and western blot. Results: The expression level of miR-423-5p was dramatically increased in DCM patients. Introduction of miR-423-5p significantly suppressed the proliferation and induced apoptosis of HCM cells. Luciferase reporter assay identified the 3'-UTR of Grb2-associated binders 1 (Gab 1) mRNA contained a complementary sequence for miR-423-5p. Gab 1 re-introduction could reverse the anti-proliferation and pro-apoptosis role of miR-423-5p. Conclusions: Our study provided a better understanding of miR-423-5p function in DCM development, which may also be benefit for the development of miRNA-directed diagnostic and therapeutic against DCM.

Keywords: Dilated cardiomyopathy, microRNAs, human cardiomyoblast cell, Gab 1, proliferation, apoptosis

Introduction

Dilated cardiomyopathy (DCM) is the third most common inherited type of heart disease [1], which is characterized by ventricular enlargement and systolic dysfunction [2]. It affects approximately 35.6 in 100,000 people and results in sudden cardiac death [3, 4]. Treatment of DCM is mainly on minimizing the effect of heart failure on the patient and delaying disease progression [5]. However, the five year mortality rate for patients with DCM is approximately 50%. Thus, it's an urgent need to develop new effective treatments.

MicroRNAs (miRNAs) are short (~22 nucleotides (nt) in length) non-coding RNAs (ncRNAs) that regulate gene expression by binding to the 3’ untranslated region (UTR) of target messenger RNA (mRNA) and promoting their degradation and/or translational repression [6, 7]. Numerous studies have demonstrated that some miRNAs are not only found intracellularly, but also detectable outside cells, including various body fluids such as blood, saliva, urine and etc [8]. Recent evidences reveal that these circulating miRNA or c-miRNAs can be detected in peripheral blood make them potentially useful to aid diagnosis or to guide therapy in human disease, such as cardiovascular disease [9]. Takahashi R et al first found in mice that the plasma level of the cardio-specific miR-208a is increased after myocardial injury induction [10]. In a study conducted by Widera et al, Patients with myocardial infarction presented higher levels of miR-1, miR-133a and miR-208b compared with patients with unstable angina [11]. Tijsen et al evaluated the plasma levels of several miRNAs in patients with acute HF, and revealed that the increase of miR-423-5p was a
strong diagnosis predictor of HF [12]. However, the mechanisms involved in increasing circulating levels of miR-423-5p in patients with DCM are unknown.

Grb2-associated binders (Gab) genes encoding mammalian Gab 1, which is a member of the insulin receptor substrate 1 (IRS1)-like multi-substrate docking adaptor protein family [13, 14]. Gab 1 is tyrosine-phosphorylated in response to many growth factors (including vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), nerve growth factor (NGF)) [13-15], propagating signals that are essential for cell proliferation and motility. Research suggests that Gab 1 is essential for cardioprotection against I/R oxidative injury [16]. In addition, it has been reported that Gab 1 may have the redundant roles for maintenance of cardiac function via neuregulin-1/ErbB signaling [17].

In this study, we hypothesized that miR-423-5p suppressed cardiomyocytes proliferation and induced cells apoptosis through directly regulating Gab 1 expression in HCM cells.

**Materials and methods**

**Participants and samples**

We recruited 12 DCM patients (47 ± 12 years, 33% males) with New York Heart Association (NYHA) function classes IeIV (1/Class I, 5/Class II, 4/Class III, 2/Class IV), and a control group of 12 healthy volunteers (47 ± 11 years, 33% males) in the study. Serum samples were obtained from patients and volunteers. All of these patients agreed to participate in this study and gave written informed consent. Both the study and consent were complied with the Declaration of Helsinki. The Institutional Review Board of the Zhejiang Province Hospital of Integrated Traditional Chinese and Western Medicine Hospital approved the study protocol.

**Cell lines and cell culture**

HCM human cardiomyoblast cell line was purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. They cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Bioind, Israel) and 1% penicillin/streptomycin sulfates. Cells were incubated in a humidified incubator supplemented with 5% CO₂ at 37°C.

**Plasmid transfection**

Hsa-miR-423-5p mimics (pGCMV/EGFP) and empty vector (NC) were synthesized by GenePharma (Shanghai, China). Human Gab 1 gene was constructed into pcDNA3.1+HA empty vector by Life Technologies (Invitrogen, CA), and the empty vector was served as the negative control. Plasmids were transfected into subconfluent cells according to the manufacturer’s instructions using Lipofectamine 2000 (Invitrogen-Life Technologies, CA) (DNA/Lipofectamine 2000=1/2.5). Stable transfection expression of cell lines were established after cells had been incubated in complete DMEM with blasticidin (12 µg/mL) (Sigma, Shanghai, China) or G418 (500 mg/ml) (Sigma, Shanghai, China) for 15 days. We verified clones using western blot and real-time quantitative polymerase chain reaction (RT-PCR) and pooled the successful clones for further experiments.

**Cell proliferation assay**

The Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay was used for cell proliferation analysis following the manufacturer’s instruction. HCM cells with established stable expression (NC, miR-423-5p mimics, miR-423-5p mimics+pcDNA3.1+HA empty vector, miR-423-5p mimics+pcDNA3.1+HA-Gab 1) were seeded at a density of 5×10³ cells per well in 96-well plates and incubated for various periods of time (0 h, 24 h, 48 h, 72 h). The absorbance at 450 nm was measured using an electroluminescence immunosorbent assay reader (Thermo Fisher Scientific, Waltham, MA).

**Flow cytometry analysis of cell apoptosis**

Cells were collected and washed twice with cold phosphate-buffered saline solution (PBS) to remove floating cells before analysis by the Annexin V-APC Apoptosis Detection Kit (KeyGEN Biotech, Nanjing, China). Apoptosis were evaluated with a flow cytometry analyzer (BD Biosciences, San Jose, CA). Data were analyzed by BD Accuri C6 software.

**Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling assay**

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) (Roche, Shanghai, China) assay has been designed to detect apoptotic cells that undergo extensive...
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DNA degradation during the late stages of apoptosis [18]. Cells were fixed with 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and incubated with the In situ cell death detection kit from Roche at 37°C for 1 h. After that, the samples were mounted in mounting media containing DAPI. Fluorescent images were captured using fluorescence microscope at 20X magnification. The total number of DAPI positive cells and total number of TUNEL positive cells were counted from at least five images from each sample.

Caspase-3 activity analysis for cell apoptosis

Caspase-3 activity was determined using the caspase-3 colorimetric activity assay kit (Beyotime, Shanghai, China) according to the manufacturer’s instructions. Briefly, cells were collected and washed twice with cold PBS and lysed with the cell lysis buffer included in the kit. Equal amounts of protein were incubated with Ac-DEVD-AMC, a caspase-3 substrate, at 37°C for 2 h. Caspase-3 activity was determined by measuring the absorbance at 405 nm using an electro luminescence immunosorbent assay reader.

Isolation of RNA and quantitative polymerase chain reaction analysis

Total RNA was extracted from serum or cultured cells using TRIzol (Invitrogen, Shanghai, China) following the manufacturer’s protocols, miRNA-specific RT primers (Ribobio, Guangzhou, China) for miR-423-5p and random primer (TaKaRa, Dalian, China) for Gab 1. Reverse-transcribed cDNA was measured by quantitative polymerase chain reaction (qPCR) using an Applied Biosystems 7500 Fast Sequence Detection System and SYBR Green PCR Kit (QIAGEN, Shanghai, China) under the following conditions: denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing and extension at 60°C for 30 seconds. The relative miRNA and mRNA expression levels were normalized by U6 and β-actin, respectively.

Reverse transcription primer miR-423-5p

5’-GTCGTATCCAGTGCTGTCGTGGAGTCGGCAATTGCACTGGATACGACAAAGTCTC-3’. The qPCR primers

miR-423-5p: 5’-GCCTGAGGGGCAGAGAGC-3’ (forward), 5’-CCACGTGTCGGTGGAC-3’ (reverse);

Gab 1: 5’-ATCAGAAACGCCAGCGAAGA-3’ (forward), 5’-TCAGATACCACAAGCACCACCA-3’ (reverse).

Western blotting

Cells were subjected to lysis in the presence of a protease and phosphatase inhibitor mixture (3 µL) and then homogenized. Collected the supernatant fraction and measured the protein concentration with bicinchoninic acid protein assay kit (Beyotime, Hangzhou, China). An aliquot of 20 µg of denatured protein from each sample was subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane, followed by blocking with 5% nonfat milk and incubation of membranes at 4°C overnight with primary antibody (1:1000 dilution), the membranes then were incubated with the secondary antibody, rabbit horseradish peroxidase-conjugated anti-goat IgG (1:2000 dilution; both, Cell Signaling Technology, Boston, MA) for 1 h at ambient temperature. The blots were then incubated with enhanced chemiluminescence solution for 1 min. Final signals were detected by the ChemiDoc XRS+ Chemiluminescence imaging system (Bio-Rad, Hercules, CA) and processed by Image Lab Software (both, Bio-Rad, Inc., Hercules, CA, USA). The gray value of each band in the imaging data was analyzed by Quantity One software (Bio-Rad, Inc. CA).

Luminescent reporter gene transfection and luciferase assays

The potential miR-423-5p binding sites of Gab 1 were predicted by using the TargetScan and miRanda database. For the luciferase activity assay, the mRNA 3’-UTR sequences of the Gab 1 (WT) was PCR amplified and inserted downstream of the Renilla luciferase gene in a Renilla/firefly luciferase reporter plasmid, psiCHECK-2 (GenePharma, Shanghai, China). The forward primer sequences for the mRNA 3’-UTR of Gab 1 was 5’-ATCAGAAACGCCAGCGAAGA-3’ and reverse primer sequences was 5’-TCAGATACCACAAGCACCACCA-3’. Gab 1 mRNA 3’-UTR contained sequences with mutations (MUT) in the putative binding sites of miR-423-5p was chemically synthesized by GenePharma. HCM cells were transfected with WT or MUT constructed reporter plasmids and miR-423-5p mimics or mimics control by using Lipofectamine 2000. 24 h after transfection, Renilla/firefly luciferase activity was measured by a dual luciferase reporter assay (Promega, Madison, WI).
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in an automatic microplate reader (Thermo Scientific, Waltham, MA).

Statistical analyses

Data were presented as the mean value ± SD and analyzed with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance was determined by an analysis of variance (ANOVA) or two-tailed Student t-test. Statistical graphs were used GraphPad 6.0 software. A $p$-value <0.05 was considered statistically significant. All experiments were performed at least three independent times.

Results

Expression level of miRNAs in DCM

The expressions of miRNAs were investigated by Quantitative real-time PCR methodologies in our patients. We selected four miRNAs (namely miR-155, miR-146a, miR-126 and miR-423-5p), which were reported to be aberrant expressed in serum of DCM patient [19-21]. The results revealed no significant difference in the expressions of miR-155, miR-146a and miR-126 ($P=0.517$, $P=0.109$, $P=0.052$, respectively) (Figure 1A). However, the expression of miR-423-5p in DCM showed significant up-regulation compared with healthy control ($P<0.01$) (Figure 1B).

Figure 1. The expression level of miRNAs in DCM serums. A-D: Analysis of the expressions of four miRNAs (miR-155, miR-146a, miR-126 and miR-423-5p) in DCM patient’s serums or the healthy control by qPCR. The expressions of miRNAs in the DCM serums were normalized by a calibrator sample from the healthy control. U6 was used as an internal reference, **$P<0.01$.

MiR-423-5p overexpression inhibits the proliferation of HCM cells

As shown in the Figure 2A, the expression of miR-423-5p was significantly increased in HCM cells after treatment with miR-423-5p mimics ($P<0.001$). CCK8 results revealed that ectopic over-expression of miR-423-5p could dramatically inhibit proliferation ability of HCM cells at 48h and 72 h (all panels, $P<0.01$), while no sig-
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Figure 2. MiR-423-5p overexpression inhibits the proliferation of HCM cells. A. After transfection with miR-423-5p mimics, the expression of miR-423-5p in HCM cells was significantly decreased compared with the controls (NC) (P<0.05) (Figure 2B). In addition, we also found that restoration of miR-423-5p decreased the protein expression levels of PCNA and ki-67 in HCM cells (Figure 2C).

Flow cytometry results showed that both early and late apoptotic cell populations were increased when miR-423-5p was overexpressed in HCM cells (all panels, P<0.01) (Figure 3A, 3B). Apoptosis was also measured using TUNEL assay, overexpression of miR-423-5p could significantly increase percentage of TUNEL positive cells compared with NC (all panels, P<0.01) (Figure 3C, 3D). Meanwhile, Caspase-3 activity and the protein expression levels of NF-κB, Bcl-2 and Bax in HCM cells were all increased dramatically after transfection with miR-423-5p mimics (all panels, P<0.01) (Figure 3E, 3F).

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Significant difference was found at 24 h compared with the controls (NC) (P>0.05) (Figure 2B). In addition, we also found that restoration of miR-423-5p decreased the protein expression levels of PCNA and ki-67 in HCM cells (Figure 2C).

Gab 1 is a direct target of miR-423-5p

TargetScan and miRanda predicted the putative target genes of miR-132 in human cells. Among which, we found that 3’-UTR of Gab 1 mRNA harbored a potential target site for miR-423-5p (Figure 4A). Luciferase reporter assay was then performed and result showed that miR-423-5p significantly suppressed the luciferase activity of the wild type (WT) but not the mutant (MUT) 3’-UTR of Gab 1 (P<0.05) (Figure 4B). Moreover, qPCR and western blot analyses revealed that over-expression of miR-423-5p significantly decreased the expression of Gab 1 in HCM cells (P<0.01) (Figure 4C-E). Therefore, our results suggest that Gab 1 might be a target of miR-423-5p in HCM cells.

Gab 1 contributes to miR-423-5p increased proliferation of HCM cells

PcDNA3.1+HA-Gab 1 were transfected to increase the expression of Gab 1 in HCM cells after transfection of miR-423-5p mimics. QPCR and western blot showed that the Gab 1 was increased in the cells transfected with pcDNA3.1+HA-Gab 1 compared with the pcDNA3.1+HA-empty plasmid (P<0.01) (Figure 5A-C).

As shown in Figure 5D, transfection of miR-423-5p mimic into HCM cells led to a decrease of cell proliferation, whereas Gab 1 re-introduction reversed the anti-proliferation role of miR-423-5p. Additionally, Western blot examined that overexpression of Gab 1 increased protein expression levels of PCNA and ki-67 in HCM cells transfected with miR-423-5p mimics (P<0.01) (Figure 5E).

Gab 1 contributes to miR-423-5p suppressed apoptosis of HCM cells

Gab 1 re-introduction reversed the pro-apoptosis role of miR-423-5p. Both apoptotic cell populations in Flow cytometry and percentage of TUNEL positive cells TUNEL assay were all decreased significantly when HCM cells transfected with miR-423-5p mimics+pcDNA3.1+HA-
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Figure 3. MiR-423-5p overexpression induces HCM cells apoptosis. HCM cells transfected with miR-423-5p mimics or empty vector. A: Early apoptotic cells (lower right, LR) and late apoptotic cells (upper right, UR) were shown. B: Histograms represent quantification of the rate of apoptosis. C: Representative images of the TUNEL assay. Blue bars represent TUNEL positive cells, and green bars represent DAPI positive cells. D: Apoptosis index represent the percentage of TUNEL positive cells in HCM cells. E: Histograms represent quantification of the rate of Caspase-3 activity. F: The protein expression levels of NF-κB, Bcl-2 and Bax in HCM cells. APC: Allophycocyanin; PI: propidium iodide. **P<0.01.

Gab 1 (all panels, P<0.01) (Figure 6A-D). Moreover, Caspase-3 activity and the protein expression levels of NF-κB, Bcl-2 and Bax in HCM cells were also decreased dramatically after transfected with miR-423-5p mimics+ pcDNA3.1+HA-Gab 1 (all panels, P<0.01) (Figure 6E, 6F).

Discussion

In the present study, we showed that the serum levels of miR-423-5p were significantly increased in DCM patients compared with healthy controls. Over-expression of miR-423-5p inhibited proliferation and induced apoptosis of HCM cells. More importantly, we identified Gab 1 as a direct target gene of miR-423-5p in HCM cells. Restoration of Gab 1 partly reversed miR-423-5p induced anti-proliferation and pro-apoptosis of HCM cells.

Previous reports demonstrated a potential pathophysiological link between expression of miRNAs (miR-155, miR-146a, miR-126 and
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related with circulating miRNAs in our study and miRNAs from myocardial tissues in theirs. Goren et al reported that plasma levels of miR-423-5p were higher in HF patients than in controls [12]. In addition, Tijsen et al found that

Figure 4. Gab 1 is a direct target of miR-423-5p. A: The predictable binding sites between miR-423-5p and Gab 1 was shown. B: MiR-423-5p inhibited the luciferase activity in the Gab 1 with WT binding sites 3’UTR, whereas, luciferase activity was not decreased in the MUT binding sites 3’UTR of Gab 1 mRNA. C-E: qPCR and Western blotting analyzes were used to detect the expression levels of Gab 1 expression after transfected with miR-423-5p mimics or mimics control in HCM cells. WT: wild type; MUT: mutant. **P<0.01.

Figure 5. Gab 1 contributes to miR-423-5p increased proliferation of HCM cells. A-C: After transfection with miR-423-5p mimics+ pcDNA3.1+HA-Gab 1, the expression of Gab 1 mRNA and protein. D: Cell proliferation ability was detected by CCK8 assay in HCM cells transfected with miR-423-5p mimics or miR-423-5p mimics+pcDNA3.1+HA-Gab 1 or miR-423-5p mimics+pcDNA3.1+HA empty vector. E: The protein expression levels of PCNA and ki-67 in HCM cells transfected with miR-423-5p mimics or miR-423-5p mimics+pcDNA3.1+HA-Gab 1 or miR-423-5p mimics+pcDNA3.1+HA empty vector. **P<0.01.

miR-423-5p) and human DCM [19, 20, 22]. In our study, we did not confirm dysregulation of miR-155, miR-146a and miR-126 serum levels in DCM patients, which is in contrast to the prior studies [23, 24]. We suspect this might be
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miR-423-5p was able to distinguish heart failure from healthy controls and patients with dyspnea attributable to other causes [25]. These findings were consistent with our results.

In our study, Over-expression of miR-423-5p significantly inhibited proliferation and induced apoptosis of HCM cells. Luciferase reporter assay identified the 3'-UTR of Gab 1 mRNA contained a complementary sequence for miR-423-5p. Up-expression of miR-423-5p significantly decreased the expression of Gab 1. Additionally, restoration of Gab 1 partly reversed miR-423-5p functions in HCM cells. So we thought that Gab 1 may be involved in miR-423-5p mediated HCM proliferation and apoptosis. Sun et al reported that cardiomyocyte-specific Gab 1 knockout mice exhibited an increase in infarct size and a decrease in cardiac function after ischemia/reperfusion (I/R).

Figure 6. Gab 1 contributes to miR-423-5p suppressed apoptosis of HCM cells. HCM cells transfected with miR-423-5p mimics or miR-423-5p mimics+pcDNA3.1+HA-Gab 1 or miR-423-5p mimics+pcDNA3.1+HA empty vector. A: Early apoptotic cells and late apoptotic cells were shown. B: Histograms represent quantification of the rate of apoptosis. C: Representative images of the TUNEL assay. D: Apoptosis index represent the percentage of TUNEL positive cells. E: Histograms represent quantification of the rate of Caspase-3 activity. F: The protein expression levels of NF-κB, Bcl-2 and Bax in HCM cells. **P<0.01.
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Previous research revealed that Gab 1 may be involved in maintenance of cardiac function via neuregulin-1/ErbB signaling [17]. These findings are consistent with our data.

Gab 1 can be recruited to activate receptor tyrosine kinases (RTKs) through direct and indirect mechanisms. Interactions between Gab 1 and effector molecules were found to be critical for transducing Gab-mediated signaling [26], in which Gab-p85 association plays an important role in activating the PI3K/Akt pathway in mammalian cells. Once activated, PI3K/Akt pathway could promote cell proliferation and inhibit apoptosis mediated by nuclear factor kappa B (NF-κB) activation. In addition, it regulates Bcl-2 family members, promoting the BCL-2 resistance to apoptosis and inhibition BAX promoting apoptosis. Moreover, PI3K/Akt activation makes caspase3 phosphorylation and result in anti-apoptosis. These findings are confirmed in our study.

In summary, our study indicated that miR-423-5p was up-regulated in DCM patients. Overexpression of miR-423-5p inhibited proliferation and induced apoptosis of HCM cells by targeting Gab 1. Our study provided a better understanding of miR-423-5p function in DCM development, which may also be benefit for the development of miRNA-directed diagnostic and therapeutic against DCM. However, this must be verified in further studies, including animal trials and prospective clinical studies.

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Disclosure of conflict of interest

None.

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