Expression and function of lncRNA ANRIL in a mouse model of acute myocardial infarction combined with type 2 diabetes mellitus

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Abstract

Background: This study intends to explore whether lncRNA ANRIL has an influence on type 2 diabetes mellitus (T2DM) complicated with acute myocardial infarction (MI) and to further investigate the underlying mechanism.

Methods: The ANRIL level in peripheral blood from patients was detected by qRT-PCR. A T2DM mouse model was established by intraperitoneal injection of streptozocin (STZ). MI was induced by ligation of the left anterior descending coronary artery. Cardiac function parameters were measured using echocardiography. Triphenyltetrazolium chloride (TTC) staining was performed to determine the infarct size, and Masson staining was conducted to delineate the area of fibrosis in the myocardium. TUNEL staining was used to detect myocardiak cell apoptosis. The expression of the myocardial fibrosis-related proteins TGF-β1, collagen I and collagen III was analysed using Western blot.

Results: ANRIL was upregulated in peripheral venous blood from patients with T2DM-MI and in myocardial tissues from the established T2DM-MI model mice. Furthermore, ANRIL overexpression caused cardiac dysfunction and increased the heart/body weight rate and infarct size in the T2DM-MI mice. Moreover, ANRIL overexpression caused myocardial fibrosis and myocardial cell apoptosis, and it increased the expression of the myocardial fibrosis-related proteins TGF-β1, collagen I and collagen III in the T2DM-MI mice. However, ANRIL knockdown exerted the opposite effects.

Conclusion: ANRIL may be involved in the progression and development of T2DM-MI, which might provide novel ideas for the prevention and treatment of cardiovasculardiseases.

Keywords: Acute myocardial infarction; lncRNA ANRIL; Type 2 diabetes mellitus

1. INTRODUCTION

Diabetes mellitus (DM) is an independent risk factor for acute myocardial infarction (MI) in European and Asian individuals.1 DM patients were reported to have a larger infarct size, atypical ischaemic symptoms, and more post-infarct complications than non-diabetic patients.2 It is well established that the clinical outcomes of acute MI in patients with type 2 diabetes mellitus (T2DM) are worse than those in non-diabetic patients.3 It is reported that mortality, morbidity and re-infarction rates are higher following MI in diabetic than in non-diabetic patients.4 To date, the increased risk has been primarily attributed to hyperglycaemia, dyslipidaemia, a prothrombotic state in the pathogenesis of T2DM and its complications.4

Further elucidation of the molecular mechanism underlying T2DM complicated with MI is of great importance for clinical treatment.

Long non-coding RNAs (lncRNAs) are mRNA-like transcripts longer than 200 bp that have no protein-coding potential.5 LncRNAs play important regulatory roles in cancer cell growth, cell differentiation, cell metastasis and cell fate decision.6,7 Antisense non-coding RNA in the INK4 locus (ANRIL), a 3.8 kb lncRNA located at the 9p21.3 locus within a gene cluster named INK4B-ARF-INK4A in the antisense direction, plays a role in the tumourigenesis of gliomas, breast cancer, and several other cancers.4–11 The chromosome (Chr) 9p21 risk locus is associated with the severity, extent, and progression of coronary artery disease, indicating a role for this locus in influencing atherosclerosis and its progression.11 ANRIL is essential in mediating Chr9p21 associations and has been reported to play an important role in many diseases, including Alzheimer disease, glaucoma, endometriosis, periodontitis, and particularly, T2DM.11 In addition, ANRIL expression is associated with a risk for atherosclerosis at Chr9p21.14 ANRIL may be implicated in the atherosclerotic process such as in thrombogenesis, vascular remodelling and/or repair, and plaque stability.15 Furthermore, ANRIL is expressed in smooth muscle cells, endothelial cells and inflammatory cells known to be stimulated by atherosclerosis.16–18 In addition, variants in ANRIL exons were found to contribute to MI risk in a Chinese Han population.19 However, the expression and function of ANRIL in patients with T2DM complicated with acute MI (T2DM-MI) remain not fully elucidated.

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Therefore, this study aimed to investigate whether ANRIL expression has an influence on patients with T2DM-MI and to further explore the underlying mechanism. Here, we first detected the level of ANRIL in peripheral venous blood from T2DM-MI patients. A T2DM-MI mouse model was then constructed, and the effects of ANRIL on cardiac function, pathological changes, myocardial fibrosis, and myocardial cell apoptosis in the T2DM-MI mice were evaluated.

2. METHODS

2.1. Patients

Patients were recruited from the Daqing Oilfield General Hospital, including healthy controls (control group, n = 26), patients suffering from T2DM but not acute MI (T2DM group, n = 24), patients suffering from acute MI but not T2DM (MI group, n = 25), and patients suffering from acute MI and T2DM (T2DM-MI group, n = 34). The diagnosis of MI was made according to contemporary guidelines, and the inclusion criteria for this study were described in a previous study. The exclusion criteria were as follows: 1) impaired glucose tolerance, 2) diabetes due to secondary causes, and 3) past history of ischaemic heart disease. This research was approved by the local research ethics committee and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained before any research procedures.

2.2. Animals

C57BL/6 male mice (aged 6–7 weeks, weighing 20 ± 2 g) were purchased from the animal laboratory of the Academy of Medical Sciences, Beijing, China. All mice used in this experiment were approved by the Ethics Committee of the Daqing Oilfield General Hospital. They were kept in separate cages and had free access to food and water in a room with 12 h light–dark cycles maintained at a temperature of 23 ± 1 °C and humidity of 50%.

2.3. Induction of T2DM mice

After being fasted overnight, mice (aged 6–7 weeks) received a single intraperitoneal (IP) injection of streptozocin (STZ, 50 mg/kg; Sigma–Aldrich, St Louis, MO, USA) for 5 days as previously described. STZ is widely used in the induction of T2DM mouse models. STZ solution was prepared fresh by dissolving it in 0.1 M citrate buffer (pH 5.5) and was terminally sterile-filtered. The normal group was injected with an equal amount of citrate buffer for the T2DM control and then received sham surgery for MI, T2DM-MI group (T2DM and MI mice), T2DM-MI + vector group (T2DM and MI mice that were injected with 800 ng/kg empty plasmid into the infarcted area of the left ventricular wall), T2DM-MI + pcDNA-ANRIL group (T2DM and MI mice that were injected with 800 ng/kg ANRIL overexpression plasmid into the infarcted area of the left ventricular wall), and T2DM-MI + shRNA-ANRIL group (T2DM and MI mice that were injected with 800 ng/kg ANRIL knockdown plasmid into the infarcted area of the left ventricular wall). The pcDNA3.1-InhRNA ANRIL (high expression plasmid) and pcDNA3.1-shLncRNA ANRIL (low expression plasmid) were made by Shanghai Genechem Co., Ltd. (Shanghai, China). The heart weight (mg)/body weight (g) ratio was calculated for all mice in each group as described previously.

2.6. Echocardiography

At week 4 after the surgery, a non-invasive transthoracic echocardiography method was used to evaluate the structure and function of the left ventricle of each mouse as previously described. In brief, mice were anaesthetized, and the parameters of heart structure and function were assessed in the two-dimensional ultrasound-guided M-curve. The parameters, including the left ventricular end-diastolic inner diameter (LVIDd), left ventricular end-systolic inner diameter (LVIDd), left ventricular end-systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV), ejection fraction (EF), fractional shortening (FS) and left ventricular mass (LVM), were automatically recorded. A Sino-Japanese AloCa5000 colour ultrasound diagnostic apparatus was used. Echocardiography was performed by a technician who was blinded to the experiment.

2.7. Triphenyltetrazolium chloride (TTC) staining

TTC was used for infarct size determination. Mice were anaesthetized, and then, the heart tissues were taken out and immediately frozen for 5 min at −20 °C. The hearts were sectioned from the apex to the base into five slices. One piece of tissue was sliced into 3 mm-thick sections, and these slices were put into 2% TTC solution and incubated at 37 °C for 20 min. The stained slices were immersed in 4% paraformaldehyde and fixed for 10 h. The non-infarcted myocardium was stained red, while the infarcted myocardium appeared white. The slices were photographed and analysed using Image-Pro Plus software (Media Cybernetics Inc, Silver Spring, MD, USA). Infarct size was expressed as a percentage of the mass of the whole myocardium.

2.8. Haematoxylin-eosin (HE) staining

The paraffin-embedded tissues were sliced into serial sections, after which they were stained with HE by using a routine staining procedure. The pathologic changes in these tissues were observed under light microscopy after HE staining. The slices were photographed and analysed using computerized Image-Pro Plus software.

2.9. Masson staining

Masson staining was performed to delineate the area of fibrosis in the myocardium. The paraffin-embedded tissues were sliced into serial sections and stained with Masson's trichrome. The fibrotic area was stained blue, while the viable area was stained red. Photographs were obtained, and the slides were observed under a stereomicroscope with the whole field view (8x).
2.10. TUNEL staining
Heart tissues were harvested and embedded with paraffin. The 4-μm-thick tissues were subjected to TUNEL staining according to the manufacturer’s instructions for an In Situ Cell Death Detection Kit (Cat. 11684817910, Roche Diagnostics, Indianapolis, IN, USA).

2.11. Western blot analysis
Protein was collected from myocardial tissues that were lysed in radioimmunoprecipitation assay buffer (RIPA) containing protease inhibitors for at 4 °C for 30 min. Cell lysates were prepared with a RIPA lysis buffer kit (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Subsequently, proteins were separated on 8% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Amersham; GE Healthcare, Chicago, IL, USA). After blocking in 5% fat-free milk (Merck, Darmstadt, Germany) overnight at 4 °C, the transferred membranes were incubated with the following primary antibodies: TGF-β1 (1:160), collagen I (1:500), collagen III (1:100) and GAPDH (1:1000) overnight at 4 °C. After washing with TBST three times, the membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (1:1000, Goldenbridge Biotechnology Co., Ltd., Beijing, China; cat. A50-106P) at room temperature for 1 h. The protein was analysed using an enhanced chemiluminescence kit (ECL; Wuhan Booute Biotechnology Co., Ltd., Beijing, China; cat. A50-106P).

2.12. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)
Total RNA was isolated from peripheral blood or the myocardial tissues using TRIzol (Invitrogen) according to the manufacturer’s protocol. Then, the isolated RNA was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). RT-qPCR was carried out using a SYBR® Premix Ex Taq™ Kit (TaKaRa Bio, Inc., Otsu, Japan) according to the manufacturer’s instructions. The primer pairs used were as follows: lncRNA ANRIL, forward, 5′-TTATGCTTTTGCAGCACCCTGTTGCTGTA-3′, and reverse, 5′-GTTCTGCCACAGCTTTGATCT-3′, and GAPDH, forward, 5′-ACACAGTCTATGCCCCATCA-3′, and reverse, 5′-TCACAAGACTTGTGCTGTA-3′. The relative expression of ANRIL was normalized to the internal control GAPDH. The comparative 2−ΔΔCq method was used to quantify gene expression levels.

2.13. Statistical analysis
All statistical analyses were performed using SPSS version 19.0 software (SPSS Inc., Chicago, Illinois, USA). Data are presented as the means ± standard deviation (SD) (x ± s). Statistical differences between two independent groups were determined using Student’s t test. For multiple groups, the statistical analyses were performed by one-way analysis of variance (ANOVA). p < 0.05 was considered to indicate a statistically significant difference.

3. RESULTS

3.1. LncRNA ANRIL was upregulated in patients with T2DM-MI
As shown in Fig. 1, the serum expression of ANRIL from peripheral venous blood was significantly increased in T2DM patients compared with that in the controls (p < 0.05). The ANRIL expression in peripheral venous blood was also significantly increased in MI patients compared with that in the controls (p < 0.05). Importantly, the expression of ANRIL was significantly increased in peripheral venous blood from T2DM-MI patients compared with that from T2DM patients (p < 0.05) or MI patients (p < 0.001). These results indicated that increased expression of ANRIL is associated with the progression and development of T2DM complicated with MI.

3.2. Successful establishment of the T2DM-MI mouse model
C57BL/6 mice were fed a high-fat diet and injected intraperitoneally with STZ for 5 consecutive days to establish a model of T2DM. The normal control group was injected with an equal amount of citrate buffer. After STZ injection, the fasting blood glucose of each mouse was measured for 3 days. Mice with blood glucose higher than 11.1 mmol/L were confirmed as T2DM mice (Table 1). Furthermore, we measured insulin resistance-related parameters before MI surgery and confirmed that the T2DM mice were insulin resistant (Supplementary Table). After the establishment of the T2DM model, the MI model was established. Then, the cardiac function parameters in each group were measured using echocardiography. As shown in Table 2, LVdD, LVIDd, LVEF, LVESV, LVEDV and LVM were significantly higher, but EF and FS were markedly lower in the T2DM-MI mice than in the normal control group.

Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Before STZ treatment</th>
<th>After STZ treatment</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>6.75 ± 0.86</td>
<td>6.51 ± 0.90</td>
</tr>
<tr>
<td>T2DM</td>
<td>6.53 ± 0.92</td>
<td>13.67 ± 1.05</td>
</tr>
</tbody>
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* p < 0.05, the T2DM+ after STZ treatment group versus the Control+ before STZ treatment group; **p < 0.05, the T2DM+ after STZ treatment group versus the T2DM+ before STZ treatment group.

The normal control group was injected with an equal amount of citrate buffer. C57BL/6 mice were fed with high fat diet and injected intraperitoneally with streptozocin (STZ) for 5 days consecutively to establish model of T2DM.
control group \( (p < 0.05) \). Collectively, these results indicated that the T2DM-MI mouse model was successfully established.

### 3.3. Effects of IncRNA ANRIL on cardiac function and pathological changes in the T2DM-MI mice

The expression of ANRIL in myocardial tissues of the T2DM-MI mice was significantly upregulated compared with that of the control group \( (p < 0.001) \). ANRIL was then overexpressed or knocked down in the mice \( (p < 0.05) \), and this change in expression was confirmed by qRT-PCR. Furthermore, the effects of ANRIL expression on cardiac function and pathological changes were determined. As shown in Table 2, ANRIL overexpression significantly increased LViDd, LViD, LVESV, LVEDV, and LVM but decreased EF and FS in the T2DM-MI mice \( (p < 0.05) \). In contrast, ANRIL knockdown significantly decreased LViDd, LViD, LVESV, LVEDV, and LVM but increased EF and FS in the T2DM-MI mice \( (p < 0.05) \). These results indicated that ANRIL overexpression caused cardiac dysfunction in the T2DM-MI mice.

Comparison of pathological changes in each group was then conducted \( (p < 0.05) \). We found that myocardial fibrosis was found in the control group. In the T2DM-MI and T2DM-MI + vector groups, we found that myocardial fibrosis was more severe than that in the control group \( (p < 0.05) \). Moreover, ANRIL overexpression significantly increased the infarct size compared with that in the T2DM-MI + vector group \( (p < 0.01) \). By contrast, ANRIL knockdown significantly decreased the infarct size in the T2DM mice \( (p < 0.01) \) (Fig. 3C).

### 3.4. Effects of IncRNA ANRIL on myocardial pathology and fibrosis in the T2DM-MI mice

HE staining was performed to determine myocardial pathology \( (p < 0.01) \), and Masson's trichrome staining was conducted to delineate the area of fibrosis in the myocardium \( (p < 0.01) \). The data revealed that the myocardial tissue structure was complete and that no myocardial fibrosis was found in the control group. In the T2DM-MI and T2DM-MI + vector groups, we found that myocardial fibres were in a disordered arrangement, the gap was narrowed, and inflammation in the necrotic area was evident. In the T2DM-MI + pcDNA-ANRIL group, myocardial fibres were also in a disordered arrangement, a large number of fibroblasts were observed in the myocardial vasculature and mesenchyme, and hyperplasia of the fibrous connective tissue was severe. In the T2DM-MI + sh-ANRIL group, cardiomyocyte staining was basically uniform, the appearance was basically normal, and slight hypertrophy with a small amount of myocardial necrosis and mild inflammatory infiltration were found in the infarct area. In addition, the blue-stained collagen fibres were more severe in the T2DM-MI and T2DM-MI + vector groups than in the control group. In addition, ANRIL overexpression aggravated collagen fibres and ANRIL knockdown alleviated collagen fibres compared with the collagen fibres in the T2DM-MI + vector group.

### 3.5. Effects of IncRNA ANRIL on myocardial cell apoptosis in the T2DM-MI mice

TUNEL staining was used to detect the effects of ANRIL on myocardial cell apoptosis in the T2DM-MI mice. As shown
Fig. 3 Effects of lncRNA ANRIL on cardiac function and pathological changes in the T2DM-MI mice. (A) Comparison of the ischemic infarct area between groups by TTC staining. Normal tissues are red, and ischemic infarct areas are pale white. Compared with the T2DM + vector group, the ANRIL overexpression group had an increased infarct area, and the ANRIL knockdown group had a decreased infarct area. (B) ANRIL overexpression significantly increased the heart/body weight ratio compared to that in the T2DM + vector group. ANRIL knockdown exerted the opposite effect. (C) The infarct size in the T2DM-MI mice was significantly increased compared with that in the control group. ANRIL knockdown exerted the opposite effect. n = 10 in each group. *p < 0.05 and **p < 0.001 vs. control group, #p < 0.05 and ##p < 0.01 vs. T2DM + vector group.

Fig. 4 Effects of lncRNA ANRIL on myocardial pathology and fibrosis in T2DM-MI mice. (A) HE staining was performed to determine myocardial pathology in the groups. (B) Masson's trichrome staining was performed to delineate the area of fibrosis in the myocardium of the groups. The fibrotic area was stained blue and viable area was stained red. n = 10 in each group. At 4 weeks after the surgery, hearts were sectioned from the apex to the base into five slices for staining, n = 10 in each group.
in Fig. 5, the percentage of apoptotic myocardial cells in the T2DM-MI group was significantly increased compared with that in the control group ($p < 0.001$). We also found that ANRIL overexpression significantly increased the apoptotic index compared with that in the T2DM-MI + vector group ($p < 0.01$), and ANRIL knockdown exerted the opposite effect ($p < 0.01$).

3.6. Effects of lncRNA ANRIL on the expression of myocardial fibrosis-related proteins in the T2DM-MI mice

TGF-β1 was reported to induce the proliferation of cardiac fibroblasts and promote the generation of extracellular matrix. $^{21}$ In addition, fibrillar collagen types I and III are the major components of the myocardial collagen matrix. $^{26}$ Here, the effects of ANRIL on the expression of the myocardial fibrosis-related proteins TGF-β1, collagen I and III in the T2DM-MI mice were analysed. As shown in Fig. 6, the protein expression of TGF-β1, collagen I and collagen III in the T2DM-MI group was significantly increased compared with that in the control group ($p < 0.01$, $p < 0.05$, and $p < 0.01$, respectively). We also found that ANRIL overexpression significantly increased the protein expression of TGF-β1, collagen I and III compared with that in the T2DM-MI + vector group.

Fig. 5 Effects of lncRNA ANRIL on myocardial cell apoptosis in the T2DM-MI mice. TUNEL staining was used to detect the effects of ANRIL on myocardial cell apoptosis in T2DM-MI mice. ANRIL overexpression significantly increased the apoptotic index compared with that in the T2DM-MI + vector group, and ANRIL knockdown exerted the opposite effect. $n = 10$ in each group. ***$p < 0.001$ vs. control group and ##$p < 0.01$ vs. T2DM + vector group.

Fig. 6 Effects of lncRNA ANRIL on the expression of myocardial fibrosis-related proteins in the T2DM-MI mice. ANRIL overexpression significantly increased the protein expression of TGF-β1, collagen I and III in myocardial tissues compared with that in the T2DM-MI + vector group, and ANRIL knockdown exerted the opposite effect. $n = 10$ in each group. *$p < 0.05$, **$p < 0.01$ vs. control group, ***$p < 0.05$ and ###$p < 0.01$ vs. T2DM + vector group.
group ($p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively). ANRIL knockdown exerted the opposite effects ($p < 0.05$, $p < 0.05$, and $p < 0.05$, respectively).

4. DISCUSSION

In this study, we first found that ANRIL was upregulated in peripheral venous blood from patients with T2DM-MI. In the established T2DM-MI mouse model, we also found that ANRIL was upregulated in myocardial tissues. Consistent with our results, ANRIL expression was increased in the mice with T2DM and cerebral infarction. In addition, variants in comprehensive estimates of the association of some genetic susceptibility to coronary artery disease and diabetes is encoded by distinct, tightly linked single-nucleotide polymorphisms (SNPs) in the ANRIL locus on Chr9p. ANRIL is essential in mediating Chr9p21 associations and has been reported to play an important role in many diseases, including T2DM. A meta-analysis also provided accurate and comprehensive estimates of the association of some genetic variants at Chr9p21 with T2DM. In addition, variants in ANRIL were found to contribute to MI risk in a Chinese Han population.

We then analysed the effect of ANRIL on cardiac function and pathological changes in the T2DM-MI mice. The results indicated that ANRIL overexpression caused cardiac dysfunction and increased the heart/body weight rate and infarct size in the T2DM-MI mice. In contrast, ANRIL knockdown exerted the opposite effects. Pfeffer et al. indicated that rats with infarctions greater than 46% had congestive heart failure, with reduced cardiac output, elevated filling pressures, and a minimal capacity to respond to pre- and after-load stresses. The heart/body weight coefficient has been used to characterize myocardial hypertrophy and could be used to assess myocardial hypertrophy. Thus, our results suggested that ANRIL contributed to heart failure and myocardial hypertrophy in the T2DM-MI mice.

Adult cardiac myocytes are terminally differentiated cells that have lost their ability to divide. A large number of studies found that apoptosis in cardiac myocytes is involved in the transition from cardiac compensation to decompensated heart failure. Myocardial fibrosis provides a structural basis for the appearance of diastolic dysfunction. In addition, it is the quality rather than the quantity of tissue that accounts for pathological left ventricular hypertrophy with associated adverse cardiovascular events and cardiac death. Fibriellar collagen types I and III are the major components of the myocardial collagen matrix. It has been found that collagen type I represents nearly 80% of the total collagen protein, while collagen III is present in lower proportions (approximately 11%). The fibrillar collagens serve as tethers between muscle cells, muscle fibres, and blood vessels and provide a scaffolding that supports the muscular and vascular compartments. In this study, in ANRIL-overexpressed T2DM-MI mice, myocardial fibres in a disordered arrangement, and a large number of fibroblasts in the myocardial vasculature and mesenchyme and severe hyperplasia of fibrous connective tissue were also observed. Furthermore, ANRIL overexpression significantly increased the apoptotic index in the T2DM-MI mice. Moreover, overexpression of ANRIL caused myocardial cell apoptosis and increased the expression of the myocardial fibrosis-related proteins TGF-β1, collagen I and III in the T2DM-MI mice. Based on these findings, it seems that ANRIL overexpression leads to myocardial fibre cell apoptosis and myocardial fibrosis by enhancing the components of the myocardial collagen matrix in T2DM-MI mice.

In conclusion, our study indicated that ANRIL was upregulated in peripheral venous blood from patients with T2DM-MI and in myocardial tissue from the established T2DM-MI mice. ANRIL overexpression caused cardiac dysfunction and led to heart failure, myocardial hypertrophy, myocardial fibre cell apoptosis, and myocardial fibres in the T2DM-MI mice. Our findings demonstrated that ANRIL may be involved in the progress and development of T2DM-MI, which might provide novel ideas for the prevention and treatment of cardiovascular diseases.

REFERENCES


