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MiR-449 improves cardiac function by regulating HDAC1 and cTnI

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Abstract. – OBJECTIVE: To investigate the effect of microRNA-449 (miRNA-449) on cTnI and cardiac function and reveal the mechanism of Histone deacetylase 1 (HDAC1)-mediated histone deacetylation in cardiomyocytes.

MATERIALS AND METHODS: First, we used biochemical analysis and Dual-Luciferase reporter gene assay to confirm that HDAC1 and miR-449 having the binding site. Then, the effect of miR-449 inhibited HDAC1 on cTnI gene transcription was observed. *In vivo,* the effect of histone acetylation on cTnI expression and cardiac function in heart was observed in elderly mice with low expression of cTnI through miR-449 agomiR intervention.

RESULTS: This study revealed miR-449 can sponge with HDAC1. HDAC1-mediated histone deacetylation was involved in the regulation of cTnI gene expression by HDAC1-mediated acetylation of H3K4 and H3K9 in cTnI promoter regions. In addition, HDAC1-mediated histone deacetylation regulated the binding of the transcription factor GATA4 to the GATA element in the cTnI promoter region and improved cardiac function in elderly mice with low expression of cTnI

CONCLUSIONS: MiR-449 can regulate the acetylation of the histones H3K4 and H3K9 of the GATA element in the cTnI promoter region, thereby recruiting the transcription factor GATA4 to the cTnI promoter region, upregulating the cTnI gene expression, and improving cardiac function in elderly mice with low expression of cTnI.

Key Words:
Mir-449, HDAC1, cTnI, Cardiac function.

Introduction

MicroRNAs (miRNAs) are a collection of small, endogenous, non-coding RNA molecules that contain 19 to 25 nucleotides and are widely found in eukaryotes[1]. Many miRNAs found in human cells are in the intron regions of protein-coding or non-coding genes. The remaining miRNAs are often found in the transcript of the genome or in the 3 'non-coding region of mRNA. MiRNAs are produced by transcription of genes encoding miRNAs in the nucleus, with fragments ranging from hundreds to thousands of nucleotides[2]. The function of miRNA is mainly to regulate the metabolism of mRNA. Mature miRNAs bind to corresponding sites on the mRNA and are divided into two mechanisms to negatively regulate gene expression based on whether the gene sequences of the two are completely complementary. When they are not completely complementary, miRNAs inhibit protein translation, if they are completely complementary, the binding of these miRNAs often results in mRNA degradation. MiRNA has a variety of functions under physiological effects, including promoting cell proliferation, differentiation, and even apoptosis[3,4]. In addition, miRNAs play their regulatory role after transcriptional regulation through the 3 'non-coding region of mRNA. MiRNA-449, which can bind to RNA-mediated silencing complexes, can regulate target mRNA degradation or translation inhibition through the miRNA recognition site of target mRNA 3′-untranslated region (3′-UTR)[5].

Histone deacetylase 1 (HDAC1), an analog of the yeast protein Rpd3p, is the earliest histone deacetylase and belongs to the class I histone deacetylase[6]. HDAC1 and HDAC2 are similar in structure. HDAC1 is more important for maintaining the activity of the HDAC1/HDAC2 complex and determines the direction of embryonic stem cell differentiation[7]. HDAC removes acetylated histone lysine sites and increases the

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electrostatic potential of nucleosome interactions, thereby tightening chromatin and inhibiting gene transcription. Additionally, HDAC1 knockout mice die early in the embryo[8].

The Troponin I (TnI) family contains three subtypes: fast skeletal troponin I (fsTnI), slow skeletal troponin I (ssTnI), and myocardium troponin I (cardiac TnI, cTnI). Among them, cTnI is only expressed in myocardial tissue[9]. cTnI is synthesized under the guidance of the TNNI3 gene. It is combined with cardiac troponin T (cTnT) and cardiac troponin C (cTnC) to form a troponin complex, which is involved in the cardiac muscle cell excitation-contraction coupling[10]. In a mouse animal model of cTnI gene knockout, cardiomyocyte sarcomere shortened, intracellular mitochondria increased, and volume increased[11]. However, the cTnIR193H gene mutation model mice showed diastolic dysfunction, such as prolonged isovolumic diastole and decreased E/A ratio. It has been also detected that cTnI gene mutations can cause familial hypertrophic cardiomyopathy and familial restricted cardiomyopathy. However, the expression level of cTnI is not constant *in vivo*. In the embryonic period, troponin I in the heart is mainly ssTnI, which is gradually replaced by cTnI after birth. After old age, cTnI gradually decreases and diastolic dysfunction occurs[12]. It can be seen that the normal expression of cTnI has an important role in maintaining the normal diastolic function of the heart. Moreover, cTnI gradually decreases and diastolic dysfunction occurs after old age. In elderly mice with low expression of cTnI, the level of HDAC1 binding in the cTnI promoter region increases[13], suggesting that HDAC1 may be involved in cTnI expression regulation.

GATA4 is one of the most noticed cardiac transcription factors. It belongs to the zinc-finger protein and is the subtype of the GATA family that is most closely related to cardiac development. Mice with GATA4 gene deletion die early in the embryo, mutations at different sites in the GATA4 gene can cause different types of congenital heart disease[14]. In addition, in the regulatory network of cardiac developmental transcription factors, GATA4 plays an important role in the differentiation of fibroblasts into cardiomyocytes. However, the inactivation of GATA4 after birth can cause serious cardiac dysfunction and even develop into heart failure[15,16]. It can be seen that the normal expression of GATA4 is essential for heart development and normal function maintenance.

A large number of studies have shown that miRNA may be involved in regulating gene expression. Based on the above analysis, we hypothesized that miRNA-449 promotes cTnI gene expression by binding to HDAC1 and thus plays a role in regulating diastolic function.

Materials and Methods

Cell Culture and Transfection

To extract primary cardiomyocytes from suckling rats (Capital Medical University), and the cells incubated in a carbon dioxide incubator, with 15% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) and antibiotics (Gibco, Rockville, MD, USA). We replaced the medium every 1-2 days. The miR-449 mimics and NC, HDAC1-siRNA and HDAC1 NC were transfected with the cardiomyocytes (Ribobio, Shanghai, China). Three days after transfection was considered as the best harvest time.

Real Time-Polymerase Chain Reaction (RT-PCR)

TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract total RNA from cardiomyocytes, and strictly followed the instructions of TaKaRa-RNA quantification and reverse transcription kit (TaKaRa, Komatsu, Japan) instructions. The internal reference was glyceraldehyde 3-phosphate dehydrogenase (GAPDH). and the quantitative analysis was performed based on the quantitative amount of the target gene ($2^{-\Delta\Delta Ct}$). All the primers were listed in Table I.

Western Blot

We loaded the sample according to the total amount of protein 50 μg, ran at 80 V for 50 min, and ran at 120 V for 1 h. Then, we took the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) into methanol for activation. We, then, transferred the membrane to 300 mA on ice for 90 min, blocked on a shaker for 2 h, and used primary antibody (cTnI, 1:2000, Abcam, Cambridge, MA, USA, Rabbit, 1:2000; HDAC1, 1:1000, Abcam, Cambridge, MA, USA, Rabbit, 1:2000; β-actin, Abcam, Cambridge, MA, USA, 1:2000) incubated at 4°C overnight. The next day, we selected the corresponding secondary antibody and incubated the PVDF membrane

Table I. Real Time-PCR primers.

Gene name	Forward (5'>3')	Reverse (5'>3')
MiR-449	AGGGTGGCAGTGTATTGTTA	GAGAGGAGAGGAAGAGGGAA
HDAC1	AGGGGCGACTGTGTCTT	ACGGGGCAGTGTAAGTTTT
cTnI	ATGGCGGATGAGAGCAG	GTTTTCTGGAGGCGGAGA
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

qRT-PCR, quantitative Reverse Transcription-Polymerase Chain Reaction.

for 1 h. Then, we used the enhanced chemiluminescence (ECL) developer solution (Corning, Corning, NY, USA) to expose in a dark room. Image J v1.8.0 (NIH, Bethesda, MD, USA) was used to analyze the gray value of the developed bands, and then β-actin was used as an internal reference to calculate the relative expression of the target protein.

Luciferase Assay

In a 24-well plate with 20,000 293T cells per well, miR-449 mimics, miR-449 NC, HDAC1-WT, and HDAC1-Mut plasmids were transfected at the same time after 24 hours. The enzyme reporter gene kit (Jian Cheng, Nanjing, China) measures the fluorescence intensity of each group.

Chromatin Immunoprecipitation (ChIP)

First, we used CHIP kit (Abcam, Cambridge, MA, USA) to extract nuclear chromatin, configured the reaction system according to the instructions, added the reaction plate, blocked it with the original film, and shook at room temperature for 90 min. Then, we washed the plate with Wash Buffer and DNA Release Buffer. Next, we added working solution, and incubated at 65°C for 15 min, then, we transferred it to a new Eppendorf (EP) tube, incubated at 95°C for 10 min, and stored in -20°C refrigerator. At the same time, we configured the Input group. Finally, the quantitative PCR detection was performed, and the input was used as the internal reference to calculate the relative amount of DNA collected according to the ($2^{-\Delta\Delta Ct}$) method.

Animal Experiment

Healthy female rats at the age of 12 months were kept in the Capital Medical University for 3 months, drinking and eating freely. The rats were then randomly divided into 3 groups: saline group (NS) and miR-449 agomiR group, miR-449 agomiR NC group, and 3 groups of rats were injected with NS and miR-449 agomiR and miR-449 agomiR NC (80 mg/kg) *via* tail vein at a fixed time every day for 3 consecutive days. This study was approved by the Animal Ethics Committee of Capital Medical University Animal Center.

Echocardiography

Mice were intraperitoneally injected with sodium pentobarbital (Capital Medical University) according to 5 μL/g. After anesthesia, hair removal cream was applied to remove the chest and upper abdomen hair. Mice were laid flat on the foam board, and their limbs were firmly fixed on the foam board with adhesive tape. Then, we applied an appropriate amount of coupling agent to the chest of the mouse, rotated the probe counterclockwise about 15° at the left margin of the sternum to find the section of the long axis of the left ventricle, switched the mode to m-mode ultrasound, and measured respectively: we calculated the left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV), left ventricular shortening fraction (FS), ejection fraction (EF). Then, the probe was placed in the apex of the heart to find the four-chamber heart section, and the mode was switched to the pulse doppler (PW) mode to measure respectively the maximum mitral flow velocity (E wave) in early diastole and the maximum mitral flow velocity (A wave) in late diastole, and the E/A ratio was calculated based on the above results.

Statistical Analysis

Using Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA), all data were expressed as mean ± standard deviation (mean ± SD). The differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was

done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

MiR-449 As a Sponge of HDAC1

First, we found that miR-449 has a binding site (Figure 1A) with HDAC1 through TargetScan software analysis. Then, we transfected the primary cardiomyocytes miR-449 mimics and NC, PCR showed the expression of HDAC1 decreased in the miR-449 mimics group (Figure 1B). At the same time, WB also verified that miR-449 and HDAC1 may have a binding site (Figure 1C). Finally, we constructed the WT and Mut plasmids of HDAC1 and transfected 293T cells, respectively, and found that the fluorescence activity was markedly reduced in the miR-449 mimics + HDAC1-WT group (Figure 1D). The above experiments have indicated that miR-449 may as a sponge of HDAC1.

MiR-449 Regulates the Expression of cTnI By Binding to HDAC1

First, we transfected miR-449 mimics and NC in primary cardiomyocytes, and found that miR-449 mimics can be markedly overexpressed (Figure 2A), while PCR detection found that the expression of cTnI in the miR-449 mimics group was significantly increased (Figure 2B). At the same time, WB experiments also showed that cTnI protein expression in the miR-449 mimics group was markedly increased (Figure 2C). Then, miR-449 inhibitor and NC were transfected into primary cardiomyocytes respectively, and it was found that miR-449 inhibitor significantly inhibited miR-449 (Figure 2D). PCR detection revealed that the expression of cTnI in miR-449 inhibitor group was markedly reduced (Figure 2E). WB experiments also indicated that the expression of cTnI protein in miR-449 inhibitor group was significantly reduced (Figure 2F).

MiR-449 Regulates cTnI Through HDAC1-Mediated Histone Deacetylation

By comparing with Input, the relative amount of co-immunoprecipitated DNA was calculated. The results showed that the overall acetylation level of histone H3 in the cTnI promoter region of miR-449 mimics group was increased, and the acetylation levels of histone H3 specific sites H3K4 and H3K9 were increased. The acetylation level of H3K27 specific site of protein H3 was not different from that of the control group. The overall acetylation level of histone H3 in the cTnI promoter region of miR-449 inhibitor group was re-

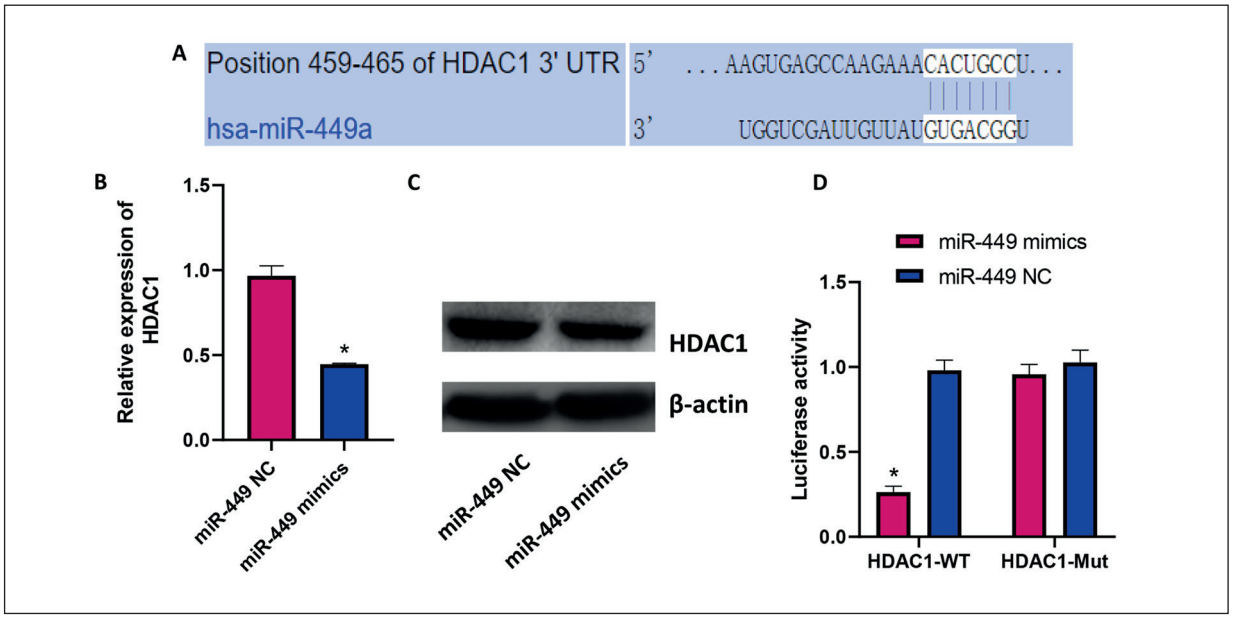

Figure 1. MiR-449 as a sponge of HDAC1. **A,** Predict the upstream miRNA of HDAC1 on Targetscan. **B,** The expression levels of HDAC1 in cardiomyocytes of miR-449 NC and miR-449 mimics groups ("*" indicates that compared with the miR-449 NC group $p < 0.05$). **C,** Western blot bands of HDAC1 in cardiomyocytes of miR-449 NC and miR-449 mimics groups. **D,** The luciferase report assay ("*" indicates that compared with the miR-449 NC + HDAC1-WT group $p < 0.05$).

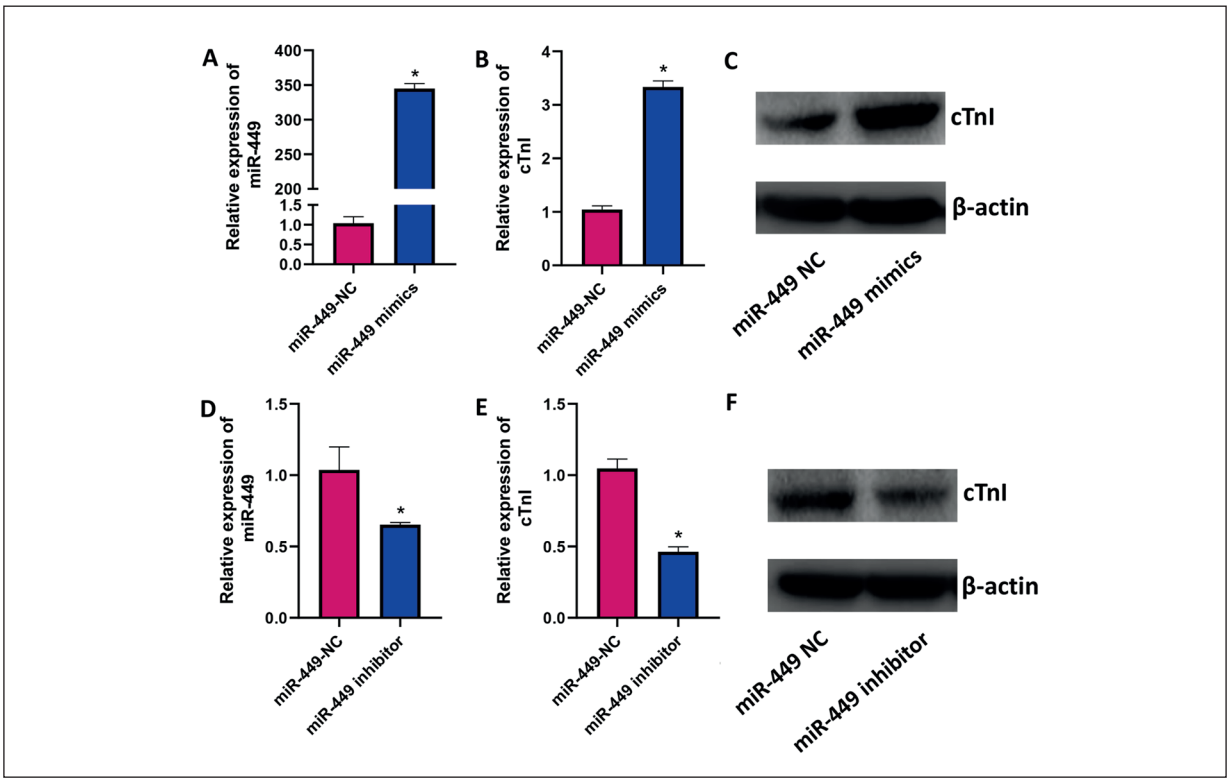

Figure 2. MiR-449 regulates the expression of cTnI by binding to HDAC1. **A,** The expression levels of miR-449 in cardiomyocytes of miR-449 NC and miR-449 mimics groups ("*" indicates that compared with the miR-449 NC group $p < 0.05$, respectively). **B,** The expression levels of cTnI in cardiomyocytes of miR-449 NC and miR-449 mimics groups ("*" indicates that compared with the miR-449 NC group $p < 0.05$). **C,** Western blot bands of cTnI. **D,** The expression levels of miR-449 in cardiomyocytes of miR-449 NC and miR-449 inhibitor groups ("*" indicates that compared with the miR-449 NC group $p < 0.05$). **E,** The expression levels of cTnI in cardiomyocytes of miR-449 NC and miR-449 inhibitor groups ("*" indicates that compared with the miR-449 NC group $p < 0.05$). **F,** Western blot bands of cTnI.

duced. However, the specific site H3K27 acetylation level was not different from the control group (Figure 3A). ChIP-qPCR was used to detect the binding level of HDAC1 to the cTnI promoter GATA element. The results showed that the level of binding between HDAC1 and cTnI promoter GATA elements in miR-449mimics group was reduced (Figure 3B). Similarly, the level of GATA4 binding to the cTnI promoter GATA element in the miR-449 mimics group increased (Figure 3C). Then, we divided them into miR-449 NC + HDAC1-NC, miR-449 NC + HDAC1-siRNA, miR-449 mimics + HDAC1-siRNA, miR-449 inhibitor + HDAC1-siRNA 4 group, and found that the expression of HDAC1 in miR-449 inhibitor + HDAC1-siRNA group was higher than that of miR-449 NC + HDAC1-siRNA group, indicating that miR-449 can offset the effect of HDAC1-siRNA on HDAC1 (Figure 3D). The overall acetylation level of histone H3 in the cTnI promoter region and histone H3-specific sites H3K4 and H3K9 in the miR-449 mimics+ HDAC1-siRNA group was the highest, while the acetylation level of histone H3-specific sites H3K27 was also not different from other groups (Figure 3E). ChIP-qPCR was used to detect the binding level of HDAC1 to the cTnI promoter GATA element. The results showed that the miR-449 mimics + HDAC1-siRNA group had the lowest GATA element binding level between HDAC1 and cTnI promoter region, and the highest GATA element binding level between GATA4 and cTnI promoter region (Figure 3F and 3G). In summary, miR-449 plays a regulatory role on cTnI through HDAC1-mediated histone deacetylation.

Effect of Mir-449 on cTnI Expression and Regulating Diastolic Function

We used miR-449 agomiR to intervene in elderly mice with low cTnI expression and observe the effect of histone acetylation on cTnI expression and heart function in the heart, so as to veri-

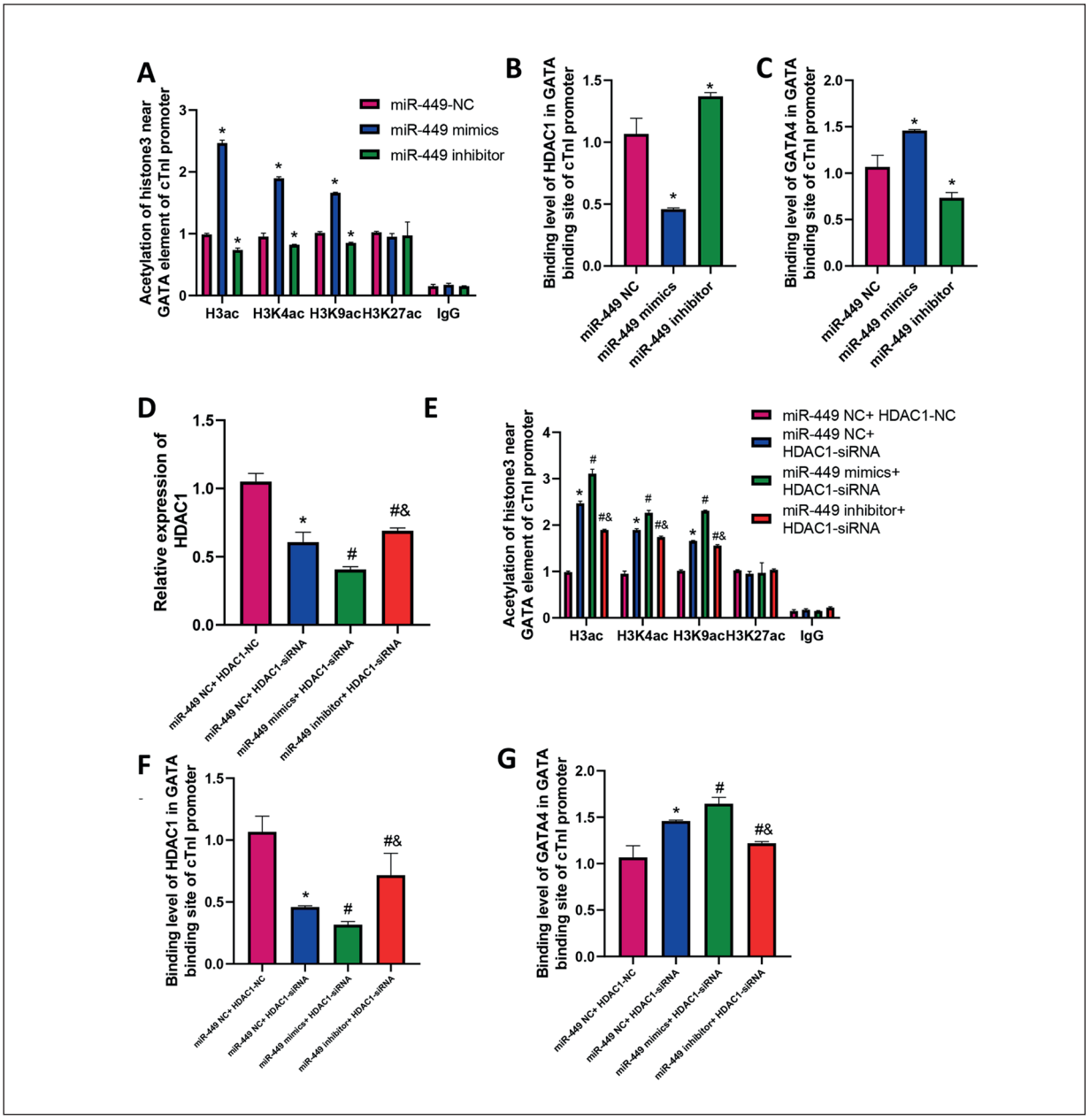

Figure 3. MiR-449 regulates cTnI through HDAC1-mediated histone deacetylation. **A,** Acetylation of histone near GATA element of cTnI promoter after miR-449 mimics and inhibitor ("*" indicates that compared with the miR-449 NC group $p < 0.05$). **B,** Binding level of HDAC1 in GATA binding site of cTnI promoter ("*" indicates that compared with the miR-449 NC group $p < 0.05$). **C,** Binding level of GATA4 in GATA binding site of cTnI promoter ("*" indicates that compared with the miR-449 NC group $p < 0.05$). **D,** Relative expression of HDAC1 in the miR-449 NC + HDAC1-NC, miR-449 NC + HDAC1-siRNA, miR-449 mimics + HDAC1-siRNA, miR-449 inhibitor + HDAC1-siRNA 4 groups ("*" indicates statistical difference from the miR-449 NC + HDAC1-NC group $p < 0.05$; "#" indicates statistical difference from the miR-449 NC + HDAC1-siRNA group; "&" indicates statistical difference from the miR-449 mimics + HDAC1-siRNA group $p < 0.05$). **E,** Acetylation of histone near GATA element of cTnI promoter ("*" indicates statistical difference from the miR-449 NC + HDAC1-NC group $p < 0.05$; "#" indicates statistical difference from the miR-449 NC + HDAC1-siRNA group; "&" indicates statistical difference from the miR-449 mimics + HDAC1-siRNA group $p < 0.05$). **F,** Binding level of HDAC1 in GATA binding site of cTnI promoter ("*" indicates statistical difference from the miR-449 NC + HDAC1-NC group $p < 0.05$; "#" indicates statistical difference from the miR-449 NC + HDAC1-siRNA group; "&" indicates statistical difference from the miR-449 mimics + HDAC1-siRNA group $p < 0.05$). **G,** Binding level of GATA4 in GATA binding site of cTnI promoter ("*" indicates statistical difference from the miR-449 NC + HDAC1-NC group $p < 0.05$; "#" indicates statistical difference from the miR-449 NC + HDAC1-siRNA group; "&" indicates statistical difference from the miR-449 mimics + HDAC1-siRNA group $p < 0.05$).

fy the role of histone acetylation in the regulation of cTnI gene expression and the improvement of heart function in elderly mice with low cTnI expression. Echocardiographic examination of the heart function of elderly mice in each group revealed that the E/A ratio of the miR-449 agomiR group was higher than that of the miR-449 NC group, while the LVESD, FS, and EF were not significantly changed compared to the miR-449 NC group (Figure 4A). By comparing with the reference gene GAPDH, the cTnI mRNA expression was calculated. The results showed that after miR-449 agomiR intervention, cTnI mRNA expression increased (Figure 4B). At the same time,

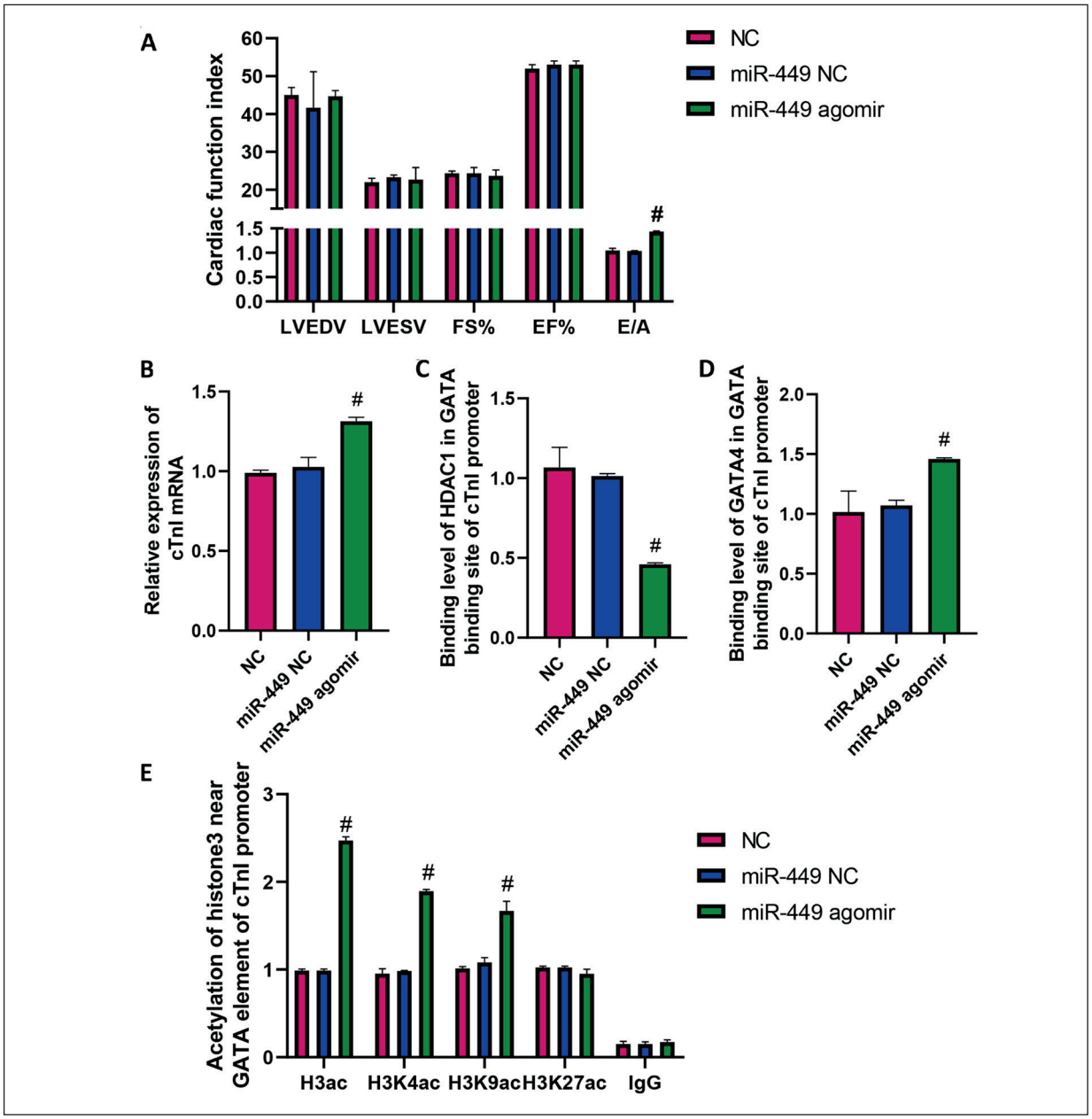

Figure 4. Effect of Mir-449 on cTnI expression and regulating diastolic function. **A,** LVEDV, LVESV, FS%, EF% and E/A detected by echocardiography ("#" indicates that compared with the miR-449 NC group $p < 0.05$). **B,** Relative expression of cTnI in the NC, miR-449 NC and miR-449 agomiR groups ("#" indicates that compared with the miR-449 NC group $p < 0.05$). **C,** Binding level of HDAC1 in GATA binding site of cTnI promoter ("#" indicates that compared with the miR-449 NC group $p < 0.05$). **D,** Binding level of GATA4 in GATA binding site of cTnI promoter ("#" indicates that compared with the miR-449 NC group $p < 0.05$). **E,** Acetylation of histone near GATA element of cTnI promoter ("#" indicates that compared with the miR-449 NC group $p < 0.05$).

by comparing with Input, the relative amount of co-immunoprecipitated DNA was calculated. The results showed that after miR-449 agomiR intervention, HDAC1 and cTnI promoter region GATA element binding level decreased (Figure 4C), GATA4 and cTnI promoter region GATA element binding level increased (Figure 4D). By contrast, the overall acetylation level of histone H3 in the cTnI promoter region was increased, the histone H3 specific sites H3K4, H3K9 were increased. However, the histone H3 specific site H3K27 acetylation level was not different from the miR-449 NC group (Figure 4E). Therefore, miR-449 may promote cTnI gene expression and improve cardiac function in elderly mice with low cTnI expression.

Discussion

cTnI is an important structural and functional protein of the heart. It participates in contraction and relaxation of the heart muscle by binding and dissociating with calcium ions in cardiac muscle cells. The present study found that cTnI gene knockout and R193H mutation model indicated that cTnI is essential for maintaining normal diastolic function[17]. The expression level of cTnI is not constant *in vivo*. During the embryonic period, troponin I in the heart is mainly ssTnI, which is gradually replaced by cTnI after birth[18]. cTnI gradually decreases and diastolic dysfunction occurs after old age. In elderly mice with low expression of cTnI, the level of HDAC1 binding in the cTnI promoter region increases, suggesting that HDAC1 may be involved in the cTnI expression regulation. GATA4 is expressed sequentially in the heart development of mouse embryos, and the expression gradually increases from 11.5 days to 14.5 days in embryo, and then, remains unchanged until birth[19], In recent years, it has been found[20] that histone acetylation can activate gene expression and affect the development of embryonic organs. Histone acetylation enables the N-terminal site of histone lysine in promoter region and open chromatin spatial structure. In different cells, different histone lysine sites have different effects on gene expression[21]. In this study, we detected the acetylation of the whole histone H3 in GATA4 promoter region and the acetylation levels of H3K4, H3K9 and H3K27. We detected that the decreased expression of HDAC1 could inhibit the histone H3 acetylation and the level of H3K4 and H3K9 acetylation in GATA4 promoter region. In this study, we observed that miR-449 has a binding site with HDAC1, and miR-449 inhibits HDAC1 gene expression, and found that HDAC1 can regulate the acetylation levels of GATA elements H3K4 and H3K9 in the cTnI promoter region and affect cTnI expression. As an important structural and functional protein of the heart, cTnI is involved in the excitation-contraction coupling of the heart muscle. It has been discovered that in the elderly mice with low cTnI expression, the level of acetylation of the cTnI promoter region was lower than that of the young and middle-aged mice, and the binding level of the HDAC1 and the GATA component of the cTnI promoter region was significantly higher than that of the young and middle-aged mice[21]. So, can histone acetylation be involved in the regulation of cTnI expression *in vivo* and improve the heart function of elderly mice with low cTnI expression? Therefore, we further explore its mechanism in aged mice. In recent years, more attention has been given to the therapeutic effects of histone deacetylase inhibitors on environmental-related diseases. Histone acetylation can promote the binding of transcription factors to the promoter region of the target gene and activate the transcription of the target gene[22,23]. Therefore, histone acetylation is involved in regulating the expression of the cTnI upstream gene GATA4, and can recruit GATA4 to the cTnI promoter Region, promoting cTnI gene expression.

Conclusions

MiR-449 can regulate the acetylation of the histones H3K4 and H3K9 of the GATA element in the cTnI promoter region, thereby recruiting the transcription factor GATA4 to the cTnI promoter region, upregulating the cTnI gene expression, and improving cardiac function in elderly mice with low expression of cTnI. This will provide new treatment opportunities for diastolic dysfunction associated with low expression of cTnI. In this study, by studying the mechanism of miRNA regulating the expression of cardiac transcription factor GATA4 and structural protein cTnI through histone acetylation. It is the first time to be verified the mechanism of histone acetylation participating in the regulation of cTnI expression by recruiting the transcription factor GATA4. This study has laid a foundation for

in-depth study of the epigenetic mechanisms of cardiac development and diseases, and provided a new intervention target for the precise treatment of cardiovascular diseases.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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