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ORIGINAL ARTICLE

# Long noncoding RNA linc-UBC1 promotes tumor invasion and metastasis by regulating EZH2 and repressing E-cadherin in esophageal squamous cell carcinoma

Guangcai Niu[1], Huijie Zhuang[1], Bingqiang Li[2], Gang Cao[1]
[1]Department of Oncological Surgery and [2]Department of Gastrointestinal Surgery, Xuzhou Central Hospital, Xuzhou, China

## Summary

***Purpose:*** *Recent reports have shown that long noncoding (lnc) RNAs are critical during tumorigenesis. This study focused on the influence of linc-UBC1 on the metastasis of esophageal squamous cell carcinoma (ESCC) and the underlying mechanism.*

***Methods:*** *In 50 ESCC tissues and 5 ESCC cell lines, linc-UBC1 expression was detected by RT-qPCR. Moreover, correlation analysis was conducted between linc-UBC1 expression level and clinicopathological features. Overall survival of these ESCC patients was analyzed by Kaplan-Meier method. In addition, wound healing assay and cell invasion assay were utilized to identify whether linc-UBC1 could affect the migration and invasion ability of ESCC cells. Western blotting and luciferase assay were used to explore the potential mechanism.*

***Results:*** *In ESCC tissues, linc-UBC1 expression level was significantly higher and was remarkably related with clinical features such as TNM stage and nodal metastasis. Meanwhile, overall survival of ESCC patients with high expression of linc-UBC1 was significantly worse than that of patients with low expression. Besides, the migration and invasion ability of ESCC cells was inhibited via knockdown of linc-UBC1. Further study showed that knockdown of linc-UBC1could suppress the protein level of EZH2 and promote the protein level of E-cadherin.*

***Conclusions:*** *The results indicate that linc-UBC1 is a novel oncogene in tumorigenesis and could promote the metastasis via EZH2 and E-cadherin, which may offer a possible therapeutic target in ESCC.*

***Key words:*** *E-cadherin, esophageal squamous cell carcinoma, EZH2, linc-UBC1, long noncoding RNA*

## Introduction

ESCC, as an ordinary malignancy, ranks 9th among the most common carcinomas worldwide [1,2]. In China, esophageal cancer (EC) ranked 5th in incidence and 4th in mortality of all cancer types, among which ESCC was the main pathological subgroup of EC [3]. However, most ESCC patients develop advanced metastatic disease with overall 5-year survival rate less than 10% [4]. Despite the intensive research in the diagnosis and therapy over the past 30 years, the low survival rate is still one of the greatest challenge. Therefore, there is an urgent need to figure out the mechanisms involved in the metastasis of ESCC.

LncRNAs contain more than 200 bps nucleotides and function in many biological processes including tumorigenesis. For example, lncRNA XIST is upregulated in nasopharyngeal carcinoma and functions as an oncogene [5]. LncRNA SNHG12 inhibits the apoptosis of human colorectal cancer cells and enhances cell proliferation [6]. Moreover, lncRNA TUSC7 is identified as a tumor suppressor in colorectal cancer and suppresses cell growth via mir-211 [7]. Besides, the expression of lncRNA HOTAIR is significantly associated with ESCC prognosis and progression [8]. A recent study demonstrated that HOTAIR acts as an oncogene

*Correspondence to:* Gang Cao, MD. Department of Oncological Surgery, Xuzhou Central Hospital, 199 Jiefang South Rd, Xuzhou, Jiangsu 221006, China.
Tel/Fax: +86 018112007561, E-mail: cao1987cn@aliyun.com
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and promotes metastasis of ESCC [9]. Therefore, it is meaningful to find novel markers for diagnosis and treatment of ESCC.

LncRNA linc-UBC1, firstly found in bladder cancer, is reported to correlate with lymph node metastasis and prognosis [10]. However, the function of linc-UBC1 in ESCC has not been studied so far.

The primary purpose of this study was to investigate the influence of linc-UBC1 on the metastasis of ESCC and the underlying mechanism.

## Methods

*Patients and specimens*

Cancer tissues were acquired from 50 ESCC patients subjected to operation at Xuzhou Central Hospital. ESCC tissues were stored at –80°C. Table 1 shows the clinicopathological features of ESCC patients. The study conformed to the requirements of the Ethics Committee of Xuzhou Central Hospital. Written informed consent was signed by all ESCC patients.

*Cell culture and culture conditions*

The Institute of Basic Medical Sciences of Chinese Academy of Medical Sciences (Beijing, China) provided us Eca-109, Te-13, TE-2, TE-12and Te-1ESCC cell lines, normal human esophageal epithelial-1cell (HEEC) and 293T embryonic kidney cell line. Culture medium containing penicillin, RPMI 1640 medium (Invitrogen Life Technologies, CA, USA) and 10% fetal bovine serum (FBS; Invitrogen Life Technologies, CA, USA) were used for cell culture. An incubator (Thermo Fisher Scientific, Waltham, MA, USA) contained 5% $CO_2$ and was maintained at 37°C.

*Lentiviral small hairpin RNA construction and infection*

Lentiviral small hairpin RNA (shRNA) targeting linc-UBC1 was synthesized and then cloned into the pLenti-EF1a-EGFP-F2A-Puro vector (Biosettia Inc., San Diego, CA, USA). Then, 293T cells were used for packaging the viruses, the linc-UBC1 lentiviruses (sh-linc-UBC1) and the empty vector (sh-ctrl).

*RNA preparation and qRT-PCR*

Total RNA in tissues and ESCC cells was extracted via TRIzol reagent (Invitrogen, CA, USA). PrimeScript RT reagent kit and SYBR Premix Ex Taq (Takara, Dalian, China) was used for qRT-PCR. Besides, linc-UBC1 mRNA level was detected using the primers below: linc-UBC1, forward 5'-CCTGCTTGGAAACTAATGACC-3' and reverse 5'-AGGCTCAACTTCCCAGACTCA-3'; EZH2, forward 5'-AATCAGAGTACATGCGACTGAGA-3' and reverse 5'-GCTGTATCCTTCGCTGTTTCC-3'; GAPDH, forward 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse 5'-GAAGATGGTGATGGGATTTC-3'. RT qPCR was performed accompanied with ABI 7500 system (Applied Biosystems, MA, USA). The thermal cycles were: 30 sec at 95°C, 5 sec at 95°C for 40 cycles, 35 sec at 60°C.

*Wound healing assay*

These cells were transferred into 6-well plates and cultured overnight in RPMI-1640 medium. The cell layer, grown to 80% confluence, was scratched by a

**Table 1.** Correlation between the expression of linc-UBC1 and clinicopathological characteristics in ESCC patients

| *Characteristics* | *Patients* | *Expression of lncRNA linc-UBC1* | | *p value* |
|---|---|---|---|---|
| | | *High expression* | *Low expression* | |
| Total | 50 | 26 | 24 | |
| Age (years) | | | | 0.817 |
| ≤60 | 30 | 16 | 14 | |
| >60 | 20 | 10 | 10 | |
| Gender | | | | 0.982 |
| Male | 27 | 14 | 13 | |
| Female | 23 | 12 | 11 | |
| Differentiation | | | | 0.706 |
| Well/moderately | 32 | 16 | 16 | |
| Poorly | 18 | 10 | 8 | |
| Tumor location | | | | 0.402 |
| Lower 1/3 | 26 | 15 | 11 | |
| Upper and middle 1/3 | 24 | 11 | 13 | |
| TNM stage | | | | 0.038 |
| T1-2 | 30 | 12 | 18 | |
| T3-4 | 20 | 14 | 6 | |
| Lymph node metastasis | | | | 0.003 |
| No | 31 | 11 | 20 | |
| Yes | 19 | 15 | 4 | |

20μl tip and then the medium was exchanged. Wound closure was viewed at different time points. Each assay was independently repeated in triplicate.

*Transwell assays*

For the invasion assays, 50 μg Matrigel (BD Biosciences, CA, USA) was added to the upper chamber of an insert (8 μm pore size; Millipore, NJ, USA) before adding 200 μL serum-free RPMI-1640 medium containing $5\times10^4$ cells. The lower chamber was filled with RPMI-1640 medium with 10% FBS. Twenty-four hrs later, the upper surface of chambers was wiped with cotton buds. Then, the chambers were stained with 0.1% crystal violet for 30 min. Images of the migrated and invaded cells were obtained via a light microscope (DFC500; Leica, Wetzlar, Germany). The data for migration and invasion was counted from three fields per membrane. Each transwell assay was independently conducted thrice.

*Western blotting analysis and antibodies*

A protein assay (Bio-Rad, Beijing, China) was conducted for measuring the total protein concentration. The target proteins were placed to polyvinylidene fluoride (PVDF) membrane, which was then blocked in 5% dry milk at 37°C for 1 hr after being fractionated by SDS-PAGE. Then, the membrane was immunostained with antibodies (Cell Signaling Technology, CST, MA, USA) overnight at 4°C: 1:1000 rabbit anti-E-cadherin, 1:1000 rabbit anti-EZH2 and 1:5000 rabbit anti-GAPDH. Subsequently, 1:1000 goat anti-rabbit secondary antibody was used for culturing. The bands were measured using ChemiDoc XRS imaging system and Image J software.

*Statistics*

Quantitative data was presented as mean ± SD. Data analysis was carried out with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Chi-square test was performed to evaluate the association between lnc RNA WIF1-1 levels and clinicopathological parameters. The method of $2^{-\Delta\Delta CT}$ was used to measure the relative expression of mRNA. Overall survival was analyzed with Kaplan-Meier method and statistical significance was calculated by the log-rank test. Only $p<0.05$ was considered to be statistically significant.

## Results

*Higher level of linc-UBC1 was detected in ESCC tissues*

First, RT-qPCR was performed in 50 pairs of ESCC tissue specimens and adjacent tissues to detect linc-UBC1 expression. The results showed

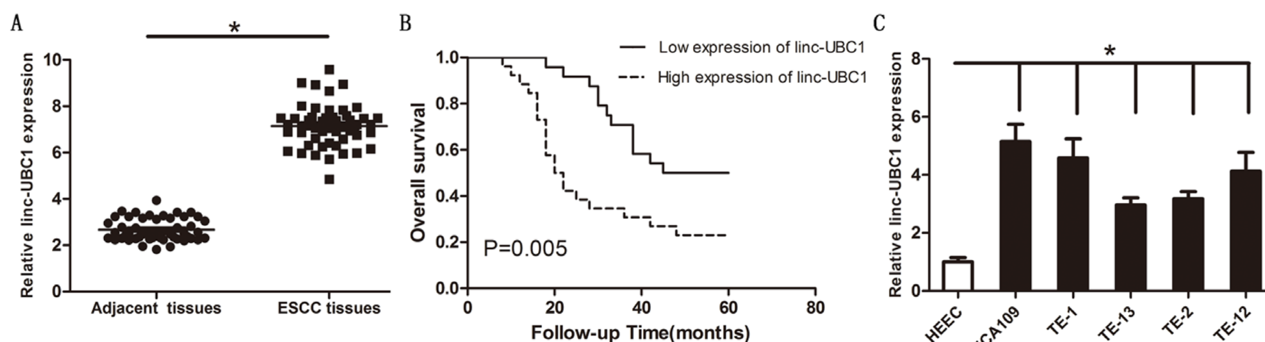


**Figure 1.** Expression levels of linc-UBC1were higer in ESCC tissues and cell lines. **(A)** linc-UBC1 expression was significantly increased in the ESCC tissues compared with adjacent tissues. **(B)** Kaplan-Meier survival curves of ESCC patients after esophagectomy. The overall survival of patients in the linc-UBC1 low-expression group was significantly better than that of patients in the high-expression group (log-rank test, p=0.005). **(C)** Expression levels of linc-UBC1 were detected in the human ESCC cell lines and normal human esophageal epithelial cells (HEEC) by RT-qPCR. Data are presented as the mean ± standard error of the mean. *p<0.05.

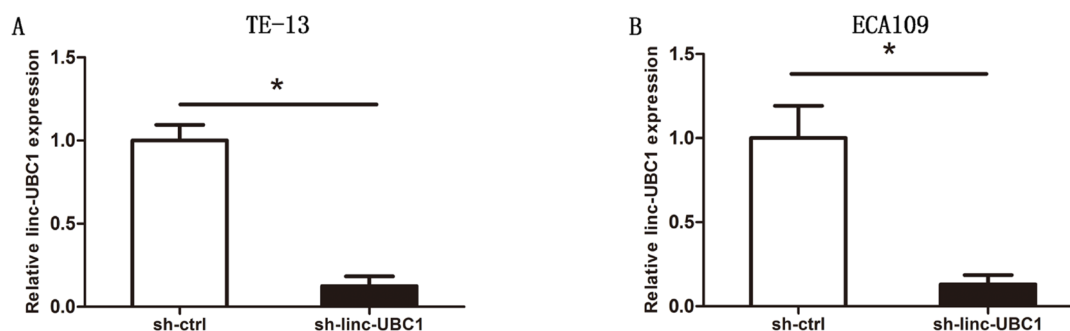


**Figure 2.** The efficiency of Linc-UBC1 knockdown in ESCC cells. **(A)** Linc-UBC1 expression in TE-13 cells transduced with control shRNA vector(sh-ctrl) or linc-UBC1 shRNA (sh-linc-UBC1) was detected by RT-qPCR. **(B)** Linc-UBC1 expression in ECA109 cells transduced with control shRNA vector (sh-ctrl) or linc-UBC1 shRNA (sh-linc-UBC1) was detected by RT-qPCR. *p<0.05 as compared with the control cells.

that higher linc-UBC1 levels were monitored in ESCC tissue samples (Figure 1A). Subsequently, investigated was the association between linc-UBC1 expression and clinical characteristics. Although linc-UBC1 expression was not associated with age, gender, tumor differentiation and tumor location, high linc-UBC1 expression was positively associated with lymph node metastasis as well as with TNM stage (Table 1). Furthermore, overall survival of ESCC patients with high expression of linc-UBC1 was significantly worse compared with patients with low expression (Figure 1B).

*Knockdown of linc-UBC1 inhibited migration and invasion of ESCC cells*

The results of RT-qPCR showed that ESCC cells had higher expression of linc-UBC1 compared with human esophageal epithelial-1 cells (HEEC) (Figure 1C). Based on the results above, we chose TE-13 and ECA109 cells for knockdown of linc-UBC1 (sh-linc-UBC1). Besides, sh-ctrl referred to the cells transfected by the empty vector (Figure 2A-2B). Wound healing assay revealed that cell migration was inhibited in sh-linc-UBC1 cells compared with sh-ctrl cells (Figure 2A). In parallel, transwell assay demonstrated that the number of invaded cells was significantly reduced in sh-linc-UBC1 cells compared with sh-ctrl cells (Figure 2B).

*Linc-UBC1 inhibited E-cadherin and promoted EZH2 in ESCC cells*

The results revealed that knockdown of linc-UBC1 suppressed the metastasis of ESCC in vitro (Figure 3). To further understand the underlying mechanism, we investigated whether linc-UBC1 could function on EMT-related protein. The results showed that E-cadherin was upregulated and EZH2 was downregulated in ESCC cells after linc-UBC1 was knocked down (Figure 4).

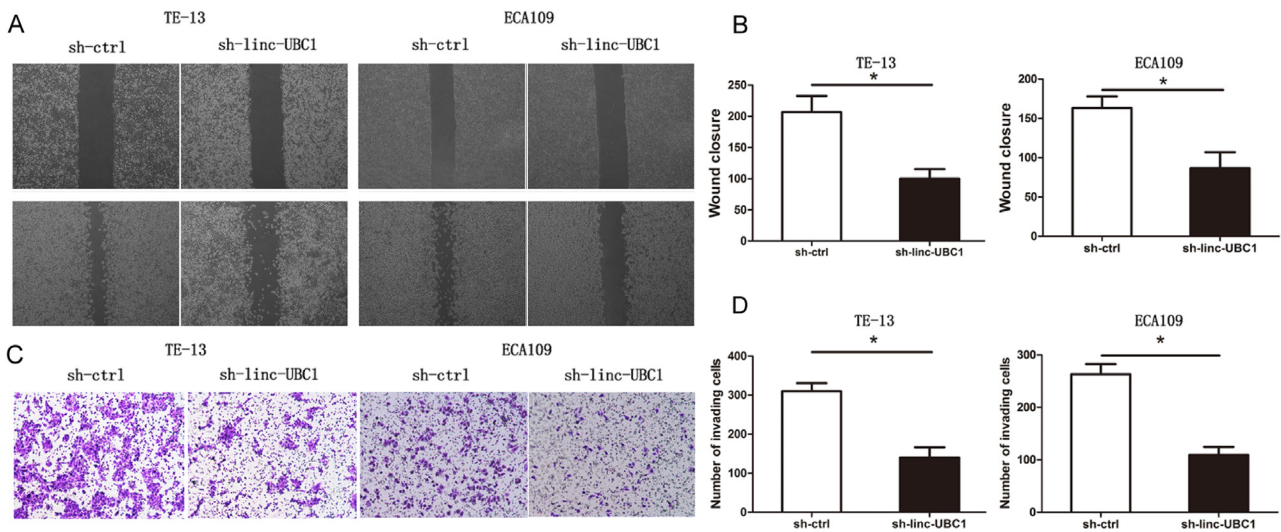


**Figure 3.** Linc-UBC1 inhibition suppressed migration and invasion of ESCC cells. **(A-B)** Wound healing assay showed that knockdown of linc-UBC1 significantly suppressed the migration ability in sh-linc-UBC1 cells compared with sh-ctrl cells. **(C-D)** Transwell assay demonstrated that the number of invaded cells was significantly reduced in sh-linc-UBC1 cells compared with sh-ctrl cells. The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with the control cells.

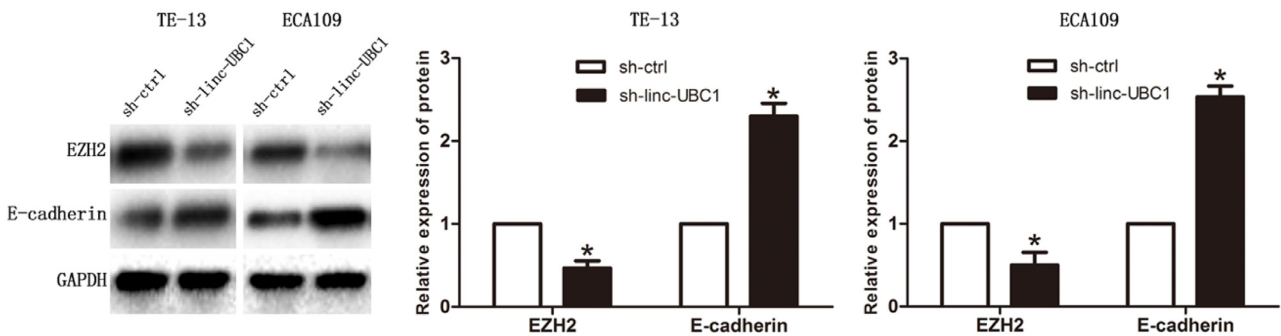


**Figure 4.** Knockdown of linc-UBC1 suppressed EZH2 and promoted E-cadherin at protein level in ESCC cells. The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with the control cells.

## Discussion

Evidence has identified that lncRNAs participate in many important biological processes including tumorigenesis and cancer metastasis, providing thus potential therapeutic targets for most cancers [10-13]. A latest study showed that linc-UBC1 enhances the growth and metastasis of colorectal cancer cells [14]. In our study linc-UBC1 was found higher expressed in tissue samples and ESCC cells. Moreover, linc-UBC1 expression level was tightly associated with lymph node metastasis, TNM stage and prognosis.

Furthermore, cell migration and invasion was inhibited in ESCC cells after linc-UBC1 was knocked down. The above results suggest that linc-UBC1 might function as an oncogene in tumorigenesis and promote migration and invasion of ESCC cells. However, the mechanism via which linc-UBC1 regulated the metastasis of ESCC remained unknown.

EZH2 is an important oncogene connected with tumor metastasis, which can be regulated by lncRNAs. For example, the function of lncRNA LET, as a tumor suppressor in nasopharyngeal carcinoma, can be repressed by EZH2 [15]. Besides, lncRNA SPRY4-IT1 enhanced cell growth and invasion via activation of EZH2 in hepatocellular carcinoma [16]. Moreover, knockdown of lncRNA-ANCR inhibited metastasis of tumor cells via suppressing EZH2 in colorectal cancer [17]. Furthermore, a recent study showed that lncRNA EBIC interacts with EZH2 and represses E-cadherin in cervical cancer, which further promotes invasion of tumor cells [18]. LncRNA H19 enhances the migration and invasion ability of bladder cancer cells via regulating EZH2 and E-cadherin [19]. Therefore, we further explored whether linc-UBC1 could regulate EZH2 and E-cadherin and then influence metastasis of ESCC in vitro. In this study, the results of Western blotting assay demonstrated that the expression of EZH2 was decreased and the expression of E-cadherin was increased after knockdown of linc-UBC1. These data indicated that linc-UBC1 had an impact on ESCC metastasis via regulating EZH2 and E-cadherin.

## Conclusions

To sum up, linc-UBC1 was higher expressed in ESCC tissue samples and its expression was positively correlated with lymph node metastasis, TNM stage and prognosis of ESCC patients. In addition, linc-UBC1 could promote cell migration and invasion of ESCC through regulating E-cadherin and EZH2. Our study provides a new potential target for diagnosis and therapy of ESCC.

## Conflict of interests

The authors declare no conflict of interests.

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