MicroRNA-322-5p Protects Against Myocardial Infarction through Targeting BTG2

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Abstract

Objective: A large cohort of studies have addressed the therapeutic importance of microRNA (miR) in the treatment of myocardial infarction (MI). The current paper gives prominence to the role of miR-322-5p in MI by regulating B-cell translocation gene 2 (BTG2).

Methods: In a rat model of MI miR-322-5p and BTG2 expression was estimated. Adenovirus that altered miR-322-5p or BTG2 expression was injected into MI rats. After that, cardiac function, inflammation, myocardial injury, pathological condition, apoptosis, and the NF- κ B pathway-related genes in the myocardial tissue of MI rats after targeted treatment were evaluated. The targeting relationship between miR-322-5p and BTG2 was assessed.

Results: miR-322-5p was lowly expressed and BTG2 was highly expressed in the myocardial tissue of MI rats. Restored miR-322-5p improved cardiac function, relived inflammation and myocardial injury, suppressed pathological condition and apoptosis and inactivated NF- κ B pathway in MI rats. BTG2 expression was negatively mediated by miR-322-5p. Overexpressed BTG2 rescued miR-322-5p-induced cardioprotection on MI rats.

Conclusion: It is evident that miR-322-5p protects against MI through suppressing BTG2 expression.

Introduction

Myocardial infarction (MI) is defined pathologically as the death of cardiomyocytes due to long-term ischemia [1]. Acute MI may be sympathetically activated by cocaine, emotional stress, sexual intercourse and physical activity which could increase heart rate, blood pressure, pulse wave, myocardial contractility and oxygen demand, and results in coronary vasoconstriction and hypercoagulability [2]. Symptoms of MI include abnormal heartbeat, chest pain spreading from the left arm to the neck, vomiting, shortness of breath, sweating, fatigue and nausea. Immediate administration of aspirin prevents blood clotting and that of nitroglycerin relieves chest pain and oxygen inhalation at the onset of MI [3]. Multiple imaging modalities have been developed in MI, including positron emission tomography, cardiac magnetic resonance imaging, CT and echocardiography [4]. It is critical to underlie the molecular mechanism associated with MI in the treatment of the disease.

MicroRNA (miR) refers to a class of RNA molecules that do not encode proteins. Mechanistically, the induction or inhibition of miRs after MI triggers downstream events in a cell-type-specific manner, and interference with endogenous miR expression may regulate overall cardiac function [5]. For example, miR-21 exerts anti-fibrotic function [6] and miR-133 has therapeutic potential to recover cardiac function in the setting of MI [7]. Definitely, miR-322 is a pathogenic factor of plaque instability in atherosclerosis [8]. miR-322 programming could engineer the process of angiogenesis in MI [9] and induction of miR-322/503 could protect from ischemia-reperfusion injury (IR/I) [10]. miR-322-5p has been shown to be involved in myocardial hypertrophy [11], but the specific mechanism related to miR-322-5p in MI has yet to be proven. B-cell translocation gene 2 (BTG2) is the first identified gene in the BTG/TOB gene family. In our

preliminary experiment, BTG2 was assessed as a target of BTG2 online. Actually, T-box 20 (Tbx20)-mediated suppression of BTG2 is an inducer for proliferation of neonatal cardiomyocytes [12], but overexpressed BTG2 notoriously represses the survival of cardiomyocytes under hypoxic conditions [13]. Also, BTG2 could reprogram the apoptosis of cardiomyocytes induced by doxorubicin [14]. Based on the references provided, the regulatory mechanism of miR-322-5p/BTG2 axis was emphasized in our design.

Methods And Materials

Ethics statement

Animal experiments were followed by the Animal ethic committee of Beijing Anzhen Hospital, Capital Medical University.

Experimental animals

Male Sprague-Dawley rats (6–7 weeks old, 230 ± 20 g) were provided by the Department of Cardiology, Beijing Anzhen Hospital, Capital Medical University. All rats were housed under controlled conditions (60% humidity, 22°C and 12-h light/dark alternate) and supplied with enough foodstuff.

Rat modeling and treatments

After anesthesia by intraperitoneal injection with pentobarbital sodium (30 mg/kg), rats were subjected to ligation of left anterior descending coronary artery (LAD). The chest was opened in the fourth left intercostal space, and the LAD was sutured with 6 – 0 silk suture 1 mm below the tip of the left atrial appendage. The success of the ligation was confirmed by the color change of the myocardium. After 28 d, the rats were euthanized, and the myocardial tissue and blood samples were stored at -80°C.

Forty-eight hours before surgery, the adeno-associated virus 9 (AAV9) carrying miR-322-5p agomir/antagomir, overexpression (oe)-BTG2, or the corresponding negative control (Hanbio, Shanghai, China) was injected into rats ($n = 10$ per treatment) through the tail vein at 2×10^{11} vg. Sham-operated rats were not treated with ligation of LAD [15, 16].

Echocardiography

Rats were given intraperitoneal injection with ketamine at 100 mg/kg for anesthesia. Ejection fraction (EF) was impaired, left ventricular end diastolic diameter(LVIDd) and left ventricular end systolic diameter (LVIDs) were tested using an Acuson Sequoia 256c ultrasound system equipped with a 13MHz linear transducer from Vevo 2100 echocardiography (VisualSonics, Canada) [17, 18].

Enzyme-linked immunosorbent assay (ELISA)

With the ELISA kit (E-EL-R0015c, E-EL-R0012c and E-EL-R2856c; Elabscience Biotechnology, Wuhan, China), the levels of inflammatory factors interleukin (IL)-6, IL-1 β and tumor necrosis factor- α (TNF- α) in myocardial tissues were measured. The absorbance at 450 nm on a Labserv K3 Touch microplate reader (Thermo Fisher Scientific, MA, USA) was recorded [15, 19].

Detection of myocardial injury markers

Myocardial tissues were homogenized with lysis buffer containing protease inhibitor, centrifuged at $14,000 \times g$, and the supernatant was assayed to test creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH) and cardiac troponin I (cTnI) by ELISA kits (E-EL-H1434c, E-EL-R0338c and E-EL-R1253c; Elabscience Biotechnology) [15].

Hematoxylin-eosin (H&E) staining

Myocardial tissues were fixed in 4% formaldehyde overnight, followed by dehydration, permeabilization and paraffin embedding. Then, the tissue samples (5 μm) were deparaffinized in xylene, rehydrated in gradient ethanol, and stained with hematoxylin and eosin. The histopathological changes were observed under an optical microscope [19, 20].

Masson staining

Paraffin-embedded myocardial tissues were stained using the Masson Tricolor Staining Kit (BA4079B, Baso, China). The samples were viewed under a M205 stereo microscope (Leica, Germany) and analyzed the image-Pro Plus 6.0 software. The fibrotic area was dyed blue, and the normal area was dyed red [21].

Transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining

Myocardial tissues were deparaffinized and exposed to 3% H_2O_2 . After that, the sample was digested with pepsin and combined with TUNEL reaction mixture. Subsequently, the sample was oxidized with hematoxylin, and the number of apoptotic cells (TUNEL-positive cells) was evaluated by an optical microscope [16].

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

After the total RNA in myocardial tissues was obtained through Trizol reagent (Invitrogen, CA, USA), for miRNA, cDNA was synthesized using Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and amplified using TaqMan Micro Assay Mix; for BTG2, cDNA was collected using a reverse transcription kit (Invitrogen) and processed by Sybr Premix EX TAQ II (Takara, Dalian, China). Gene levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or U6, and calculated by the $2^{-\Delta\Delta\text{Ct}}$ method. Table 1 shows the primer sequences [18].

Table 1
Primer sequences

Genes	Primer sequences (5'-3')
miR-322-5p	F: AAACGGCTACCACATCCAAG
	R: CCTCCAATGGATCCTCGTTA
BTG2	F: GCGCGGGCTCTTCCTCTTTG
	R: AAGGAAGGCTGGAAGAGTGC
GAPDH	F: CGGAGTCAACGGATTTGGTCGTAT
	R: AGCCTTCTCCATGGTGGTGAAGAC
U6	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT
Note: F, forward; R, reverse; miR-322-5p, microRNA-322-5p; BTG2, B-cell translocation gene 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.	

Western blot assay

Protein samples were extracted from myocardial tissues using radio-immunoprecipitation assay lysis buffer (Beyotime, Shangha, China) containing 0.1 mmol/L phenylmethylsulfonyl fluoride. Then, the sample was isolated by 10% sodium lauryl sulfate polyacrylamide gel electrophoresis, electro-blotted on polyvinylidene fluoride membrane (Millipore, MA, USA) and sealed with 5% skim milk in Tris-HCl buffered saline. The membrane was supplemented with primary antibodies BTG2 (1:800, Abcam, MA, USA), IkappaB kinase α (IKK α), p-IKK α , IkappaB kinase β (IKK β), p-IKK β and GAPDH (1:1000, Abcam) for incubation overnight, and with the corresponding secondary antibody (1:5000, Abcam) for 120 min. After development, the protein bands were observed [21].

Dual luciferase reporter gene assay

On the bioinformatics website (<http://www.targetscan.org>), the binding site of miR-322-5p and BTG2 was estimated. Dual luciferase reporter gene experiment was carried out. The corresponding sequence was inserted into the luciferase reporter vector pGL3 (Promega, WI, USA) to construct BTG2-wld type (WT) and BTG2-mutant (MUT). The constructed vector was co-transfected with miR-322-5p agomir or miR-322-5p NC into HEK293T cells for 48 h. The dual luciferase assay system (Promega) was used for detection of luciferase activity [18].

RNA immunoprecipitation (RIP) assay

RIP assay was performed using Magna RIP™ kit (Millipore). HEK293T cells were lysed with RIP buffer, and then incubated with RIP buffer containing Ago2 (1:1000, ab5072, Abcam) and IgG (1:200, sc-2025, Santa Cruz Biotechnology, CA, USA) conjugated magnetic beads. After interacting with proteinase K, the immunoprecipitated RNA was analyzed by RT-qPCR [22].

Statistical analysis

SPSS 21.0 (IBM, NY, USA) was adopted to statistical analysis. Measurement data were expressed as mean $\pm$ standard deviation and followed a normal distribution. Two sets of data were evaluated by independent samples t test, multi-sets of data by one-way analysis of variance (ANOVA) and Tukey's post-hoc test. Repeated measures ANOVA evaluated data comparison at different time points, followed by Bonferroni post-hoc test. At $P < 0.05$, statistically significance was established.

Results

miR-322-5p is lowly expressed and BTG2 is highly expressed in MI rats

miR-322-5p has protective effects on hypertrophied myocardium [21]. To confirm the success of MI modeling, various assays were performed. Echocardiogram observed that ejection fraction (EF) was impaired, left ventricular end diastolic diameter (LVIDd) and left ventricular end systolic diameter (LVIDs) were increased after MI modeling in rats (Fig. 1A). The levels of pro-inflammatory factors TNF- α , IL-1 β and IL-6, as well as myocardial injury markers (LDH, CK-MB and cTnI) were increased in rats after MI surgery (Fig. 1B-E). Also, pathological changes in myocardial tissues of rats after MI surgery were observed by H&E staining and Masson staining, and the myocardium was severely injured (Fig. 1F, G). Besides, TUNEL staining reflected that MI modeled rats had augmented apoptosis rate in the myocardial tissue (Fig. 1H).

In our study, miR-322-5p and BTG2 expression was estimated by RT-qPCR and Western blot. Indeed, miR-322-5p level was reduced while BTG2 level was increased in rats after MI surgery (Fig. 1I-K). In short, MI model was successfully established.

Restored miR-322-5p eases MI, but depleted miR-322-5p worsens MI in rats

In attempting to unearth miR-322-5p's function in MI, AAV9 carrying miR-322-5p NC, miR-322-5p agomir or antagomir was injected into MI rats. miR-322-5p agomir induced miR-322-5p expression to upregulate (Fig. 2A). In MI rats with upregulated miR-322-5p, the cardiac function was improved, the level of pro-inflammatory factors and myocardial injury markers was suppressed, the pathological condition was relived and apoptosis was decreased (Fig. 2B-I). Oppositely, miR-322-5p antagomir treatment lowered miR-322-5p expression, eliciting the opposite effects of miR-322-5p agomir on MI rats. Shortly, restored miR-322-5p eases MI, but depleted miR-322-5p has the opposite effects.

BTG2 expression is negatively mediated by miR-322-5p

Based on the expression trend of miR-322-5p and BTG2 in MI rats, BTG2 was speculated to be a target of miR-322-5p. On the targetscan website, the binding site was found between miR-322-5p and BTG2 (Fig.

3A). Through luciferase activity experiment, the luciferase activity of BTG2-WT was depressed after transfection with miR-322-5p agomir (Fig. 3B). By RIP experiment, it was detected that the Ago2 immunoprecipitated had higher miR-322-5p and BTG2 expression (Fig. 3C).

To further verify the targeting relationship between miR-322-5p and BTG2, BTG2 expression alternation was observed after mediating miR-322-5p. The findings presented low BTG2 expression in myocardial tissues of MI rats after injection with AAV9 loading miR-322-5p agomir, but high BTG2 expression after injection with AAV9 loading miR-322-5p antagomir (Fig. 3D, E). Clearly, BTG2 expression is negatively mediated by miR-322-5p.

miR-322-5p inactivates NF-κB pathway

Inhibition of NF-κB pathway can attenuate the production of oxidative stress and inflammatory factors in cardiomyocytes [23]. In MI rats, NF-κB pathway-related genes were analyzed by Western blot. It turned out that MI rats had increased p-IKKα and p-IKKβ protein expression, as well as increased ratio of p-IKKα/IKKα and p-IKKβ/IKKβ. After treatment with miR-322-5p agomir, the protein expression of NF-κB pathway-related genes was reversed. However, miR-322-5p antagomir had a promotive role to activate NF-κB pathway (Fig. 4A, B). Evidently, miR-322-5p inactivates NF-κB pathway.

Overexpressed BTG2 rescues miR-322-5p-induced cardioprotection on MI rats

In order to further determine the mechanism by which miR-322-5p targeting BTG2 affects MI, miR-322-5p agomir + oe-NC or miR-322-5p agomir + oe-BTG2 adenovirus was injected into MI rats. As the results indicated, oe-BTG2 possessed strong ability to restore BTG2 mRNA expression that had been suppressed by miR-322-5p agomir (Fig. 5A), thereby impairing cardiac function, worsening inflammation and myocardial injury, and augmenting pathological injury and apoptosis in myocardial tissues, and elevated increased p-IKKα/IKKα and p-IKKβ/IKKβ in MI rats (Fig. 5B-K).

Discussion

MI is a subset of the acute coronary syndromes with negative effect on morbidity and mortality of patients. With regard to miR-322-5p/BTG2 axis, the molecular mechanism of MI was partly identified. The main conclusion in our design stressed that upregulating miR-322-5p directly suppressed BTG2 and conferred cardioprotection for rats with MI.

The reduction of miR-322-5p expression has been testified in the heart in a rat model with pulmonary arterial hypertension [24]. Also, miR-322-5p expression is obviously repressed in Ang-II-conditioned cardiomyocytes in response to hypertrophy [11]. In fact, in macrophages conditioned to lipopolysaccharide, upregulated miR-322 could inhibit the level of inflammatory cytokines and enhance that of anti-inflammatory cytokines [25]. Besides, Wei Dong *et al.* have proposed that recovering the suppressed miR-322/503 expression during the process of IR/I could narrow infarct size, limit apoptosis and induce proliferation [10]. Intriguingly, miR-322 from cardiac progenitor cell-derived exosomes protects

against acute IR/I through suppressing apoptosis and promoting angiogenesis [9]. From an innovative study, it is well established that elevating miR-322 expression is efficient in protecting the heart from high fat diet effect and improving cardiac function [26]. Collectively, the specific role of miR-322-5p has not been completely uncovered yet in MI, thus our study concerning to the role of miR-322-5p was implemented. At start, in the rat model of MI, miR-322-5p expression was found to lowly express in myocardial tissues, which shows coincidence with the former researches. In our experiments, miR-322-5p expression was altered in rats through injection with miR-322-5p agomir or antagomir, thus to change the pathological injury in the myocardial tissue. The experimental observations eventually summarized that restoring miR-322-5p restrained the levels of pro-inflammatory indices and myocardial injury markers, improved cardiac function, attenuated the situation of the injured myocardium and repressed apoptosis. On the other hand, the impacts of silenced miR-322-5p on MI rats were opposite to those of upregulated miR-322-5p.

miR-322-5p is one of the core RNAs involved in NF- κ B pathway [27] and miR-322 could negatively regulate NF- κ B1 expression [28]. Similarly, our research obtained the finding that miR-322-5p negatively regulated the activation of NF- κ B pathway. Indeed, NF- κ B pathway is activated in MI [29] and blockade of NF- κ B pathway actively obstructs apoptosis and inflammation in cardiomyocytes in the course of MI [30]. The downstream of NF- κ B pathway was not sufficiently explored in our study, which is a future study direction.

BTG2 expression is high in asthma [31]. Also, in patients with ulcerative colitis, highly expressed BTG2 has been examined in colonic mucosa [32]. In an experimental model of central retinal artery ligation/reperfusion injury, BTG2 expression climbs to a high level [33]. In consistence with previous researches, our study revealed that BTG2 was upregulated in the myocardial tissue of MI rats. Functionally, it has been illustrated that suppression of BTG2 by binding with Tbx20 at least augments proliferation of neonatal cardiomyocytes and induces cardiac function recovery after MI [12]. On the contrary, induction of BTG2 in hypoxia-suffered cardiomyocytes leads to suppressed viability and promoted apoptosis [13]. To overcome doxorubicin-induced toxicity, Zhongyi Tong *et al.* have verified that treatment with miR-21 could reduce apoptosis of cardiomyocytes through inhibiting BTG2 expression [14]. In the current study design, serious assays were conducted to prove that miR-322-5p transcriptionally regulated BTG2, and miR-322-5p targeting BTG2 was involved in the process of MI. Finally, it was proved that overexpressed BTG2 mitigated the miR-322-5p-induced protection against MI in rats.

To shortly conclude, endogenous expression of miR-322-5p prevents injury from MI in rats, which is facilitated by downregulation of BTG2. Our analysis has renewed the knowledge about miR-322-5p/BTG2 axis-regulated MI progression, and offered a basis for developing potential therapeutics in MI. Our study is based on animal trails in the absence of clinical data, which is the main limitation of our research.

Declarations

Conflict of interest

The authors declare no competing interests.

Funding

The authors did not receive support from any organization for the submitted work.

Ethics approval and consent to participate

Animal experiments were followed by the Animal ethic committee of Beijing Anzhen Hospital, Capital Medical University.

Availability of data and material

The raw/processed data required to reproduce these findings cannot be shared at this time due to legal or ethical reasons.

Consent for publication

The participant has consented to the submission of the case report to the journal.

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Figures

Figure 1

miR-322-5p is lowly expressed and BTG2 is highly expressed in MI rats. A. EF, LVIDd and LVIDs in rats after MI modeling; B. TNF- α , IL-1 β and IL-6 levels in rat myocardial tissue after MI modeling; C-E. LDH, CK-MB and cTnl levels in rat myocardial tissue after MI modeling; F. HE staining of rat myocardial tissue after MI modeling (400 $\times$); G. Masson staining of rat myocardial tissue after MI modeling (200 $\times$); H. TUNEL staining of rat myocardial tissue after MI modeling (400 $\times$); I. miR-322-5p expression level in rat myocardial tissue after MI modeling; J-K. BTG2 expression level in rat myocardial tissue after MI modeling; Measurement data were expressed as mean $\pm$ standard deviation of 10 individuals; * $P < 0.05$ vs. the sham group.

Figure 2

Restored miR-322-5p eases MI, but depleted miR-322-5p worsens MI in rats. A. miR-322-5p expression level in rat myocardial tissue after injection with miR-322-5p agomir or antagomir; B. EF, LVIDd and LVIDs in rats after upregulating or downregulating miR-322-5p; C. TNF- α , IL-1 β and IL-6 levels in rat myocardial tissue after upregulating or downregulating miR-322-5p; D-F. LDH, CK-MB and cTnl levels in rat myocardial tissue after upregulating or downregulating miR-322-5p; G. HE staining of rat myocardial tissue after upregulating or downregulating miR-322-5p (400 $\times$); H. Masson staining of rat myocardial tissue after upregulating or downregulating miR-322-5p (200 $\times$); I. TUNEL staining of rat myocardial tissue after upregulating or downregulating miR-322-5p (400 $\times$); Measurement data were expressed as mean $\pm$ standard deviation of 10 individuals; * $P < 0.05$ vs. the agomir NC group; # $P < 0.05$ vs. the antagomir NC group.

Figure 3

BTG2 expression is negatively mediated by miR-322-5p. A. The binding site of miR-322-5p and BTG2 on the bioinformatics website; B. The luciferase activity of BTG2-WT and BTG2-MUT after transfection with miR-322-5p agomir; C. The enrichment of miR-322-5p and BTG2 in Ago2; D-E. BTG2 expression level in rat myocardial tissue after regulating miR-322-5p; Measurement data were expressed as mean $\pm$ standard deviation of 10 individuals; * $P < 0.05$ vs. the agomir NC group; # $P < 0.05$ vs. the antagomir NC group.

Figure 4

miR-322-5p inactivates NF-κB pathway. A-B. p-IKKα, IKKα, p-IKKβ and IKKβ protein expression in rat myocardial tissue after regulating miR-322-5p; Measurement data were expressed as mean ± standard deviation of 10 individuals; * P < 0.05 vs. the sham group; # P < 0.05 vs. the agomir NC group; & P < 0.05 vs. the antagomir NC group.

Figure 5

Overexpressed BTG2 rescues miR-322-5p-induced cardioprotection on MI rats. A. BTG2 mRNA expression in rat myocardial tissue after overexpressing miR-322-5p and BTG2; B. EF, LVIDd and LVIDs in rats after overexpressing miR-322-5p and BTG2; C. TNF- α , IL-1 β and IL-6 levels in rat myocardial tissue after overexpressing miR-322-5p and BTG2; D-F. LDH, CK-MB and cTnI levels in rat myocardial tissue after overexpressing miR-322-5p and BTG2; G. HE staining of rat myocardial tissue after overexpressing miR-322-5p and BTG2 (400 $\times$); H. Masson staining of rat myocardial tissue after overexpressing miR-322-5p

and BTG2 (200 ×); I. TUNEL staining of rat myocardial tissue after overexpressing miR-322-5p and BTG2 (400 ×); J-K. p-IKK α , IKK α , p-IKK β and IKK β protein expression in rat myocardial tissue after overexpressing miR-322-5p and BTG2; Measurement data were expressed as mean $\pm$ standard deviation of 10 individuals; * P < 0.05 vs. the agomir NC + oe-NC group; # P < 0.05 vs. the miR-322-5p agomir + oe-NC group.