MicroRNA-208a-3p participates in coronary heart disease by regulating the growth of hVSMCs by targeting BTG1

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Abstract. Human vascular smooth muscle cells (hVSMCs) are crucial in the progression of coronary heart disease (CHD). The present study aimed to investigate the role of microRNA-208a-3p (miR-208a-3p) in hVSMCs. Reverse transcription quantitative-PCR was performed to detect the levels of miR-208a-3p in the peripheral blood samples of patients with CHD and healthy volunteers. The results showed that miR-208a-3p was significantly upregulated in peripheral blood samples from patients with CHD compared with healthy volunteers. Bioinformatics analysis and dual-luciferase reporter assays indicated that B-cell translocation gene 1 (BTG1) was a direct target gene of miR-208a-3p, and was downregulated in the peripheral blood samples of patients with CHD. Furthermore, this study also suggested that miR-208a-3p served an inhibitory role in the proliferation of hVSMCs, induced cell apoptosis, promoted the protein expression of Bax and reduced Bcl-2 protein expression; however, these effects were reversed by BTG1 silencing. In addition, the role of the PI3K/AKT pathway in mediating hVSMC apoptosis was examined via western blot analysis. Results indicated that inhibition of miR-208a-3p decreased phosphorylated (p)-AKT protein expression levels and the ratio of p-AKT/AKT in hVSMCs; however, BTG1-small interfering RNA abolished these effects. Taken together, these findings revealed that miR-208a-3p served a critical role in CHD development, regulating hVSMC function via targeting of BTG1, which was associated with the PI3K/AKT signaling pathway. Therefore, downregulated miR-208a-3p may serve as an ideal therapeutic target for CHD diagnosis and therapy.

Introduction

Cardiovascular disease, in particular coronary heart disease (CHD), has become the leading cause of death worldwide (1,2). Emerging evidence has demonstrated that dysfunction of human vascular smooth muscle cells (hVSMCs) serves an important role in the progression of angioardiopathy, including CHD (3,4). Numerous studies have indicated that abnormal regulation of VSMCs is crucial in the progression of CHD (3-5). Furthermore, a number of reports have indicated that microRNAs (miRNAs/miRs) are indispensable regulators of VSMC function (6,7).

miRNAs, a family of small non-coding RNAs (~22 nucleotides), can regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of target mRNAs (8). miRNA functions have been reported in various biological behaviors, including cell proliferation, invasion and apoptosis (9). Previous reports have demonstrated that miRNAs are important regulators in the intricate biological progression of various cardiovascular diseases, including cardiac hypertrophy, heart failure, myocardial ischemia and reperfusion (10-14). However, to the best of our knowledge, little is known concerning the function of miR-208a-3p in the development of cardiovascular diseases in VSMCs. miR-208a-3p, the main sequence of miR-208a, has been reported to be associated with cardiovascular disease (15). Montgomery et al (16) have reported that downregulation of miR-208a improved cardiac function. In addition, miR-208 has been reported to be involved in various diseases, including colorectal cancer, gastric cancer and coronary artery disease (15,17,18). However, the molecular mechanism of miR-208a-3p in CHD remains to be elucidated.

B-cell translocation gene 1 (BTG1) is member of the BTG anti-proliferative protein family, which may promote cytochrome c translocation in mitochondria (19). In addition, it is a vital cofactor regulating certain biological behaviors, including cell proliferation and apoptosis (19). However, the potential role of BTG1 in VSMCs remains unclear.

The aim of the present study was to investigate the role of miR-208a-3p in CHD by examining its functional role in the regulation of hVSMC proliferation, and further examine the molecular mechanism. This study may provide a novel diagnostic and treatment target in CHD therapy.

Materials and methods

Clinical specimen collection. Peripheral blood samples (2 ml/individual) were extracted from 30 patients with CHD (age range, 43-75 years; 20 males and 10 females) and 30 healthy donors (age range, 46-77 years; 20 males and 20 females).

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10 females) at Shanxi Dayi Hospital between February 2017 and August 2018. The samples were immediately frozen and stored at -80°C until further use. Written informed consent was provided from all participants approving the use of their samples in the present study. All study protocols were approved by the Ethics Committee of Shanxi Bethune Hospital (Taiyuan, China). The inclusion criteria were as follows: Coronary angiography suggested that at least one main coronary artery or main branch had obvious coronary artery stenosis ≥50%, and the conclusion was judged by at least two experienced cardiologists. The exclusion criteria were as follows: i) Patients with myocardial bridge, cardiomyopathy, valvular heart disease or acute myocardial infarction; ii) patients with hypothyroidism, hyperthyroidism, hypothyalamic or pituitary disease and other endocrine diseases; iii) patients with cancer, acute cerebrovascular disease, severe infection, liver or kidney dysfunction and hereditary hyperlipidemia; and iv) patients with history of mental illness or family history of mental illness.

Cell culture. hVSMCs were purchased from American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium (HyClone; Cytiva) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C with 5% CO₂.

Cell transfection and reagents. A total of 100 nM miR-208a-3p inhibitor (5'-ACAAGCUUUUGUCUGUCUUAAU-3'; Shanghai GenePharma Co., Ltd.), 100 nM inhibitor control (5'-CAGUACUUUGUGAUAGCAAA-3'; Shanghai GenePharma Co., Ltd.), 1 µM control-siRNA, or 0.5 µM BTG1-siRNA was transfected into hVSMCs (5x10⁵ cells/well) or 100 nM miR-208a-3p mimic (sense, 5'-UAUAGACGACGAAAGAACCUUUGA-3'; and antisense, 5'-AGCUUUUUGUCUGUCAUUU-3'; Shanghai GenePharma Co., Ltd.) or 100 nM mimic control (sense, 5'-UUCUCGAGAGUGUCAGGUTT-3'; and antisense, 5'-ACUGUACACGUGCCGAAAT-3'; Shanghai GenePharma Co., Ltd.) using Lipofectamine 2000 according to the manufacturer’s protocols. Following 48 h of transfection, luciferase activity was detected using a Dual-Luciferase Reporter Assay System (Promega Corporation). Firefly luciferase activities were normalized to Renilla luciferase activity.

RNA isolation and RT-qPCR. Total RNA from cultured cells or blood specimens was extracted using TRizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. Total RNA (200 ng) was used to produce cDNA using PrimeScript RT Master Mix (Takara Biotechnology Co., Ltd.) using the RT primer 5'-CTCAACTGGTGCCTGTTGAGTCGGCAAATTCACGTGAGACCAACGCTT-3'. A SYBR PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd.) was used to evaluate the miR-208a-3p and BTG1 expression levels. The relative expression of mRNA or miR-208a-3p was normalized to GAPDH or U6, respectively. The thermostabilizing conditions were as follows: Initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 15 sec, 50°C for 30 sec, 72°C for 30 sec; and final extension at 72°C for 10 min. Primer sequences for PCR were listed as follows: miR-208a-3p forward, 5'-GGGCCATAAGACGACGAA-3' and miR-208a-3p reverse, 5'-CTCAACTGGTGCCTGTTGAGTCGGCAAATTCACGTGAGACCAACGCTT-3'; GAPDH forward, 5'-GCACGGTGTGCACCACAGG-3' and reverse, 5'-GACCTTGGAGTACGAGGCAG-3'; 18s forward, 5'-GGGCCATAAGACGACGAA-3'; and reverse, 5'-AGCTTGGAGTACGAGGCAG-3'. The results were calculated using the 2⁻ΔΔCq method (20).

Western blot analysis. hVSMCs were harvested and lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology). The concentration of total protein was calculated using a BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). The protein

Dual-luciferase reporter assay. Bioinformatics analysis using TargetScan (version 7.2; http://www.targetscan.org/vert_72/) was performed to predict the target gene of miR-208a-3p. The miR-208a-3p binding site was cloned into a pmirGLO dual-luciferase vector (Promega Corporation) to conduct the pmirGLO-BTG1 wild-type plasmid (BTG1-WT). The mutant (MUT) 3'-UTR of BTG1 was also cloned into pmirGLO to generate BTG1-MUT plasmids. 1 µg BTG1-WT and 1 µg BTG1-MUT were co-transfected into hVSMCs (5x10⁴ cells) with 100 nM miR-208a-3p mimic (sense, 5'-AUAAAGACGCAAAAAGUUGUCAUUU-3'; and antisense, 5'-AGCUUUUUGUCUGUCAUUU-3'; Shanghai GenePharma Co., Ltd.) or 100 nM mimic control (sense, 5'-UUCUCGAGAGUGUCAGGUTT-3'; and antisense, 5'-ACUGUACACGUGCCGAAAT-3'; Shanghai GenePharma Co., Ltd.) using Lipofectamine 2000 according to the manufacturer’s protocols. The luciferase reporter activity was performed using a Dual-Luciferase Reporter Assay System (Promega Corporation). The results were calculated using the 2⁻ΔΔCq method (20).
fraction were subsequently mixed with 5X SDS, boiled and centrifuged at 10,000 x g at 4°C for 5 min. A total of 40 µg proteins per lane were subjected to 10% SDS-PAGE. The proteins were transferred onto PVDF membranes and were subsequently incubated with primary antibodies against BTG1 (cat. no. ab151740; Abcam), Bel-2 (cat. no. ab185002; Abcam), Bax (cat. no. ab32503; Abcam), phosphorylated (p)-AKT (cat. no. 4060; Cell Signaling Technology, Inc.), AKT (cat. no. 4691; Cell Signaling Technology, Inc.) and GAPDH (cat. no. 5174; Cell Signaling Technology, Inc.) at a dilution of 1:1,000 at 4°C overnight. After washing with PBS-0.1% Tween 20, the membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 1 h at room temperature. The protein bands were quantified using Image Lab™ Software (version 5.2.1; Bio-Rad Laboratories, Inc.).

Statistical analysis. Each experiment was repeated in triplicate. Data were presented as the mean ± standard deviation. Statistical analysis was carried out using SPSS software version 16.0 (SPSS, Inc.). The differences between two groups were assessed by Student's t-test, and comparisons between multiple groups were detected using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-208a-3p expression is upregulated in the peripheral blood of patients with CHD. In order to explore the clinical relevance of miR-208a-3p in CHD, the expression level of miR-208a-3p was first determined in the peripheral blood samples of 30 patients with CHD and 30 healthy volunteers via RT-qPCR analysis. The results showed that significantly higher miR-208a-3p expression was detected in the peripheral blood samples of patients with CHD compared with healthy donors, indicating that miR-208a-3p may participate in CHD development (Fig. 1).

miR-208a-3p directly targets to the 3’-UTR of BTG1. Bioinformatics analysis was used to identify potential miR-208a-3p target genes. It was found that miR-208-3p has hundreds of potential target genes, including BTG1, with a putative binding site within its 3’-UTR for miR-208a-3p (Fig. 2A). As abnormal growth of VSMCs is crucial in the progression of CHD (5), and BTG1 serves important roles in regulating cell proliferation (17). However, the potential mechanism of BTG1 in VSMCs remains unclear. Thus, BTG1 was selected for further investigation. A luciferase reporter assay was subsequently conducted to verify whether miR-208a-3p could directly target to BTG1. Results indicated that miR-208a-3p mimic significantly enhanced miR-208a-3p expression in hVSMCs compared with the mimic control (Fig. 2B). Furthermore, miR-208a-3p overexpression significantly inhibited the luciferase activity of BTG1-WT. However, the luciferase activity of BTG1-MUT was not notably affected by co-transfection with miR-208-3p mimic compared with mimic control, suggesting that miR-208a-3p directly targeted the 3’-UTR of BTG1 (Fig. 2C).

To further investigate BTG1 expression in patients with CHD, RT-qPCR analysis was conducted to evaluate the mRNA expression levels of BTG1 in the blood samples of patients with CHD. The findings suggested that the BTG1 mRNA level was significantly downregulated in patients with CHD compared with healthy individuals (Fig. 2D). Therefore, BTG1 was hypothesized to serve as a functional target of miR-208a-3p in CHD.

miR-208a-3p negatively regulates BTG1 expression in hVSMCs. The effect of miR-208a-3p on BTG1 expression was subsequently investigated in hVSMCs. Control-siRNA, BTG1-siRNA, miR-208a-3p inhibitor or inhibitor control were transfected into hVSMCs for 48 h and transfection efficiency was evaluated via RT-qPCR analysis. As shown in Fig. 3A, miR-208a-3p inhibitor significantly suppressed miR-208a-3p levels in hVSMCs compared with the control group. In addition, in hVSMCs, BTG1-siRNA significantly downregulated the mRNA expression of BTG1 compared with the control group (Fig. 3B). Further experiments demonstrated that miR-208a-3p inhibitor was able to upregulate BTG1 at both the mRNA and protein level, and this upregulation was reversed by BTG1-siRNA (Fig. 3C-E). These findings suggested that miR-208a-3p negatively regulated BTG1 expression in hVSMCs.

BTG1-siRNA abolishes the effect of miR-208a-3p inhibitor on cell viability and apoptosis in hVSMCs. To further assess the role of miR-208a-3p in hVSMCs, MTT and flow cytometry assays were performed to determine hVSMC viability and apoptosis, respectively. miR-208a-3p inhibitor, inhibitor control, miR-208a-3p inhibitor + control-siRNA or miR-208a-3p inhibitor + BTG1-siRNA were transfected into hVSMCs. MTT assay results suggested that miR-208a-3p inhibitor significantly inhibited cell viability compared with the control group, while miR-208a-3p inhibitor and BTG1-siRNA co-transfection significantly promoted VSMC viability compared with the miR-208a-3p inhibitor group (Fig. 4A). Furthermore, flow cytometry analysis was carried out to evaluate the effect of miR-208a-3p inhibitor on hVSMC apoptosis. The results

Figure 1. miR-208a-3p is upregulated in the peripheral blood samples of patients with CHD. N=30/group. *P<0.01 vs. healthy control. CHD, coronary heart disease; miR-208a-3p, microRNA-208a-3p.
demonstrated that miR-208a-3p inhibitor induced hVSMC cell apoptosis compared with the inhibitor control, while these effects were significantly attenuated by inhibiting BTG1 (Fig. 4B and C). Western blot analysis was also performed to investigate apoptosis-associated proteins, such as Bcl-2 and Bax. As shown in Fig. 4D-F, miR-208a-3p inhibitor significantly reduced Bcl-2 protein expression levels compared with the control, while it increased the expression level of Bax. However, BTG1-siRNA efficiently reversed the effects of miR-208a-3p inhibitor on Bax and Bcl-2 protein expression in hVSMCs. These results suggested that miR-208a-3p was involved in hVSMC proliferation and apoptosis by targeting BTG1.
miR-208a-3p inhibitor affects the apoptosis of hVSMCs by regulating the PI3K/AKT pathway. The potential molecular mechanism of miR-208a-3p inhibitor was further investigated in terms of inducing cell apoptosis. Western blot analysis was conducted to detect relative protein expression levels, including p-AKT and AKT in hVSMCs (Fig. 5A). The ratio of p-AKT/AKT levels (Fig. 5B) was significantly suppressed in the miR-208a-3p inhibitor group compared with the control group; BTG1-siRNA significantly reversed these effects.

Effects of BTG1-siRNA on cell viability, apoptosis and the PI3K/AKT pathway in hVSMCs. To further explore the role of BTG1 in hVSMCs, the effect of BTG1-siRNA transfection alone on cell viability, apoptosis and PI3K/AKT activity were investigated in hVSMCs. It was observed that compared with the control group, BTG1-siRNA significantly promoted cell viability (Fig. 6A), inhibited cell apoptosis (Fig. 6B and C), and enhanced AKT phosphorylation (Fig. 6D and E) in hVSMCs. These findings further
suggested that BTG1 served a role in CHD development through effects on hVSMC function.

Discussion

It has been previously reported that abnormal regulation of hVSMCs may lead to the development of cardiovascular disease (21). Studies have demonstrated that miRNAs participate in the function of VSMCs, such as miRNA-21 (22) and miRNA-214 (23). Therefore, it is necessary to identify the specific miRNAs associated with the disease and their respective targets to further understand their role and examine novel therapeutic strategies for disease treatment. Previous reports have indicated that miR-208a-3p is involved in a number of diseases, including atrial fibrillation (24) and pathological cardiac hypertrophy (25). In addition, a previous study has suggested that miR-208a-3p is involved in acute cardiac injury (26). However, to the best of our knowledge, there are limited number of reports investigating the expression levels and roles of miR-208a-3p in CHD.
To understand the function of miR-208a-3p in CHD, the expression levels of miR-208a-3p were initially determined in the peripheral blood samples of 30 patients with CHD and healthy volunteers. The results showed that miR-208a-3p expression levels were significantly upregulated in patients with CHD compared with healthy individuals, suggesting that miR-208a-3p may serve important roles in CHD progression. This study also revealed that miR-208a-3p directly targeted the BTG1 3'-UTR by performing a dual-luciferase reporter assay. BTG1, which is a member of the pro-apoptotic Bcl-2 family, has been reported to be involved in multiple biological behaviors, including apoptosis and factor translocation regulation (27). Studies have shown that BTG1 plays an important role in regulating cell proliferation and apoptosis (19,28), and it has been found to be a target gene of multiple miRNAs (28,29). Furthermore, the present study found that BTG1 was downregulated in patients with CHD. Thus, it was hypothesized that miR-208a-3p may participate in CHD by regulating hVSMC function via targeting of BTG1.

Then, the present study examined the functional mechanism through which miR-208a-3p regulated hVSMCs. The results demonstrated that miR-208a-3p inhibitor significantly inhibited cell viability and promoted apoptosis, whereas downregulation of BTG1 reversed these effects. Furthermore, miR-208a-3p participated in the regulation of apoptosis-related proteins, including Bcl-2 and Bax (30). These results suggested that miR-208a-3p may regulate VSMC proliferation and apoptosis in CHD. Therefore, the present study examined potential signaling pathways involved in miR-208a-3p-regulated apoptosis in hVSMCs, and the PI3K/AKT pathway (31) was analyzed in this study. PI3K/AKT signaling has also been reported to be involved in CHD development (32-34). For example, miR-26a-5p inhibition induced endothelial cell apoptosis by inhibiting the PI3K/AKT pathway (33). Decoy receptor-3 could regulate inflammation and apoptosis by regulating the PI3K/AKT signaling pathway in a mouse model of coronary artery disease (33). The results of the present study indicated that miR-208a-3p inhibitor could inhibit the PI3K/AKT signaling pathway in hVSMCs, and in turn, this inhibition was abolished by BTG1-siRNA.

Finally, to further investigate the reversal effect of BTG1-siRNA on miR-208a-3p inhibitor-transfected hVSMCs, the effect of BTG1-siRNA on hVSMCs was determined. The data suggested that BTG1-siRNA could promote cell viability, inhibit cell apoptosis and promote the PI3K/AKT signaling pathway in hVSMCs. The data further suggested that miR-208a-3p may serve a role in CHD development by targeting BTG1.

In summary, these findings provided novel insight into the involvement of miR-208a-3p in the progression of CHD by regulating the proliferation of hVSMCs via downregulation of BTG1. This study provided a basis for understanding the functional mechanism of miR-208a-3p in CHD. However, this study is only a preliminary study of miR-208-3p in CHD. In order to verify the role of miR-208-3p in CHD, further in-depth studies are required. For example, the effect of BTG1-siRNA on hVSMCs requires further investigation. The role of miR-208-3p/BTG1 in CHD should also be investigated in vivo. The association between the expression of miR-208-3p/BTG1 and the clinicopathological features of patients with CHD also requires further exploration. In addition, examining other targets of miR-208a-3p in CHD therapy is critical.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
DW contributed to data collection, statistical analysis, data interpretation and manuscript preparation. CY contributed to data collection and data interpretation. DW and CY confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
Written informed consent was provided from all participants approving the use of their samples in the present study. All study protocols were approved by the Ethics Committee of Shanxi Bethune Hospital (Taiyuan, China).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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