Biomedicine & Pharmacotherapy 105 (2018) 789–797

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

LncRNA XIST accelerates cervical cancer progression via upregulating Fus through competitively binding with miR-200a

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ARTICLE INFO

Keywords:
LncRNA XIST
Cervical cancer
miR-200a
Fus

ABSTRACT

As one of the commonest gynecological malignancies in the world, cervical cancer brings great threat for public health. Long non-coding RNAs (LncRNAs) have been proved to be closely related to the progression of various cancers, including cervical cancer. As a tumor promoter, lncRNA XIST has been reported in various malignant tumors. In this study, we aim to explore the specific mechanism and biological function of XIST in cervical cancer. At first, the expression levels of XIST were examined in both tissues and cell lines with qRT-PCR. XIST was extremely overexpressed in cervical cancer tissues and cell lines. Kaplan Meier method was then applied to analyze the correlation between XIST expression and overall survival of cervical cancer patients. Loss-of-function assays were designed and conducted to verify the oncogenic function of XIST on cervical cancer progression. Additionally, the results of mechanistic experiments indicated that XIST upregulated Fus through competitively binding with miR-200a. Finally, rescue assays were conducted to demonstrate the regulatory function of XIST-miR-200a-Fus axis in cervical cancer progression. Collectively, XIST served as a ceRNA in cervical cancer progression through modulating miR-200a/Fus axis.

1. Introduction

As one of the commonest cancers in the world, cervical cancer (CC) causes high death rate worldwide [1]. Despite of the development and effectiveness of early screening tests for CC, there are still more and more women died of CC [2,3]. Although the molecular mechanisms and treatment for CC have been widely investigated, the overall 5-year survival rate of CC patients is still unsatisfied [4]. Therefore, it is urgent to find novel therapeutic pathways for cervical cancer.

With the development of human genome project, more and more mammalian genomes have been recognized to produce non-coding RNAs (ncRNAs) [7]. Among ncRNAs, long non-coding RNAs (lncRNAs) have been widely studied in recent years [8]. LncRNAs can regulate various biological behaviors in human cancers [9–12]. LncRNAs can modulate gene expression through exerting functions of ceRNAs [13–15]. LncRNA XIST is a main regulator of X inactivation in mammals [16]. It has been reported to be dysregulated in various cancers [17–19]. In addition, XIST also can act as a ceRNA to regulate tumorigenesis and progression in human cancers [20]. For example, XIST can promote EMT progression in lung cancer by mediating miR-367/141-ZEB2 axis [21]. In this study, the authors demonstrated that lncRNA XIST facilitated lung cancer progression through upregulating ZEB2 by competetively binding with miR-367/141. Similarly, in this study, XIST was firstly found to be highly expressed in CC tissues and cell lines. The high expression of XIST predicted unfavorable prognosis of CC patients. Loss-of function assays were performed to demonstrate the oncogenic role of XIST in cervical cancer progression.

As we all know, miRNAs can be regulated by lncRNAs in human cancers [22]. MiR-200a has been reported in various malignant tumors for its anti-oncogenic function [23–26]. However, the specific biological role of miR-200a is still not clarified in human cervical cancer. MiR-200a was demonstrated to be the target of XIST through bioinformatics analysis and luciferase reporter analysis.

Fus is a member of the TET protein family [36,37]. It was initially defined as a fusion protein (FUS-CHOP) for the reason of chromosomal translocations in human liposarcoma [38–40]. Fus has been reported to be targeted by miR-141 in human neuroblastoma [41]. Additionally, Fus can participate in a ceRNA pathway [42]. In this study, Fus was found to be upregulated by XIST. According to the results of Mechanism experiments, XIST could upregulate Fus through competitively binding with miR-200a in cervical cancer. All these findings might help to provide novel therapeutic target for cervical cancer.

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https://doi.org/10.1016/j.biopha.2018.05.053
Received 21 March 2018; Received in revised form 4 May 2018; Accepted 9 May 2018

2. Materials and methods

2.1. Tissue samples

All CC tissues and adjacent normal tissues used in this study were obtained from Department of Obstetrics and Gynecology, Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University. This study was approved by the ethics committee of Shanghai Jiao Tong University. The written informed consents had been acquired from all patients before this study. All participators didn't have any therapy before the operation.

2.2. Cell culture

Normal cell line GH329 and four human CC cell lines (Hela, Caski, C4-1 and Siha) were purchased from BeNa culture collection, (Beijing, China). All cell lines were incubated in Dulbecco's modified Eagle's medium (DMEM) medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) which were added with 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA) and 1% penicillin-streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin) in a moist atmosphere with 5% of CO_2 at 37 °C.

2.3. Quantitative real-time PCR (qRT-PCR)

Under the commendations of instructions, RNAs were isolated from frozen cells using TRIzol® reagent (Invitrogen, USA). High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used for lncRNA XIST and Fus reverse transcription. According to previous description [45], qRT-PCR reactions were performed using SYBR Premix DimerEraser (Takara, Dalian, China), and human GAPDH was used as an endogenous control for mRNA detection. Primers used in this study were illustrated in Supplementary Table 1. The relative expression levels were determined using the $2^{-\Delta\Delta Ct}$ method.

2.4. Plasmid construction and transfection

To knockdown XIST, shRNA against XIST (sh-XIST) and control shRNA were stably transfected into cervical cancer cells. To upregulate Fus, the full-length sequence of Fus was cloned into the pcDNA 3.0 vector (Realgene, Shanghai, China). The sequence of Fus was connected to the pcDNA 3.0 vector for about 2 h at room temperature. The constructed plasmid and the negative control pcDNA 3.0 empty vector (pcDNA-NC) was transfected into CC cells with Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). To overexpress or silence miR-200a, miR-200a mimics/inhibitors and corresponding negative control (NC) were obtained from GenePharma Co. Ltd (Shanghai, China).

Table 1
Correlation between LncRNA XIST expression and clinical features (n = 52).

Variable	LncRNA XIST Low High	P value
Age(years)	29 12	0.074
< 40	4 7	
≥40		
Tumor Size(cm)	32 10	< 0.001***
≥4	1 9	
< 4		
FIGO Stage	23 7	0.04*
I-II	10 12	
III-IV		
Lymphatic Metastasis	21 8	0.157
Yes	12 11	
No		
Distant Metastasis	6 9	0.027*
Yes	27 10	
No		

Low/high by the sample mean. Pearson χ^2 test. *P < 0.05 was considered statistically significant.

2.5. MTT assay

Cells were planted in 96-well plates (Sigma-Aldrich; Merck KGaA) at a density of 1×10^4 cells/well in 100 μl DMEM medium, with 5 replicates for each group. Then they were incubated with 5 mg/ml MTT solution (10 μl) at 37 °C in a 5% CO_2 atmosphere for 4 h. Following incubation, the medium was removed, 150 μl DMSO (Sigma-Aldrich; Merck KGaA). The absorbance at 490 nm was detected using a spectrophotometer (Thermo Fisher Scientific, Inc.), and cell viability was measured once per day for 7 days.

2.6. Colony formation assay

After required transfection, CC cells were lysed and seeded onto a 6-well plates and cultured in a normal temperature at 37 °C. After two weeks, the cells were fixed with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet for 30 min. Finally, the visible colonies were precisely calculated under an Olympus CK2phase-contrast inverted microscope (Olympus, Tokyo, Japan).

2.7. Transwell assay

For invasion assay, CC cell lines were seeded into the upper chamber of Transwell Permeable Support (24-well, Costar, USA) which was coated with or without Matrigel (BD Biosciences, USA). The lower chamber was filled with 500 medium containing 10% FBS. Cells were cultured in an atmosphere with 5% CO_2 at 37 °C. After the supernatants were removed, cells stayed in the upper chamber were scrapped with a cotton swab. Subsequently, cells invaded into the membrane were fixed with methanol and stained with hematoxylin. Finally, number of invaded cells were calculated under an inverted microscope.

2.8. Flow cytometry analysis

According to the reccomandations of manufacturer, cervical cancer cells were treated with fluorescein isothiocyanate (FITC), Annexin V and propidium iodide (PI) in a dark room at normal temperature. Apoptosis condition was assessed with FACScan®.

2.9. Western blot

According to the guidance of manufacturer, RIPA kit (Beyotime, Shanghai, China) was used to extract protein lysates from tissues or cells. As for protein concentrations were tested by utilizing an enhanced BCA Protein Assay kit (Beyotime). Loading buffer was employed to mix the samples containing equal amounts of protein. And then, the samples were separated by SDS-PAGE (Thermo Scientific, Shanghai, China) and were transferred to the PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with a buffer containing 5% defatted milk and were treated with primary antibodies (anti-cleaved caspase3, anti-caspase3, anti-cleaved caspase9, anti caspase9, anti-β–actin, anti-E-cadherin, anti-*N*-cadherin and anti-Fus) at 4 °C overnight and with peroxidase-conjugated secondary antibodies. All primary antibodies were treated at a dilution of 1:1000 and were acquired from Epitomics (Burlingame, CA, USA). The secondary antibodies were bought from Cell Signaling Technology (Danvers, MA, USA). The blot images were visualized by using chemiluminescence (Beyotime).

2.10. Subcellular fractionation

Cytoplasmic and Nuclear RNA Purification Kit (Norgen, Thorold, ON, Canada) were separately used to extract the cytoplasm and nuclear fractions. Next, qRT-PCR assays were applied to analyze the expression