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Original Article

LINC01133 contributes to the malignant phenotypes of non-small cell lung cancer by targeting miR-30b-5p/FOXA1 pathway

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Article Info	Abstract
	This study aimed to explore the mechanism of action of LINC01133 in non-small cell lung cancer. LINC01133 expression in NSCLC patient tissues and cells was detected by qRT-PCR. After transfecting siRNA-LINC01133 in NSCLC cells, the proliferation and invasive migration ability of the cells were assessed via CCK-8 and Transwell assay, respectively. The sublocalization of LINC01133 in NSCLC cells was analyzed by bioinformatics prediction and nucleoplasm separation assay and RNA-FISH assay. Analysis of the binding relationship between LINC01133, FOXA1 and miR-30b-5p was all through bioinformatics website analysis, dual-luciferase reporter and RNA Pulldown assay. Functional rescue experiments confirmed the character of miR-30b-5p and FOXA1 in LINC01133 regulating the NSCLC cells biological behavior. LINC01133 high expressions were found in NSCLC tissues and cells. siRNA-LINC01133 treatment inhibited NSCLC cells malignant behavior. Mechanistically: LINC01133 promoted FOXA1 expression through adsorption binding of miR-30b-5p. Knocking down miR-30b-5p expression or up-regulating FOXA1 expression was able to reverse siRNA-LINC01133 inhibitory effect of tumor cell malignant behavior. LINC01133 promoted FOX1 expression by competitively binding miR-30b-5p, which attenuated the targeting inhibitory effect of miR-30b-5p on FOXA1 and ultimately promoted proliferation and invasive migration of NSCLC cells.

1. Introduction

In 2020, there were 19.3 million new cases of all types of cancer in the entire world. Lung cancer cases accounted for 11.4%, which was the second-highest number of all cancers. European and American countries rank first in the incidence and mortality rates of lung cancer [1]. Lung cancer is usually classified into 2 categories--non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Non-coding RNAs take up 98% of the human genome [2]. It often acts as a regulatory factor in multiple life processes such as embryogenesis, neurodevelopment, and DNA damage.

LncRNAs are non-coding RNAs over 200 nucleotides in length and splice structures similar to mRNA but do not encode proteins [3]. LncRNAs play a prominent part, especially in regulating transcription and translation. LncRNAs are able to regulate mRNA expression by completely combining with miRNAs in the cytoplasm [4]. A part of lncRNAs owns a "sponge" effect on miRNAs, which can isolate miRNAs and prevent them from regulating the translation of target mRNAs, thus weakening the effect of miRNAs, i.e. ceRNA effect [5]. Dysregulation of non-coding RNA leads to many diseases, including reproductive disorders and cancer [6]. Researchers have found

that lncRNAs can interact with miRNAs as a competitive endogenous RNA (ceRNA) and participate in the regulation of mRNA expression [7]. lncRNAs and miRNAs abnormally expressed have oncogenic or oncogenic roles in human lung cancer. They are important during cancer development and prognosis, and have possible function as biomarkers for disease diagnosis [6].

miRNAs are a class of small non-coding RNAs of 18-25 nt in length, derived from pri-miRNAs, hairpin precursor molecules of animals, plants, protozoa and viruses, which often act on the 3'UTR region of target mRNAs to inhibit translation. Most miRNAs are transcribed from DNA sequences into pri-miRNAs, processed into pre-miRNAs, and finally into mature miRNAs. miRNAs statistically regulate more than half of protein translation and are involved in cellular senescence, proliferation, cycling, differentiation metabolism and neuronal formation [7]. miRNAs are gradually becoming new clinical biomarkers with potential diagnoses and prognostics. miR-21 affects NSCLC cell growth behavior by regulating PTEN transcripts, while miR-451a regulates lung cancer cell migration and invasion by targeting ATF2 [8]. miRNAs may become a molecular target for lung cancer therapy.

This study focused on investigating the effect of

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LINC01133 in NSCLC on the malignant phenotype of NSCLC through ceRNA mechanism.

2. Materials and methods

2.1. NSCLC tissue collection

Postoperative cancerous and paracancerous normal tissue came from patients (N=87) with pathological resection performed by the Oncology Department from February 2015 to April 2020. NSCLC patients' ages ranged from 43 to 69 years. Inclusion criteria: patients' clinical data were complete; all patients did not suffer from pneumonia and had not received anti-inflammatory treatment during the sample collection and had not received preoperative oncological treatment. All procedures followed ethical requirements.

2.2. Cell culture

We purchased human NSCLC cell lines A549, NSCLC827, NCI-H23, NCI-H1155 and human normal lung epithelial cell line BEAS2B from ATCC cell bank (ATCC, Manassas, VA, USA). All cells were cultured in DMEM medium (Gibco Carlsbad, CA, USA) containing 10% fetal bovine serum, 1% P/S (penicillin and streptomycin, Gibco Carlsbad, CA, USA) at 37°C with 5% CO_2 in a constant temperature incubator.

2.3. Cell transfection

A549 cells were inoculated in 6-well plates (2×10^5/well) and then incubated for 24 hours. siRNA-NC/LINC01133#1/LINC01133#2/LINC01133#3, miR-3b-5p/NC mimic, miR-30b-5p/NC inhibitor, pcDNA3.1-FOXA1/NC (Shanghai Genechem Co., Ltd., Shanghai, China) were transfected into the cells, and the operation was performed following the instructions. Follow-up procedures were operated 48 hours after transfection.

2.4. qRT-PCR

Total RNA was isolated from clinical sample tissues or cultured cells using Trizol reagent (Invitrogen). 1 ug of RNA was reverse transcribed to cDNA using PrimerScript RT Master Mix (Takara, Dalian, China). qRT-PCR was then performed according to the instructions using SYBR Premix Ex Taq (Takara, Dalian, China) on ABI 7500 (ABI, America) for qRT-PCR experiments. Internal reference: GAPDH, U6. Gene relative expression calculation method: the $2^{-\Delta\Delta Ct}$ method [9]. The primer sequences are shown in Table 1.

2.5. CCK-8

The cell suspension was prepared in conformity with the provided instructions of the CCK-8 kit (Signalway Antibody, P002, College Park, USA) and the number of cells in each group was adjusted to 1×10^5 cells/well. Cells (100 μL per well) were seeded in 96-well culture plates and incubated at 37°C with 5% CO_2. 10 μL of CCK-8 reaction reagent (Beyotime Institute of Biotechnology, Haimen, China) was added to each well at different incubation periods. After 4-hour incubation at 37°C, cell proliferation ability was assessed by 450 nm absorbance.

2.6. Transwell assay

A549 cells were collected after 48 h of transfection (cell density: 1×10^6 cells/mL). For invasion experiments, matrigel gel was spread on the bottom of the chambers. For migration experiments, no gel was spread. Added cell suspension to the upper Transwell chamber. After 24 h, the chambers were removed, and the chambers were washed with PBS solution three times, and then wiped the Matrigel gel and cells on the upper layer of the microporous membrane of the chambers, followed by fixation with 40 g/L paraformaldehyde for 15 min, staining with 1 g/L crystal violet for 10 min and washing with PBS solution, and finally the chambers were placed under an inverted microscope for observation and counting.

2.7. Nucleocytoplasmic separation

The nuclei were separated from the cytoplasm using the Nucleus and Cytoplasm Protein Extraction Kit (Beyotime, China). Cells were collected into 200 μL of cytoplasmic protein extractant A/protease inhibitor buffer, and ice bath for 15 min later. Using 10 μL cytoplasmic protein extractant B for cell incubation, then centrifuged (13,000 g, 10 min, 4°C) to separate nuclei and cytoplasm. Nuclear protein was extracted from ice with nucleoprotein extractor, and then centrifugation (13,000 g, 10 min, 4°C). Treating supernatant as nuclear extract and analyzed by qRT-PCR.

2.8. Dual-luciferase reporter gene assay

The binding sites of LINC01133 and miR-30b-5p were predicted using Starbase (https://starbase.sysu.edu.cn/index.php) and miR-30b-5p and FOXA1 were predicted using Targetscan (http://www.targetscan.org/vert_72/). Both wild-type (LINC01133-wt and FOXA1-wt) and mutant-type (LINC01133-mut and FOXA1-mut) luciferase plasmids were constructed by luciferase vector pGL3 (Pro-mega, Madison, WI, USA). Luciferase activity was assessed using Dual-Lucy Assay Kit (Solarbio) 24 h following cotransfection of each plasmid with miR-30b-5p/NC mimic.

Table 1. Primer sequences.

Name of primer	Sequences
LINC01133-F	GGCAAGGTGAACCTCAAAAA
LINC01133-R	TTCCTGCAAGAGGAGAAAGC
U6-F	GCTTCGGCAGCACATATACTAAAAT
U6-R	CGCTTCACGAATTTGCGTGTCAT
miR-30b-5p-F	CCGAAACATCCTACACTCAGCTAA
miR-30b-5p -R	CAGTGCGTGTCGTGGAGT
GAPDH-F	TGCACCACCAACTGCTTAGC
GAPDH-R	GGCATGCACTGTGGTCATGAG
FOXA1-F	GAAGATGGAAGGGCAT
FOXA1-R	GCCTGAGTTCATGTTGCTGA

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2.9. RNA Pull down

We used the 3' End Biotinylation Kit (Thermo, Waltham, MA) to label LINC01133 and standard RNAs. For the detection of interacting miRNAs, Biotin-labeled RNA was stirred with streptavidin magnetic beads and placed in a Biotin-labeled RNA pull-down assay (Thermo, Waltham, MA). The labeled RNA was captured with 100 mL of streptavidin magnetic beads in RNA Capture Buffer for 30 minutes at room temperature. On a spinner at 4°C for one hour, cell lysates were mixed with LINC01133-bound magnetic beads. A qRT-PCR analysis was conducted after the magnetic beads were fully washed and the RNA bound by magnetic beads was separated.

2.10. Statistical analysis

Using SPSS22.0 software (IBM Corp., Armonk, NY, USA), all data were processed. Data complied with normal distribution and homogeneous variance. Comparisons between two groups were analyzed by independent sample-t test, and comparisons between multiple groups were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test. p is a two-sided test, the difference of $p < 0.05$ was considered statistically significant.

3. Results

3.1. High LINC01133 expression in NSCLC patients cancer tissues and NSCLC cell lines

To find out LINC01133 role in NSCLC, we collected cancerous tissues from NSCLC patients with normal tissues adjacent to cancer and detected the expression level of LINC01133 by qRT-PCR, which showed LINC01133 expression was significantly increased in NSCLC cancerous tissues compared with normal tissues adjacent to cancer (Figure 1A, $p < 0.01$). In addition, we selected A549, NSCLC827, NCI-H23, NCI-H1155 and BEAS2B, and detected the difference expression of LINC01133 by qRT-PCR. We found that LINC01133 expression was significantly higher in NSCLC cell lines than that in BEAS2B9 cells (Figure 1B, all $p < 0.01$). The above results indicated that LINC01133 might be associated with the development of NSCLC.

3.2. LINC01133 down-regulation inhibits malignant behavior of NSCLC cells

To investigate the function of LINC01133 in the cell malignant behavior, based on the results in Figure 1B, we selected the A549 cell line with relatively highest LINC0113 expression and inhibited the expression of LINC01133 in cells by transfecting siRNA-LINC01133 with transfecting siRNA-NC as a control. Firstly, the expression of LINC01133 was significantly reduced in cells after transfection of LINC01133 (Figure 2A, $p < 0.01$), and we selected si-LINC01133#2 with the best interference efficiency for subsequent experiments. Next, CCK-8 assay results found that downregulation of LINC01133 significantly inhibited A549 cell proliferation (Figure 2B, $p < 0.01$). The migration and invasion ability of NSCLC cells were also inhibited after transfecting siRNA-LINC01133 (Figure 2C, $p < 0.01$). It indicated that down-regulation of LINC01133 could inhibit NSCLC cell malignant behavior.

3.3. LINC01133 regulates FOXA1 expression by binding miR-30b-5p

To further understand LINC01133 potential mecha-

nism, we first analyzed the sublocalization of LINC01133 in A549 cells by prediction and nucleoplasmic separation assay through bioinformatics website (lncatlas.crg.eu). In A549 cells, LINC01133 was mainly found in the cytoplasm (Figure 3A-B, $p < 0.01$). In this regard, we hypothesized that LINC01133 may act as a competitive endogenous RNA (ceRNA) in the biological process of NSCLC. According to the Starbase database, LINC01133 binds miR-30b-5p and miR-30b-5p binds FOXA1 (Figure 3C). It has been shown that miR-30b-5p is involved in the biological behavior of NSCLC [9]. FOXA1 may be useful for treating NSCLC [10]. In addition to its luciferase digestion reporter assay, RNA pull-down assay was also used to validate its binding relationship (Figure 3D-E, $p < 0.01$). qRT-PCR analysis showed that si-LINC01133#2 significantly up regulated the expression of miR-30b-5p and down regulated FOX1 expression level in A549 cells
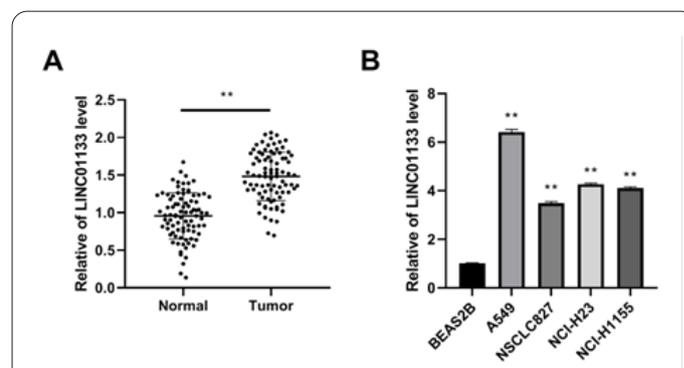
Fig. 1. High LINC01133 expression in NSCLC patients' cancer tissues and NSCLC cell lines. A-B, the expression levels of LINC01133 were detected by qRT-PCR. The cell experiment was independently repeated 3 times. Data were expressed as mean ± standard deviation. Data in panel A were analyzed using an independent sample-t test, and data in panel B were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test. ** $p < 0.01$.
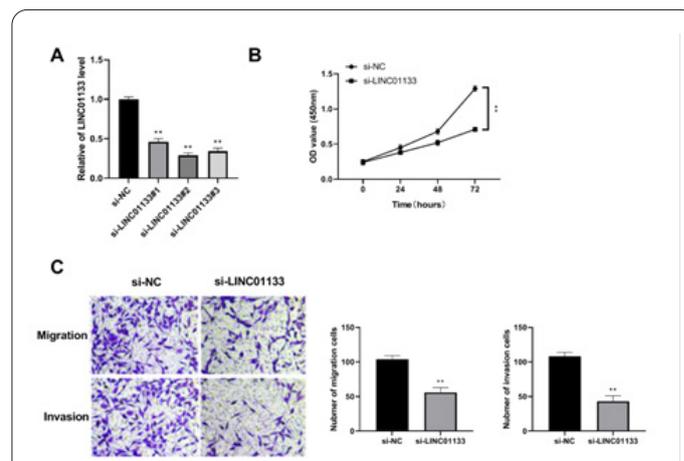
Fig. 2. LINC01133 down-regulation inhibits malignant behavior of NSCLC cells. siRNA of LINC01133 (si-LINC01133#1, si-LINC01133#2, si-LINC01133#3) was transfected in A549 cells, and si-NC was transfected as a control. A, qRT-PCR validated the intervention effect of si-LINC01133. B-C, cell proliferation, migration and invasion ability of cells were measured by CCK-8 and Transwell assays. Cell experiments were conducted 3 times. Data in panel A were analyzed using one-way ANOVA, and data in panel B/C were analyzed using an independent sample-t test, followed by Tukey's multiple comparisons test.
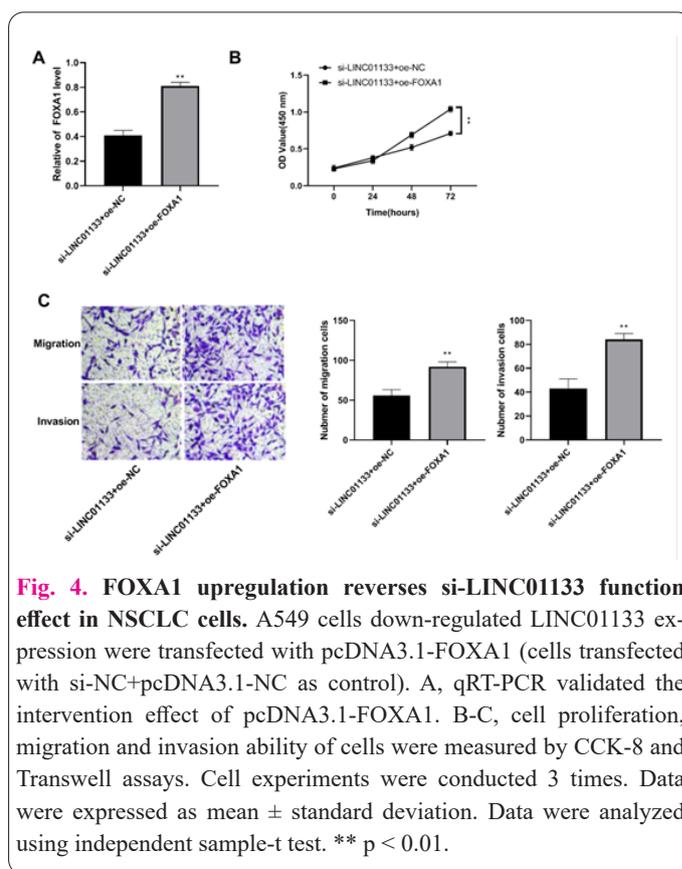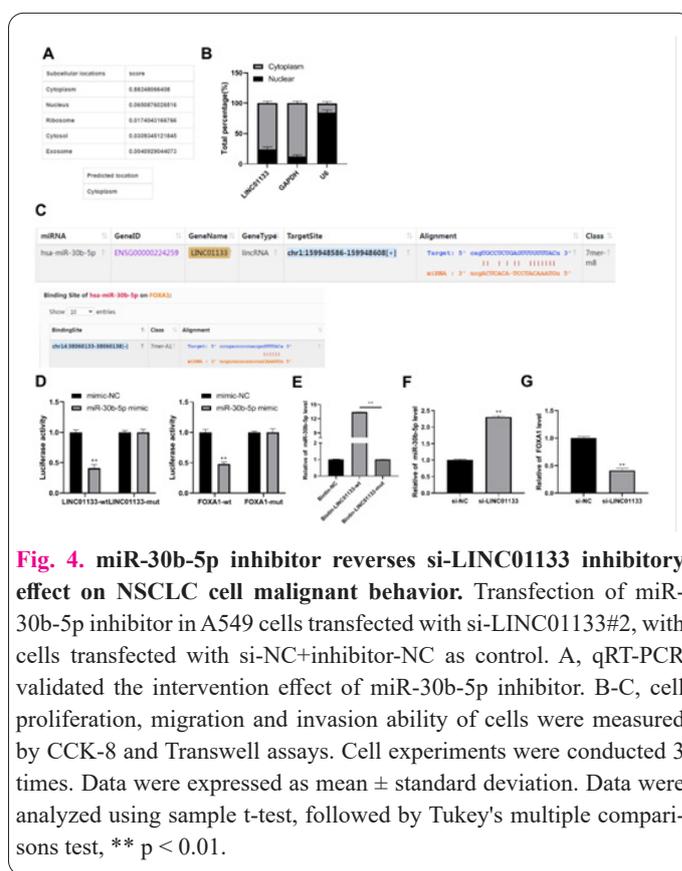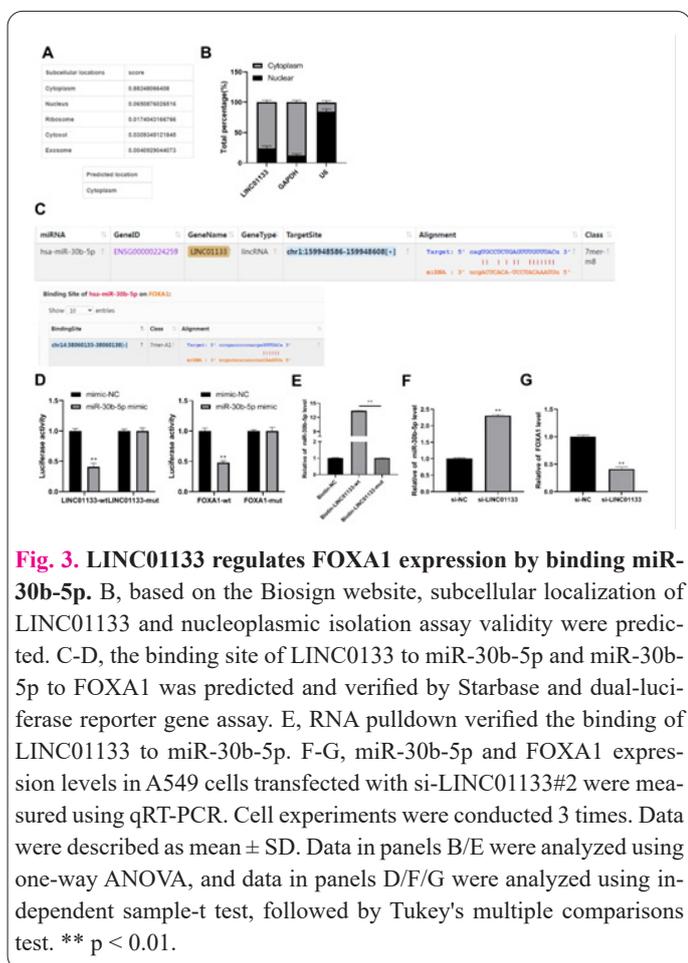
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compared with si-NC (Figure 3F-G, p < 0.01). It indicated that LINC01133 could competitively bind miR-30b-5p, thus promoting the expression of FOXA1.

3.4. miR-30b-5p inhibitor reverses si-LINC01133 inhibitory effect on NSCLC cell malignant behavior

To confirm that LINC01133 can competitively bind miR-30b-5p to regulate the NSCLC cell biological behavior, we performed a rescue assay for functional defects by knock down miR-30b-5p expression in A549 cells with low LINC01133 expression. In cells transfected with miR-30b-5p inhibitor, qRT-PCR revealed a significant decrease in miR-30b-5p expression (Figure 4A, p < 0.01), and then CCK-8 assay discovered that downregulation of miR-30b-5p reversed the changes caused by si-LINC01133#2 (Figure 4B, p < 0.01). Transwell assay found that miR-30b-5p increases cell invasion and migration ability (Figure 4C, p < 0.01). The above results hinted LINC01133 could competitively bind to repress miR-30b-5p expression and thus promote NSCLC cell biological behavior.

3.5. FOXA1 upregulation reverses si-LINC01133 function effect in NSCLC cells

For validating the ceRNA mechanism of LINC01133/miR-30b-5p/FOXA1 in NSCLC cells biological behavior, we again investigated the changes in the effect of si-LINC01133#2 on the cell biological behavior after intervention of FOXA1 expression by functional rescue experiments. After NSCLC cells transfected with pcDNA3.1 FOXA1, a significant increase in FOXA1 expression was detected (Figure 5A, p < 0.01). Then overexpression of FOXA1 was found to reverse the si-LINC01133#2-mediated change in cell proliferation capacity by CCK-8 assay

Fig. 4. **miR-30b-5p inhibitor reverses si-LINC01133 inhibitory effect on NSCLC cell malignant behavior.** Transfection of miR-30b-5p inhibitor in A549 cells transfected with si-LINC01133#2, with cells transfected with si-NC+inhibitor-NC as control. A, qRT-PCR validated the intervention effect of miR-30b-5p inhibitor. B-C, cell proliferation, migration and invasion ability of cells were measured by CCK-8 and Transwell assays. Cell experiments were conducted 3 times. Data were expressed as mean ± standard deviation. Data were analyzed using sample t-test, followed by Tukey's multiple comparisons test, ** p < 0.01.

Fig. 3. **LINC01133 regulates FOXA1 expression by binding miR-30b-5p.** B, based on the Biosign website, subcellular localization of LINC01133 and nucleoplasmic isolation assay validity were predicted. C-D, the binding site of LINC0133 to miR-30b-5p and miR-30b-5p to FOXA1 was predicted and verified by Starbase and dual-luciferase reporter gene assay. E, RNA pulldown verified the binding of LINC01133 to miR-30b-5p. F-G, miR-30b-5p and FOXA1 expression levels in A549 cells transfected with si-LINC01133#2 were measured using qRT-PCR. Cell experiments were conducted 3 times. Data were described as mean ± SD. Data in panels B/E were analyzed using one-way ANOVA, and data in panels D/F/G were analyzed using independent sample-t test, followed by Tukey's multiple comparisons test. ** p < 0.01.

Fig. 4. **FOXA1 upregulation reverses si-LINC01133 function effect in NSCLC cells.** A549 cells down-regulated LINC01133 expression were transfected with pcDNA3.1-FOXA1 (cells transfected with si-NC+pcDNA3.1-NC as control). A, qRT-PCR validated the intervention effect of pcDNA3.1-FOXA1. B-C, cell proliferation, migration and invasion ability of cells were measured by CCK-8 and Transwell assays. Cell experiments were conducted 3 times. Data were expressed as mean ± standard deviation. Data were analyzed using independent sample-t test. ** p < 0.01.

(Figure 5B, p < 0.01). The invasive and migratory abilities of the cells were similarly reversed by Transwell assay (Figure 5C, p < 0.01). Combined with the results of previous experiments, we could determine that LINC01133 combines miR-30b-5p through ceRNA mechanism. This upregulated FOXA1 expression, which ultimately promoted the malignant behavior of NSCLC cells.

4. Discussion

LncRNAs have been of great importance in a variety of biological processes. LncRNAs were in tissue-specific expression related to cancer type and correlate with the genes affecting cell cycle, survival and metastasis, and their aberrant expression plays a role in tumorigenesis development. LncRNAs have also been studied as markers for cancer diagnosis and therapeutic targets. As proof, LncRNA PCA3 is used as a biomarker for prostate cancer [11] and LncRNA HOTAIR is regarded as a new therapeutic direction for primary breast, colon and lung cancers [12, 13].

To further investigate the function and regulatory mechanism of LINC01133 in NSCLC, we predicted and determined that miR-30b-5p could directly target LINC01133 by starbase and a dual luciferase reporter assay. Lin et al. [14] suggested that miR-30b-5p could inhibit the gastric cancer cells growth properties, and low miR-30b-5p expression was associated with 5-FU drug resistance. In this study, miR-30b-5p had in lower expression level in NSCLC cell line than in normal lung epithelial cells by qRT-PCR. The malignant phenotype of A549 cells was enhanced after knockdown of miR-30b-5p expression. This result is consistent with multiple studies showing miR-30b-5p like a tumor suppressor [15-17]. miR-30b-5p has a role in suppressing the development of NSCLC. Lately, ceRNA mechanisms in cancer have been increasingly covered to be of considerable significance in post-transcriptional regulation and to be involved in tumorigenesis. miRNAs move to target mRNAs by activating the RISC (RNA-induced silencing complex) and induce translation inhibition by RISC and miRNAs, while ceRNAs can compete with miRNAs through miRNA response elements to affect the gene silencing caused by miRNAs. LncRNAs have been reported to work as ceRNAs for miRNAs [18]. Our study indicated that LINC01133 served as ceRNA to regulate miR-30b-5p and promoted the malignant phenotype of lung cancer cells A549. LINC01133 was inversely correlated with miR-30b-5p. Functional rescue assays revealed that siRNA-LINC01133 lessened the increase of ability of A549 cells proliferation, migration, and invasion caused by knockdown of miR-30b-5p.

FOXA1, a pioneer transcription factor, is essential for the normal development of some endodermal-derived organs [19-22]. Duan et al's [23] study showed that FOXA1 could be used as a biomarker for ovarian cancer, while Liu et al's [24] study showed that FOXA1 could promote prostate cancer tumor progression. These are consistent with the results obtained in this study that FOXA1 promotes the malignant progression of A549.

5. Conclusion

In summary, this study revealed the interaction and regulation of LINC01133 and miR-30b-5p in the NSCLC cell line A549. LINC01133 expression was significantly upregulated in A549 and contributed cells malignant phenotype. miR-30b-5p expression was decreased in the cells and inhibiting its expression could promote the malignant phenotype. LINC01133 can regulate FOXA1 to promote the malignant phenotype of A549 cells through a ceRNA mechanism that competitively binds to miR-30b-5p.

Conflict of interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
XX conducted the experiments and wrote the paper; LYS, LY, JX, YY, YG, LH and LX analyzed and organized the data; YS conceived, designed the study and revised the manuscript.

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