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Long non-coding RNA SNHG15 accelerates the progression of non-small cell lung cancer by absorbing miR-211-3p

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Abstract. – OBJECTIVE: The aim of this study was to investigate whether lncSNHG15 promoted the proliferation and migration of non-small cell lung cancer (NSCLC) cells by binding to miR-211-3p, thereby participating in the development of NSCLC.

PATIENTS AND METHODS: Expressions of lncSNHG15 and miR-211-3p in NSCLC tissues and para-cancerous tissues were detected by quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR). LncSNHG15 and miR-211-3p expression in NSCLC cell lines were determined as well. Furthermore, cell counting kit-8 (CCK-8) and transwell assays were performed to evaluate the effects of lncSNHG15 and miR-211-3p on cell proliferation and migration, respectively. The binding relationship between miR-211-3p and ZNF217, as well as between miR-211-3p and lncSNHG15, were further verified by the Luciferase reporter gene assay. In addition, rescue experiments were performed to verify whether lncSNHG15 promoted the proliferation and migration of NSCLC cells by degrading miR-211-3p.

RESULTS: The expression of lncSNHG15 in NSCLC tissues was significantly higher than that of para-cancerous tissues. In particular, the expression of lncSNHG15 in NSCLC patients with stage III-IV was higher than those with stage I-II. Furthermore, lncSNHG15 over-expression remarkably promoted the proliferation and migration of NSCLC cells (A549 and H358). The Luciferase reporter gene assay further indicated that lncSNHG15 could bind to miR-211-3p. Simultaneously, miR-211-3p expression in NSCLC tissues was significantly lower than that of para-cancerous tissues. The over-expression of miR-211-3p could inhibit the proliferation and migration of A549 and H358 cells. Meanwhile, the Luciferase reporter gene assay indicated that ZNF217 was the target of miR-211-3p. In addition, the over-expression of ZNF217 reversed the inhibitory effect of miR-211-3p on the proliferative and migratory potentials of A549 and H358 cells.

CONCLUSIONS: High expression of lncSNHG15 promoted the proliferation and migration of NSCLC cells by upregulating ZNF217 by adsorbing miR-211-3p.

Key Words:
Gastric cancer, microRNA, miR-198, Toll-like receptor 4 (TLR4).

Introduction

Non-small cell lung cancer (NSCLC) is one of the most common malignant tumors with the highest morbidity and mortality in the world[1]. In the past decade, individualized treatment based on pathological characteristics of NSCLC has made great progress. However, the five-year survival of NSCLC is far from satisfactory[2,3]. A great number of studies[4] have shown that long non-coding RNA (lncRNA) plays an important role in tumorigenesis and development. A previous study has shown that the expression of lncRNA H19 in gastric cancer cells and tissues is significantly higher than controls. The over-expression of H19 significantly promotes the growth of gastric cancer AGS cells. This indicates that lncRNA H19 is involved in the development of gastric cancer[5]. Furthermore, the expression of MALAT1 increases with the elevated recurrent risk of hepatocellular carcinoma. Meanwhile, cell viability and invasiveness are significantly reduced by inhibiting MALAT1 expression in hepatocellular carcinoma cells. These findings indicate that MALAT1 has the potential to be a new therapeutic target[6]. In addition, lncSNHG15 is highly expressed in pancreatic cancer. It also promotes the proliferation of pancreatic cancer cells, thereby stimulating tumor development[7]. Moreover, lncSNHG15

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is also highly expressed in osteosarcoma. A functional experiment has shown that SNHG15 may participate in osteosarcoma progression by promoting the proliferation and invasion of osteosarcoma cells[8]. Despite massive studies on SNHG15 in different cancers, few researches have focused on the role of SNHG15 in NSCLC. The aim of this work was to explore whether SNHG15 exerted biological functions in NSCLC. As evidenced by multiple previous studies, in addition to direct involvement in the disease development, lncRNAs affect the expressions of target genes by adsorbing miRNAs. This may finally lead to the onset of diseases[9]. These lncRNAs are also known as competing endogenous RNA (ceRNA). LncRNA FER1L4 has been reported to be down-regulated in gastric cancer. As a ceRNA, FER1L4 regulates the mRNA expressions of miR-106a-5p and its target gene PTEN, thereby promoting the proliferation of tumor cells and participating in gastric cancer occurrence[10]. Furthermore, other studies have indicated that lncRNA HOTAIR targets miR-331-3p in the manner of ceRNA in gastric cancer. Furthermore, it regulates the expression of oncogene HER2, activates EGF-related signaling pathways and eventually promotes the growth and proliferation of gastric cancer[11]. In addition, lncRNA-ATB induces epithelial-mesenchymal transition of hepatocellular carcinoma cells by up-regulating ZEB1 and ZEB2 through competitive binding with members in miR-200 family. On the other hand, lncRNA-ATB promotes metastasis of hepatocellular carcinoma cells through combining with IL-11. This can eventually lead to a cascade reaction of invasion-migration in hepatocellular carcinoma cells[12]. Previous bioinformatics analysis has revealed that miR-211-3p is a potential target gene for lncSNHG15. Meanwhile, ZNF217 is a potential target for miR-211-3p[13]. Therefore, it was hypothesized that lncSNHG15 could serve as a ceRNA to regulate the expression of ZNF217 by adsorbing miR-211-3p, thus participating in the NSCLC development.

Patients and Methods

Patients

Fresh NSCLC tissues and adjacent normal tissues were collected from 24 NSCLC patients who underwent surgical treatment. Enrolled patients received no preoperative treatments, and the patients declared no family history. NSCLC was confirmed by pathological examination. Signed informed consent was obtained from each patient before the research. This study was approved by the Ethics Committee of Beijing Shijitan Hospital, Capital Medical University. All collected specimens were stored in liquid nitrogen for further experiments.

Cell Culture

Normal cell line (HBEC3) and NSCLC cell lines (H358, H1299, H23 and A549) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 0.1 mg/mL streptomycin, and maintained in an incubator with 5% CO_2 volume at 37°C.

Cell Transfection

An appropriate number of cells were first inoculated into culture plates or bottles. When the density was up to 70%-80%, cell transfection was performed. Furthermore, cells were transfected with miR-211-3p mimics, pcDNA-lncSNHG15, pcDNA-ZNF217 or corresponding negative control, respectively.

Total RNA Extraction

50 mg tissue was collected, ground in liquid nitrogen and incubated with 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Cells were harvested and washed with Phosphate-Buffered Saline (PBS) once, followed by incubation with 1 mL TRIzol reagent. Subsequently, prepared tissues or cells were evenly homogenized, transferred to a 1.5 mL centrifuge tube and incubated for 5 min. After that, chloroform was added to the lysates at a ratio of 1:5, gently mixed and maintained at room temperature for 5 min. Afterward, the lysates were centrifuged at 12000 rpm/min and 4°C for 10 min. The supernatant was transferred to another new 1.5 mL centrifuge tube, and an equal volume of isopropanol was added. Then the mixture was centrifuged at 12000 rpm/min and 4°C for another 10 min. The precipitate was washed with 1 ml 75% ethanol, centrifuged and air dried. Extracted RNA was diluted in diethyl pyrocarbonate water (DEPC; Beyotime Biotechnology, Shanghai, China) and preserved for subsequent use.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

RNA samples were quantified and reversely transcribed into complementary deoxyribose nucleic acid (cDNA). PCR reaction system was prepared based on the addition of SYBR Green (Applied Biosystems, Foster City, CA, USA), including premixed liquid, template, upstream and downstream primers and DEPC water. PCR amplification reaction was carried out by the Real-Time-PCR instrument. The relative gene expressions were quantitatively analyzed by PCR according to the instructions of the miRNA RT Kit (Tiangen Biotech, Beijing, China). Primer sequences used in this study were listed as follows: MiR-211-3p (F: 5'-ACACTCCAGCTGGGGCAGGGACAGCAAAG-3', R: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCCCCACG-3'); U6 (F: 5'-CTCGCTTCGGCAGCAGCACATATA-3', R: 5'-AAATATGGAACGCTTCACGA-3'); LncSNHG15 (F: 5'-GCTGAGGTGACGGTCTCAAA-3', R: 5'-GCCTCCCAGTTTCATGGACA-3'); GAPDH (F: 5'-GAAGAGAGAGACCCTCACGCTG, R: 5'-ACTGTGAGGAGGGGAGATTCAGT-3'); ZNF217 (F: 5'-GAGAAGCGAATGGTGAAAGC-3', R: 5'-CAGCGCTCAAGTATGCAAAA-3').

Luciferase Reporter Gene Assay

The transcript 3'UTR sequence of ZNF217 gene was cloned into vector pGL3 containing Luciferase reporter gene, namely ZNF217 WT group. The mutative plasmid was constructed by site-directed mutagenesis kit as ZNF217 MUT group. Meanwhile, lncSNHG15 WT group and lncSNHG15 MUT group were constructed as well. Cells in each group were co-transfected with Renilla Luciferase reference plasmid (Promega, Madison, WI, USA) and miR-211-3p mimics. After co-transfection for 24 h, the cells were lysed, followed by centrifugation at 10,000 g/min for 5 min. 100 μL lysate supernatant was collected for subsequent determination. Renilla Luciferase was used as an internal control. RLU values measured by firefly Luciferase were calculated as RLU values determined by Renilla Luciferase.

Cell Migration

The transwell chamber was first placed in 24-well culture plates. Transfected NSCLC cells were suspended in a serum-free medium, and cell density was adjusted to 1×10^5/ml. The cell suspension was added to the upper transwell chamber; meanwhile, the culture medium containing 10% FBS was added to the lower chamber. After 48 hours of culture, the cells were washed with PBS twice and the transwell chamber was then removed. Penetrating cells were fixed with 4% paraformaldehyde for 30 min, stained with 0.5% crystal violet for 10-15 min and washed with PBS. Finally, cells were observed under a microscope and the number of cells was counted.

Cell Proliferation

Transfected NSCLC cells were inoculated into 96-well plates at a density of 1×10^4/well. Subsequently, the cells were cultured for 6, 24, 48, 72 and 96 h, respectively. 10 μL cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) was added to each well, followed by incubation at 37° C for 1 h in the dark. The optical density (OD) value at 450 nm was detected by a microplate reader. 5 repeated wells were set in each group.

Western Blot

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel at an appropriate concentration was prepared according to the molecular weight of target proteins. An amount of 50 μg proteins was loaded in each electrophoretic lane and the electrophoretic voltage was set at 80 V for 40 min and was transferred to 120 V for 60-80 min. Proteins were transferred onto nitrocellulose membranes (Thermo Fisher Scientific, Waltham, MA, USA) by wet transfer method at the voltage of 100 V for 90 min on ice. After blocking with 5% skimmed milk powder for 1 h, the membranes were incubated with primary antibodies overnight at 4°C. Subsequently, the membranes were incubated with diluted secondary antibody at room temperature for 1 h. Chemiluminescence imaging system was used for development, followed by photography using an imaging system (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Measurement data were expressed as mean ± standard deviation (Mean ± SD). *t*-test was used to compare the difference between the two groups. ROC curve was introduced to evaluate the diagnostic sensitivity of SNHG15 in NSCLC. The Kaplan-Meier method was performed to evaluate the overall survival of NSCLC based on the SN-

HG15 expression. Pearson correlation analysis was performed to determine the relationship between two gene expressions in NSCLC. $p < 0.05$ was considered statistically significant.

Results

LncSNHG15 Was Highly Expressed in NSCLC

We first detected the expression of lncSNHG15 in para-cancerous tissues and NSCLC tissues by qRT-PCR. The lncSNHG15 expression in NSCLC tissues was significantly higher than that of para-cancerous tissues (Figure 1A). In particular, lncSNHG15 expression in NSCLC tissues with stage III-IV was remarkably higher than those with stage I-II. This indicated that the expression of lncSNHG15 was positively correlated with pathological grade of NSCLC (Figure 1B). Furthermore, the survival curve was introduced by analyzing the clinical information of enrolled NSCLC patients. The results showed that the area under the ROC curve was 0.8811 and the cut-off value was 0.03233 (Figure 1C). These results suggested that lncSNHG15 might be a potential biomarker for NSCLC. Simultaneously, the results indicated that the five-year survival rate of NSCLC patients with high lncSNHG15 expression was remarkably lower than those with low lncSNHG15 expression (p = 0.0081, HR = 3.298; Figure 1D). These results suggested that lncSNHG15 might be involved in the occurrence of NSCLC.

LncSNHG15 Promoted Proliferation and Migration of NSCLC Cells

Owing to the significant high expression of lncSNHG15 in NSCLC tissues, we further explored its expression in NSCLC cells (Figure 2A).

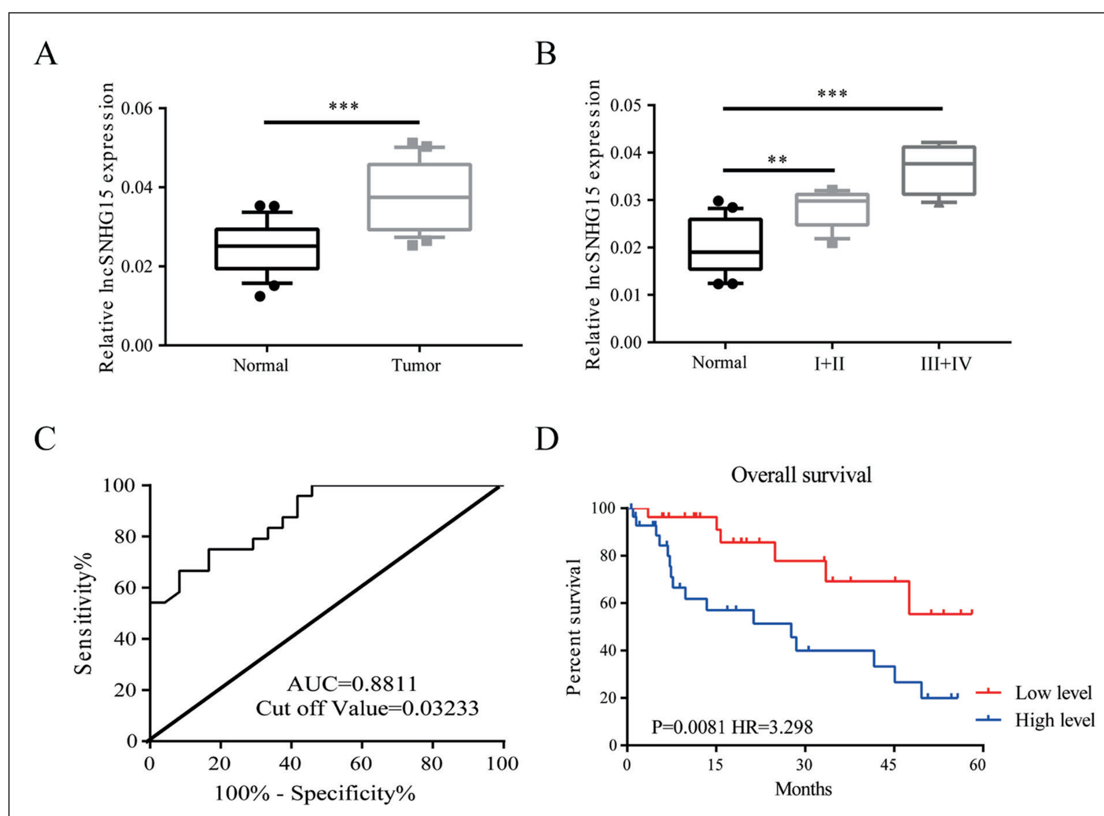

Figure 1. LncSNHG15 was highly expressed in NSCLC. ***A,*** The expression of lncSNHG15 was detected in 24 pairs of para-cancerous tissues and lung cancer tissues. The results showed that the expression of lncSNHG15 was significantly higher in lung cancer tissues. ***B,*** The expression of lncSNHG15 was markedly higher in NSCLC tissues with stage III-IV than those with stage I-II. ***C,*** The survival curve of lncSNHG15 was analyzed. The area under the ROC curve was 0.8811, and the cut-off value was 0.03233. ***D,*** The five-year survival of NSCLC patients with high expression of lncSNHG15 was significantly lower than those with low expression (p = 0.0081, HR = 3.298). *p < 0.05, **p < 0.01, ***p < 0.001.

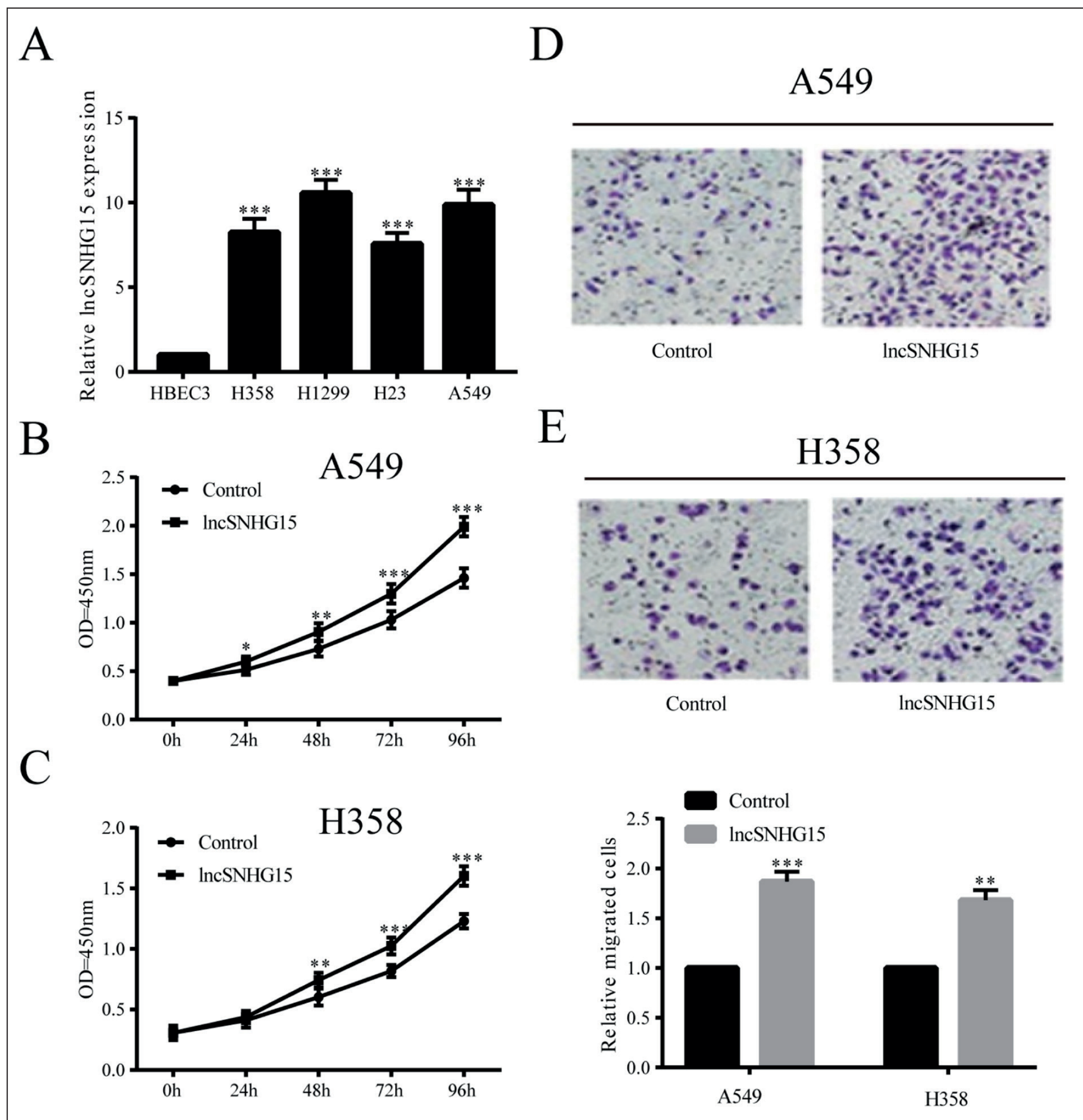

Figure 2. LncSNHG15 promoted the proliferation and migration of NSCLC cells. ***A,*** LncSNHG15 was highly expressed in lung cancer cell lines. ***B, C,*** The over-expression of lncSNHG15 significantly promoted the proliferation of A549 and H358 cell lines. ***D, E,*** The over-expression of lncSNHG15 enhanced the migration of A549 and H358 cell lines. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

The results showed that lncSNHG15 was highly expressed in selected NSCLC cell lines. Due to the moderate expression of lncSNHG15, A549 and H358 cell lines were selected for cell function experiments. Over-expression plasmid of lncSNHG15 was transfected into A549 and H358 cell lines. Subsequently, the effect of lncSNHG15 on cell proliferation was explored by the CCK-8 assay. The results showed that lncSNHG15 over-expression significantly promoted cell proliferation (Figure 2B, 2C). At the same time, the cell migration ability was detected by transwell assay. The results demonstrated that the over-expression of lncSNHG15 remarkably enhanced the migration of NSCLC cells (Figure 2D, 2E). The above results suggested that lncSNHG15 accelerated the progression of NSCLC by promoting cell proliferation and migration.

MiR-211-3p Could Combine LncSNHG15

Bioinformatics analysis predicted that miR-211-3p was a target gene of lncSNHG15. There-

fore, we speculated that the regulatory effect of lncSNHG15 on NSCLC development might depend on adsorbing miR-211-3p. The expression of miR-211-3p in NSCLC tissues was detected. The results showed that miR-211-3p was lowly expressed in NSCLC (Figure 3A). Similar results were also observed in NSCLC cell lines (Figure 3B). The correlation analysis based on Pearson correlation coefficient indicated that lncSNHG15 was negatively correlated with miR-211-3p expression in NSCLC (Figure 3C), suggesting a regulatory relationship between lncSNHG15 and miR-211-3p. Subsequent Luciferase reporter assay revealed that miR-211-3p could bind to lncSNHG15 (Figure 3D). QRT-PCR further verified that miR-211-3p expression was downregulated in A549 and H358 cells overexpressing lncSNHG15 (Figure 3E). This indicated that lncSNHG15 could bind to miR-211-3p and downregulate its expression. Subsequently, to further verify whether lncSNHG15 exerted its role *via* miR-211-3p, miR-211-3p and lncSNHG15 were co-overexpressed in A549 and H358 cells. The CCK-8 assay indicated that the promotive effect of lncSNHG15 on cell proliferation was reversed by miR-211-3p overexpression (Figure 3F, 3G). The transwell assay also showed that miR-211-3p overexpression reversed the promotive effect of lncSNHG15 on cell migration (Figure 3H).

ZNF217 Was the Target Gene of MiR-211-3p

In this study, online prediction software predicted that ZNF217 was a potential target gene for miR-211-3p. The expression level of ZNF217 was subsequently detected in NSCLC tissues. The re-

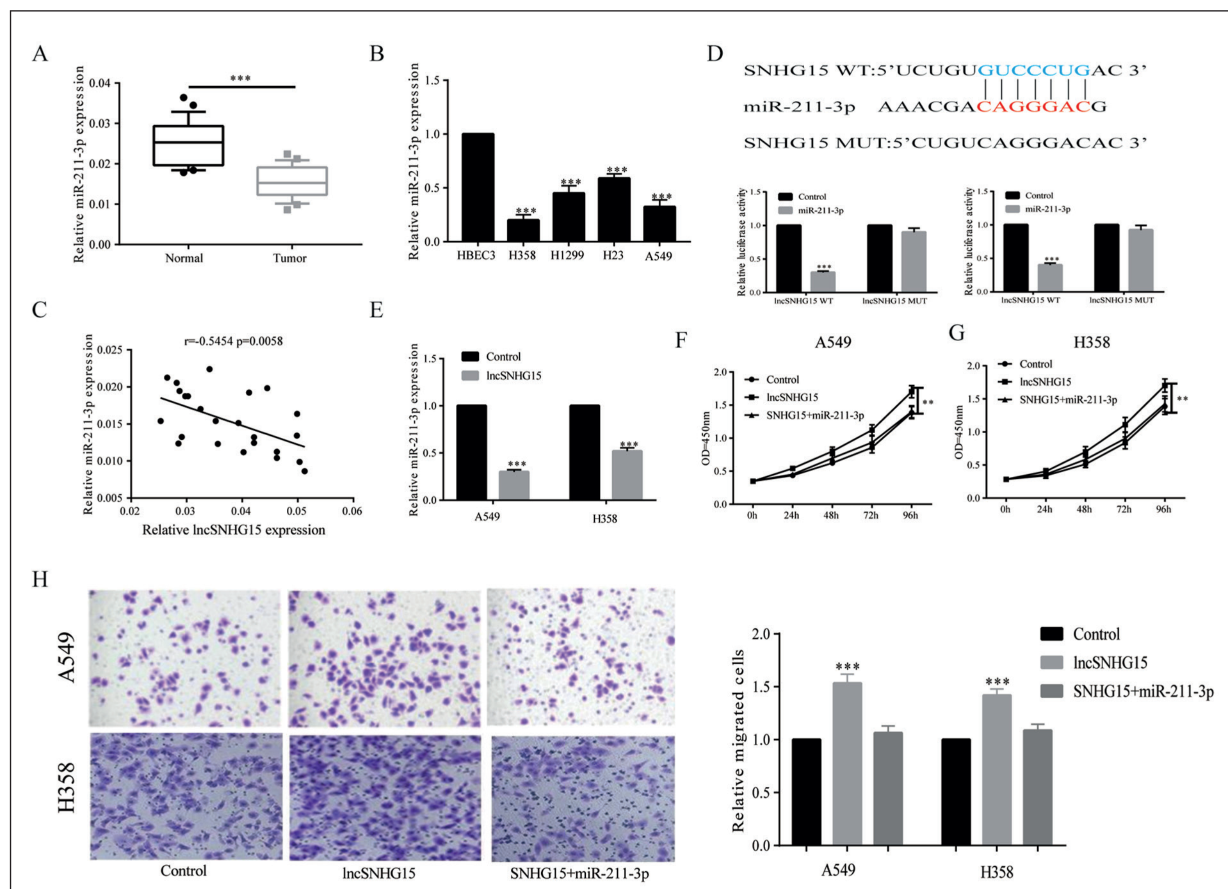

Figure 3. MiR-211-3p could combine lncSNHG15. ***A,*** The expression of miR-211-3p was significantly lower in lung cancer tissues. ***B,*** The expression of miR-211-3p was significantly lower in lung cancer cell lines. ***C,*** The expression of lncSNHG15 was negatively correlated with miR-211-3p expression ($R^2 = 0.2975$, $p = 0.0058$). ***D,*** Luciferase reporter gene assay showed that miR-211-3p could bind to lncSNHG15. ***E,*** The expression of miR-211-3p was significantly decreased after over-expressing lncSNHG15 in A549 and H358 cell lines. ***F, G,*** The over-expression of lncSNHG15 remarkably promoted the proliferation of A549 and H358 cell lines, which was reversed by miR-211-3p over-expression. ***H,*** The over-expression of lncSNHG15 markedly advanced the migration of A549 and H358 cell lines, which was reversed by miR-211-3p over-expression. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

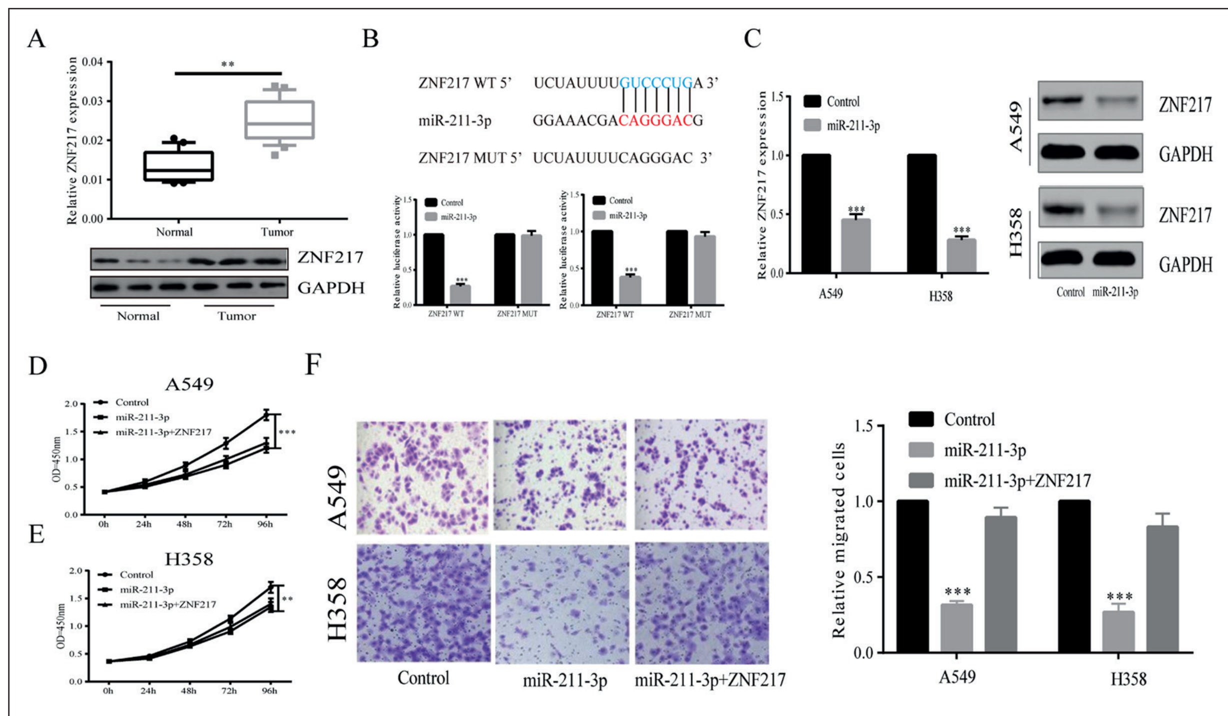

Figure 4. ZNF217 was the target gene of miR-211-3p. ***A,*** ZNF217 expression was significantly higher in lung cancer tissues at both mRNA and protein levels. ***B,*** Luciferase reporter gene assay revealed that ZNF217 could bind to miR-211-3p. ***C,*** The over-expression of miR-211-3p significantly decreased the expression of ZNF217 at mRNA and protein levels in A549 and H358 cell lines. ***D, E,*** In A549 and H358 cell lines, the over-expression of miR-211-3p significantly inhibited cell proliferation, which was reversed by the over-expression of ZNF217. ***F,*** The over-expression of miR-211-3p markedly reduced the migration of A549 and H358 cells, which was reversed by over-expression of ZNF217. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

sults showed that ZNF217 was highly expressed in NSCLC tissues at both mRNA and protein levels (Figure 4A). To verify the binding condition between ZNF217 and miR-211-3p, Luciferase reporter assay was performed. The results showed that ZNF217 could bind to miR-211-3p (Figure 4B). Afterward, ZNF217 expression was determined in A549 and H358 cells over-expressing miR-211-3p. QRT-PCR and Western blotting results showed that the over-expression of miR-211-3p could significantly inhibit the expression of ZNF217 at both mRNA and protein levels (Figure 4C). These results suggested that miR-211-3p could bind to and degrade ZNF217. Therefore, we speculated that miR-211-3p played a role by targeting ZNF217. To verify the above hypothesis, A549 and H358 cells were co-overexpressed with miR-211-3p and ZNF217. The over-expression of ZNF217 reversed the inhibitory effect of miR-211-3p on the proliferation of A549 and H358 cells (Figure 4D, 4E). Furthermore, inhibited migration of A549 and H358 cells induced by miR-211-3p overexpression was reversed by ZNF217 over-expression (Figure 4F). The above results indicated that miR-211-3p exerted its role in NSCLC by targeting ZNF217.

Discussion

In recent years, lncRNA has gradually become a new research hot-spot. Previous studies have shown that lncRNA plays a significant role in regulating cell differentiation, proliferation and metastasis[12]. LncRNAs also exert their biological functions by interacting with miRNAs. For example, by competitive binding to members in the miR-181 family, lncRNA MEG3 inhibits the proliferation, migration and invasion of gastric cancer cells[14]. Researches have also reported that lncRNA APF regulates the expression level of ATG7, and further regulates the autophagy of cardiac myocytes and myocardial infarction by targeting miR-188-3p[15]. In this study, lncSNHG15 was highly expressed in NSCLC tissues. Therefore, we speculated that

lncSNHG15 might be crucial in the progression of NSCLC. The effect of lncSNHG15 on the proliferation and migration of NSCLC cells were verified by over-expressing lncSNHG15 in A549 and H358 cells. The results proved the promotive effects of lncSNHG15 on proliferative and migratory potentials of NSCLC cells. Since lncRNA plays a role by adsorbing miRNAs, therefore, it was hypothesized in our work that lncSNHG15 functioned by targeting miRNAs. Bioinformatics analysis found that miR-211-3p was a potential target gene of lncSNHG15. The expression of miR-211-3p was significantly decreased in NSCLC. The Luciferase reporter gene assay further demonstrated the binding condition between lncSNHG15 and miR-211-3p. After over-expression of lncSNHG15, miR-211-3p expression was significantly downregulated. This indicated that miR-211-3p was negatively regulated by lncSNHG15. To verify whether the function of lncSNHG15 was mediated by miR-211-3p, miR-211-3p and lncSNHG15 were co-overexpressed in NSCLC cell lines. Rescue experiment results showed that the over-expression of miR-211-3p could partially reverse the effect of lncSNHG15 on the growth of NSCLC cells, which supported our speculation. MiRNA regulates gene expression by targeting its target genes, thereby participating in tumor progression[16]. Previous evidence has revealed that the expressions of miRNAs may be positively correlated with E-cadherin in pancreatic cancer. Meanwhile, patients with high expression of miR-200c present a higher survival rate. Nevertheless, the proliferation ability is significantly increased after the over-expression of miR-200c, whereas cell invasion is decreased[17]. In addition, other studies have found miR-21 promotes the protrusion-like changes of colon cancer cells by down-regulating Sprouty2, which is an inhibitory factor with dendrite-like growth. This may eventually enhance cell migration[18]. In epithelial ovarian cancer, it has been proved that miR-211 alleviates ovarian cancer development by inhibiting the proliferation and cell cycle of ovarian cancer cells by down-regulating Cyclin D1 and CDK6[19]. Furthermore, miR-211 shows an inhibitory effect on the proliferation and invasion of gastric cancer cells by down-regulating the expression of SOX4. Therefore, miR-211 provides a new target for the prevention and treatment of gastric cancer[20]. In this study, we found that miR-211-3p was lowly expressed in NSCLC. ZNF217 was predicted to be a potential target gene of miR-211-3p. ZNF217 plays a significant role in promoting the progression of cancers[21]. In our work, the miRNA and protein expressions of ZNF217 in NSCLC were significantly increased. Furthermore, ZNF217 expression was significantly decreased after the over-expression of miR-211-3p. Luciferase reporter gene assay results revealed a successful binding relationship between ZNF217 and miR-211-3p. To further explore whether the function of miR-211-3p was mediated by ZNF217, the over-expression of miR-211-3p was constructed in NSCLC cell lines. Subsequent results showed that the proliferation and migration of cells were significantly inhibited, which could be partially reversed by the over-expression of ZNF217. Furthermore, we confirmed that the function of miR-211-3p in NSCLC was achieved through the participation of ZNF217. To sum up, this study concluded that lncSNHG15 promoted the occurrence and development of NSCLC through the lncSNHG15/miR-211-3p/ZNF217 axis, which served as a ceRNA to sponge miR-211-3p. Our results provided a theoretical basis for the treatment and prevention of NSCLC.

Conclusions

We found that high expression of lncSNHG15 promoted the proliferation and migration of NSCLC cells by upregulating ZNF217 expression by adsorbing miR-211-3p.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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