LncRNA-MALAT 1 regulates cardiomyocyte scorching in diabetic cardiomyopathy by targeting NLRP3

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ABSTRACT

Diabetic cardiomyopathy (DCM) is a common complication of diabetes mellitus, often leading to heart failure, arrhythmias and sudden death. Long-stranded non-coding RNA (LncRNA), an endogenous non-coding long-stranded RNA, is associated with diabetic cardiomyopathy. However, the mechanism of LncRNA regulation of cellular scorching in diabetic cardiomyopathy remains unclear. This study aimed to elucidate whether MALAT 1 affects cardiomyocyte scorching in DCM patients. Methods: Streptozotocin (STZ) at 35 mg/Kg was used to induce diabetes in rats. H9C2 cardiomyocytes and primary cardiomyocytes (PCM) were cultured at 5.5 and 50 mmol/L glucose, respectively. The expression levels of MALAT 1 and scorch death-related genes were detected by RT-PCR using plasmids to suppress or overexpress the related genes, respectively. Immunofluorescence, RT-PCR and Western blot were used to detect the extent of cell scorch death. RESULTS: MALAT 1 expression was elevated in diabetic and high glucose-induced cardiomyocytes and myocardial tissue from diabetic mice (p<0.001). Silencing of MALAT 1 alleviated cardiomyocyte scorch death by targeting NLRP3. Furthermore, silencing MALAT 1 reduced cell death and improved cardiac function and morphology. CONCLUSIONS: MALAT 1 is overexpressed in DCM and silencing MALAT 1 inhibits cell scorch death by affecting NLRP3 expression. We clarify for the first time that MALAT 1 may be a new therapeutic target for DCM.

Materials and methods
Samples
Serum samples from 20 diabetic patients and 20 healthy subjects were obtained from Wuhan Xinzhou District People's Hospital. All patients were free from coronary artery disease, hypertension or other
cardiac diseases. All patients participating in this study signed an informed consent form. The study was approved by the Wuhan Xinzhou District People's Hospital Ethics Committee.

**Experimental animals and model construction**

Wistar rats (180–220 g) were housed at an ambient temperature of 22°C and humidity of 55±5%, with food and water available ad libitum. Rats were randomly divided into 4 groups: control (Control), diabetic (DM), MALAT 1 expression silencing shRNA group (DM+ MALAT 1-shRNA) and NLRP3 inhibitor group (DM+ MCC950). Citrate buffer (pH=4.6) containing 35 mg/kg of streptozotocin (STZ, Sigma, St. Louis, MO) was administered intraperitoneally to rats for 5 consecutive days. After 1 week, blood glucose levels in the tail vein blood of rats were measured using a glucometer from Roche (Germany), and detection of blood glucose levels above 16.7 mmol/L was considered evidence of the successful establishment of a diabetic model. Subsequently, some of the diabetic mice were treated with MALAT 1-shRNA or NLRP3 inhibitor. MALAT 1-shRNA was synthesized at Jikai gene Biological Company and 1 x 109 TU of MALAT 1-shRNA was dissolved in 50 μL of saline and injected into the tail vein of the diabetic rats. DM+ NLRP3 I group rats were injected intraperitoneally daily with 100 mg/kg NLRP3 inhibitor MCC950.

**Cell culture and plasmid transfection**

Primary Cardiomyocytes (PCM) were extracted from 1 ~ 3-day old neonatal rat hearts. The cells were cultured with 5.5 mM glucose (control) or 50 mM glucose (HG) in Dulbecco's modified Eagle's medium (DMEM) for 24 hours with 10% fetal bovine serum (Biological Industries, Beit Haemek, Israel) at 37°C and 5% CO2. The HG group of PCM was treated with the NLRP3 inhibitor MCC950 100 μM to inhibit NLRP3 expression and to detect the apoptotic regulation of NLRP3 on cardiomyocytes in the HG group. H9C2 cardiomyocytes and PCM were reproduced using plasmid siRNA-MALAT 1 and pcDNA3.1- NLRP3 and the corresponding negative plasmid as control.

**RNA isolation and RT-PCR**

Total serum RNA extraction was performed using a serum extraction kit (Life Technologies, US) according to the experimental procedure provided (12). RT-PCR was used to detect the expression of LncRNA-H19 in serum. Reverse transcription reaction experiments were performed using a Revert Aid kit (Thermo Fisher Scientific, US) with a reverse transcription system consisting of 10 μl of total RNA extract, 4 μl of 5X reaction buffer, 2 μl of 10 mM dNTP, 1 μl of primers 18 primers, 1 μl of Revert Aid RT, 1 μl of ribonuclease inhibitor and 1 μl of ribonuclease-free water. The mixed system was incubated at 42°C for 60min and 70°C for 5min. The synthesised cDNA was stored at 80°C and set aside for use. All reactions were performed using a Roche LightCycler 480 (Roche, Switzerland) under the following conditions: 30 sec at 95°C, 5 sec at 95°C and 30 sec at 60°C for a total of 45 reactions. The appropriate product amplification was determined by melting curve analysis. Primer sequences are presented in Table1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>Lnc-RNA MALAT 1</td>
<td>Forward 5'-TAAGGTCAAGAGAAGTGCAG-3'; Reverse 5'-AAGACCTGCAGACCATCGT-3'</td>
</tr>
<tr>
<td>NLRP3</td>
<td>Forward 5'-GTGGGATCTTCTGAAGGCTC-3'; Reverse 5'-CAGGATCTCTCTTCCTGGGATC-3'</td>
</tr>
<tr>
<td>caspase-1</td>
<td>Forward 5'-AAGGTCTGAGGGGCAAAGAG-3'; Reverse 5'-GTGGTGAGATAATGAGGG-3'</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward 5'-CCCTGCAGCTGGAGGTTTGG-3'; Reverse 5'-TGTGTCGCTGTCGAGGCTG-3'</td>
</tr>
<tr>
<td>IL-18</td>
<td>Forward 5'-ACAACCCGAAGATCCTGAGG-3'; Reverse 5'-TGGGCTGCTGCTGAGGCTGCTG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward 5'-CAGTGCCAGGTCTGCTCT-3'; Reverse 5'-AGGGCCATCCACAGTCCTC-3'</td>
</tr>
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**Protein extraction and Western blot analysis**

Protein samples (25 μg/lane) from extracted heart tissue were injected into SDS-PAGE gel and then transferred to polyethylene difluoride membranes. The membranes were incubated with 5% milk TBST for 1 h at room temperature, followed by overnight incubation with the appropriate antibody at 4°C, then washed three times with PBS containing 0.5% Tween 20, incubated at room temperature using a suitable secondary antibody (1:5000; Jackson ImmunoResearch) coupled to horseradish peroxidase, washed and visualized (Amersham Biosciences) by ECL Western Blotting detection reagent. After stripping with Western blotting antibody stripping buffer (Pierce) for 20 min at room temperature, the membranes were incubated at 4°C for 1 h and washed three times with PBS containing 0.5% Tween 20.
temperature, the membranes were treated similarly with anti-actin antibody as a control.

**Cell scorch level assay**
Caspase-1 positive cells (caspase-1+) and PI positive (PI+) cells were labelled using the caspase-1 activity assay kit and PI staining kit respectively. 5 X 10^4 labelled cells were screened using Bio-rad flow cytometry and the number of double positive (caspase-1 + PI +) cells was counted. Repeat more than 4 times and perform statistical analysis.

**Data analysis**
SPSS 17.0 software was used for analysis and expressed as mean standard deviation (SD). The unpaired student-t test and one-way ANOVA were used to compare the differences between two or different groups. A two-tailed P<0.05 was considered a statistically significant difference. Graphs were made using GraphPad Prism 8.

**Results and discussion**
**LncRNA-MALAT1 and NLRP3 pathway-related genes are overexpressed in high glucose-induced cardiomyocytes**
We found that the expression of LncRNA-MALAT1 in the serum of diabetic patients was significantly higher than that of healthy controls (Figure 1A, p<0.001). To determine the expression of MALAT 1 in cultured cells in vitro, we used 5.5 mmol/L glucose-treated human cardiomyocytes H9C2 as the control group (Control) and 50 mmol/L glucose-treated H9C2 cells as the high-glucose group (HG), respectively, to simulate the hyperglycemic state of DCM. The results revealed that MALAT 1 expression was significantly increased in the HG group compared to the Control group (Figure 1B, p<0.001). The expression level of MALAT 1 was also elevated in the PCM treated with high glucose compared to the control group (Figure 1C, p<0.001). In addition, the expression of NLRP3, caspase-1, IL-1β and IL-18, genes related to the NLRP3 pathway, was also significantly elevated (Figure 1D, p<0.001, p<0.005). Western blot analysis showed that the expression of NLRP3 protein was significantly elevated in the HG group compared to the control group (Figure 1E). Thus, we determined that MALAT 1 was highly expressed in the DCM model and that the cellular scorch death pathway was activated in high glucose-treated cardiomyocytes.

**The siRNA silencing of MALAT 1 expression inhibits cardiomyocyte scorch death in the high glucose group**
To further investigate the effect of MALAT 1 on cardiomyocyte scorching, we used siRNA plasmids to specifically silence MALAT 1 expression in cardiomyocytes under high glucose conditions, and then examined the scorching of cardiomyocytes. siRNA MALAT 1 was able to successfully inhibit MALAT 1 expression (Figure 2A). In contrast, the mRNA and protein expression levels of NLRP3 were significantly down-regulated (Figure 2B and C). And the transfection of Si-RNA MALAT 1 plasmid also reduced the level of scorch death in H9C2 cells cultured in high glucose (Figure 2D). The same results were also verified in PCM (Figure 2E-H). MALAT 1 was associated with cardiomyocyte scorch death. These results suggest that NLRP3 levels are elevated in H9C2 cells cultured in serum and high glucose from diabetic patients and that reducing MALAT 1 expression could affect NLRP3 expression, thus acting as a regulator of cell scorching.
Figure 2. siRNA silencing of MALAT 1 inhibits the scorching effect of H9C2 cells and PCM. (A and B) RT-PCR to detect the mRNA expression levels of MALAT 1 and NLRP3 in H9C2 cells cultured in high glucose and after transduction of Si-RNA-MALAT 1. (C) Western Blotting to detect NLRP3 protein expression in H9C2 cells. (D) Silencing of MALAT 1 by siRNA can inhibit the scorching effect of H9C2 cells. (E and F) RT-PCR to detect mRNA expression levels of MALAT 1 and NLRP3 in PCM cells cultured in high glucose and after transduction of Si-RNA-MALAT 1. (G) Western Blotting to detect NLRP3 protein expression in PCM cells. (H) Silencing of MALAT 1 by siRNA can inhibit the scorching effect of PCM cells. (*P<0.05, **P<0.01, ***P<0.001, n.s.=no difference)

LncRNA-MALAT 1 regulates focal death by targeting NLRP3 in H9C2 cells

To determine that LncRNA-MALAT 1 regulates cardiomyocyte scorch death by targeting NLRP3, we validated the NLRP3 pathway using Si-RNA-MALAT 1 and pc-DNA 3.1 NLRP3 plasmids.

RT-PCR results showed that the expression levels of caspase-1, IL-1β and IL-18 were significantly down-regulated in high glucose cultured H9C2 cells after transfection with Si-RNA MALAT 1 (Figure 3A, ***p<0.01, ***p<0.001), while western blotting results similarly showed a significant decrease in caspase-1, IL 1β and IL-18 protein levels were significantly reduced (Figure 3B). In contrast, the transfection of pc DNA 3.1 NLRP3 prevented the down-regulation function of Si-RNA MALAT, while it did not affect MALAT 1 expression (Figure 3A and C, ***p<0.01). Meanwhile, cell scorching assay revealed that transfection of Si-RNA MALAT 1 effectively reduced cell scorching in H9C2 cells cultured with high glucose, while the pc DNA 3.1 NLRP3 plasmid redeemed the cell scorching state of H9C2 (Figure 3D).

Figure 3. Silencing of MALAT 1 can inhibit the NLRP3 signaling pathway in cardiomyocytes. (A) Si-RNA-MALAT 1 can inhibit mRNA expression of NLRP3 signaling pathway-related genes, while the pc DNA 3.1 NLRP3 plasmid can redeem the effect of Si-RNA-MALAT 1. (B) Si-RNA-MALAT 1 can inhibit the protein expression of NLRP3 signaling pathway-related genes. (C) The pc DNA 3.1 NLRP3 plasmid can increase the expression of NLRP3 but not MALAT 1. (D) Si-RNA-MALAT 1 can reduce the level of cell scorch death, while pc DNA 3.1 NLRP3 plasmid can redeem the effect of Si-RNA-MALAT 1. (*P<0.05, **P<0.01, ***P<0.001, n.s.=no difference)

NLRP3 inhibitor reduces PCM scorch death but does not affect MALAT 1 expression

We found that inhibition of MALAT 1 reduced NLRP3 expression. Next, we determined whether inhibition of NLRP3 could form a negative feedback network to regulate MALAT 1 expression in PCM. High glucose-induced PCM were given 100 μM/L NLRP3 inhibitors or transfected with Si-RNA MALAT. The concentration of NLRP3 inhibitor was shown not to affect cell activity and could effectively inhibit high NLRP3 expression in PCM cultured with high glucose, but had no effect on MALAT 1 expression (Figure 4A-C, ***p<0.001). The effect on NLRP3 pathway-related genes was the same as the transfection effect of Si-RNA MALAT, both of which reduced the expression levels of caspase-1, IL-1β and IL-18 in PCM (Figure 4D, **p<0.01, ***p<0.001). The above results indicate that the regulation of NLRP3 by MALAT 1 was unidirectional and failed to form a negative feedback regulation.
Figure 4. Inhibition of NLRP3 expression does not affect MALAT 1 expression in primary cardiomyocytes. (A) The number of live cells was determined by MTT assay. (B) The amount of NLRP3 mRNA expression. (C) NLRP3 inhibitor does not affect MALAT 1 expression. (D) NLRP3 inhibitor can reduce the expression of NLRP3 pathway-related genes. (*P<0.05, **P<0.01, ***P<0.001, n.s.=no difference)

Silencing MALAT ameliorates cellular scorching in diabetic rats

To further validate the role of MALAT, we established a type I diabetes model in Wistar rats to test the effect of silencing MALAT with the lentiviral vector shRNA MALAT in DCM (DM+ shRNA MALAT). In addition, we treated diabetic rats with an NLRP3 inhibitor (DM+ MCC950) to determine the effect of DCM on cellular scorching in vivo. m-mode echocardiography showed significant deterioration in left ventricular systolic and diastolic function, ejection fraction (EF) and shortening fraction (FS) in diabetic rats, whereas the DM+ shRNA MALAT and DM+ MCC950 groups showed significant improvement (Figure 5A, **p<0.01). RT-PCR results showed that MALAT was upregulated in diabetic rats and significantly downregulated after shRNA MALAT injection, while inhibitor MCC950 treatment had no effect on MALAT expression (Figure 5B), and NLRP3 was downregulated upon injection of both shRNA MALAT and inhibitor MCC950 (Figure 5C). In addition, the expression levels of other genes in the NLRP3 pathway were significantly upregulated in diabetic mice, and this upregulation was partially reversed by shRNA MALAT and MCC950 inhibitors (Figure 5D). These data suggest that MALAT can regulate cardiac function and structure in vivo via the MALAT/NLRP3 pathway.

Figure 5. MALAT 1 can regulate DCM focal death in vivo. (A) Inhibition of MALAT 1 or NLRP3 can improve ejection fraction (EF) and shortening fraction (FS) in all groups of rats. (B) shRNA MALAT 1 can reduce MALAT 1 expression in rats. (C) Inhibition of MALAT 1 or NLRP3 can reduce the expression of NLRP3 in rats. (D) Inhibition of MALAT 1 or NLRP3 can decrease the expression of NLRP3-related pathways in rats. (*P<0.05, **P<0.01, ***P<0.001, n.s.=no difference)

LncRNAs have an important role in the development of many diseases, including cardiovascular disease, diabetes and related complications, but the mechanistic relationship between LncRNAs and cellular scorching in DCM is unclear (3, 13, 14). In the present study, we demonstrate that LncRNA MALAT is upregulated in serum from diabetic patients, in cardiomyocytes cultured with high glucose and in myocardial tissue from diabetic mice. Silencing MALAT can attenuate cell scorch death by targeting NLRP3, thereby reducing cell death in vitro and improving cardiac function and morphology in vivo. In this paper we have for the first time fully elucidated the effect of MALAT on DCM focal death.

Previous studies have shown that many LncRNAs are involved in the process of DCM. For example, LncRNA H19 is down-regulated in DCM and affects apoptosis through miR-675 and VDAC1 (15, 16). LncRNA MIAT is upregulated in high glucose-treated cardiomyocytes and STZ-induced mice, and regulates DAPK2 expression via sponge cell miR-22-3p (17). In addition, emerging studies have shown that MALAT has an important role in many diseases (18-22). However, reports on MALAT and cellular scorch death in DCM have not been reported. In our study,
MALAT is significantly upregulated in DCM. The siRNA and lentiviral shRNA silencing of MALAT significantly can reduce scorch death and improve cardiac function in DCM patients. Thus, MALAT may be a novel mechanism and therapeutic target for DCM.

LncRNAs exert biological roles through a variety of mechanisms, including genetic imprinting, chromatin remodelling, cell cycle control, splicing regulation, mRNA decline and translational regulation (23-25). MALAT is a widely studied LncRNA that regulates multiple genes in the MALAT domain by recruiting chromatin and DNA-modifying proteins (26). MALAT has also been shown to act as a ceRNA, targeting miRNAs to regulate mRNA expression (18).

In our study, MALAT can regulate cardiomyocyte scorch death via NLRP3. We first illustrate the upregulation of MALAT in high glucose-treated cardiomyocytes and the effect of MALAT on DCM via NLRP3.

Cell scorching is a form of programmed cell death associated with inflammation and is involved in the development of DCM (11, 27). High glucose induction activates the reactive oxygen species (ROS) pathway which in turn can trigger the formation of NLRP3 inflammatory vesicles and promote the production of caspase-1 cleavage, thereby accelerating the release of IL-1β and IL-18 (10). This process induces mitochondrial oxidative stress, promotes apoptosis and affects the abnormal metabolism of sugar and adipose tissue (9, 28, 29). Also, inflammation is a common mechanism for accelerating other pathologies (5). A growing number of studies have shown that inhibition of inflammation can reduce cardiomyocyte death and cardiac cytoskeletal structure (3, 5, 6). Our study reveals that silencing MALAT can improve cardiomyocyte scorch death and maintain cardiomyocyte integrity via NLRP3. We hypothesise that this is closely related to the inhibition of inflammation. In this study, we have described the mechanism of MALAT’s role in DCM in several ways. In the future, more studies are needed to determine whether silencing MALAT can regulate other pathological processes in DCM, such as ROS, mitochondrial dysfunction and metabolic abnormalities.

References
10. Fan Y, Du L, Fu Q et al. Inhibiting the NLRP3 inflammasome with MCC950 ameliorates isoflurane-induced pyroptosis and cognitive impairment in aged mice.


