LNCRNA-XIST PROMOTES CELL APOPTOSIS IN PATIENTS WITH CHRONIC HEART FAILURE BY REGULATING MIR-126

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ABSTRACT

Objective: *To explore the role of lncRNA-XIST (XIST) in promoting cell apoptosis in patients with chronic heart failure (CHF) by regulating miR-126.*

Methods: *A total of 114 CHF patients treated in our hospital were enrolled as a research group, and 92 healthy people in physical examination during the same period were enrolled as a control group. Myocardial cell were purchased. A polymerase chain reaction (PCR) assay was conducted to quantify XIST and miR-126 in the cells of CHF patients, and the correlation of XIST expression with clinicopathological features and prognosis of the research subjects was analyzed. The influences of XIST on the biological functions of CHF cells were also analyzed, and the regulatory relationbetween XIST and miR-126 in CHF cells was studied.*

Results: *XIST was over-expressed in the serum of CHF patients, while miR-126 was lowly expressed in it, and the expression of the two was negatively correlated. Multivariate Cox analysis revealed that high XIST expression, age, renal insufficiency, smoking history, and New York Heart Association classification were independent factors for prognosis of CHF patients. Overexpression of miR-126 and silence of XIST suppressed invasion and proliferation of CHF cells, and promoted apoptosis of them. Moreover, co-transfection of XIST-inhibitor + miR-126-mimics inhibited the invasion and proliferation of AC19 more significantly and strengthened the apoptosis of them more strongly. Furthermore, inhibition of XIST could increase the expression of miR-126, thus weakening the invasion and proliferation of CHF cells and strengthening their apoptosis.*

Conclusion: *Inhibition of XIST can weaken the proliferation and invasion of CHF cells and strengthen the apoptosis of them by up-regulating miR-126.*

Keywords: *XIST, miR-126, chronic heart failure, biological function.*

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Introduction

Chronic heart failure (CHF) is a chronic inflammatory disease[1], which causes dyspnea and respiratory failure by lowering the ventricular compliance and myocardial contractility via reducing the cardiac output[2]. Although progress has been made in clinical drugs and technical equipment, the occurrence and prevalence of CHF are still increasing[3], and about 50% of patients still face a risk of death within 5 years[4]. Therefore, we tried to classify relevant mechanisms of CHF and find new potential and reliable markers with high sensitivity and sensitivity to improve the prognosis of CHF patients.

LncRNA can regulate the expression of nearby genes through RNA-protein complexes, and it can be used as a regulator of the body and is related to the expression of nearby genes[5, 6]. XIST is one of the caner-related lncRNAs found first, which affects the

development and progression of various tumors and is found to abnormally express in cancers including hepatocellular carcinoma, breast cancer, colorectal cancer, cervical cancer, and ovarian cancer[7, 8].

There are some studies indicating that XIST heaveily affects the genome of human cells, and there are also some studies indicating that XIST shows expression dysregulation in human cancers, and such a dysregulation has been proved to have pathological effect[9]. For example, one study by Zhou T et al.[10] revealed that XIST was overexpressed in myocardial cells, and high expression of XIST could accelerate apoptosis of myocardial cells and suppress proliferation of them. MiR-126 is clinically considered to be the main regulator of physiological angiogenesis. Enriched in endothelial cells and endothelial progenitor cells, miR-126 accelerates the maturation of endothelial cells and participates in the signal induction of angiogenesis[11]. Furthermore, miR-126 promotes vascular remodeling and reduces fibrosis, and it has been taken as an important factor in the onset of cardiovascular diseases[12]. One previous study revealed that lncRNA could act as a sponge for miRNA to weaken the regulatory effect on mRNA[13]. According to one other study by Cheng Z, et al.[14], XIST can act as a competitive endogenous RNA of miR-126, and miR-126 is the direct target gene of XIST in glioblastoma cells.

Therefore, this study was designed to analyze the influences of regulating miR-126 by XIST on the biological functions of cells of CHF patients, so as to provide new therapeutic targets for CHF patients.

Methods and materials

Collection of clinical samples

A total of 114 CHF patients treated in our hospital from April 2017 to December 2018 were enrolled as a research group, and 92 healthy people in physical examination in the same time period were included in a control group.

The research group consisted of patients between 58 and 69 years old, with a mean age of (55.05±8.57) years, and the control group consisted of people between 55 and 70 years old, with a mean age of (54.11±8.51) years, so no significant difference was seen between the two groups in age. This study was carried out under the approval of the Ethics Committee of our hospital, and all subjects provided their signature on informed consent forms after understanding the study.

Cell transfection and culturing

Serum of each subject from the research group and the control group was sampled, placed into EDTA test tubes, and centrifuged in a centrifuge at 4°C and 1500× g for 10 min to collect plasma.

Myocardial cells (AC16) purchased from ACTT (number: 35427) was cultured in DMEM containing 10% fetal bovine serum (FBS) in a 5% CO_2 incubator at 37°C.

Instruments and reagents

DMEM (Shanghai Bohu Biotechnology Co., Ltd., BH-S3208), TRIzol kit (Zhen Shanghai and Shanghai Industrial Co., Ltd., hz81027-501), reverse transcription kit (GeneCopoeia, the United States), PrimeScript RT Master Mix (Beijing Biolab Technology Co., Ltd., YT378-XQM), radio immunoprecipitation assay (RIPA) buffer (Shanghai Shifeng Biological Technology Co., Ltd., R1176), bicinchoninic acid (BCA) kit (Shanghai Jingke Chemical Technology Co., Ltd., JK-201), Lipofectamine™ 2000 kit (Shanghai Mito Biotechnology Co., Ltd., 11668019), dual luciferase reporter gene assay kit (Wuhan Chundu Biotechnology Co., Ltd., CDLG-4997), Pierce™ Magnetic RNA-Protein Pull-Down Kit (Shanghai Bei Nuo Biotechnology Co., Ltd., 20164), AnnexinV-FITC (Shanghai Kemin Biotechnology Co., Ltd., DXT-130-097-928), CCK8 kit (Shanghai Yubo Biological Technology Co., Ltd., IC-CCK8-Hu), Transwell kit (Shanghai BioGenius Biotechnology Co., Ltd., Transwell), flow cytometry (BD Company, the Untied States, FACS Canto II), multi-function microplate reader (BioTek, the United States, DLK0001622). All primers were provided by Shanghai Sangon Biotech Co., Ltd.

PCR assay

The total RNA of the collected plasma was extracted using the TRIzol kit, and the integrity, purity and concentration of it were determined by an ultraviolet spectrophotometer. Reverse transcription was carried out to XIST and miR-126 in strict according with kit instructions.

The PCR reaction system consisted of 20 μl of total volume with 10 μl of 2×Talent qPCR PreMix, 1.25 μl of upstream and downstream primers, respectively, 100 ng of cDNA, and water to adjust the volume, and the PCR conditions were as follows: Pre-denaturation at 94°C for 30 s, followed by 40 cycles of denaturation at 94°C for 5 s and annealing and extension at 60°C for 30 s. The data in this

experiment were analyzed using the $2^{-\triangle\triangle ct}$ with GADPH as the internal reference for PVT1 and U6 as the internal reference for miR-190.

Western blot assay

The cultured cells were pyrolyzed with RIPA buffer to take the total protein, and the protein was centrifuged in a centrifuge at 4°C and 1500× g for 10 min to collect the supernatant as protein sample. The concentration of the supernatant was determined with the BAC kit, and then adjusted to 4 μg/μL.

Afterwards, the protein was separated through 12% SDS-PAGE, and then placed on a 0.22 μm polyvinylidene fluorid (PVDF) membrane, blocked with 5% skim milk powder for 2 hours. Subsequently, the protein was added with IGF-1 and β-actin primary bodies at a ratio of 1: 1000, and blocked at 4°C overnight. The membrane was washed to remove the primary antibodies, and then it was incubated at 37°C for 1 hour with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1: 5000), and rinsed with PBS 3 times, 5 min each time, A filter paper was used for the membrane to remove excess liquid on it, and then the membrane was made to be luminescent with ECL and developed.

The protein band was scanned, and the gray value was calculated using Quantity One under the internal reference of GAPDH.

Cell proliferation detection by CCK-8 assay

Cells transfected for 24 hours were collected and transferred into a 96-well plate at $4*10^6$ cells/ well, and each well was added with 10 μL of CCK-8 solution and 90 μL of basic DMEM at 24 h, 48 h, 72h, and 96 h after culturing, respectively.

After addition of the solution each time, the plate was cultured at 37°C for 2 hours. The optical density of every well at 450 nm was measured with a enzyme mark instrument.

Detection of cell invasion by a transwell assay

Cells transfected for 24 hours were collected, transferred into a 6-well plate at $5*10^4$ cells / well, and washed with PBS two times. Afterwards, the upper compartment was added with 200 μL of DMEM, and the lower compartment was added with 500 mL DMEM with 20% FBS.

The plate was incubated at 37°C for 48 h, and the substrate and cells not penetrating the microporous membrane in the upper compartment were wiped off. The plate was washed 3 times, fixed with paraformaldehyde for 10min, and washed with double distilled water 3 times, and stained with 0.5% crystal violet after being dried out. Then the cell invasion in the plate was analyzed using a microscope.

Cell apoptosis assay

The transfected cells were processed with 0.25% trypsin. After digestion, the cells were washed with PBS 2 times, and then added with 100 μL of binding buffer to prepare $1*10^6$ cells /mL suspension. Then it was added with AnnexinV-FITC and PI in order, cultured at indoor temperature in the dark for 5 min, and finally detected using the FC500MCL flow cytometer system.

The experiment was repeated three times and the obtained data were averaged as results.

Target gene detection

Starbase 3.0 was employed to predict potential target genes of XIST, and the Lipofectamine™ 2000 kit was utilized to clone XIST on pmirGLO dual-luciferase target expression vectors. The constructed pmirGLO-XIST-3'UTR wild type (Wt) and pmirGLO-XIST-3'UTR mutant type (Mut) were transfected into the downstream of the luciferase reporter genes to sequence and identify the constructed plasmids.

Correctly sequenced plasmids along with miR-126-mimics or miR-NC were co-transfected into AC16 cells.

Statistical analysis

In this study, the collected data were analyzed statistically using SPSS20.0, and visualized into required figures using the GraphPad 7. Comparison between groups was conducted with the independent-samples T test, and comparison among multiple groups was conducted using the one-way anova.

Post hoc pairwise comparison was carried out through the LSD-t test, and comparison of expression in different time points was conducted using the variance of repeated measures.

Figures about the diagnostic value of XIST in CHF were drawn using receiver operating characteristic (ROC) curves, and Pearson's correlation analysis was conducted to analyze the relationship between miR-126 and XIST expression in patients' serum and tissues.

In addition, the Log-rank test and multivariate Cox regression analysis were performed to analyze the prognosis of patients. $P<0.05$ suggested a bigdifference.

Results

The expression of serum XIST and miR-126 in CHF patients

The qRT-PCR assay results revealed that the two groups had difference in the expression of serum XIST and miR-126: The expression of serum XIST in the research group was higher than that in the control group, while the expression of miR-126 in the research group was lower than that in the control group (both P<0.05). Figure 1.

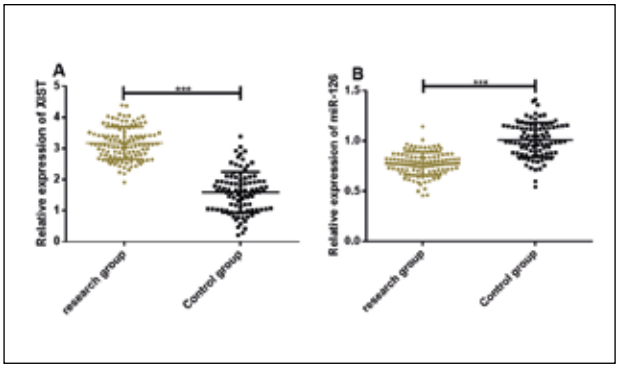

Figure 1: The expression of serum XIST and miR-126 in CHF patients. The expression of serum XIST in CHF patients increased significantly (A). The expression of serum miR-126 in CHF patients decreased significantly (B).
*Note: *indicates P<0.05.*

Relationship between XIST and clinicopathological features of CHF patients

We conducted a multivariate Cox regression to analyzethe relation between pathological data of CHF patients and serum XIST in them, finding that high XIST expression, age, renal insufficiency, smoking history, and New York Heart Association classification were independent factors for prognosis of CHF patients. Tables 1-2.

Diagnostic value of XIST and miR-126 expression in CHF

We drew ROC curves of serum XIST and miR-126 for diagnosis of CHF, and the ROC curves displayed that the AUC of serum XIST in diagnosing CHF, sensitivity, specificity, and the best cut-off value of it were 0.831, 73.68, 86.97, and 2.361, respectively, and the AUC of serum miR-126 in diagnosing CHF, sensitivity, specificity, and the best cut-off value of it were 0.857, 76.09, 89.47, and 0.933, respectively, Pearson's correlation analysis revealed that the expression of serum XIST and miR-126 in CHF patients was negatively correlated (P< 0.05). Table 3 and Figure 2.

Factor	n	XIST		t-value	P-value
		High expression group (n=57)	Low expression group (n=57)		
Age (Y)				4.950	0.023
≥60	62	24 (38.71)	38 (61.29)		
<60	52	31 (59.62)	21 (40.38)		
Sex				0.891	0.345
Male	66	34 (51.52)	32 (48.48)		
Female	48	29 (60.42)	19 (39.58)		
Renal insufficiency				4.784	0.029
Yes	72	41 (56.94)	31 (43.06)		
No	42	15 (35.71)	27 (64.29)		
Smoking history				7.396	0.007
Yes	69	44 (63.77)	25 (36.23)		
No	45	17 (37.78)	28 (62.22)		
Drinking history				0.331	0.565
Yes	65	38 (58.46)	27 (41.54)		
No	49	26 (53.06)	23 (46.94)		
Comorbid with diabetes mellitus				0.460	0.498
Yes	51	36 (55.38)	29 (44.62)		
No	63	24 (48.98)	25 (51.02)		
New York Heart Association classification				15.542	0.001
Level II	62	20 (32.26)	42 (67.74)		
Level III	31	21 (67.74)	10 (32.26)		
Level IV	21	15 (71.43)	6 (28.57)		

Table 1: Relationship between XIST and clinicopathological features of CHF patients.

Factor	Univariate Cox			Multivariate Cox		
	P-value	HR	95 CI%	P-value	HR	95 CI%
Age	0.048	2.182	1.042-4.364	0.023	2.856	1.153-7.072
Sex	0.664	1.116	0.685-1.232			
Renal insufficiency	0.038	1.245	0.486-2.469	0.030	1.004	0.023-2.008
Smoking history	0.048	1.815	1.012-4.120	0.036	2.006	1.047-3.843
Drinking history	0.663	1.116	0.681-1.83			
Comorbid with diabetes mellitus	0.775	0.931	0.571-1.518			
New York Heart Association classification	<0.001	6.151	2.773-13.644	<0.001	5.339	2.378-11.984

Table 2: Cox regression analysis.

Diagnostic index	AUC	95%CI	Standard error	Cut-off	Sensitivity (%)	Specificity (%)
XIST	0.831	0.775-0.887	0.029	2.361	73.68	86.97
MiR-126	0.857	0.801-0.913	0.028	0.933	76.09	89.47

Table 3: Diagnostic value of serum XIST and miR-126 expression in CHF.

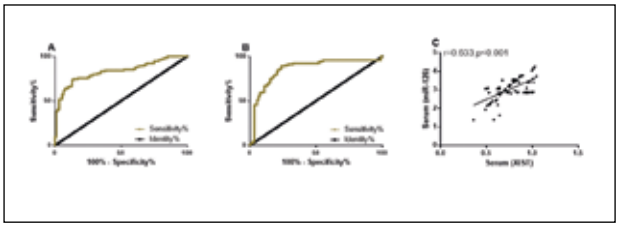

Figure 2: Diagnostic value of XIST and miR-126 expression in CHF. ROC curve of serum XIST in diagnosing CHF (A). ROC curve of serum miR-126 in diagnosing CHF (B). The expression of serum XIST and miR-126 was negatively correlated (C).

Effects of XIST expression in transfected myocardial cells on the biological functions of cells of CHF patients

We determined the expression of XIST in transfected AC16 cells, finding that the XIST expression in cells transfected with XIST-inhibitor was much lower than that in cells transfected with si-NC (P<0.05). We also measured the proliferation, invasion, and apoptosis of transfected cells, finding that cells transfected with XIST-inhibitor showed significantly weaker proliferation and invasion and significantly stronger apoptosis than those transfected with si-NC (all P<0.05). Figure 3.

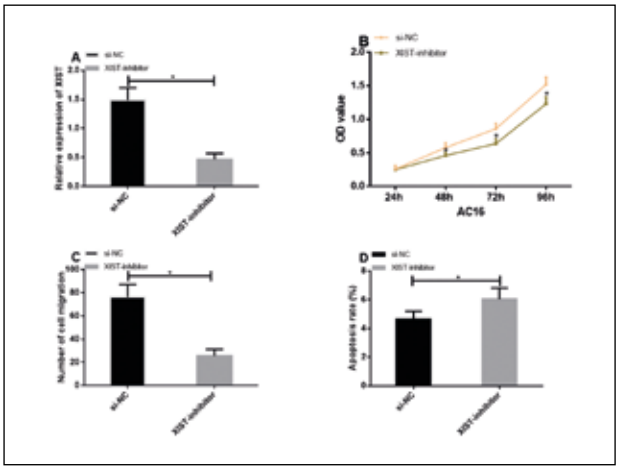

Figure 3: Influences of XIST expression in transfected myocardial cells on the biological functions of cells of CHF patients. The XIST expression in transfected AC16 cells (A). Proliferation of transfected AC19 cells (B). Invasion of transfected AC19 cells (C). Apoptosis of transfected AC19 cells (D).
*Note: *indicates P<0.05.*

Effects of miR-126 expression in transfected myocardial cells on the biological functions of cells of CHF patients

We quantified miR-126 in transfected AC16 cells, finding that the expression of miR-126 in cells transfected with miR-126-mimics was greatly higher than that in those transfected with miR-NC (P<0.05). We also measured the proliferation, invasion, and apoptosis of transfected cells, finding that cells transfected with miR-126-mimics showed significantly weaker proliferation and invasion and significantly stronger apoptosis than those transfected with miR-NC (P<0.05). Figure 4.

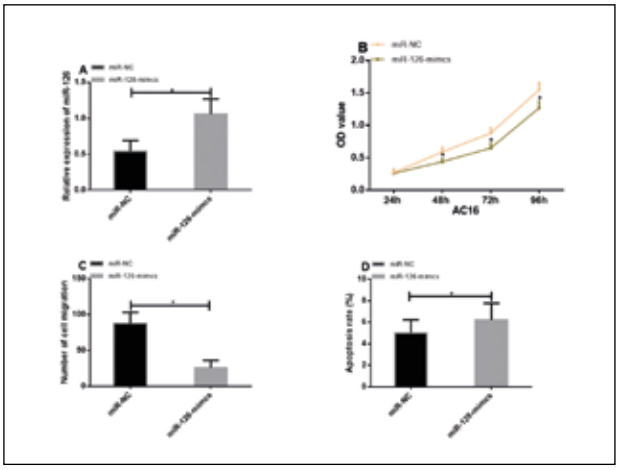

Figure 4: Influenes of miR-126 expression in transfected myocardial cells on the biological functions of cells of CHF patients. The miR-126 expression in transfected AC16 cells (A). Proliferation of transfected AC19 cells (B). Invasion of transfected AC19 cells (C). Apoptosis of transfected AC19 cells (D).
*Note: *indicates P<0.05.*

Gene identification of XIST

We predicted that there were targeted binding loci between miR-126 and XIST by predicting potential targets of XIST through starbase3.0.

In order to verify it, we carried out a dual luciferase reporter assay, finding that miR-126 overexpression strongly lowered the luciferase activity of XIST-3'UT Wt (P<0.05), but exerted no effect on that of XIST-3'UTR Mut (P>0.05).

The PCR assay results revealed that AC16 cells transfected with XIST-mimics showed significantly decreased expression of miR-126, and those transfected with XIST-inhibitor showed significantly increased miR-126 expression (all P<0.05). Figure 5.

Rescue experiment

We measured the biological functions of AC16 cells co-transfected with XIST-inhibitor+miR-

126-mimics, finding that the cells transfected with XIST-inhibitor+miR-126-mimics were not different from those transfected with si-NC in proliferation, invasion, and apoptosis, and those transfected with XIST-inhibitor showed much stronger proliferation and invasion and much weaker apoptosis than those transfected with si-NC. Figure 6.

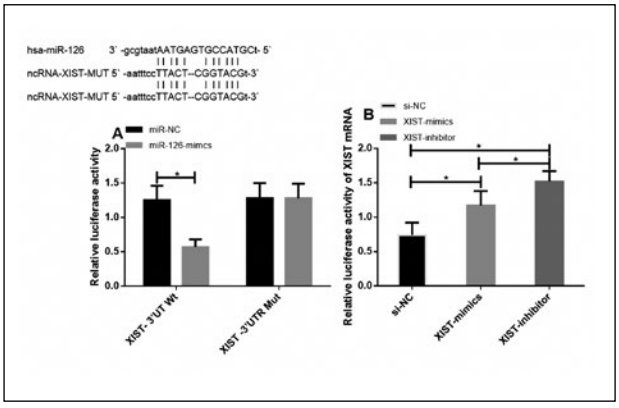

Figure 5: Gene identification of XIST. Binding loci between XIST and miR-126, and relative luciferase activity - dual luciferase reporter assay results (A). Relative expression of XIST protein in transfected AC19 cells (B).
*Note: *indicates P<0.05.*

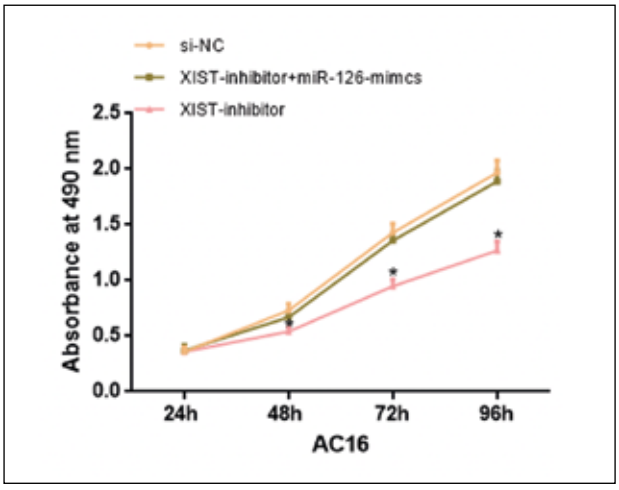

Figure 6: Variation in the biological functions of AC19 cells after co-transfection of XIST-inhibitor + miR-126-mimics. Cells transfected with XIST-inhibitor+miR-126-mimics were not different from those transfected with si-NC in proliferation, and those transfected with XIST-inhibitor showed significantly stronger proliferation than those transfected with si-NC.

Discussion

CHF is a disease harmful to myocardial relaxation and systolic function[15]. It often gives rise to congestion of the circulatory system and progressive debilitation of patients[16]. Although the survival rate of CHF patients has been improved with the improvement of disease therapy at present, these surviving patients still need hospitalization and face a risk of recurrence[17]. Therefore, it is of great significance to find biomarkers affecting the prognosis of CHF in improving the prognosis and survival rate of CHF patients. With a regulatory function in various cells, lncRNA participates in cell fate determination and cell genome imprinting, and plays a crucial role in cell development, metabolism and differentiation.[18, 19].

Previous studies have revealed that lncRNA participates in various human diseases, and it has been identified in myocardial cells as extremely important for heart development[20]. For example, one study by Deng H et al.[21] showed that lncRNA-GASL1 was down-regulated in CHF patients, and its overexpression could inhibit apoptosis of myocardial cells through inactivation of TGF-β1 to relieve CHF. In addition, some studies have uncovered that lncRNA can act as an endogenous sponge to regulate the function of miRNA, and both lncRNA and miRNA are related to the pathological process of vascular diseases[22, 23]. For example, one study by Zheng J et al.[24] concluded that lncRNA-PVT1 affected angiogenesis in microvascular cells of glioma patients by regulating the expression of miR-186, and one other study by Xiong Y et al.[25] pointed out that lncRNA-XIST could regulate the growth and invasion of bladder cancer cells via the dependence of miR-124. However, it is not clear whether XIST can regulate the biological functions of cells of CHF patients by targeting miR-126. Therefore, we first analyzed the expression of XIST and miR-126 in CHF patients and evaluated their clinical value in CHF in our study. The results revealed that the expression of serum XIST in the research group was above than that in the control group, while the expression of miR-126 in the research group was less than that in the control group.

We carried out a Pearson's correlation analysis, finding that the expression of serum XIST and miR-126 was negatively correlated. We also drew ROC curves and analyzed them. The AUC of XIST and miR-126 was 0.831 and 0.857, respectively, which suggested that XIST and miR-126 had good diagnostic value for CHF. Afterwards, we conducted a multivariate Cox analysis to analyze the relationship between pathological data of patients and XIST, finding that high XIST expression, age, renal insufficiency, smoking history, and New York Heart Association classification were independent factors for prognosis

of CHF patients. We speculated that XIST and miR-126 participated in the development and progression of CHF, so we carried out cell biology experiments to verify it. We determined the XIST and miR-126 in myocardial cells (AC19), and it came out that the XIST expression was up-regulated in AC19 cells, while the miR-126 expression decreasedin them. Further, we transfected XIST-inhibitor and miR-126-mimics plasmids into AC19 cells, respectively, finding that transfection of XIST-inhibitor strongly suppressed the proliferation and invasion of cells and strongly strengthened apoptosis of them, which implied that down-regulation of XIST expression could inhibit cell proliferation and invasion, and promote cell apoptosis. Moreover, transfection of miR-126 also inhibited proliferation and invasion of cells and strengthened apoptosis of them.

Those results implied that XIST and miR-126 could be used as potential targets for treatment of CHF. In order to understand the correlation between XIST and miR-126, we carried out a rescue experiment. It came out that CHF cells transfected with XIST-inhibitor+miR-126-mimics were not different from those transfected with si-NC in proliferation, invasion, and apoptosis, while those transfected with XIST-inhibitor showed significantly strengthened proliferation and invasion and significantly weakened apoptosis, which suggested that there was a certain regulatory relation between XIST and miR-126. We conducted a dual luciferase reporter assay to verify the regulatory relation, and found that overexpression of miR-190 strongly decreased the luciferase activity of XIST-3UT Wt, but exerted no effect on that of XIST-3'UTR Mut, and transfection of XIST-mimics strongly decreased the expression of miR-190 in AC19 cells, but transfection of XIST-inhibitor significantly increased the expression of in AC19 cells. The results implied a targeted regulatory relation between XIST and miR-126. Namely, inhibition of XIST could weaken proliferation and invasion of CHF cells and strengthen apoptosis of them by increasing the expression of miR-126.

This study has confirmed that XIST could suppress the proliferation of miR-126-mediated cells, but it still has certain limitations. First of all, we have not carried out a nude mouse tumorigenesis assay, so whether the role of XIST in experiments in vivo is consistent with that in cell experiments still needs further verification. We hope to carry out more basic research in the future, understand more potential mechanisms of XIST through bioinformatics analysis, and collect more types (cell lines) and different types (serum) of samples to verify our research results.

To sum up, inhibition of XIST can weaken the proliferation and invasion of CHF cells and strengthen the apoptosis of them by increasing of miR-126 expression.

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