lncRNA HOXB-AS3 protects doxorubicin-induced cardiotoxicity by targeting miRNA-875-3p

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Received October 14, 2019; Accepted December 12, 2019

DOI: 10.3892/etm.2019.8335

Abstract. Protective role of lncRNA HOXB-AS3 in doxorubicin (DOX)-induced cardiotoxicity and its mechanism were studied. Viability of PC and H9c2 cells treated with different doses of DOX was determined through CCK-8 assay. Relative level of HOXB-AS3 in DOX-treated cardiomyocytes was detected. Regulatory effect of HOXB-AS3 on the proliferative ability of DOX-treated cardiomyocytes was assessed. Through dual-luciferase reporter gene assay, the binding relationship between HOXB-AS3 and miRNA-875-3p was verified. Rescue experiments were conducted to explore the role of HOXB-AS3/miRNA-875-3p in influencing the proliferation of DOX-treated cardiomyocytes. The proliferative ability of cardiomyocytes was dose-dependently downregulated after DOX treatment. Relative level of HOXB-AS3 was upregulated in DOX-treated cardiomyocytes. Silence of HOXB-AS3 in cardiomyocytes undergoing DOX treatment markedly elevated their proliferative ability. miRNA-875-3p was the direct target of HOXB-AS3. Knockdown of miRNA-875-3p reversed the role of HOXB-AS3 in regulating the proliferative ability of cardiomyocytes. HOXB-AS3 protects DOX-induced suppression in the proliferation of cardiomyocytes through targeting and downregulating miRNA-875-3p.

Introduction

Doxorubicin (DOX) is an anthracycline, broad-spectrum anti-tumor drug. It has a remarkable therapeutic effect on cancers, including acute lymphoblastic leukemia, lung cancer and breast cancer. However, the endotoxicity of DOX restricts its clinical application. With the accumulation of DOX in the body, cardiotoxicity eventually leads to apoptosis and necrosis of cardiomyocytes, mainly manifesting as dilated cardiomyopathy and heart failure (1-3). Studies have shown that the cumulative amount of 450-500 mg/m^2 DOX leads to 4-5% incidence of myocardial toxicity, which is up to 18% at 550-660 mg/m^2. So far, the mechanism of myocardial damage caused by DOX has not been fully elucidated. Effective methods for preventing and treating DOX-induced cardiotoxicity are lacking (4).

lncRNA is >200-bp long and widely present in organisms (5-7). It is generally considered to be dark substance in the human genome without protein-encoding functions (8,9). Most of lncRNAs are produced in a similar way to that of mRNAs through cleavage, folding, capping and polyadenylation. They are involved in regulating chromosome silencing, epigenetic mediation, genomic imprinting, intranuclear and transport (10-13). In recent years, lncRNAs were identified to regulate gene expression at transcriptional and post-transcriptional levels. In the progression of cardiac diseases, lncRNAs serve as potential targets for cardiac regeneration and repair (14,15). lncRNA HOXB-AS3 encodes an HOXB-AS3 polypeptide that blocks the tricarboxylic acid cycle of tumor cells. It acts as a switch in the carbohydrate metabolism pathway. Hence, HOXB-AS3 polypeptide is a candidate anti-tumor drug, exerting a promising clinical application (16).

miRNAs are extensively involved in the regulation of gene expression. Approximately 1/3 of human genes could be regulated by miRNAs. They exert crucial functions in cellular behavior and tumorigenesis (17). It is reported that miRNA-875-3p regulates expression of functional genes during the synthesis of casein by mediating arginine (18). It prevents the binding of arginine to the exon of PKM mRNA, thereby producing PKM1 splicing instead of PKM2, which in turn mediates the tricarboxylic acid metabolic pathway (16).

The biological role of lncRNA HOXB-AS3 in influencing tumor progression has been discovered. However, its role in DOX-induced cardiotoxicity remains unclear. This study mainly investigated the role of HOXB-AS3 in protecting DOX-induced cardiotoxicity and the potential mechanism.

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Key words: DOX, HOXB-AS3, miRNA-875-3p, cardiotoxicity

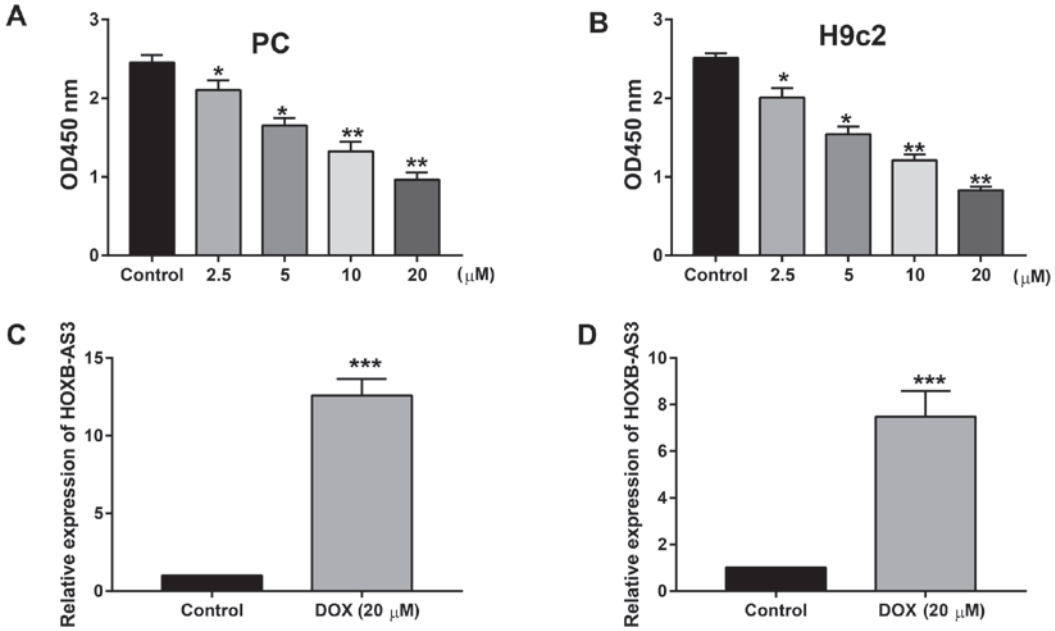

Figure 1. HOXB-AS3 is upregulated in DOX-treated cardiomyocytes. (A) Viability in PC cells treated with 0, 2.5, 5, 10 and 20 μM DOX for 24 h. (B) Viability in H9c2 cells treated with 0, 2.5, 5, 10 and 20 μM DOX for 24 h. (C) Relative level of HOXB-AS3 in PC cells treated with 0 or 20 μM DOX for 24 h. (D) Relative level of HOXB-AS3 in H9c2 cells treated with 0 or 20 μM DOX for 24 h. DOX, doxorubicin. Compared with control, *P<0.05, **P<0.01, ***P<0.001.

Materials and methods

Cell culture and transfection. PC and H9c2 cells were purchased from American Type Culture Collection (ATCC). Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 containing 10% FBS (Life Technologies), 100 U/ml penicillin and 100 μg/ml streptomycin. Until 70-80% confluence, cells were transfected with the vector using Lipofactamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Fresh medium was replaced at 6 h. Sequences of transfection vectors were as follows: sh-HOXB-AS3 1#, 5'-GGUAAACUCGCACCUCUUATT-3'; sh-HOXB-AS3 2#, 5'-GGGUCGUCUGUAUCAAUUUTT-3'; miRNA-875-3p inhibitor, 5'-CACAACCUCGUGUUUCCATT-3'.

RNA extraction. Cells ($5x10^6$) were lysed in 1 ml of TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated with 0.2 ml of chloroform. After 5 min of standing time, the mixture was centrifuged at 4˚C, 10,500 x g for 10 min. The precipitant was transferred to a new tube, incubated with the isodose isopropanol and centrifuged again at 4˚C, 10,500 x g for 10 min. The precipitant was washed with 75% ethanol and air dried. Finally, the purified RNA was dissolved in diethyl-pyrocarbonate (DEPC) water (Beyotime Institute of Biotechnology).

Quantitative real-time polymerase chain reaction (qRT-PCR). RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT reagent (Takara Bio). The obtained cDNA underwent qRT-PCR using SYBR® Premix Ex Taq™ (Takara Bio) at 95˚C for 2-min pre-denaturation, and 40 cycles at 95˚C for 1 min, 60˚C for 1 min and 72˚C for 1 min. Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. Primer sequences were as follows: GAPDH, F, 5'-AGAAGGCTGGGGCTCATTTG-3'; R, 5'-AGGGGC CATCCACAGTCTTC-3'. CACNA1G-AS1, F, 5'-CGTCCA GCTGCGAGCCAGC-3'; R, 5'-AGCCTTCCTGTGACCTC ATC-3'.

5-Ethynyl-2'- deoxyuridine (EdU) assay. Cells were inoculated into 96-well plates with $1x10^5$ cells per well, and labeled with 100 μl of EdU reagent (50 μM) per well for 2 h. After washing with phosphate-buffered saline (PBS), the cells were fixed in 50 μl of fixation buffer, decolored with 2 mg/ml glycine and permeated with 100 μl of penetrant. After washing with PBS once, cells were stained with 1X Hoechst 33342 in the dark for 30 min. EdU-positive ratio was determined under a fluorescent microscope.

Cell Counting Kit (CCK-8). Cells were seeded in a 96-well plate with $5x10^3$ cells per well. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories) for depicting the viability curve.

Statistical analysis. Statistical Product and Service Solutions (SPSS) 13.0 (SPSS Inc.) was used for data analyses. Data are expressed as mean ± standard deviation. Intergroup differences were analyzed by the t-test. Kaplan-Meier method was introduced for survival analysis. Two-tailed P<0.05 was considered as statistically significant.

Results

HOXB-AS3 is upregulated in DOX-treated cardiomyocytes. PC and H9c2 cells were treated with 0, 2.5, 5, 10 and 20 μM DOX for 24 h. The viability of cardiomyocytes was dose-dependently downregulated after DOX treatment (Fig. 1A and B).

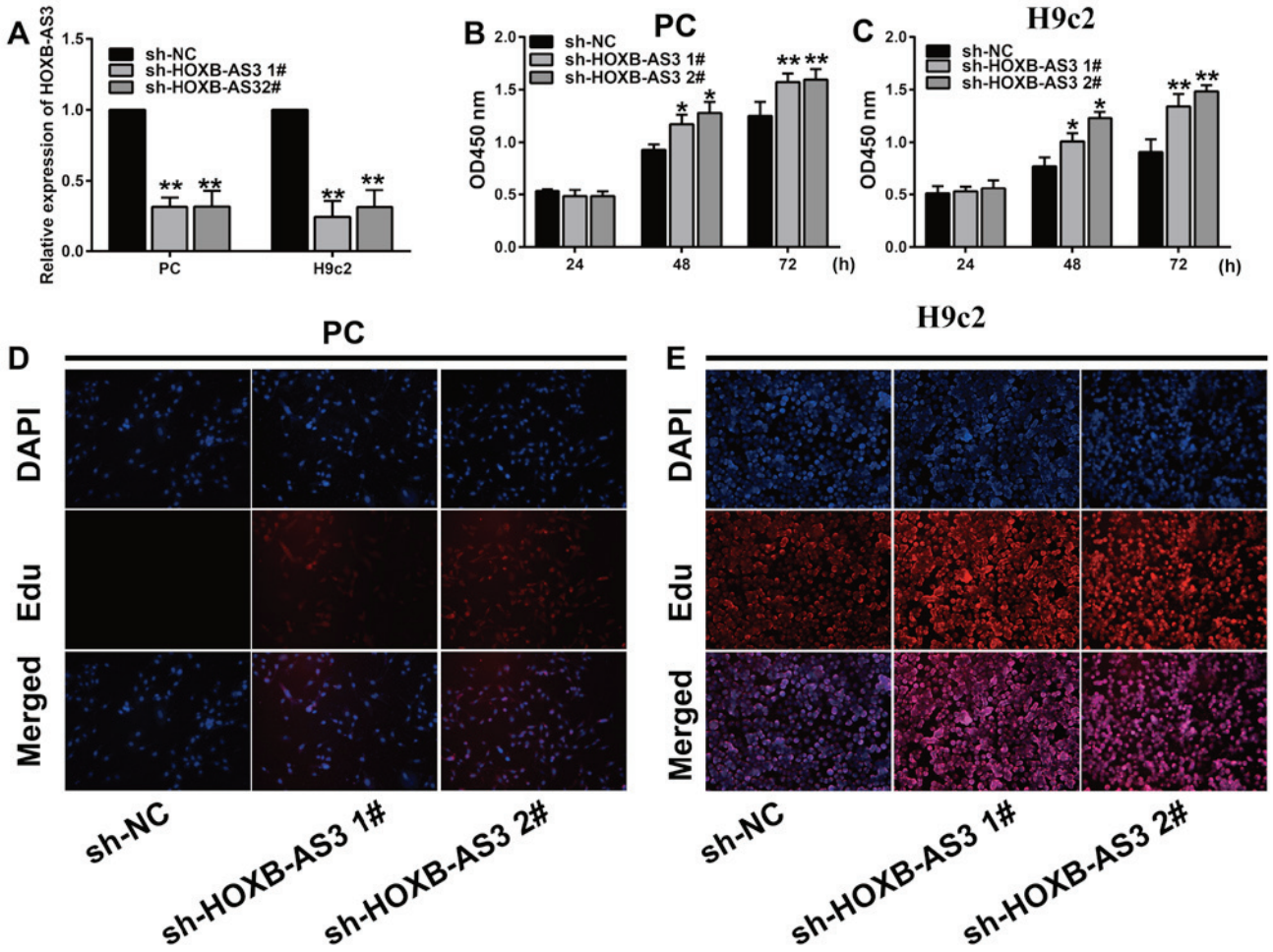

Figure 2. Overexpression of HOXB-AS3 suppresses cardiomyocyte proliferation. (A) Transfection efficacies of sh-HOXB-AS3 1# and sh-HOXB-AS3 2# in PC and H9c2 cells. (B) Viability in PC cells transfected with sh-NC, sh-HOXB-AS3 1# or sh-HOXB-AS3 2# for 24, 48 and 72 h. (C) Viability in H9c2 cells transfected with sh-NC, sh-HOXB-AS3 1# or sh-HOXB-AS3 2# for 24, 48 and 72 h. (D and E) EdU-positive ratio in PC and H9c2 cells transfected with sh-NC, sh-HOXB-AS3 1# or sh-HOXB-AS3 2#. Compared with sh-NC, *P<0.05, **P<0.01.

After 20 μM DOX treatment for 24 h, HOXB-AS3 level in PC and H9c2 cells was markedly upregulated (Fig. 1C and D). We speculate that upregulated HOXB-AS3 may serve as a protective effect on DOX-induced cardiotoxicity.

Overexpression of HOXB-AS3 suppresses cardiomyocyte proliferation. To further investigate the biological function of HOXB-AS3, sh-HOXB-AS3 1# and sh-HOXB-AS3 2# were constructed. Transfection of sh-HOXB-AS3 1# or sh-HOXB-AS3 2# markedly downregulated HOXB-AS3 level in PC and H9c2 cells (Fig. 2A). At 48 and 72 h, the viability in DOX-treated PC and H9c2 cells transfected with sh-HOXB-AS3 1# or sh-HOXB-AS3 2# was markedly elevated compared to those in the controls (Fig. 2B and C). Similarly, EdU-positive ratio was enhanced after transfection of sh-HOXB-AS3 1# or sh-HOXB-AS3 2# (Fig. 2D and E). It is concluded that silence of HOXB-AS3 markedly enhanced the proliferative ability in DOX-treated cardiomyocytes.

miRNA-875-3p is the target of HOXB-AS3. miRNA-875-3p was found to be downregulated after 20 μM DOX treatment in PC and H9c2 cells (Fig. 3A). Through bioinformatics prediction, binding sequences between miRNA-875-3p and HOXB-AS3 were discovered (Fig. 3B). Dual-luciferase reporter gene assay showed declined luciferase activity after co-transfection of HOXB-AS3-WT and miRNA-875-3p mimics (Fig. 3C). Moreover, a negative correlation was identified between expression of miRNA-875-3p and HOXB-AS3. Transfection of miRNA-875-3p mimics downregulated HOXB-AS3 level in PC and H9c2 cells and conversely, transfection of miRNA-875-3p inhibitor upregulated the level of HOXB-AS3 (Fig. 3D and E).

miRNA-875-3p partially reverses the regulatory effect of HOXB-AS3 on cardiomyocytes. It is speculated that miRNA-875-3p may be involved in HOXB-AS3-mediated cardiomyocyte proliferation. In PC and H9c2 cells transfected with sh-HOXB-AS3 1#, miRNA-875-3p level was markedly upregulated, which was partially downregulated by co-transfection of miRNA-875-3p inhibitor (Fig. 4A). Interestingly, the elevated EdU-positive ratio in PC cells transfected with sh-HOXB-AS3 1# was partially reversed by knockdown of miRNA-875-3p (Fig. 4B). Hence, HOXB-AS3/miRNA-875-3p complex was determined to protect cardiomyocytes from DOX-induced cardiotoxicity.

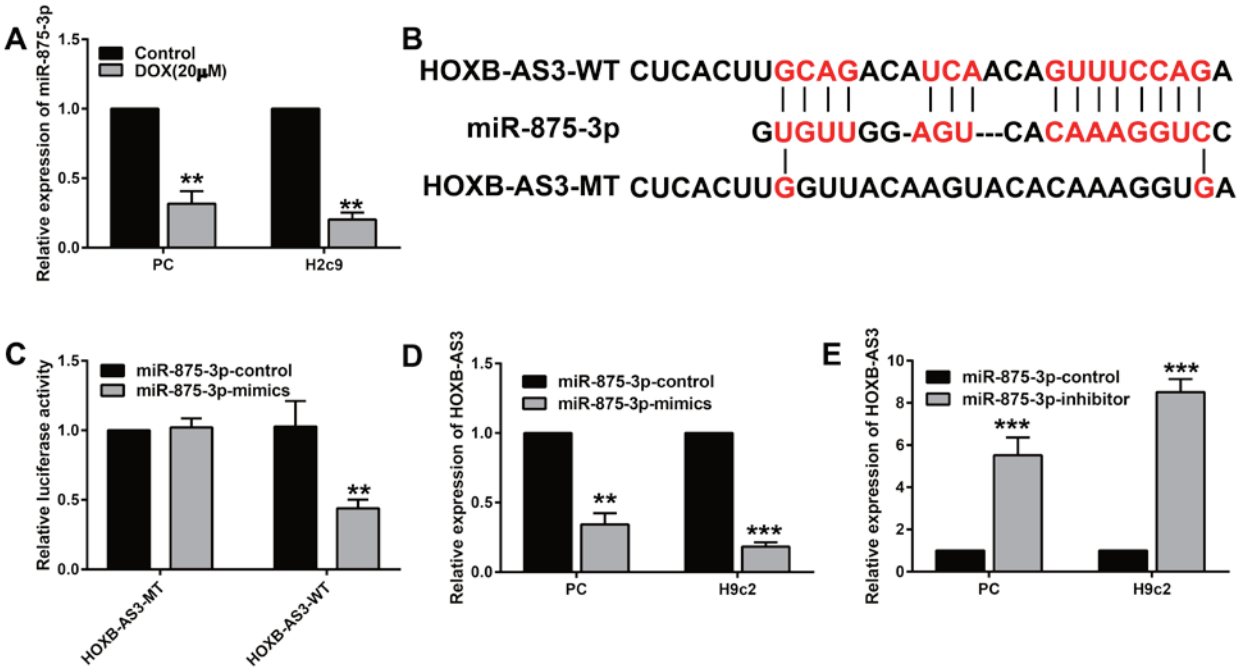

Figure 3. miR-875-3p is the target of HOXB-AS3. (A) Relative level of HOXB-AS3 in PC and H9c2 cells treated with 0 or 20 μM DOX for 24 h. (B) Binding sites between HOXB-AS3 and miR-875-3p. (C) Luciferase activity in PC cells co-transfected with HOXB-AS3-MT/HOXB-AS3-WT and miR-875-3p mimics/negative control. (D) Relative level of HOXB-AS3 in PC and H9c2 cells transfected with miR-875-3p mimics or negative control. (E) Relative level of HOXB-AS3 in PC and H9c2 cells transfected with miR-875-3p inhibitor or negative control. DOX, doxorubicin. Compared with control or miR-875-3p-control, **P<0.01, ***P<0.001.

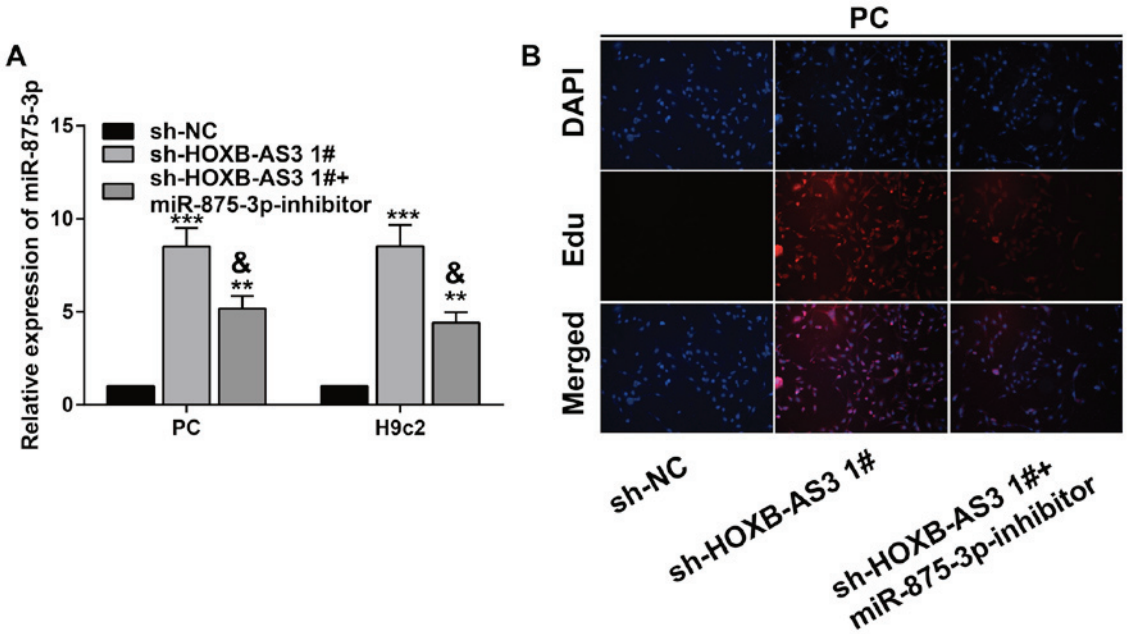

Figure 4. miR-875-3p partially reverses the regulatory effect of HOXB-AS3 on cardiomyocytes. (A) Relative level of miR-875-3p in PC and H9c2 cells transfected with sh-NC, sh-HOXB-AS3 1# or sh-HOXB-AS3 1# + miR-875-3p inhibitor. (B) EdU-positive ratio in PC cells transfected with sh-NC, sh-HOXB-AS3 1# or sh-HOXB-AS3 1# + miR-875-3p inhibitor. Compared with sh-NC, **P<0.01, ***P<0.001; Compared with sh-HOXB-AS3 1#, $^{\&}$P<0.05.

Discussion

DOX is one of the important chemotherapeutic drugs applied in the treatment of various solid tumors. However, dose-dependence and cardiotoxicity of DOX severely restrict its clinical application (19). Currently, lncRNAs are found to be abnormally expressed in patients with myocardial diseases, and able to alleviate cardiomyocyte apoptosis and improve cardiac function (20). Li *et al* (21) reported that lncRNA NRF regulates the programmed necrosis of cardiomyocytes, which may be utilized as a target for the treatment of cardiac remodeling following myocardial infarction. In non-ischemic cardiac diseases (such as DOX-induced myocardial necrosis), lncRNAs also has crucial functions.

It negatively regulates the expression of target genes by binding to the mRNAs. Studies have demonstrated that miRNAs are differentially expressed in tissues under pathological conditions, which are widely applied as disease markers (22). Serum level of miRNA-875-3p is found to be downregulated in children with primary dilated heart disease (23). In this study, DOX-induced cardiotoxicity upregulated the level of HOXB-AS3 and downregulated miRNA-875-3p.

Genomic sequencing and bioinformatics analysis technology contribute to identification of the differentially expressed lncRNAs in tumor cells, which help to develop therapeutic targets for tumor diseases (22). Previous studies illustrated the switch function of HOXB-AS3 in glycolysis pathway (19-21). Our study showed that DOX treatment remarkably suppressed the viability in cardiomyocytes, confirming the cardiotoxicity of DOX. Silence of HOXB-AS3 enhanced the viability and EdU-positive ratio in DOX-treated cardiomyocytes, indicating an elevated proliferation. Hence, we considered that HOXB-AS3 protected cardiotoxicity induced by DOX treatment. Subsequently, miRNA-875-3p was verified to be the direct target of HOXB-AS3. Notably, knockdown of miRNA-875-3p could reverse the regulatory effect of HOXB-AS3 on the cardiomyocyte proliferation. As a result, HOXB-AS3/miRNA-875-3p complex was proved to alleviate DOX-induced cardiotoxicity.

In conclusion, HOXB-AS3 protects DOX-induced suppression in the proliferation of cardiomyocytes through targeting and downregulating miRNA-875-3p. HOXB-AS3/miRNA-875-3p complex shows potential as drug targets for alleviating cardiotoxicity.

Acknowledgements

Not applicable.

Funding

This study was supported by the Science and Technology Foundation of Shannxi Province (nos. 2016HM-04, 2016JM-8038 and S2017-ZDYF-ZDCXL-SF-0054).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

QL and JH designed the study and performed the experiments, QL and PL collected the data, LB and AM analyzed the data, QL prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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