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LINC01535 promotes proliferation and inhibits apoptosis in esophageal squamous cell cancer by activating the JAK/STAT3 pathway

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Abstract. – OBJECTIVE: To detect the expression of long intergenic non-coding ribonucleic acid (LINC) 01535 in esophageal squamous cell cancer (ESCC) tissues and cells, and to investigate the influences of LINC01535 on the proliferation and apoptosis of ESCC cells and the possible mechanism.

PATIENTS AND METHODS: Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to determine the relative expression of LINC01535 in 43 cases of ESCC tissues and human esophageal cancer cells (KYSE30, EC9706, TE-13, and Ecal09) compared with human esophageal mucosal epithelial cells (HET-1A). The esophageal cancer cells with the highest expression were selected and transfected with small interfering RNA (si)-LINC01535 (experimental group) or si-negative control (NC) (control group). The interference efficiency was measured *via* qRT-PCR assay. Regulatory effects of LINC01535 on cell proliferative capacity was examined through colony formation assay and cell proliferation assay [Cell Counting Kit-8 (CCK-8)]. Cell cycle and apoptosis influenced by LINC01535 were detected *via* flow cytometry. Western blotting was applied to determine the expression changes in the molecular markers of the Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) signaling pathway.

RESULTS: LINC01535 expression in ESCC tissues and cells was remarkably higher than that in para-carcinoma tissues and esophageal mucosal epithelial cells. Knockdown of LINC01535 decreased proliferative capacity, arrested cell cycle in G1/G0 phase, and increased apoptotic rate of ESCC cells. The expressions of the molecular markers of the JAK/STAT3 signaling pathway were altered after knockdown of LINC01535. The above results illustrated that LINC01535 accelerated the proliferation but repressed the apoptosis of ESCC cells by regulating the JAK/STAT3 signaling pathway.

CONCLUSIONS: The expression of LINC01535 is up-regulated in ESCC tissues and cells, and the highly expressed LINC01535 promotes the proliferation and inhibits the apoptosis of ESCC cells by regulating the JAK/STAT3 signaling pathway. Our findings provide new directions for the diagnosis and treatment of esophageal cancer.

Key Words:
LINC01535, Esophageal squamous cell cancer, Biological function, JAK/STAT3 signaling pathway.

Introduction

Esophageal squamous cell cancer (ESCC) is one of the tumors with a high morbidity rate and poor prognosis in the world. China has a high incidence of ESCC, with a morbidity rate up to 29.81/100,000 in some regions[1]. With the changes in people's eating habits in recent years, the morbidity rate of ESCC is on the rise in partial provinces and cities in China, posing a huge threat to human health[2]. In spite of great progress in the diagnosis and treatment of ESCC, the prognosis is still far from satisfactory, which is largely attributed to the unclear potential molecular mechanism of the malignant phenotype formation of ESCC. Therefore, it is particularly important to investigate the molecular mechanism[3].

Various long non-coding ribonucleic acids (lncRNAs) play crucial roles in the occurrence and development of tumors[4]. As a category of non-coding RNAs with a length of about 200 nt, lncRNAs participate in a variety of physiological and pathological processes through the epigenetic regulation of gene expressions. LncRNAs have vital functions and significant diagnostic values in digestive system neoplasms, such as gastric cancer, colorectal cancer, and liver cancer[5-7].

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The potential relationship between lncRNAs and esophageal cancer in China and foreign countries has been reported. Chen et al[8] revealed that the expression of SET binding factor 2-antisense RNA 1 (SBF2-AS1) is markedly up-regulated in ESCC cells, and the proliferative and invasive abilities of ESCC cells are weakened significantly after silencing SBF2-AS1. Therefore, SBF2-AS1 is expected to become a potential therapeutic target of ESCC. Chu et al[9] found that lncRNA MNX1-AS1 sponges the expression of microRNA (miR-34a) to control the expression of SIRT1, thus facilitating the formation of malignant phenotype of ESCC. The aforementioned studies implied that the differential expressions of lncRNAs are involved in the occurrence and development of esophageal cancer. However, the expression and biological function of long non-coding RNA (LINC) 01535 in ESCC have not been reported yet. Our analysis uncovered that LINC01535 expression is up-regulated in ESCC, and it promotes the proliferation and inhibits the apoptosis of ESCC cells. The potential molecular mechanism of LINC01535 in the genesis and progression of ESCC was preliminarily explored in this research, thus providing promising targets for the clinical treatment of ESCC patients.

Patients and Methods

Tissue Specimens

A total of ESSC 43 patients who received surgical resection in Zhongshan Hospital, Fudan University from January 2015 to December 2016 were selected. ESSC tissues and normal para-carcinoma tissues (> 6 cm away from the tumor) were obtained within 30 min after the specimens were isolated, and they were immediately stored in liquid nitrogen. This research was approved by the Ethics Committee of the hospital, and all the patients signed the informed consent.

Cell Culture

Human esophageal cancer cells (KYSE30, EC9706, TE-13, and Eca109) and human esophageal mucosal epithelial cells (HET-1A) were purchased from Cell Bank of Type Culture Collection Committee of China Academy of Sciences (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone; South Logan, UT, USA). The cells were cultured in an incubator with 5% CO_2 at 37°C and subcultured regularly.

Cell Transfection

Esophageal cancer cells in the logarithmic growth phase were seeded into a 6-well plate. Until 40-50% confluence, cells were transfected using the Lipofectamine 2000 transfection reagents (Invitrogen, Carlsbad, CA, USA) according to the instructions. In this experiment, control group [small interfering RNA (si)-negative control (NC)] and experimental group (si-LINC01535, Shanghai Genechem Co., Ltd., Shanghai, China) were set, with a final concentration of transfection at 100 nmol/L. 24 h later, the medium was replaced with a fresh one. LINC01535 siRNA: F 5'-GUUCAUAAGCACGUAUUGAAA-3', R: 5'-UCAAUACGUGCUUAUGAACA A-3'.

RNA Extraction, Reverse Transcription and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNAs were extracted *via* TRIzol reagent method (Invitrogen, Carlsbad, CA, USA) in accordance with the instructions, and the purity and concentration of the RNAs were measured using NanoDrop ultraviolet spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA). Then, 1.5 μg of RNAs were reversely transcribed into cDNAs by PrimeScript RT Master Mix, followed by preservation at –20°C. Later, qRT-PCR assay was conducted in ABI 7300 system (API; Applied Biosystems, Foster City, CA, USA) using SYBR Select Master Mix (API; Applied Biosystems, Foster City, CA, USA), with the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the normalized internal reference. The expression levels of related RNAs were calculated by the $2^{-\Delta\Delta Ct}$ method. The primers were as follows: GAPDH: Forward 5'-CCAGCCGAGCCACATCGCTC-3'; Reverse 5'-ATGAGCCCCAGCCTTCTCCAT-3', Linc01535: Forward 5'-GGGCGGCAGGTCACTGACAC-3'; Reverse 5'-GCCAGCAGCCGCTGGCTTAG-3'.

Colony Formation Assay

At 48 h after transfection, the cells were digested with trypsin, collected, and then adjusted into single-cell suspension. After that, the cells were inoculated into new 6-well plates (1,000 cells/well) for continuous culture for 10 d. Next, cells were fixed in 1 mL of paraformaldehyde for 15 min and stained in 0.1% crystal violet solution for 15 min. After washing with phosphate-buffered saline (PBS) twice, visible colonies were captured.

Cell Counting Kit-8 (CCK-8) Assay

The cells were digested with trypsin and harvested at 48 h after transfection, and then the cell concentration was adjusted to 1.5×10^4/mL. Later, the cells were seeded into a 96-well plate at 3,000 cells/well. 10 μL of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added into each well for incubation at 37°C with 5% CO_2 for 2 h. Then, the absorbance at the wavelength of 450 nm (A_{450} value) in every well was detected using a microplate reader at 0, 24, 48, 72, and 96 h for plotting growth curves.

Detection of Cell Cycle Via Flow Cytometry

The cells were digested with trypsin and then collected at 48 h after transfection. Next, the cells collected were fixed in 70% ethanol overnight according to the operations of cell cycle detection kits (AmyJet Scientific Inc., Wuhan, China). Cells were incubated with 100 μL of propidium iodide (PI) in the dark at 4°C for 30 min, and cell cycle distribution was detected using a flow cytometer.

Detection of Apoptosis Via Flow Cytometry

At 48 h after transfection, the cells were digested in trypsin, harvested and resuspended in 200 μL of PBS. Cells were incubated with 10 μL of FITC and 1 μL of PI in the dark at 4°C for 20 min. Finally, 300 μL of PBS was added and mixed, and the mixture was examined using the flow cytometer.

Western Blotting

Following trypsin digestion and collection at 48 h after transfection, the cells were lysed with lysis buffer to extract the total proteins. After denaturation through a water bath, the protein loading buffer was added, and the proteins were separated *via* electrophoresis using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) *via* electroblotting and sealed in 5% bovine serum albumin at room temperature for 1 h. Subsequently, primary antibodies (diluted at 1:1000) was added for incubation at 4°C overnight, and secondary for incubation antibodies at room temperature for 2 h, followed by image development by virtue of enhanced chemiluminescence (ECL) developer.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was employed for statistical analysis. The measurement data were expressed by mean ± standard deviation ($\chi \pm s$), and the *t*-test was adopted for comparison between two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

LINC01535 Expression Was Up-Regulated in ESCC

First of all, the expression level of LINC01535 in 43 cases of ESCC tissues was detected by qRT-PCR assay. Compared with that in para-carcinoma tissues, the expression of LINC01535 was up-regulated in 37 cases of ESCC tissues (Figure 1A). Next, the expression level of LINC01535 in ESCC cells was measured, and it was shown that the expression of LINC01535 was highly expressed in comparison with that in human esophageal mucosal epithelial HET-lA cells (Figure 1B). Two types of cells with the highest fold change of expression were utilized for subsequent experiments. ESCC cells were transfected with si-LINC01535, and the interference efficiency was determined through qRT-PCR 48 h later (Figure 1C and 1D).

Biological Functions of LINC01535 in ESCC Cells

After the transient transfection of si-LINC01535 or si-NC into the ESCC cells, CCK-8 assay was performed to investigate the changes in the cell proliferative capacity. The results manifested that the cell proliferative capacity declined after knockdown of LINC01535 (Figure 2A and 2B). To verify the results of CCK-8 assay, the colony formation assay was implemented, and the same results were obtained (Figure 2C and 2D). Subsequently, the impact of LINC01535 on the ESCC cell apoptosis was probed, and it is indicated that the apoptosis rate was raised in experimental group compared with that in control group (Figure 2E and 2F).

LINC01535 Regulated the Janus Kinase/Signal Transducer and Activator of Transcription 3 (JAK/STAT3) Signaling Pathway

The influence of LINC01535 on cell cycle distribution of ESCC cells was detected *via* flow cytometry.

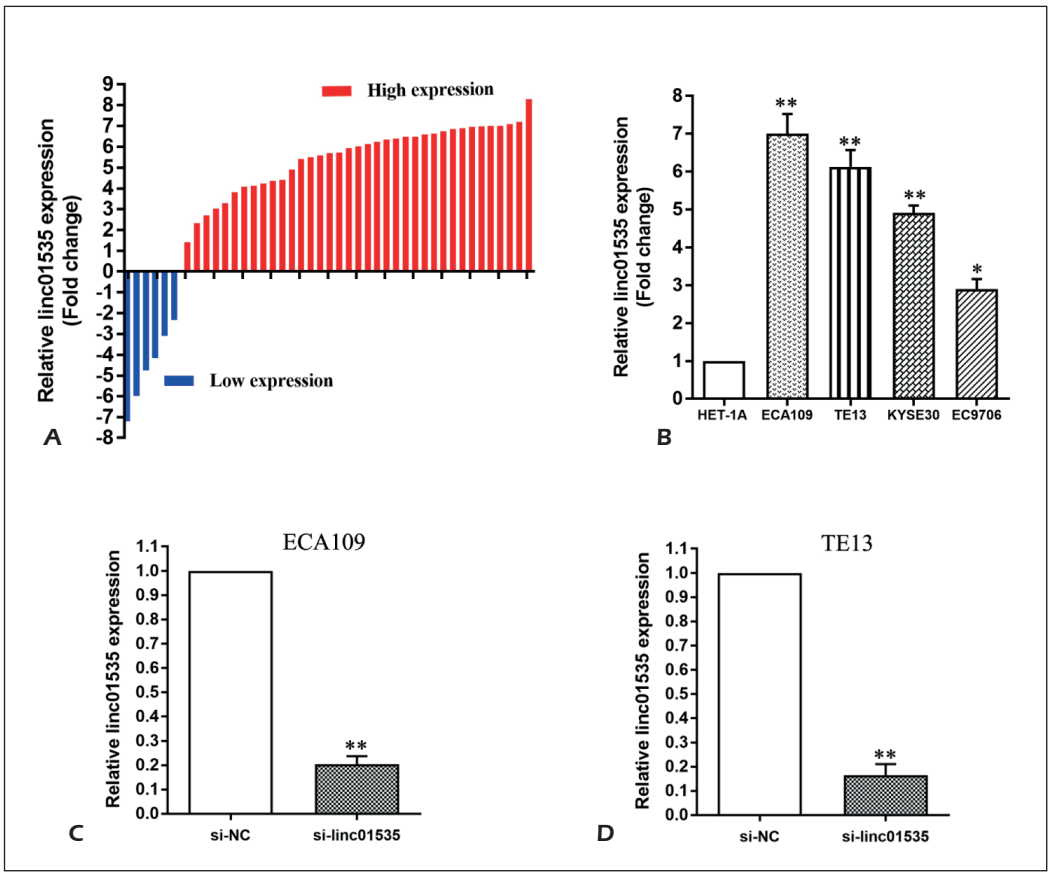

Figure 1. LINC01535 expression is up-regulated in ESCC. **A**, Expression level of LINC01535 in 43 cases of ESCC tissues and para-carcinoma tissues detected via qRT-PCR. It is up-regulated in 37 cases, with GAPDH as the internal reference. **B**, Relative expression level of LINC01535 in ESCC cells measured through qRT-PCR. **C-D**, Interference efficiency determined by qRT-PCR after transient transfection of si-LINC01535 or si-NC into ESCC cells.

The results manifested that cell cycle progression was arrested at the G1/G0 phase after knockdown of LINC01535 (Figure 3A and 3B). After that, the effect of LINC01535 on its downstream signaling pathway was investigated. According to the Western blotting results, the expressions of the molecular markers of the JAK/STAT3 signaling pathway were altered after knockdown of LINC01535 compared with those in control group (Figure 3C and 3D). The above results illustrated that LINC01535 accelerated the proliferation but repressed the apoptosis of ESCC cells by regulating the JAK/STAT3 signaling pathway.

Discussion

The cancer-related mortality caused by esophageal cancer, one of the malignant tumors with high invasiveness, ranks sixth in the world[10]. China is a high-incidence country of esophageal cancer. About 50% of global new esophageal cancer cases occur in China, and ESCC accounts for 90%[11]. The malignant proliferation and anti-apoptosis of esophageal cancer are the most important causes of tumor deaths. Hence, deeply studying the pathogenesis and the specific molecular mechanism of malignant phenotypes of esophageal cancer can provide bases for the early diagnosis and prognosis assessment of esophageal cancer.

Studies[12-14] have demonstrated that abnormally expressed lncRNAs are associated with the occurrence and development of tumors and many researchers have reported that lncRNAs with abnormal expressions in ESCC tissues act as oncogenes or tumor suppressors, including UCA1, AFAP1-AS1, and SPRY4-IT1[15-17]. The expression of LINC01535, first reported in cervical cancer by Song et al[18], is up-regulated in cervical cancer and promotes the proliferation and metastasis of the disease by modulating EZH2 expression through absorbing miR-214. It was found by this

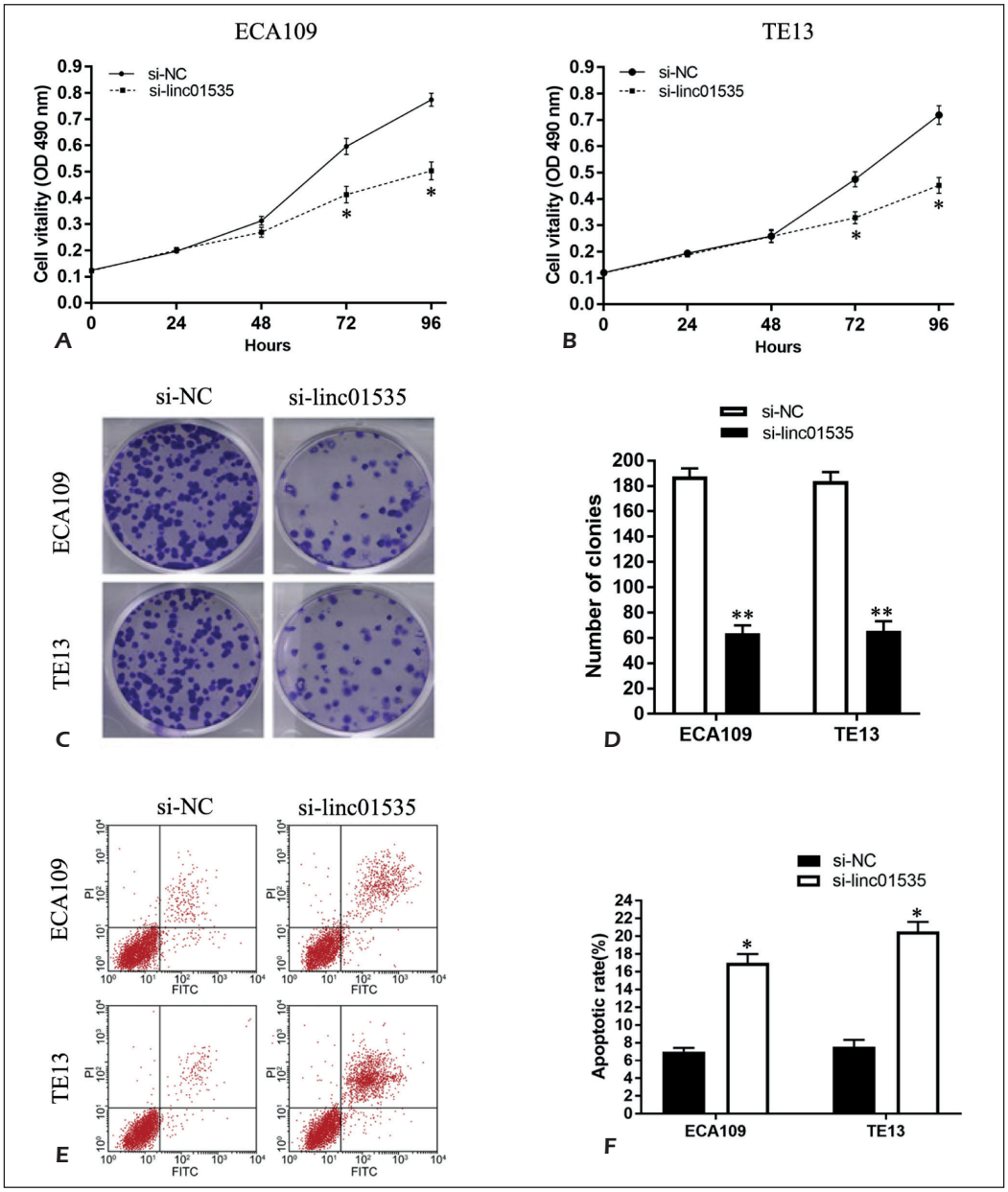

Figure 2. Biological functions of LINC01535 in ESCC cells. **A-B**, Influence of silenced LINC01535 on ESCC cell proliferative capacity tested by CCK-8 assay. **C-D**, Changes in proliferative capacity of ESCC cells after interference in LINC01535 expression in ESCC cells determined through colony formation assay (40X). **E-F**, Changes in apoptosis rate of ESCC cells at 48 h after knockdown of LINC01535 expression in ESCC cells measured via flow cytometry.

research group for the first time that LINC01535 was highly expressed in ESCC tissues and cells, and it promoted the proliferation and inhibited the apoptosis of ESCC cells.

The JAK/STAT signaling pathway is a cell signaling pathway activated by JAK, an epidermal growth factor receptor, which mainly contains the JAK family and the STAT family. The activation/inactivation of the JAK/STAT signaling pathway is related to the development of multiple tumors, such as liver cancer and ESCC[18,19]. LncRNAs can participate in the regulation of the JAK/STAT3 signaling pathway as crucial regulatory factors. LncRNA FEZF1-AS1 can stimulate the occurrence and progression of ovarian cancer by activating the JAK/STAT3 signaling pathway[20]. Here, we have verified that LINC01535 was able to regulate key genes in the JAK/STAT3 signaling pathway.

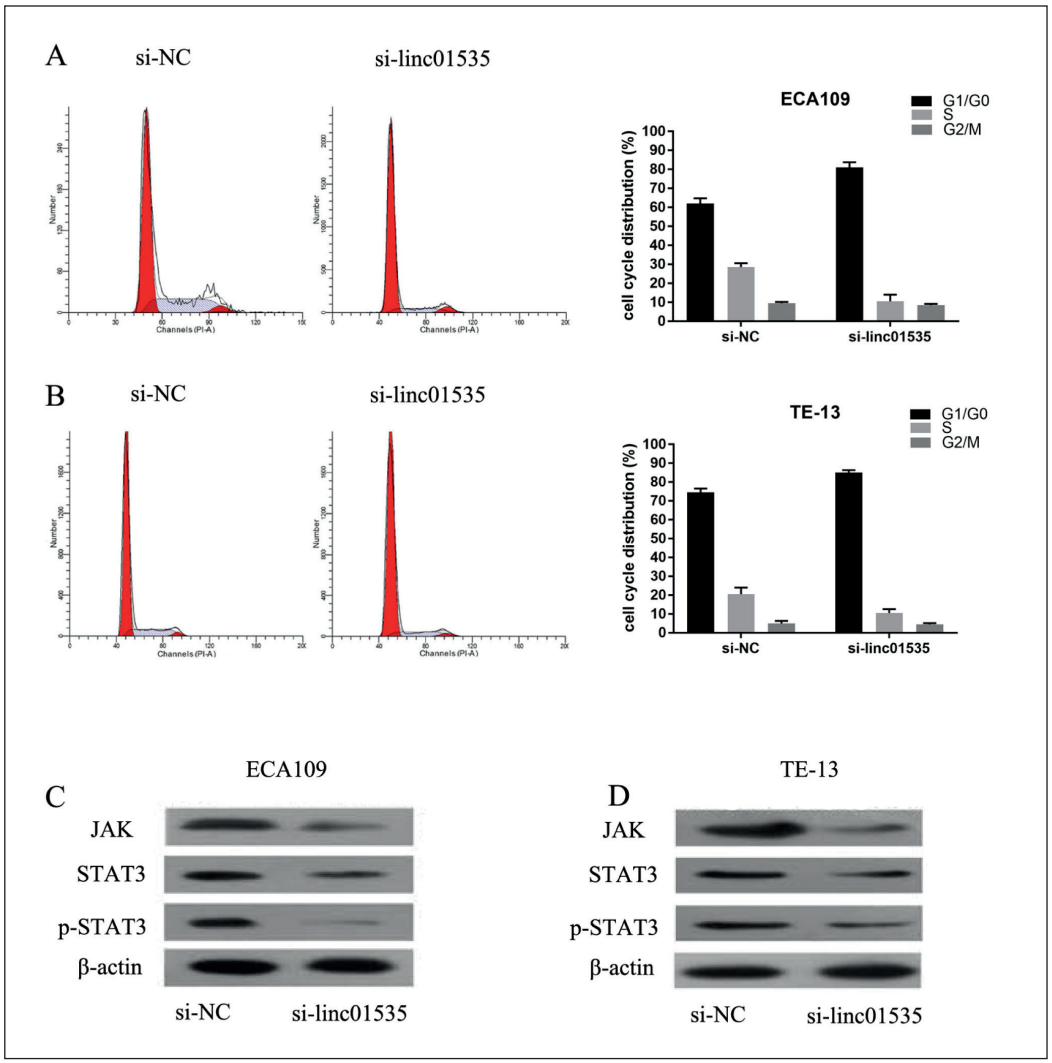

Figure 3. LINC01535 controls the JAK/STAT3 signaling pathway. ***A-B***, Influence of silenced LINC01535 on ESCC cell cycle distribution detected via flow cytometry. ***C-D***, Changes in expressions of molecular markers of the JAK/STAT3 signaling pathway after knockdown of LINC01535 expression examined by Western blotting assay.

Conclusions

This research group was the first to reveal that LINC01535 facilitated the proliferation and suppressed the apoptosis of ESCC cells by modulating the JAK/STAT3 signaling pathway. LINC01535/JAK/STAT3 could become potential targets for the gene therapy of esophageal cancer.

Conflict of Interests

The Authors declare that they have no conflict of interests.

References

1) Xu LJ, Yu XJ, Wei B, Hui HX, Sun Y, Dai J, Chen XF. Long non-coding RNA DUXAP8 regulates proliferation and invasion of esophageal squamous cell cancer. Eur Rev Med Pharmacol Sci 2018; 22: 2646-2652.
2) Blackham AU, H NS, Schell MJ, Jin W, Gangi A, Almhanna K, Fontaine JP, Hoffe SE, Frakes J, Venkat P, Pimiento JM. Recurrence patterns and associated factors of locoregional failure following neoadjuvant chemoradiation and surgery for esophageal cancer. J Surg Oncol 2018; 117: 150-159.
3) Noordman BJ, Verdam M, Lagarde SM, Shapiro J, Hulshof M, van Berge HM, Wijnhoven B, Nieuwenhuijzen G, Bonenkamp JJ, Cuesta MA, Plukker

J, Spillenaar BE, Steyerberg EW, van der Gaast A, Sprangers M, van Lanschot J. Impact of neoadjuvant chemoradiotherapy on health-related quality of life in long-term survivors of esophageal or junctional cancer: results from the randomized CROSS trial; CROSS Study Group. Ann Oncol 2018; 29: 445-451.

4) Talebi A, Masoodi M, Mirzaei A, Mehrad-Majd H, Azizpour M, Akbari A. Biological and clinical relevance of metastasis-associated long noncoding RNAs in esophageal squamous cell carcinoma: a systematic review. J Cell Physiol. 2019 Jul 16. doi: 10.1002/jcp.29083. [Epub ahead of print]

5) Cheng C, Wang Q, Zhu M, Liu K, Zhang Z. Integrated analysis reveals potential long non-coding RNA biomarkers and their potential biological functions for disease free survival in gastric cancer patients. Cancer Cell Int 2019; 19: 123.

6) Pan H, Pan J, Song S, Ji L, Lv H, Yang Z. Identification and development of long non-coding RNA-associated regulatory network in colorectal cancer. J Cell Mol Med 2019; 23: 5200-5210.

7) Wu DM, Zheng ZH, Zhang YB, Fan SH, Zhang ZF, Wang YJ, Zheng YL, Lu J. Down-regulated lncRNA DLX6-AS1 inhibits tumorigenesis through STAT3 signaling pathway by suppressing CADM1 promoter methylation in liver cancer stem cells. J Exp Clin Cancer Res 2019; 38: 237.

8) Chen R, Xia W, Wang X, Qiu M, Yin R, Wang S, Xi X, Wang J, Xu Y, Dong G, Xu L, De W. Upregulated long non-coding RNA SBF2-AS1 promotes proliferation in esophageal squamous cell carcinoma. Oncol Lett 2018; 15: 5071-5080.

9) Chu J, Li H, Xing Y, Jia J, Sheng J, Yang L, Sun K, Qu Y, Zhang Y, Yin H, Wan J, He F. LncRNA MNX1-AS1 promotes progression of esophageal squamous cell carcinoma by regulating miR-34a/SIRT1 axis. Biomed Pharmacother 2019; 116: 109029.

10) Miller KD, Nogueira L, Mariotto AB, Rowland JH, Yabroff KR, Alfano CM, Jemal A, Kramer JL, Siegel RL. Cancer treatment and survivorship statistics, 2019. CA Cancer J Clin 2019; 69: 363-385.

11) Ferlay J, Colombet M, Soerjomataram I, Mathers C, Parkin DM, Pineros M, Znaor A, Bray F. Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. Int J Cancer 2019; 144: 1941-1953.

12) Svoboda M, Slyskova J, Schneiderova M, Makovicky P, Bielik L, Levy M, Lipska L, Hemmelova B, Kala Z, Protivankova M, Vycital O, Liska V, Schwarzova L, Vodickova L, Vodicka P. HOTAIR long non-coding RNA is a negative prognostic factor not only in primary tumors, but also in the blood of colorectal cancer patients. Carcinogenesis 2014; 35: 1510-1515.

13) Zhao X, Liu Y, Li Z, Zheng S, Wang Z, Li W, Bi Z, Li L, Jiang Y, Luo Y, Lin Q, Fu Z, Rufu C. Linc00511 acts as a competing endogenous RNA to regulate VEGFA expression through sponging hsa-miR-29b-3p in pancreatic ductal adenocarcinoma. J Cell Mol Med 2018; 22: 655-667.

14) Zhang JX, Chen ZH, Chen DL, Tian XP, Wang CY, Zhou ZW, Gao Y, Xu Y, Chen C, Zheng ZS, Weng HW, Ye S, Kuang M, Xie D, Peng S. LINC01410-miR-532-NCF2-NF-kB feedback loop promotes gastric cancer angiogenesis and metastasis. Oncogene 2018; 37: 2660-2675.

15) Wang X, Gao Z, Liao J, Shang M, Li X, Yin L, Pu Y, Liu R. LncRNA UCA1 inhibits esophageal squamous-cell carcinoma growth by regulating the Wnt signaling pathway. J Toxicol Environ Health A 2016; 79: 407-418.

16) Zhou XL, Wang WW, Zhu WG, Yu CH, Tao GZ, Wu QQ, Song YQ, Pan P, Tong YS. High expression of long non-coding RNA AFAP1-AS1 predicts chemoradioresistance and poor prognosis in patients with esophageal squamous cell carcinoma treated with definitive chemoradiotherapy. Mol Carcinog 2016; 55: 2095-2105.

17) Cui F, Wu D, He X, Wang W, Xi J, Wang M. Long noncoding RNA SPRY4-IT1 promotes esophageal squamous cell carcinoma cell proliferation, invasion, and epithelial-mesenchymal transition. Tumour Biol 2016; 37: 10871-10876.

18) Song H, Liu Y, Jin X, Liu Y, Yang Y, Li L, Wang X, Li G. Long non-coding RNA LINC01535 promotes cervical cancer progression via targeting the miR-214/EZH2 feedback loop. J Cell Mol Med 2019; 23:6098-6111.

19) Fang J, Chu L, Li C, Chen Y, Hu F, Zhang X, Zhao H, Liu Z, Xu Q. JAK2 inhibitor blocks the inflammation and growth of esophageal squamous cell carcinoma in vitro through the JAK/STAT3 pathway. Oncol Rep 2015; 33: 494-502.

20) Zhao X, Cheng Z, Wang J. Long noncoding RNA FEZF1-AS1 promotes proliferation and inhibits apoptosis in ovarian cancer by activation of JAK-STAT3 pathway. Med Sci Monit 2018; 24: 8088-8095.