MicroRNA-423-5p targets O-GlcNAc transferase to induce apoptosis in cardiomyocytes

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Abstract. Congestive heart failure (CHF) remains the single most prevalent cause of mortality and morbidity; therefore, the identification of novel biomarkers is required for the diagnosis of CHF. The aim of the present study was to examine whether microRNA (miR)-423-5p is a novel biomarker for CHF, which was defined by the circulatory biomarker pro-brain natriuretic peptide (BNP). Samples of plasma from patients with CHF and healthy controls were collected and used for quantitative polymerase chain reaction analysis. In addition, the O-GlcNAc transferase (OGT) 3' untranslated region was cloned and used for a luciferase assay. The effects of the expression of miR-423-5p in cardiomyocytes were determined using western blotting and terminal deoxynucleotidyl transferase-deoxyuridine triphosphate nick-end labeling analyses. The results demonstrated that miR-423-5p was associated with CHF and the expression levels of proBNP; in addition, OGT was found to be a direct target of miR-423-5p. The expression of miR-423-5p significantly regulated the expression of OGT and its associated downstream targets and induced apoptosis in the cardiomyocytes. Therefore, the results of the present study indicated that miR-423-5p was involved in CHF via the direct targeting of OGT and the induction of apoptosis in cardiomyocytes.

Introduction

The post-translational β-O-linkage of N-acetylglucosamine (O-GlcNAc) to cellular proteins represents one signaling pathway, which has been implicated in the pathophysiology of cardiovascular disease (1). O-GlcNAc transferase (OGT), which adds O-GlcNAc to proteins, has been demonstrated to be required for cell division and embryogenesis (2). Cardiomyocytes ablation of OGT induces heart failure and OGT is required as part of the endogenous compensatory response to infarct-induced heart failure (3). In developed countries, the single most prevalent cause of mortality and morbidity is congestive heart failure (CHF) (4). CHF accounts for ~5,000,000 hospitalizations annually in developed nations, which results in the treatment of CHF being a major expenditure in the health care budgets of all developed nations, and is also becoming an increasing expense for those of numerous developed nations. CHF is known to occur as a common result of a wide range of etiologies; however, the propensity to develop CHF varies significantly following a given insult (4,5). This is considered to be due to responses, including inherited variation in myocardial and vascular responses. However, the factors underlying the ultimate development of CHF remain to be fully elucidated. The clinical management of CHF is enabled by the identification of circulating biomarkers, including brain natriuretic peptide (BNP) (6,7). However, there remains a requirement for the identification of novel circulating biomarkers as objective indicators of CHF, which can be readily and reliably measured.

MicroRNAs (miRNAs) were first identified in mammals as a large class of evolutionarily conserved small, non-coding RNAs. These are involved in the regulation of gene expression at the post-transcriptional level through targeted interactions with the 3'-untranslated region (UTR) of messenger RNA (mRNA) transcripts (8). miRNAs are important regulators of a wide range of biological processes by affecting protein translation (8). In addition, miRNAs can be efficiently suppressed for prolonged periods using antisense technologies, which has been of increasing interest due to its potential therapeutic use in cardiovascular diseases through the targeted inhibition of specific miRNAs (9). A study in 2008 revealed that miRNAs were also present in blood, where they were detected in plasma, platelets, erythrocytes and nucleated blood cells. Plasma miRNAs were found to remain stable, even under conditions of stress, including boiling, low or high pH, long-term storage at room temperature and multiple freeze-thaw cycles (10-12). These characteristics suggest that miRNAs may be a suitable biomarker for disease diagnosis.

In previous years, miR-423-5p has been investigated widely as a potential biomarker for heart diseases. Array investigations have reported that miR-423-5p is upregulated in human failing myocardium (13). Tijsen et al (14) demonstrated that circulating levels of miR-423-5p are increased in patients with clinical heart failure, defined by the Framingham criteria and elevated...
pro-brain (pro)BNP levels. This indicated that the levels of miR-423-5p levels were associated with proBNP and ejection fraction in this patient group (14). However, the association between miR-423-5p and CHF and the underlying mechanism remain to be elucidated. Therefore, elucidating the direct target of miR-423-5p and the associated pathways in cardiomyocytes is essential for understanding the association between miR-423-5p and heart disease, and to determine why miR-423-5p fails as a biomarker for systemic ventricular and left ventricular remodeling (15,16).

The present study aimed to examine the role of miR-423-5p in the development of CHF through investigating the expression of miR-423-5p in the plasma of patients with CHF and healthy volunteers. The expression levels of miR-423-5p, proBNP, OGT and the downstream targets of OGT, and apoptosis in the cardiomyocytes were examined.

Materials and methods

Clinical study. The present study was approved by the ethics committee of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital (Chengdu, China). Human plasma samples were obtained with informed consent under a general waiver by the Academic Medical Center institutional review board for the proper secondary use of human material. Plasma samples were obtained from the patients and healthy volunteers at the People's Hospital of Sichuan Province and experiments were performed on samples at the Sichuan Academy of Medical Science. Written informed consent was obtained from the patients and volunteers.

Definition of CHF diagnosis. The subjects were classified as CHF cases in accordance with the Framingham criteria for CHF diagnosis (17) and if they had circulating proBNP levels of >1,000 pg/ml. The subjects were classified as non-CHF cases if the clinical diagnosis excluded CHF and if circulating proBNP levels were below the age-associated cut-off points. In total, 51 of the 74 subjects screened for CHF fulfilled the criteria.

Cell culture and transfection. Mouse cardiomyocytes were obtained from Shanghai Cell Bank (Shanghai, China). The cardiomyocytes were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco-BRL, Carlsbad, CA, USA). The cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. Cell transfection was performed using FuGENE® HD Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions. Briefly, the cardiomyocytes were seeded into six-well plates at a density of 2x10⁵ cells/well and cultured for 24 h to reach 70-80% confluence. Plasmids (2 µg), encoding mouse (m)-miR-423-5p and miR-Ctrl, were diluted in 100 µl media without serum. Subsequently, 5 µl FuGENE® HD Transfection Reagent was added to tubes containing the diluted DNA, mixed, incubated with the transfection complex for 15 min at room temperature and then added to the six-well plates. A total of 24 or 48 h post-transfection the cells were collected for total mRNA and protein extraction. miR-Ctrl was transfected for 48 h. Medium (DMEM containing 10% FBS) alone was used as the blank agent. Cardiomyocytes was placed in a six-well plate at a density of 2x10⁵ cells/well. After 24 h, puromycin (Beyotime Biotechnology, Shanghai, China) was used to treat cardiomyocytes at 0, 5, 10, 20 or 40 µM. The cell vitality was observed in the following 48-72 h. The terminal concentration was found to be 20 µM. Consequently, after plasmid transfection for 48 h, 20 µM puromycin was added to treat cardiomyocytes. The surviving cells were selected as the stable cells which expressed the plasmid.

Bioinformatics analysis. To analyze the potential targets of miR-423-5p, online miRNA databases were used. TargetScan (http://www.targetscan.org/) and microRNA.org (http://www.microrna.org/microrna/getGeneForm.do) were used to predict the potential targets of miR-423-5p, and O-GlcNAc was identified as a target. Further study was performed to determine whether O-GlcNAc is the direct target of miR-423-5p.

Luciferase assays. The miR-423-5p binding site was synthesized and cloned into an Ambion pMIR-REPORT vector (Ambion Life Technologies, Carlsbad, CA, USA) to generate pMiRluc-423-5p. Briefly, each miRNA and plasmid oligonucleotide were annealed at 90°C for 3 min, cooled to 37°C, and incubated for 1 h. The annealed dsDNA oligonucleotides were ligated between HindIII and SacI (Fermentas, Thermo Scientific, Waltham, MA, USA) sites on the pMIR-REPORT vector. The resulting recombiant plasmids were named pMiR-miR-423-5p and pMiR-miR-Ctrl. The two constructs were verified by DNA sequencing (Invitrogen Life Technologies, Carlsbad, CA, USA). Plasmids were extracted using EndoFree Plasmid Giga kits (Qiagen GmbH, Hilden, Germany) from DH5α (Geneviz, Suzhou, China) Escherichia coli transformants and stored at -20°C until further use. The concentration was determined by measuring the A260/A280 ratio using a Thermo ND 2000 spectrophotometer (Thermo Scientific). The 3'-UTRs of O-GlcNAc transferease (OGT) containing miR-423-5p binding sites were amplified and cloned into the same vector to generate pMiRluc-OGT. The reporter was co-transfected with a cytomegalovirus β-galactosidase vector using FuGENE HD® (Roche Diagnostics). Luciferase activity was measured 4 h later using a Luciferase Reporter assay (Promega Corporation, Madison, WI, USA). Values were normalized to the activity of β-galactosidase.

26S proteasome activity assay. The function of the 26S proteasome was assayed, as described previously (17). Briefly, the cells were seeded at a density of 2x10⁵ cells/well and cultured for 24 h, until they had reached 70-80% confluence. The cells were then washed with phosphate-buffered saline (ZsBio, Beijing, China), and then with buffer I, containing 150 mM Tris (pH 7.4), 2 mM diithio- retiol, 5 mM MgCl₂, and 2 mM adenosine triphosphate (ATP), and pelleted by centrifugation (Mini Centrifuge #166-0623; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 200 x g for 5 min. Glass beads (Beyotime Biotechnology) and homogenization buffer, containing 50 mM Tris (pH 7.4), 1 mM diithiothriol, 5 mM MgCl₂, 2 mM ATP and 250 mM sucrose, were added and vortexed (MixPlus; Aibenseng Bio, Hefei, China) for 1 min. The beads and cell debris were removed by centrifugation at 1,000 x g for 5 min and then at 10,000 x g for 20 min. The protein concentrations were determined using a bicinchoninic acid assay (Pierce Biotechnology, Inc., Rockford, IL, USA), according to the manufacturer's instructions. The protein from each sample (100 µg) was diluted with buffer I to a final volume of 1,000 µl, and Suc-LLVY-7-amido-4-methylcoumarin fluoro-
genic proteasome substrate (chymotrypsin-like; Sigma-Aldrich, St., Louis, MO, USA) was added to a final concentration of 80 µM in 1% dimethyl sulfoxide (Sigma-Aldrich). In order to access the 20S function, the buffer I was replaced with an ATP-free buffer containing 20 mM HEPES (pH 7.8) 0.5 mM EDTA and 0.03% SDS (18). Cleavage activity was monitored continuously by detection of free 7-amido-4-methylcoumarin (AMC) using a fluorescence plate reader (Gemini; Molecular Devices, Sunnyvale, CA, USA) at 380/460 nm and 37°C. As controls, the AMC (2 µM) was incubated with the drugs in buffer I without the cell extracts, and measurements of proteasome function were corrected when necessary.

Quantitative polymerase chain reaction (qPCR). Total RNA was extracted from each experimental group using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instructions. RNA concentration was assessed spectrophotometrically at 260 nm (Thermo ND 2000; Thermo Scientific). Reverse transcription was performed on the isolated total RNA using a Reverse Transcription kit (Takara Bio, Inc., Otsu, Japan), and PCR was carried out using a Real Time PCR kit (Takara Bio, Inc.). Reverse transcription was performed at 65°C for 5 min, 30°C for 10 min, 42°C for 10-30 min and 92°C for 3 min. PCR conditions were as follows: Denaturation at 94°C for 2 min; amplification for 30 cycles at 94°C for 0.5 min, annealing at 60°C for 0.5 min, and extension at 72°C for 1 min; followed by a terminal elongation step at 72°C for 10 min, and was performed on a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, Inc.). U6 was amplified as an internal control and the Ct value of each PCR product was calculated, and the fold change was analyzed. The m-miR-423-5p, h-miR-423-5p, m-U6 and h-U6 primers were supplied by Ribio Biotechnology (Guangzhou, China); the sequences were not supplied due to the rules of the company.

Detection of miR-423-5p downstream targets using western blot analysis. The cardiomyocytes were transfected with n-miR-423-5p or miR-Ctrl for 24 or 48 h, as described above, following which the total protein was collected. The cells were lysed on ice for 30 min using radioimmunoprecipitation assay lysis buffer, containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM NaVO₃ and 1 mM NaF. The proteins (20 µg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Beyotime Biotechnology) and electronically transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). Following blocking with 5% skim milk powder in tris-buffered saline with Tween, the membranes were incubated with recommended dilution primary antibodies against OGT (rabbit polyclonal; 1:800 for 1 h at 37°C; cat. no. sc-32921; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), phosphorylated (p)-adenosine monophosphate (AMP)-activated protein kinase (AMPK; cat. no. 4188; 1:1,000), AMPK (cat. no. 2532; 1:1,000) p53 (cat. no. 2527; 1:1,200) and caspase 3 (cat. no. 9662; 1:600) for 1 h at 37°C (all purchased from Cell Signaling Technology, Inc., Danvers, MA, USA), and GAPDH (cat. no. sc-25778; 1:5,000; Santa Cruz Biotechnology, Inc.). This was followed by incubation with peroxidase-conjugated secondary antibodies (Abcam, Cambridge, MA, USA). The peroxidase-labeled bands were visualized using an enhanced chemiluminescence kit (Pierce Biotechnology, Inc.). The ratio of OGT, p-AMPK, AMPK, p53 and caspase 3/GAPDH were calculated using densitometry.

Terminal deoxynucleotidyl transferase-deoxyuridine triphosphate nick-end labeling (TUNEL) assay. In order to detect apoptotic cells in cardiomyocytes following transfection with miR-423-5p and miR-Ctrl, a TUNEL assay was performed using a DeadEnd® Fluorometric TUNEL system (Promega Corporation), according to the manufacturer’s instructions. Cell nuclei with dark green fluorescent staining were defined as TUNEL-positive nuclei, which were visualized using a fluorescence microscope (DTX500; Nikon Corporation, Tokyo, Japan). In order to quantify the TUNEL-positive cells, the number of green fluorescence-positive cells were counted in randomly selected fields at x200 magnification. The cell nuclei were then counter-stained with 4,6-diamidino-2-phenylindole (Beyotime Biotechnology).

Statistical analysis. Statistical comparisons of all the results were analyzed using a one-way analysis of variance. Statistical analyses were performed using SPSS version 19.0 (IBM SPSS, Armonk, NY, USA). Values are expressed as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-423-5p is upregulated in patients with CHF. In order to investigate whether the expression of miR-423-5p was altered in patients with CHF, plasma samples from patients and healthy controls were collected and qPCR was performed to determine...
the expression levels of circulating miR-423-5p in the patients with CHF and the healthy controls. The results demonstrated that the expression of miR-423-5p was upregulated in the patients, with CHF compared with the healthy controls (Fig. 1A). The circulating protein levels of the proBNP biomarker of CHF were also determined and the results indicated that the circulating levels of miR-423-5p were positively correlated with the expression of proBNP and the CHF classification (Fig. 1B).

OGT is a target of miR-423-5p. To determine the targets of miR-423-5p, a number of potential target proteins in a database library were screened to identify potential miRNA binding seed sequences within the 3'-UTR. Using bioinformatic analyses (Targetscan, microRNA.org and microRNASeq), it was found that OGT was a potential candidate target for miR-423-5p (Fig. 2A). In order to verify this, OGT-3'UTR containing an miR-423-5p binding site were cloned downstream of the reporter construct.
of the luciferase open reading frame. The m-OGT-3’UTR mutant, which contained a mutated m-miR-423-5p binding site, was also introduced into the luciferase construct. The plasmid expressing m-miR-423-5p was transfected into the cardiomyocytes, and puromycin (Beyotime Biotechnology) was used to select the stable expression cells. qPCR analysis confirmed that m-miR-423-5p was upregulated in these cells (Fig. 2B). The luciferase-OGT-3’UTR and luciferase-OGT-3’UTR mutant constructs were then transfected into the 293 cells stably expressing m-miR-423-5p. Following 4-6 h transfection, the expression of luciferase in the OGT-3’UTR constructs was significantly downregulated compared with that of the miR-Ctrl, whereas luciferase activity was unaffected in the cells transfected with the mutant construct (Fig. 2C).

m-miR-423-5p regulates the expression levels of OGT and downstream targets of OGT in cardiomyocytes. The effects of m-miR-423-5p transfection were investigated in cardiomyocytes from mice due to the limited use of human cardiomyocytes in investigations. The plasmid encoding m-miR-423-5p was transfected into the mouse cardiomyocytes and incubated for 24 or 48 h. The cells were then collected and qPCR analysis confirmed that miR-423-5p was upregulated in these cells (Fig. 2B). The luciferase-OGT-3’UTR and luciferase-OGT-3’UTR mutant constructs were then transfected into the 293 cells stably expressing m-miR-423-5p. Following 4-6 h transfection, the expression of luciferase in the OGT-3’UTR constructs was significantly downregulated compared with that of the miR-Ctrl, whereas luciferase activity was unaffected in the cells transfected with the mutant construct (Fig. 2C).

Figure 4. miR-423-5p induces apoptosis in cardiomyocytes. (A) miR-423-5p was transfected into cardiomyocytes and total protein was collected 24 and 48 h post-transfection. Western blotting and densitometric analyses were used to determine the protein expression levels of p53 and caspase-3. GAPDH was used as a loading control. (B) Cardiomyocyte apoptosis was determined using a TUNEL assay following transfection with miR-423-5p for 48 h. Positive cells are indicated in green and DAPI staining indicates the whole cells (magnification, x200). (C) Quantitative analysis of apoptosis (apoptotic index = number of apoptotic cells/total number of cells). Data are presented as the mean ± standard error of the mean. **P<0.01, vs. miR-Ctrl, *P<0.05 and ##P<0.01, vs. miR-423-5p transfected for 24 h. miR-423-5p, microRNA-423-5p; miR-Ctrl, control plasmid; TUNEL, terminal deoxynucleotidyl transferase-deoxyuridine triphosphate nick-end labeling; DAPI, 4',6-diamidino-2-phenylindole.
Discussion

The results of the present study demonstrated that the expression of miR-423-5p was positively correlated with CHF diagnosis and the plasma levels of proBNP. In addition, the results indicated that OGT was a target of miR-423-5p; therefore, it was hypothesized that miR-423-5p directly targeted the OGT 3’-UTR, inhibiting the expression of OGT and inducing apoptosis in the cardiomyocytes. Furthermore, the inhibition of OGT by miR-423-5p significantly decreased the expression of p-AMPK and upregulated the activity of the 26S proteasome and the protein expression levels of pro-apoptotic p53 and caspase-3. The TUNEL analysis also revealed increased apoptosis in the cardiomyocytes following transfection with miR-423-5p.

Previous evidence suggests that circulating miRNAs may be used as stable blood-based biomarkers in cancer (12). In particular, the expression levels of miR-1 and miR-208 cardiac miRNAs are elevated in plasma following myocardial injury, which has been suggested to occur due to the release of these miRNAs from the damaged cardiac cells (20,21). The results of a previous array study reported that miR-423-5p is upregulated in the failing human myocardium (13) and circulating levels of miR-423-5p have been associated with proBNP and ejection fractions in this patient groups (14). However, the use of miR-423-5p as a biomarker for systemic ventricular and left ventricular remodeling has been unsuccessful (15,16). The results of the present study demonstrated that circulating levels of miR-423-5p were upregulated in the CHF patients and were associated with the expression of proBNP. In addition, the expression levels of miR-423-5p in the plasma may also provide novel biomarker for CHF. However, further clinical investigation is required in order to confirm this hypothesis.

The post-translational β-O-linkage of N-acetylgalactosamine (O-GlcNAc) to cellular proteins is an important signaling pathway, which has been implicated in the pathophysiology of cardiovascular disease (22). OGT enables the addition of O-GlcNAc to proteins and is required for cell division and embryogenesis (23). Cardiomyocyte ablation of OGT induces heart failure, and OGT is required for the endogenous compensatory response to infarct-induced heart failure (24). AMPK is present in all mammalian cells and is a sensor of cellular energy (25) and cellular redox status (26). As a downstream target of OGT, the reduction of p-AMPK by OGT in endothelial cells upregulates proteasome activity (27). The results of the present study indicated that OGT was a target of miR-423-5p; and that the expression of miR-423-5p in mouse cardiomyocytes significantly inhibited the expression of OGT and phosphorylation of the downstream target, AMPK. Of note, 26S proteasome activity and the expression levels of the p53 and caspase-3 pro-apoptotic proteins were markedly increased. Furthermore, according to the molecular changes observed in the TUNEL assay, the apoptotic rate of cells was increased in cardiomyocytes transfected with miR-423-5p.

References