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MiR-141 promotes cell proliferation and invasion in non-small cell lung cancer by targeting KLF9

Y.-J. KONG[1], X.-X. TAN[2], Y. ZHANG[3], Q.-J. HE[3], L. ZHAO[4], Q. MENG[5]

[1]School of Public Health and Management, Weifang Medical University, Weifang, China
[2]Department of Respiratory Medicine, Linyi No. 3 People's Hospital, Linyi, China
[3]Department of Respiratory Medicine, the People's Hospital of Zhangqiu Area, Jinan, China
[4]Department of Respiratory Medicine, People's Hospital of Rizhao, Rizhao, China
[5]ICU, Jining First People's Hospital, Jining, China

Abstract. – OBJECTIVE: Non-small cell lung cancer is the cancer with the highest mortality rate in the whole world. MicroRNA-141 (miR-141) has been reported to be an abnormal expression in multiple tumors including in non-small cell lung cancer. The aim of this study was to verify the potential roles of miR-141 in non-small cell lung cancer and evaluate the effects on cell proliferative and invasive abilities.

PATIENTS AND METHODS: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and transwell assays were conducted to calculate the tissues and cell lines' proliferative and invasive abilities. Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) and Western blotting were utilized to evaluate the mRNA and protein levels of specific genes.

RESULTS: MiR-141 was significantly upregulated, while krüppel-like factor 9 (KLF9) downregulated in non-small-cell lung cancer (NSCLC) tissues and cell lines. MiR-141 and KLF9 mRNA levels had a negative correlation in NSCLC tissues. The overexpression of miR-141 promoted the proliferation and invasion of A549 cells, while caused contrast results when knockdown miR-141. In addition, KLF9 was a direct target gene of miR-141 and KLF9 partially reversed the roles of miR-141 in A549 cells. MiR-141 promoted the proliferation and invasion by binding to KLF9 in NSCLC.

CONCLUSIONS: MiR-141 promoted the proliferation and invasion by targeting the KLF9 in non-small cell lung cancer, and the newly identified miR-141/KLF9 axis provides novel insight into the pathogenesis of non-small cell lung cancer.

Key Words:
MiR-141, KLF9, Proliferation, Invasion, Non-small cell lung cancer.

Abbreviations

FBS: Fetal Bovine Serum; KLF9: Krüppel-Like Factor 9; NSCLC: Non-Small Cell Lung Cancer; 3'-UTR: 3'-untranslated region; RT-qPCR: Real Time-Quantitative Polymerase Chain Reaction; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide; MicroRNAs: MiRNAs; ATCC: American Type Culture Collection; RPMI-1640: Roswell Park Memorial Institute-1640; cDNA: Complementary Deoxyribose Nucleic Acid; GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase; RIPA: Radioimmunoprecipitation Assay; PMSF: Phenylmethylsulfonyl Fluoride; BCA: Bicinchoninic Acid; SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis; PVDF: Polyvinylidene Difluoride; HRP: Horseradish Peroxidase; SPSS: Statistical Product and Service Solutions.

Introduction

Lung cancer is the most common malignant tumor, divided into small cell carcinoma and non-small-cell lung cancer (NSCLC). NSCLC accounts for 85% of all the lung cancer that included squamous cell carcinoma, adenocarcinoma and large cell carcinoma[1-3]. In recent years, the incidence of NSCLC has continued to increase and has the highest mortality rate in the whole world. Thus, to explore the biomarkers for the diagnosis and treatment of NSCLC is necessary.

MicroRNAs (miRNAs), a class of short non-coding RNAs that usually act as gene expression regulators, relate to the regulation of diverse biological functions and pathological implications by binding to the 3'-untranslated region (3'-UTR) of target mRNAs at the post-transcriptional level[4,5]. Increasing evidence elucidated that miRNAs mediated tumor progress, such as metabolism, proliferation, invasion and apoptosis[6-8]. MiR-141 was a member of the miR-200 family that has been reported to be upregulated in miscellaneous tumors, including esophageal squamous cell carcinoma, prostate cancer, renal cell carcinoma and breast cancer[9-12]. Ding

Corresponding Author: Qiang Meng, MD; e-mail: bsfy09@163.com

et al[13] revealed that miR-141 promoted the proliferation in colon cancer. What's more, miR-141 has been reported to promote the proliferation, cell cycle, migration and invasion, and inhibited the apoptosis in gastric cancer[14]. Thus, we wonder the function of miR-141 on the proliferation and invasion of NSCLC cells.

Krüppel-like factor 9 (KLF9), a transcription factor that belongs to the SP/KLF family, regulate the gene expression by binding to GC box elements located at the promoter[15]. KLF9 was abnormally expressed in multiple tumors, including multiple myeloma, prostate cancer, esophageal squamous cell carcinoma and hepatocellular carcinoma[16-19]. Brown et al[20] has been discovered that KLF9 was a haploinsufficient suppressor of colon tumorigenesis, by repression of ISG15 and the latter's antiapoptotic function. Ji et al[21] has been indicated that the overexpression of KLF9 inhibited the proliferation and clone formation in pancreatic ductal adenocarcinoma. Qiao et al[18] showed that KLF9 inhibited the growth, migration and invasion in esophageal squamous cell carcinoma. Therefore, we strongly believe that the miR-141 mediated the proliferation and invasion by targeting to KLF9 in NSCLC.

Patients and Methods

Clinical Specimens

We obtained 55 pairs of NSCLC tissues and matched adjacent normal tissue specimens from NSCLC patients who received surgery at the Weifang Medical University. All tissues were frozen in liquid nitrogen and stored immediately at -80°C refrigerator. Neither radiotherapy nor chemotherapy therapy was used in any patients before surgery. Informed consent was obtained from all patients, and the studies were approved by the Ethics Committee of the Weifang Medical University.

Cell Lines and Culture Condition

Two human lung cancer cell lines (A549 and H460) and a normal embryonic lung fibroblast cell line MRC-5 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) was employed to culture the cells in a 37°C incubator in an atmosphere consisting of 5% CO_2.

Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

The RNAiso for Small RNA (TaKaRa, Dalian, China) and the RNAiso Plus (TaKaRa, Dalian, China) were conducted to extract the total miRNAs and total RNAs, respectively. The reverse transcription was performed by the One Step PrimeScript miRNA complementary deoxyribose nucleic acid (cDNA) Synthesis Kit (TaKaRa, Dalian, China) and then the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China) was utilized to perform and synthesis the first cDNA chain. The SYBR Green PCR Kit (TaKaRa, Dalian, China) was employed to perform the qPCR using ABI 7500 FAST Real Time-Polymerase Chain Reaction System (Applied Biosystems, Foster City, CA, USA). The normalization of miR-141 and KLF9 were U6 small nuclear RNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. The primers used were as followed: miR-141 forward, 5'-CACATCCACCTCCTCCACATC-3' and reverse, 5'-AATGCGGCCGCAACTCAATCAACATCACCAT-3'; U6 forward, 5'-CTCGCTTCGGCAGCACATATACT-3' and reverse, 5'-ACGCTTCACGAATTTGCGTGTC-3'; GAPDH forward, 5'-AAGGGAAGGTTGCTGGATAGG-3' and reverse, 5'-CACATCCACCTCCTCCACATC-3'. PCR cycling conditions were as follows: an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 10 sec, annealing and synthesis at 60°C for 60 sec.

Plasmids and Transfection

We used RiboBio (RiboBio, Guangdong, China) to design and synthesize the miR-141 mimic, the miR-141 inhibitor as well as their controls. The 3'-UTR of KLF9 were amplified and cloned into a pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA) to overexpress KLF9, which was designated as pcDNA3.1-KLF9. The A549 cells were seeded in 6-well plates and cultured in a phenol-red free medium, which can reduce the presence of hormones at 37°C overnight. According to the manufacturer's instructions, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was conducted to transfect the vectors into A549 cells.

Western Blotting

Radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) along with phenylmethylsulfonyl difluoride (PMSF; Sigma-Aldrich, St. Louis, MO, USA) was utilized to lyse the cells. The Bicinchoninic acid (BCA)

Protein Assay Kit (Pierce, Waltham, MA, USA) was employed to determine the concentration of proteins. Proteins with equal quantity were separated by 10% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in 5% skimmed milk, the membranes were incubated by the primary antibodies against KLF9 (1:1000, Abcam, Cambridge, MA, USA) and GAPDH (1:3000, Sigma-Aldrich, St. Louis, MO, USA). After washing three times with Tris-Buffered Saline with 0.5% Tween 20, the membranes were incubated by Horseradish Peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. The chemiluminescence action with HRP substrate was utilized to measure the signals.

Transwell Assay

Transwell inserts (8 μm pores; Corning Incorporated, Lowell, NY, USA) covered with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were placed in 24-well plate and then generated upper and lower chambers. For invasion assay, 200 μL of resuspended cells were added into the upper chamber followed by adding 500 μL of normal medium containing 20% FBS in the lower chamber, which acted as chemoattractant. After incubating at 37°C for 48 h, the non-invaded cells were wiped off by cotton swabs. The invaded cells were fixed by 4% paraformaldehyde and sequentially stained by 10% crystal violet. The cells in five randomly selected visual fields were photographed and counted under the microscope (Olympus Corporation, Tokyo, Japan).

Luciferase Report Assay

The PCR was performed to amplify the 3'-UTR sequences of KLF9 from human genomic DNA. The QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was employed to mutate the miR-141 binding sequences on KLF9 3'-UTR, which was deemed to act as a control. The wild-type and the mutated sequences were both cloned into the pmirGlo vector (Promega, Madison, WI, USA). Briefly, the cells at 80% confluence would be co-transfected two vectors, one of that were containing the KLF9 3'-UTR (wild-type or the mutant), and the other was contained the miR-141 mimic or NC mimic, and that were transfected using Lipofectamine 2000. After 48 h, the cells were harvested and evaluated the Luciferase activities using the Dual-GLO Luciferase Assay System (Promega, Madison, WI, USA) with Renilla activities as the internal reference.

Statistical Analysis

The data were expressed as the mean ± standard deviation from at least three independent experiments. The Statistical Product and Service Solutions (SPSS) software (version 16.0; SPSS Inc., Chicago, IL, USA) was applied to perform the statistical analysis. The statistical significance between the two groups was determined using the two-tailed Student's *t*-test. Nevertheless, the comparison between three or more groups was made using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered to indicate statistically significant differences.

Results

The Correlation Between the Expression of MiR-141 and KLF9 in NSCLC Tissues

The expression of miR-141 and KLF9 in 55 pairs of NSCLC and matched adjacent non-cancerous lung tissues were evaluated by RT-qPCR. The expression of miR-141 was up-regulated in NSCLC tissues compared to the matched adjacent non-cancerous lung tissues ($p<0.0001$; Figure 1A). On the contrary, the expression of KLF9 was dramatically lower in NSCLC tissues than matched adjacent non-cancerous tissues ($p=0.0007$; Figure 1B). Thus, the connection between miR-141 and KLF9 expression were measured and we discovered that miR-141 had a negative correlation with KLF9 in NSCLC tissues ($p=0.0020$, $r=-0.4086$) (Figure 1C). Apart from tissues samples, the expression of miR-141 was higher in two lung cancer cell lines NCI-H460 ($p=0.0037$) and A549 ($p=0.0004$) cells than normal lung fibroblast cell line MRC-5 (Figure 1D).

Moreover, the connection between the expression of miR-141 and clinicopathological features of 55 NSCLN patients was assessed. We discovered that the expression of miR-141 in non-small cell lung cancer tissues had connection with TNM stage ($p=0.038$) and lymph node metastasis ($p=0.020$), while had a tendency to had association with tumor size ($p=0.061$); however, the expression of miR-141 had no connection with age ($p=0.243$) and gender ($p=0.201$; Table I).

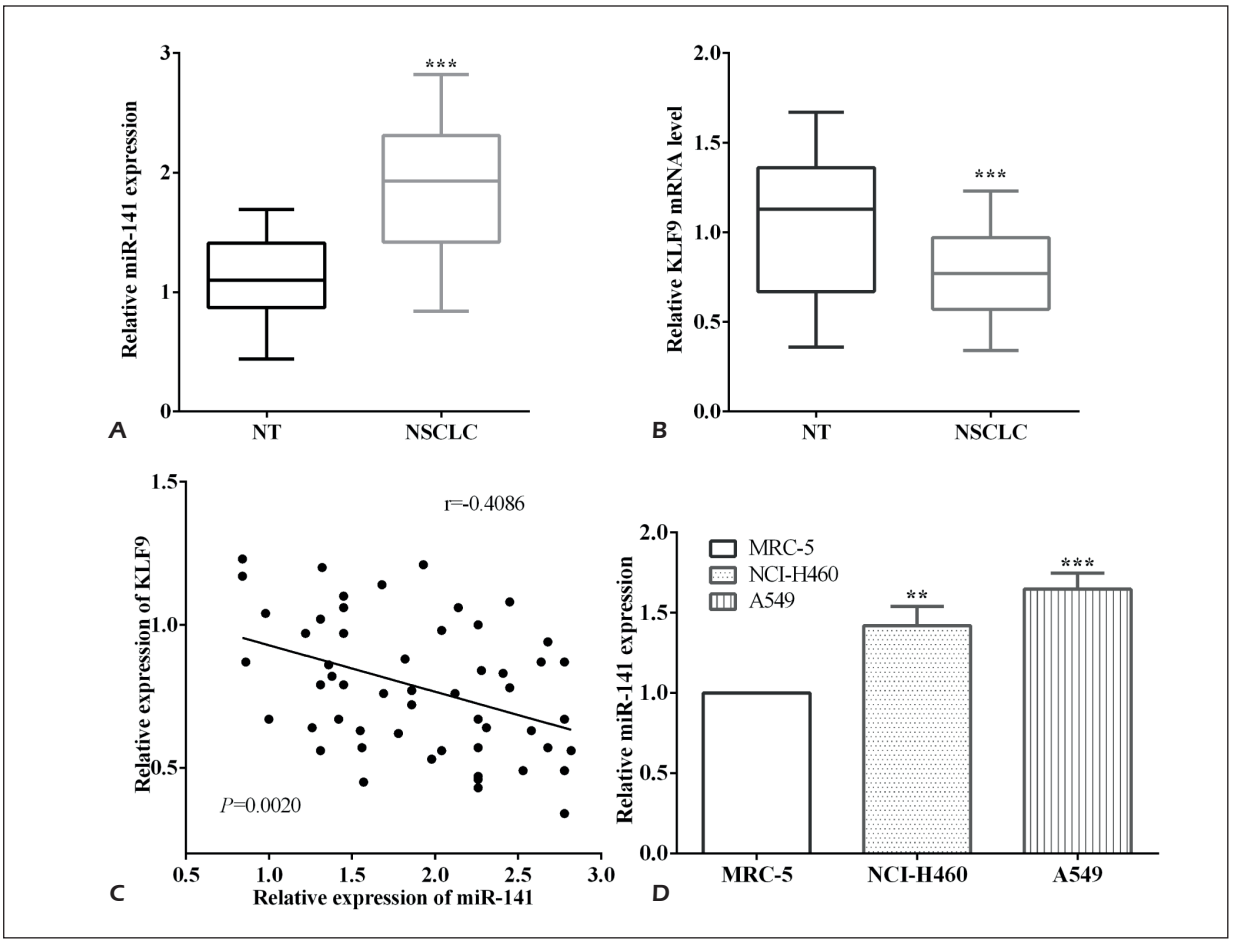

Figure 1. The correlation of miR-141 and KLF9 in NSCLC tissues. **A**, The expression of miR-141 was up-regulated in NSCLC tissues (n=55) compared to the matched adjacent non-cancerous lung tissues (n=55). **B**, The expression of KLF9 was lower in NSCLC tissues (n=55) than matched adjacent non-cancerous tissues (n=55). **C**, The expression of miR-141 had negative connection with KLF9 in NSCLC. **D**, The expression of miR-141 was higher in lung cancer cells NCI-H460 and A549 cells than normal lung fibroblast cell line MRC-5.

Table I. Correlations between miR-141 expression and clinicopathological features in 55 non-small cell lung cancer.

Clinicopathological features	Cases (No. =55)	miR-141 expression		*p*-value*
		26 High (%)	29 Low (%)	
Age (years)				0.243
≤50	23	9 (39.1)	14 (60.9)	
>50	32	18 (56.3)	15 (43.7)	
Gender				0.201
Male	31	17 (54.8)	14 (45.2)	
Female	24	9 (37.5)	15 (62.5)	
Tumor size (mm)				0.061
≤5.0	22	7 (31.8)	15 (68.2)	
>5.0	33	19 (57.6)	14 (42.4)	
TNM stage				0.038*
I-II	25	8 (32.0)	17 (68.0)	
III-IV	30	18 (60.0)	12 (40.0)	
Lymph-node metastasis				0.020*
0-2	29	18 (62.1)	11 (37.9)	
>2	26	8 (23.1)	18 (76.9)	

**p*-values are calculated with Chi-square test.

MiR-141 Enhanced the Proliferation and Invasion of NSCLC Cells

To determine the great roles of miR-141, A549 cells were selected and transiently transfected with the miR-141 mimic/inhibitor or the corresponding negative controls respectively. RT-qPCR assay revealed that the mRNA level of miR-141 was upregulated (p=0.0013) when transiently transfected with miR-141 mimic, while it was statistically downregulated (p=0.0080) transiently transfected with a miR-141 inhibitor as compared with the level in cells transfected with the control (Figure 2A). MTT assays revealed that the overexpression of miR-141 dramatically promoted (p=0.0060 and 0.0015 of 72 h and 96 h) the proliferation of A549 cells, whereas it had the opposite effect (p= 0.0334, 0.0092 and 0.0083 of 48 h, 72 h and 96 h) when knockdown of miR-141 (Figure 2B). What's more, transwell assay showed that miR-141 upregulation improved (p=0.0037) the invasive ability of A549 cells, in contrast, knockdown of miR-141 inhibited (p=0.0030) cell invasion (Figure 2C). All the results illuminated that miR-141 promoted the proliferation and invasion of NSCLC.

MiR-141 Targeted to KLF9 and Inhibited its Expression

TargetScan was used to identify the target genes of miR-141, and we discovered KLF9 maybe a target gene of miR-141. To verify miR-141 binding to KLF9, the complementary sequences of miR-141 on KLF9 were mutated from ACAGUGUU to ACAGUCAA, and then cloned in pmirGlo Luciferase vector, which were designated as pmirGlo-KLF9-WT and pmirGlo-

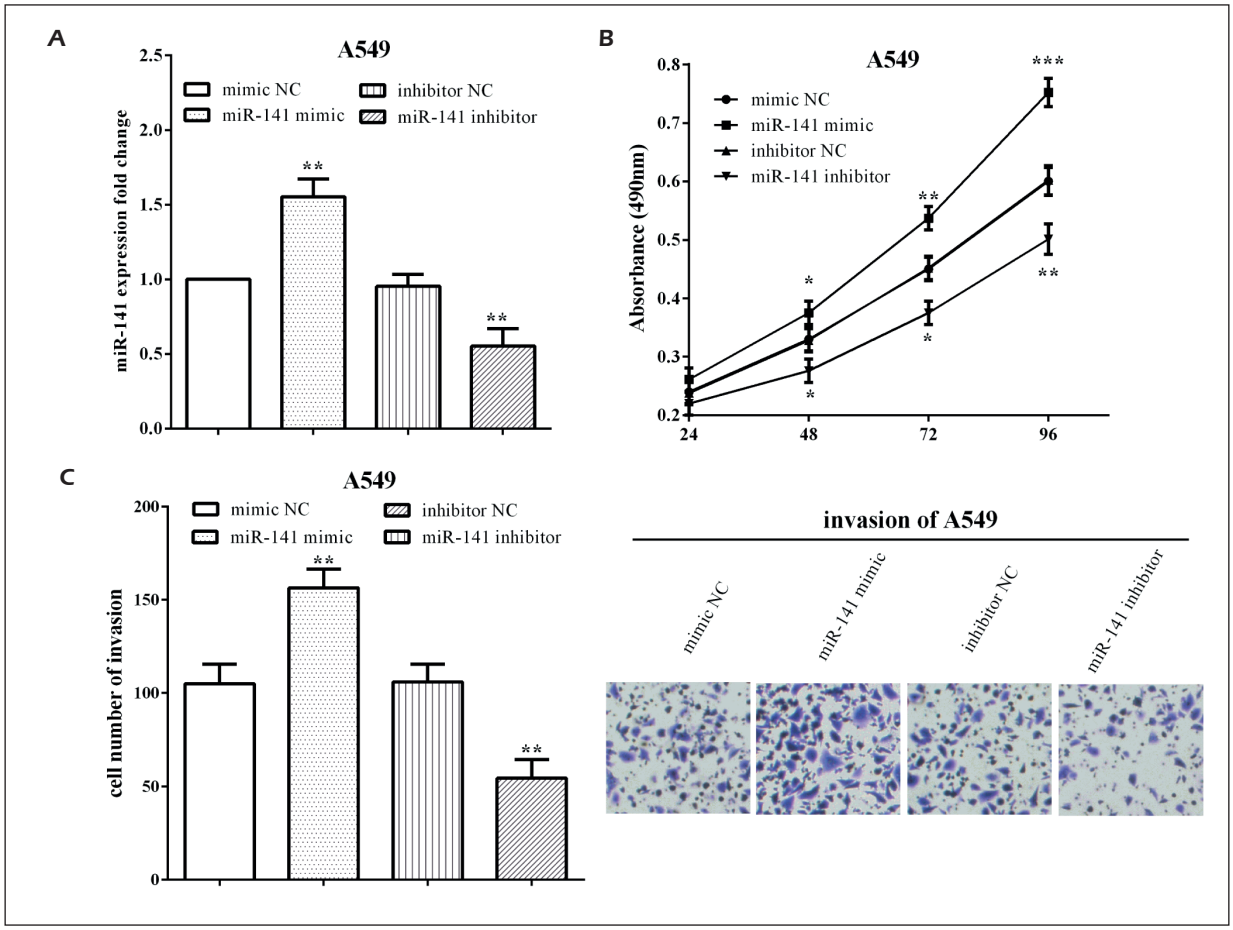

Figure 2. MiR-141 promoted the proliferation and invasion of NSCLC cells. **A**, RT-qPCR revealed the level of miR-141 was upregulated when transfected with miR-141 mimic, while downregulated when transfected with miR-141 inhibitor. **B**, MTT assays revealed that the overexpression of miR-141 promoted the proliferation of A549 cells, whereas it had the opposite effect when knockdown of miR-141. **C**, Transwell assay showed that upregulation of miR-141 improved the invasive ability of A549 cells, whereas knockdown of miR-141 inhibited the invasion (Magnification × 40).

KLF9-MUT, respectively (Figure 3A). As expected, miR-141 suppressed (p=0.0013) KLF9 Luciferase activity, which could be abrogated (p=0.7545) by mutations in A549 cells (Figure 3B). Moreover, the mRNA level of KLF9 was decreased (p=0.0012) in miR-141-overexpressed A549 cells; however, it was increased (p=0.0042) after transfected miR-141 inhibitor (Figure 3C). All the findings indicated that KLF9 was a direct target of miR-141.

KLF9 Could Partially Reverse the Function of MiR-141 on the Proliferation and Invasion

To confirm that the promoting function of miR-141 on the proliferation and invasion attributable to the expression of KLF9 decreased (p=0.0003), we restored (p=0.0089) the KLF9 expression in miR-141-overexpressing A549 cells evaluated by RT-qPCR and Western blot (Figure 4A). MTT assay indicated that KLF9 re-introduction increased the proliferation of miR-141-overexpressed A549 cells (p=0.0225 and 0.0062 of 72 h and 96 h; Figure 4B). Moreover, transwell assay revealed that KLF9 re-transfection increased (p=0.0282) the invasive ability, and that was reduced (p=0.0023) by the miR-141 mimic in A549 cells (Figure 4C), which suggests that KLF9 reversed partial function of miR-141. All the results demonstrate that miR-141 inhibited the proliferation and invasion by binding to KLF9.

Discussion

Non-small-cell lung cancer, accounting for 85% of all the lung cancer, has the highest mortality rate in the whole world[1,2]. Thus, it is urgent to explore the biomarkers for the diagnosis and treatment of NSCLC.

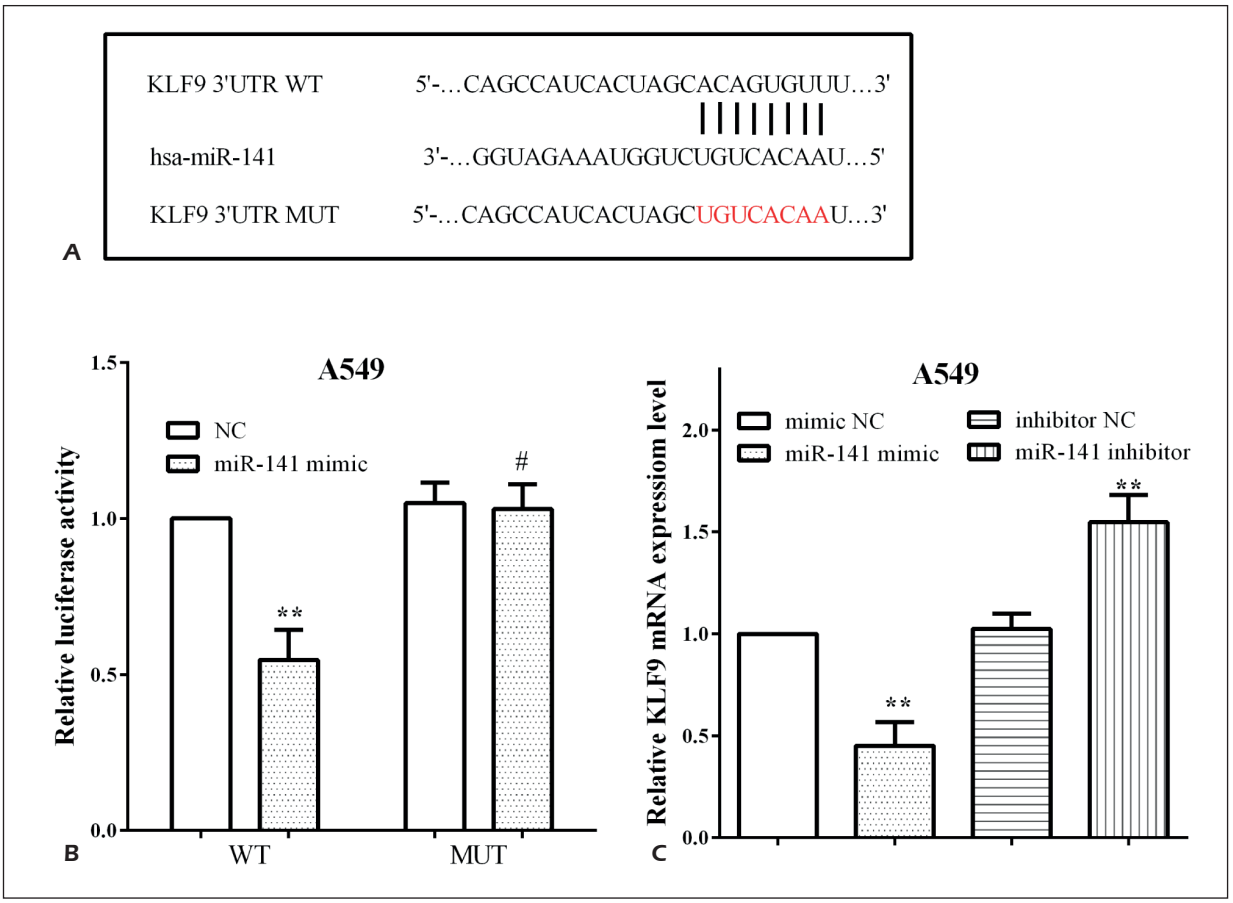

Figure 3. MiR-141 targeting to KLF9 and inhibits its expression. **A**, TargetScan was used to identify the target genes of miR-141, and we discovered KLF9 maybe one. **B**, MiR-141 suppressed the KLF9 Luciferase activity, which could be abrogated by mutations in A549 cells. **C**, The mRNA level of KLF9 was decreased in miR-141-overexpressed A549 cells, but was increased after transfected miR-141 inhibitor.

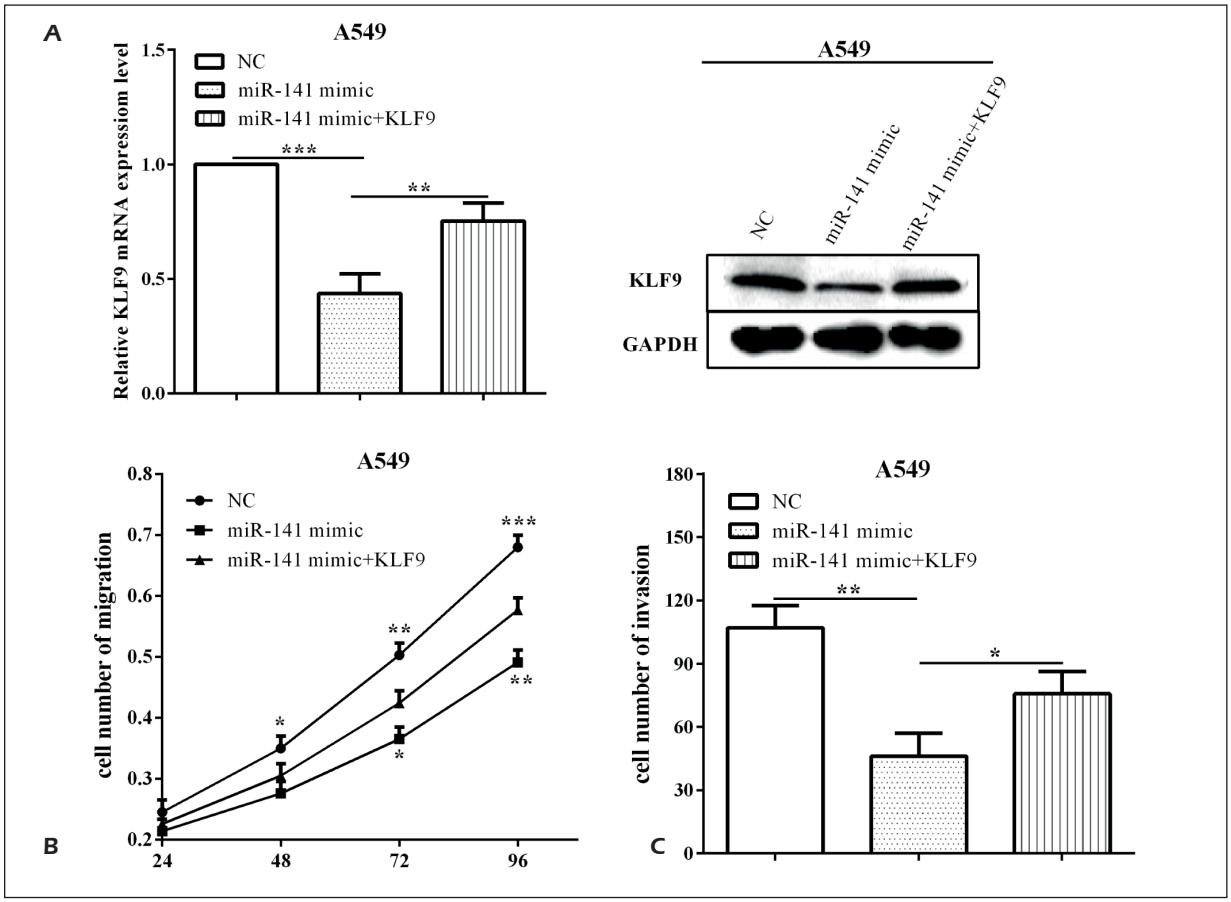

Figure 4. KLF9 could reverse partial function of miR-141 on the proliferation and invasion. **A**, RT-qPCR and Western blot were performed to evaluate the effect of re-transfection of KLF9 expression in miR-141-overexpressing A459 cells. **B**, MTT assay indicated that re-introduction of KLF9 increased the proliferation of miR-141-overexpressed A459 cells. **C**, KLF9 reversed partial function of miR-141 on the invasion.

MiRNAs possess diverse biological functions and pathological implications by binding to the 3'-UTR of targeted mRNAs at posttranscriptional level[4,5]. Liu et al[22] has been elucidated that miR-141 promoted the proliferation, colony formation and inhibited the apoptosis of nasopharyngeal carcinoma. However, Xiong et al[23] have indicated that miR-141 inhibited the proliferation and that predicted poor prognosis in human glioblastoma. Our results were consistent with Liu et al[22]; we found that miR-141 was overexpressed in NSCLC tissues and cells. MTT assay and transwell assay revealed that miR-141 upregulation promoted the proliferation and invasion, while knockdown of miR-141 caused the opposite results.

KLF9, regulating gene expression by binding to GC box elements, was a haploin sufficient suppressor of colon tumorigenesis[20]. In breast cancer, KLF9 has been reported to be low expressed and suppressed the invasion[24]. KLF9 was a direct target gene of multiple miRNAs that includes miR-378, miR-21, miR-570 and miR-141[25-28]. Li et al[28] discovered that miR-141 promoted the proliferation through KLF9 in prostate cancer. Consistent with Li et al[28], we validated that KLF9 was a direct target gene of miR-141 in NSCLC. KLF9 was downregulated in NSCLC tissues and lung cancer cells; also, the expression of KLF9 and miR-141 has an inverse connection in NSCLC tissues. We first proposed that miR-141 mediated the expression of KLF9 through direct complementary pairing in NSCLC. Moreover, KLF9 reversed partial functions of miR-141 on the proliferation and invasion in A549 cells.

Conclusions

The above data showed that miR-141 was low expressed in NSCLC tissues and cells compared

to the negative control, while the KLF9 appeared the opposite results. MiR-141 had a negative connection with the mRNA level of KLF9 in NSCLC tissues. MiR-141 overexpression promoted the proliferation and invasion of A549 cells, while caused contrast results when knockdown miR-141. In addition, KLF9 was a direct target gene of miR-141 and KLF9 reversed partial roles of miR-141 in A549 cells. MiR-141 promoted the proliferation and invasion by binding to KLF9 in NSCLC.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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