miRNA-9 Inhibits Proliferation and Migration of Lung Squamous Cell Carcinoma Cells by Regulating NRSF/EGFR

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Abstract
Background: To investigate the mechanism of microRNA9 in inhibiting proliferation and migration of lung squamous cell carcinoma cells via neuron-restricted silencing factor/epidermal growth factor receptor. Material and Methods: Detection of microRNA9, neuron-restricted silencing factor, and epidermal growth factor receptor expression levels in lung cancer patients’ tissues and lung cancer cells by Western blotting and quantitative polymerase chain reaction. Detection of cell proliferation by colony formation assay and cell counting kit-8 assay. Detection of cell migration by wound-healing assay and Transwell assay. And detection of the regulatory effect between neuron-restricted silencing factor and epidermal growth factor receptor by Luciferase reporter gene system. Subcutaneous implantation mouse models of NCI-H520 cells were constructed to detect cell proliferation in vivo, and Kaplan–Meier method calculated patient survival. Results: The expression of microRNA9 and epidermal growth factor receptor was higher in lung cancer tissues than in normal lung tissues, while the expression of neuron-restricted silencing factor was lower in lung cancer tissues than in normal lung tissues. MicroRNA9 higher expression was strongly related to tumor size, and TNM stage and predicted showed reduced overall survival in patients with lung cancer. Further loss of function and enhancement experiments revealed that inhibition of microRNA9 could significantly inhibit lung squamous carcinoma cell proliferation and migration. Luciferase reporter assay demonstrated that microRNA9 could bind to NRSF messenger RNA and inhibit its expression, neuron-restricted silencing factor overexpression also exerted inhibitory effects on cell proliferation and migration. Moreover, Luciferase reporter assay showed that neuron-restricted silencing factor downregulate epidermal growth factor receptor expression levels by binding to epidermal growth factor receptor promoter regions, and Pearson’s correlation analysis indicated that the levels of microRNA9 in lung cancer tissues were correlated with neuron-restricted silencing factor and epidermal growth factor receptor. Combined microRNA9 with neuron-restricted silencing factor or epidermal growth factor receptor to predict the prognosis of patients with lung cancer may be more accurate. Conclusion: MicroRNA9 inhibits proliferation and migration of lung squamous cell carcinoma cells by inhibiting neuron-restricted silencing factor/epidermal growth factor receptor axis. MicroRNA9 can be a new prognostic marker and therapeutic target for lung squamous cell carcinoma.

Keywords
miRNA9, lung squamous carcinoma, NRSF/EGFR, proliferation, migration

Abbreviations
CCK-8, cell counting kit-8; CDNA, complementary DNA; DMEM, Dulbecco’s modified eagle medium; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; miRNA, microRNA; mRNA,
Background

Lung cancer is the most common malignant tumor in the world, with an increasing trend year by year. Small cell lung carcinoma (SCLC) and non-small cell lung cancer (NSCLC) account for 75% to 85% of the total number of patients with NSCLC, and squamous cell carcinoma (SCC) is one of the major types of NSCLC. At present, most of the patients with NSCLC are advanced at the time of their first diagnosis, and the treatment and prognosis are very poor. About 30% of the patients with lung cancer are prone to distal metastasis after operation, and the 5-year survival rate is less than 20%. Therefore, the study of the pathogenesis of lung cancer is still of great significance.

MicroRNA (miRNA) is a large group of small noncoding RNAs that can inhibit nearly a third of human genes at the transcriptional and translational levels by binding to the 3-untranslated region (UTR) region of the target gene messenger RNA (mRNA). More and more studies have shown that miRNA mediates many biological processes, including the onset of tumors. Recent studies have shown that miRNA9 is unusually high in many malignancies, including lung cancer, but its role in tumors is controversial, for example, in promoting breast cancer but can inhibit the progression of melanoma. In addition, it has been reported that miRNA9 can influence the development of liver cancer. But at present, the effect of miRNA9 on lung cancer has not been reported.

Current studies have shown that epidermal growth factor receptors (EGFRs) promote division and proliferation and is upregulated in many malignancies, including lung cancer, gastric cancer, and colorectal cancer. The upregulation of EGFR is closely related to the development of malignant tumor, drug resistance, and prognosis. In patients with SCC, more than 60% of patients had high EGFR expression in tumor tissues. Therefore, EGFR was a new potential target for developing drugs to treat lung cancer currently. However, there are so many mutations in patients with SCC with EGFR that the current drugs are ineffective.

Material and Methods

Clinical Sample Collection

Tumor tissues and paired non-cancerous tissues were collected from 60 patients with lung cancer who underwent surgery in our hospital from February 1, 2016, to December 1, 2019, all the patients included in the study were first diagnosed, no obvious distal metastatic lesions, without radiotherapy or preoperative chemotherapy, and the pathology was identified as NSCLC. Clinicopathological data, including age, gender, tumor size, and TNM stage, were collected and tumor stage was assessed according to international TNM classification (Table 1). The experiment was approved by the ethics committee of our hospital. Written informed consent was obtained from all the participants in accordance with the Declaration of Helsinki. Each pair of normal tissues and cancer tissues were from the same patient.

Cell Culture and Transfection

Human lung cancer cell lines, NCI-H520, NCI-H1915, and SK-MES-1, and normal human lung epithelial cell line BEAS-2B, placed in moist 5% CO2, 37°C cell incubator, using a Dulbecco’s modified eagle medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 lg/mL streptomycin into the culture. MicroRNA 9 mimic and inhibitor and plasmid were transfected using Lipo2000 transfection reagent according to the instructions.

In Vivo Tumorigenesis Assay

A 6 to 8 weeks male BALB/c nude mouse (Viton Lihua) was purchased and the mice were prepared to undergo tumor-bearing experiments 3 to 5 days after resting in the animal room. A total of 1 × 10⁶ cells were subcutaneously injected under the armpit of the mouse. The same mouse injected 2 different cells, respectively, under the left and right armpits to exclude the difference between different individuals. After 30 days, the mice were sacrificed and tumors were harvested and measured. The animal experiment has been approved by the Ethics Committee of Biomedical Research in our hospital. All experimental procedures were approved by the animal protection and use committee of ZheJiang University and complied with National Institute of Health (NIH)’s criteria for laboratory animal protection and safety. Under standard ambient conditions (temperature: 22-25 °C, humidity: 45-50%, and a light-dark cycle for 12 hours), all animals were raised separately with unrestricted access to food and water.
Table 1. The Relationship Between Expression Level of miRNA9 and Clinical Characteristics in Lung Cancer (n = 60).

| Variables   | All cases | Low | High | P value |
|-------------|-----------|-----|------|---------|
| Age at surgery (years) |           |     |      |         |
| <60         | 25        | 10  | 15   | .4386   |
| ≥60         | 35        | 18  | 17   |         |
| Gender      |           |     |      |         |
| Male        | 38        | 14  | 24   | .7884   |
| Female      | 22        | 9   | 13   |         |
| Tumor size (cm) |          |     |      |         |
| <5          | 27        | 11  | 16   | .0396\(^a\) |
| ≥5          | 33        | 5   | 28   |         |
| TNM         |           |     |      |         |
| I + II      | 29        | 14  | 15   | .0044\(^b\) |
| III + IV    | 31        | 4   | 27   |         |

\(^a\)P < .05. \(^b\)P < .01.

Quantitative Real-Time Polymerase Chain Reaction

Using Trizol reagents (15596026, Invitrogen) to extract the total RNA, using the PrimeScript RT regent Kit (RR047A, Takara) instruction to reverse transcribe the RNA complementary DNA (cDNA), using the FastSyBR Green PCR kit (Applied Biosystems) to prepare the reaction system for the synthesized cDNA, the abipr ism7300 RT-PCR system (Applied biosystems) was performed for quantitative real-time polymerase chain reaction detection. Three repeats per sample. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. MiRNA expression was detected using PrimeScript miRNA RT_PRCC kit (Takara Bio-technology co, ltd) with u6 as within the miRNA. The primers were as follows: NRST, F5'; R5'-CTTTGTCCCTTATCTCAAGTTCTCG-3'; EGFR, F5'-GGATGAGTCAGTCAG-3' R5'-TGTTCTATTGTCGTCAGGT-3'; GAPDH, F5'-TGCAACAACACTTAG-3', R5'-GGACTGTGTGTGTGTG-3'; and miRNA-9, F5'-TCCCTTGGATCTCTCCTGCT-3'.

Western Blot

Cells were collected, and protease inhibitors were added according to the number of cells (Roche) to radio immunoprecipitation assay lysis buffer, with 30 minutes on ice. The lysate was obtained after centrifugation of 13 000 rpm for 20 minutes. The protein concentration was assessed using BCA Protein Assay kit (Beyotime Institute of Biotechnology). Reducing loading buffer was added according to the need of the experiment. The sample was boiled for 10 minutes and then sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed. After electrophoresis is finished, the protein was transferred to the polyvinylidene fluoride membrane (Millipore) at a constant current of 1.2 ma/cm, 21hours, by a semi-dry membrane transducer. The membranes were then incubated in 5% skim milk-TBST at room temperature for 1 hour, the corresponding first antibody was added and incubated at 4 °C shaking table. The first antibody was recovered the next day, the Tris Buffered saline Tween (TBST) was rinsed for 5 minutes for 3 times, the second antibody coupled with horseradish peroxidase was incubated for 1 hour in a room temperature shaking table, and then rinsed with TBST for 5 minutes for 3 times. Finally, the chemogenic reaction was carried out by adding Enhanced Chemiluminescence (ThermoFisher Scientific) imager Chemi-Scope mini imaging system (Clinx Science). The band strength was quantified by Image J software (NIH).

The antibodies used in the experiments included: NRST (1:100; Abcam), EGFR and GAPDH (1:100; Cell signaling Technology).

Dual Luciferase Reporter Gene

We imported the synthesized NRST and EGFR 3’ UTR gene fragments into the pmir-reporter gene (Beijing Huayueyang Biotechnology). NRST-mut and EGFR-wt design binding site mutations based on NRST-mu and EGFR-mu. Epidermal growth factor receptor-WT and MUT were co-transfected into HEK293 T cells with NRST (Shanghai Beinuo Biotechnology). After 48 hours of transfection, cells were collected and cleaved using a luciferase detection kit (K801-200; Biovision).

Cell Counting Kit-8 Assay

Cell proliferation was determined using cell counting kit-8 (CCK-8; Dojindo Laboratories). The cells with logarithmic growth were digested with 0.25% trypsin and gently blown into a single cell.\(^4\) Cells were collected and cell viability was detected by CCK-8 method. After culture, 10 µL CCK-8 reagent was added to each well and incubated at 37°C for another 4 hours. Optical density values were measured at 450 nm.

Colony Formation Assay

Cells in the logarithmic phase were digested with 0.25% trypsin and gently blown into a single cell. The cells were counted and the cell density was adjusted to 1 × 10⁶ cells/mL. Then the cells of each group were reseeded at a density of 50, 100, and 200 cells/mL into 10 cm dish containing 10 mL of preheated medium at 37 °C and incubated for 2 to 3 weeks, the cell culture medium was refreshed every 3 days. It is often observed to terminate the culture when a naked-eye clone appears in the dish. Discard the supernatant and wash 2 carefully with phosphate-buffered saline (PBS). Five milliliters of 4% paraformaldehyde was added to the fixed cells for 15 minutes, then washed with PBS twice, and dyed with proper amount of GIEMSA (Invitrogen) staining solution for 10 to 30 minutes, then slowly washed with running water to remove the staining solution and dried in air. The plate was placed under an inverted microscope (Leica DMI8-M) to observe the number.
of cell clones and calculate the clone formation rate = the number of formed clones/inoculated cells.

**Wound Healing Assay**

A 1 × 10⁶/mL cells were cultured with 10% FBS of DMEM and inoculated in a plug-in for Culture-Insert 2 Well μ-Dish (Ibidi) of 100 μs per slot. After about 24 hours, the cells were completely adherent to the wall, growing to a confluent monolayer, using tweezers to carefully remove the plug-in, leaving a uniform width between the cells. The cells were washed with PBS for 2 to 3 times to remove the original medium as well as the floating cells, and the medium was replaced with a DMEM containing 1% FBS. The migration process of the cells to the wound was photographed under the microscope, and the time points were 0 and 24 hours.

**Transwell Assay**

Add 600 μL DMEM with 10% FBS in the lower chamber; and then 5 × 10⁴ cells were resuspended in 200 μL DMEM and seeded in the upper well of the transwell chamber. After incubation at 37 °C for 24 hours, the cells in the upper surfaces of the filter were removed with cotton swabs, and cells that had migrated onto the lower surfaces of the filter were fixed with 4% formaldehyde for 20 minutes and then stained with 0.1% crystal violet. The number of migrated cells was obtained by counting in 5 random fields under a light microscope. Experiments were independently repeated 3 times.

**Statistical Analysis**

The statistical analysis of the data in this study were all divided by SPSS 21.0 (IBM) statistical software. The measurement data are expressed as mean ± standard deviation; first of all, the normality and variance homogeneity test are carried out, the test accords with the normal distribution and the variance is uniform, the non-paired T test is used between groups, and the one-way analysis of variance and Tukey’s are compared between groups for ex post-test. The survival rate of patients was calculated by Kaplan–Meier method, and the Log-rank test was used for a single factor score. Pearson correlation analyses the correlation of observational indicators. **P < .01.

**Results**

**High Expression of miRNA9 in Lung Cancer**

The mRNA expression levels of miRNA9 were compared between lung cancer tissues and adjacent noncancerous tissues using real time-polymerase chain reaction. The results indicated that miRNA9 is upregulated in lung cancer tissues (Figure 1A). The present study also examined the mRNA levels of miRNA9 in human lung cancer cell lines. The results demonstrated the mRNA levels of miRNA9 were significantly higher in NCI-H520, NCI-H1915, and SK-MES-1 human lung cancer cell lines, compared with the BEAS-2B cells (Figure 1B). To investigate the functions of miRNA9, miRNA9 inhibitor was added into NCI-H520. The results showed that the miRNA9 inhibitor efficiently inhibits cell proliferation (Figure 1C and D) and migration (Figure 1E and F). The mRNA expression levels of miRNA9 were positively correlated with TNM stage (Figure 1G).
Figure 2. Significantly lower expression of NRSF in lung cancer cells and associated with cancer malignancy. A, Luciferase activity in cells cotransfected with miRNA9 mimics and luciferase reporters containing NRSF or mutant transcripts. Data are presented as the relative ratio of firefly luciferase activity of renilla luciferase activity. B, Western blot analysis of NRSF expression in lung cancer and paired adjacent normal lung tissues. C, Quantitative real time-polymerase chain reaction analysis of NRSF expression in lung cancer and paired adjacent normal lung tissues. D, Western blot analysis of NRSF expression in human lung cancer cells and lung epithelial cells. E, Quantitative real time-polymerase chain reaction analysis of NRSF expression in human lung cancer cells and lung epithelial cells. F, Pearson correlation scatter plots of miRNA9 and NRSF in lung cancer. G, Quantitative real time-polymerase chain reaction analysis of NRSF expression in 60 pairs of human lung cancer and adjacent normal lung tissues. H, Quantitative real time-polymerase chain reaction analysis of NRSF expression in lung cancer (T1, T2, T3, and T4) and adjacent normal lung tissues (normal). I, Survival analysis of 60 patients with lung cancer. miRNA indicates microRNA; NRSF, neuron-restricted silencing factor.
Figure 3. Overexpression of NRSF inhibits proliferation and migration of lung cancer cells. Empty vector and NRSF were transfected into NCI-H520. A, Colony formation was assessed and representative images of cell colony formation are shown. B, Cell counting kit-8 assay was used to detect cell viability. C and D, Wound-healing assay and Transwell assay were conducted to evaluate cell migration. E, NCI-H520 cells overexpressing NRSF or control vector were subcutaneously injected in nude mice. Tumor volume and weight were evaluated. NRSF indicates neuron-restricted silencing factor.
Figure 4. Neuron-restricted silencing factor was able to inhibit EGFR expression. A, Western blot analysis of EGFR expression in lung cancer and paired adjacent normal lung tissues. B, Quantitative real time-polymerase chain reaction analysis of EGFR expression in lung cancer and paired adjacent normal lung tissues. C, Western blot analysis of EGFR expression in human lung cancer cells and lung epithelial cells. D, Quantitative real time-polymerase chain reaction analysis of EGFR expression in human lung cancer cells and lung epithelial cells. E and F, Western blot analysis of EGFR and NRSF in NCI-H520 cells transduced with NRSF OE or sh NRSF. G, Western blot analysis of EGFR and NRSF in NCI-H520 cells treated with/without miRNA9 inhibitor. H, Luciferase activity in cells transduced with NRSF OE and luciferase reporters containing EGFR or mutant transcripts. Data are presented as the relative ratio of firefly luciferase activity of renilla luciferase activity. I, Quantitative real time-polymerase chain reaction analysis of EGFR expression in 60 pairs of human lung cancer and adjacent normal lung tissues. EGFR indicates epidermal growth factor receptor; miRNA, microRNA; NRSF, neuron-restricted silencing factor.
Figure 5. Neuron-restricted silencing factor affects proliferation and migration of lung cancer cells by regulating EGFR. Empty vector and EGFR were transfected into NRSF overexpressed NCI-H520 cells. A, Colony formation was assessed and representative images of cell colony formation are shown. B, Cell counting kit-8 assay was used to detect cell viability. C and D, Wound-healing assay and Transwell assay were conducted to evaluate cell migration. E, Neuron-restricted silencing factor-overexpressed NCI-H520 cells transfected with EGFR or control vector were subcutaneously injected in nude mice. Tumor volume and weight were evaluated. EGFR indicates epidermal growth factor receptor; NRSF, neuron-restricted silencing factor.
Significantly Lower Expression of NRSF in Lung Cancer Cells and Associated With Cancer Malignancy

MicroRNA 9 is able to bind to NRSF and inhibit its expression (Figure 2A). The levels of NLSF and mRNA in cancer tissue and squamous cell line of lung cancer were measured by western blotting and quantitative polymerase chain reaction. The results showed that the levels of NLSF and mRNA in cancer tissue were significantly lower than those in adjacent tissues (Figure 2B-C) and that in lung SCC cells were significantly lower than those in normal lung epithelial cells (Figure 2D-E). Furthermore, to confirm the low expression of NRSF in lung cancer, we collected 60 clinical lung cancer samples, the NRSF mRNA level was significantly reduced, NRSF was negatively correlated with miRNA 9 expression and the degree of cancer malignancy (Figure 2F-H). In addition, the low expression of NRSF means that patients have a shorter survival time (Figure 2I).

Overexpression of NRSF Inhibits Proliferation and Migration of Lung Cancer Cells

To further confirm the function of NRSF on lung cancer cells, NCI-H520 lung cancer cells were transduced with vector control or NRSFOE lentivirus. The results showed that overexpression of NRSF inhibited cell colony formation (Figure 3A), cell proliferation (Figure 3B), cell migration (Figure 3C and D). To explore whether NRSF affected tumor growth of lung cancer in vivo, the experiment was produced by subcutaneous injection of nude mice with NCI-H520 cells overexpressing NRSF or vector. Compared with the vector cells, overexpression of NRSF significantly inhibited lung cancer growth (Figure 3E).

Neuron-Restricted Silencing Factor Could Inhibit EGFR Expression

Epidermal growth factor receptor is important for the development of lung cancer, and bioinformatics analysis showed that, EGFR may be the downstream target of NRSF. We then investigate the function of EGFR in lung cancer. The results showed that EGFR expression in lung cancer tissues and lung cancer cells was upregulated (Figure 4A-D, and I). Overexpression of NRSF was able to downregulate EGFR expression, whereas knockdown of NRSF was able to upregulate EGFR (Figure 4E-G). Luciferase reporter gene experiments showed that overexpression of NRSF was able to significantly inhibit EGFR wild-type luciferase activity but had no significant effect on EGFR binding mutants (Figure 4H).

Neuron-Restricted Silencing Factor Affects Proliferation and Migration of Lung Cancer Cells by Regulating EGFR

Moreover, to further explore the association between NRSF and EGFR, we overexpressed EGFR expression in NCI-H520 lung cancer cells with NRSF overexpression. Overexpression of EGFR can abrogate the inhibitory effects of NRSF overexpression on the proliferation (Figure 5A and B) and migration of cells (Figure 5C and D). In vivo experiment also showed that overexpression of EGFR can rescue the inhibitory effects of NRSF overexpression on the tumor growth (Figure 5E).

Clinical Relevance of miRNA9 and EGFR in Patients With Lung Cancer

In 60 clinical samples, EGFR mRNA levels was negatively correlated with miRNA9 expression levels. In addition, high levels of miRNA9 and high levels of EGFR mean that patients
have poorer survival while low levels of miRNA9 and low levels of EGFR could prolong the survival time (Figure 6).

Discussion
Lung cancer is the world’s highest incidence and mortality of malignant tumors seriously affect this human health, due to the current limitations of diagnosis and treatment, lung cancer treatment and poor prognosis, in the treatment and care of patients with lung cancer with a large amount of social capital. Therefore, studying the mechanism of occurrence and development of lung cancer and developing new drug targets have become a major scientific problem to be solved in the world.

A growing number of studies have shown that miRNA plays an important role in tumorigenesis and development. In this study, we found that miRNA9 is highly expressed in lung cancer and inhibition of miRNA9 can inhibit tumor proliferation and migration. Therefore, miRNA9 may be a potential target for intervention in tumorigenesis.

As an important silencing factor, NRSF plays a key role in the establishment and maintenance of neurons. Currently, research on NRSF focuses on neuro-related diseases, including neurodegenerative diseases, epilepsy, neuralgia, and so on. As the research progresses, researchers have found that NRSF may play an important role in the development of brain cancer by maintaining tumor stem cell properties. It has also been reported that some specific shear variant of NRSF may be used as a biomarker for small cell lung cancer in clinical diagnosis.

In this study, we found that miRNA9 could inhibit the expression of NRSF, which was lower in cancer tissues in patients with pulmonary squamous cell carcinoma, and found that its low level of expression meant higher cancer malignancy and less patient survival. Furthermore, we found in vivo and in vitro experiments that overexpression of NRSF in cancer cells inhibited tumor cell proliferation and migration, suggesting that NRSF regulates the occurrence and development of lung SCC. We examined EGFR expression levels in patients with pulmonary SCC and found EGFR was increased in cancer tissues. Further found that NRSF was able to inhibit the expression of EGFR. In lung SCC, due to the low expression of NRSF, the inhibition of EGFR was relieved, resulting in the high expression of EGFR, which in turn promoted the occurrence and development of lung SCC.

Conclusion
In this study, miRNA9/NSFR/EGFR was first proposed to promote the development of lung SCC. This has a guiding effect on the research and development of lung cancer drugs. As present, the therapeutic effect of lung cancer drugs is far from satisfactory, and the targeting treatment of EGFR is mainly aimed at enzyme activity. Because of the large number of mutations in EGFR in patients with lung cancer, the current drug treatment effect is not so useful. In this work, we found for the first time that miRNA9 can increase the expression level of EGFR by inhibiting NSFR and then the expression level of EGFR. These results indicated a new strategy for lung cancer treatment by inhibiting miRNA9 activity and upregulating the expression level of NSFR in lung cancer cells, thus inhibiting the overall expression level of EGFR, which may improve the therapeutic effect of patients with lung cancer.

Authors’ Note
This study was approved by the Sichuan Cancer Hospital, Cancer Hospital Affiliated to School of Medicine, University of Electronic Science and Technology of China Ethical Committee (approval no: 2018-0302-03). All patients provided written informed consent prior to enrollment in the study, the ethical issues and handling methods involved in all animal studies are in accordance with the regulations of relevant institutions at home and abroad.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by the Sichuan Science and Technology Project (No. 2014JY0251).

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