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LncRNA LINC02418 regulates proliferation and apoptosis of non-small cell lung cancer cells by regulating miR-4677-3p/SEC61G

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Abstract. – OBJECTIVE: Increasing studies indicated that long non-coding RNA (lncRNA) has crucial roles in cancer development, including non-small cell lung cancer (NSCLC). LINC02418 was reported to promote colorectal cancer development. However, whether LINC02418 has a role in NSCLC remains to be explored.

MATERIALS AND METHODS: First, expression level of LINC02418 in NSCLC tissues and normal tissues was analyzed at ENCORI. Moreover, expression level of LINC02418 in NSCLC cells and normal cell was analyzed with quantitative real-time PCR. Cell counting kit-8 assay, transwell invasion assay, and flow cytometry assay were used to analyze cell proliferation, cell invasion, and cell apoptosis.

RESULTS: LINC02418 was found as upregulated expression in both NSCLC tissues and cells. Functional assays showed that LINC02418 knockdown suppressed NSCLC cell proliferation and invasion but promoted cell apoptosis, while the overexpression of LINC02418 exerts opposite effects. Mechanistically, we showed LINC02418 could interact with microRNA-4677-3p (miR-4677-3p) to regulate Sec61 gamma subunit (SEC61G) expression.

CONCLUSIONS: These results indicated that LINC02418 functions as an oncogene, and regulated miR-4677-3p/SEC61G axis to accelerate NSCLC progression.

Key Words:
LINC02418K, MiR-4677-3p, SEC61G, NSCLC, CeRNA.

Introduction

Lung cancer ranks first among newly diagnosed and cancer-related deaths cases worldwide in 2018[1]. Lung cancer with squamous cell carcinoma (LUSC) and adenocarcinoma (LUAD) are the main sub-types of Non-small cell lung cancer (NSCLC)[2]. Overall survival for NSCLC remains poor despite recent advances in NSCLC treatment methods. Hence, a deep understanding of mechanisms behind NSCLC development is necessary to improve the prognosis of NSCLC patients.

Long non-coding RNAs (lncRNAs) are non-coding RNAs with length of over 200 nucleotides[3]. For a long time, lncRNA was regarded as junk gene as the biological roles of lncRNA are unclear[3]. In recent years, lncRNAs were revealed to play crucial roles in regulating cellular processes, such as gene expression and cellular behaviors. Some studies[4] showed that aberrant lncRNA expression is associated with cancer progression. Among these lncRNAs, few of them were functionally characterized.

The proposal of competing endogenous RNA (ceRNA) theory by Salmena et al[5] has extended our understanding of the roles of lncRNAs and provided novel insights into the mechanisms of gene expression regulation. LINC02418 was a lncRNA that recently reported to be significantly elevated expression in colorectal cancer tissues and cell lines, and was able to distinguish colorectal cancer patients from normal patients[6]. In addition, knockdown of LINC02418 was found it could inhibit colorectal cancer cell proliferation by arresting cell cycle and promoting cell apoptosis *via* the microRNA-1273g-3p/maternal embryonic leucine zipper kinase (MELK) axis[6]. To date, the roles of LINC02418 in NSCLC remain unclear and therefore further investigations are needed.

In this work, we characterized the expression pattern of LINC02418 in NSCLC tissues at ENCORI. In addition, LINC02418 expression level in NSCLC cells was explored. Effects of LINC02418 expression on NSCLC cell proliferation, invasion, and apoptosis were investigated using Cell Counting Kit-8 (CCK-8) assay, transwell invasion assay,

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and flow cytometry assay, respectively. At length, the mechanisms underlying the LINC02418-mediated effects of NSCLC cell behaviors were explored.

Materials and Methods

NSCLC Cell Lines

Shanghai Cell Collection Center (Shanghai, China) provided NSCLC cells A549, PC-9, and human normal bronchial epithelial cell (16HBE) used in this work. These cells were incubated at Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in supplement with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and antibiotic, and maintained at a 37°C humidified incubator containing 5 % of CO_2.

Cell Transfection

NSCLC cells were transfected with small interfering RNA (siRNA) targeting LINC02418 (si-LINC02418) or pcDNA3.1 contains full sequence of LINC02418 to manipulate LINC02418 expression using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The microRNA-4677-3p (miR-4677-3p) inhibitor and negative control (miR-NC) were purchased from RiboBio (Guangzhou, Guangdong, China). SiRNA targeting Sec61 gamma subunit (SEC61G, si-SEC61G) was also bought from RiboBio.

LINC02418 Expression Level Exploration at ENCORI

Expression level of LINC02418 in two major subtypes of NSCLC was explored at ENCORI[7], a database which contains gene expression data at Pancancer.

Quantitative Real Time Polymerase Chain Reaction (RT-qPCR)

RNA extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was reverse transcribed into complementary DNA (cDNA) with PrimeScript RT Kit (TaKaRa, Dalian, Liaoning, China). RT-qPCR was conducted at StepOne Real-time PCR system (Applied Biosystems; Foster City, CA, USA) using SYBR Green (TaKaRa, Dalian, Liaoning, China) with the following primers: LINC02418: 5'-CCTTCCTTTCCAGCAGGACTT-3' (forward) and 5'-GAGCAGAACCTGCCCAAAATG-3' (reverse); Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5'-ACCCACTCCTCCACCTTTGAC-3' (forward) and 5'-TGTTGCTGTAGCCAAATTCGTT-3' (reverse); miR-4677-3p: 5'-CTGTGAGACCAAAGAACTACTCGC-3' (forward), 5'-CTCTACAGCTATATTGCCAGCCAC-3' (reverse); U6 small nuclear RNA (U6 snRNA): 5'-CTCGCTTCGGCAGCACA-3' (forward), and 5'-AACGCTTCACGAATTTGCGT-3' (reverse). The $2^{-\Delta\Delta Ct}$ method was utilized to measure the expression level of target genes.

CCK-8 Assay

Cell proliferation rate in NSCLC cells was analyzed with CCK-8 reagent (Beyotime, Haimen, Jiangsu, China). 3×10^3 cells were seeded in 96-well plate and incubated for indicated time. Then, CCK-8 reagent was added to the well and further incubation for 4 h. Microplate reader was used to analyzed optical density at 450 nm.

Transwell Invasion Assay

3×10^4 cells in serum-free medium was filled into top chamber of Matrigel (BD Biosciences; San Jose, CA, USA) pre-coated 8 μm insert. DMEM contains 10% FBS was added to lower chamber. After incubation for 48 h, non-invasive cells were wiped, while invasive cells were fixed with paraformaldehyde, stained with crystal violet for 30 min, and observed using microscope (magnification 200×).

Flow Cytometry Assay

Cells were trypsinized and then stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI; Beyotime, Shanghai, China) in the dark and according to the provided protocols. Then, these stained cells were analyzed at FACSCalibur (BD Biosciences; San Jose, CA, USA) to measure the cell apoptosis rate.

Western Blot

Proteins extracted from cells with radio immunoprecipitation assay lysis buffer (RIPA; Beyotime, Shanghai, China) were quantified with the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Equal amounts of samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene difluoride (PVDF; Millipore, Billerica, MA, USA) membrane. After blocked with fat-free milk, membranes were incubated with primary antibodies (anti-SEC61G: NBP2-13291, Novus Biologicals, Shanghai, China, anti-GAPDH: ab181602, Abcam, Cambridge,

MA, USA) overnight at 4°C. Subsequently, membranes were incubated with suitable secondary antibody (ab6721, Abcam, Cambridge, MA, USA) for 1 h at room temperature. Band signals were visualized with BeyoECL kit (Beyotime, Shanghai, China).

Bioinformatic Analysis

ENCORI was used to analyze the miRNA that can target LINC02418, and we found miR-4677-3p was a putative target for LINC02418. Subsequently, TargetScan was used to analyze the targets of miR-4677-3p. Among these predicted targets, SEC61G was selected for further analyses.

Luciferase Activity Reporter Assay

Luciferase reporter vectors contain the wild-type (wt) sequence of LINC02418 or SEC61G were built and named as wt-LINC02418 or wt-SEC61G. Mutant (mt) luciferase reporter vectors were built using site-direct mutagenesis kit (TaKaRa, Dalian, China) and designated as mt-LINC02418 or mt-SEC61G. Cells were co-transfected with synthetic miRNAs or luciferase reporter vectors using Lipofectamine 2000. Relative luciferase activity was analyzed with Dual-Luciferase activity system (Promega, Madison, WI, USA) after transfection for 48 h.

Statistical Analysis

Statistical Product and Service Solutions version 20.0 (IBM, Armonk, NY, USA) was used for all data analyses and then presented as the manner of mean ± standard deviation. Student's *t*-test and one-way analysis of variance (ANOVA) and Tukey post-hoc test were utilized to analyze difference in groups. *p*-value less than 0.05 was considered as statistically significant.

Results

LINC02418 Expression Was Elevated in NSCLC

First, we analyzed LINC02418 expression in two major subtypes of NSCLC at ENCORI. As presented in Figure 1A and 1B, we found LINC02418 expression was significantly elevated in tumor tissues compared with normal tissues. Moreover, by utilizing RT-qPCR, we showed LINC02418 expression in NSCLC cell lines was significantly higher than in normal cell line (Figure 1C).

Knockdown of LINC02418 Inhibits NSCLC Cell Proliferation, Invasion but Promotes Apoptosis

Loss-of-function experiments were performed on A549 cell line. We showed the introduction of si-LINC02418 significantly decreased the levels of LINC02418 (Figure 2A). CCK-8 assay and transwell invasion assay indicated that knockdown of LINC02418 inhibited NSCLC cell proliferation and invasion (Figure 2B and 2C). Flow cytometry assay showed that LINC02418 downregulation promoted cell apoptosis (Figure 2D).

Overexpression of LINC02418 Promotes NSCLC Cell Proliferation, Invasion but Inhibits Apoptosis

Subsequently, gain-of-function experiments were conducted on PC-9 cell line. A significant increase of LINC02418 level was observed in

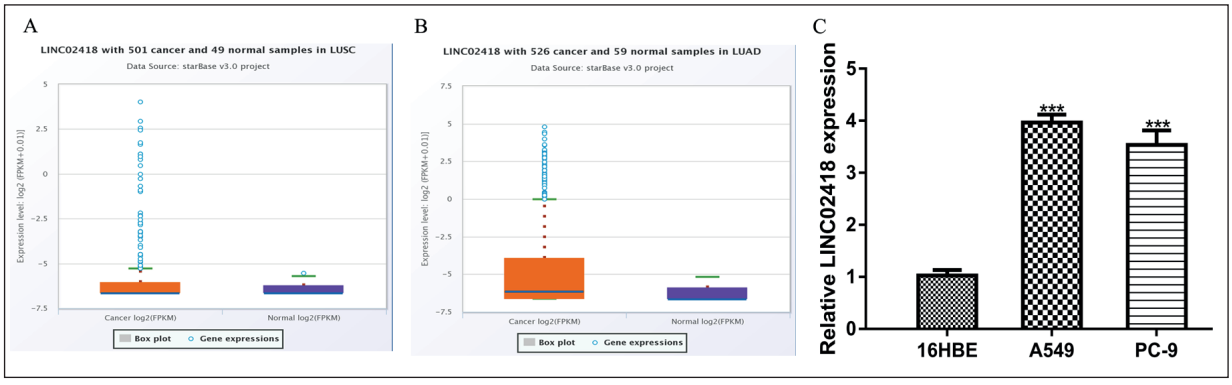

Figure 1. LINC02418 expression was elevated in NSCLC. Expression of LINC02418 in (**A**) lung squamous cell carcinoma and (**B**) Lung adenocarcinoma. **C**, Expression of LINC02418 in NSCLC cells and normal cell was examined by RT-qPCR. LINC02418: long non-coding RNA 02418; NSCLC: non-small cell lung cancer; RT-qPCR: Quantitative Real Time Polymerase Chain Reaction.

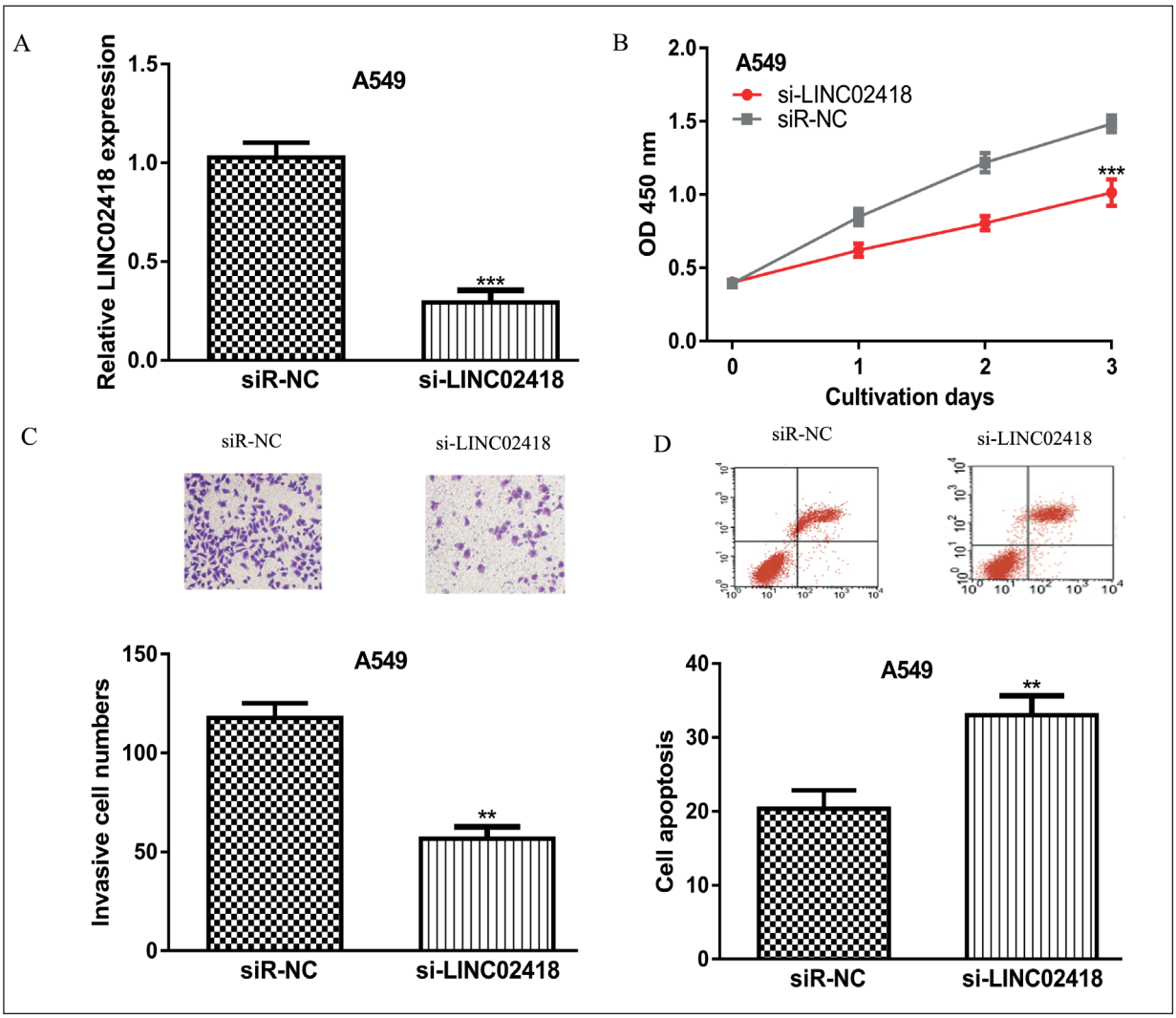

Figure 2. Knockdown of LINC02418 inhibits NSCLC cell proliferation, invasion but promotes apoptosis. **A**, LINC02418 expression, (**B**) Cell proliferation, (**C**) Cell invasion (magnification 200×), and (**D**) Cell apoptosis in NSCLC cells with si-LINC02418 or siR-NC transfection. LINC02418: long non-coding RNA 02418; NSCLC: non-small cell lung cancer; si-LINC02418: small interfering RNA targeting LINC02418; siR-NC: negative control small interfering RNA.

pLINC02418 transfection group (Figure 3A). As shown in CCK-8 assay and transwell invasion assay, LINC02418 overexpression significantly increased cell proliferation and invasion (Figure 3B and 3C). Not surprisingly, we found LINC02418 overexpression inhibited NSCLC cell apoptosis (Figure 3D).

MiR-4677-3p Directly Target LINC02418 and SEC61G

To understand the biological roles of LINC02418, bioinformatic analyses algorithms were used to predict the miRNA that can target LINC02418. As showed in Figure 4A, we found miR-4677-3p could bind with LINC02418. Luciferase activity reporter assay conducted in A549 cell line showed miR-4677-3p inhibitor transfection increased the luciferase activity in wt-LINC02418 transfected group (Figure 4B). We showed that miR-4677-3p expression level in NSCLC cell can be downregulated by miR-4677-3p inhibitor (Figure 4C). Furthermore, we analyzed the targets of miR-4677-3p using TargetScan. We found SEC61G was a putative target for miR-4677-3p (Figure 4D). Not surprisingly, the introduction of miR-4677-3p inhibitor elevated the luciferase activity in cells with wt-SEC61G transfection (Figure 4E).

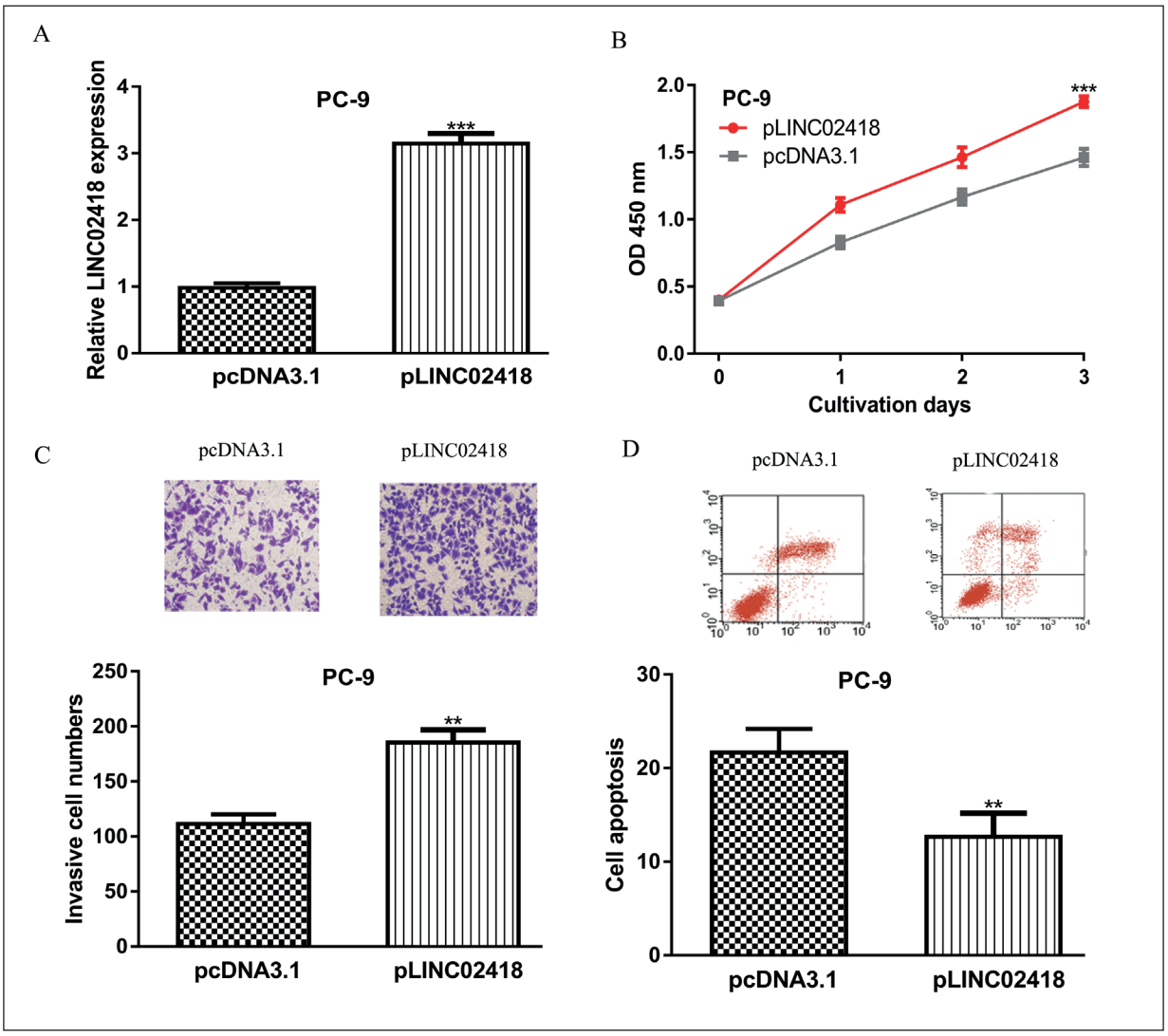

Figure 3. Overexpression of LINC02418 promotes NSCLC cell proliferation, invasion but inhibits apoptosis. **A**, LINC02418 expression, (**B**) Cell proliferation, (**C**) Cell invasion (magnification 200×), and (**D**) Cell apoptosis in NSCLC cells with pLINC02418 or pcDNA3.1 transfection. LINC02418: long non-coding RNA 02418; NSCLC: non-small cell lung cancer.

LINC02418 Regulates the NSCLC Cell Behaviors Via Targeting MiR-4677-3p/SEC61G Axis

At length, we investigated whether LINC02418 controls NSCLC cell events *via* miR-4677-3p/SEC61G axis using rescue experiments. We found SEC61G expression was increased by miR-4677-3p inhibitor but decreased by si-LINC02418 and si-SEC61G (Figure 5A). *In vitro* functional assays demonstrated that knockdown of SEC61G inhibited NSCLC cell proliferation, invasion but promoted apoptosis (Figure 5B-5D). In addition, we showed knockdown of miR-4677-3p promoted cell proliferation, invasion but inhibited apoptosis (Figure 5B-5D). More importantly, we showed the effects of miR-4677-3p on NSCLC cell behaviors can be partially reversed by si-SEC61G (Figure 5B-5D).

Discussion

Numerous genes, including protein-coding genes and non-protein-coding genes have been identified to be abnormally expressed in NSCLC[7,8]. For instance, formin protein formin-like 1 (FMNL1) was found significantly elevated in NSCLC tissues and correlated with bone metas-

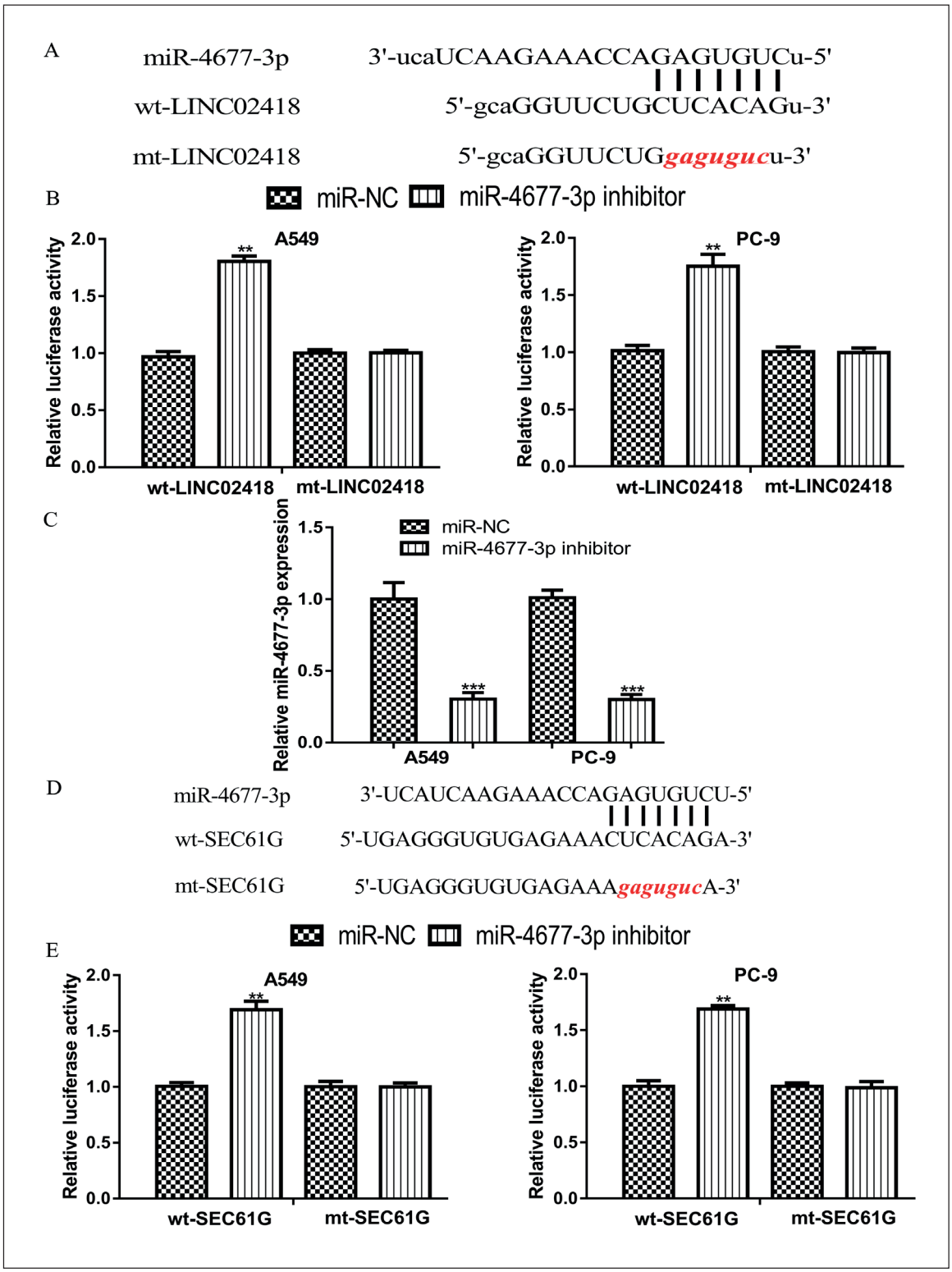

Figure 4. MiR-4677-3p direct target LINC02418 and SEC61G in NSCLC. **A**, Binding site between miR-4677-3p and LINC02418. **B**, Luciferase activity in NSCLC cell with synthetic miRNAs or LINC02418 luciferase vectors transfection. **C**, MiR-4677-3p expression in NSCLC cell with synthetic miRNAs transfection. **D**, Binding site between miR-4677-3p and SEC61G. **E**, Luciferase activity in NSCLC cell with synthetic miRNAs or SEC61G luciferase vectors transfection. LINC02418: long non-coding RNA 02418; NSCLC: non-small cell lung cancer; miR-4677-3p: microRNA-4677-3p; SEC61G: Sec61 gamma subunit; wt: wild-type; mt: mutant.

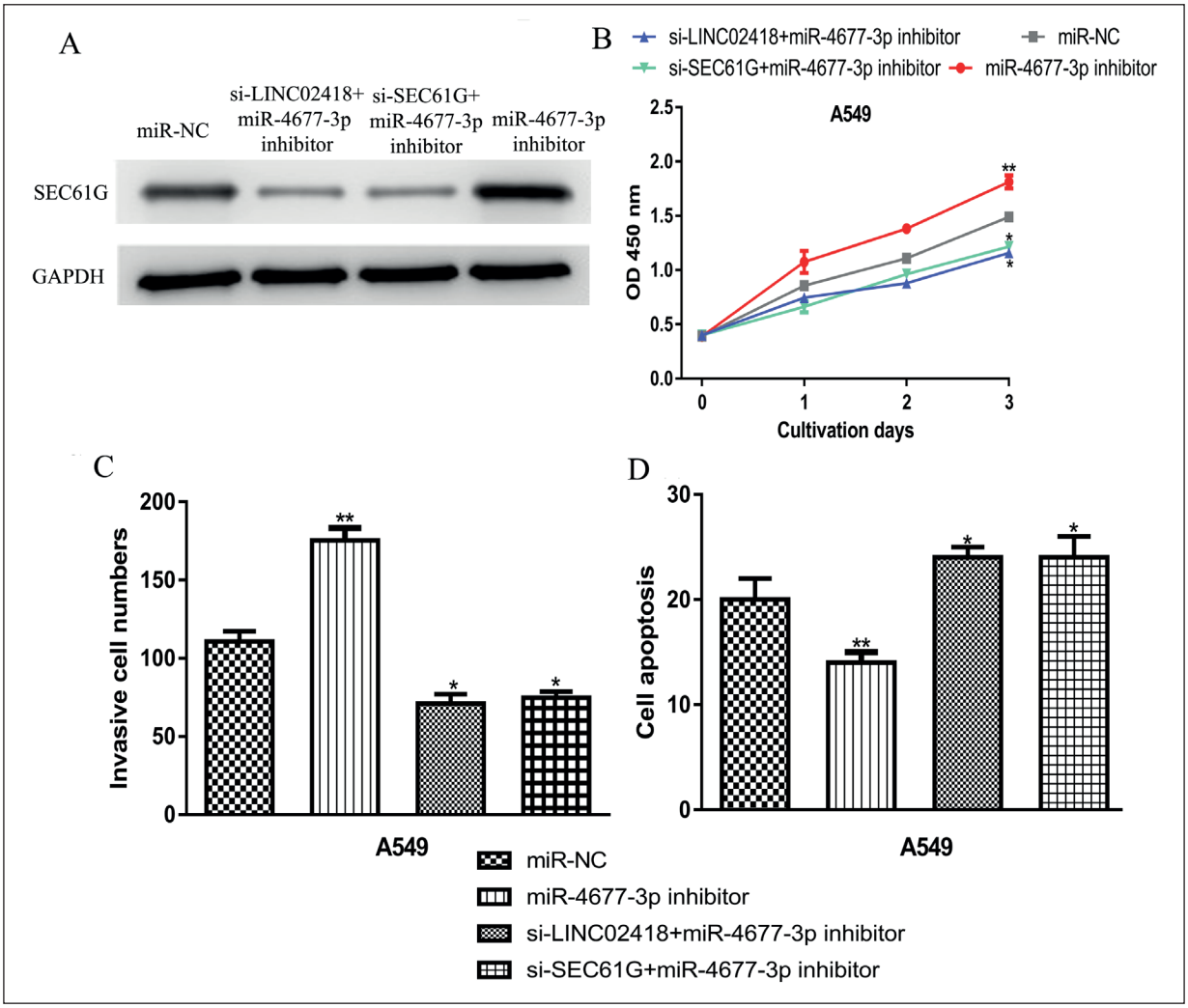

Figure 5. LINC02418 regulates NSCLC cell behaviors *via* targeting miR-4677-3p/SEC61G axis. **A**, SEC61G expression, (**B**) Cell proliferation, (**C**) Cell invasion, and (**D**) Cell apoptosis in NSCLC cell with miR-4677-3p inhibitor, si-SEC61G, miR-4677-3p inhibitor and si-LINC02418, or miR-4677-3p inhibitor and si-SEC61G transfection. LINC02418: long non-coding RNA 02418; NSCLC: non-small cell lung cancer; miR-4677-3p: microRNA-4677-3p; SEC61G: Sec61 gamma subunit.

tasis[9]. Knockdown of FMNL1 decreases NSCLC cell proliferation, migration, and invasion *via* regulating transforming growth factor-β1 signaling pathway[9]. MiR-451a overexpression suppressed NSCLC cell proliferation and migration *via* regulating liver kinase B1 (LKB1) and activating adenosine monophosphate-activated protein kinase[10]. Moreover, they found the knockdown of miR-451a will cause opposite effects on NSCLC cells[10]. As regard to lncRNA, lncRNA cancer susceptibility 19 (CASC19) was found elevated expression in both NSCLC tissues and cells, and correlated with poorer overall survival[11]. Functional assays showed CASC19 downregulation hindered NSCLC cell growth and metastasis *via* sponging miR-130b-3p[11]. Zoli et al[12] showed LINC00472 was reduced expression in NSCLC and regulated cancer progression *via* miR-149-3p and miR-4270.

In this research, we showed LINC02418 expression level was elevated in both NSCLC tissues and cell lines. Through gain and loss-of-function experiments, we showed that LINC02418 could promote NSCLC cell proliferation, invasion but inhibit apoptosis. These studies indicated that LINC02418 functions as an oncogene in NSCLC, which is consistent with its role in colorectal cancer[6]. In colorectal

cancer, LINC02418 was revealed to function as a ceRNA for MELK and hence to influence cancer progression. Therefore, we are also interested in investigating the molecules mediated by LINC02418 in NSCLC. By online bioinformatic prediction tools, we showed miR-4677-3p has the potential to target both LINC02418 and SEC61G. Luciferase activity reporter assays confirmed LINC02418 and SEC61G were targets for miR-4677-3p.

MiR-4677-3p is a recently identified miRNA that aberrantly expressed in lung adenocarcinoma and regulated by lncRNA TTN antisense RNA 1 (TTN-AS1)[13]. Also, the TTN-AS1/miR-4677-3p/zinc finger E-box binding homeobox 1 axis was shown to influence the migration and invasion of lung adenocarcinoma cells[13]. SEC61G is one of the subunits of Sec61 complex and involved in the regulation of multiple biological processes[14]. SEC61G was also found overexpressed in gastric cancer, breast cancer, and glioblastoma[15-17]. In the rescue assays, we showed LINC02418 regulates NSCLC cell proliferation, invasion, and apoptosis *via* regulating miR-4677-3p/SEC61G axis.

Conclusions

Based on these results, we showed LINC02418 regulates SEC61G expression by sponging miR-4677-3p to affect NSCLC cell behaviors. We emphasize the value of LINC02418 in human cancers, and may provide potential therapeutic target for NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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