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MiRNA-20a-5p accelerates the proliferation and invasion of non-small cell lung cancer by targeting and downregulating KLF9

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Abstract. – OBJECTIVE: To uncover the role of microRNA-20a-5p (miRNA-20a-5p) in the progression of Non-small cell lung cancer (NSCLC) and the underlying mechanism.

PATIENTS AND METHODS: MiRNA-20a-5p level in NSCLC tissues and cell lines was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Its level in NSCLC patients with larger or smaller tumor size, and either with lymphatic metastasis or not was examined as well. Regulatory effects of miRNA-20a-5p on viability, cell cycle, and invasiveness of A549 and PC9 cells were assessed. The interaction between miRNA-20a-5p and KLF9 was explored by Dual-Luciferase Reporter Gene Assay and Spearman correlation test. At last, the role of miRNA-20a-5p/KLF9 axis in influencing the progression of NSCLC was determined.

RESULTS: MiRNA-20a-5p was upregulated in NSCLC tissues and cell lines. Its level was much pronounced in NSCLC patients with larger tumor size or accompanied with lymphatic metastasis. Overexpression of miRNA-20a-5p in A549 cells enhanced viability, cell ratio in S phase, and invasiveness, while the knockdown of miRNA-20a-5p in PC9 cells achieved the opposite trends. KLF9 was confirmed to be the direct target of miRNA-20a-5p. There was a negative correlation between the expression levels of miRNA-20a-5p and KLF9 in NSCLC tissues. In addition, KLF9 overexpression could reverse the promotive effects of upregulated miRNA-20a-5p on the proliferation and invasiveness of A549 cells. On the contrary, the knockdown of KLF9 reversed the inhibitory effects of downregulated miRNA-20a-5p on cellular behaviors of PC9 cells.

CONCLUSIONS: MiRNA-20a-5p stimulates NSCLC to proliferate and invade by targeting KLF9.

Key Words:
MiRNA-20a-5p, KLF9, Non-small cell lung cancer (NSCLC).

Introduction

Non-small cell lung cancer (NSCLC) is a malignancy with high mortality, accounting for 85% of all lung cancer cases[1]. About 67% of NSCLC patients are older than 65 years[2]. In recent years, the increased incidence and poor prognosis of NSCLC with a low overall survival of 20% seriously affect life qualities of affected people[3]. Hence, it is of great significance to uncover the molecular mechanism of NSCLC.

MicroRNAs (MiRNAs) are non-coding, single-strand RNAs widely distributed in mammals, plants, and viruses, participating in various biological processes[4,5]. They[6-8] have a crucial function in the occurrence and progression of tumors. MiRNAs are considered to be novel non-invasive hallmarks for diagnosing, treating, and prognosticating NSCLC[7]. Wang et al[8] demonstrated that the overexpression of miR-616 could stimulate NSCLC to proliferate, invade, and metastasize. Liu et al[9] suggested the role of miR-661 in influencing EMT of NSCLC, thus triggering the distant metastasis of tumor cells. MiRNA-20a-5p is a 23-nucleotide miRNA upregulated in gastric cancer and hepatocellular cancer[10-12]. The role of miRNA-20a-5p in NSCLC, however, remains unclear.

Kruppel-like factors (KLFs) belong to a type of transcription factor with a zinc finger structure, with a typical structural feature of three C_2H_2 zinc fingers at its carboxyl terminus[13]. KLFs are extensively involved in cellular performances and embryonic development[14]. KLF9 is one of the members of KLFs family, which influences the proliferation and apoptosis of many types of tumor cells[15,16].

This study mainly explored the biological role of miRNA-20a-5p in the progression of NSCLC,

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and its interaction with KLF9. Our results provide a new idea for improving the therapeutic efficacy of NSCLC.

Patients and Methods

Sample Collection

NSCLC tissues and adjacent normal tissues were surgically resected from 40 NSCLC patients treated in Shanghai Pulmonary Hospital from January 2016 to December 2018. They did not receive preoperative anti-tumor therapy and had no history of other malignancies. The clinical data of enrolled NSCLC patients were collected. All subjects volunteered to participate in the study and signed written informed consent. This study was approved by the Ethics Committee of Shanghai Pulmonary Hospital.

Cell Culture and Transfection

Lung cancer cell lines (A549, PC9, and H1299) and human bronchial epithelial cell line (16HBE) were provided by Cell Bank (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 Ul/mL penicillin, and 0.1 mg/mL streptomycin, at 37°C in a 5% CO_2 incubator.

The cells were cultured until 60% of confluence and subjected to transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 6 hours later, the complete medium was replaced. The transfected cells for 24-48 h were harvested for *in vitro* experiments.

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

The cells were lysed for harvesting the total RNA and subjected to reverse transcription for extracting complementary deoxyribose nucleic acid (cDNA). The cDNA was used for conducting qRT-PCR using the SYBR® PrimeScript™ RT-PCR (TaKaRa, Dalian, China) on the ABI 7300 PCR system. The relative levels were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences were as follows: miRNA-20a-5p: F: 5'-GCCCGCTAAAGTGCTTATAGTG-3', R: 5'-CCAGTGCAGGGTCCGAGGT-3'; KLF9: F: 5'-ACAGTGGCTGTGGGAAAGTC-3', R: 5'-TCACAAAGCGTTGGCCAGCG-3'. U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'. GAPDH: F: 5'-AGGTCGGTGTGAACGGATTTG-3', R: 5'-TGTAGACCATGTAGTTGAGGTCA-3'.

Cell Counting Kit-8 (CCK-8)

The cells were seeded in the 96-well plate with 1×10^4 cells per well and cultured overnight. Absorbance (A) at 450 nm was recorded at the appointed time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curves.

Cell Cycle Determination

1×10^6 cells were collected, washed with phosphate-buffered saline (PBS) twice and fixed in pre-cold 70% ethanol. Subsequently, the cells were incubated with RNase for 15 min and 50 μg/mL propidium iodide for 30 min. The cell cycle was determined using flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Transwell

The cell density was adjusted to 1×10^6 cells/mL. 200 μL of suspension was applied in the upper side, while 600 μL of medium containing 5% FBS was applied in the bottom of a transwell chamber (8 μm in pore size, Corning, Corning, NY, USA). After 24 h of incubation, the cells penetrated to the bottom side were fixed in methanol for 15 min, stained with crystal violet for 20 min, and counted using a microscope. The number of invasive cells was counted in 5 randomly selected fields per sample (magnification 100×).

Western Blot

The total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) and quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The protein sample was loaded for electrophoresis and transferred on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% skim milk for blocking the non-specific sites. Two hours later, the membranes were subjected to incubation with primary and secondary antibodies. The bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Dual-Luciferase Reporter Gene Assay

Wild-type and mutant-type Luciferase plasmids of KLF9 were constructed. The cells seeded

in the 24-well plate with 2×10^5 cells per well were co-transfected with 0.5 μg wild-type/mutant-type KLF9 and 20 nM miRNA-20a-5p mimic/NC. 48 hours later, the cells were lysed for determining the relative Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 16.0 (SPSS Inc., Chicago, IL, USA) was used for data analyses. The data were expressed as mean ± standard deviation. The intergroup differences were analyzed by the *t*-test. The Chi-square test was performed to identify the correlation between the miRNA-20a-5p level and pathological indexes of NSCLC patients. The Spearman correlation test was used for assessing the relationship between miRNA-20a-5p and KLF9. $p<0.05$ was considered as statistically significant.

Results

Upregulation of MiRNA-20a-5p in NSCLC

The miRNA-20a-5p level was determined in 40 matched NSCLC tissues and adjacent normal tissues by qRT-PCR. It is shown that miRNA-20a-5p was markedly upregulated in NSCLC tissues relative to those in adjacent normal ones (Figure 1A). In addition, miRNA-20a-5p was highly expressed in lung cancer cell lines (Fig-

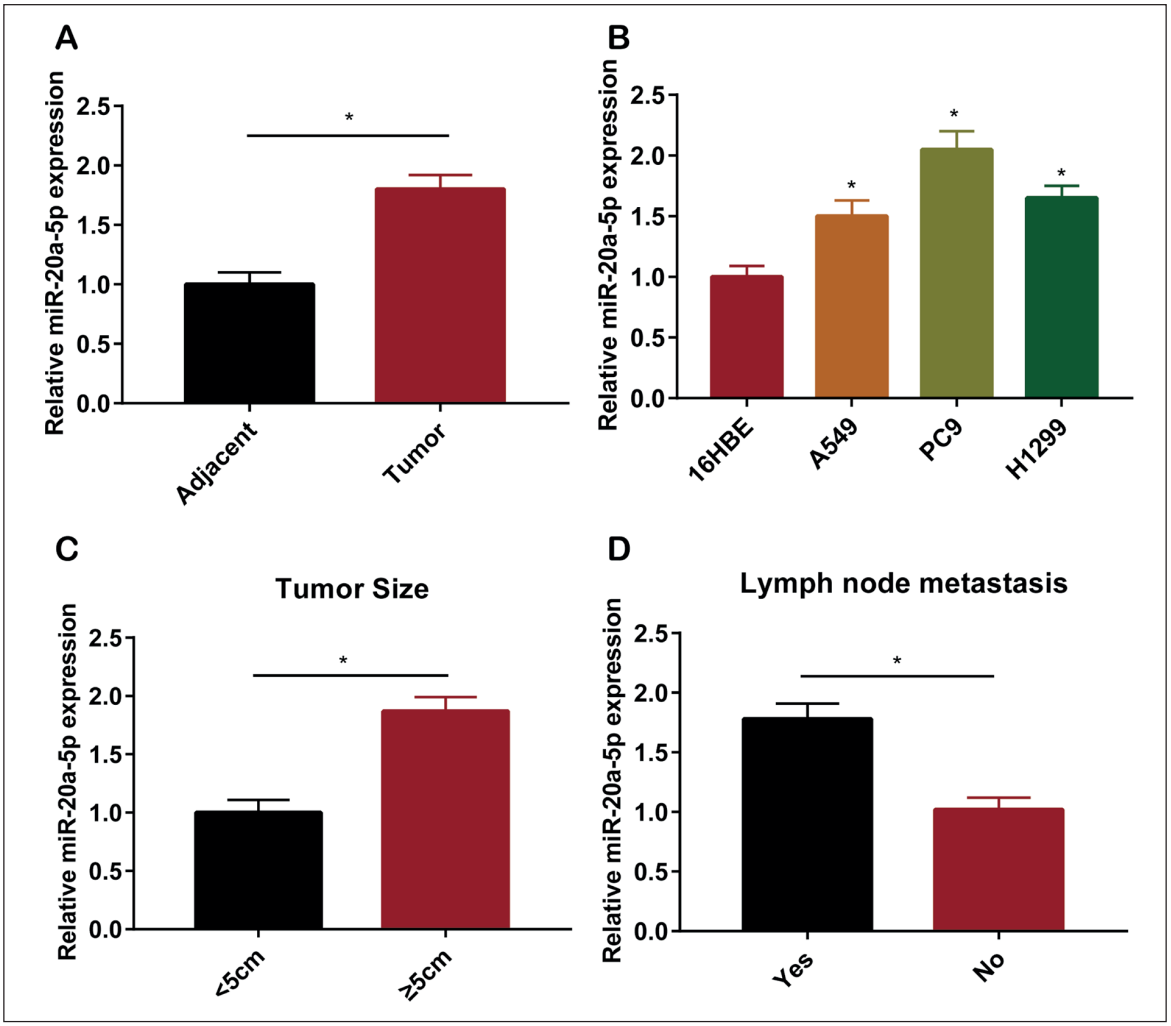

Figure 1. Upregulation of miR-20a-5p in NSCLC. **A,** MiR-20a-5p level in adjacent normal tissues and NSCLC tissues. **B,** MiR-20a-5p level in 16HBE, A549, PC9, and H1299 cells. **C,** MiR-20a-5p level in NSCLC patients with <5 cm or ≥ 5 cm in tumor size. **D,** MiR-20a-5p level in NSCLC patients either with lymphatic metastasis or not.

ure 1B). A549 and PC9 cells expressed the lowest and highest level of miRNA-20a-5p among the three selected NSCLC cell lines, which were selected for the following experiments. According to the clinical data of enrolled NSCLC patients, we further analyzed the relationship between the miRNA-20a-5p level and pathological indexes of NSCLC patients. The Chi-square test suggested that miRNA-20a-5p level was positively correlated with tumor size and lymphatic metastasis of NSCLC patients (Table I). Moreover, higher abundance of miRNA-20a-5p was observed in NSCLC patients with ≥ 5 cm in tumor size or accompanied with lymphatic metastasis (Figures 1C, 1D). The above data illustrated the potential involvement of miRNA-20a-5p in NSCLC.

MiRNA-20a-5p Accelerated Viability and Invasiveness of NSCLC

MiRNA-20a-5p overexpression and knockdown models were constructed in A549 and PC9 cells, respectively. The transfection efficacies of miRNA-20a-5p mimic and inhibitor were verified by performing qRT-PCR (Figure 2A). In A549 cells transfected with miRNA-20a-5p mimic, their viability, cell ratio in S phase, and invasiveness were remarkably enhanced (Figure 2B left, Figure 2C left, and Figure 2D upper). Transfection of miRNA-20a-5p inhibitor in PC9 cells decreased the viability, cell ratio in S phase, and invasiveness (Figure 2B right, Figure 2C right, and Figure 2D bottom).

KLF9 Was the Direct Target of MiRNA-20a-5p

Through bioinformatics prediction in TargetScan, it is found that miRNA-20a-5p could bind to KLF9 3'UTR (Figure 3A). Luciferase activity was markedly reduced by the co-transfection of KLF9-WT and miRNA-20a-5p mimic, verifying the binding relationship between miRNA-20a-5p and KLF9 (Figure 3B). KLF9 was downregulated in NSCLC tissues relative to adjacent normal ones (Figure 3C). Besides, a negative correlation was identified in the expression levels between miRNA-20a-5p and KLF9 (Figure 3D). The overexpression of miRNA-20a-5p in A549 cells downregulated both mRNA and protein levels of KLF9, while the knockdown of miRNA-20a-5p in PC9 cells upregulated KLF9 level (Figure 3E, 3F).

MiRNA-20a-5p Regulated Cellular Behaviors of NSCLC by Targeting KLF9

We constructed pcDNA-KLF9 and si-KLF9 to further uncover the role of KLF9 in NSCLC. Their transfection efficacies were verified in A549 and PC9 cells, respectively (Figure 4A). KLF9 overexpression could reverse the promotive effects of upregulated miRNA-20a-5p on the proliferation and invasiveness of A549 cells (Figure 4B left, Figure 4C left, and Figure 4D upper). On the contrary, the knockdown of KLF9 reversed the inhibitory effects of the downregulated miRNA-20a-5p on cellular behaviors of PC9 cells (Figure 4B right, Figure

Table I. Correlation between miR-20a-5p level and pathological indexes of NSCLC patients (n=40).

Clinicopathologic features	Number of cases	miR-20a-5p expression		*p*-value
		Low (n = 20)	High (n = 20)	
Age (years)				0.525
≤ 60	18	8	10	
> 60	22	12	10	
Gender				0.519
Male	24	11	13	
Female	16	9	7	
Tumor size				0.010*
< 5 cm	16	12	4	
≥ 5 cm	24	8	16	
TNM stage				0.057
I-II	18	6	12	
III-IV	22	14	8	
Lymph node metastasis				0.043*
Yes	21	8	13	
No	19	12	7	

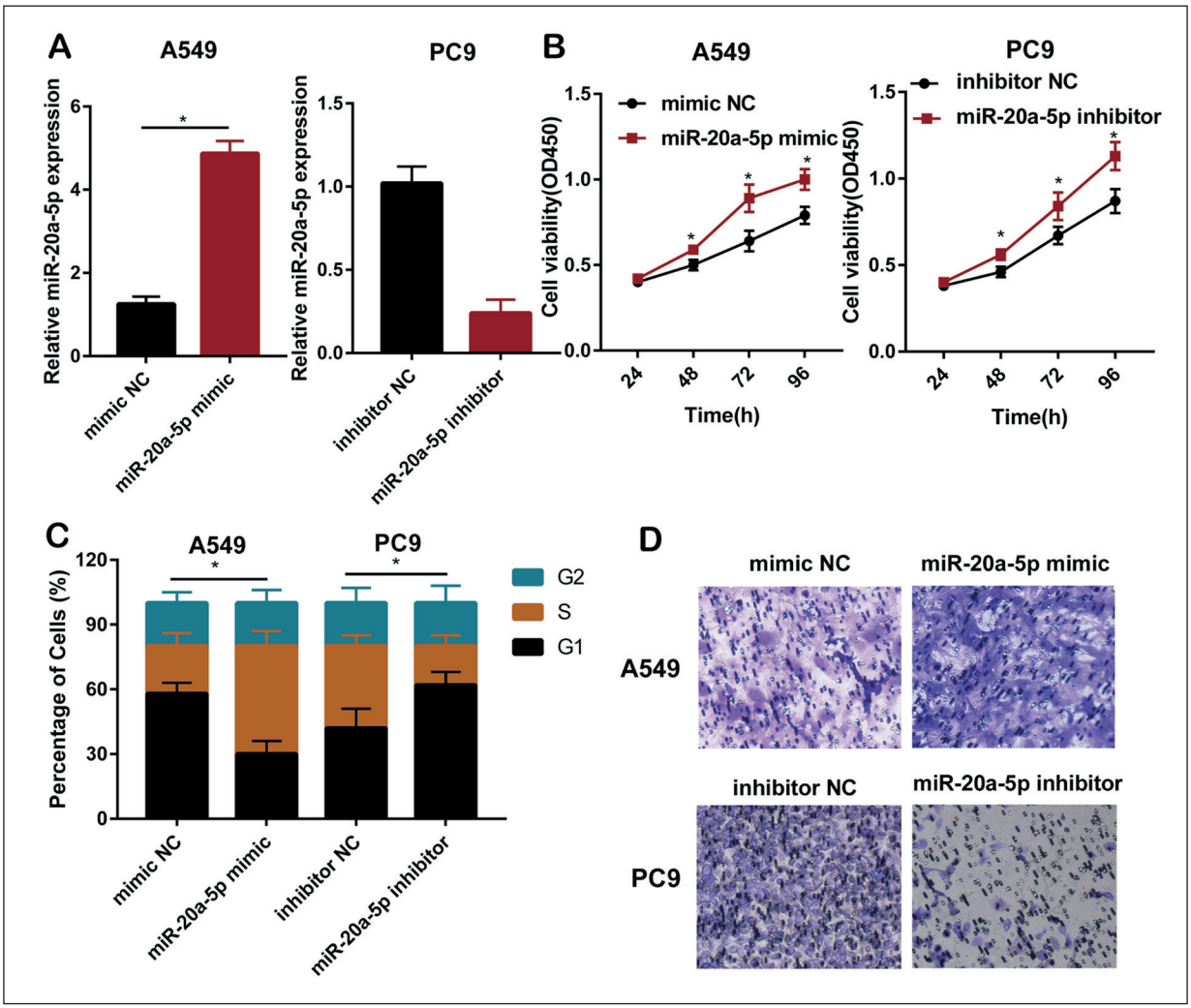

Figure 2. MiR-20a-5p accelerated viability and invasiveness of NSCLC. **A,** The transfection efficacies of miR-20a-5p mimic and inhibitor in A549 and PC9 cells, respectively. **B,** Viability in A549 cells transfected with mimic NC or miR-20a-5p mimic (left). Viability in PC9 cells transfected with inhibitor NC or miR-20a-5p inhibitor (right). **C,** Percentage of cells in G2, S, and G1 phase in A549 cells transfected with mimic NC or miR-20a-5p mimic (left). Percentage of cells in G2, S, and G1 phase in PC9 cells transfected with inhibitor NC or miR-20a-5p inhibitor (right). **D,** Invasiveness in A549 cells transfected with mimic NC or miR-20a-5p mimic (upper). Invasiveness in PC9 cells transfected with inhibitor NC or miR-20a-5p inhibitor (bottom) (magnification: 40×).

4C right, and Figure 4D bottom). Collectively, miRNA-20a-5p was able to promote the progression of NSCLC by negatively regulating KLF9 level.

Discussion

Lung cancer is a malignancy with the highest morbidity and mortality throughout the world. The death number of lung cancer accounts for 30% of all tumor deaths[17]. NSCLC is the major subtype of lung cancer, which is affected by multiple complex factors (i.e., genetics, smoking, virus infection, etc.). During the pathogenesis of NSCLC, multiple genes and signaling pathways are dysregulated.

MiRNAs are mainly expressed in eukaryotes. They could recognize and specifically target mRNA 3'UTR, thus degrading mRNAs or inhibiting their translation to mediate the post-transcriptional regulation[18,19]. Plenty of abnormally expressed miRNAs are found in NSCLC. These miRNAs are closely related to the occurrence and progres-

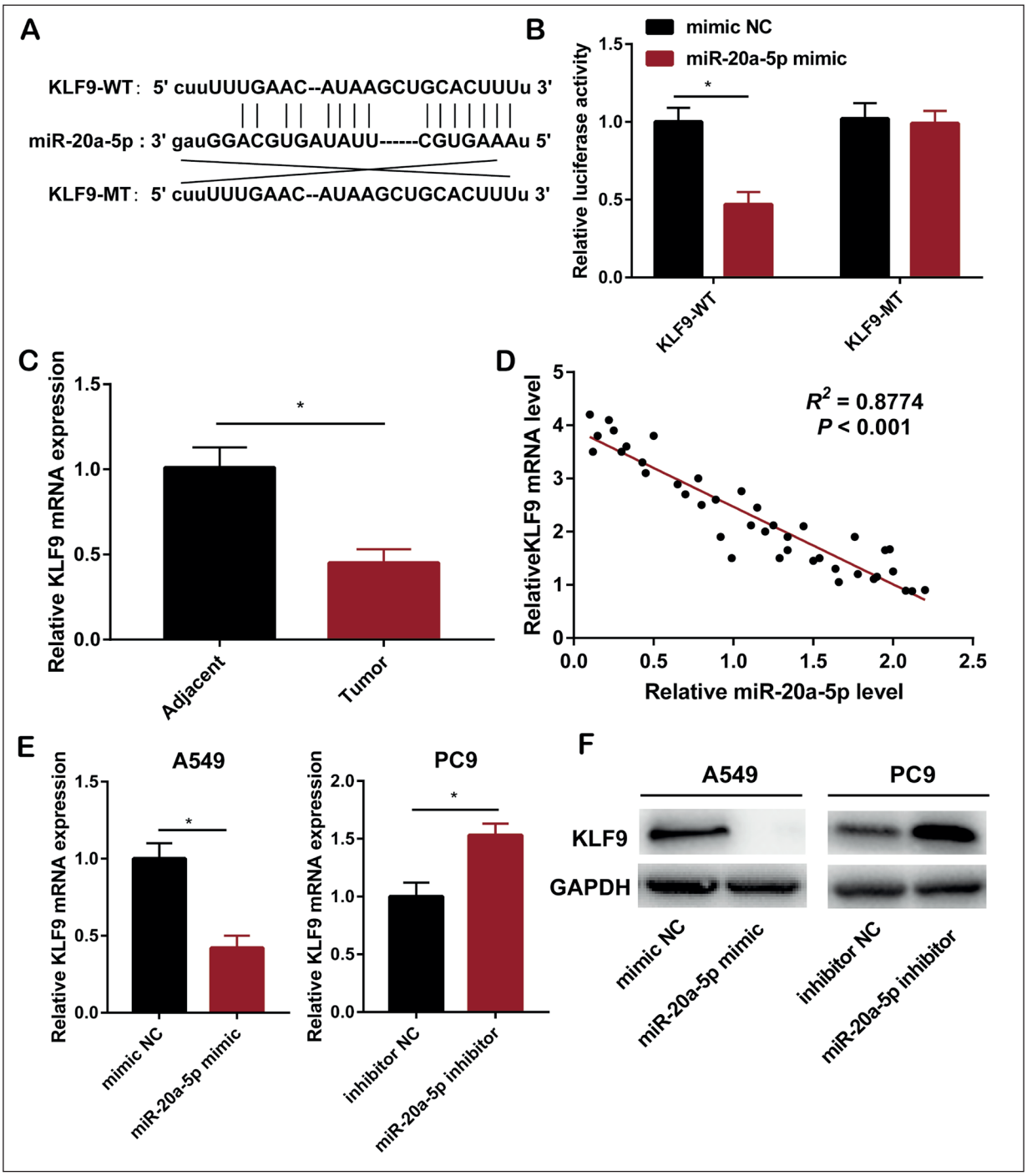

Figure 3. KLF9 was the direct target of miR-20a-5p. **A,** Binding sites in the promoter region of KLF9 and miR-20a-5p. **B,** Luciferase activity in 293T cells co-transfected with KLF9-WT/KLF9-MT and mimic NC/miR-20a-5p mimic. **C,** KLF9 level in adjacent normal tissues and NSCLC tissues. **D,** A negative correlation between the expression levels of miR-20a-5p and KLF9. **E,** The mRNA level of KLF9 in A549 cells transfected with miR-20a-5p mimic and PC9 cells transfected with miR-20a-5p inhibitor. **F,** The protein level of KLF9 in A549 cells transfected with miR-20a-5p mimic and PC9 cells transfected with miR-20a-5p inhibitor.

sion of NSCLC, such as miR-675-5p[20], miR-504[21], and miR-193a-3p[22]. In this paper, we observed that miRNA-20a-5p was highly expressed in NSCLC tissues and cell lines. This overexpression accelerated A549 cells to proliferate and invade, as well as decreased cell ratio in G1 phase. Con-

versely, the knockdown of miRNA-20a-5p yielded the opposite results. Therefore, miRNA-20a-5p was believed to be an oncogene in NSCLC.

It is reported that miRNAs could exert certain regulatory effect by directly regulating the target gene expressions[23]. Through online prediction, a presence of binding sites in the promoter region of miRNA-20a-5p and KLF9 was observed. Later, the Dual-Luciferase Reporter Gene Assay further confirmed their binding relationship. KLF9 locates on human chromatin 9q13[24]. KLF9 is identified to be a potential tumor-suppressor gene, which is downregulated in liver cancer[25] and colorectal cancer[26]. Sun et al[27] uncovered the downregulated KLF9 in liver cancer tissues, which is closely related to p53 level. Besides,

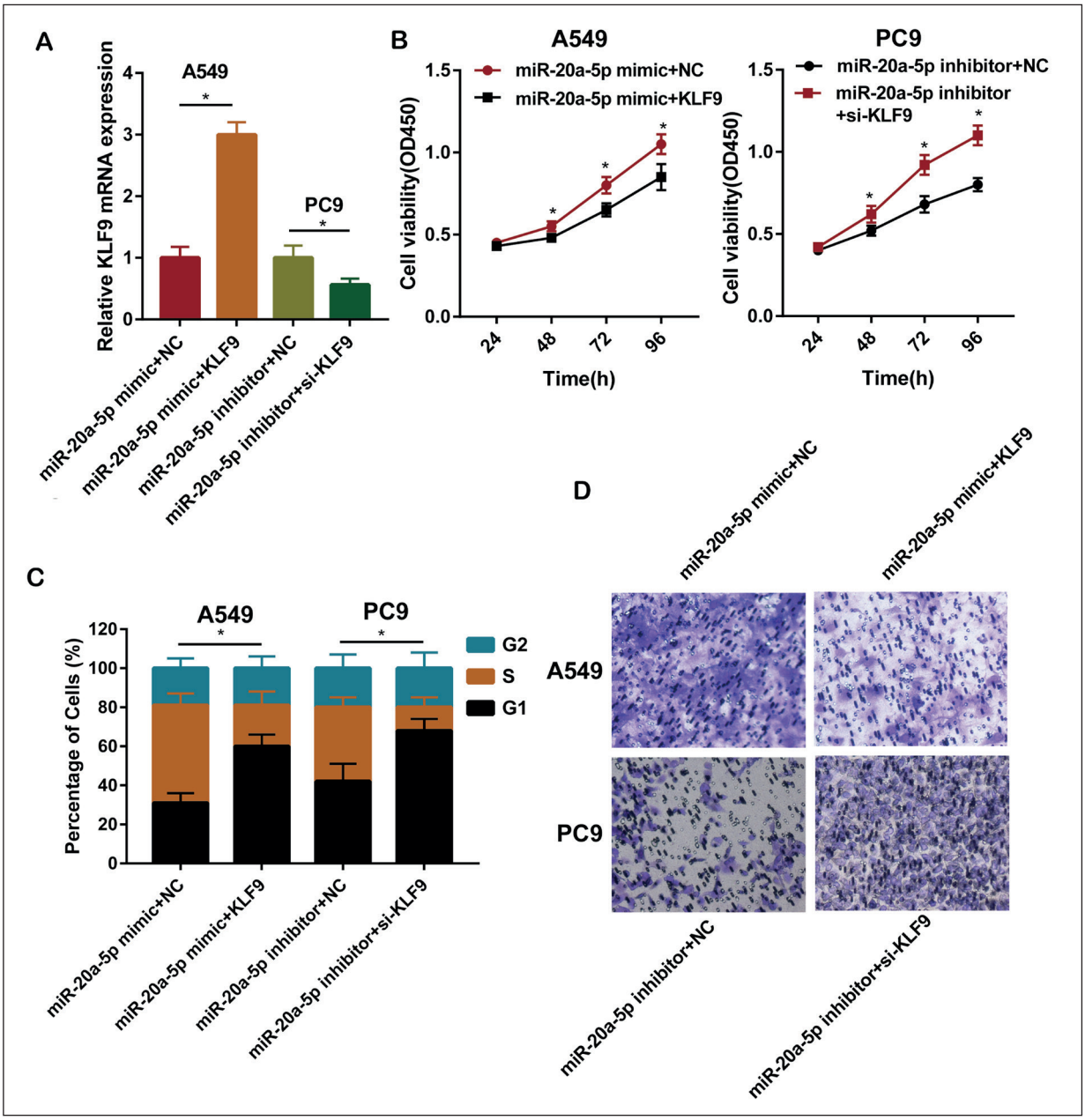

Figure 4. MiR-20a-5p regulated the cellular behaviors of NSCLC by targeting KLF9. A549 cells were transfected with miR-20a-5p mimic+NC or miR-20a-5p mimic+pcDNA-KLF9. PC9 cells were transfected with miR-20a-5p inhibitor+NC or miR-20a-5p inhibitor+si-KLF9. **A,** KLF9 level in A549 and PC9 cells. **B,** Viability in A549 and PC9 cells at 24, 48, 72, and 96 h. **C,** Percentage of cells in G2, S, and G1 phase. **D,** Invasiveness in A549 and PC9 cells (magnification: 40×).

KLF9 could inhibit the malignant growth of prostate cancer by suppressing the AKT pathway[26]. A relevant study demonstrated that miR-889 influences the proliferative and invasive capacities of NSCLC by targeting KLF9[28]. Therefore, KLF9 is of clinical significance in NSCLC. Our results showed that KLF9 was downregulated in NSCLC, and negatively regulated by miRNA-20a-5p. Moreover, KLF9 could reverse the regulatory effect of miRNA-20a-5p on the viability and invasiveness of lung cancer cells. Collectively, miRNA-20a-5p exerted the carcinogenic role in NSCLC by targeting and downregulating KLF9.

Conclusions

The above results indicated that miRNA-20a-5p is upregulated in NSCLC, which accelerates the proliferation and invasiveness of NSCLC cells by targeting KLF9.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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