Long Noncoding RNA (lncRNA) n379519 Promotes Cardiac Fibrosis in Post-Infarct Myocardium by Targeting miR-30

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Background: Abnormally expressed long noncoding RNAs (lncRNAs) are recognized as one of the key causes of cardiac diseases. However, the role of lncRNA in cardiac fibrosis remains largely unknown.

Material/Methods: The experiment was divided into 4 groups: a sham operation group, a myocardial infarction (MI) group, a lentivirus group (LV-si-n379519), and a lentivirus control (LV-NC) group. The adenovirus expression vectors LV-si-n379519 and LV-NC were constructed and transfected into mice. Echocardiography, HE staining, and Masson staining were performed to detect the heart function and collagen volume fraction in each group. RT-PCR was used to detect the expression level of n379519, miR-30, collagen I, and collagen III. In vitro, cardiac fibroblasts (CFs) were cultured and the relationship between n379519 and miR-30 was verified using luciferase reporter vector, n379519 siRNA, and miR-30 inhibitor.

Results: The expression of n379519 was markedly upregulated in the hearts of mice with MI and in the fibrotic CFs. Knockdown of endogenous n379519 by its siRNA improved the heart function and reduced collagen deposition and the process of cardiac fibrosis. Further experiments showed the opposite trend of expression between n379519 and miR-30. Bioinformatics analysis and luciferase reporter assay indicated that n379519 directly binds to miR-30. Moreover, miR-30 inhibitor abrogated the collagen synthesis inhibition induced by n379519.

Conclusions: These findings reveal a novel function of n379519-miR-30 axis as a negative regulator for the treatment of MI-induced cardiac fibrosis and the associated cardiac dysfunction.

MeSH Keywords: Endomyocardial Fibrosis • MicroRNAs • Myocardial Infarction • RNA, Long Noncoding
Background

Myocardial infarction (MI) is an ischemic cardiomyopathy caused by the lesion of the coronary arteries or its branches, leading to stenosis or occlusion of the lumen. This occurs despite a wide appreciation that cardiomyocytes necrosis triggers inflammatory cell infiltration, which in turn activates proliferation of fibroblasts, transforms into myofibroblasts, producing large amounts of collagen fibers, eventually leading to cardiac fibrosis and ventricular remodeling [1]. The essence of cardiac fibrosis is the imbalance between extracellular matrix (ECM) synthesis and degradation, resulting in increased collagen deposition, the imbalance between various types of collagen (collagen I and collagen III increased), and the disordered tissues [2]. However, anti-fibrotic drug therapy (guideline-recommended for chronic heart failure treatment drugs, including ACE inhibitor, beta blockers and aldosterone receptor antagonists) in clinical practice has little effect [3] and the underlying mechanism of cardiac fibrosis is still unclear. Considering the important role of cardiac fibroblasts (CFs) differentiating into myofibroblasts in the progression of fibrosis [4], a more in-depth study of CF gene program and regulatory mechanism is needed to slow the progression of this disease.

IncRNAs are defined as RNA transcripts of >200 nucleotides that lack protein-coding potential [5], but base-pair with DNA or RNA in a sequence-specific manner, thus regulating gene expression and biological progress. Many studies have revealed that IncRNAs adjust the origination and progression of cardiac disease through transcriptional and posttranscriptional control and by competitively-binding miRNAs [6–8]. However, research on the biological role of IncRNAs in cardiac fibrosis is still in its infancy, and only a few articles have reported on the association between IncRNAs and cardiac fibrosis [9,10]. Therefore, further research on the role of IncRNAs in myocardial fibrosis is necessary to better understand the regulation of cardiac disease.

Members of the miR-30 family (including miR-30a, miR-30b, miR-30c-1, miR-30c-2, miR-30d, and miR-30e) are expressed in various tissues and act as indispensable regulators in different cancers [11]. miR-30 has been proved to be downregulated in hypertrophic myocardium and fibroblasts, whereas overexpressed miR-30 can decrease connective tissue growth factor levels and reduce collagen deposition [12]. In animal models, the repression of pro-fibrotic genes was enhanced when the expression of miR-30 decreases [13]. In addition, the expression level of miR-30 in human serum was found to be significantly correlated with collagen volume fraction [14].

In this study, n379519, an IncRNA that is pro-fibrotic and regulates the expression and function of ECM, as reported by Huang et al. [15], was used to further explore whether silencing it can affect specific binding to the 3′-UTR of miR-30 to improve cardiac function and reduce the process of cardiac fibrosis in MI rats.

Material and Methods

Animal experiment

Male Sprague-Dawley rats (age 8–10 weeks, weight 200–250 g) were purchased from Vital River Laboratory Animal Company (Beijing, China) and housed for 1 week at room temperature. This study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. Sixty rats (each group n=15) were divided into a sham group, an MI group, a lentivirus group (LV-si-n379519), and a lentivirus control (LV-NC) group. Rats were anesthetized and intubated, then connected to a standard limb lead II electrocardiogram (ECG). The heart was exposed by thoracotomy and pericardiotomy at the 3rd to 4th ribs. A segment of saline-soaked 6–0 suture was looped around the left anterior descending coronary artery. When the left ventricular myocardial turns white and the ECG ST segment is greater than 0.1 mv, the model is successfully established. The sham operation group did not receive ligation of the anterior descending branch. Twenty minutes after surgery, a lentivirus vector plasmid (Genepharma, Shanghai, China) 40 μl of 1×10^9 UT/ml carrying si-n379519 or si-NC (Genepharma, Shanghai, China) was injected into myocardium of rats. At the second week and the fourth week after the operation, 3 rats in the sham operation group and the MI group were sacrificed to detect the expression of miR-30, and the remaining rats were fed until the fourth week for further study.

Cardiac function and histopathological examination

Four weeks after surgery, small-animal transthoracic echocardiography (Visualsonics, Inc., Toronto, Canada) was used to take the M-ultrasonography of the heart to detect left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS). After echocardiography, the rats were sacrificed and the hearts were removed and placed 10% formalin, then tissues were embedded in paraffin for Masson Trichrome staining and HE staining.

Isolation and culture of CFs cells

The ventricular tissues of 1–3-day-old SD neonatal rats were cut into 1.0-mm pieces and placed in D-Hank’s mixed solution of 0.03% trypsin and 0.05% collagenase for 10 min. Subsequently, cells in the supernatant were isolated by centrifugation (1000 rpm, 5 min) at room temperature and cultured in Dulbecco’s modified Eagle’s medium (DMEM, 11965092, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA).
LncRNA n379519 siRNA (si-n379519), miR-30 mimics, and miR-30 inhibitor/NC inhibitor were synthesized by GenePharma Co., Ltd (Shanghai, China). The sequences were: si-n379519, 5'-CCTCTCATCTCTCATCTTCTTA-3'; miR-30 mimics, 5'-GACUGGAGCAGCAGCU-3'; miR-30 inhibitor, 5'-UCUCGGAGACCUGACGU-3'; NC inhibitor, 5'-UCCUCGGACCCUGAGCU-3'. Cells were transfected with indicated nucleotides or plasmid using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. After a 36 h transfection, CFs were harvested for further experiments.

Luciferase assays

The 3'-UTR of n379519 contained the conserved miR-30 binding sites and was amplified using PCR. The 3'-UTR sequenc- es were amplified and cloned downstream of the firefly luciferase gene in the pMIR-Report luciferase vector (Ambion, Cambridge, MA, USA) to construct a luciferase reporter vector. Wild-type (WT) or mutant (Mut) fragments of the 3'-untranslated region (3'-UTR) of the n379519 containing the predicted miR-30 binding sites. Cells were transfected with miR-30 inhibitor/NC with Lipofectamine 2000 (Invitrogen, America) for 48 h. Luciferase activity was measured using the Bright-Glo™ Luciferase Assay System (Promega, USA) and normalized to that of Renilla.

RNA extraction and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted from tissues or cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription polymerase chain reaction was performed using the PrimeScript™ RT reagent Kit (TAKARA, Otsu, Shiga, Japan). Reverse transcription was performed according to the reagent instructions. The expression levels of n379519 and miR-30 were detected using SYBR Premix Ex Taq (TAKARA, Otsu, Shiga, Japan). The amplification conditions were: at 94°C for 15 min, followed by 40 cycles (94°C for 10 s; 60°C for 32 s; 72°C for 60 s). GAPDH was used as an internal control and the relative expressions of genes were calculated using the 2^−ΔΔCT method. The primers are presented in Supplementary Table 1.

Western blotting

The peri-infract regions of myocardial or CFs were lysed in RIPA buffer containing PMSF (Beyotime, Shanghai, China). After being homogenized and centrifuged (12 000 rpm, 10 min), the supernatant protein was transferred to another tube and subjected to standard BCA assay (Solarbio, Beijing, China). We separated 50 μg of proteins to SDS-PAGE on 12% polyacrylamide gels, transferred it to a PVDF microporous membrane (Millipore, Bedford, MA, USA), then blocked it with buffer containing TBST and 5% fat-free milk for 2 h at room temperature. The PVDF membrane was incubated in the primary antibodies (diluted by TBST with 0.1% tween) at 4°C overnight. After washing 3 times, the PVDF membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit (1: 1000, Cell Signaling Technology, Inc., Danvers, MA, USA) for 2 h at room temperature. High-sensitivity chemiluminescence (Bio-Rad, Hercules, CA, USA) was used to detect the protein bands. The relative band densities were normalized to β-actin (1: 1000, Cell Signaling Technology, Inc., Danvers, MA, USA). The primary antibodies Anti-Collagen I (ab34710, 1: 1000) and Anti-Collagen III (ab7778, 1: 1000), purchased from Abcam (USA).

Statistical analysis

Data are expressed as the mean ±SD. Statistical analyses were performed using SPSS 19.0 (IBM, Armonk, NY, USA). The differences between individual groups were analyzed by one-way ANOVA followed by Fisher’s LSD test. P<0.05 was considered statistically significant.

Results

LncRNA n379519 is up-regulated in infarcted myocardium and TGF-β1-induced CFs

As shown in Figure 1, the myocardium in the left ventricle anterior turned pale and the ST segment elevation in the lead II of ECG showed that the model was successful. Compared with the sham group, the expression of n379519 was significantly upregulated at 2 and 4 weeks following MI (Figure 1B). After the induction by TGF-β1, the expression of n379519 was significantly increased, consistent with the in vivo experimental results (Figure 1C, 1D).

Suppression of n379519 improved heart function and attenuated fibrotic changes in myocardium.

To investigate the role of n379519 in the process of ventricular remodeling in vivo, we injected a siRNA-containing lentiviral plasmid into rat myocardium to suppress the n379519 gene. The echocardiography showed that knockdown of n379519...
notably restored the LVEF and LVFS (Figure 2A–2C). Meanwhile, pathological staining indicated that transfection of n379519 siRNA resulted in a smaller infarct size and a more robust left ventricular wall (Figure 2 D–2F). Furthermore, the collagen volume fraction was significantly decreased in the Lv-sin379519 group (Figure 2G). Similarly, collagen I and collagen III expression in the Lv-sin379519 group were reduced at the transcriptional level (Figure 2H, 2I).

LncRNA n379519 function as an miR-30 sponge in myocardial fibrosis

Our study revealed the fibrosis-promoting effect of n379519 in MI rat myocardium. Next, we wanted to determine the potential mechanism of n379519 in cardiac fibrosis. Through in vivo and in vitro experiments, we consistently observed significant decreases of miR-30, and this down-regulation was reversed when we injected Lv-sin379519 into the peri-infarct region of myocardial and TGF-β1 treatment CFs. Data are expressed as mean ±SD. * P<0.05, ** P<0.01 vs. sham operation group or control group. * P<0.05, ** P<0.01 vs. MI group or TGF-β1 group.

Our data demonstrate that collagen I and collagen III were significantly upregulated at the transcriptional level in MI hearts (Figure 2H, 2I) after TGF-β1 treatment of CFs (Figure 4A, 4B). However, knockdown of n379519 obviously reversed collagen deposition. To explore the potential mechanism, CFs were incubated with miR-30 inhibitor and the result indicated that miR-30 inhibitor reversed the anti-collagen deposition effect of n379519 silencing at the protein level (Figure 4C, 4D).

**Discussion**

As the main component of the myocardial cell mesenchyme, type I collagen accounts for 80–85%, functioning to maintain the structural strength of the heart, and type III collagen accounts for 10–12%, which can maintain the elasticity of the myocardium.
Figure 2. Suppression of n379519 improved heart function and attenuates fibrotic changes of myocardium. (A) Echocardiography images of each group. (B) Ejection fraction. (C) Left ventricular fractional shortening. (D) HE staining of cross sections of the heart. (E) Comparison of infarct size. (F) Comparison of left ventricular wall thickness. (G) Masson staining and Collagen Volume Fraction were used to compare the severity of myocardial fibrosis. (H) The mRNA expression level of collagen I. (I) The mRNA expression level of collagen III. Data are expressed as mean ±SD. * P<0.05, ** P<0.01 vs. sham operation group. # P<0.05, ## P<0.01 vs. MI group.
and tension of the myocardium [17]. The primary pathological changes of post-myocardial infarction are the activation, proliferation, and conversion of fibroblasts into myofibroblasts, which then synthesize and secrete large amounts of extracellular matrix proteins [18]. Hence, Masson staining and collagen are the common detection index of myocardial fibrosis.

Long noncoding RNAs are not only critical for regulating cell proliferation, differentiation, metabolism, apoptosis, and other important biological processes, but are also related to the occurrence, development, and diagnosis of diseases [19]. Although many studies have reported the regulating function of lncRNA on cardiovascular disease, little is known about myocardial fibrosis. Wisper has been shown act as a specific regulator of cardiac fibroblasts proliferation, migration, survival, and extracellular matrix deposition [20]. Liang et al. found that lncRNA n379519 is cardioprotective and improves myocardial interstitial fibrosis and systolic function.

In lncRNAs can be used as competitive endogenous RNAs to regulate multiple biological functions of cells. They often contain complements of the sequence domain of miRNAs that allow them to act as natural sponges to bind with multiple miRNAs [22]. lncRNA n379519 has been shown to be abundantly expressed in human left ventricle myocardium and mouse CFs, and knockdown of n379519 significantly reduced α-SMA and fibrosis genes (collagen 3A1, collagen 8A1, and fibronectin) expression [15]. n379519 and miR-30 have been found to be related with TGF-β signaling pathway [23,24]. Therefore, we explored whether n379519 regulates TGF-β to further regulate the expression of miR30. We demonstrated that alignment of complementary regions between n379519-3’UTR and miR-30 indicated a putative miR-30 target site in the n379519 gene. Dual-luciferase reporter assay identified the interaction between n379519 and miR-30. Furthermore, rescue experiments showed that miR-30 inhibitor reverses the increased expression of collagen caused by silencing n379519. These results provide powerful evidence that n379519 interacts with miR-30.

![Figure 3](image-url)
Figure 4. IncRNA n379519 regulated myocardial collagen deposition by targeting miR-30. (A, B) CFs were co-transfected with si-n379519 and miR-30 inhibitor. Expressions of collagen I and collagen III were detected using RT-PCR. (C, D) Protein expressions of collagen I and collagen III in CFs transfected with si-n379519 and miR-30 inhibitor were measured by Western blot. Data are expressed as mean ±SD. * P<0.05, ** P<0.01 vs. control group. # P<0.05, ## P<0.01 vs. TGF-β1 group. & P<0.05 vs. si-n379519 group.

Conclusions

Our results reveal the role and mechanism of n379519/miR-30 axis in the regulation of cardiac fibrosis after MI and provide advanced insight into the pathogenesis of IncRNAs in cardiac fibrosis.

Conflict of interest

None.
Supplementary Table

Supplementary Table 1. Sequence of primers used for RT-PCR.

| RNA name | Sequence |
|----------|----------|
| GAPDH    | Forward: 5'-TGTGTCGGTCGTGGATCTGA-3'  
Reverse: 5'-CTTCTCACCCACACCTTCCTGA-3' |
| n379519  | Forward: 5'-TTTAGTGGGTAGGGACATT-3'  
Reverse: 5'-TTATAGTGGGATGGGCAGTT-3' |
| miR-30   | Forward: 5'-TGAAACATCCTCGAC-3'  
Reverse: 5'-ACATCCAGTGTAGCATA-3' |
| Collagen I| Forward: 5'-TGACAGCAGTGTGCAAGTA-3'  
Reverse: 5'-TGGCACAGCAGTCCAACGTA-3' |
| Collagen III | Forward: 5'-AAGGACAGATCCTCGAGTACACAGA-3'  
Reverse: 5'-CTTCACTCCTGCAAATGTGTT-3' |

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