European Review for Medical and Pharmacological Sciences 2018; 22: 5946-5953

LncRNA H19 serves as a ceRNA and participates in non-small cell lung cancer development by regulating microRNA-107

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Abstract. – OBJECTIVE: To investigate whether lncRNA H19 can regulate NF1 expression through competitive binding to microRNA-107, thereby participating in the occurrence and development of non-small cell lung cancer (NSCLC).

PATIENTS AND METHODS: Expression levels of H19 and NF1 in NSCLC tissues, paracancerous tissues and NSCLC cell lines were detected by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). The binding condition of microRNA-107, H19 and NF1 was detected by dual-luciferase reporter gene assay. Corresponding lentiviruses of H19 were constructed. The regulatory effects of H19 on proliferative and migratory abilities of A549 cells were detected by cell counting kit-8 (CCK-8) and transwell assay, respectively. Rescue experiments were conducted to explore the regulatory interaction between H19 and microRNA-107 in A549 cells.

RESULTS: H19 and NF1 were highly expressed in NSCLC tissues and NSCLC cell lines (A549 and HCC823) than those of controls. Overexpressed H19 increased proliferative and migratory abilities of A549 cells. Dual-luciferase reporter gene assay demonstrated that H19 regulates NF1 expression through competitive binding to microRNA-107, thereafter participating in NSCLC development.

CONCLUSIONS: H19 is highly expressed in NSCLC, which promotes NSCLC development by regulating NF1 via competitive binding to microRNA-107.

Key Words:
H19, MicroRNA, Non-small cell lung cancer, CeRNA.

Introduction

Lung cancer is one of the most common malignancies in the world, which has become the leading cause of death in China[1]. Non-small cell lung cancer (NSCLC) is pathologically divided into squamous cell carcinoma, adenocarcinoma and large cell carcinoma. Compared with small cell carcinoma, NSCLC is manifested as slower growth and division, as well as less frequent diffusion and metastasis[2]. NSCLC accounts for about 80% of all cases of lung cancer. More seriously, about 75% of NSCLC patients are already in advanced stage when they are diagnosed. The 5-year survival rate of NSCLC is very low because of its high incidence, rapid growth, high mortality, and poor prognosis[3]. The latest data reported that the global incidence and mortality of NSCLC rank the first among malignancies. Death number due to lung cancer exceeds the sum of deaths from breast cancer, prostate cancer, and colorectal cancer[4]. Hence, NSCLC has become a serious public health problem. Recent works[5] have proved that proliferation and migration of tumor cells are important pathogenic factors in NSCLC development.

Non-coding RNAs (ncRNAs) account for about 98% of gene transcripts, including microRNA (miRNA) and long non-coding RNA (lncRNA). MicroRNAs are a class of non-coding small RNAs with approximately 22 nucleotides in length. They regulate target genes at post-transcriptional level. The dysregulated microRNAs are related to the occurrence and development of various diseases, including tumors and digestive diseases[6,7]. The regulatory roles of microRNAs in various types of tumors have been widely reported, such as liver cancer and lung cancer[8,9]. LncRNAs are non-coding RNA molecules with over 200 nt in length, which are widely present in the nucleus and cytoplasm. LncRNA could not or barely encode proteins since the open reading frame is lacked[10]. How-

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ever, lncRNAs exert a crucial role in biological processes, such as cell proliferation, cell cycle, differentiation, and apoptosis[11]. For example, lncRNA MIAT participates in the development of breast cancer by regulating the proliferation of breast cancer cells. LncRNA CCAT1 participates in the development of thyroid cancer by promoting the proliferation and migration of thyroid cancer cells[12,13]. In recent years, many investigations[14,15] have found that lncRNA is served as a competing endogenous RNA (ceRNA). CeRNAs, also known as the molecular sponges of miRNA, regulate downstream function through absorbing target miRNAs.

H19 is discovered with important regulatory effect on tumors. Previous studies[16,17] have shown that H19 can not only promote tumorigenesis, but also inhibit tumorigenesis. The specific role of H19 in NSCLC, however, still needs to be further elucidated.

Patients and Methods

Sample Collection

36 pairs of NSCLC tissues and paracancerous tissues were surgically resected and preserved in liquid nitrogen. Enrolled patients were pathologically diagnosed as NSCLC. This study was approved by the Hospital Ethics Committee and all patients were informed consent.

Cell Culture

BEAS-2B, A549 and HCC823 cells were cultured in RPMI-1640 (Roswell Park Memorial Institute-1640, Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained in a 5% CO_2 incubator at 37°C for regular observation.

Cell Transfection

Lentiviruses were constructed by co-transfection of pMagic 4.1 containing GFP (green fluorescent protein), pCD/NLBH*DDD and pLTR-G in 293T cells. A549 cells were centrifuged at 1000 r/min for 5 min and resuspended in 1 mL of TrypLE Express for digestion. Cells were then centrifuged at 1000 r/min for another 5 min. Cells were collected and seeded in 24-well plates with 5×10^5 cells per well. Cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded in the 96-well plates at the density of 1×10^6/mL. After culturing for 24 h, serum-free medium was replaced. 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) were added into each well. Absorbance values at the wavelength of 450 nm were detected by the microplate reader (Bio-Rad, Hercules, CA, USA). Each experiment was repeated for 5 times.

Transwell Assay

Cell density was adjusted to 2×10^5/mL with serum-free medium. Briefly, 100 μL of cell suspension and 600 μL of medium containing 10% FBS were added in the upper and lower chamber, respectively. 24 h later, cells were fixed with formaldehyde for 30 min and stained with crystal violet for 15-30 min. Penetrating cells were observed and captured.

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

The TRIzol kit (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA, which was then reversely transcribed into complementary deoxyribose nucleic acid (cDNA). After the cDNA was amplified, qRT-PCR was performed to detect the expressions of related genes. Primers used in this study were as follows: LncRNA H19: F: 5'-GTGATCATGACTGGGACCCA-3', R: 5'-GGGATGTTTCTGCAGGCAAA-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): F: 5'-AGCCACATCGCTCAGACAC-3', R: 5'-GCCCAATACGACCAAATCC-3'; NF1: F: 5'-AGATGAAACGATGCTGGTCAAA-3', R: 5'-CCTGTAACCTGGTAGAAATGCGA-3'. The relative expression was calculated using $2^{-\Delta\Delta CT}$ method.

Dual-Luciferase Reporter Gene Assay

The binding site of microRNA-107 and NF1 was predicted by Target Scan. Besides, the binding site of H19 and microRNA-107 was predicted by Starbase. Wild-type NF1, mutant-type NF1, wild-type H19 and mutant-type H19 were constructed by Ruizhen (Nanjing, China). MicroRNA-107 mimic and negative control were constructed by GenePharma (Shanghai, China). After cells were co-transfected with relative sequences, luciferase activity was detected using the relative commercial kit (Promega, Madison, WI, USA).

Western Blot

The total protein was extracted by the RIPA (radioimmunoprecipitation assay) lysate (Yeasen, Shanghai, China). The concentration of each protein sample was determined by a BCA (bicinchoninic acid) kit (Abcam, Cambridge, MA, USA). Briefly, total protein was separated by a SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gel under denaturing conditions and then transferred to PVDF (polyvinylidene difluoride) membranes (Merck Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk for 1 h, followed by the incubation of specific primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight. After washing with TBST (Tris-buffered saline and Tween, Yeasen, Shanghai, China) for 3 times, membranes were incubated with the secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence method (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) were used for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x} \pm s$). Prognostic value of H19 in NSCLC was analyzed by ROC (receiver operating characteristics) curve. The *t*-test was used for comparing differences between the two groups. $p < 0.05$ was considered statistically significant.

Results

H19 was Highly Expressed in NSCLC

H19 expression in NSCLC tissues and paracancerous tissues was detected by qRT-PCR. The data showed a higher expression of H19 in NSCLC tissues that are larger than 3 cm in diameter compared with those smaller than 3 cm (Figure 1A). Besides, H19 expression was positively correlated to tumor stage of NSCLC (Figure 1B). ROC curve showed that H19 has a significant diagnostic value in NSCLC (Figure 1C). We subsequently detected H19 expression in NSCLC cells and normal pulmonary epithelial cells. H19 was also highly expressed in NSCLC cell lines, especially in A549 cells, which were selected for the following experiments (Figure 1D).

H19 Regulated Proliferation and Migration of NSCLC Cells

LV-shH19 and LV-H19 were first constructed and their transfection efficacies in A549 cells were verified by qRT-PCR (Figure 2A). CCK-8 assay showed decreased proliferative ability in A549 cells transfected with LV-shH19 compared with those transfected with LV-Vector (Figure 2B). The regulatory effect of H19 in cell migration of A549 cells was detected by transwell assay. The results demonstrated that migratory ability was decreased in A549 cells transfected with LV-shH19 compared with those transfected with LV-Vector (Figure 2C).

H19 was the Molecular Sponge of microRNA-107

We found that H19 was mainly distributed in cytoplasm (Figure 3A). Subsequently, microRNA-107 was predicted to bind to H19 by bioinformatics. In our study, microRNA-107 was lowly expressed in NSCLC tissues than those of paracancerous tissues (Figure 3B). To further verify the binding condition of microRNA-107 and H19, dual-luciferase reporter gene assay was performed. Wild-type H19 and mutant-type H19 were first constructed (Figure 3C). The data showed that luciferase activity was decreased in A549 cells co-transfected with microRNA-107 mimic and wild-type H19 compared with those co-transfected with microRNA-107 mimic and mutant-type H19 (Figure 3D).

H19 Regulated the Target Gene of microRNA-107

After online prediction and function analysis, NF1 was screened out to be the target gene of microRNA-107. Our data demonstrated that the mRNA level of NF1 was higher in NSCLC tissues than those of paracancerous tissues (Figure 4A). Protein expression of NF1 showed the similar trend in NSCLC tissues (Figure 4B). Subsequently, wild-type NF1 and mutant-type NF1 were constructed (Figure 4C). The data showed that luciferase activity was decreased in A549 cells co-transfected with microRNA-107 mimic and wild-type NF1 compared with those co-transfected with microRNA-107 mimic and mutant-type NF1 (Figure 4D). Notably, we found the decreased mRNA and protein levels of NF1 induced by LV-shH19 transfection were reversed after microRNA-107 knockdown (Figure 5A and 5B).

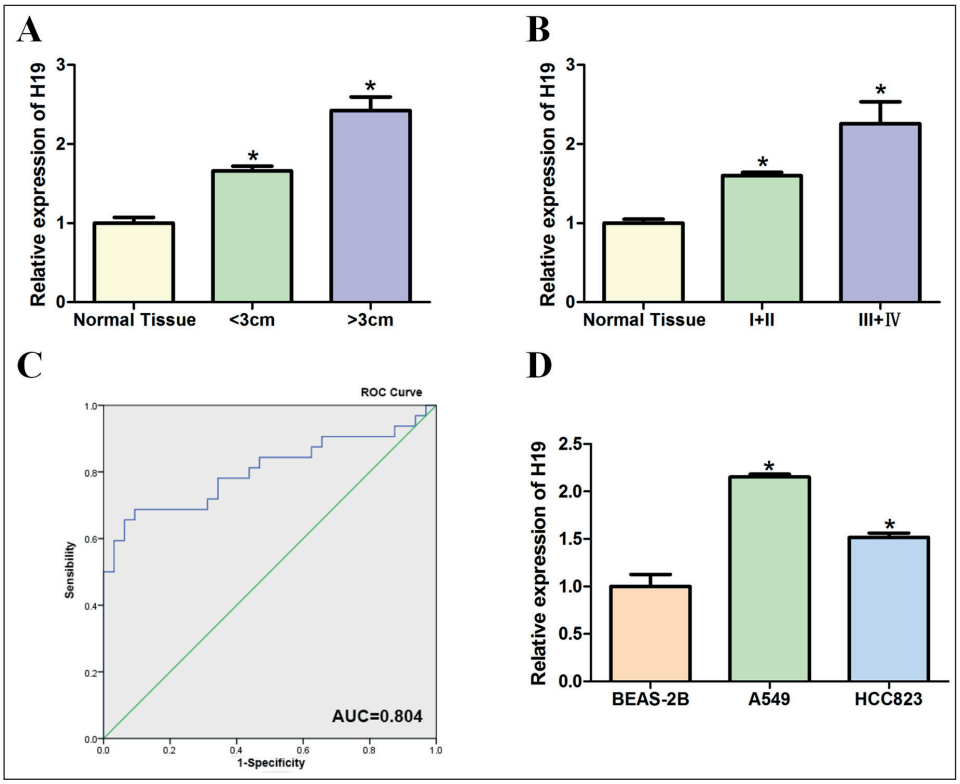

Figure 1. H19 was highly expressed in NSCLC. ***A,*** H19 expression was higher in NSCLC tissues larger than 3 cm in diameter compared with those smaller than 3 cm. ***B,*** H19 expression was positively correlated to tumor stage of NSCLC. ***C,*** ROC curve showed that H19 has a significant diagnostic value in NSCLC. ***D,*** H19 was highly expressed in NSCLC cell lines.

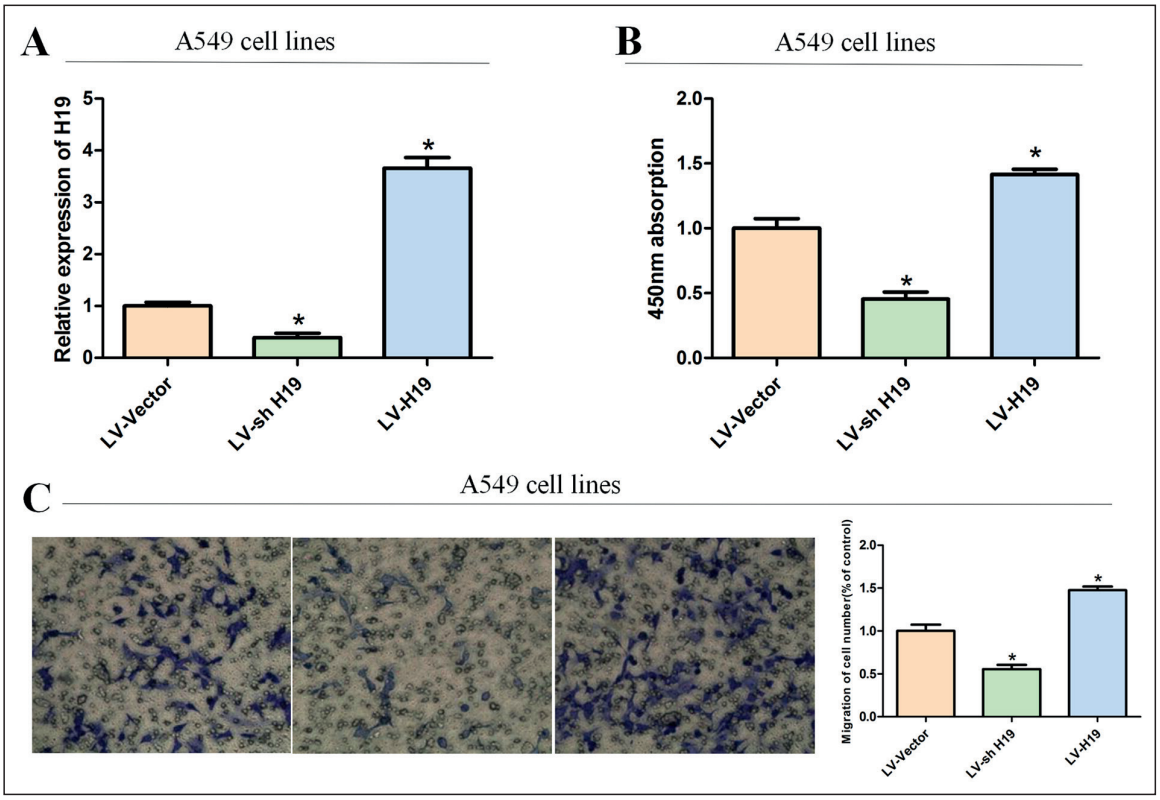

Figure 2. H19 regulated proliferation and migration of NSCLC cells. ***A,*** Transfection efficacies of LV-shH19 and LV-H19. ***B,*** CCK-8 assay showed decreased proliferative ability in A549 cells transfected with LV-shH19 compared with those transfected with LV-Vector. ***C,*** Transwell assay demonstrated that decreased migratory ability in A549 cells transfected with LV-shH19 compared with those transfected with LV-Vector.

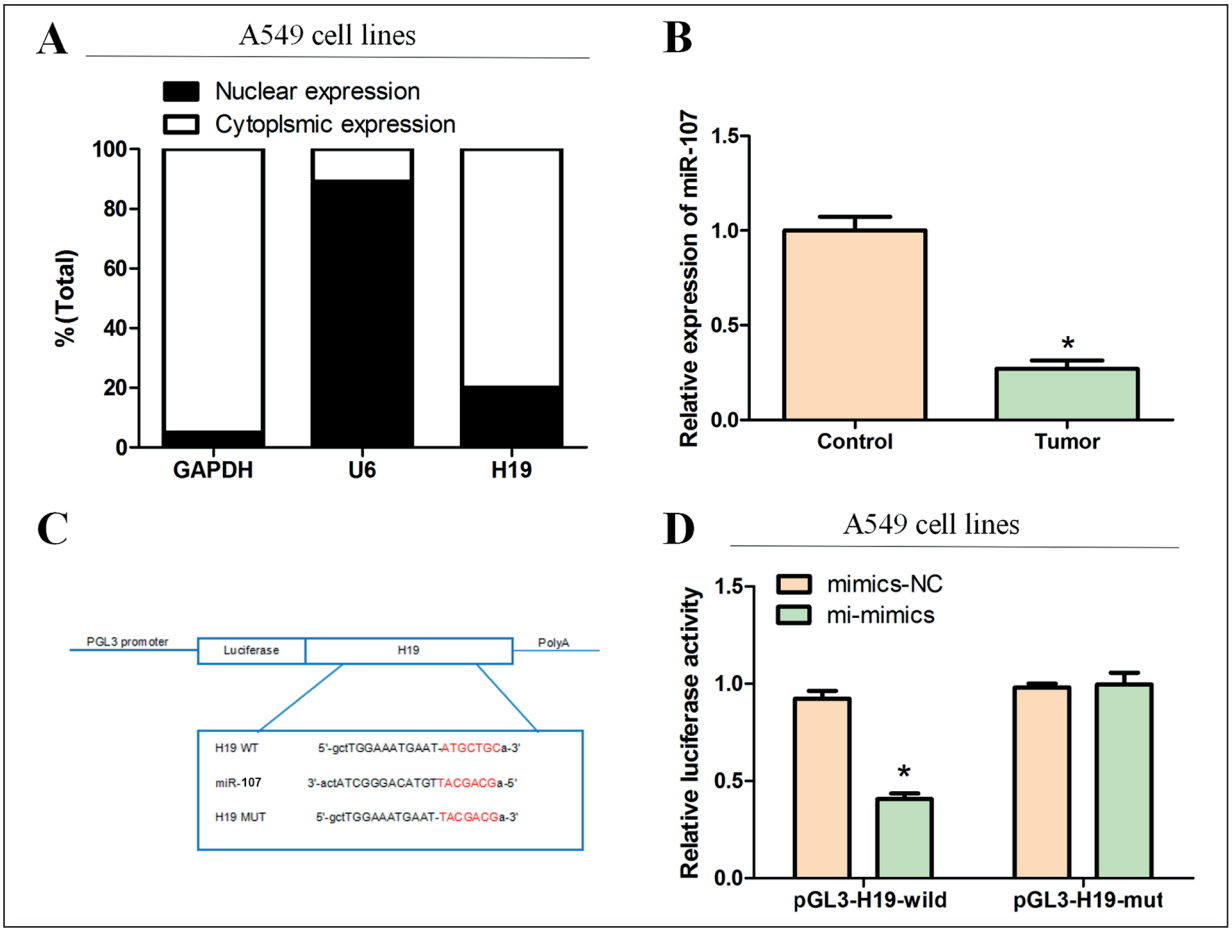

Figure 3. H19 was the molecular sponge of microRNA-107. ***A,*** H19 was mainly distributed in cytoplasm. ***B,*** MicroRNA-107 was lowly expressed in NSCLC tissues than those of paracancerous tissues. ***C,*** Construction of wild-type H19 and mutant-type H19. ***D,*** Luciferase activity was decreased in A549 cells co-transfected with microRNA-107 mimic and wild-type H19 compared with those co-transfected with microRNA-107 mimic and mutant-type H19.

H19/microRNA-107 Regulated Proliferation and Migration of NSCLC Cells

To explore whether microRNA-107 could regulate proliferation and migration of A549 cells, rescue experiments were carried out. Our results demonstrated that decreased proliferation and migration of A549 cells induced by LV-shH19 transfection could be reversed after microRNA-107 knockdown (Figure 5C and 5D).

Discussion

Epigenetics is a branch of biology studying the genetic changes without alteration of DNA sequence. Epigenetics is essential for cell growth and differentiation and tumor development. The main mechanisms of epigenetics include DNA methylation, histone modifications, and newly discovered non-coding RNAs. Non-coding RNAs do not exert the protein-encoding function. Abundant evidences have demonstrated that non-coding RNAs exert a vital role in epigenetic regulation. LncRNAs belong to non-coding RNAs with relative long chains. It is reported that lncRNA expression is closely related to tumor development[18]. Disordered lncRNAs have an important effect on cellular homeostasis. H19 is located on human chromosome 11p15.5. Studies have found that H19 serves as an oncogene that is overexpressed in various malignancies. A large number of studies[19,20] have shown that H19 overexpression can upregulate the proliferation of tumor cells. Overexpressed H19 can also promote tumor cell migration[21,22]. In recent years, many studies have found that lncRNAs could be

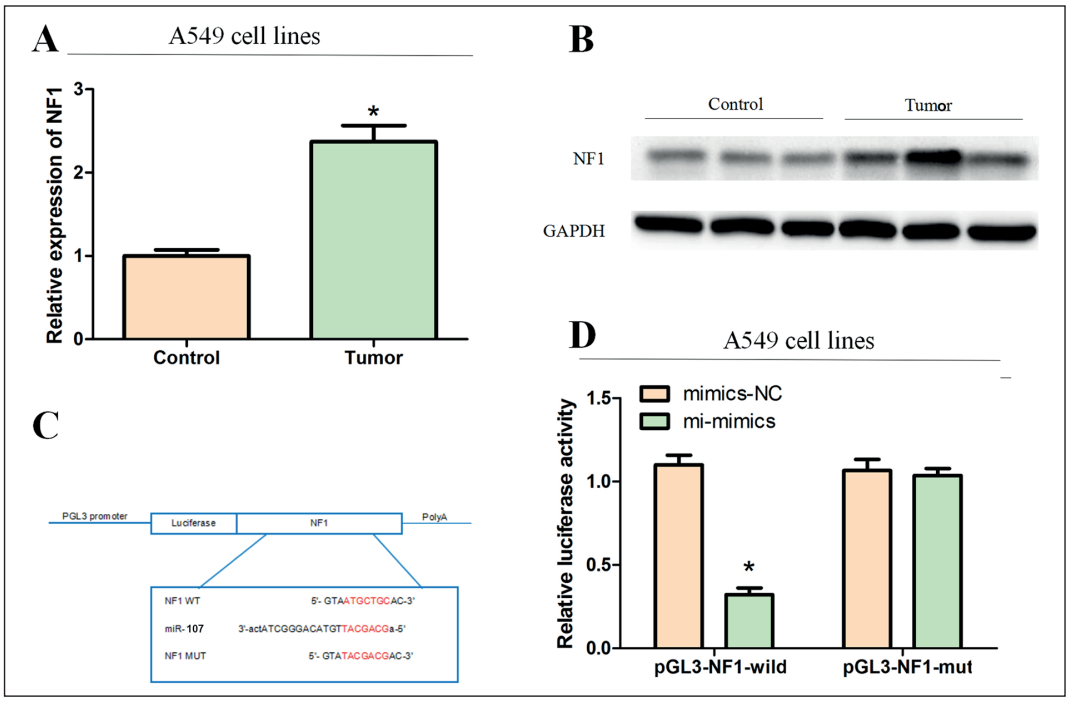

Figure 4. H19 regulated the target gene of microRNA-107. ***A,*** The mRNA level of NF1 was higher in NSCLC tissues than those of paracancerous tissues. ***B,*** Protein expression of NF1 was higher in NSCLC tissues than those of paracancerous tissues. ***C,*** Construction of wild-type NF1 and mutant-type NF1. ***D,*** Luciferase activity was decreased in A549 cells co-transfected with microRNA-107 mimic and wild-type NF1 compared with those co-transfected with microRNA-107 mimic and mutant-type NF1.

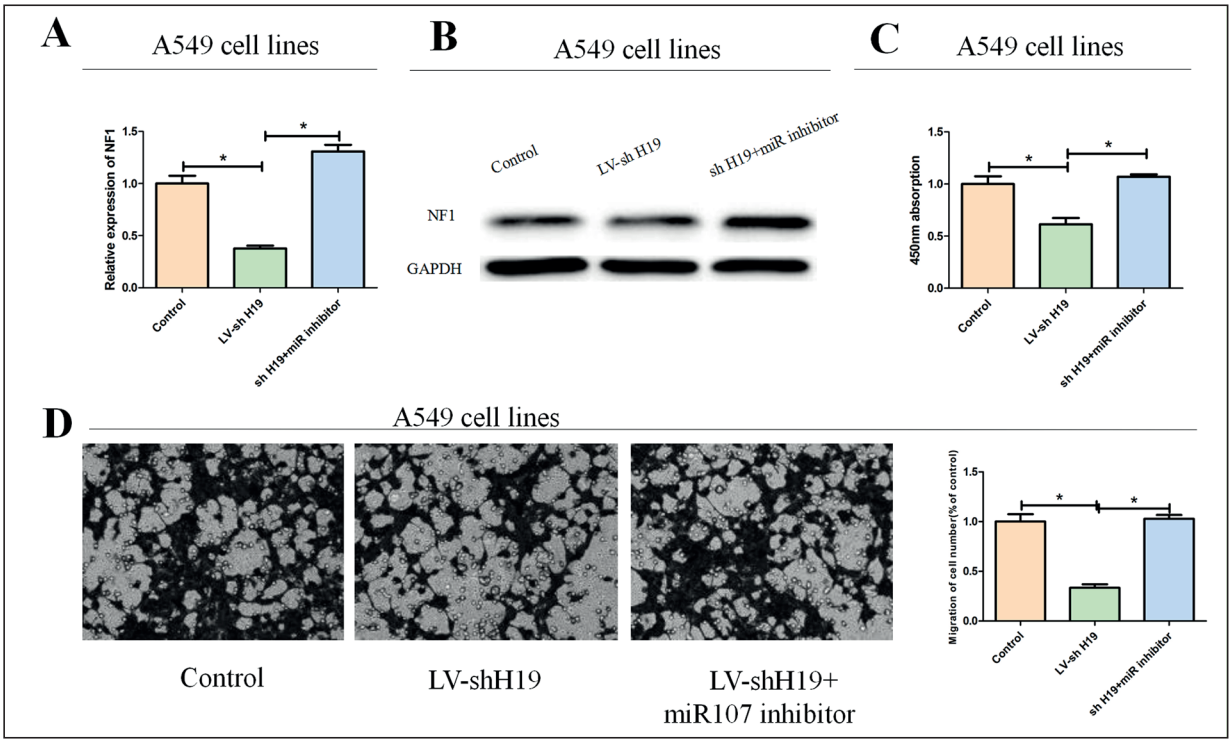

Figure 5. H19/microRNA-107 regulated proliferation and migration of NSCLC cells. ***A, B,*** Decreased mRNA and protein levels of NF1 induced by LV-shH19 transfection were reversed by microRNA-107 knockdown. ***C, D,*** Decreased proliferation and migration of A549 cells induced by LV-shH19 transfection could be reversed by microRNA-107 knockdown.

served as competing endogenous RNAs (ceRNAs) to regulate target gene expressions. Functionally, ceRNAs participate in proliferation, apoptosis, angiogenesis, invasion and metastasis of tumor cells. For example, MEG3 is served as a ceRNA to regulate the progression of gastric cancer[23]. In this study, overexpressed H19 promoted proliferation and migration of NSCLC cells. Dual-luciferase reporter gene assay demonstrated that H19 could directly bind to microRNA-107. NF1 gene products are served as negative mediators in Ras pathway. NF1 mutation is associated with type 1 neurofibromatosis, juvenile myelomonocytic leukemia and Watson syndrome. Li et al[24] have shown that NF1 is involved in the occurrence of liver cancer via regulating metastasis of liver cancer cells. In this study, we found that the expression level of NF1 was significantly upregulated in NSCLC. Furthermore, dual-luciferase reporter gene assay showed that NF1 can bind to microRNA-107. In addition, microRNA-107 knockdown could reverse the downregulated NF1 expression induced by H19 knockdown. Our data indicated H19 can competitively bind to microRNA-107, thereby inhibiting the effect of microRNA-107 on NF1 degradation.

Conclusions

We demostrated that H19 was highly expressed in NSCLC, which promotes NSCLC development by regulating NF1 via competitive binding to microRNA-107.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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