Long non-coding RNA NEAT1 modulates hypoxia/reoxygenation-induced cardiomyocyte injury via targeting microRNA-520a

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Received June 7, 2018; Accepted January 18, 2019

DOI: 10.3892/etm.2019.7788

Abstract. In the present study, a hypoxia/reoxygenation (H/R) model of cardiomyocytes was established to investigate the effects of long non-coding RNA (LncRNA) Nuclear Enriched Abundant Transcript 1 (NEAT1) and microRNA (miR)-520a on H/R-induced cardiomyocyte apoptosis. Flow cytometry and terminal deoxynucleotidyl transferase dUTP nick end labeling staining were used to evaluate cell apoptosis. Luciferase activity assay was used to investigate whether miR-520a targets NEAT1. Results revealed that NEAT1 was significantly upregulated and miR-520a was downregulated in the ischemia/reperfusion myocardium and the cardiomyocytes that received H/R treatment. Further study demonstrated that knockdown of NEAT1 and overexpression of miR-520a serves a protective role against H/R-induced cardiomyocyte apoptosis. miR-520a directly targets NEAT1 and its expression level is negatively correlated with that of NEAT1. The findings suggested that NEAT1 and miR-520a may protect cardiomyocytes from apoptosis through regulating apoptotic proteins B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein, and altering cleaved caspase3 expression levels.

Introduction

Long non-coding RNAs (LncRNAs) are a novel class of non-coding RNA, which are longer than 200 nucleotides. Increasing evidence have suggested that LncRNAs serve a crucial role in various pathological and physiological processes, including cell proliferation, differentiation and metabolism. Furthermore, LncRNAs participate in various key regulatory processes, which are not limited to X-chromosome silencing, genomic imprinting, chromatin modification, transcriptional activation, transcriptional interference and intranuclear transport (1).

Ischemia/reperfusion (I/R) is a pathological process characterized by a reduction of blood supply to tissues, followed by the subsequent restoration of perfusion and concomitant re-oxygenation (2). Through a multitude of mechanisms, the re-establishment of blood flow causes secondary damage to the ischemic tissue, producing an I/R injury. I/R injury predominantly contributes to the morbidity and mortality of coronary artery disease. Myocardial infarction, and the subsequent acute loss of viable myocardium, is the leading cause of mortality in industrialized countries, and has been reported to account for >400,000 fatalities a year in the United States (3). Even if a patient survives the acute phase of myocardial infarction, the subsequent injury to cardiac function accompanied by myocardial remodeling will notably lower the quality of life.

The pathogenesis of I/R injury is complex, and a combination of several mechanisms act either independently or together (3). LncRNAs are known to have various functions binding proteins or microRNAs (miRs). Numerous studies have demonstrated that LncRNAs may function as competing endogenous RNAs that can crosstalk with mRNAs by competitively binding to their common miRs (4-6). Among these LncRNAs, Nuclear Enriched Abundant Transcript 1 (NEAT1) is a critical structural component of paraspeckles and is essential for paraspeckle formation (7). NEAT1 has two isoforms that share the same transcription start site. With its structural characteristics, NEAT1 has been reported to contribute to the progression of various of types of cancer including breast and prostate cancer. However, there are relatively few studies, which have examined the association between NEAT1 and ischemic injury.

In the present study, a hypoxia/reoxygenation (H/R) cell model was established to explore the biological effect of NEAT1 and miR-520a. The expression level of NEAT1 in the H/R-induced cardiomyocyte injury model was evaluated.

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Key words: long non-coding RNA nuclear enriched abundant transcript 1, hypoxia/reoxygenation, cardiomyocytes, microRNA

Subsequently, a loss of function study was used to investigate the role of NEAT1 in myocardial injury. Furthermore, the underlying molecular mechanism of NEAT1 in myocardial injury was explored. It was identified that NEAT1 could be a potential therapeutic biomarker for the diagnosis and treatment of ischemia heart disease. A novel explanation for the mechanism of myocardial injury was suggested.

Materials and methods

Cell culture and low oxygen treatments. The H9c2 cardiomyocyte cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 12% fetal bovine serum (FBS) and 1% antibiotics (streptomycin and penicillin) in a humidified incubator atmosphere containing 5% CO_2 at 37˚C. To establish the H/R model, H9c2 cells were cultured in a humid atmosphere containing 5% CO_2 and 95% N_2 for 12 h followed by incubation under normal conditions (5% CO_2) for 0, 3, 6 and 12 h.

I/R model. A total of 40 Sprague Dawley male rats (age, 8 weeks; weight, 230-250 g) were randomly divided into five groups (n=8/group): Sham, 0 min post-I/R, 30 min post-I/R, 60 min post-I/R and 120 min post-I/R. Rats had access to food and water *ad libitum* and were maintained under controlled conditions (temperature, 22-25˚C; 50-60% humidity; 12-h light-dark cycle, with lights on at 0700 h). Rats were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and artificially ventilated. Following this, the left anterior descending coronary arteries (LADs) were exposed and 6-0 silk sutures were used to ligate the LADs. Cyanosis in the anterior ventricular walls was used to confirm ligation. Ischemia was induced using small vinyl tubes that were threaded through the ligatures. Following 30 min of ischemia, tubes were translocated and coronary circulation was restored for 0, 30, 60 or 120 min, as previously described (8). Following reperfusion, the blood vessel connecting the heart was severed. The hearts were then removed. Subsequently, the infarct area of myocardium tissues and blood samples were collected for further analysis. Sham control animals were subjected to the entire surgical procedure without LAD ligation. All procedures were performed following the Declaration of Helsinki of the World Medical Association. The research protocol was approved by the Ethics Committee of The First Affiliated Hospital of Jiaxing University (Jiaxing, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from H9c2 cells and heart tissue from I/R rats using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. RNA concentration was detected using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using QuantiTect Reverse Transcription kit (Qiagen China Co., Ltd., Shanghai, China) using the following thermocycling conditions: 50˚C for 60 min followed by 70˚C for 15 min. The relative expression levels of lncRNAs and miRs were detected using the SYBR Green qPCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and a 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The following primer pairs were used for the qPCR: NEAT1 forward, 5'-GCTCTGGGACCTTCGTGACTCT-3' and reverse, 5'-CTGCCTTGGCTTGGAAATGTAA-3'; GAPDH forward, 5'-CCTCGTCCCGTAGACAAAATG-3' and reverse, 5'-TCTCCACTTTGCCACTGCAA-3'; miR-520a forward, 5'-ACACTCCAGCTGGGAAAGTGCTTCCC-3' and reverse, 5'-CTCAACTGGTGTCGTGGA-3'; and U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The following thermocycling conditions were used for the qPCR: Initial denaturation at 50˚C for 2 min, 95˚C for 10 min; 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. lncRNA and miR levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to GAPDH and U6, respectively (9).

Adenovirus construction. The short hairpin (sh)RNA sequence targeting NEAT1 was 5'-GCCAUCAGCUUUGAAUAAAUU-3', the shRNA control sequence was 5'-CCATGACTTCGGATCGGGTCG-3', the sequence of miR-520a was 5'-AAAGUGCUUCCCUUUGGACUGU-3' and the control scramble sequence of miR-520a was 5'-UUCUCCGAACGUGUCACGUTT-3' were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Adenovirus-mediated overexpression of NEAT1 was performed using the following primer pairs: NEAT1 forward, 5'-CTTCCTCCCTTTAACTTATCCATTCAC-3' and reverse, 5'-CTCTTCCTCCACCATTACCAACAATAC-3'; and for control forward, 5'-ACTTTATATGCCGCTACCTACTAT-3' and reverse, 5'-CCTATACTGTGGCTATGGCAGTACT-3'. These primers were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The adenovirus construction system including the pHB-U6-CMVMCS-PGK-ZsGreen-pur, pSPAX2 and pMD2G vectors were kindly provided by Tianjin Medical University (Tianjin, China). The shRNA sequences were cloned into the pHB-U6-CMVMCS-PGK-ZsGreen-puro vector followed by the transduction of 293T cells with the packaging plasmid pSPAX2 and the envelope plasmid pMD2G using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following 6-h transfection, the medium was replaced with DMEM supplemented with 10% FBS. Following a 72-h incubation, the 293T lentiviral supernatant (Ad-shNEAT1, Ad-shctrl, Ad-miR-520 and Ad-ctrl) was harvested following 72-h for centrifugation at 1,000 x g for 2 h at 4˚C to remove cell debris.

To determine the viral titer, 293T cells were seeded into 96-well plates at a density of $5x10^3$ cells/well. Lentiviral supernatant was serially diluted three times and 10 μl of serially diluted lentiviral vector was added to each corresponding well, respectively. DMEM culture medium was added to a total volume of 100 μl. Following 48-h incubation, the medium was replaced with 100 μl complete DMEM medium supplemented with 1.5 μg/ml puromycin. Following 24-h incubation, the medium was replaced with complete medium without puromycin. Fluorescence was observed under a fluorescent microscope(Zeiss GmbH, Jena, Germany). Viral titer was calculated using the following formula: viral titer (TU/ml)= cell number x fluorescence percentage x MOI x virus dilution multiplier x 10^3.

Transfection with shRN lentiviral plasmid. In the *in vitro* study, H9c2 cells were seeded into six-well plates and incubated for 24 h. Following incubation, the adenovirus solution was used to transfect cells at a multiplicity of infection (MOI) of 0. 1 for 24 h. Transfection efficiency was examined following 24-h transfection. Cells were observed under a fluorescence microscope (magnification, x100, Zeiss GmbH). The ratio of green (positive) cells compared with the total number of cells indicated the infection efficiency.

In the *in vivo* study, 3 and 7 days prior to the establishment of the I/R model, rats received a tail vein injection with the adenovirus at a MOI of 0.6. The efficiency of adenovirus knockdown and gene overexpression was evaluated using RT-qPCR.

Luciferase assay. Bioinformatics analysis was performed using TargetScanHuman (www.targetscan.org/vert_72) to predict NEAT1 as a target gene of miR-520a and the luciferase assay was performed as previously described (10). The 3'-untranslated region (UTR) of NEAT1, harboring either the wild-type or mutant miR-520a binding site was cloned into the psiCHECK-2 vector (Promega Corporation, Madison, WI, USA) immediately downstream of the stop codon of the luciferase gene to generate the psiCHECK-PDK1-3'-UTR luciferase reporter plasmid. Plasmid DNA and miR-520a mimics or control miRs (Shanghai GenePharma Co., Ltd., Shanghai, China) were co-transfected into 293T cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 72 h. Luciferase activities were measured with a Dual-Glo Luciferase Assay System (Promega Corporation). Firefly luciferase activity was normalized to *Renilla* luciferase activity and all experiments were performed in triplicate.

H/R model. H9c2 cells were cultured in a modular incubator (Model 3131; Thermo Fisher Scientific, Inc.) containing 1% O_2, 94% N_2 and 5% CO_2. Following hypoxia for 24 h, the cells were exposed to 95% air, 5% CO_2 and 37˚C for 12 h. Cells in the normal control group were cultured with 5% CO_2 at 37˚C.

Flow cytometry assay. Cells were trypsinized and re-suspended in PBS. Cell apoptosis was analyzed using the Annexin- V-FITC Apoptosis Detection kit (cat. no. C1062S; Beyotime Institute of Biotechnology, Haimen, China). Briefly, following 48-h transfection, $1x10^5$ H9c2 cells were harvested with trypsin, washed with PBS and resuspended in 100 μl binding buffer. Cells were subsequently stained with 5 μl Annexin V-FITC in the dark for 10 min at room temperature, followed by incubation with 5 μl PI solution for 5 min at room temperature. Cell apoptosis was detected using a FACSVerse flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo software (version 7.6.5; BD Biosciences).

Western blotting. Total protein was extracted from cells using radioimmunoprecipitation assay buffer (Tianjin Bioco Biochemical Co., Ltd., Tianjin, China) containing 0.1 mM proteinase inhibitor. Total protein was quantified and 40 mg protein/lane was separated via SDS-PAGE on 10% gels. The separated proteins were transferred onto polyvinylidene fluoride membranes (EMD Millipore Corporation, Billerica, MA, USA) and blocked for 2 h at room temperature with 5% skimmed milk. The membranes were incubated with primary antibodies against B-cell lymphoma-2 (Bcl-2, 1:200; cat. no. ab196495), Bcl-2-associated x protein (Bax, 1:200; cat. no. ab182734), cleaved Caspase-3 (1:200; cat. no. ab214430), Caspase-3 (1:200; cat. no. ab90437), GAPDH (1:200; cat. no. ab181602; all Abcam, Cambridge, MA, USA) overnight at 4˚C. Membranes were washed three times in Tris-buffered saline with Tween 20. Following primary incubation, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology) for 2 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence reagent (Tianjin Bioco Biochemical Co., Ltd.). Protein expression was quantified using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA) with GAPDH as an internal control.

Rescue experiments. To further confirm the association between miR-520a and NEAT1, a rescue experiment was performed using adenovirus-mediated overexpression of miR-520a and NEAT1. H9c2 cells were infected with miR-520a and NEAT1 overexpression adenoviruses for 48 h. Following 48-h infection, cells were subjected to H/R treatment. Flow cytometry and Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) staining were performed to evaluate apoptosis.

TUNEL staining. Heart specimens were embedded in paraffin and cut into 5-μm-thick sections. Following deparaffinization and rehydration, the paraffin sections were washed in PBS three times. Subsequently, the sections were incubated with Proteinase K working solution (10 μg/ml in 10 mM Tris/HCl, pH 7.5-8.0) at 37˚C for 20 min and then treated with TUNEL working solution (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Following this, sections were counter stained with 4', 6-diamidino-2-phenylindole for 5 min at 37˚C.

Statistical analysis. All data were expressed as the mean ± standard deviation, and $P<0.05$ was considered to indicate a statistically significant difference. A two-tailed unpaired Student's t-test or one-way analysis of variance (ANOVA) was used to compare two or more than three groups, respectively. ANOVA with Scheffe's post hoc test was used to evaluate the statistical significance of differences between groups. Pearson correlation analysis was used to examine the association between miR520a and NEAT1.

Results

NEAT1 is upregulated in the myocardium and cardiomyocytes subjected to I/R or H/R injury. A rat I/R injury model and a H/R-induced cardiomyocyte injury model were established. RT-qPCR was performed to evaluate the expression of NEAT1. NEAT1 was significantly upregulated in the myocardium and cardiomyocytes subjected to I/R and H/R injury (Fig. 1).

NEAT1 knockdown inhibits cardiomyocyte apoptosis induced by H/R. The cardiomyocyte H/R model was established to investigate the effect of NEAT1. Annexin V-FITC/PI staining

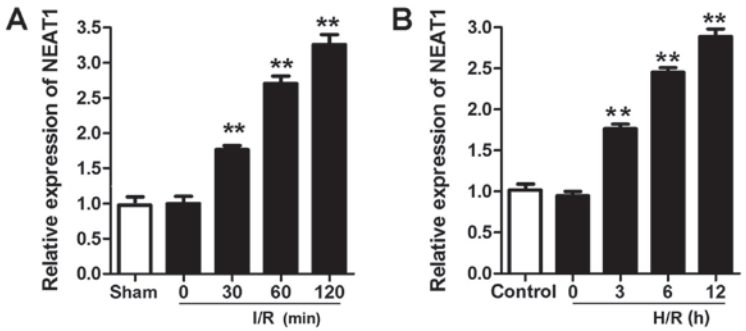

Figure 1. LncRNA NEAT1 is upregulated in myocardium and cardiomyocytes subjected to I/R and H/R. Reverse transcription-quantitative polymerase chain reaction was used to determine the expression of LncRNA NEAT1 in the (A) myocardium post-I/R and in (B) cardiomyocytes post-H/R. Data were presented as the mean ± standard deviation (n=6). **P<0. 01 vs. Sham/Control. LncRNA, long non-coding RNA; NEAT1, Nuclear Enriched Abundant Transcript 1; I/R, ischemia/reperfusion; H/R, hypoxia/reoxygenation.

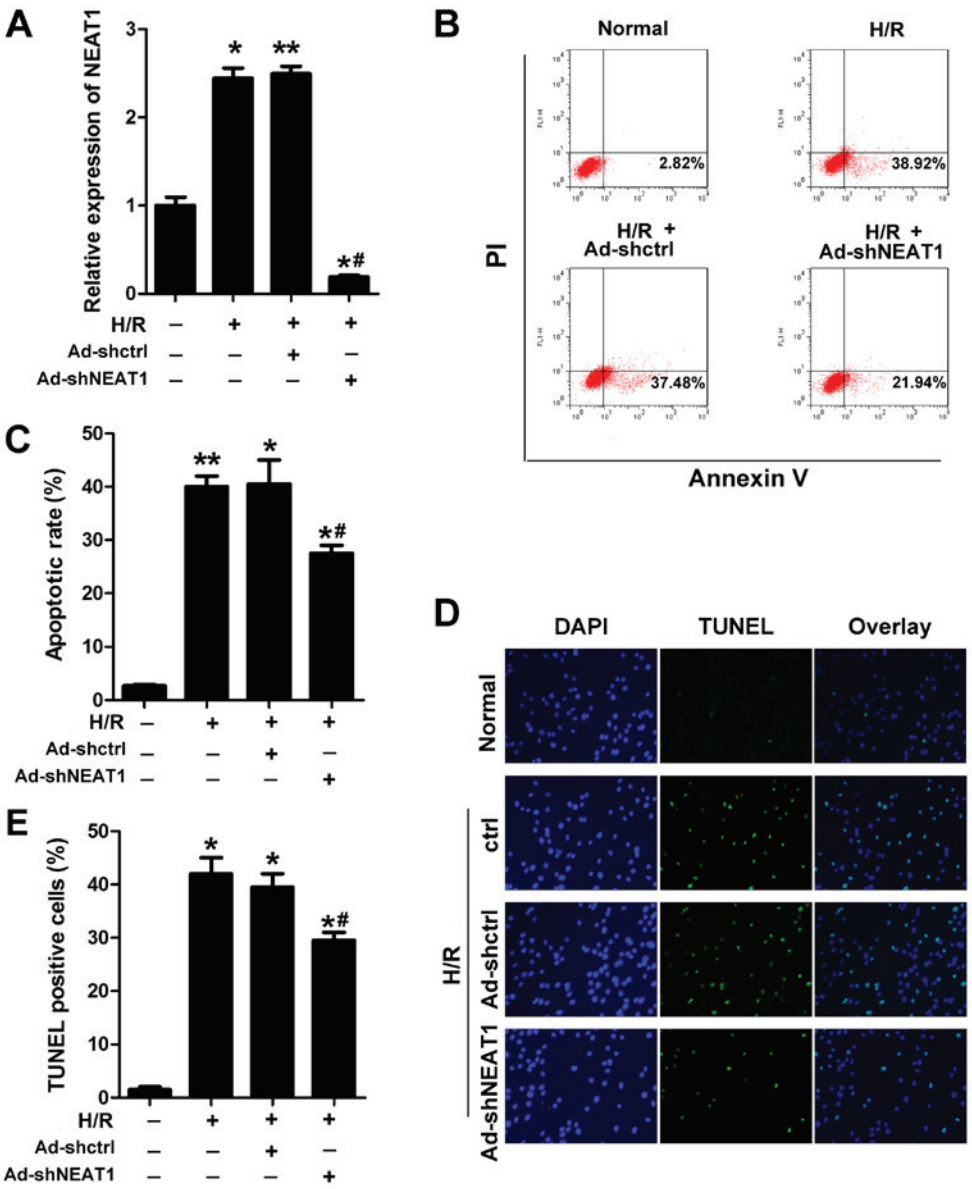

Figure 2. NEAT1 knockdown inhibits cardiomyocyte apoptosis induced by H/R. (A) Reverse transcription-quantitative polymerase chain reaction was used to determine the expression of LncRNA NEAT1 in cardiomyocytes post-H/R with Ad-shNEAT1 treatment. (B and C) Flow cytometry was used to evaluate the apoptotic rates of cardiomyocytes with H/R injury that received Ad-shNEAT1 treatment. (D and E) TUNEL assay was used to detect cardiomyocyte apoptosis with H/R injury that received ad-shNEAT1 treatment (magnification, x100). Data were presented as the mean ± standard deviation (n=6). *P<0. 05 and **P<0. 01 vs. Control; $^{\#}$P<0. 05 vs. Hypoxia. TUNEL, Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling; LncRNA, long non-coding RNA; NEAT1, Nuclear Enriched Abundant Transcript 1; H/R, hypoxia/reoxygenation; DAPI, 4', 6-diamidino-2-phenylindole; Ad-shNEAT1, NEAT1 knockdown adenovirus; Ad-shctrl, control knockdown adenovirus.

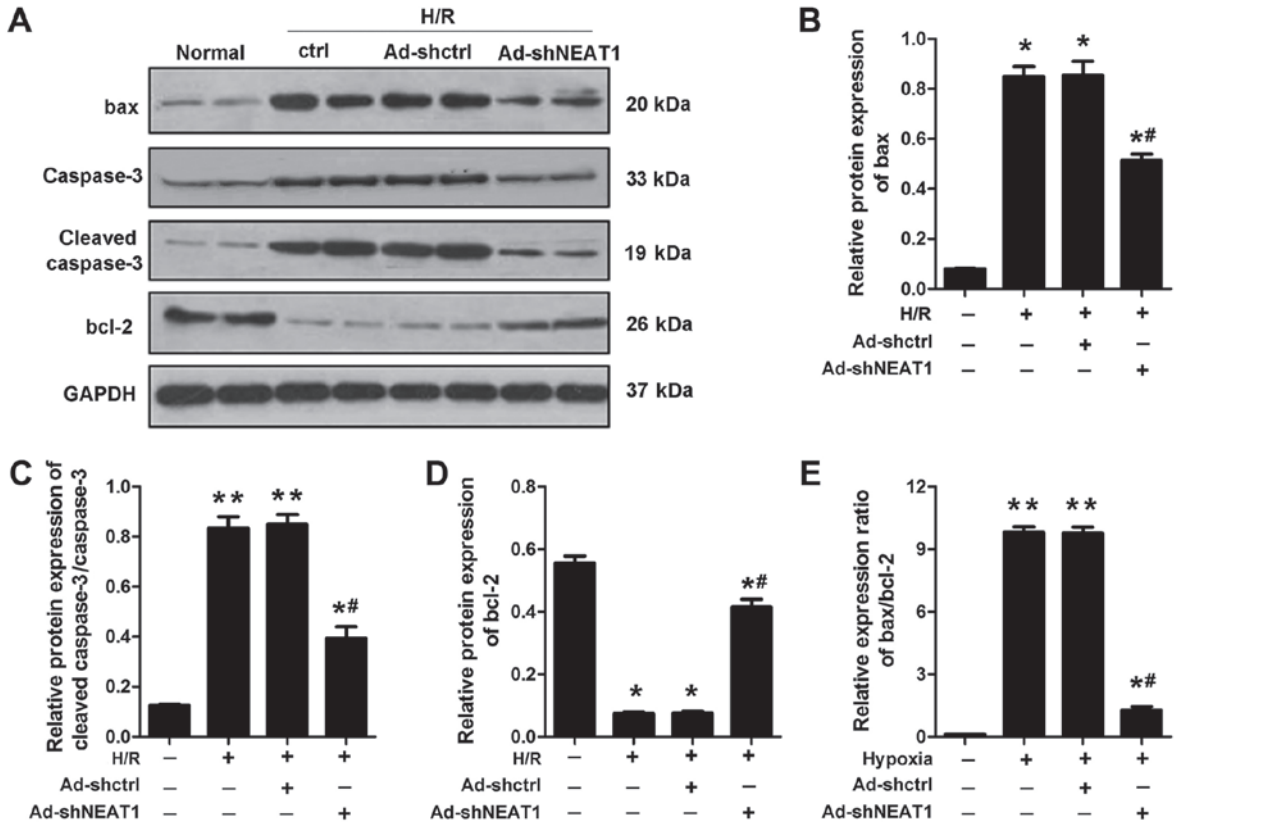

Figure 3. NEAT1 knockdown regulates the expression of apoptotic proteins. (A-E) Western blotting was performed to detect the expression of apoptotic proteins Bax, Bcl-2, caspase3 and cleaved-caspase3. Results were presented as the mean ± standard deviation (n=6). *P<0. 05 and **P<0. 01 vs. Control, #P<0. 05 vs. Hypoxia. NEAT1, Nuclear Enriched Abundant Transcript 1; Ad-shNEAT1, NEAT1 knockdown adenovirus; Ad-shctrl, control knockdown adenovirus; H/R, hypoxia/reoxygenation.

with flow cytometry and TUNEL staining were performed to evaluate the apoptosis of cardiomyocytes. Ad-shNEAT1 was used to knock down the expression of NEAT1, which was effective (Fig. 2A). NEAT1 knockdown with the application of Ad-shNEAT1 significantly reduced the rate of apoptosis of cardiomyocytes subjected to H/R (Fig. 2B-E).

NEAT1 knockdown regulates the expression of apoptotic proteins. To further determine that NEAT1 regulates the apoptosis of cardiomyocytes, the relative protein expression levels of apoptotic proteins Bax, Bcl-2 and cleaved-caspase3 were assessed. H/R significantly elevated the expression levels of Bax, caspase3 and cleaved caspase-3, but inhibited the expression level of Bcl-2 compared with the control group. The knockdown of NEAT1 significantly reduced the expression levels of Bax, caspase3 and cleaved-caspase3 that were elevated by H/R. Furthermore, knockdown of NEAT1 significantly increased the expression levels of Bcl-2 that were inhibited by H/R (Fig. 3).

NEAT1 is the target of miR-520a. LncRNA participates in cell processes through various mechanisms, including sequestering targeting miRs. Bioinformatics analysis was used to predict NEAT1 as a target gene of miR-520a (Fig. 4A). Luciferase activity was assessed to determine whether NEAT1 binds to miR-520a. miR-520a was successfully overexpressed in cardiomyocytes transfected with Ad-miR-520a (Fig. 4B). The expression of NEAT1 was reduced in cardiomyocytes transfected with Ad-miR-520a (Fig. 4C). Furthermore, miR-520a was indicated to directly target NEAT1 (Fig. 4D-F).

miR-520a overexpression inhibits cardiomyocyte apoptosis induced by H/R, and NEAT1 reverses this effect. The effect of miR-520a on cardiomyocyte apoptosis was assessed. Ad-miR-520a and Ad-NEAT1 were used to elevate miR-520a and NEAT1 expression, respectively (Fig. 5A and B). Flow cytometry and TUNEL assay results indicated that miR-520a overexpression resulted in a similar effect as knockdown of NEAT1 and significantly reduced the apoptosis rate of cardiomyocytes subjected to H/R (Fig. 5C-F). NEAT1 overexpression significantly reversed the effects of miR-520a on cardiomyocyte apoptosis (Fig. 5C-F).

miR-520a is downregulated in I/R myocardium and H/R cardiomyocytes and is negatively correlated with the expression of NEAT1. As it was demonstrated that miR-520a possesses a protective role in H/R-induced cardiomyocytes, the miR-520a expression levels in ischemic myocardium and cardiomyocytes subjected to I/R and H/R were evaluated. miR-520a was significantly downregulated in ischemic myocardium and cardiomyocytes subjected to I/R and H/R (Fig. 6A and B). Pearson correlation analysis was performed to examine the association between NEAT1 and miR-520a. The results indicated that the relative expression of NEAT1 was negatively correlated with the relative expression of miR-520a (Fig. 6C).

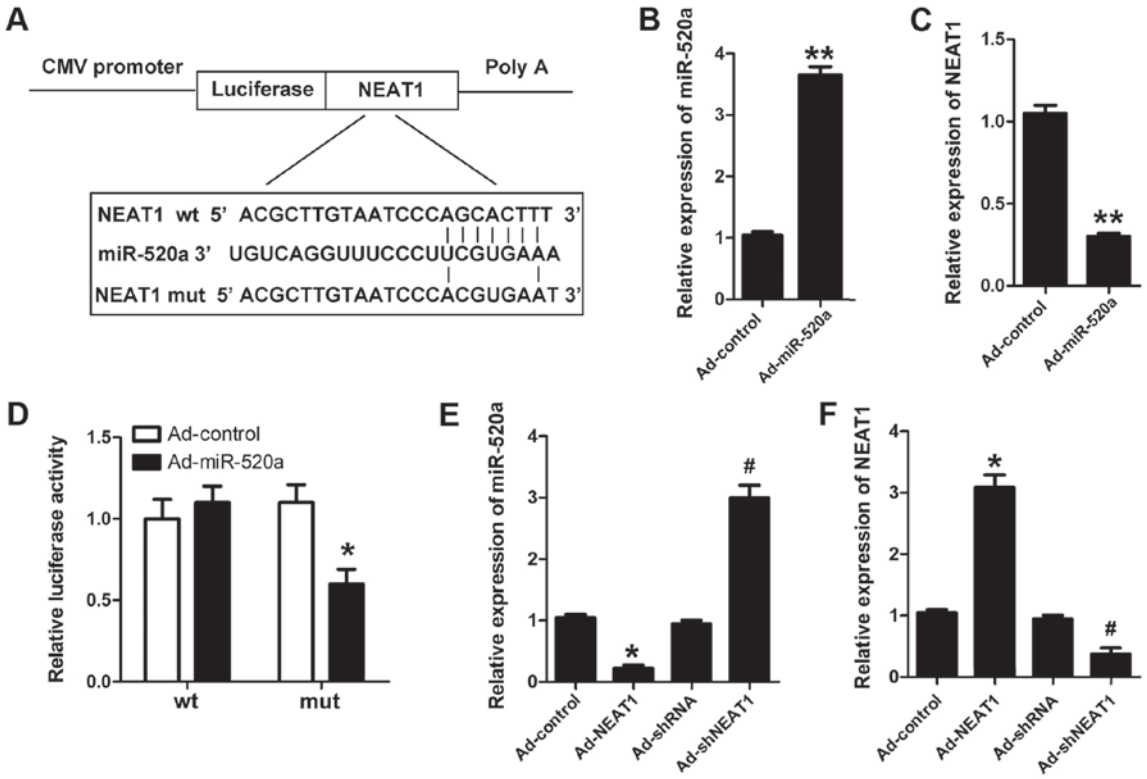

Figure 4. NEAT1 targets miR-520a in cardiomyocytes. (A and B) Bioformatics analysis predicted miR-520a as a target of NEAT1. Expression levels of (B) miR-520a and (C) NEAT1 were detected by RT-qPCR. (D) The luciferase assay was performed to determine whether NEAT1 binds to miR-520a. (E) RT-qPCR was performed to evaluate the gene expression of miR-520a in different groups. (F) RT-qPCR was used to evaluate the expression of NEAT1 in different groups. Results were presented as the mean ± standard deviation (n=6). *P<0. 05 and **P<0. 01 vs. Ad-control; $^{\#}$P<0. 05 vs. Ad-shRNA. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Ad-NEAT1, NEAT1 overexpression adenovirus; NEAT1, Nuclear Enriched Abundant Transcript 1; miR, microRNA; Ad-control, control overexpression adenovirus; Ad-shNEAT1, NEAT1 knockdown adenovirus; Ad-shRNA, control knockdown adenovirus.

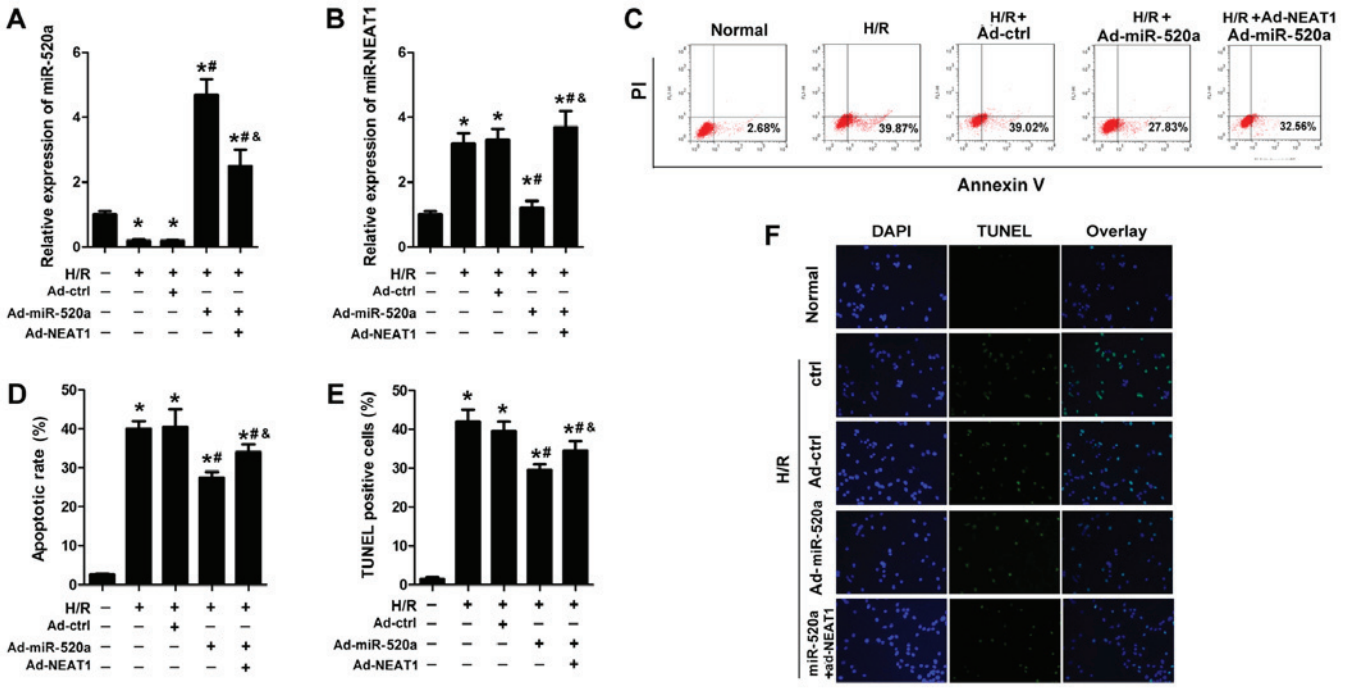

Figure 5. miR-520a overexpression inhibits cardiomyocyte apoptosis induced by H/R. (A) The expression level of miR-520a and NEAT1 were detected by reverse transcription-quantitative polymerase chain reaction in cardiomyocytes with the application of Ad-miR-520a and Ad-NEAT1. (C and D) Flow cytometry was used to evaluate the apoptotic rates of cardiomyocytes subjected to H/R injury and treatment with Ad-miR-520a. (E and F) TUNEL assay was used to detect cardiomyocyte apoptosis subjected to H/R injury with the treatment of Ad-miR-520a (magnification, x100). Data were presented as the mean ± standard deviation (n=6). *P<0. 05 and **P<0. 01 vs. Control; $^{\#}$P<0. 05 vs. hypoxia; $^{\&}$P<0. 05 vs. H/R+Ad-miR-520a. TUNEL, Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling; NEAT1, Nuclear Enriched Abundant Transcript 1; miR, microRNA; Ad-miR-520a, miR-520a overexpression adenovirus; Ad-ctrl, control overexpression adenovirus; H/R, hypoxia/reoxygenation; PI, propidium iodide.

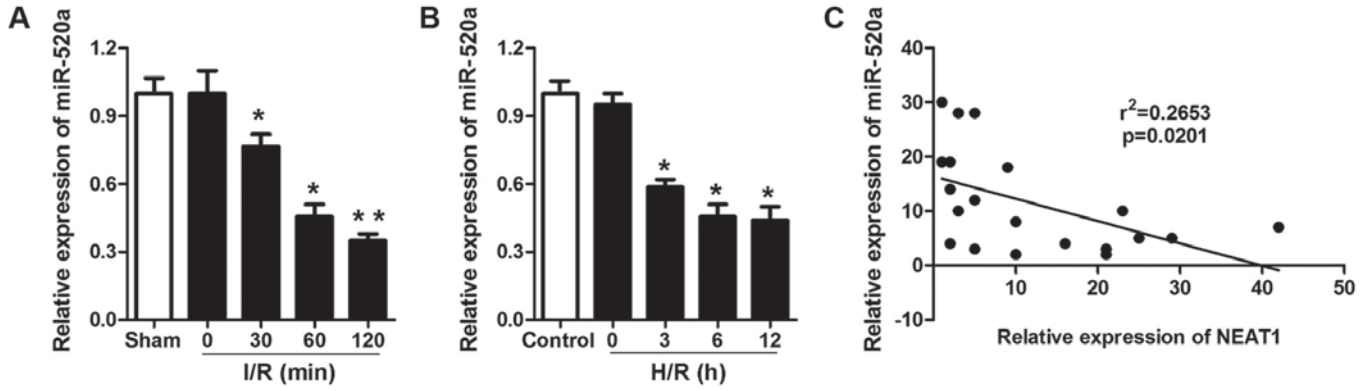

Figure 6. Correlation between NEAT1 and miR-520a. Reverse transcription-quantitative polymerase chain reaction was used to determine the expression of miR-520a in the (A) myocardium following I/R and in (B) cardiomyocytes subjected to H/R. (C) Pearson analysis was performed to evaluate the correlation between NEAT1 and miR-520a. Data were presented as the mean ± standard deviation (n=15). *P<0.05 and **P<0. 01 vs. Sham/Control. NEAT1, Nuclear Enriched Abundant Transcript 1; miR, microRNA; H/R, hypoxia/reoxygenation; I/R, ischemia/reperfusion.

Discussion

Ischemic injury is a severe cardiovascular disease, a serious threat to human life which can lead to congestive heart failure and malignant arrhythmia (11). Thus, in order to provide valuable benefits to current therapies it is necessary to distinguish novel, highly sensitive and specific biomarkers for the early diagnosis of acute myocardial infarction (AMI) and to identify possible regulatory targets of AMI.

Apoptosis is characterized by cell shrinkage, chromosomal DNA and nuclear fragmentation (12,13). It is a type of programmed cell death that contributes to cardiac diseases, including myocardial infarction, heart failure and I/R injury. Cardiomyocyte apoptosis was notably increased in the ischemic hearts in recent studies and subsequently lead to continuous cardiomyocyte loss (14). Several studies have demonstrated that inhibiting apoptosis of cardiomyocytes protected against I/R injury and improved cardiac dysfunction significantly (15,16). Furthermore, it has been demonstrated that LncRNAs may regulate cell apoptosis through altering the pro-apoptotic or anti-apoptotic protein expression. However, how LncRNAs regulate the process of cell apoptosis remains unclear. The major signaling pathways mediating apoptosis involve the death receptor, mitochondrial and endoplasmic reticulum-stress signaling pathways.

Mitochondrial cell death is regulated by pro-apoptotic proteins, such as Bax, and anti-apoptotic proteins, such as Bcl-2. Intracellular stress, including oxidative stress, serum deprivation and DNA damage, contributes to mitochondrial membrane permeability changes and subsequent release of several pro-apoptotic factors such as cytochrome *c*, apoptosis-inducing factor, endonuclease G and second mitochondria-derived activator of caspases (Smac/Diablo). These pro-apoptotic factors result in the initiation of apoptosis.

In the present study, NEAT1 was upregulated in the myocardium and cardiomyocytes following I/R and H/R, respectively. Further study demonstrated that the knockdown of NEAT1 inhibited the apoptosis of cardiomyocytes. Additionally, NEAT1 knockdown elevated the expression levels of Bax and cleaved caspase3, and reduced the expression level of Bcl-2.

LncRNAs regulate molecules through multiple mechanisms, including epigenetic regulation, genomic imprinting, RNA stability, RNA alternative splice and miR regulation (1,17-20). miRs are small, non-coding RNAs that regulate the expression of protein-encoding genes at post-transcriptional level. Accumulating evidence has reported the key roles of miRs in the process of cardiac disease, including hypoxia-induced myocardial injury. In the present study, the use of bioinformatics analysis predicted that human NEAT1 could bind to miR-520a. Luciferase activity was also assessed, which confirmed this prediction. Following this, it was investigated whether miR-520a was involved in I/R injury in the present study. The results indicated that miR-520a overexpression also inhibited apoptosis induced by H/R. The results revealed that infection of Ad-control alone did not significantly alter the expression of miR-520a or NEAT1, nor did it significantly impact cell apoptosis compared with the control group. For this reason, co-infection of Ad-miR-520a and Ad-control as an additional control in the rescue experiment was not performed.

In conclusion, the present study indicated the role of NEAT1 and miR-520a in H/R and I/R injury. Knockdown of NEAT1 and overexpression of miR-520a protected cardiomyocytes from H/R-induced cell apoptosis. Furthermore, it was revealed that NEAT1 can directly bind to miR-520a. However, miRs participate in various physical and pathological processes by regulating the level of target genes. miRs may participate in the regulation of signal pathways involved in cell apoptosis which include the PI3K/AKT signaling pathway. Cardiomyocyte apoptosis was induced by altering the expression levels of bax, bcl-2 and cyto-c, which are relevant to the mitochondria. However, the mitochondrial membrane potential was not examined and this may be considered a potential limitation of the current study.

Taken together, the present findings may aid in the design of an adenovirus vector for clinical use to knockdown NEAT1 expression, which may be protective in ischemia. Further studies concerning the target of miR-520a and downstream genes are required.

Acknowledgements

Not applicable.

Funding

This study was supported by grants from the 2015 Zhejiang Province Medical and Health Science and Technology Research Fund (grant no. 2015KYB387), 2015 Zhejiang Province Traditional Chinese Medicine Scientific Research Fund (grant no. 2015ZA203), 2016 Zhejiang Province Traditional Chinese Medicine Scientific Research Fund (grant no. 2016ZA191), 2016 Zhejiang Province Medical and Health Science and Technology Research Fund (grant no. 2016KYB287), 2016 Jiaxing Science and Technology Project Fund (grant no. 2016AY23038), 2017 Jiaxing Science and Technology Project Fund (grant no. 2017AY33021), Jiaxing Cardiovascular Key Discipline Fund (grant no. 2014-F07), Jiaxing Key Innovation Team Fund (grant no. 2015-CX1) and the Class General Financial Grant from the China Postdoctoral Science Foundation (grant no. 2017M611587).

Availability of data and materials

All datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HW and GT performed the experiments and data collection. WS, MH and HP performed the data analysis. CZ prepared the manuscript. GQ contributed to the design of the study design and reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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