Novel long non-coding RNA LINC02323 promotes epithelial-mesenchymal transition and metastasis via sponging miR-1343-3p in lung adenocarcinoma

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Keywords
Epithelial-mesenchymal transition; LINC02323; lung adenocarcinoma; miR-1343-3p; TGF-β receptor 1.

Abstract
Background: We have previously developed a unique metastasis-associated signature consisting of six long non-coding RNAs (lncRNAs), including a novel lncRNA, namely LINC02323. In the present study, we aimed to investigate the underlying roles of LINC02323 in the migration, invasion and TGF-β-induced epithelial-mesenchymal transition (EMT) of lung adenocarcinoma (LUAD) cells.

Methods: The distribution of LINC02323 was detected by the nuclear-plasma separation experiment. Cell proliferation was assessed by MTT assay, and cell migration and invasion were detected by transwell assays. EMT was detected by RT-qPCR and western blotting. Interaction between miRNA and LINC02323 was predicted by starBase v2.0 and confirmed by the double luciferase reporting system.

Results: LINC02323 was distributed in the cytoplasm and nucleus. The overexpression or deletion of LINC02323 did not affect the proliferation of LUAD cells, while significantly affected the migration and invasion of LUAD cells. TGF-β-induced EMT process was significantly affected by both RNA interference (RNAi) and overexpression of LINC02323. The predicted results showed that there were binding sites between LINC02323 and miR-1343-3p. The expression of LINC02323 was found to be negatively correlated with miR-1343-3p in LUAD by analyzing The Cancer Genome Atlas (TCGA) database. The double luciferase reporting system, RT-qPCR and western blotting experiments confirmed that LINC02323 could bind to miR-1343-3p, which bound to TGF-β receptor 1 (TGFBR1). Inhibition of miR-1343-3p reversed LINC02323 silencing-mediated suppression of migration, invasion and EMT.

Conclusions: LINC02323 acts as a competing endogenous RNA (ceRNA), which sponged miR-1343-3p to upregulate the TGFBR1 expression and promote the EMT and metastasis in LUAD.

Key points
Significant findings of the study: LINC02323 promotes epithelial-mesenchymal transition and metastasis via sponging miR-1343-3p in lung adenocarcinoma.
What this study adds: LINC02323 is a key molecule in the process of invasion and metastasis of LUAD and might be used as a potential target in metastatic cancer.
Introduction

Lung carcinoma (LC) is the most common malignant tumor worldwide. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of LC cases, and lung adenocarcinoma (LUAD) is the most common subtype of NSCLC. Moreover, LUAD patients are usually diagnosed at advanced stages with local or distant organ metastasis, leading to poor clinical outcomes.

Tumor metastasis is a predominate cause of cancer-associated deaths and determines survival outcomes of cancer patients. A wealth of evidence suggests that epithelial-mesenchymal transition (EMT) plays pivotal roles in migration and metastasis of tumor cells. Transforming growth factor-β (TGF-β) is a major inducer of EMT, which is known to be expressed in most adenocarcinomas including LUAD and associates with cancer cell metastasis. Long non-coding RNAs (lncRNAs) are defined as transcripts of length > 200 nucleotides with a low coding potential. Emerging data support the involvement of lncRNAs in carcinogenesis and cancer progression. Many of these lncRNAs are regulated by TGF-β. LncRNAs play essential roles in the migration, invasion and EMT and are discovered to act as miRNA sponges to participate in cancer progression. Our previous study has shown that six lncRNAs, including LINC02323, are associated with metastasis and prognosis of LUAD. Herein, we hypothesized that LINC02323-miRNA-target pathway might participate in the process of EMT and metastasis of LUAD. However, the exact mechanisms and pathways still remain unclear.

In the present study, we investigated the potential roles of LINC02323 in LUAD metastasis and TGF-β-induced EMT by gain-and-loss analysis in vitro. The interaction between LINC02323 and corresponding miRNA was also analyzed. Additionally, the effects of LINC02323-miRNA-target on LUAD metastasis and TGF-β-induced EMT were further explored.

Methods

Microarray data analysis

The lncRNA sequencing data were obtained from the Cancer Genome Atlas (TCGA) database. The edgeR package in R software was used to calculate the expression of LINC02323 in LUAD patients with and without metastasis. X-tile analytic tool (version 3.6.1, Yale University, School of Medicine) was used to determine the optimum cutoff of LINC02323 expression and classify LUAD patients into high-LINC02323 and low-LINC02323 expression groups. Kaplan-Meier analysis and log-rank test were performed to estimate relapse-free survival (RFS) of LUAD patients.

Cell culture and subcellular fractionation

Recombinant human TGF-β and its receptor antagonist SB-431542 were purchased from Peprotech and Caymanchem, respectively. Four human LUAD cell lines (A549, SPC-A-1, H1975 and H1299) and one human bronchial epithelial cell line (HBE) were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China). HBE cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA), 50 U/mL penicillin and 0.1 mg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO2. LUAD cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, USA) supplemented with 10% FBS, 50 U/mL penicillin and 0.1 mg/mL streptomycin at 37°C with 5% CO2.

For subcellular fraction, SPC-A-1 cells were cultured in DMEM containing 10% FBS at 37°C with 5% CO2. Subsequently, one-fifth of cells were resuspended in 300 μL Triton (Invitrogen, USA), while the remaining cells were resuspended in 300 μL buffer A (10 mM HEPE, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 1mM DTT) on ice for 10 minutes, followed by incubation with Triton X-100 at a final concentration of 0.1%. The cell lysates were centrifuged at 1300 g for five minutes at 4°C, and the supernatants were collected, followed by centrifugation at 12000 g for 10 minutes at 4°C. The precipitates were washed twice with buffer A, centrifuged at 1300 g for five minutes at 4°C, lysed with 300 μL NETN buffer (3 mM EDTA, 0.2 mM EGTA and 1 mM DTT) for three minutes and centrifuged at 16000 g for 25 min at 4°C. Finally, the supernatants were collected and stored at −80°C.

Cell transfection

Four types of small interfering RNA (siRNA) targeting LINC02323 were designed and respectively synthesized (Oligobio, Beijing, China). Scrambled sequence was used as a negative control siRNA (si-NC). Overexpression vector of LINC02323 was built using pcDNA3.1 plasmid (Invitrogen, USA). The miR-1343-3p mimics and inhibitors were obtained from Oligobio Company (Beijing, China). The pmirGLOdual-luciferase plasmid was purchased from Biosune Biotechnology (Shanghai, China). The cells were seeded into a six-well plate at a density of 1.8 × 104/well and cultured at 37°C for 24 hours. The cells were then maintained in DMEM supplemented with 10% FBS in the absence of antibiotics and transfected with corresponding plasmid vectors for 48 hours. Subsequently, the cells were harvested and washed with PBS for qRT-PCR. The sequence information of siRNA, miR-1343-3p mimics and inhibitors are listed in Table S1.
Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from four LUAD cell lines, HBE cell line, cytoplasmic and nuclear fractions, and transfected cells. Form RNA and lncRNA expression detection, RNA templates were reversely transcribed to cDNA using Super RT cDNA synthesis kit (Toyobo, Japan). The qRT-PCR was carried out by specific primers for mRNA and lncRNA. GAPDH was selected as a housekeeping gene. For miRNA expression detection, cDNA synthesis and quantitation were conducted using the Bulge-Loop miRNA qRT-PCR Starter Kit (Ribobio, Guangzhou, China). U6 was used as the internal reference. The amplifications were performed on 7500 Fast Real-Time PCR System (Applied Biosystems, USA). The relative expressions of LINC02323, miRNA and mRNA were calculated using the 2−ΔΔCt method. The primers used are listed in Table S1.

Cell viability assay

The cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cells with overexpression or deletion of LINC02323 were collected, seeded into a 96-well plate at and maintained at 37°C with 5% CO2. After incubation for 2–3 hours, 20 μL MTT solution (Beyotime, Shanghai, China) was added into each well, and the cells were incubated at 37°C for four hours. Subsequently, cells were resuspended in 200 μL formazan solution and vortexed for 10 minutes. Finally, the absorbance at a wavelength of 570 nm was determined by an enzyme-linked immunosorbent assay reader (Thermo, USA).

Transwell assay

A transwell assay was performed to evaluate cell migratory and invasive abilities. Briefly, the upper and bottom transwell chambers were coated with 100 μL serum-free medium and 600 μL complete medium containing 10% FBS, respectively. For cell invasion assay, the upper chamber was also pre-coated with Matrigel matrix (BD Biosciences). Subsequently, nearly 2 × 10^5 LINC02323-depleted SPC-A-1 cells or 2 × 10^5 H1975 cells overexpressing LINC02323 were seeded into the upper chamber, followed by incubation at 37°C for 24 hours. The cells that did not migrate to the lower surface of the membranes were removed from the upper surface of chamber by a cotton swab. Those migrated cells were stained with 0.1% crystal violet and counted by a digital microscope (Olympus IX81, Japan).

Western blotting analysis

SPC-A-1 cells were cultured in the absence or presence of TGF-β at various concentrations (0 ng/mL, 0.5 ng/mL, 1 ng/mL, 2 ng/mL, 5 ng/mL and 10 ng/mL) for 48 hours. The total proteins were extracted, subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Beyotime, Shanghai, China) gel and transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). The relative expressions of LINC02323, miRNA and mRNA were calculated using the 2−ΔΔCt method. The primers used are listed in Table S1.

Prediction of LINC02323-miRNA relationship

StarBase v2.0 was used to predict miRNAs that interacted with LINC02323. In addition, based on the expressions

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Figure 1 LINC02323 overexpression represents poor prognosis in LUAD patients. (a) Kaplan-Meier survival curve of LINC02323 expression with survival of LUAD patients (—) High, (—) Low. The red line represents the high expression of LINC02323, while the blue line represents the low expression of LINC02323. (b) The expression of LINC02323 in four LUAD cell lines (H1299, H1975, A549 and SPC-A-1) and one HBE cell line was determined by qRT-PCR.
of LINC02323 and miRNAs in LUAD patients of TCGA database, the correlation of the LINC02323 and miRNA was identified by Spearman analysis. \( P < 0.05 \) was considered statistically significant.

**Dual-luciferase assay**

LINC02323 and TGFBR1 3’UTR wild-type (WT) or mutant (MUT) luciferase plasmid were constructed using the pmiR-GLO dual-luciferase reporter vector. SPC-A-1 cells were seeded into 96-well plates and cultured for 24 hours. The reporter gene and miR1343-3p mimics/NC or control plasmid were cotransfected with Lipofectamine 2000 reagent. After 48 hours, the cells were lysed and centrifuged (12,000 g, 10 minutes), and the supernatant was collected. Next, the supernatant (20 μL per well) and Luciferase Assay Reagent II (100 μL per well) were added into a white opaque 96-well microtiter plate. After thorough mixing, the intensity of the luciferase reaction was determined using a fluorescent luminometer (Thermo Scientific Varioskan Flash, USA).

**Statistical analysis**

All data in this study were analyzed using R software (R 3.4.3; http://www.Rproject.org) and GraphPad Prism 5.0 software. The experimental data were expressed as mean ± standard deviation, and each experiment was repeated three times. The Kolmogorov-Smirnov test was used to evaluate the distribution of experimental data in each group. The statistical differences were assessed using the Mann-Whitney U test and Student’s t-test. The Kruskal-Wallis test was used for comparison among multiple groups. \( P < 0.05 \) was considered statistically significant.

![Figure 2](image)

**Figure 2** LINC02323 is expressed in cytoplasm and nucleus and is not related to cell viability. (a) LINC02323 was in the cytoplasmic and nuclear components of SPC-A-1 cells as determined by qRT-PCR. The GAPDH and U1 were respectively used as the internal controls (red nuclear, blue cytoplasmic). (b) The siRNA depletion efficiency in SPC-A-1 cells was assessed by qRT-PCR. (c) The LINC02323 overexpression efficiency in H1975 cells was assessed by qRT-PCR. (d–e) The effect of LINC02323 on the viability of SPC-A-1 cells transfected with si-L1 or si-L4 was respectively evaluated by MTT assay (••) si-NC, (■–■) si-L1, (●–●) si-L4. (f) The effect of LINC02323 on the viability of H1975 cells transfected with pcDNA3.1-LINC02323 was evaluated by MTT assay (●–●) pcDNA3.1, (●–●) pcDNA3.1-LINC02323. Error bars indicate the mean ± SD. **\( P < 0.01 \), *** \( P < 0.001 \).
Results

High LINC02323 expression is correlated with poor survival

Our results revealed that the patients with high expression of LINC02323 had a shorter RFS compared with the patients with low expression of LINC02323 (P < 0.001; Fig 1a), suggesting that LINC02323 was a predictor for the survival outcome of LUDA patients. Besides, we found that LINC02323 was highly expressed in SPC-A-1 cells, while its expression was barely detected in H1299 and H1975 cells (Fig 1b). Therefore, the SPC-A-1 and H1975 cell lines were selected to evaluate the effects of LINC02323 depletion and overexpression on cellular processes, respectively.

LINC02323 is expressed in cytoplasm and nucleus and not related to cell proliferation

To explore the underlying roles of LINC02323 in LUAD progression, the localization of LINC02323 expression in SPC-A-1 cells was first determined. We found that LINC02323 was localized in cytoplasmic and nuclear extracts (Fig 2a). We then transfected SPC-A-1 cells with four specific siRNAs and discovered that si-L1 and si-L4 had higher transfection efficiency, which would be used for following assays (Fig 2b). Additionally, LINC02323 was highly expressed in H1975 cells with LINC02323 overexpression vector (Fig 2c). Interestingly, H1975 cells were gradually increased in length following overexpression of LINC02323.
LINC02323, showing mesenchymal morphology (Fig S1). Notably, MTT assay indicated that overexpression or deletion of LINC02323 did not obviously affect the cell viability (Fig 2d-f).

**LINC02323 knockdown inhibits migration and invasion of LUAD cells**

Overwhelming evidence has demonstrated that tumor migration and invasion are closely related to tumor development. Therefore, we further concentrated on investigating whether LINC02323 affected the migration and invasiveness of LUAD cells. By transwell analysis, we found that the migratory and invasive abilities were obviously lower in SPC-A-1 cells transfected with si-L1 and si-L4 compared with the control group (Fig 3a). Meanwhile, the migration and invasiveness of H1975 cells overexpressing LINC02323 were enhanced compared with the control group (Fig 3b).

**LINC02323 plays a crucial role in TGF-β-induced EMT process in LUAD cells**

Recently, Lai et al. have highlighted that lncRNAs are associated with TGF-β signaling-regulated EMT, migration and invasion of LC cells. Here, we found that 5 ng/mL TGF-β could effectively induce EMT, while such induction was inhibited by equivalent TGF-β receptor antagonist SB431542 (Fig 4a and b). Moreover, there was a significant increase of LINC02323 expression in SPC-A-1 cells in the presence of TGF-β. However, the LINC02323 expression was not changed in SPC-A-1 cells in the presence of 5 ng/mL TGF-β and SB431542 (Fig 4c). The mesenchymal and spindle-like morphology induced by TGF-β in SPC-A-1 cells was changed after LINC02323 depletion (Fig S2). Additionally, the expressions of two mesenchymal markers (vimentin and N-cadherin) were elevated, while the expression of epithelial marker (E-cadherin) was decreased, which were also discovered in H1975 cells overexpressing LINC02323 (Fig 4d and e).

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**Figure 4** LINC02323 was requisite for EMT. (a) Expressions of vimentin, N-cadherin and E-cadherin in SPC-A-1 cells in the presence of TGF-β as determined by western blotting analysis. (b) Expressions of vimentin and E-cadherin in SPC-A-1 cells in the presence of TGF-β and its receptor antagonist SB431542 as determined by western blotting analysis. (c) Expression of LINC02323 in SPC-A-1 cells in the presence of TGF-β and SB431542 as determined by RT-qPCR. (d) Expressions of EMT markers in si-NC or si-L1/si-L4 transfected SPC-A-1 cells in the presence of TGF-β as determined by western blotting analysis. (e) Expressions of EMT markers in pcDNA3.1-LINC02323-transfected H1975 cells as determined by western blotting analysis. Error bars indicate the mean ± SD. ***P < 0.001.
LINC02323 strongly interacts with miR-1343-3p

LncRNAs play critical roles in tumor invasion and metastasis via targeting miRNAs. Our results showed that LINC02323 was targeted by miR-1343-3p and miR-6783-3p. Remarkably, Yao et al. have pointed out that miR-6783-3p is highly expressed in LUAD tissues, and its overexpression promotes the migratory and invasive capacity of LUAD cells. However, these findings are contradictory to our study. Herein, miR-1343-3p was considered as the interest of following analysis. Bioinformatics analysis predicted the binding sites between LINC02323 and miR-1343-3p sequences. The expression of LINC02323 was negatively correlated with the miR-1343-3p expression in LUAD patients (r = -0.114, P < 0.05; Fig 5b). MiR-1343-3p expression was high in A549 and H1975 cells but low in SPC-A-1, HBE and H1299 cells (Fig 5c). Notably, the expression of miR-1343-3p was higher in SPC-A-1 cells with miR-1343-3p mimics compared with the control group, while the expression of miR-1343-3p was lower in SPC-A-1 cells with miR-1343-3p inhibitors compared with the control group (Fig 5d). The expression of LINC02323 was significantly decreased in SPC-A-1 cells treated with miR-1343-3p mimics (Fig 5e). MiR-1343-3p expression was significantly reduced in SPC-A-1 cells in the presence of TGF-β (Fig 5f). Additionally, the miR-1343-3p expression was dramatically elevated in SPC-A-1 cells transfected with si-L4 (Fig 5g).

LINC02323 regulates the TGFBR1 expression by targeting miR-1343-3p

Existing evidence has demonstrated that lncRNA-miRNA-gene target regulatory axis as a novel biomarker is associated with the pathogenesis of LC, including LUAD. Therefore, we next investigated the associations among
LINC02323, miR-1343-3p and gene target. The sequences of LINC02323 (WT and MUT) with miR-1343-3p were constructed and cloned into pmirGLO vector (Fig 6a). The relative luciferase activity was detected in SPC-A-1 cells after cotransfection of miR-1343-3p mimics and luciferase plasmids. The results suggested that overexpression of miR-1343-3p significantly decreased the luciferase activity in SPC-A-1 cotransfected with pmirGLO-LINC02323-WT and miR-1343-3p mimics, which could be reversed by mutation of binding sites between miR-1343-3p and LINC02323 (Fig 6b). These results verified the inhibitory effect of LINC02323-miR-1343-3p. A previous research has indicated that miR-1343-3p can directly target and inhibit TGFBR1 expression. We found that luciferase activity was markedly decreased after cotransfection of miR-1343-3p mimics and pmirGLO-TGFBR1-3’UTR-WT vector, which was dramatically increased in SPC-A-1 cells after cotransfection of miR-1343-3p mimics and pmirGLO-TGFBR1-3’UTR-MUT vector (Fig 6c). Collectively, LINC02323 had an indirect regulatory effect on the TGFBR1 expression via sponging miR-1343-3p.

**Discussion**

Tumor metastasis or recurrence is a key contributor to unfavorable prognosis for LUAD patients. We have previously reported that LINC02323 and another five lncRNAs (LINC01819, ZNF649-AS1, HNF4A-AS1, FAM222A-AS1 and LINC00672) could be used as both a marker of metastasis and a predictor for poor prognosis in LUAD patients. In the present study, our data showed that LINC02323 silencing markedly inhibited the migration and invasion of SPC-A-1 cells, while LINC02323 overexpression significantly enhanced the migratory and invasive abilities of H1975 cells. We also proved that LINC02323 did not affect the proliferative ability of SPC-A-1 and H1975 cells. These findings suggest that...
LINC02323 plays a critical role in tumor metastasis and then results in poor prognosis in LUAD. Advanced cancers are well known to secrete TGF-β, which promotes EMT and metastasis due to contextual changes that occurred in the tumor cells. The general paradigm of TGF-β signaling entails a complex of membrane-bound type I and type II receptors. TGF-β can bind to TGFBR to activate and initiate downstream proteins. Our results showed that LINC02323 depletion inhibited the EMT induced by TGF-β1. Similarly, overexpression of LINC02323 could enhance the migration and invasiveness of SPC-A-1 cells. This evidence implied that LINC02323 was involved in the migration, invasion and TGF-β1-induced EMT of SPC-A-1 cells. Intriguingly, we also found that LINC02323 was upregulated by TGF-β1, which is consistent with the LINC02323-mediated metastasis.

LncRNAs, as competing endogenous RNAs (ceRNAs), are involved in the initiation and progression of several tumors, including LUAD. Herein, our predictive analysis indicated that LINC02323 was negatively correlated with the miR-1343-3p. Meanwhile, there was a negative association between miR-1343-3p and TGFBR1, which was consistent with an earlier research. These results indicated that LINC02323 could modulate the expression of TGFBR1 via sponging miR1343-3p. Kim et al. have previously highlighted that the decreased expression of miR-1343-3p could partly reverse the suppression of vascular invasion in LUAD patients. Herein, our results demonstrated that the inhibition of miR-1343-3p could partly reverse the suppression of...
migration, invasion and TGF-β-induced EMT mediated by LINC02323 silencing in SPC-A-1 cells. We inferred that LINC02323-miR-1343-3p-TGFBR1 axis was involved in the molecular mechanism of LUAD metastasis, and LINC02323 in cytoplasm might play a leading role in the molecular mechanism of LUAD metastasis, and LINC02323-silencing in SPC-A-1 cells. We inferred that it was a key molecule in the process of invasion and metastasis of LUAD and might be used as a potential target in metastatic cancer.

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Disclosure

The authors declare that there are no conflicts of interest.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

**Table S1** The sequence information of siRNA, miR-1343-3p mimics and inhibitors.

**Figure S1** Morphology of pcDNA3.1 or pcDNA3.1-LINC02323 transfected H1975 cells. **Figure S2** Morphology of SPC-A-1 cells transfected with si-NC or si-L1/si-L4 and TGF-β stimulation.