Original Article
MicroRNA-18b* induces apoptosis in cardiomyocytes through targeting Topoisomerase 1 (TOP1)

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Abstract: miR-18b* was up-regulation in the plasma of Heart failure (HF) patients. However, the role of miR-18b* in Congestive Heart failure (CHF) is still unclear. In the present study, plasma from CHF patients and healthy controls was collected and used for miR-18b* detection by real-time PCR. Pearson analysis was performed to analyze the correlation between miR-18b* and proBNP. Furthermore, luciferase assay and western blotting were used to determine the potential target of miR18b*. Finally, the apoptosis cell in cardiomyocytes after miR-18b* transfection was determined by TUNEL assay. Our results firstly indicated that miR-18b* is up-regulated in the plasma from CHF patients and positively correlates with proBNP expression levels. Luciferase assay demonstrated that TOP1 is a direct target of miR-18b*. Over-expression of miR-18b* significantly inhibits TOP1 expression, induces the followed pro-apoptosis protein expression and apoptosis in cardiomyocytes. Collectively, miR-18b* corrects with CHF through direct targeting TOP1 and inducing apoptosis in cardiomyocytes. It maybe a novel target for CHF diagnosis and therapy.

Keywords: Congestive heart failure, miR-18b*, TOP1, cardiomyocytes

Introduction

Congestive Heart failure (CHF) is one of the most cause of mortality and morbidity all over the world [1]. And more than 5 million patients were hospitalization for CHF treating [1, 2]. Clinical management of CHF is facilitated by circulating biomarkers like brain natriuretic peptide (BNP) [3, 4]. However, novel biomarker is needed for simple and reliable measurement of CHF.

MicroRNAs (miRs) are endogenous, about 22-nucleotide (nt) ssRNAs that directly bind and suppress multiple mRNA targets through base-pairing of their "seed" sequences to the 3-9 untranslated region (UTR) or, less commonly, the coding regions [5]. By governing translation, they have been found to influence nearly every normal and pathological process examined [6, 7]. miRs are also found in blood, where they were detected in plasma, platelets, erythrocytes, and nucleated blood cells [8-10]. It was notably that plasma miRs are remarkably stable even under conditions as harsh as boiling, low or high pH, long-time storage at room temperature, and multiple freeze-thaw cycles [8-10]. Thus, miRNAs may be the best biomarker for disease diagnosis.

In the previous study by Tijsen, circulating levels of miR-18b* are increased in subjects with clinical heart failure defined by the Framingham criteria and elevated proBNP levels, and miR-18b* levels are related to proBNP and ejection fraction in this patient group [11]. However, the role of miR-18b* under CHF developing was still unclear. Thus, defining the direct target of miR-18b* and the related pathway in cardiomyocytes is essential for understanding the role of miR-18b* in CHF.

In the present study, we found that miR-18b* expression was higher in the CHF group, compared with the healthy group. The Pearson correlation analysis indicated there was a positively correlation between miR-18b* and proBNP expression in the plasma. Further results demonstrated TOP1 is a direct target of miR-18b* and over-expression miR-18b* inhibited TOP1 expression and induced followed pro-apoptosis protein expression in cardiomyocytes. Further

TUNEL assay also demonstrated that miR-18b* induces apoptosis in cardiomyocytes. Collectively, these findings indicate that miR-18b* corrects with CHF through direct targeting TOP1 and inducing apoptosis in cardiomyocytes.

Materials and methods

Clinical study

The clinical 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 (n=38) and healthy volunteers (n=13) 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 and if they had circulating proB-NP 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, 38 of the 51 subjects screened for CHF fulfilled the criteria.

Cell culture and transfection

Human cardiomyocytes were obtained from Shanghai Cell Bank (Shanghai, China) and cultured in Dulbecco's Modifed 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% CO_2 at 37°C. FuGENE® HD Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA) was used for cell transfection as previous study indicated [12]. At 24 or 48 h post-transfection, the transfected cells were collected for total mRNA and protein extraction. After transfection for 48 h, 20 μM puromycin (Beyotime Biotechnology, Shanghai, China) was used to treat cardiomyocytes. The surviving cells were selected as the stable cells which expressed the miR-18b*.

Quantitative polymerase chain reaction (qPCR)

TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) was performed to extract total RNA from each experimental group using, according to the manufacturer's instructions. RNA concentration was assessed by using Thermo ND 2000 (Thermo Scientific). 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 by using the Reverse Transcription kit (Takara Bio, Inc., Otsu, Japan). Real Time PCR kit (Takara Bio, Inc., Otsu, Japan) was employed for PCR. 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 58°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 h-miR-18b*, and h-U6 primers were supplied by Riob Bio Technology (Guangzhou, China); the sequences were not supplied due to the rules of the company.

Bioinformatics analysis and luciferase assay

The online miRNA databases TargetScan (http://www.targetscan.org/) and microRNA.org (http://www. microrna.org/microrna/get-GeneForm.do) were used to predict the potential targets of miR-18b*, and TOP1 was identified as a target of miR-18b*. Luciferase assay was performed to determine whether TOP1 is the direct target of miR-18b*. The miR-18b* binding site was synthesized and cloned into an Ambion pMIR-REPORT vector (Ambion Life Technologies, Carlsbad, CA, USA) to generate pMiR-miR-18b*. Plasmids were extracted using EndoFree Plasmid Giga kits (Qiagen GmbH, Hilden, Germany) from DH5α (Genewiz, Suzhou, China). The concentration was determined by Thermo ND 2000 spectrophotometer (Thermo Scientific). The 3'-UTRs of TOP1 containing miR-423-5p binding sites were amplified and cloned into the same vector to generate pMiR-luc-TOP1. 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.

Table 1. Information of Healthy Control and HF patients

Characteristic	Healthy controls	HF cases
Age	58 (range, 47-66)	61 (range, 52-71)
Gender		
Women	5 (38.5%)	17 (44.7%)
Men	8 (61.5%)	21 (55.3%)
proBNP (ng/L)	650 (range, 207-982)	2241 (range, 1029-4687)
miR-18b*	1.47 (range, 1.00-2.19)	2.44 (range, 0.95-4.29)
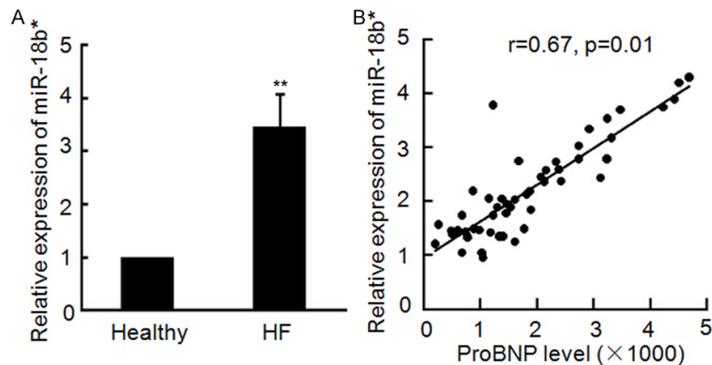
Figure 1. Expression of miR-18b* in plasma from CHF patients. A. Plasma from 38 CHF patients and 13 healthy controls is collected and used to determine miR-18b* by real-time PCR analysis. Data are shown as means ± SEM. **, P<0.01 compared to healthy controls; B. Pearson correlation between miR-18b* and proBNP. n=51; r=0.67; P=0.01.

Western blotting

Western blotting was performed as the previous study indicated [13]. Briefly speaking, the cells after treatment were lysed on ice for 30 min using radioimmunoprecipitation assay lysis buffer and protease inhibitor PMSF (Beyotime, Beijing, China). The proteins (10 μg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Beyotime Biotechnology) and electronically transferred onto a polyvinylidene difuoride membrane (Millipore, Bedford, MA, USA). The membranes were incubated with recommended dilution primary antibodies against TOP1, PARP, p53, cleaved caspase-9 (CC9) and cleaved caspase-3 (CC3) for 1 h at 37°C (all purchased from Cell Signaling Technology, Inc., Danvers, MA, USA), and GAPDH (Santa Cruz Biotechnology, Inc.). This was followed by incubation with peroxidase-conjugated secondary antibodies (Zsbio, Beijing, China). The peroxidase-labeled bands were visualized using an enhanced chemiluminescence kit (Millipore, Bedford, MA, USA). The ratio of TOP1/GAPDH was calculated using densitometry.

Terminal deoxynucleotidyl transferase-deoxyuridine triphosphate nick-end labeling (TUNEL) assay

In order to detect apoptotic cells in cardiomyocytes following transfection, a TUNEL assay was performed using a DeadEndTM Fluor metric 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 (DTX-500; Nikon Corporation, Tokyo, Japan). The number of green fluorescence-positive cells were counted in randomly selected fields at ×200 magnification. The cell nuclei were then counter-stained with 4',6-di-amidino-2-phenylindole (Beyotime Biotechnology).

Statistical analysis

Statistical comparisons of all the results were analyzed using Students'-test by 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. The correlation between ProBNP and relative expression of miR-18b* was analyzed using the Pearson correlation analysis using SPSS version 19.0 (IBM SPSS, Armonk, NY, USA). The r value and *P* value were both showed.

Results

MiR-18b is up-regulated in CHF patients*

To investigate the expression of miR-18b* in the plasma of CHF and healthy volunteers (**Table 1**), the total RNA in the plasma were exacted and used for further real-time PCR. As shown in **Figure 1A**, miR-18b* was up-regulated in CHF patients, compare with the healthy controls (**Figure 1A**). Furthermore, proBNP expression, as the biomarker of CHF patients, was also determined. The Pearson analysis indicated that circulating levels of miR-18b* was positively related with proBNP expression and CHF classification (**Figure 1B**).
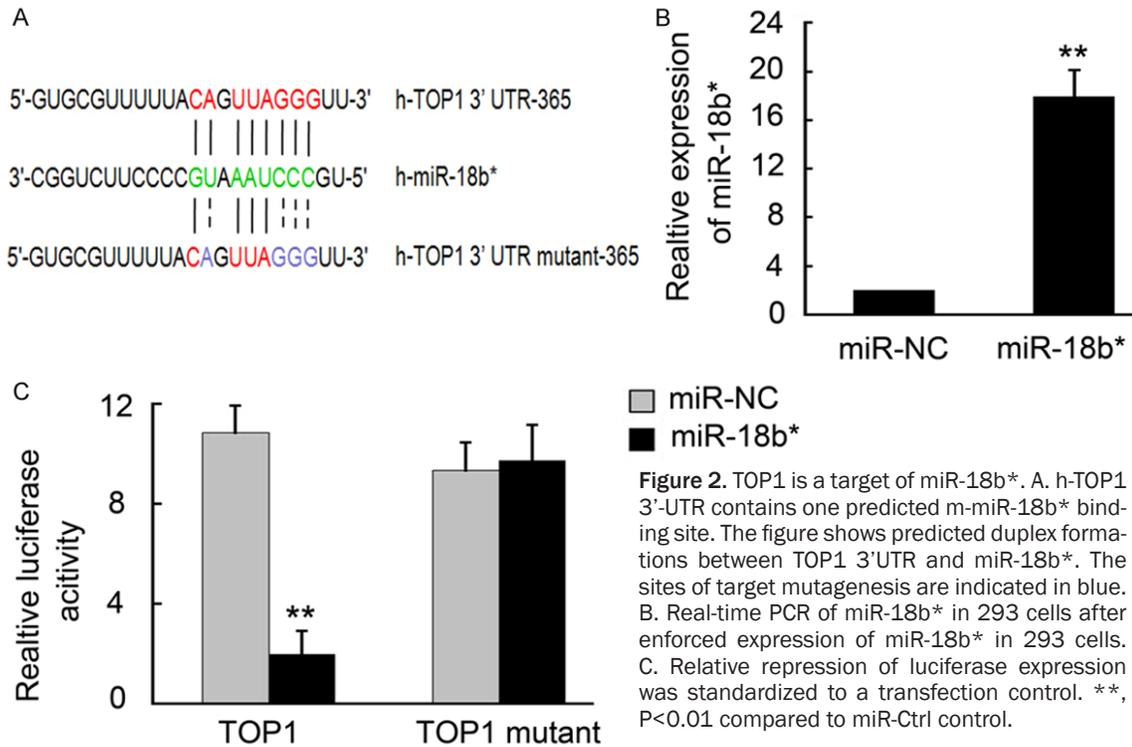
Figure 2. TOP1 is a target of miR-18b*. A. h-TOP1 3'-UTR contains one predicted m-miR-18b* binding site. The figure shows predicted duplex formations between TOP1 3'UTR and miR-18b*. The sites of target mutagenesis are indicated in blue. B. Real-time PCR of miR-18b* in 293 cells after enforced expression of miR-18b* in 293 cells. C. Relative repression of luciferase expression was standardized to a transfection control. **, $P < 0.01$ compared to miR-Ctrl control.

TOP1 is a target of miR-18b*

Through bioinformatic analyses (Targetscan micro, RNA.org, microRNASeq), TOP1 was predicated as a target for miR-18b* (**Figure 2A**). To determine this possibility, we constructed the luc-TOP1-3'UTR vector, containing miR-18b* binding site, and luc-TOP1-3'UTR mutant vector, containing mutated miR-18b* binding site. The plasmid expressing miR-18b* was transfected in 293 cells, and puromycin was used to select the stable expression cells. Real-time PCR analysis confirmed the over-expression of miR-18b* in the cells (**Figure 2B**). Then, the luc-TOP1-3'UTR and luc-TOP1-3'UTR mutant constructs were transfected into 293 cells stable expression miR-18b* and miR-NC, separately. And 4-6 hrs later, luciferase identification indicated that luciferase expression in TOP1-3'UTR constructs was significantly inhibited (**Figure 2C**). Whereas, no observable reduction of luciferase expression was found between miR-NC and miR-18b* cells transfected with luc-TOP1-3'UTR mutant plasmid (**Figure 2C**). These data indicated that TOP1 is a direct target of miR-18b*.

MiR-18b* regulated TOP1 and downstream targets expression in cardiomyocytes

To explore the effects of miR-18b* in cardiomyocytes, the plasmid encoding miR-18b* was transfected into the cardiomyocytes. 24 and 48 hrs later, cells was collected and real-time PCR indicated that miR-18b* expression was significant up-regulated from 24 hrs and last to 48 hrs post transfection (**Figure 3A**). Western blotting analysis indicated that over-expression of miR-18b* significantly inhibited TOP1 expression in cardiomyocytes (**Figure 3B**), both at 24 and 48 h post transfection. Further results indicated that the pro-apoptosis protein PARP, p53, cleaved caspase-3, cleaved caspase-9 expression was significantly induced by miR-18b* in cardiomyocytes (**Figure 3C** and **3D**). Collectively, miR-18b* inhibited TOP1 expression and regulated TOP1-related protein expression in cardiomyocytes.

MiR-18b* induced apoptosis in cardiomyocytes

Cardiomyocytes apoptosis was the main cause of CHF. So, in the next study, apoptosis was examined by TUNEL analysis. As shown in
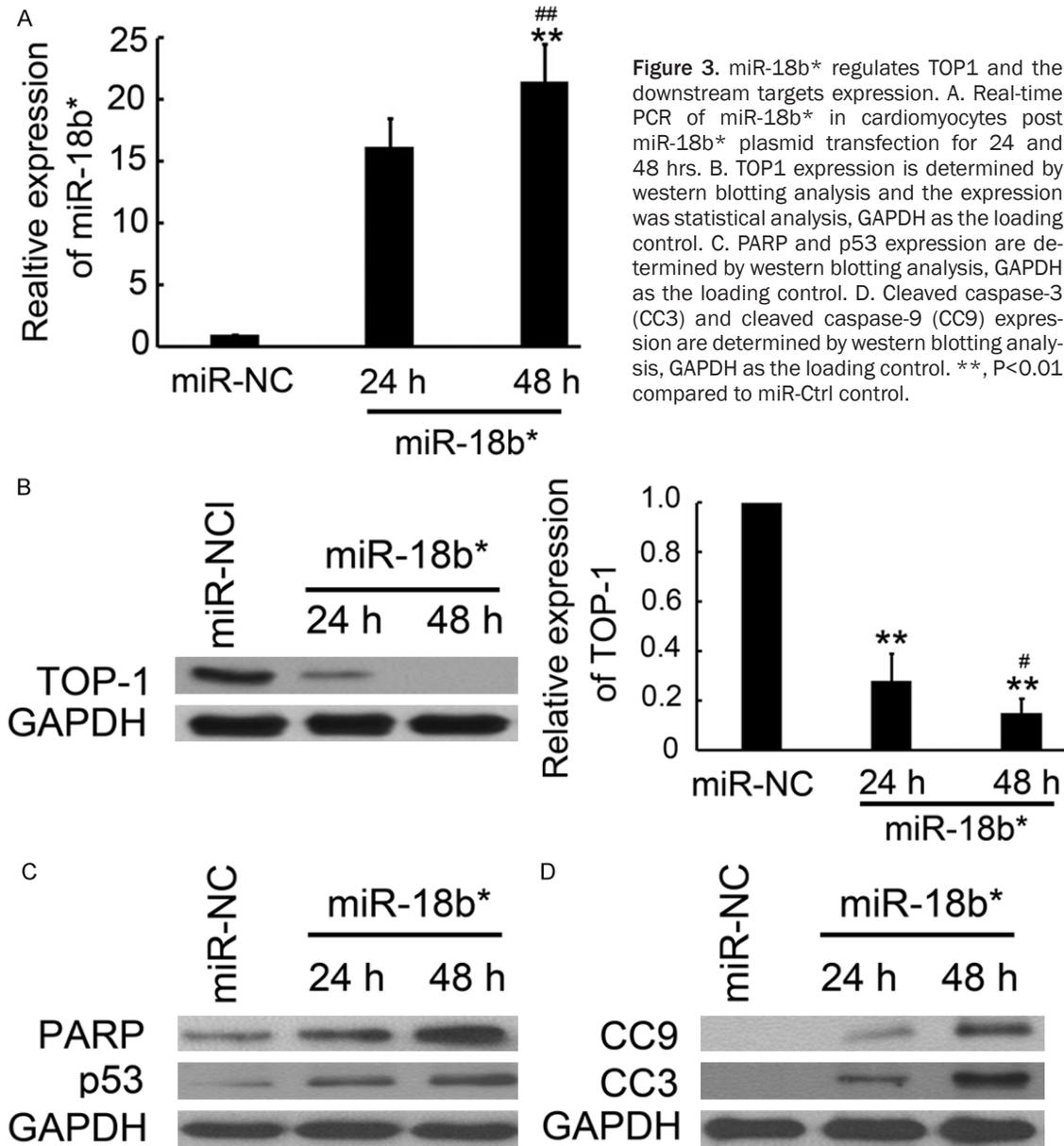
Figure 3. miR-18b* regulates TOP1 and the downstream targets expression. A. Real-time PCR of miR-18b* in cardiomyocytes post miR-18b* plasmid transfection for 24 and 48 hrs. B. TOP1 expression is determined by western blotting analysis and the expression was statistical analysis, GAPDH as the loading control. C. PARP and p53 expression are determined by western blotting analysis, GAPDH as the loading control. D. Cleaved caspase-3 (CC3) and cleaved caspase-9 (CC9) expression are determined by western blotting analysis, GAPDH as the loading control. **, P<0.01 compared to miR-Ctrl control.

Figure 4A and **4B**, more apoptosis cells were found in miR-18b* transfected cardiomyocytes, compare with the miR-Ctrl group. Collectively, these data demonstrated that up-expression of miR-18b* in cardiomyocytes significantly induce cell apoptosis.

Discussion

In this study, we firstly demonstrated that miR-18b* expression is positively corrects with CHF and proBNP expression in plasma. Luciferase analysis indicated that miR-18b* directly targets TOP1-3'-UTR to inhibit TOP1 expression.

Further results identified the induction role of miR-18b* in pro-apoptosis protein expression in cardiomyocytes, which were the downstream targets of TOP1. As the direct evidence, more apoptotic cardiomyocytes were found in the miR-18b* transfected group.

Various studies have demonstrated that circulating miRNAs might be used as the stable blood-based biomarkers for cancer and other diseases [10]. miR-1 and miR-208, are elevated in plasma following myocardial injury [14, 15]. Furthermore, miRNA array identified six miR-NAs that are elevated in patients with HF,

A

DAPI TUNEL

miR-NC

miR-18b*

B
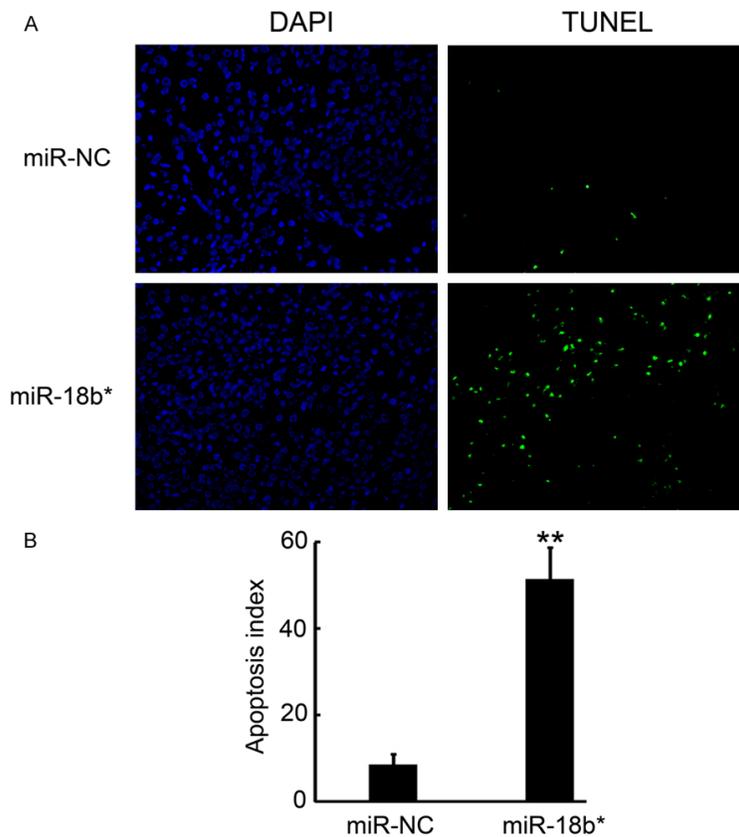
Figure 4. miR-18b* induce apoptosis in cardiomyocytes. A. Cardiomyocytes apoptosis after transfection for 48 hrs is determined by TUNEL assay, followed the kit instructions. The positive cells are indicated in green and DAPI indicate the total cells. B. Statistical analysis the apoptosis index (apoptosis index = the number of apoptosis cell/the number of total cell). **, P<0.01 compared to miR-Ctrl control. Original modification 200×.

DNA topological entanglements [18]. Furthermore, various studies indicated that TOP1 participated in the apoptosis and chemotherapy resistant in several cancer [19-21]. Inhibition of TOP1 with camptothecin significantly induced apoptosis in human embryonic stem cells [22]. In the present study, we firstly demonstrated that TOP1 is a direct target of miR-18b* by luciferase assay and indicated the inhibition role of miR-18b* on TOP1 expression in cardiomyocytes. Furthermore, western blotting results provided solid evidence for the induction role of miR-18b* on the pro-apoptosis protein, PARP, p53, cleaved caspase-3, cleaved caspase-9 expression, which were the downstream targets of TOP1. The direct evidence was supplied by TUNEL assay, more apoptotic cardiomyocytes were found in the miR-18b* transfected group.

Disclosure of conflict of interest

None.

including miR-423-5p, miR-18b*, miR-129-5p, miR-622, miR-1254 and HS_202.1 [11, 16]. Our previous study indicated that higher expression of miR-423-5p was found in the plasma from CHF patients, compared with the healthy group [12]. In this study, we firstly proved that circulating levels of miR-18b* are upregulated in CHF patients and positively correlated with proBNP expression. It is indicated that the expression level of miR-18b* in plasma maybe used as the novel biomarker for CHF diagnosis. But, more clinical samples are needed in the further study.

TOP1 is essential for embryonic development in mammals [17]. It binds to the regions of the pre-replicative complex in cells during the M, early G1, and G1/S phases of the cell cycle to control the firing of replication origins [18]. In addition, TOP1 also has functions in transcription that are independent of its role in resolving

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