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LncRNA FBXL19-AS1 promotes proliferation and metastasis via regulating epithelial-mesenchymal transition in non-small cell lung cancer

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Abstract. – OBJECTIVE: To detect the relative expression of long non-coding ribonucleic acid (lncRNA) F-box and leucine-rich repeat protein 19-antisense RNA 1 (FBXL19-AS1) in tissues and cells of non-small-cell lung cancer (NSCLC), and investigate the mechanism of lncRNA FBXL19-AS1 in promoting NSCLC cell proliferation and metastasis by regulating epithelial-mesenchymal transition (EMT) via *in vitro* experiments.

PATIENTS AND METHODS: The relative expression of lncRNA FBXL19-AS1 in NSCLC tissues and cells was detected via quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The colony formation assay was performed to study the impact of interference with lncRNA FBXL19-AS1 expression on NSCLC cell proliferation. The flow cytometry was applied to determine the influence of si-FBXL19-AS1 on the cycle distribution of NSCLC cells. After the interference with lncRNA FBXL19-AS1 expression, the transwell assay was utilized to measure the changes in the migratory and invasive abilities of NSCLC cells, while the expression changes in EMT-related molecular markers was detected via Western blotting.

RESULTS: The results of qRT-PCR showed that the expression of lncRNA FBXL19-AS1 in NSCLC tissues and cells was up-regulated. According to the results of the colony formation assay, the proliferative capacity of NSCLC cells was decreased after the interference with lncRNA FBXL19-AS1 expression. In flow cytometry, it was indicated that the cell cycle was arrested at the G0/G1 phase in the experimental group compared with that in the control group. The transwell assay results showed that the migratory and invasive abilities of NSCLC cells were weakened after the interference with lncRNA FBXL19-AS1 expression. The results of the Western blotting assay revealed that the expressions of EMT-related molecular markers (E-cadherin, N-cadherin, etc.) were changed.

CONCLUSIONS: The expression of lncRNA FBXL19-AS1 in NSCLC tissues and cells is up-regulated, and the highly expressed lncRNA FBXL19-AS1 can promote NSCLC cell proliferation and metastasis by regulating the EMT.

Key Words:
Non-small cell lung cancer, LncRNA FBXL19-AS1, Epithelial-mesenchymal transition, Proliferation, Metastasis.

Introduction

Lung cancer is a malignant tumor with the highest incidence and mortality rate in the world. According to the data from the International Agency for Research on Cancer, WHO[1], there were nearly 1.82 million new cases of lung cancer in 2012 around the world, ranking the first among all the malignant tumors. Non-small cell lung cancer (NSCLC) is a major type of lung cancer, accounting for approximately 80% of all cases[2]. Most cases of NSCLC are in the late stage when discovered due to the lack of clinical manifestations in the early stage, and lymphatic and distant metastases of tumor cells have occurred. Therefore, the prognosis of NSCLC patients is poor, and the 5-year survival rate is usually lower than 20%[3], seriously threatening the life and health of human beings. Hence, it is crucial for the diagnosis and treatment of NSCLC to explore the mechanisms of the occurrence and development of the disease.

Long non-coding ribonucleic acids (lncRNAs), a category of non-coding RNAs, are named for their transcript length of over 200 nucleotides and disability to encode proteins,

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which play vital roles in numerous life activities such as epigenetic regulation and cell differentiation regulation[4]. Discovered Zhang et al[5] that lncRNAs are abnormally expressed in multiple tumors, and the lncRNAs with dysregulated expressions can serve as tumor promoting or suppressive factors. Moreover, they possess a variety of complex regulatory mechanisms, including the interfering mechanisms with the expressions of the downstream genes, mediating chromatin remodeling and histone modification and binding to specific proteins[6,7]. Studies on NSCLC and lncRNAs have manifested that lncRNAs are able to influence many signaling pathways and exert important effects in the formation and progression of NSCLC. For example, lncRNA HOXD-antisense RNA 1 (AS1) stimulates the proliferation and invasion of NSCLC cells by regulating the micro RNA (miR)-133/MMP9 axis[8]. However, there has been no report about the expression and the role of lncRNA F-box and leucine-rich repeat protein 19 (FBXL19)-AS1 in NSCLC yet.

According to literature, Shen et al[9] first discovered that lncRNA F-box and leucine-rich repeat protein 19-antisense RNA 1 (FBXL19-AS1) has an up-regulated expression and promotes the cell proliferation and invasion by sponging miR-203 in colorectal cancer. Similarly, the expression of lncRNA FBXL19-AS1 is up-regulated in osteosarcoma and breast cancer, and it can accelerate the occurrence and development of tumors by sponging miRNAs[10,11]. In this research, it was discovered that through *in vitro* experiments lncRNA FBXL19-AS1 manifested an up-regulated expression in NSCLC tissues and cells and promoted the proliferation and metastasis of NSCLC cells by regulating epithelial-mesenchymal transition (EMT).

Patients and Methods

Clinical Specimens

A total of 52 pairs of carcinoma and para-carcinoma tissues were obtained from NSCLC patients via surgical resection in China-Japan Union Hospital of Jilin University from January 2010 to December 2016, which were quickly frozen in liquid nitrogen at -180°C until use. This research was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University, and written informed consent was obtained from all the participants.

Cell Culture

Five kinds of NSCLC cell lines (A549, H1299, H520, SPCA1, and H358) and normal lung epithelial cells (16HBE) were provided by the Cell Bank of Shanghai Institutes for Biological Sciences, CAS (Shanghai, China). All the cell lines were cultured in an incubator with 5% CO_2 at 37°C, with sufficient Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA), 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin.

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

The total RNA was extracted from the tissues and cells using the total RNA extraction reagent according to the instructions. The reverse transcription reaction was conducted using PrimeScript™ RT Master Mix (TaKaRa, Tokyo, Japan), and the qRT-PCR was performed using SYBRTM Select Master Mix on ABI 7500 flourescence qRT-PCR instrument (Applied Biosystems, Foster City, CA, USA). The reaction conditions were: polymerase activation at 95°C for 5 min, followed by denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s for 40 cycles. With glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal reference, the primer sequences are as follows: FBXL19-AS1: 5'-AGGAGTCTACCTGGAAATG-3' and 5'-GGACAGAGTTAAACGCAAC-3'; GAPDH: 5'-TCAAGAAGGTGGTGAAGCA-3' and 5'-AGGTGGAGGAGTGGGTGT-3'. Finally, the fold change in the expression of FBXL19-AS1 was calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell Transfection

The sequences of si-FBXL19-AS1 and si-negative control (NC) include 5'-GCAGUCUGUUCUGAACAUA-3' and 5'-GUCUGCAGCUGAGAGUAT-3'. The transfection procedures are as follows: the NSCLC cells in logarithmic growth phase were seeded into a 6-well plate with adequate complete medium and then, when the fusion reached 60%, transfected with 50 nmoL si-FBXL19-AS1 and si-NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the instruction. 48 h later, the transfected cells were collected to detect the transfection efficiency.

Colony Formation Assay

The cells were inoculated into a 6-well plate (100 cells/well), with 3 duplicated wells set for

each group, and then cultured in the incubator for 14 d. The medium was changed, and the cell state was observed every 3 d. Next, the culture was terminated when the cloned cells grew into a suitable size, and the cells were washed with phosphate-buffered saline (PBS) twice and fixed with cold methanol at 4°C for 15 min, followed by washing in PBS twice and staining with crystal violet solution (1 mL/well) for 20 min.

Flow Cytometry

The cells in the experimental group and control group were collected and fixed with 75% ethanol at 4°C overnight. On the next day, the cells were incubated with RNase at 37°C for 30 min and then, stained with propidium iodide for 30 min. After that, the cultures were collected to analyze the cell cycle using a flow cytometer. The data were presented as the percentage of cells distributed at the G0/G1, S, and G2/M phases of the cell cycle.

Wound-Healing Assay

The transfected NSCLC cells were seeded into the 6-well plate at 1×10^6 cells/well and cultured to 80% confluence. Later, the monolayers were scratched using the tip of a 200 μL pipette, and the culture was continued in the FBS-free medium. Subsequently, the wound healing was observed under an inverted microscope and photographed at 0 and 48 h.

Transwell Assay

The transfected NSCLC cells were resuspended in 200 μL serum-free DMEM and plated in the upper chamber of the transwell device (or coated with Matrigel), with 5×10^4 cells/well. The lower chamber was added with 600 μL complete medium as the chemical attractant. After incubation in the incubator at 37°C for 48 h, the cells and Matrigel in the upper chamber were removed, and the cells invading in the lower chamber were fixed with formaldehyde.

Western Blotting Assay

Total protein samples were extracted from the prepared NSCLC cells using radioimmunoprecipitation assay (RIPA) buffer and then quantified by virtue of a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). Next, the proteins were separated by 10% polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), followed by sealing in Tris-Buffered Saline-Tween 20 (TBST) solution containing 5% skim milk at room temperature for 2 h and incubation with antibodies (E-cadherin, N-cadherin, and Vimentin) at 4°C overnight. After that, the membrane was washed with TBST solution for 3 times and incubated with horseradish peroxidase-conjugated antibodies at room temperature for 2 h.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was employed for statistical analysis of the data. The measurement data were expressed by ($\bar{\chi}\pm s$), and *t*-test or analysis of variance was used for comparison. Least Significant Difference (LSD) *t*-test was adopted for the pairwise comparison after the analysis of variance. $p<0.05$ suggested that the difference was statistically significant.

Results

Expression of LncRNA FBXL19-AS1

According to the results of qRT-PCR for the 52 pairs of carcinoma and para-carcinoma tissues of NSCLC, the expression of lncRNA FBXL19-AS1 was up-regulated in 41 cases (78.8%) and down-regulated in 11 cases (21.2%) of carcinoma tissues compared with that in para-carcinoma tissues (Figure 1A). Next, the expression level of lncRNA FBXL19-AS1 in NSCLC cells was determined, and it was indicated that, in comparison with that in normal lung epithelial cells (16HBE), the expression of lncRNA FBXL19-AS1 was up-regulated in the 5 kinds of NSCLC cell lines (A549, H1299, H520, SPCA1, and H358) (Figure 1B). In order to study the biological functions of lncRNA FBXL19-AS1 in NSCLC cells, the interference sequences were synthesized, and the interference efficiency was detected via qRT-PCR (Figures 1C and 1D).

Biological Functions of LncRNA FBXL19-AS1

To explore the impact of lncRNA FBXL19-AS1 on NSCLC cell proliferation, the NSCLC cells were transfected with si-FBXL19-AS1 and si-NC. The results of the colony formation assay showed that the experimental group declined the proliferative capacity compared with the control group (Figures 2A and 2B). Subsequently, the influence of lncRNA FBXL19-AS1 on the cell cycle was investigated. The cells in the experimental

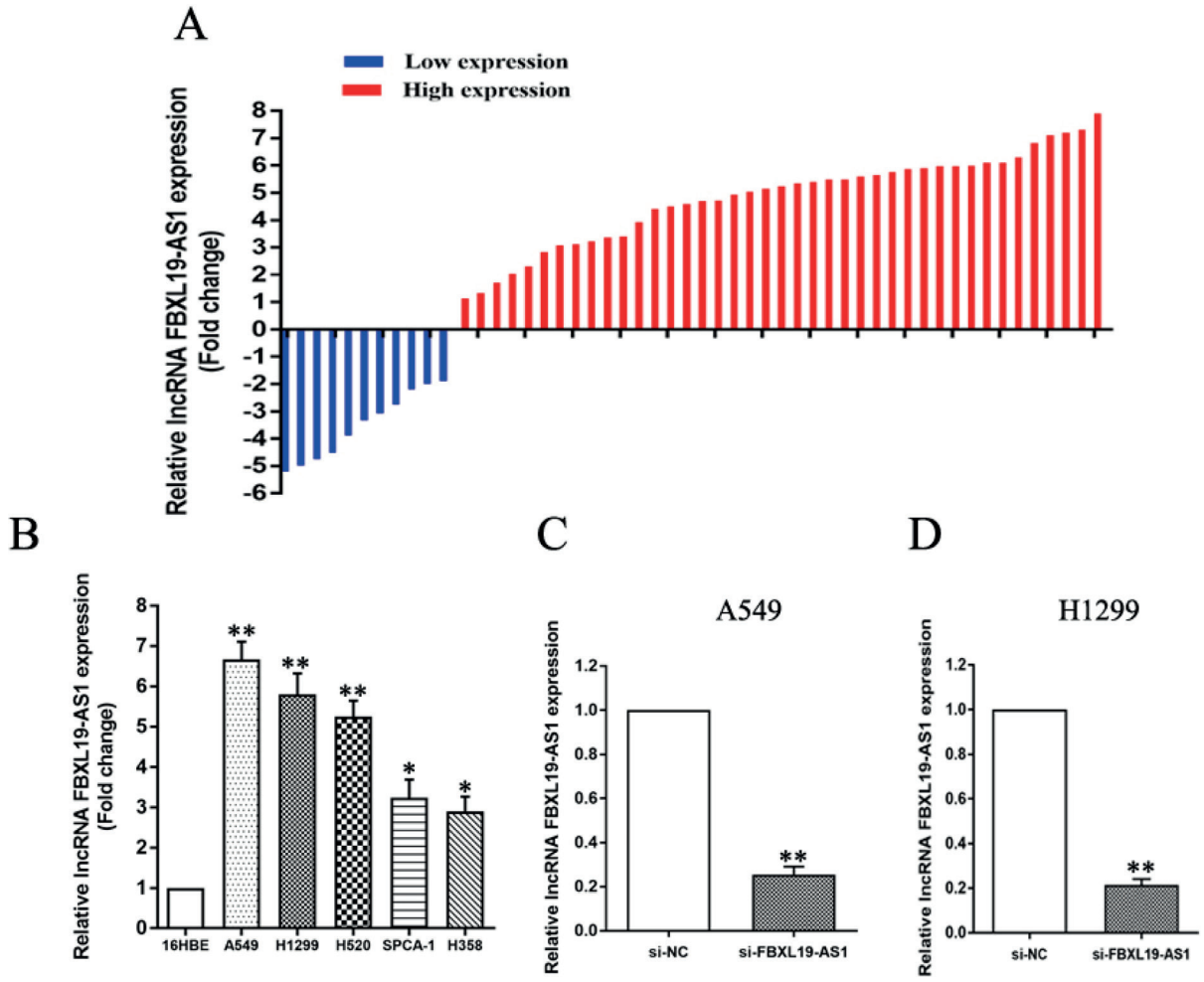

Figure 1. Expression of lncRNA FBXL19-AS1. ***A,*** Expression of lncRNA FBXL19-AS1 in 52 cases of NSCLC tissues detected via qRT-PCR. It is shown that the expression of lncRNA FBXL19-AS1 is up-regulated in 41 cases and down-regulated in 11 cases. ***B,*** Relative expression of lncRNA FBXL19-AS1 in NSCLC cells to 16HBE detected via qRT-PCR. ***C-D,*** Interference efficiency of si-FBXL19-AS1 detected via qRT-PCR (**p<0.01, *p<0.05).

group and control group were treated as the aforementioned methods and collected at 48 h after transfection. It was found via flow cytometry that the interference with lncRNA FBXL19-AS1 expression arrested the cell cycle at the G0/G1 phase (Figures 2C and 2D).

LncRNA FBXL19-AS1 Regulated the EMT

The wound-healing assay results indicated that the migratory ability of NSCLC cells was weakened after the interference with lncRNA FBXL19-AS1 expression (Figures 3A and 3B). Later, the transwell assay was performed to investigate the impacts of lncRNA FBXL19-AS1 on the migration and invasion of NSCLC cells. It was manifested that the migratory and invasive abilities of the cells were markedly decreased in the experimental group (Figures 3C and 3D). The potential mechanism of lncRNA FBXL19-AS1 in influencing the migration and invasion was preliminarily studied using the Western blotting. It was revealed that the protein expressions of EMT-related molecular markers (E-cadherin, N-cadherin, and Vimentin) were changed after the interference with lncRNA FBXL19-AS1 expression (Figures 3E and 3F).

Discussion

In recent years, the incidence and death rates of lung cancer are constantly rising, and studies on the pathogenesis of lung cancer are continuously conducted. With the rapid development and ap-

plication of such techniques as deep sequencing, gene chip, qRT-PCR, and in situ hybridization, the in-depth investigations about lncRNAs are also performed, and much attention has been paid to the roles of lncRNAs in the occurrence and development of lung cancer.

The lncRNAs exert crucial effects in NSCLC induced by exposure to hazardous substances in the environment. According to reports, lncRNAs such as CCAT1, CAR10, and MEG3 can trigger the malignant transformation of bronchial epithelial cells, thus forming tumors[12,13].

Liang et al[14] studied and discovered that the growth arrest-specific transcript 5 (GAS5) in the plasma of NSCLC patients is stable, and its expression is down-regulated. The diagnostic sensitivity and specificity are 82.2% and 72.7%, respectively, thereby confirming the relationship between GAS5 and NSCLC diagnosis. It has been reported in some studies that lncRNAs can act as pro-oncogenic factors. For instance, Yeung et al[15] discovered that lncRNA XLOC_008466 is highly expressed in NSCLC patients, and its inhibition can suppress the proliferative and invasive abilities of cells and promote apoptosis. However, the correlation between lncRNA FBXL19-AS1 and NSCLC has not been reported yet. Therefore, it was found in this research for the first time that

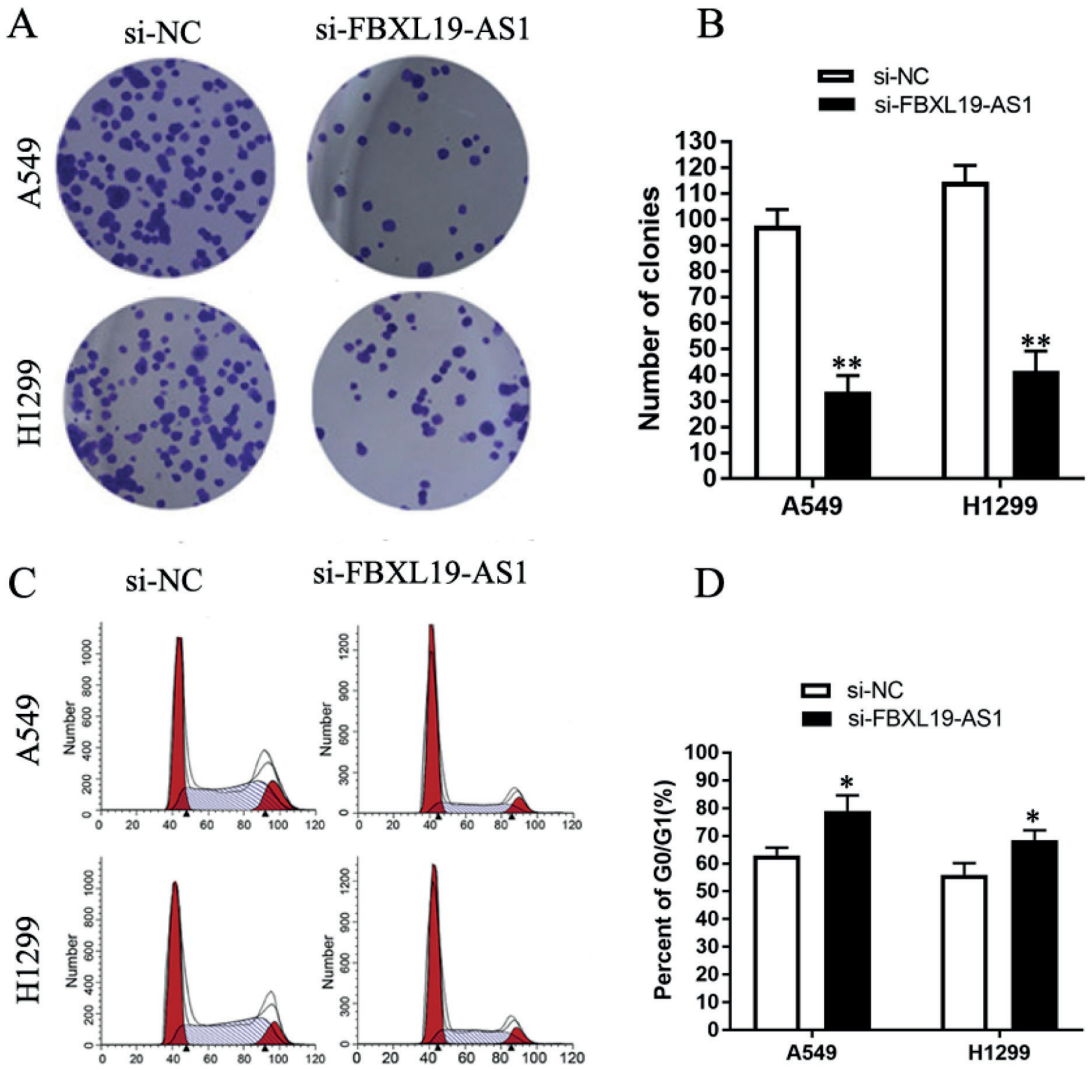

Figure 2. Impacts of lncRNA FBXL19-AS1 on proliferation cycle. ***A, B,*** Impact of si-lncRNA FBXL19-AS1 on proliferative capacity of NSCLC cells detected via colony formation assay (magnification: 10×). ***C, D,*** Impact of si-FBXL19-AS1 on the distribution of NSCLC cell cycle detected via flow cytometry (**$p<0.01$, *$p<0.05$).

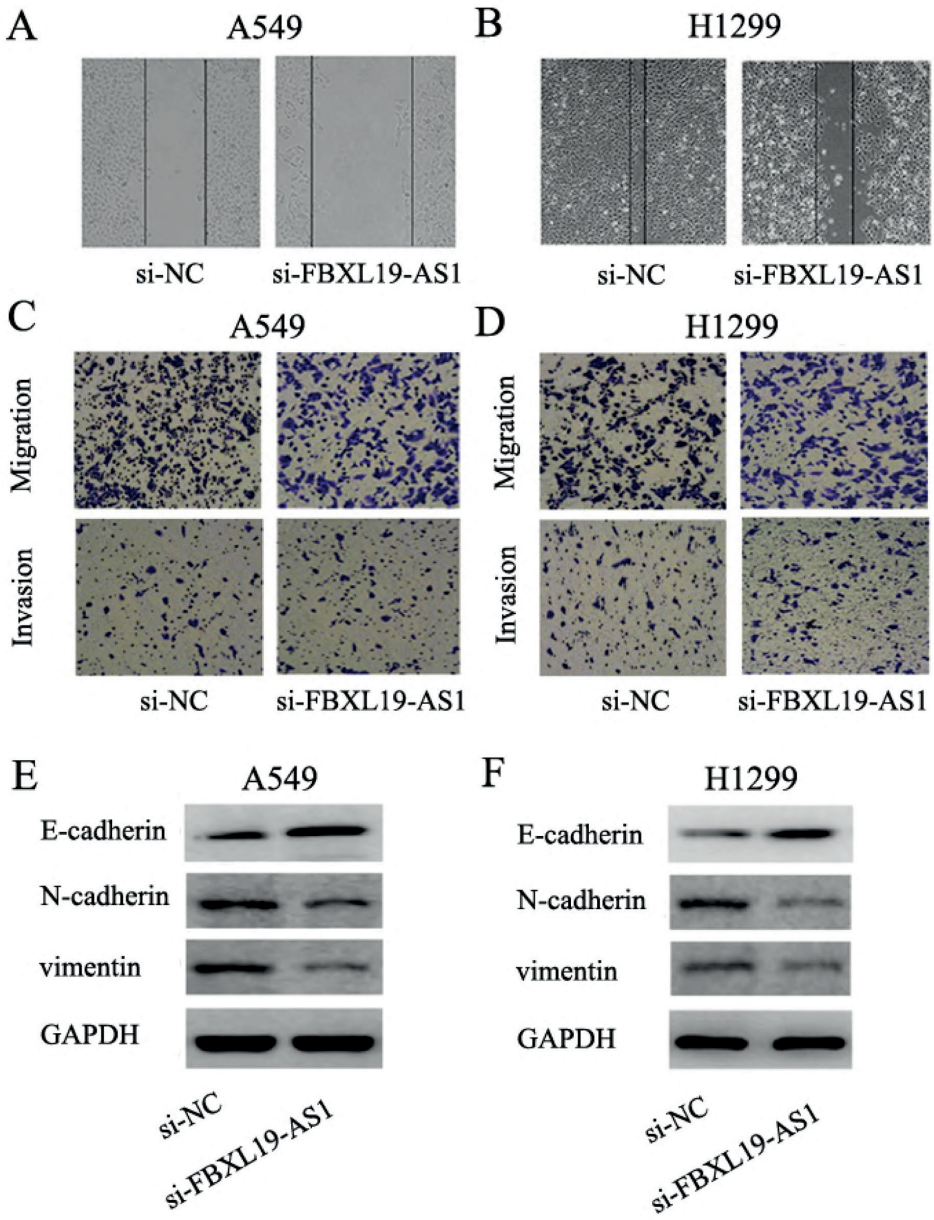

Figure 3. LncRNA FBXL19-AS1 promotes invasion and migration of NSCLC cells by regulating EMT. ***A-B,*** Impact of si-lncRNA FBXL19-AS1 on migratory ability of NSCLC cells detected via wound-healing assay. ***C-D,*** Impacts of si-lncRNA FBXL19-AS1 on migratory and invasive abilities of NSCLC cells detected via transwell assay (magnification: 40×). ***E-F,*** Changes in the expressions of EMT-related molecular markers after interference with lncRNA FBXL19-AS1 expression detected via Western blotting.

the expression of lncRNA FBXL19-AS1 was up-regulated in NSCLC tissues and cells, which accelerated the proliferation and metastasis of NSCLC cells.

The proliferation and metastasis are the leading causes of the high mortality rate of cancers, and the EMT is closely related to the high invasiveness and metastasis of cancer cells[15]. When the epithelial cells lose connexins (e.g., E-cadherin) and acquire mesenchymal marker proteins (e.g., N-cadherin and Vimentin), they play critical roles in the promotion of the EMT[16]. There is growing evidence that lncRNAs are involved in tumor invasion and metastasis by regulating the EMT. For example, the overexpressed lncRNA ATB stimulates the EMT process by up-regulating

zinc-finger E-box-binding homeobox 1 (ZEB1) and ZEB2, ultimately promoting the invasion and metastasis of liver cancer cells[17]. Moreover, lncRNA LINC01186 can inhibit the migration and invasion by reducing the EMT of lung cancer cells, while lncRNA SOX2OT can accelerate the migration and invasion of NSCLC cells by inducing the EMT process[18-20].

Conclusions

We showed that the protein expressions of N-cadherin and Vimentin were remarkably down-regulated, while that of E-cadherin was up-regulated after transfection with si-FBXL19-AS1, suggesting that the interference with lncRNA FBXL19-AS1 represses the metastasis of NSCLC cells, mainly by reducing the EMT.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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