Long Noncoding RNA LINC00634 Functions as an Oncogene in Esophageal Squamous Cell Carcinoma Through the miR-342-3p/Bcl2L1 Axis

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Abstract
Many long noncoding RNAs reportedly have tumor suppressive roles or are oncogenic in esophageal cancer. We have previously performed a chip-based expression analysis of primary esophageal cancer tissues and found that the expression of LINC00634 in these tissues was higher than that in nontumor tissues. Quantitative real-time–polymerase chain reaction, cell counting kit-8, flow cytometry, caspase3/7 assay, dual-luciferase reporter assay, and restore assay were used to detect the proliferative and apoptotic effects of LINC00634 in esophageal cancer cells. The results showed that the expression of LINC00634 in these tissues was higher than that in nontumor tissues and associated with tumor–node–metastasis (TNM) stage of patients. Knockdown of LINC00634 decreased cell viability and increased cell apoptosis levels in EC9706 and EC1 cells. LINC00634 could target Bcl2L1 through miR-342-3p. In this study, we show that LINC00634 is upregulated in esophageal cancer. We also show that the knockdown of LINC00634 decreased cell viability and increased cell apoptosis levels in EC9706 and EC1 cells through the miR-342-3p/Bcl2L1 axis.

Keywords
esophageal cancer, IncRNA, miR-342-3p, Bcl2L1

Introduction
Esophageal cancer (EC) is a malignant cancer with a high incidence worldwide. Approximately 400 000 people die of EC each year. China is one of the high prevalence areas of this disease in the world, with more than 150 000 deaths each year. The 5-year overall survival rate of patients with EC is 9.5% to 45%. Further exploration of the mechanisms of the genomic changes involved in EC is urgently required.

More than 70% of the human genome is transcribed as non-coding RNA (ncRNA), while only about 2% of the human genome is transcribed as protein-coding RNA. NcRNA can be classified as short and long ncRNA (IncRNA), which is defined as noncoding transcripts with more than 200 nucleotides in length and limited protein-coding potential. Many IncRNAs are known to regulate many aspects of key cell pathophysiological processes. Accumulating evidence has revealed that IncRNAs play important roles in the biological behaviors of various cancer cells, including cell proliferation,
apoptosis, and migration.  Furthermore, many lncRNAs are reportedly dysregulated in various cancer and are associated with diagnosis and prognosis. Many lncRNAs have been reported to have tumor suppressive roles or to be oncogenic in EC. We have previously performed a chip-based expression analysis of primary EC tissues and found that the expression of LINC00634 in these tissues was higher than that in nontumor tissues. However, there is no existing research focused on LINC00634 in EC, and the expression, roles, and mechanisms of action of LINC00634 in EC remain unknown.

In this study, we investigated the expression pattern of LINC00634 in EC and the biological functions of LINC00634 in EC to confirm that LINC00634 may be used as a new target for the treatment of EC.

Materials and Methods

Patients and Tissues

A total of 41 tissues sample of patients with EC in the First Affiliated Hospital of Zhengzhou University were collected from 2016 to 2018. Diagnosis by pathology experts, the tissues were stored in liquid nitrogen immediately. All patients signed an informed in advance and signed explicit informed consent. This study was approved by the ethics committee of Zhengzhou University.

Cell Lines and Cell Transfection

The human esophageal epithelial cell line (Het-1a) and the EC cell lines EC9706, Eca109, EC1, KYSE30 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences. Esophageal cancer cell lines were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS; Gibco) and were incubated at 37% CO2. Het-1a cell line was maintained in Dulbecco’s Modified Eagle medium with high glucose. The small interfering RNA, microRNA (miRNA) mimics, and inhibitors were purchased from Shanghai GenePharma Co, Ltd and were transfected into cells with the BTX ECM 2001 square wave electroporator according to the BTX Molecular Delivery Systems protocol.

RNA Isolation and Quantitative Real-Time–Polymerase Chain Reaction

Total RNA from EC tissues was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA was extracted using the Total RNA Kit I (Omega) according to the manufacturer’s instructions. Then, RNA was reverse transcribed to complementary DNA with reverse transcription Kit (Thermo).

SYBR Green real-time PCR assay was used to detect the expression of LINC00634, miR-342-3p, and Bcl2L1 mRNA, with the reaction conditions set according to the manufacturer’s instructions. Primers were designed using Primer Premier 5.0 software. PCR reaction conditions were as follows: 50 °C, 2 minutes; 95 °C 2 minutes; 95 °C 15 seconds; 55 °C, 30 seconds; 72 °C, 30 seconds, for 35 cycles. The 2−ΔΔCt method was used to evaluate the relative expression of the targets.

Cell Counting Kit-8 Assay

To detect the effects of LINC00634 on proliferation in EC cells, cell counting kit-8 (CCK-8) assay was performed in EC9706 and EC1 cells. Cells were seeded into 96-well plates at a density of 3 × 10⁴/well. At 0, 24, 48, and 72 hours, using Cell Counting Kit-8 kit (Dojindo) to detect absorbance of different group cells at 450 nm by Microplate Reader (Bio-Rad). The experiment was repeated 3 times.

Apoptosis Assay

The different groups of EC9706 and EC1 cells were harvested and resuspended at 10⁷ cells/mL in 1× binding buffer. After double staining with Fluorescein-5-isothiocyanate (FITC)-Annexin V and propidium iodide of FITC-Annexin V Apoptosis Detection Kit (BestBio), cells were analyzed using FACScan flow cytometer (BD Biosciences) equipped with Cell Quest software (BD Biosciences).

Caspase 3/7 Activity Assay

Caspase activity in EC9706 and EC1 cells was measured using a caspase activity assay kit (Beyotime). Cellular extracts and substrates were kept in 96-well plates for 2 hours at 37 °C. Absorbance values were measured using a microplate reader at 405 nm (Infinite M200).

Plasmid Construction and the Luciferase Reporter Assay

Wild type (WT) and mutant type (MT) LINC00634 and Bcl2L1 3′-untranslated region (3′-UTR) fragments were created by 2 single-strand annealing (Sangon). These fragments were, respectively, cloned into pmirGLO vector (Promega) downstream of luciferase gene to generate recombinant vectors WT/MT-LINC00634 pmirGLO and WT/MT-Bcl2L1 3′-UTR pmirGLO.

For the luciferase reporter assay of LINC00634 and miR-342-3p, HEK293 cell were cotransfected with matched reporter vectors (WT-LINC00634 pmirGLO or MT-LINC00634 pmir-GLO) and miRNA (miR-342-3p mimics or miRNA negative control [miR-NC]) with BTX ECM 2001 square wave electroporator. For the luciferase reporter assay of miR-342-3p and Bcl2L1, HEK293 cell were cotransfected with matched reporter vectors (WT-Bcl2L1 3′-UTR pmirGLO or MT-Bcl2L1 3′-UTR pmirGLO) and miRNA (miR-342-3p mimics or miR-NC) with BTX ECM 2001 square wave electroporator. Luciferase activity was measured at 24 hours posttransfection using the Dual-Luciferase Reporter Assay System kit (Promega) according to the manufacturer’s instructions.
Western Blotting

Total protein was extracted using RIPA Lysis buffer, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PAGE) gel electrophoresis, and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking, the membranes were successively incubated with diluted primary antibodies and horseradish peroxidase-conjugated immunoglobulin G. Signals were determined with an ECL detection kit (Beyotime). β-actin served as the endogenous reference.

Statistical Analysis

Statistical analysis was performed using SPSS 21.0. The Student t test and 1-way analysis of variance were used to test differences among groups. The difference was statistically significant when \( P < .05 \).

Results

Upregulation of LINC00634 in EC

Using adjacent normal tissues as a reference, LINC00634 expression in EC tissues was found to be significantly upregulated \( (P < .05; \text{Figure 1A}) \). Additionally, LINC00634 expression in EC tissues was associated with tumor–node–metastasis (TNM) stage, the LINC00634 expression of EC tissues with TNM III was higher than that with TNM I or II \( (P < .05; \text{Figure 1B}) \). Quantitative real-time polymerase chain reaction (qRT-PCR) assay was used to detect the LINC00634 expression levels in EC cells. LINC00634 expression of EC cells (EC9706, Eca109, EC1, and KYSE30) was found to be significantly higher than that of esophageal epithelial cell Het-1a \( *(P < .05) \) compared to the control group.

Downregulation of LINC00634 Inhibited Cell Proliferation and Promoted Cell Apoptosis

The levels of LINC00634 after transfection of the si-LINC00634 in EC9706 and EC1 cells are shown in Figure 2A. LINC00634 expression levels of si-LINC00634 group cell were significantly lower than in the siRNA negative control (si-NC) group \( (P < .05; \text{Figure 2A}) \). To examine the effect of LINC00634 on proliferation in EC cells, CCK-8 assay was conducted. The results of CCK-8 assay showed that si-LINC00634 group cells’ viability was significantly lower than that of si-NC groups in EC9706 and EC1 cells \( (P < .05; \text{Figure 2B and C}) \). To examine the effect of LINC00634 on apoptosis in EC cells, flow cytometry assay was conducted. Flow cytometry results indicated that the level of apoptosis in the si-LINC00634 group was significantly higher than in the si-NC group in EC9706 and EC1 cells \( (P < .05; \text{Figure 2D}) \). These results suggest that downregulation of LINC00634 inhibited cell proliferation and promoted cell apoptosis in EC9706 and EC1 cells.

miR-342-3p Was Directly Targeted by LINC00634

DIANA LncBASE Predicted was used to identify the miRNAs containing complementary bases with LINC00634, and LINC00634 was predicted to harbor miR-342-3p binding sites \( \text{(Figure 3A). To verify whether LINC00634 could bind to miR-342-3p, dual-luciferase reporter assay was conducted. We established reporter vectors WT-LINC00634 pmirGLO and MT-LINC00634 pmirGLO, which were cotransfected into HEK293 cells with miR-342-3p mimics or miR-NC, respectively. The results showed that the luciferase activity of cells cotransfected with WT-LINC00634 pmirGLO and MT-LINC00634 pmirGLO, which were cotransfected into HEK293 cells with miR-342-3p mimics or miR-NC, respectively. The results showed that the luciferase activity of cells cotransfected with WT-LINC00634 pmirGLO and miR-342-3p mimics was lower than in the other 3 groups of cells \( (P < .05; \text{Figure 3B}) \). The relative miR-342-3p expression in cells that were transfected with si-LINC00634 was also analyzed by qRT-PCR assay. The results showed that the miR-342-3p expression in cells transfected with si-LINC00634 was higher than in those with si-NC in EC9706 and EC1 cells \( (P < .05; \text{Figure 3C}) \). We further detected miR-342-3p expression in EC tissues. Correlation
analysis demonstrated that miR-342-3p expression level was negatively correlated with LINC00634 expression in EC tissues (Figure 3D). Taken together, these results indicated that LINC00634 could bind to miR-342-3p.

**LINC00634 Targeted Bcl2L1 Through miR-342-3p**

TargetScan was used to predict the targets of miR-342-3p, and results showed that the 3'-UTR of Bcl2L1 harbors miR-342-3p binding sites (Figure 4A). To verify whether miR-342-3p targets Bcl2L1, dual-luciferase reporter assay was utilized. We established reporter vectors WT/MT-Bcl2L1 3'-UTR pmirGLO and WT/MT-Bcl2L1 3'-UTR pmirGLO, which were cotransfected into HEK293 cells with miR-342-3p mimics or miR-NC, respectively. The result showed that the luciferase activity of cells cotransfected with WT-Bcl2L1 3'-UTR pmirLO and miR-342-3p mimics was lower than in the other 3 groups of cells ($P < .05$; Figure 4B). Western blotting results showed that in EC9706 and EC1 cells, Bcl2L1 protein expression in cells transfected with miR-342-3p mimics was lower than in those with miR-NC or si-NC ($P < .05$; Figure 4C). The relative Bcl2L1 mRNA expression of cells that were transfected with si-LINC00634 and miR-342-3p mimics was also detected by qRT-PCR assay. Results showed that in EC9706 and EC1 cells, the Bcl2L1 mRNA expression of cells transfected with si-LINC00634 or miR-342-3p mimics was lower than in those with miR-NC or si-NC ($P < .05$; Figure 4D). We further detected Bcl2L1 mRNA expression in EC tissues. Correlation analysis demonstrated that Bcl2L1 mRNA expression level was negatively correlated with miR-342-3p and was positively correlated with LINC00634 expression in EC tissues (Figure 4E and F). Taken together, these results indicated that LINC00634 targeted Bcl2L1 through miR-342-3p.

**Downregulation of miR-342-3p Restored Function of si-LINC00634**

Cell counting kit-8 results showed that cotransfection of si-LINC00634 and miR-342-3p inhibitor led to an increase in cell viability and abrogated the effects of si-LINC00634 in EC9706 and EC1 cells ($P < .05$; Figure 5A and B). Flow cytometry (FCM) results showed that cotransfection of si-LINC00634 and miR-342-3p inhibitor led to a decrease in the number of apoptotic cells and abrogated the effects of si-LINC00634 in EC9706 and EC1 cells ($P < .05$; Figure 5C). These results indicated that downregulation of miR-342-3p restores function of si-LINC00634.
Figure 3. miR-342-3p was directly targeted by LINC00634. A, The binding sites of miR-342-3p and LINC00634. B, Luciferase activity assay showed cells cotransfected with WT-LINC00634 pmirGLO and miR342-3p mimics was lower than that of the other 3 group cells. C, The relative miR-342-3p expression of cells that were transfected with si-LINC00634 was also detected by qRT-PCR assay. D, Correlation analysis demonstrated that miR-342-3p expression level was negatively correlated with LINC00634 expression in EC tissues. *P < .05 compared to the control group.

Figure 4. LINC00634 could target Bcl2L1 through miR-342-3p. A, The binding sites of miR-342-3p and Bcl2L1 3'-UTR. B, Luciferase activity assay showed cells cotransfected with WT-Bcl2L1 3'-UTR pmirGLO and miR342-3p mimics was lower than that of the other 3 group cells. C, Western blot analysis of Bcl2L1 expression of transfected cells. D, The relative Bcl2L1 mRNA expression of transfected cells. E and F, Correlation analysis demonstrated that Bcl2L1 mRNA expression level was negatively correlated with miR-342-3p and was positively correlated with LINC00634 expression in EC tissues. *P < .05 compared to the control group.
Discussion

Different lncRNAs have been reported to have oncogenic roles in EC. We previously performed expression microarray analysis of primary EC tissues and found that the expression of LINC00634 in the tumor tissues was higher than in the adjacent normal tissues. However, there is no existing research focused on LINC00634 in EC. In this study, we analyzed the expression, roles, and mechanisms of action of LINC00634 in EC.

We first detected LINC00634 expression in EC tissues and found that LINC00634 expression in EC tissues was associated with TNM stage and that LINC00634 expression in EC tissues with TNM III was higher than in TNM I or II. We also detected the LINC00634 expression levels in EC cells and found LINC00634 expression in EC cells (EC9706, Eca109, EC1, and KYSE30) were significantly higher than in Het-1a esophageal epithelial cells. The observed aberrant expression of LINC00634 suggests that LINC00634 functions as a tumor suppressor in EC.

Then, we examined the effect of LINC00634 on proliferation in EC cells using CCK-8 assay and found that knockdown LINC00634 could decrease cell viability in EC9706 and EC1 cells. We examined the effect of LINC00634 on apoptosis in EC cells using flow cytometry and caspase3/7 assays and found that knockdown LINC00634 could increase cell apoptosis levels in EC9706 and EC1 cells. These results were consistent with the results found in EC tissues.

miR-342-3p was predicted to harbor LINC00634 binding sites using DIANA LncBASE Predicted. Dual-luciferase reporter assay was used to show relative miR-342-3p expression in cells that were transfected with si-LINC00634 and correlation analysis of miR-342-3p, and LINC00634 expression level in EC tissues indicated that LINC00634 could bind to miR-342-3p. miR-342-3p functions as a tumor suppressor in many kinds of tumors. Studies by Wang et al proved that LINC00460 regulated KDM2A to promote cell proliferation and migration by targeting miR-342-3p in gastric cancer, and Xue et al reported that miR-342-3p suppressed cell proliferation and migration by targeting AGR2 in small-cell lung cancer.

TargetScan was used to predict the targets of miR-342-3p and 3′-UTR of Bcl2L1 was predicted to harbor miR-342-3p binding sites. Dual-luciferase reporter assay, Bcl2L1 expression of EC cells and correlation analysis of Bcl2L1 mRNA with miR-342-3p and LINC00634 expression in EC tissues indicated that LINC00634 targeted Bcl2L1 through miR-342-3p. BCL2L1(Bcl-xL) belongs to the antiapoptotic Bcl-2 family member, and high Bcl-xL expression has indeed been associated with paclitaxel resistance in solid tumors. Taken together, our findings indicate that LINC00634 can act as an oncogene in esophageal squamous cell carcinoma.

Conclusions

In this study, we showed that LINC00634 is upregulated in EC. We have also shown that the knockdown LINC00634 decreased cell viability and increased cell apoptosis levels in EC9706 and EC1 cells through miR-342-3p/Bcl2L1 axis. Based on these findings, we propose that LINC00634 may act as a therapeutic agent and could potentially serve as a prognostic biomarker.

Authors’ Note

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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References

1. Koshy M, Esiashvilli N, Landry JC, Thomas CR, Jr, Matthews RH. Multiple management modalities in esophageal cancer: epidemiology, presentation and progression, work-up, and surgical approaches. Oncologist. 2004;9(2):137-146.
2. Dantoc MM, Cox MR, Eslick GD. Does minimally invasive esophagectomy (MIE) provide for comparable oncologic outcomes to open techniques? A systematic review. J Gastrointest Surg. 2012;16(3):486-494.
3. Carninci P, Kasukawa T, Katayama S, et al. The transcriptional landscape of the mammalian genome. Science. 2005;309(5740):1559-1563.
4. Djebali S, Davis CA, Merkel A, et al. Landscape of transcription in human cells. Nature. 2012;489(7414):101-108.
5. Katayama S, Tomaru Y, Kasukawa T, et al. Antisense transcripts in the mammalian transcriptome. Science. 2005;309(5740):1564-1566.
6. Wahlestedt C. Natural antisense and noncoding RNA transcripts as potential drug targets. Drug Discov Today. 2006;11(12):503-508.
7. Batista PJ, Chang HY. Long noncoding RNAs: cellular address codes in development and disease. Cell. 2013;152(6):1298-1307.
8. Liu X, Xiao ZD, Han L, et al. LncRNA NBR2 engages a metabolic checkpoint by regulating AMPK under energy stress. Nat Cell Biol. 2016;18(4):431-442.
9. Xu D, Yang F, Yuan JH, et al. Long noncoding RNAs associated with liver regeneration 1 accelerates hepatocyte proliferation during liver regeneration by activating Wnt/beta-catenin signaling. Hepatology. 2013;58(2):739-751.
10. Zhang C, Yuan J, Hu H, et al. Long non-coding RNA CHCHD4P4 promotes epithelial-mesenchymal transition and inhibits cell proliferation in calcium oxalate-induced kidney damage. Braz J Med Biol Res. 2017;51(1):e6536.
11. Li JK, Chen C, Liu JY, et al. Long noncoding RNA MRCCAT1 promotes metastasis of clear cell renal cell carcinoma via inhibiting NPR3 and activating p38-MAPK signaling. Mol Cancer. 2017;16(1):111.
12. Lin A, Hu Q, Li C, et al. The LINK-A lncRNA interacts with PtdIns(3,4,5)P3 to hyperactivate AKT and confer resistance to AKT inhibitors. Nat Cell Biol. 2017;19(3):238-251.
13. Lin A, Li C, Xing Z, et al. The LINK-A lncRNA activates normoxic HIF1alpha signalling in triple-negative breast cancer. Nat Cell Biol. 2016;18(2):213-224.
14. Parasramka M, Yan IK, Wang X, et al. BAP1 dependent expression of long non-coding RNA NEAT-1 contributes to sensitivity to gemcitabine in cholangiocarcinoma. Mol Cancer. 2017;16(1):22.
15. Zhu XT, Yuan JH, Zhu TT, et al. Long noncoding RNA glypican 3 (GPC3) antisense transcript 1 promotes hepatocellular carcinoma progression via epigenetically activating GPC3. FEBS J. 2016;283(20):3739-3754.
16. Fernando TR, Contreras JR, Zampini M, et al. The lncRNA CASC15 regulates SOX4 expression in RUNX1-rearranged acute leukemia. Mol Cancer. 2017;16(1):126.
17. Hosseini ES, Meryet-Figuierre M, Sabzalipoor H, Kashani HH, Nikzad H, Asemi Z. Dysregulated expression of long noncoding RNAs in gynecologic cancers. Mol Cancer. 2017;16(1):107.
18. Mizrahi I, Mazeh H, Grinbaum R, et al. Colon cancer associated transcript-1 (CCAT1) expression in adenocarcinoma of the stomach. J Cancer. 2015;6(2):105-110.
19. Zhang L, Yang F, Yuan JH, et al. Epigenetic activation of the MiR-200 family contributes to H19-mediated metastasis suppression in hepatocellular carcinoma. Carcinogenesis. 2013;34(3):577-586.
20. Arocho A, Chen B, Ladanvi M, et al. Validation of the 2-DeltaDeltaCt calculation as an alternate method of data analysis for quantitative PCR of BCR-ABL P210 transcripts. Diagn Mol Pathol. 2006;15(1):56-61.
21. Wang Y, Chen J, Zhang M, et al. MiR-149 sensitizes esophageal cancer cell lines to cisplatin by targeting DNA polymerase beta. J Cell Mol Med. 2018;22:3857-3865.
22. Wang F, Liang S, Liu X, Han L, Wang J, Du Q. LINC00460 modulates KDM2A to promote cell proliferation and migration by targeting miR-342-3p in gastric cancer. Onco Targets Ther. 2018;11:6383-6394.
23. Liu W, Kang L, Han J, et al. miR-342-3p suppresses hepatocellular carcinoma proliferation through inhibition of IGF-1R-mediated Warburg effect. Onco Targets Ther. 2018;11:1643-1653.
24. Xue X, Fei X, Hou W, Zang Y, Liu L, Hu R. miR-342-3p suppresses cell proliferation and migration by targeting AGR2 in non-small cell lung cancer. Cancer Lett. 2018;412:170-178.
25. Shoemaker AR, Oleksijew A, Bauch J, et al. A small-molecule inhibitor of Bcl-XL potentiates the activity of cytotoxic drugs in vitro and in vivo. Cancer Res. 2006;66(17):8731-8739.
26. Tan N, Malek M, Zha J, et al. Navitoclax enhances the efficacy of taxanes in non-small cell lung cancer models. Clin Cancer Res. 2011;17(6):1394-1404.
27. Wong M, Tan N, Zha J, et al. Navitoclax (ABT-263) reduces Bel-x(L)-mediated chemoresistance in ovarian cancer models. Mol Cancer Ther. 2012;11(4):1026-1035.