Int J Clin Exp Pathol 2017;10(2):2333-2339
www.ijcep.com /ISSN:1936-2625/IJCEP0043174

Original Article

Long non-coding RNA XIST promotes cervical cancer cell epithelial-mesenchymal transition through the Wnt/β-catenin pathway

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Received October 30, 2016; Accepted January 6, 2017; Epub February 1, 2017; Published February 15, 2017

Abstract: Background: Increasing evidences indicated that long non-coding RNAs (lncRNAs) play critical roles in tumor development and progression. However, the role of lncRNA XIST in cervical cancer remains unclear. Methods: QRT-PCR was used to explore the relative expression of XIST in cervical cancer. The biological functions of XIST on cervical cancer cell growth and mobility were determined by CCK-8 assay, transwell migration assay and transwell invasion assay. In addition, the potential mechanism of XIST in cervical cancer progression was investigated by western blot. Results: Our data showed that lncRNA XIST expression was upregulated in cervical cancer tissues and cell lines compared to normal cervical epithelium tissues and normal human cervical epithelial cell line H8. High XIST expression was significantly correlated with cervical cancer lymph node metastasis. Reduced expression of XIST suppressed cervical cancer cell proliferation, migration, invasion and epithelial-mesenchymal transition (EMT) by modulating Wnt/β-catenin signaling pathway. Conclusion: Our results revealed that lncRNA XIST could act as a novel molecule involved in cervical cancer progression, which provided a potential therapeutic target for new therapies in patients with cervical cancer.

Keywords: Long non-coding RNAs, XIST, cervical cancer, EMT, Wnt/β-catenin

Introduction

Cervical cancer is one of the most common female malignancies worldwide, with an estimated 500,000 new cases and 300,000 deaths per year [1]. Although its mortality decreased along with advances in surgery, radiotherapy, and chemotherapy, patients with advanced cervical cancer still have a poor prognosis due to tumor recurrence and metastasis [2, 3]. Therefore, it is necessary for understanding of molecular mechanisms and identifying new therapeutic strategies for the treatment of cervical cancer.

Long non-coding RNAs (lncRNA) are transcripts longer than 200 nucleotides with little or no protein-coding capacity [4]. Recent studies revealed that lncRNAs play significant roles in a large range of biological processes, including cell differentiation, proliferation, apoptosis and migration [5, 6]. Few lncRNAs have been found to be associated with human cancer progression. For example, Zhang et al showed that upregulation of lncRNA MALAT1 correlated with tumor progression and poor prognosis in clear cell renal cell carcinoma [7]. Sun et al suggested that decreased expression of lncRNA GAS5 indicated a poor prognosis and promoted cell proliferation in gastric cancer [8]. Li et al showed that overexpression of lncRNA HOTAIR leaded to chemoresistance by activating the Wnt/β-catenin pathway in human ovarian cancer [9]. Wang et al revealed that upregulated lncRNA UCA1 contributed to the progression of hepatocellular carcinoma through inhibition of miR-216b and activation of FGFR1/ERK signaling pathway [10]. These studies suggested a complex role for lncRNA in tumor progression. However, the functions of lncRNA XIST in cervical cancer are still unclear.

In the present study, we objected to explore the expression of XIST in cervical cancer and eluci-

date the underlying molecular mechanism. We found that XIST was upregulated in cervical cancer tissues and cell lines, reduced XIST expression suppressed cervical cancer cell proliferation, migration, invasion and epithelial-mesenchymal transition (EMT) by modulating Wnt/β-catenin signaling pathway. Therefore, our findings described a novel role of XIST as a tumor oncogene in cervical cancer progression.

Materials and methods

Patients and specimens

A cohort of 47 cervical cancer tissues were collected postoperatively from patients who underwent surgery at the Department of Obstetrics and Gynecology, Huaihe Hospital of Henan University. Normal cervical epithelium samples were collected from 28 patients who had hysterectomy for benign disease. Consent from all patients was obtained. All patients recruited to this study did not receive any preoperative treatments. The study methodologies met the standard set by the Declaration of Helsinki and were approved by the Human Ethics Committee of Huaihe Hospital of Henan University. Each sample was snap-frozen in liquid nitrogen and stored at -80°C before RNA isolation.

Cell culture and transfection

The normal human cervical epithelial cell line H8 and cervical cancer cell lines HeLa, HT-3, SiHa and Ca-Ski were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 Medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies) in a humidified atmosphere at 37°C with 5% CO_2.

The small interfering RNA (siRNA) sequences targeting XIST, which were designed as follows: si-XIST-1, 5'-GCTTATTATTGCTAATTCAATC-3'; si-XIST-2, 5'-CACATGCACTTATTATTTCATA-3', and a scrambled negative control siRNA (si-NC) were purchased from Invitrogen. Cells were cultured and transfected with si-XIST-1, sI-XIST-2 or si-NC by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection at a different time point, cells were harvested for following analysis.

Cell proliferation assay

Cell proliferation was measured using a Cell Counting Kit-8 (CCK-8, Dojindo). Cells were seeded into 96-well plates at 2000 cells per well. After incubation for a series of time periods, 10 μl CCK-8 was added to each well and incubated for 1 h at 37°C. Cell viability was determined by measuring the absorbance at 450 nm. All experiments were performed three times and were calculated using average results.

Transwell migration and invasion assays

Briefly, 24-well transwell chambers (Costar) with uncoated or Matrigel-coated membranes were used for migration and invasion assays in this research, respectively. The post-transfected cervical cancer cells were seeded on the upper chamber which contained the serum free RPMI-1640 medium, while the RPMI-1640 medium combined with 10% fetal bovine serum which acted as chemoattractant was supplemented to the lower chamber. After incubation for 24 h (migration assay) or 48 h (invasion assay), cells that did not migrate across the upper surface were removed using a cotton swab, while cells that adhered to the lower surface of the inserts were stained with crystal violet of 0.1% for 20 min. Finally, the complete filters were washed in water twice before being observed.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using TRIZOL (TaKaRa), according to the manufacturers' protocol. RNA was reversed transcribed into cDNAs using the Primer-Script one step RT-PCR kit (TaKaRa). The cDNA template was amplified by real-time RT-PCR using the SYBR Premix Dimmer Eraser kit (TaKaRa). Gene expression in each sample was normalized to U6 expression. The primer sequences used were as follows: XIST: forward, 5'-CGGGTCTCTTCAAGGACATTTAGCC-3' and reverse, 5'-GCACCAATACAGAGGAATGGAGGG-3'. U6: forward, 5'-TCGCTTCGGCAGCACATA-3', reverse, 5'-TTTGCGTGTCATCCTTGC-3'. QRT-PCR reactions were performed by the ABI7500 system (Applied Biosystems). The relative expression fold change of mRNAs was calculated by the $2^{-\Delta\Delta Ct}$ method.

Western blot

Cells were lysed with RIPA lysis buffer. Proteins were separated in SDS-PAGE gels and trans-

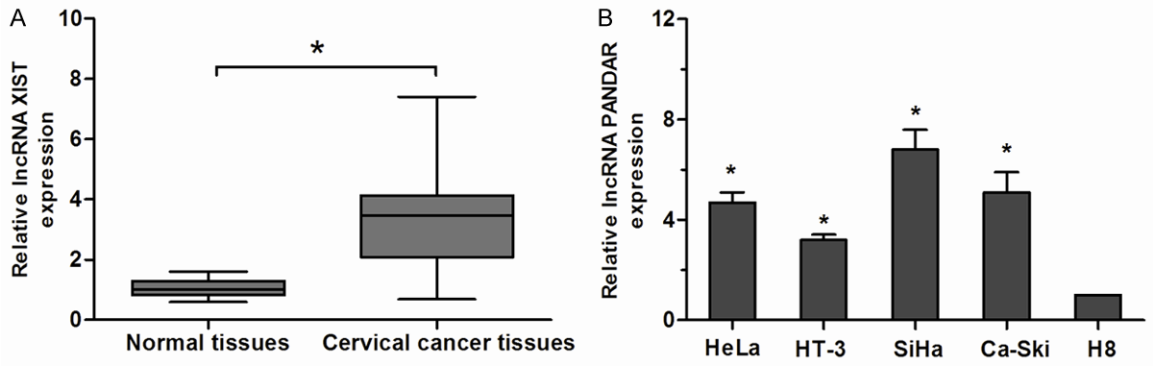

Figure 1. The expression of lncRNA XIST was significantly increased in cervical cancer tissues and cell lines. A. The expression of XIST in cervical cancer tissues and normal cervical epithelium tissues were measured by qRT-PCR. B. The expression of XIST in cervical cancer cell lines (HeLa, HT-3, SiHa and Ca-Ski) and normal human cervical epithelial cell line H8 were measured by qRT-PCR. *P < 0.05.

Table 1. Correlation between clinicopathologic features and lncRNA XIST expression in cervical patients

Characteristics	Group	Total	XIST expression		*P value*	*Chi square value*
			High	Low		
Age (years)	< 50	16	9	7	0.609	0.261
	≥ 50	31	15	16		
Tumor size (cm)	< 4	20	10	14	0.188	1.733
	≥ 4	23	14	9		
Histology	SCC	33	16	17	0.587	0.295
	AD/ASC	14	8	6		
FIGO stage	Ib-IIa	22	9	13	0.191	1.707
	IIb~IIIa	25	15	10		
Differentiation	Well+Moderate	30	13	17	0.159	1.984
	Poor	17	11	6		
Lymph nodes metastasis	Negative	35	14	21	0.010	6.715
	Positive	12	10	2		

SCC: squamous cell carcinoma; AD: adenocarcinoma; ASC: adenosquamous cell carcinoma.

ferred to PVDF membrane (Bio-Rad). The membrane was blocked with 10% skim milk solution and incubated overnight at 4°C with primary antibody (Abcam). After washing, the appropriate HRP-conjugate secondary antibody was added for 1 h incubation at room temperature. The immunoreactive proteins were visualized using an ECL system (Amersham).

Statistical analysis

All statistical analyses were performed with Statistical Product and Service Solutions (SPSS) 20.0 software, statistical analysis was performed by ANOVA, chi-squared test, or Student's t-test. Data are presented as mean ± standard deviation. P < 0.05 was regarded as statistically significant.

Results

LncRNA XIST was upregulated in cervical cancer tissues and cell lines

To explore the function of lncRNA XIST on the progression of cervical cancer, we first determine XIST expression in cervical cancer tissues and cell lines. QRT-PCR results showed that XIST expression was significantly increased in cervical cancer tissues compared to normal cervical epithelium tissues (**Figure 1A**; P < 0.05). Furthermore, we found that XIST expression was upregulated in four cervical cancer cell lines (HeLa, HT-3, SiHa and Ca-Ski) compared to normal human cervical epithelial cell line H8 (**Figure 1B**; P < 0.05). These findings

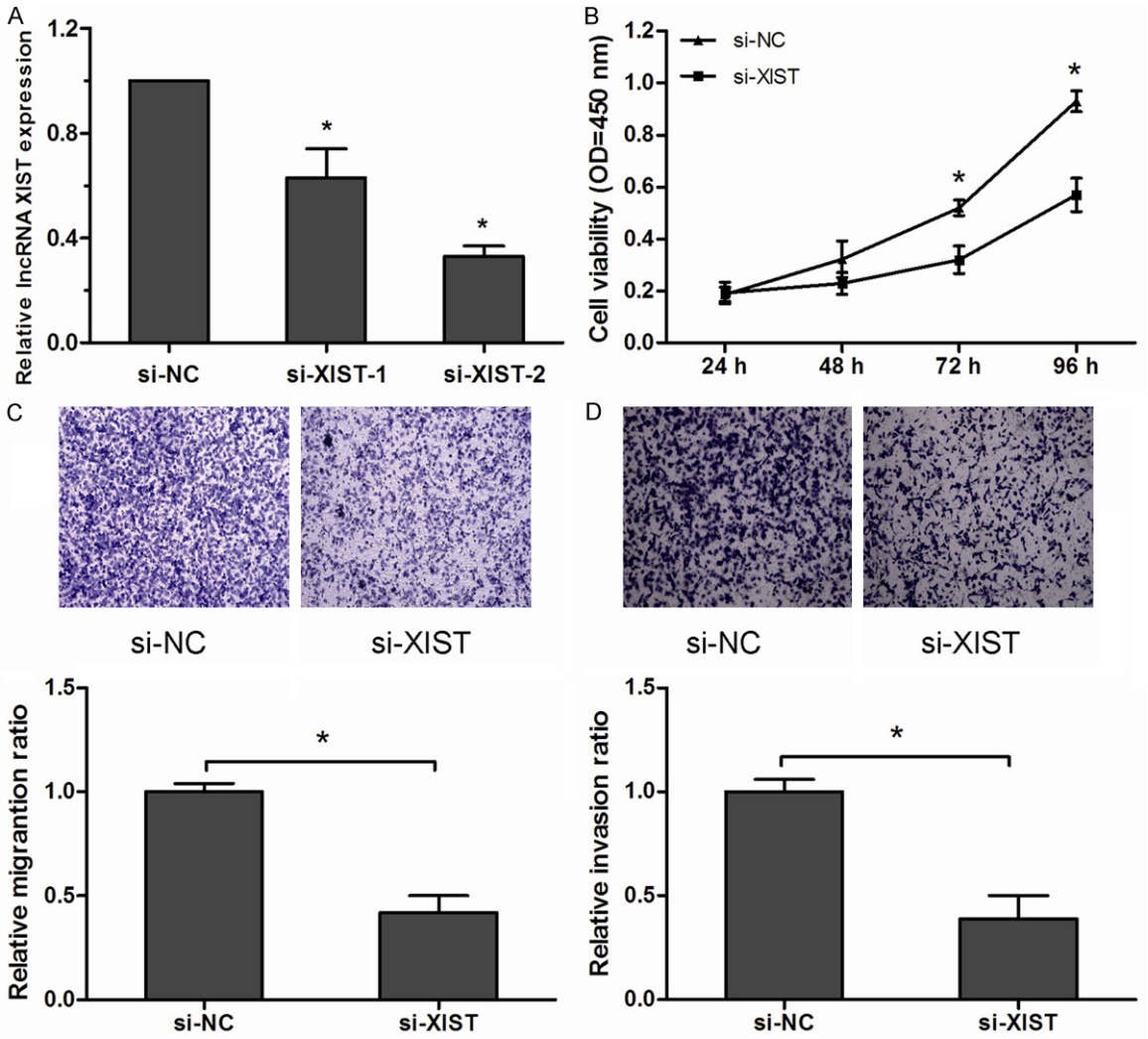

Figure 2. LncRNA XIST inhibition suppressed cervical cancer cell proliferation, migration and invasion in vitro. A. The expression level of XIST was determined by qRT-PCR after SiHa cells were transfected with si-XIST or si-NC. B. The effect of XIST on the proliferation of SiHa cells was measured by CCK-8 assay. C. The effect of XIST on the migration of SiHa cells was measured by transwell migration assay. D. The effect of XIST on the invasion of SiHa cells was measured by transwell invasion assay. *P < 0.05.

indicated that XIST might be involved in the progression of cervical cancer.

Relationship between lncRNA XIST expression and clinicopathological features of cervical cancer patients

To explore the correlation between XIST expression and clinicopathological features. 47 cervical cancer patients were classified into two groups depending on median XIST expression: high XIST expression group (XIST expression ratio ≥ median ratio) and low XIST expression group (XIST expression ratio < median ratio). We found that high XIST expression was significantly correlated with cervical cancer lymph node metastasis (**Table 1**, P < 0.05). However, there were no correlation was found between XIST upregulation and age, tumor size, histology, FIGO stage or differentiation (**Table 1**, P > 0.05). These findings imply that XIST plays important roles in cervical carcinogenesis.

LncRNA XIST inhibition suppressed cervical cancer cell proliferation, migration and invasion in vitro

To explore the possible biological significance of XIST in cervical cancer progression, si-XIST or si-NC was transfected into SiHa cells. As shown in **Figure 2A**, SiHa cells transfected with si-XIST presented a significantly decreased

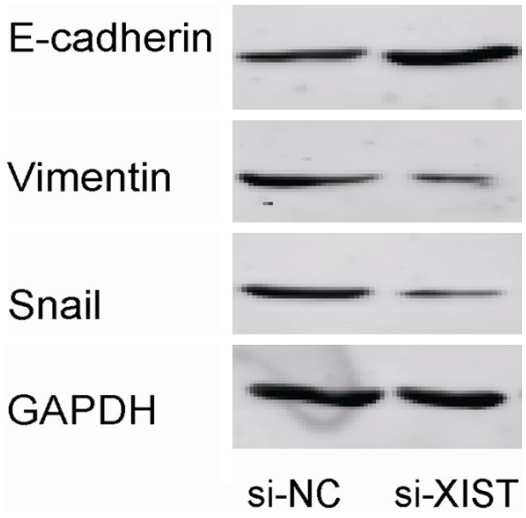

Figure 3. LncRNA XIST inhibition suppressed EMT process in SiHa cells.

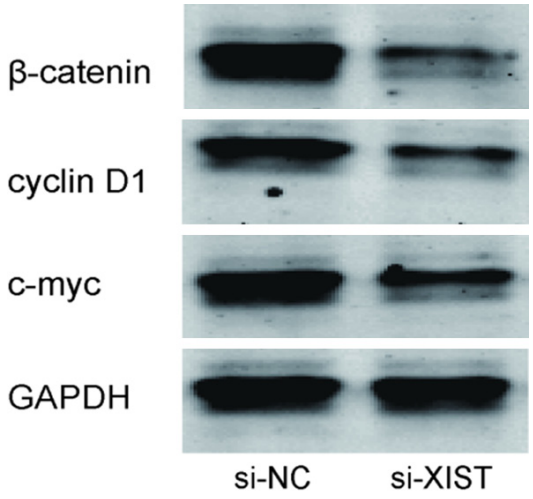

Figure 4. LncRNA XIST inhibition suppressed Wnt/β-catenin signaling pathway in SiHa cells.

mRNA expression level of XIST compared with the si-NC group ($P < 0.05$). To determine the function of XIST on cervical cancer cell proliferation, CCK-8 assay revealed that XIS T inhibition obviously suppressed the proliferation rate of SiHa cells compared to si-NC group (**Figure 2B**; $P < 0.05$). In addition, transwell assays were used to further investigated the effect of XIST on cell migration and invasion. We found that knockdown of XIST strikingly inhibited SiHa cell migration and invasion ability compared to the si-NC group (**Figure 2C**, **2D**; $P < 0.05$). These data demonstrated that reduced XIST expression could inhibit cervical cancer cell proliferation, migration and invasion in vitro.

LncRNA XIST inhibition suppressed EMT process

EMT is a main mechanism involved in cell migration and invasion of cancer cells. Therefore, we further explored the effects of XIST on EMT of SiHa cells. Western blot showed that the expression of Vimentin and Snail was significantly downregulated, while E-cadherin expression was markedly upregulated when XIST was inhibition in SiHa cells (**Figure 3**). These results suggested that XIST was involved in EMT process in cervical cancer cells.

LncRNA XIST inhibition suppressed Wnt/β-catenin signaling pathway

The Wnt/β-catenin signaling pathway is a prototypic survival pathway that plays a crucial role in various cellular functions, such as proliferation, growth, survival and metabolism [11]. Therefore, we investigate the regulative effect of XIST on Wnt/β-catenin signaling pathway. Western blot revealed that XIST inhibition reduced the protein expression of β-catenin and suppressed the protein expression of cyclin D1 and c-myc (classic downstream genes of the Wnt/β-catenin signaling pathway). These data suggested that Wnt/β-catenin signaling pathway was in-activated by knockdown XIST expression in cervical cancer (**Figure 4**).

Discussion

LncRNAs play important roles in the occurrence and development of cervical cancer. For example, Lempridee et al showed that lncRNA H19 enhanced cell proliferation and anchorage-independent growth of cervical cancer cell lines [12]. Liao et al found that lncRNA XLOC_010588 could indicate a poor prognosis and promoted proliferation through upregulation of c-Myc in cervical cancer [13]. Zhang et al reported that lncRNA MEG3 was downregulated in cervical cancer and affected cell proliferation and apoptosis by regulating miR-21 [14]. However, the function of lncRNA XIST in cervical cancer is still unclear.

The lncRNA XIST (X-inactive specific transcript) is a product of the XIST gene and the master regulator of X inactivation in mammals [15]. More and more studies indicated that XIST plays critical role in cell proliferation, differentiation, and genome maintenance. For example, Yao et al found that knockdown of lncRNA XIST

exerted tumor-suppressive functions in human glioblastoma stem cells by up-regulating miR-152 [16]. Fang et al suggested that lncRNA XIST acted as an oncogene in non-small cell lung cancer by epigenetically repressing KLF2 expression [17]. Zhou et al indicted that lncRNA XIST regulated gastric cancer progression by acting as a molecular sponge of miR-101 to modulate EZH2 expression [18]. However, up to date, there is no related study elaborating the relevance between XIST expression and cervical cancer progression. Hence, the role of XIST on cervical cancer and its potential biological mechanisms still remain to be explored.

Epithelial-mesenchymal transition (EMT) is a common morphologic transformation in cancer cells that causes loss of cell-cell adhesion and increases cell motility which plays an important role in tumor progression and metastasis [19]. EMT is induced through activation of Wnt signaling [20]. β-catenin is the key initial protein in the Wnt/β-catenin signaling. After activation, β-catenin can translocate from the cytoplasm to the nucleus, then regulate expression of several transcription factors, and subsequently induce EMT [21]. Recent studies showed that Wnt/β-catenin signaling play important roles in EMT. For example, Liang et al suggested that lncRNA MALAT1 induced tongue cancer cells' EMT and inhibits apoptosis through Wnt/β-catenin signaling pathway [22]. Yuan et al found that overexpression of lncRNA CTD903 inhibited colorectal cancer invasion and migration by repressing Wnt/β-catenin signaling and predicts favorable prognosis [23].

In the present study, our data showed that lncRNA XIST expression was significantly increased in cervical cancer tissues and cell lines. High XIST expression was associated with cervical cancer lymph node metastasis. Knockdown of XIST suppressed cervical cancer cell proliferation, migration and invasion in vitro. In addition, we found that XIST inhibition decreased protein expression of Vimentin, Snail and upregulated E-cadherin expression. Furthermore, western blot showed that decreased expression of XIST significantly inhibited β-catenin, cyclin D1 and c-myc expression. Thus, these results suggested that XIST could increase cell proliferation, migration and invasion by EMT through Wnt/β-catenin signaling pathway.

In conclusion, we found that lncRNA XIST acted as a tumor oncogene in cervical cancer progression, and decreased XIST expression in cervical cancer could suppress cell proliferation, invasion and migration by EMT through Wnt/β-catenin signaling pathway. Thus, our findings suggested that XIST could be as a novel therapeutic strategy for the treatment of cervical cancer.

Acknowledgements

This study was supported by grants from the Key Research Projects of Institutions of Higher Education in Henan Province (No. 17A320023).

Disclosure of conflict of interest

None.

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