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# LncRNA FER1L4 suppressed cancer cell growth and invasion in esophageal squamous cell carcinoma

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**Abstract. – OBJECTIVE: To investigate the regulatory effect of long non-coding ribonucleic acid (lncRNA) FER1L4 on biological behaviors of esophageal squamous cell carcinoma (ESCC) cells, such as proliferation and invasion.**

**PATIENTS AND METHODS: The expressions of FER1L4 were detected in 42 pairs of ESCC tissues and corresponding para-carcinoma tissues and 5 kinds of ESCC cell lines *via* quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Polyethyleneimine (PEI) and liposomes were used for FER1L4 expression or interference elimination assays, respectively. The proliferation and invasion of ESCC cells were detected *via* MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), apoptosis assay, cell cycle assay, and transwell chamber.**

**RESULTS: Results of qRT-PCR showed that, compared with that in normal tissues, FER1L4 was lowly expressed in ESCC tissues. Overexpression of FER1L4 could inhibit cell proliferation and invasion, promote apoptosis and increase the cell cycle distribution in G0/G1 phase. Knockout of FER1L4 could promote the proliferation and invasion of ESCC cells, inhibit apoptosis and decrease the cell cycle distribution in G0/G1 phase.**

**CONCLUSIONS: FER1L4 is involved in the occurrence and development of ESCC and plays a key role as a tumor suppressor gene in ESCC.**

Key Words
lncRNA FER1L4, Esophageal squamous cell carcinoma, Proliferation, Invasion.

## Introduction

Esophageal cancer is widespread around the world, whose incidence rate ranks 9th in tumors and mortality rate ranks 6th in cancer-related deaths. The number of male patients is larger than that of female patients[1,2]. In esophageal cancer, esophageal squamous cell carcinoma (ESCC) and adenocarcinoma are the main pathological types, the former of which accounts for more than 90% in China[3]. About 400,000 people die of esophageal cancer each year in the world, and China is one of the high-prevalence areas of esophageal cancer in the world, in which more than 150,000 people die of this disease each year on average. In recent years, studies[4-7] have shown that the expression disorder of long non-coding ribonucleic acid (lncRNA) is involved in the occurrence and development of a variety of diseases. In particular, it plays an important role in tumorigenesis. Therefore, determining the tumor-associated lncRNAs and deeply analyzing their molecular and biological functions play a vital role in clarifying the occurrence and development of tumors.

FER1L4 is located on the q23 region of chromosome 10 and encodes a tumor suppressor lncRNA. Recent studies have shown that FER1L4 is involved in the occurrence and development of a variety of tumors. FER1L4 is expressed in many normal tissues, but its expression is deleted in some human tumors, including gastric cancer[8], liver cancer[9], intestinal cancer[10], and endometrial cancer[11]. However, the correlations of FER1L4 expression in esophageal cancer with occurrence and development of esophageal cancer remain unclear.

In this paper, the effects of FER1L4 on proliferation and invasion of esophageal cancer cells were detected *via* quantitative reverse transcription-polymerase chain reaction (qRT-PCR), methyl thiazolyl tetrazolium (MTT), apoptosis assay, cell cycle assay, and transwell chamber. Also, the role of lncRNA FER1L4 in esophageal cancer was preliminarily investigated, so as to provide a certain


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experimental basis for the development of new clinical diagnostic kits for esophageal cancer in the future, and provide new therapeutic targets for the development of new drugs for esophageal cancer.

## Patients and Methods

### Tumor Samples and Cell Lines

A total of 42 cases of tumor tissues (tumor group) and 42 cases of para-carcinoma normal tissues (control group) surgically resected and collected from patients with esophageal cancer in the Pathology Department of our hospital from 2014 to 2017 were selected. Patients did not receive any other treatment means (including radiotherapy, chemotherapy, and cell therapy), and they all signed the informed consent. After surgical resection, esophageal cancer tissues were immediately cryopreserved in liquid nitrogen for subsequent extraction of RNA. This study was approved by the Ethics Committee of Gansu Province Hospital.

Human esophageal squamous cell cancer (ESCC) cell lines Eca109, EC9706, KYSE30, KYSE150, and KYSE450 and human normal esophageal epithelial cell line (HEEC) were cultured in the Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in an incubator with 5% $CO_2$ at 37°C. When 80%-90% cells were fused, they were digested with 0.25% trypsin, followed by a passage for subsequent experiments.

### RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from cells using TRIzol, and then, its purity and concentration were determined using a Nanodrop micro-quantometer. Reverse transcription reaction was performed according to instructions of the TaKaRa (Otsu, Shiga, Japan) kit to synthesize complementary deoxyribonucleic acid (cDNA). Then, the expression levels of FER1L4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were detected *via* RT-PCR with cDNA as a template and GAPDH as an internal reference. The PCR system was 20 μL, and reaction conditions are as follows: pre-denaturation at 95°C for 1 min, denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, a total of 33 cycles. The relative expression of FER1L4 was calculated using the ratio of fluorescence intensity of FER1L4 product to that of GAPDH amplification product, and each experiment was repeated for 3 times. The reaction using $ddH_2O$ as a template was used as a negative control for PCR amplification. PCR primers used in the experiment are as follows: FER1L4 forwards, 5'-ACACCTTGTGGGCAAGTTCA-3' and reverse, 5'-CACCACGTAAGGGTCTGCTT-3'; GAPDH forwards, 5'-GTCAACGGA TTTGGTCTGTAT T-3' and reverse, 5'-AGTCTTCTGGGTGGCAGTGAT-3'.

### Cells Transfection

Lipofectamine 2000 was used for small-interfering RNA (siRNA) interference experiment. 6 μL Lipofectine 2000 was added to 500 μL Opti-MEM and placed at room temperature for 5 min. Then, 5 μL siRNA of FER1L4 (5›-UUAGGUAAGAGGGACAGCUGGCUGG-3') was added to 500 μL Opti-MEM and mixed evenly. Lipofectamine 2000 and siRNA mixture were placed at room temperature for 20 min, dripped into the culture dish with KYSE30 cells and mixed evenly for culture. After 48 h of transfection, the interference efficiency of siRNA for FER1L4 was detected *via* RT-PCR.

PcDNA3-FER1L4-overexpressed plasmid: 200 μL serum-free and antibiotic-free RMPI-1640 medium was added into a 1.5 mL Eppendorf (EP) tube, and 6 μL polyethyleneimine (PEI) and 2 μg pcDNA3-FER1L4 plasmid were also added. The mixture was shaken for 10 s, placed at room temperature for 15 min, and added into cells in each group. At 12 h after transfection, the medium was replaced. At 48 h after transfection, overexpression and transfection efficiency were detected *via* RT-PCR.

### MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

Cells with FER1L4 knockout or overexpression were digested with trypsin and ethylene diamine tetraacetic acid (EDTA), prepared into the single cell suspension, and inoculated into a 96-well plate at a density of $2\times10^3$ cells per well. RMPI-1640 medium containing 10% FBS was added into each well for incubation under 5% $CO_2$ at 37°C for 24 h. After adherence, cells were starved with serum-free medium for 24 h and cultured with a normal medium for 24, 48, and 72 h. Then, 20 μL MTT (5 mg/mL) was added to each well and incubated at 37°C for 4 h, and the supernatant was discarded. After 150 μL dimethylsulfoxide (DMSO) was added to each well to dissolve the crystal violet formed, the absorbance value was measured using a microplate reader, and the relative cell proliferative activity was calculated.

### Cell Apoptosis Analysis

Cells were seeded into a 6-well plate and subjected to apoptosis assay. Cells were digested with ethylene diamine tetraacetic acid (EDTA)-free trypsin, and the digestion was stopped using complete medium. Then, cells were collected, 250 μL 1xBinding Buffer was added to each tube to re-suspend cells, and cell concentration was adjusted to $1\times10^6$/mL. After that, 100 μL cell suspension was taken, added with 5 μL Annexin V/fluorescein isothiocyanate (FITC) and 10 μL 20 μg/mL propidium iodide (PI) solution, and incubated at room temperature in darkness for 15 min. Lastly, 300 μL Binding Buffer was added to the reaction tube, and the suspension was loaded onto a flow cytometer for detection.

### Cell Cycle Analysis

Cells were inoculated into the 6-well plate and followed by cell cycle detection. Cells were collected after conventional trypsinization and fixed in 70% ethanol at 4°C for 48 h. Cells were harvested after centrifugation and stained with 500 μL PI staining solution containing RNase for 1 h. Lastly, flow cytometer was used for detection.

### Transwell Assay

The upper chamber surface of the bottom membrane of the transwell chamber was dried with 50 mg/L Matrigel with 1:8 dilution, and dried at 4°C. A diluted Matrigel (3.9 μg/μL) 60-80 μL is added to the polycarbonate film on the upper chamber, and the Matrigel is polymerized into a gel at a temperature of 37°C for 30 min. After 48 h of transfection, the cells were digested with trypsin. The cells were washed with PBS about 2 times and suspended with serum containing BSA medium. The cell density was adjusted to $5x10^4$/mL. 6 mL FBS containing medium was added to the lower chamber. After 24 h of normal culture, the basement membrane of the lower chamber was removed.

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) Statistics 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of experimental data. One-way analysis of variance was used for multiple groups of measurement data in line with normal distribution and homogeneity of variance, and rank sum test was used for the comparison of data not in line with normal distribution or homogeneity of variance. $p<0.05$ suggested that the difference was statistically significant.

## Results

### LncRNA FER1L4 Expression was Decreased in ESCC Tissues and Cell Lines

The expression level of FER1L4 in esophageal cancer tissues was significantly lower than that in normal esophageal tissues ($p<0.05$) (Figure 1A), suggesting that the down-regulation of FER1L4 expression may be related to the occurrence and development of esophageal cancer, and it may, as a tumor suppressor gene, be involved in the occurrence of esophageal cancer.

Also, the relative expression level of FER1L4 in each ESCC cell line was detected *via* qRT-PCR. Results showed that the expression levels of FER1L4 in all ESCC cell lines were decreased compared with that in HEEC, and the relative expression level of FER1L4 was the highest in KYSE30 cells and the lowest in KYSE450 cells (Figure 1B). According to these results, KYSE30 cells were selected for the FER1L4 knockout in subsequent cell function experiments, and KYSE450 cells were selected for FER1L4 overexpression (Figure 1C).

### LncRNA FER1L4 Inhibited ESCC Cell Growth In Vitro

The cell proliferative activity of KYSE450 cells with high expression of FER1L4 was detected *via* MTT assay. After overexpression of FER1L4, the proliferative activity of KYSE450 cells were significantly reduced ($p<0.05$) (Figure 2A).

To further confirm the role of FER1L4 in the proliferative activity of esophageal cancer cells, the expression of FER1L4 was interfered with siRNA transfection, and KYSE30 cell lines with low expression of FER1L4 were constructed. Results of MTT assay showed that the proliferative activity of KYSE30 cells was significantly increased after FER1L4 knockout ($p<0.05$) (Figure 2B). The above results indicate that FER1L4 has the activity of tumor suppressor gene and can inhibit the proliferation of ESCC cells.

### LncRNA FER1L4 Promoted Cell Apoptosis and Induced Cell Cycle Arrest at G0/G1 Phase

We further detected the mechanism of how FER1L4 inhibiting the proliferation of NSCLC cells with cell apoptosis and cell cycle analysis. After overexpression of FER1L4, the cell apoptosis activity of KYSE450 cells was significantly increased ($p<0.05$) (Figure 3A). Meanwhile, KYSE30 cells was significantly decreased after FER1L4 knockout ($p<0.05$).

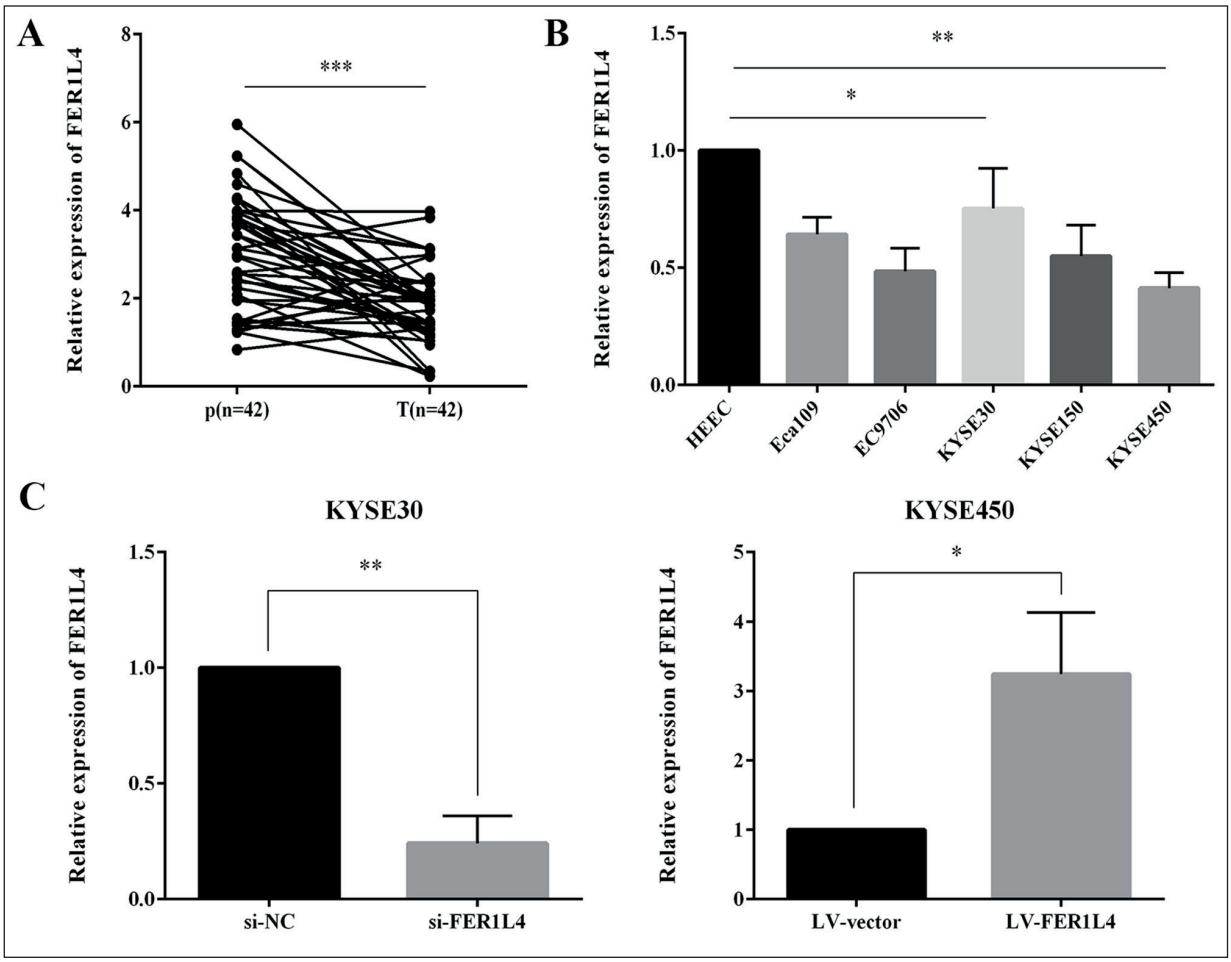


**Figure 1.** LncRNA FER1L4 expression was decreased in ESCC tissues and cell lines. **A**, Analysis of FER1L4 expression in paracarcinoma tissues (P) and tumor tissues (T); **B**, Analysis of FER1L4 expression in several ESCC cell lines and normal cell line; **C**, Analysis of transfection efficiency in KYSE30 cells and KYSE450 cells. *$p$<0.05; **$p$<0.01; ***$p$<0.001.

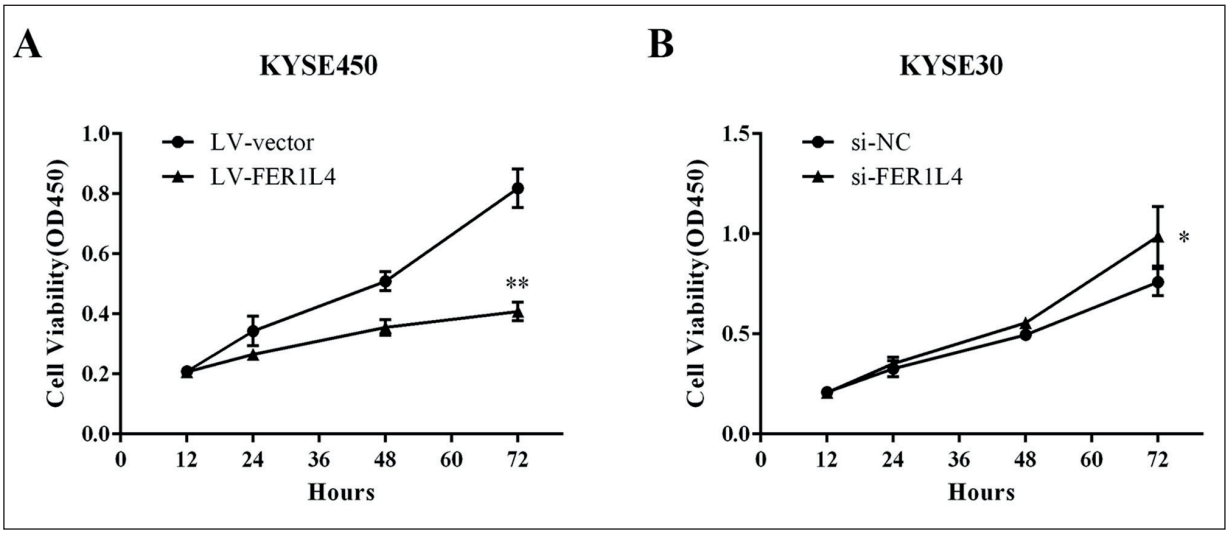


**Figure 2.** LncRNA FER1L4 inhibited ESCC cell growth in vitro. A, MTT assay was performed to determine the viability of transfected KYSE450 cells; B, MTT assay was performed to determine the viability of transfected KYSE30 cells. *$p$<0.05; **$p$<0.01.

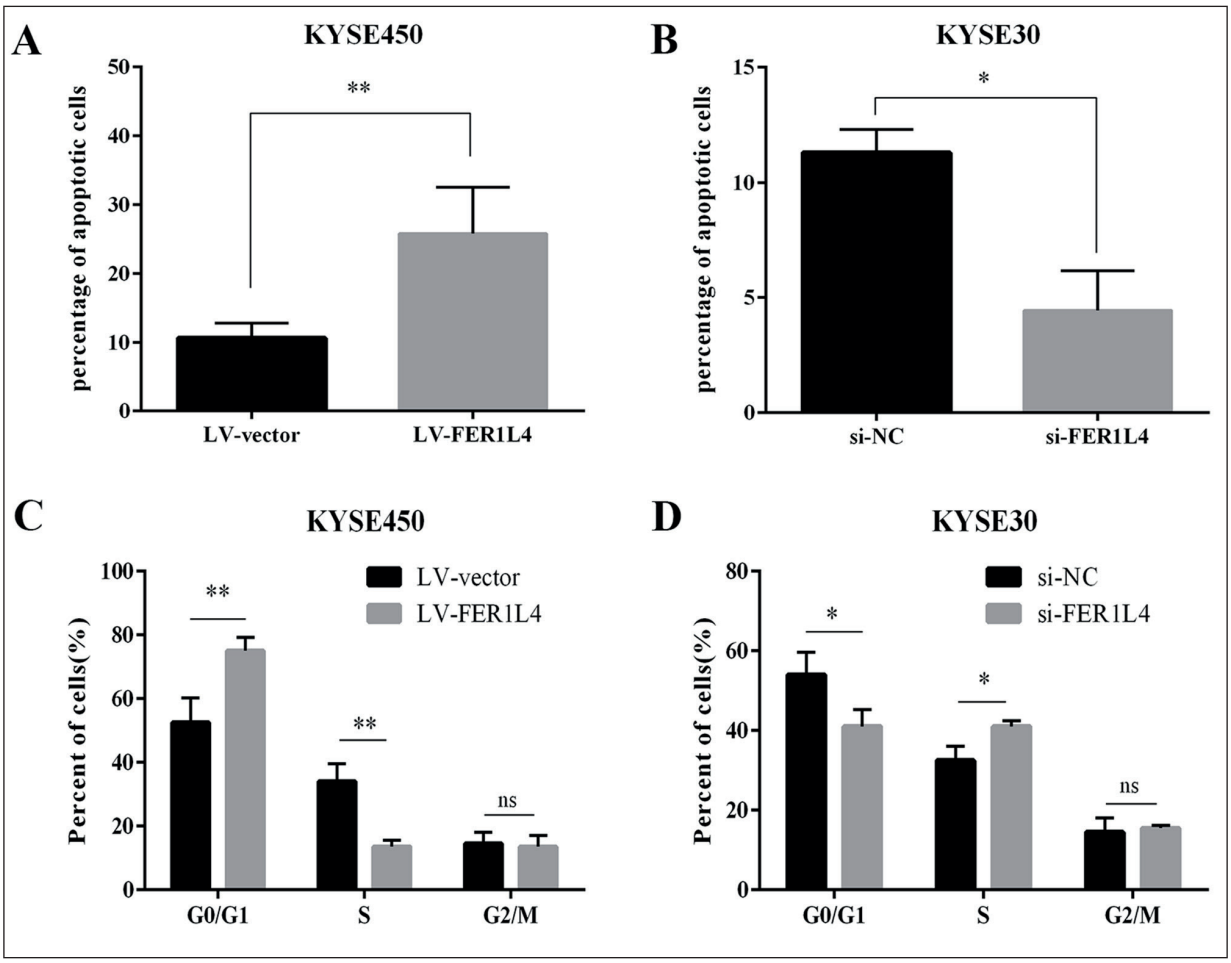


**Figure 3.** LncRNA FER1L4 promoted cell apoptosis and induced cell cycle arrest at G0/G1 phase. **A**, Flow cytometric analysis was performed to detect the apoptotic rates of transfected KYSE450 cells; **B**, Flow cytometric analysis was performed to detect the apoptotic rates of transfected KYSE30 cells; **C**, Flow cytometric analysis was performed to detect cell cycle progression of transfected KYSE450 cells; **D**, Flow cytometric analysis was performed to detect cell cycle progression of transfected KYSE30 cells. *$p$<0.05; **$p$<0.01.

As shown in Figure 3C-D, overexpressed FER1L4 induced the proportion of G0/G1 phase in KYSE450 cells, but the proportion of S phase decreased. Meanwhile, downregulated FER1L4 reduced the proportion of G0/G1 phase in KYSE30 cells, but the proportion of S phase increased. The difference was statistically significant ($p$<0.01).

These results indicated that FER1L4 could inhibit the growth of ESCC cells by inducing cell apoptosis and weakening the G0/G1 phase block of the cell cycle.

### *LncRNA FER1L4 Inhibited ESCC Cell Invasion In Vitro*

Transwell chamber assay was used to investigate the effects of overexpression of FER1L4 or interference in the FER1L4 expression on esophageal cancer cell invasion and migration, respectively. Results revealed that after overexpression of FER1L4, the invasive activity of esophageal cancer cells was reduced by about 1.5 times, and the difference was statistically significant ($p$<0.05) (Figure 4A).

At the same time, the role of FER1L4 in the proliferative activity of esophageal cancer cells was further confirmed. After knockout of FER1L4, the invasive activity of esophageal cancer cells was increased by about 2 times, and the difference was statistically significant ($p$<0.05) (Figure 4B).

In conclusion, FER1L4, as a tumor suppressor lncRNA, is involved in the regulation of invasion and metastasis capacities of ESCC.

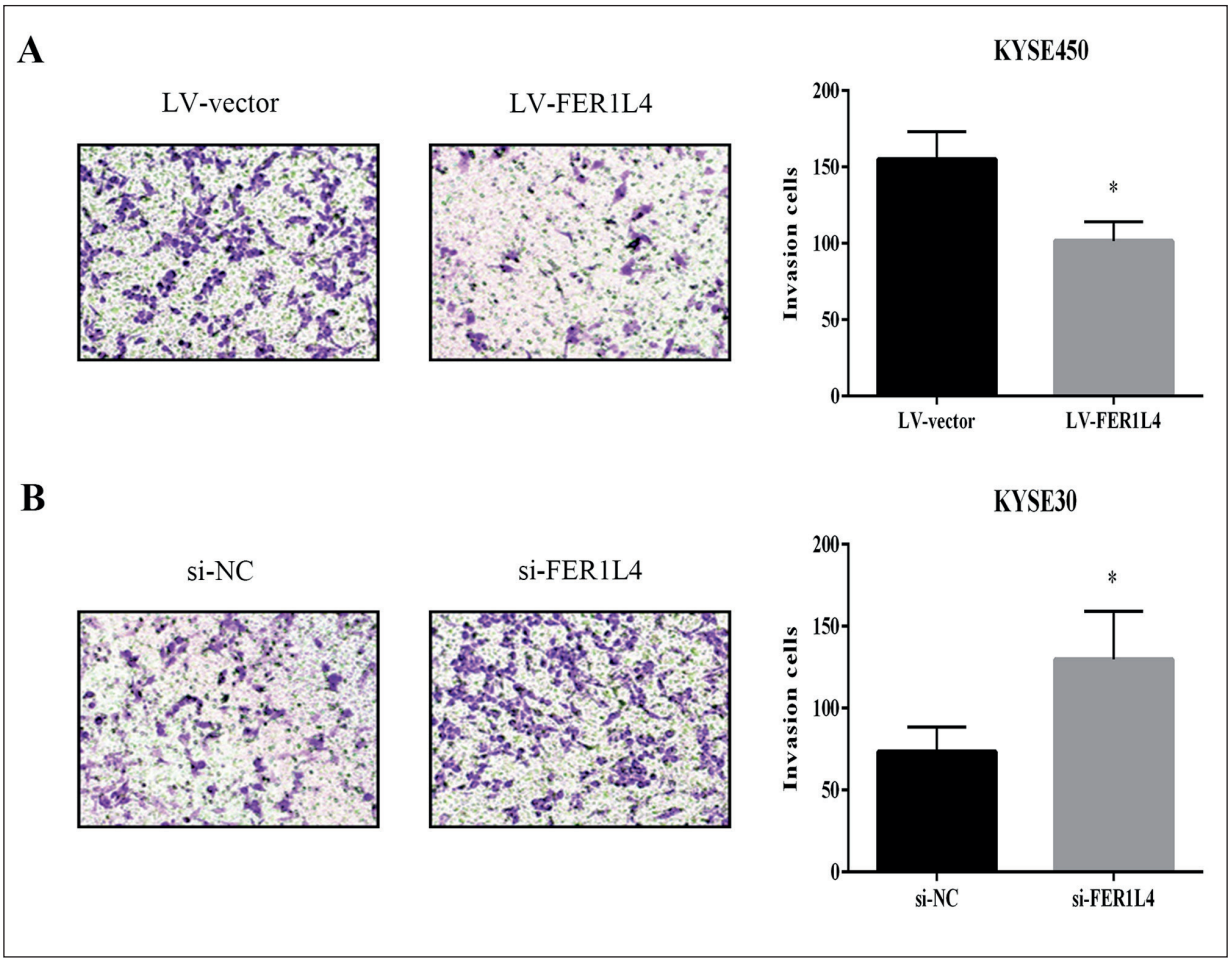


**Figure 4.** LncRNA FER1L4 inhibited ESCC cell invasion *in vitro*. **A**, Transwell assay was performed to determine the invasion of transfected KYSE450 cells; **B**, Transwell assay was performed to determine the invasion of transfected KYSE30 cells. *$p<0.05$.

## Discussion

Esophageal cancer is one of the most common human malignant tumors[2]. Although surgery, neoadjuvant chemotherapy, radiotherapy, biotherapy, etc., are combined in the treatment of esophageal cancer in recent years, the prognosis of patients with esophageal cancer is still poor, and the overall 5-year survival rate is still very low[1]. In pathological types of esophageal cancer, squamous cell carcinoma accounts for 90%. Squamous cell carcinoma is not sensitive to radiotherapy, so its prognosis is worse than that of adenocarcinoma[12]. So far, the pathogenesis of esophageal cancer and factors influencing its development and prognosis remain unclear. Therefore, it is of great significance to search the index that affects the formation and development of esophageal cancer and evaluates the prognosis of patients.

In this study, it was found that the expression of lncRNA FER1L4 in esophageal cancer tissues was significantly down-regulated compared with that in normal esophageal tissues. To verify the role of change in FER1L4 expression in the occurrence of esophageal cancer, functional experiments of FER1L4 overexpression and inhibition were performed in esophageal cancer cells. Results showed that overexpression of FER1L4 in esophageal cancer cells inhibited the proliferation and invasion of cells. However, down-regulation of FER1L4 expression promoted proliferation and invasion capacities of esophageal cancer cells. These findings indicate that FER1L4, as a tumor suppressor gene, plays a role in esophageal cancer, and its deletion or low expression is involved in the occurrence and development of esophageal cancer. Therefore, FER1L4 plays a key role in the occurrence of esophageal cancer and may serve as a therapeutic target for esophageal cancer in the future.

Recently, many lncRNAs have been found. Studies[4,7] have shown that lncRNA regulates the expressions of various genes involved in a variety of biological processes through multiple ways, such as regulating the cleavage of messenger RNA (mRNA), affecting the degradation of RNA and regulating the translation of mRNA. Moreover, the expression disorders of these lncRNAs also affect the epigenetic regulation information in cells, providing the advantages of progressive and uncontrollable cell proliferation[5]. Epigenetic modification mainly includes modification of DNA region (such as DNA methylation and demethylation modification), histone modification in the specific amino acid region (such as acetylation, methylation and phosphorylation modification), and chromatin remodeling, all of which are generally considered to be involved in the regulation of gene expression. In addition to the changes in genetic factor, the methylation of the promoter region of tumor suppressor genes plays an important role in the initiation and development of tumors. Therefore, the methylation change in tumor suppressor genes may act as a potential tumor marker. At present, epigenetic changes in some genes exert important functions and effects in the occurrence and development of esophageal cancer. For example, some genes are involved in cell cycle regulation (p16), apoptosis [death associated protein kinase gene (DAPK)] and DNA damage repair [breast cancer type 1 susceptibility gene (BRCA1) and glutathione S-transferase P1 (GSTP1)]. With the deepening of research, non-coding RNA, in addition to protein-coding genes, is also regulated by methylation, and participates in the occurrence and development of esophageal cancer. Therefore, the discovery of lncRNA provides evidence for filling the oncogene and tumor suppressor gene networks in the past.

Recent studies[13,14] have slowly unveiled the mystery of lncRNA, including the importance of lncRNA in tumorigenesis. The relatively well-known lncRNA HOX transcript antisense RNA (HOTAIR) is involved in the occurrence of a variety of tumors, and it is constitutively overexpressed in various tumors and can act as a marker for prognosis and metastasis of a variety of tumors. Researches on the regulatory mechanism of HOTAIR have shown that it can bind to polycomb repressive complex 2 (PRC2) and lysine-specific demethylase 1 (LSD1) complex through its 5' and 3' structural domains to participate in the expressions of tumor-related genes[15]. There is a growing number of evidence showing that lncRNA can be involved in the regulation of gene expression at various levels, including transcriptional level and post-transcriptional modification level. It is noteworthy that 20% human lncRNAs can interact with PRC2 to mediate chromatin epigenetic modification and participate in the regulation of gene expression. The expression of lncRNA highly up-regulated in liver cancer (HULC) is significantly up-regulated in esophageal cancer tissues and plays an important role in the occurrence and development of esophageal cancer. Studies on its mechanism have shown that HULC is homologous with the target gene of miR-372, and can competitively bind to the target gene of miR-372, thereby leading to expression disorders of target genes of the corresponding miRNAs[16]. In addition, similar investigations have found that lncRNA SPRY4-IT1 also plays an important role in the occurrence of human melanoma, and studies on the function of SPRY4-IT1 argue that inhibiting the expression of lncRNA SPRY4-IT1 leads to the deletion of cell growth and differentiation, and increases the apoptosis rate of melanoma cells[17]. However, the function of lncRNA in esophageal cancer remains unclear up to now. We found that the FER1L4 expression was significantly down-regulated in esophageal cancer tissues compared with that in normal esophageal tissues, and various cell function experiments showed that lncRNA FER1L4 could inhibit the proliferation and invasion capacities of esophageal cancer.

## Conclusions

We showed that FER1L4, as a tumor suppressor lncRNA, is involved in the occurrence and development of esophageal cancer, suggesting that FER1L4 can be used as one of the markers for early diagnosis of esophageal cancer.

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### Conflict of Interest

The authors declared no conflict of interest.

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