Tropical Journal of Pharmaceutical Research January2021; 20 (1): 89-94
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v20i1.14

Original Research Article

LncRNA SNHG1 protects the cardiac muscle cells from hypoxia/ re-oxygenation injury in vitro by targeting microRNA-21-5p and miR-30a-5p

Youbin Liu[1], Tingting Zhang[1], Ying Fan[1], Jinglong Li[1], Meng Chen[1], Anyong Chen[1], Dahao Yang[1], Xue Guan[2], Yong Cao[1,3]*

[1]Department of Cardiology, The second Affiliated Hospital of Harbin Medical University, Harbin, PR China, [2]Department of Animal Center, The Second Affiliated Hospital of Harbin Medical University, Harbin, PR China, [3]The Affiliated Hospital of Jining Medical University, Jining city, Shandon, 272000, China

For correspondence: **Email: trcv5645@126.com, **Tel**: +8618273605524*

Sent for review: 23 Mar 2020 *Revised accepted: 26 April 2020*

Abstract

Purpose: *Cardiovascular diseases are responsible for numerous deaths globally. Long noncoding RNA SNHG1 has presented its protective role in cardiomyocytes previously. Herein, we examined the underlying molecular mechanisms of SNHG1 in cardiac muscle cells from hypoxia and re-oxygenation (H/R) in vitro.*

Methods: *RT-qPCR measured expression of SNHG1, miR-21-5p and miR-30a-5p in rat cardiac muscle cell line HL-1 before and after H/R treatment and cell transfection, which was applied to regulate expression of SNHG1, miR-21-5p and miR-30a-5p for further use. The flow cytometry method was used to compare changes in cellular apoptosis, and cell viability was measured by CCK-8 method. Bioinformatics predicted the bindings ofSNHG1 and miR-21-5p / miR-30a-5p while the luciferase reporter assays further verified this.*

Results: *The outcomes revealedthat SNHG1 was downregulated and meanwhile miR-21-5p / miR-30a-5p was elevated that enhanced apoptosis and reduced cell viability in HL-1 cells. However, overexpressed SNHG1 inhibited cell apoptosis and increased cell viability brought by H/R. In addition, SNHG1 targeted at miR-21-5p/ miR-30a-5p, which contributed to the inter-regulation in between. Furthermore, interactive experiments revealed that upregulation of miR-21-5p/ miR-30a-5p added to the cell apoptosis which was induced by H/R and partially counteracted by the upregulation of SNHG1.*

Conclusion: *In this study we have demonstrated the protective role of SNHG1 in the moderation of H/R-induced HL-1 apoptosis and viability through suppression of miR-21-5p/miR-30a-5p. This offers new perspective into the molecular interpretation of cardiovascular diseases such as ischemic reperfusion injury.*

Keywords*: SNHG1; apoptosis; Hypoxia/Re-oxygenation injury;miR-21-5p; miR-30a-5p; Cardiovascular diseases*

Tropical Journal of Pharmaceutical Researchis indexedby Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Cardiovascular diseases contribute to numerous fatalities every year worldwide. Ischemia reperfusion (I/R) enhances the gravity of cardiomyocyte damage although reperfusion therapy has been widely accepted as treatment of numerous cardiovascular diseases. However, many patients suffer from irreversible myocardial ischemia/reperfusion injury (MIRI) after reperfusion therapy [1]. A large body of evidence acknowledged that long non-coding RNAs show aberrant expression in human diseases, namely neurodegenerative diseases, cancers, [2-4] and cardiovascular diseases [5]. Previously, noncoding RNAs (ncRNAs), such as long ncRNAs (lncRNAs) and micro RNAs (miRNAs), are associated in patho-physiological mechanisms in cardiovascular diseases[5]. The non-coding RNA, the myocardial infarction associated transcript 1 (MIAT1), is upregulated in acute myocardial infarction (AMI) patients and AMI animal models ,which is associated with the occurrence of AMI [6] ,enhancing cardiomyocytes apoptosis and inflammation [7] and also responsible for moderation of cardiac fibrosis [8]. Some other evidence suggests more non-coding RNAs involved in some cardiovascular diseases. For instance,MALAT1 was established as a therapeutic candidate for a broad spectrum of vascular and cardiorenal complications [9].The long noncoding RNA NKILA protects against myocardial ischemic injury by enhancing myocardin expression via suppressing the NF-κBsignalling pathway [10]. LncRNA MALAT1 enhances the apoptosis of cardiomyocytes through autophagy inhibition by regulating TSC2-mTOR signaling [11]. Down-regulation of GAS5 ameliorates myocardial ischemia/reperfusion injury via the miR-335/ROCK1/AKT/GSK-3β axis [12]. Knockdown of lncRNA AK139328 alleviates myocardial ischaemia/reperfusion injury in diabetic mice via modulating miR-204-3p and inhibiting autophagy [13]. LncRNA-ROR aggravates myocardial ischemia/reperfusion injury [14]. Inhibition of long noncoding RNA BDNF-AS rescues cell death and apoptosis in hypoxia/reoxygenation damaged murine cardiomyocyte [15]. Zhuo et al., also identified LncRNA SNHG8 as a crucial moderator of acute myocardial infarction by RNA-seq analysis [16]. Our previous research also unveiled the protective role of small nucleolar RNA host gene 1 (SNHG1) in inhibiting apoptosis of human cardiomyocytes from Hydrogen peroxide by targeting miR-195 in vitro and SNHG1 presented lower [17]. In another research, it exerted its protective role in cardiac hypertrophy through in-vitro and rat model [18].

At present, no study has reported the functional roles of SNHG1 and interactions with miRNAs in H/R-induced cardiac muscle cell models. miR-21-5p and miR-30a-5p was reported to be overexpressed in ischemic regions of the affected heart were subjected to global sequencing [19, 20]. H/R–induced cardiomyocyte apoptosis has been recognized as one of the main causes of Ischemia reperfusion (I/R) [21-23]. Therefore, the associations between expression of the three genes and cell apoptosis are mainly examined in this research. Furthermore, the interplays in between the genes were also investigated so as to discover the mechanisms beneath LNCRNA SNHG1 in shielding cardiac muscle cells from hypoxia/re-oxygenation challenge *in vitro.* .

EXPERIMENTAL

Cell culture, H/R treatment and transfection assays

Rat cardiac muscle cell (HL-1, Cell Systems, Seattle, USA) were multiplied in adapted RPMI-1640 substrate, with addition of 10 percent fetal bovine serum (FBS) and 200 µg/L of penicillin and 200 µg/L of streptomycin (Beyotime, Shanghai, China). The cells were nurtured at 37°C in a saturated environment of 5 percent CO2. The manufacturer's guideline to perform all cell culture procedures was strictly followed.

To induce reperfusion injury *in vitro*, cardiac muscle cells were tested with a hypoxia/reoxygenation (H/R) treatment in a tri-gas incubator for six-hour hypoxia stimulation (95% N2 and 5% CO2) and 12-hour re-oxygenation (95% air and 5% CO2). Cells after H/R treatment were selected for transfection so as to differentiate the expression of the genes SNHG1, miR-21-5p and miR-30a-5p in H/R-induced HL-1 cells.

Transfection was performed utilizing Lipofectamine 3000 (Beyotime, Shanghai, China) following manufacturer's guidelines. The Lv-SNHG1, miR-21-5p mimics, miR-21-5p inhibitor, miR-30a-5p mimics, miR-30a-5p inhibitors and their control plasmids were designed and synthesized by Guangzhou Fulengen Co., Ltd (Guangzhou, China). The transfection rate was measured by qRT-PCR. The experiments were conducted after 48 hours following the transfection.

Real Time-quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was removed from cardiac muscle

cells using Beyozol mixture (#R0011, Beyotime, Shanghai, China) then reverse transcription of 1 µg RNA was done for each specimen to cDNA using BeyoRT™ cDNA First Chain Synthesis Kit (#D7166, Beyotime, Shanghai, China) following guidelines provided by manufacturer. The qRT-PCR experiments were conducted by use of Beyofast™ SYBR Green QPCR Mix (Beyotime, China) and associated miRNA qRT-PCR detection kit (Beyotime, China) to observe the expression of LNCRNA SNHG1. GAPDH was normaolized control and the $2^{-\Delta\Delta CT}$ procedure was used to estimate the comparative expression level of Endo-1. The experiments were conducted in triplicates. All primers were produced by GenelilyBioTech Co., Ltd (Shanghai, China) and the sequences are summarized in Table 1.

Table 1: Primer sequences

Name	Sequences
SNHG1 (forward)	ACGTTGGAACCGAAGAGAGC
SNHG1 (reverse)	GCAGCTGAATTCCCCAGGAT
miR-21-5p (forward)	CGGCGGTAGCTTATCAGACTGA
miR-21-5p (reverse)	CTGGTGTCGTGGAGTCGGCAATTC
miR-30a-5p (forward)	TGTAAACATCCTCGACTGGAAG
miR-30a-5p (reverse)	TGCGTGTCGTGGAGTC
U6 (forward)	CTCGCTTCGGCAGCACA
U6 (reverse)	AACGCTTCACGAATTTGCGT
GAPDH (forward)	AGAAGGCTGGGGCTCATTTG
GAPDH (reverse)	AGGGGCCATCCACAGTCTTC

Flow cytometry method for apoptosis analysis

Cell apoptosis was determined using the Annexin V-FITC/PI kit (BD Bioscience, USA). Cells were collected and blotted with 5 µL of Annexin V-FITC for 10 minutes in devoid of light and tarnished with 10 µL of PI at 25°C. The apoptosis rate in every sample was examined by a flow cytometer (BD FACSC anto II) and estimated by BD FACSDiva software. Each group was examined for three times.

CCK-8 assays

Cells from all groups were collected for the cell viability assays. The cell suspension was inoculated into 96-well plates and prepared in an incubator with humidified condition at 37°C, 5% CO2. 10 µl CCK-8 solution was added into each well and incubated for 2 hours. Thereafter, a microplate reader was used to read the absorbance at 450nm wavelength at 24, 48 and 72h. Each experiment was repeated for three times.

Bioinformatics analysis

The binding sites between miR-21-5p/miR-30-5p and SNHG1 were were predicted on Starbase online database (http://www.targetscan.org/vert_72/).

Luciferase reporter assay

The target sequence of LNCRNA SNHG1 and microRNA-21-5p connecting locations were predicted and replicated into a pGL3 Dual-luciferase Target Vector (Promega, CA, USA), to construct Wild Type and Mutant Type SNHG1 plasmids. These Wild Type or Mutant Type SNHG1 membranes were transfected together into HL-1 cells along with mimic-NC or miR-21-5p mimics, miR-30a-5p mimics using Lipofectamine 3000 guided by manufacturer's protocol. Following elapsing of 48hrs, we conducted luciferase reporter assays via the dual-luciferase reporter method (Promega, USA), following protocol specified by the manufacture.

Statistical analysis

The trials were conducted in three times separately. The experimental data has been exhibited as average and standard error. The GraphPad Prism 5 software and SPSS 18.0 version analyzed the statistics of the study. Student's t-test and ANOVA analysis were applied. P-value less than 0.05 shows a difference in statistical significance.

RESULTS

H/R Downregulated expression of SNHG1 and elevated expression ofmiR-21-5p and miR-30a-5p, inducing Apoptosis in HL-1 cells

we performed RT-qPCR initially to determine the expressions of the genes treated with H/R. The outcomes revealed remarkably downregulated SNHG1 expression in the H/R group in contrast to the normoxia group in HL-1 cells (Fig.1A, $p<0.05$). In addition, the miR-21-5p/miR-30a-5p expression increased in H/R group compared with the normoxia the control group of the HL-1 cells (Figure 1B, $p<0.05$). Furthermore, the cell viability assays were performed to research the effect of H/R on the cardiac muscle cells regarding cell viability. The results showed a decrease in cell viability in H/R group in contrast to the normoxia group (Figure 1C, $p<0.05$). In

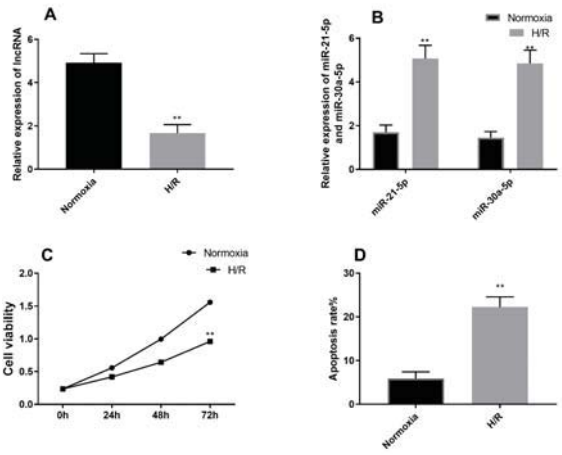

Figure 1: H/R Downregulated expression of SNHG1 and elevated expression of miR-21-5p and miR-30a-5p, inducing Apoptosis in HL-1 cells. Cells were divided into two groups, one treated with normoxia condition and the other treated with H/R. A-B. RT-qPCR measured the differential expression levels of SNHG1, miR-21-5p and miR-30a-5p in both groups with GAPDH as an internal control. C. CCK8 method detected the relative cell viability in each group. D. Flow Cytometry method was used to evaluate the cell apoptosis rates of each group. All the experiments were repeated for three times; **, P<0.05.

addition, the Flow cytometry methods were employed to evaluate the apoptosis changes and it was found that apoptosis was elevated in H/R group compared to the normoxia group (Figure 1D, p<0.05).

SNHG1 negatively modulated cell apoptosis induced by H/R

After H/R treatment, the HL-1 cells were transfected with lv-SNHG1 and sh-SNHG1. Thereafter, RT-qPCR method was used to measure expression of SNHG1 in each group and it was disclosed that SNHG1 expression was inhibited b H/R treatment and decreased more by sh-SNHG1 but enhanced by lv-SNHG1 (Figure 2A，P<0.05). Cell viability inhibited by H/R was recovered by upregulation of SNHG1 but inhibited more by knockdown of SNHG1 in HL-1 cells (Figure 2B, P<0.05). Cell apoptosis rates in all the groups were evaluated by Flow cytometry and apoptosis was suppressed by the upregulation of SNHG1 but enhanced by downregulation of SNHG1 in HL-1 cells (Figure 2C).

LNCRNA SNHG1 connects directly with miR-21-5p and miR-30a-5p in HL-1 cells

We predicted the potential binding locations between SNHG1 and miR-21-5p or miR-30a-5p using bioinformatics tool, Starbase (http://www.targetscan.org/vert_72/). The putative binding sites were shown here (Figure 3A&C). To further validate this interaction, luciferase reporter assays were performed. First, we created and generated luciferase reporter for wild type SNHG1 (WT) or mutant type SNHG1 (MUT) followed by transfection with negative control (mimic NC) or miR-21-5p mimic or miR-30a-5p mimic in HL-1 cell lines. The results indicated that miR-21-5p lessened the luciferase activity of wild-type of SNHG1 (Figure 3B, p<0.05), verifying the binding of SNHG1 and microRNA-21-5p. The similar results from Luciferase reporter assays confirmed SNHG1 targets miR-30a-5p too (Figure 3D, P<0.05)

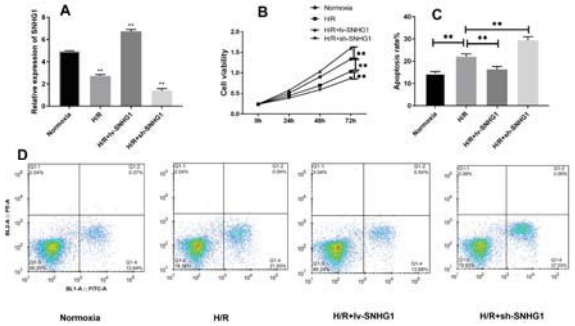

Figure 2: SNHG1 negatively modulated cell apoptosis induced by H/R. After H/R treatment, the HL-1 cells were transfected with lv-SNHG1 and sh-SNHG1. A. RT-qPCR examined the SNHG1 expression in the four groups. B.CCK-8 assays evaluated the cell viability. C. Flow cytometry assays measured the apoptosis rates in all groups. All the experiments were repeated for three times. **, P<0.05

LncRNA SNHG1 safeguarded cardiac muscle cells against H/R-induced apoptosis by targeting and inhibiting miR-21-5p and miR-30a-5p

Cells were transfected with miR-21-5p mimics, miR-21-5p inhibitor, lv-SNHG1, miR-30a-5p or miR-30a-5p after treated in H/R condition. Therefore, 8 sub-groups were divided with H/R group as a control. The expression of miR-21-5p/ miR-30a-5p and SNHG1 was measured in each group by RT-qPCR. In addition to the above findings in Figure 1-3, the results in all the groups showed that miR-21-5p and miR-30a-5p were positively associated, which is to say that the upregulation of miR-30a-5p could enhance miR-21-5p expression in HL-1 cells and vice versa while silencing miR-30a-5p could reduce miR-21-5p expression in HL-1 cells and vice versa (Figure 4A-B, P<0.05). On the other hand, overexpression of SNHG1 inhibited miR-21-5p and miR-30a-5p expression in HL-1 cells (Figure 4A-B, P<0.05). Also, upregulation of miR-21-5p or miR-30a-5p could decrease SNHG1.expres-

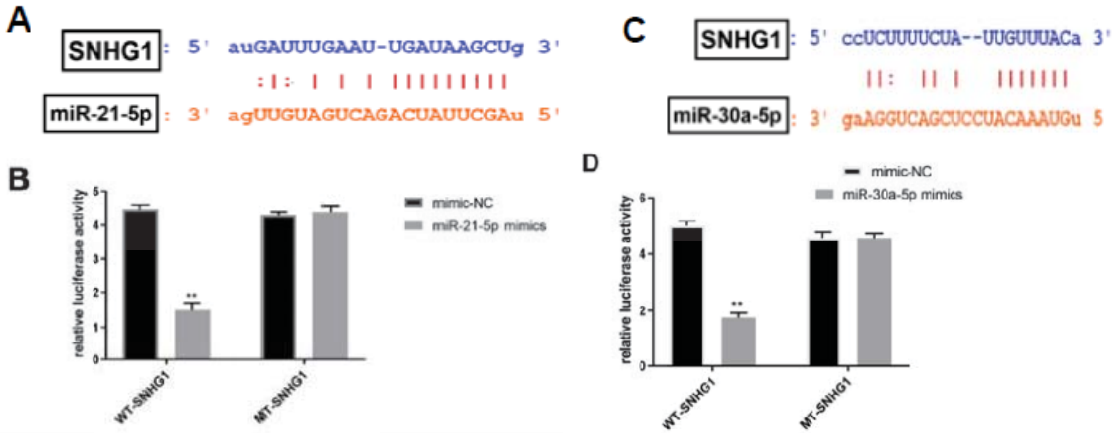

Figure 3: SNHG1 connects directly with miR-21-5p and miR-30a-5p in HL-1 cells. A&C. Starbase online tool (http://www.targetscan.org/vert_72/) was resorted when we predicted the potential targets. The putative binding sites were presented in the two sub-figures here. B&C. Luciferase reporter assays were conducted to execute the verification of the prediction after the co-transfection of miR-30a-5p/ miR-21-5p mimics or mimics NC and WT-SNHG1 or MT-SNHG1. The relative luciferase activity in each group was displayed

sion in HL-1 cells (Figure 4C, P<0.05). To investigate the impacts of the interactions among the three genes in HL-1 cells, we used CCK-8 assays to measure the cell viability and flow cytometry to evaluate the cell apoptosis. Results showed that the combined group of miR-30a-5p mimic and miR-21-5p mimic could bring about a higher decrease in cell viability compared to miR-21-5p mimic group alone while miR-30a-5p downregulation could partially counteract the viability decrease induced by miR-21-5p upregulation (Figure 4D, P<0.05). Additionally, SNHG1 upregulation could restore the viability reduced by miR-21-5p upregulation (Figure 4D, P<0.05). Downregulation of miR-21-5p could contribute to higher viability induced by SNHG1 upregulation (Figure 4D, P<0.05). Flow cytometry results showed that upregulation of miR-21-5p enhanced cell apoptosis while inhibition of miR-21-5p decreased apoptosis (Figure 4E, P<0.05). In addition, upregulation of both miR-21-5p and miR-30a-5p could lead to higher apoptosis while overexpression of SNHG1 could decrease the apoptosis significantly (Figure 4E, P<0.05). Apart from this, the combined group of inhibited miR-21-5p and elevated SNHG1 presented the lowest rate of cell apoptosis in all the groups (Figure 4E, P<0.05

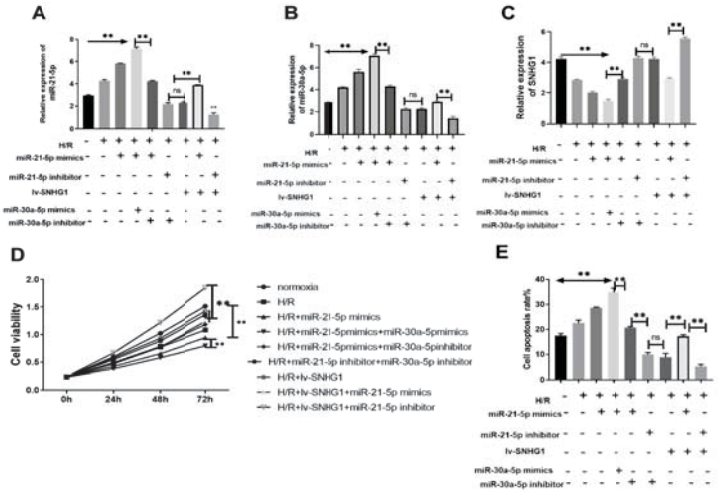

Figure 4: SNHG1 safeguarded cardiac muscle cells against H/R-induced apoptosis by targeting and inhibiting miR-21-5p and miR-30a-5p. Cells were transfected with miR-21-5p mimics, miR-21-5p inhibitor, lv-SNHG1, miR-30a-5p or miR-30a-5p after treated in H/R condition. Therefore, 8 sub-groups were divided with H/R group as a control. A-C. RT-qPCR measured the relative expression of miR-21-5p, miR-30a-5p and SNHG1 in all the nine groups. D. CCK-8 assays evaluated the relative cell viability in each group. E. Flow cytometry assays measured the apoptosis rates in all the groups. All the experiments were repeated for three times. **, P<0.05

DISCUSSION

Accumulative data substantiates and demonstrates that long non-coding RNAs can serve as defensive roles in cardiovascular diseases, for instance, HOX antisense intergenic RNA (HOTAIR), XIST and H19 [24-26]. LncRNA SNHG1 was previously reported to be protective in human cardiomyocytes from cardiac hypertrophy by targeting miR-15a-5p [18]. In this study, we have explicitly demonstrated that the expression of SNHG1 was remarkably reduced in cardiac muscle cells (HL-1) treated with H/R exposure. The cell apoptosis was elevated dramatically, which was overturned by SNHG1-overexpression, implying that SNHG1 might act as a cardio-protective marker in H/R-induced cardiac muscle cells against apoptosis.

As previously reported, Long non-coding RNA is capable of controlling numerous genes including microRNAs [27-29]. MiR-21-5p was identified to be upregulated in myocardial transcription factors GATA-binding protein 4, GATA-binding protein 6, and Krüppel-like factor 6, serving as a pro-apoptosis microRNA during myocardial ischemia in cardiomyocytes [19]. It has been evidently reported that miR-21-5p deregulation in exosomes resulting from heart failure patients damaged regenerative capability [30]. Exosomal miR-21-5p was investigated on its role in the diagnosis of Hyperacute and Acute Ischemic Stroke in which they were applicable in early-stage diagnosis as potential biomarkers for Ischemic Stroke thrombolysis therapy [31]. On the other hand, it has been widely reported that miR-30a-5p is aberrantly enhanced in patients with heart failure and can serve as a biomarker for early-stage diagnosis of heart failure[20]. Apart from this ,it has also been discovered that miR-30a-5p serves as a prognostic gene for the left ventricular dysfunction in patients after AMI[32]. Therefore, in our study , we explored the interplays among SNGH1, miR-21-5p and miR-30a-5p in HL-1 cells treated by H/R method.

We discovered that in HL-1 cells after H/R treatment, SNHG1 was downregulated and miR-21-5p and miR-30a-5p were elevated. In addition, apoptosis was induced by H/R too. Furthermore,upregulation of SNHG1 contributed to a reduction in HL-1 apoptosis induced by H/R. We verified the bindings between SNHG1 and miR-21-5p or miR-30a-5p via the predicted binding locations by Starbase online system and subsequent dual-luciferase reporter assays. It was revealed that SNHG1 negatively regulates the expression of miR-21-5p and miR-30a-5p in cardiac muscle cells HL-1. Correspondingly, miR-21-5p or miR-30a-5p likewise inversely modulated the level of SNHG1. In addition, extended investigation on the apoptosis and cell viability associated with hypoxia/Re-oxygenation treatment in HL-1 cells demonstrated that SNHG1 significantly slashed the expression of miR-21-5p and miR-30a-5p and suppressed cell apoptosis and enhanced cell viability induced by H/R. Upregulation ofmiR-21-5p reversed the effect ofSNHG1 signifying that SNHG1 could function as a protective RNA inHL-1 cells via promotion of cell survival and suppression of apoptosis through targeting miR-21-5p. Similarly, miR-30a-5p is associated with miR-21-5p in expression and presents similar functions in inducing cell apoptosis. To detail, overexpression of miR-30a-5p could inhibit apoptosis and stimulate cell survival of HL-1 cells treated by H/R. Inhibition of miR-30a-5p could restore cell apoptosis and inhibit cell survival. Therefore, we unveiled that SNHG1 protected the HL-1 cells from H/R treatment in stimulation of cell survival and reduction of apoptosis by targeting and inhibiting miR-21-5p and miR-30a-5p.

CONCLUSION

the present study puts forward novel regulatory mechanism of SNHG1 in the moderation of hypoxia/Re-oxygenation-induced cardiac muscle cell cycle apoptosis via miR-21-5p/miR-30a-5p repression. This might disclose potential therapeutics in the future in cardiovascular diseases such as ischemic reperfusion injury.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication.

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