Int J Clin Exp Med 2018;11(8):8317-8323
www.ijcem.com /ISSN:1940-5901/IJCEM0070409

Original Article

lncRNA XIST promotes aggressive tumor phenotype and is associated with poor prognosis in esophageal squamous cell carcinoma

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Received December 5, 2017; Accepted May 16, 2018; Epub August 15, 2018; Published August 30, 2018

Abstract: Background: Growing evidence indicates that long non-coding RNAs (lncRNAs) play important role in the progression of esophageal squamous cell carcinoma (ESCC). However, little is known about the role of the lncRNA XIST in ESCC. Methods: Expression of lncRNA XIST was analyzed in 186 pairs of ESCC tissues and adjacent normal tissues by real-time PCR (qRT-PCR). Correlations between lncRNA XIST expression and clinicopathological factors were evaluated. Cell proliferation and invasion ability was analyzed by CCK-8 and transwell assays. Results: lncRNA XIST was significantly overexpressed in ESCC tissues compared with adjacent normal tissues. Up-regulation of lncRNA XIST was significantly correlated with tumor size, differentiation, and distant metastasis. Higher lncRNA XIST expression level was significantly associated with worse overall survival in ESCC patients. Multivariate analysis indicated that XIST expression served as an independent predictor for overall survival. Further experiments revealed that knockdown of XIST inhibited cell proliferation and invasion in ESCC cells. Conclusions: Taken together, these results suggest that lncRNA XIST may serve as a candidate molecular biomarker for predicting the outcome of ESCC patients.

Keywords: Long non-coding RNA, XIST, ESCC, progression, prognosis

Introduction

Esophageal cancer is one of the most common human malignant diseases, ranking eighth in incidence and sixth in cancer death worldwide [1, 2]. Esophageal squamous cell carcinoma (ESCC) is the major histopathological subtype of esophageal cancer, which accounts for about 90% of all esophageal cancers [2]. Although great advances have been made in medical and surgical treatments, the prognosis of ESCC patents is still poor, and most ESCC patients are diagnosed at an advanced stage, with an overall 5-year survival ranging from 15% to 25% [3, 4]. Early detection of the primary tumors provides timely interventions and medical treatments, which can significantly improve the outcomes [4]. However, clinically used biomarkers for ESCC, such as squamous cell carcinoma antigen (SCC), carcinoembryonic antigen (CEA), and p53 are inadequate for identifying subclinical patients with early tumors and predicting disease recurrence [5].

With the recent development of genome sequencing, the human genome has been shown to be comprised of less than 2% protein coding genes while more than 90% of the genome is transcribed into non-coding RNAs (ncRNA), such as microRNAs, small interfering RNAs, and long non-coding RNAs (lncRNAs) [6]. Mounting evidence indicats that ncRNAs play important role in the development of many diseases especially in tumors [7]. lncRNAs are defined as RNA molecules that longer than 200 nucleotides in length and with no or little protein coding capacity [8]. lncRNAs are involved in the regulation of gene expression at different levels, such as the epigenetic, transcriptional, and posttranscriptional levels [9]. lncRNAs have been found to be novel tumor biomarkers for early cancer diagnosis and prognosis [10]. For instance, HOTAIR was up-regulated

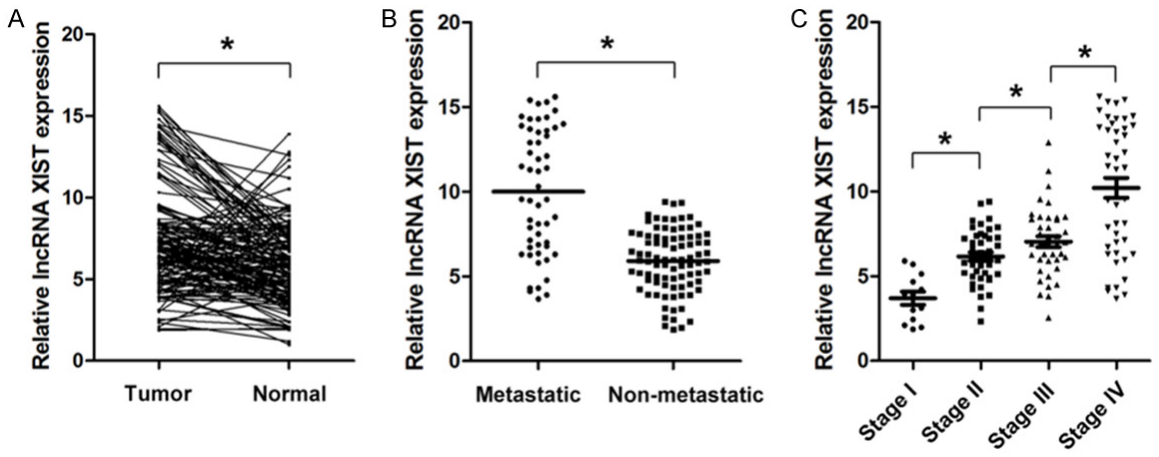

Figure 1. lncRNA XIST is significantly up-regulated in ESCC tissues. A. Real-time PCR analysis showed increased expression of lncRNA XIST in tumor tissues than normal tissues ($P < 0.001$); B. Real-time PCR analysis showed higher expression of lncRNA XIST in tissues with distant metastasis than that without distant metastasis ($P < 0.001$). C. Real-time PCR analysis showed lncRNA XIST expression significantly increased in advanced stage than in low stage ($P < 0.001$).

in colorectal cancer, and increased HOTAIR expression was significantly associated with colorectal cancer liver metastasis [11], while increased expression of MALAT1 predicted higher risk of tumor recurrence in hepatocellular carcinoma [12]. lncRNA XIST (X-inactive specific transcript) is the product of the XIST gene and the key regulator of X inactivation in mammals [13]. More and more studies have shown that lncRNA XIST is frequently deregulated in various tumors and involved in the pathology of tumors [14, 15]. However, little is known about the role of lncRNA XIST in ESCC which needs exploring.

Materials and methods

Patients and tissue samples

This study was approved by the Medical Ethics Committee of the Institutional Review Board of Wuhan University of Science and Technology. Written informed consent was obtained from all participants, and tissue specimens were obtained and handled according to ethical and legal standards. A total of 186 paired human ESCC and adjacent normal tissues were obtained from patients who underwent surgery at Hubei Cancer Hospital between July, 2012 and May, 2015. All the patients did not receive any chemotherapy or radiotherapy before surgery and were followed up regularly. The clinicopathological data (including gender, age, tumor size, tumor depth, differentiation, T stage, lymph node invasion, and Peritoneal dissemination) were recorded. Overall survival time was calculated from the date of the surgery to the date of death or last contact.

Real-time PCR analysis

Total RNA was isolated from tumor tissues r cells by using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using the Primer-Script one step RT-PCR kit (Promega, Madison, WI, USA). The PCR was performed for 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s with 1.0 μl of cDNA using the SYBR Premix Dimmer Eraser kit (Takala, Dalian, China) on a ABI7900 system (Applied Biosystems, Foster City, USA). GAPDH was used as a reference, and lncRNA XIST level were normalized to GAPDH. The primers (Invitrogen) were designed as follows: for human lncRNA XIST, the forward primer was 5'-CTCTCCATTGGGTTCAC-3' and the reverse primer was 5'-GCGGCAGGTCTTAAGAGATGAG-3'; for human GAPDH, the forward primer was 5'-CCCACTCCTCCACCTTTGAC-3' and the reverse primer was 5'-ATGAGGTCCACCACCCTGTT-3'. Relative quantification of RNA expression was calculated by using the $2^{-\Delta\Delta CT}$ method. Each sample was tested in triplicate.

Cell culture

ESCC cells lines (KYSE450, KYSE150, EC109 and EC9706) were obtained from the Institute

Table 1. Correlation between clinicopathological parameters and lncRNA XIST expression in 140 ESCC patients

Characteristics	n	High expression	Low expression	*P* value
Age				0.866
< 60	67	33	34	
≥ 60	73	37	36	
Gender				0.608
Male	81	42	39	
Female	59	28	31	
Tumor size				0.042
< 4 cm	74	31	43	
≥ 4 cm	66	39	27	
Differentiation				< 0.001
Well	28	9	19	
Moderate	63	24	39	
Poor	49	37	12	
Lymph node invasion				0.376
Absent	49	22	27	
Present	91	48	43	
Distant metastasis				< 0.001
Absent	87	33	54	
Present	53	37	16	
TNM stage				0.145
I-II	44	18	26	
III-IV	96	52	44	

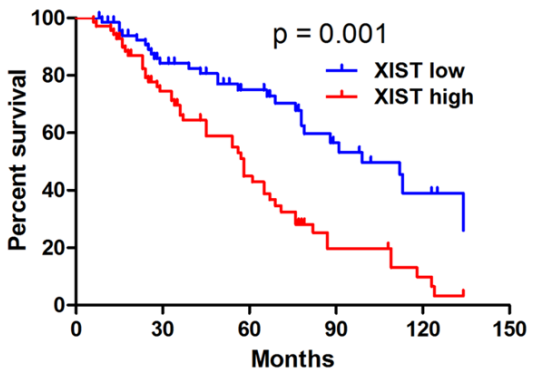

Figure 2. lncRNA XIST expression is associated with overall survival of ESCC patients, high lncRNA XIST expression is associated with worse overall survival (*P* = 0.001).

of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Human normal esophagus epithelial cell line Het-1A was purchased from American Type Culture Collection (ATCC). All cells were cultured using RPMI 1640 (Hyclone, USA) supplied with 10% fetal bovine serum (Gibco) at 37°C with 5% CO_2.

Cell transfections

ESCC cells were transfected with siRNA by using Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's protocol. siRNA targeting lncRNA XIST was provided by GenePharma (Shanghai, China). The level of lncRNA XIST expression after transfection was assayed by real-time PCR 48 hours after transfection, and then the cells were used for assays.

Cell proliferation assays

Cell proliferation was tested with MTT kit (Sigma) according to the manufacturer's instruction. For colony formation assay, ESCC cells were trypsinized to single-cell suspensions of 3 × 10^3 cells and then were plated in six-well plates and cultured with RPMI 1640 supplied with 10% FBS. The plates were incubated at 37°C in the presence of 5% CO_2 for 14 days. Colonies containing at least 50 cells were scored. The data are presented as the mean ± standard deviation of five randomly scored fields.

Invasion assays

Cell invasion was assessed by transwell assays (Corning, NY, USA) according to the manufacturer's instructions. The insert membranes were coated with diluted Matrigel (San Jose, CA, USA). Cells (1 × 10^5) were added to the upper chamber and were cultured for 24 h. The insert membranes were then cut and stained with crystal violet (0.04% in water; 100 ml), and the migrated cells were counted under an inverted microscope and were photographed.

Statistical analysis

All statistical analyses were performed using SPSS version 16.0 or GraphPad 5.0 software. Data are expressed as mean ± SD. The significance of differences between groups was analyzed by Student's t-test, X^2 test or Wilcoxon test, as appropriate. The overall survival rate was determined by using the Kaplan-

Table 2. Univariate and Multivariate analysis of various potential prognostic factors in 140 ESCC patients

Characteristics	Univariate analysis		Multivariate analysis	
	HR[b] (95% CI[c])	*P*	HR[b] (95% CI[c])	*P*
Age	0.88 (0.69-1.29)	0.154	-	-
Gender	1.11 (0.79-1.71)	0.511	-	-
Differentiation	1.05 (0.78-1.36)	0.162	-	-
Tumor size	1.03 (0.81-1.22)	0.501	-	-
Lymph node invasion	1.12 (1.02-1.80)	0.112	-	-
Distant metastasis	1.48 (1.11-2.07)	0.007[a]	1.22 (1.04-1.99)	0.107
TNM stage	1.21 (1.02-2.07)	0.012[a]	1.17 (1.03-1.72)	0.136
lncRNA XIST level	1.87 (1.42-2.77)	0.003[a]	1.49 (1.11-2.46)	0.023[a]

[a]$P < 0.05$. [b]HR: hazard ratio. [c]CI: confidence interval.

Meier method with the log-rank test. Survival data were evaluated using univariate and multivariate Cox proportional hazards model. A *P* value of < 0.05 was considered statistically significant.

Results

lncRNA XIST is significantly up-regulated in ESCC tissues

In order to explore the role of lncRNA XIST in ESCC, we first detected the expression level of lnccRNA XIST in ESCC tissues and adjacent normal tissues. As shown in **Figure 1A**, lncRNA XSIT expression was significantly up-regulated in ESCC tissues as compared with that of adjacent normal tissues. Moreover, XIST expression was significantly increased in ESCC tissues with distant metastasis than that without distant metastasis (**Figure 1B**). In addition, lncRNA XIST expression was markedly increased in advanced stage tissues than that of low stage tissues (**Figure 1C**). These data suggest that lncRNA XIST is involved in the progression of ESCC progression.

lncRNA XIST expression is significantly associated with clinicopatholoical parameters in ESCC patients

The median value of lncRNA XIST level was used as a cutoff value to divide the 186 patients into high and low expression group. ESCC patients who express lncRNA XIST at levels higher than the cutoff value were assigned to the high expression group (n = 93, lncRNA XIST expression level ≥ cutoff point), and those with expression lower than the cutoff value were assigned to the low expression group (n = 93, lncRNA XIST expression level < cutoff point). We then analyzed the association between lncRNA XIST expression and clinical and pathological parameters in ESCC patients. As shown in **Table 1**, lncRNA XIST expression was significantly correlated with tumor size ($P = 0.042$), differentiation ($P < 0.001$), and distant metastasis ($P < 0.001$). However, no association was observed between lncRNA XIST expression and age (< 60 vs. ≥ 60, $P = 0.866$), gender (female vs. male, $P = 0.437$), lymph node invasion ($P = 0.437$) and TNM stage ($P = 0.145$). These results indicate that lncRNA XIST might serve as an important regulator of ESCC development.

Overexpression of lncRNA XIST expression predicts poor prognosis in ESCC patients

Kaplan-Meier analysis with the log-rank test was performed to determine the expression of lncRNA XIST on survival of ESCC patients. The results showed that ESCC patients with high expression of lncRNA XIST tended to have worse overall survival than those patients with low expression of ESCC patients (**Figure 2A**). Furthermore, to determine whether the expression of lncRNA XIST was an independent prognostic factor for gastric cancer patients, univariate and multivariate analyses were performed. Univariate analysis demonstrated that distant metastasis ($P = 0.007$), TNM stage ($P = 0.012$) and lncRNA XIST expression ($P = 0.003$) were significantly correlated with overall survival of ESCC patients (**Table 2**). However, multivariate analysis using the Cox proportional hazards model indicated that only lncRNA XIST expression ($P = 0.023$) was an independent prognostic factor of ESCC patients (**Table 2**). These data indicate that lncRNA XIST expression might be used as a prognostic indicator in ESCC patients.

Knockdown of lncRNA XIST inhibits cell proliferation and invasion of ESCC cells

As lncRNA XIST expression is significantly upregulated in ESCC tissues, we explored the biological effect of lncRNA XIST expression in

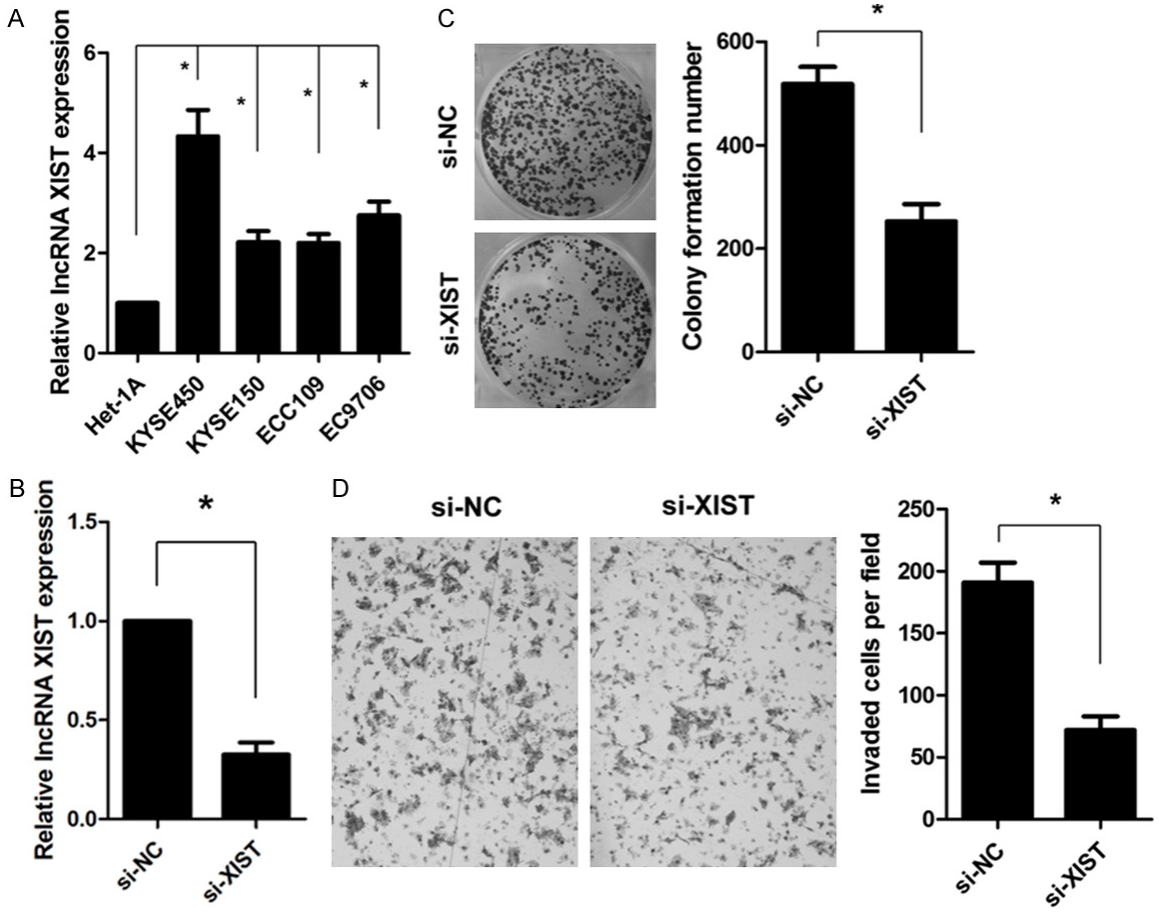

Figure 3. Knockdown of lncRNA XIST inhibits cell proliferation and invasion of ESCC cells. A. The relative expression of lncRNA XIST in ESCC cell lines ($P < 0.05$). B. Knockdown of lncRNA XIST significantly reduced the expression of lncRNA XIST in KYSE450 cells. C. Knockdown of lncRNA XIST significantly inhibited colony formation ability of KYSE450 cells ($P < 0.05$). D. Knockdown of lncRNA XIST significantly inhibited cell invasion ability of KYSE450 cells ($P < 0.05$).

ESCC cells. We first detected the expression level of lncRNA XIST in ESCC cell lines, as shown in **Figure 3A**, lncRNA XIST was significantly increased in ESCC cells than that of normal cell. Knockdown of lncRNA XIST significantly reduced its expression level in KYSE450 cells (**Figure 3B**). Knockdown of lncRNA XIST significantly inhibited cell proliferation and colony formation in KYSE450 cells (**Figure 3C**). Transwell assay showed that knockdown of lncRNA XIST significantly inhibited cell invasion ability in KYSE450 cells (**Figure 3D**). These results demonstrate that knockdown of lncRNA XIST significantly suppresses the proliferation and invasion ability in ESCC cells.

Discussion

Increasing evidence indicates that deregulation of lncRNAs is a frequent molecular event in human cancers including ESCC [16]. Recent studies have shown that the expression of lncRNA was significantly altered in ESCC tissues through screening lncRNA expression profile [17]. For instance, lncRNA 91H contributes to tumor progression and occurrence by inhibiting IGF2 expression in ESCC [18]. Long non-coding RNA TP73-AS1 expression was increased in tumor tissues and associated with tumor location and clinical stage of ESCC [19], In addition, Inhibition of lncRNA TP73-AS1 suppress cell proliferation and induce apoptosis by way of the caspase-3 dependent apoptotic signaling pathway [19]. However, there is still no report on the role of lncRNA XIST in ESCC.

In this study, we found that lncRNA XIST expression was significantly increased in ESCC tissues than that of adjacent normal tissues. High expression of lncRNA XIST was correlated with

tumor size, tumor depth, lymph node invasion and TNM stage in ESCC. Moreover, overexpression of lncRNA XIST was significantly associated with poor prognosis and was an independent prognostic factor in ESCC. lncRNA XIST is an important inhibitor of X chromosome inactivation (XCI) in female cells, which achieves dosage equilibration of X-linked genes with males [13]. Abnormal expression of lncRNA XIST might lead to tumorigenesis since XCI silences hundreds of genes, including oncogenes and tumor suppressor genes [20, 21]. In agreement with our results, lncRNA XIST has been found to act as an oncogene in several tumors. For example, lncRNA XIST promotes pancreatic cancer proliferation by regulating the miR-133a/EGFR signaling pathway [22]. In another study, it has been found that lncRNA XIST regulates PTEN expression through sponging miR-181a and stimulates hepatocellular carcinoma progression [23]. However, some other studies showed that deletion of lncRNA XIST is essential for some malignancies. For instance, Yildirim et al. reported that loss of lncRNA XIST could lead to a highly aggressive myeloproliferative neoplasm and myelodysplastic syndrome in female mice [24]. Huang et al. found that lncRNA XIST inhibits tumor development in breast cancer [25]. It seems that up-regulation of lncRNA XIST may lead to a variety of non-sex-related tumors in both humans and mice, whereas loss of lncRNA XIST might result in some female tumors. These studies indicated that lncRNA XIST may be oncogenic or tumor suppressive, depending on the cancer type and cellular context.

Considering the biological effect of lncRNA XIST in ESCC, we found that knockdown of lncRNA XIST inhibited the proliferation and invasion ability of ESCC cells. In accordance with our results, previous studies have demonstrated that lncRNA XIST is essential for the proliferation, invasion, tumoregenesis and metastasis in glioblastoma, gastric cancer, and colorectal cancer [15, 26, 27]. These data further confirmed that lncRNA XIST is involved in the development of ESCC progression and metastasis. In conclusion, our study demonstrates that overexpression of lncRNA XIST is associated with aggressive tumor phenotypes and poor overall survival in ESCC. Moreover, knockdown of lncRNA XIST inhibits ESCC proliferation and invasion capacities. Further study is needed to investigate the underlying molecular mechanism for the role of lncRNA XIST in ESCC.

Disclosure of conflict of interest

None.

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