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MiR-572 promotes the development of non-small cell lung cancer by targeting KLF2

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Abstract. – OBJECTIVE: The aim of this study was to uncover the role of miR-572 in regulating proliferative and migratory abilities in non-small cell lung cancer (NSCLC) and the possible mechanism.

PATIENTS AND METHODS: Expression levels of miR-572 in 46 matched NSCLC and paracancerous samples were detected. The relationship between miR-572 level and clinical features of NSCLC was analyzed. Subsequently, the regulatory effects of miR-572 on proliferative and migratory abilities in lung cancer cells were assessed by functional experiments. Finally, the downstream genes of miR-572 were tested by luciferase assay, and their functions in the development of NSCLC were finally explored by rescue experiments.

RESULTS: It was found that miR-572 was upregulated in NSCLC samples. High level of miR-572 predicted high rates of lymphatic and distant metastases, as well as poor prognosis in NSCLC. Besides, the knockdown of miR-572 suppressed proliferative and migratory abilities in A549 and SPC-A1 cells. KLF2 was identified to be the downstream gene of miR-572, which was involved in the regulation of NSCLC phenotypes influenced by miR-572.

CONCLUSIONS: MiR-572 is closely linked to metastasis and prognosis in NSCLC patients, and it promotes the malignant development of NSCLC via targeting KLF2.

Key Words:
MiR-572, KLF2, NSCLC, Malignant development.

Introduction

Lung cancer is the worldwide popular cancer that displays a relatively high incidence and mortality[1,2]. In China, the prevalence of lung cancer has been largely enhanced owing to the rapid development of industry, aging and severe environmental pollution[3,4]. Pathologically, lung cancer is classified into small cell lung cancer and non-small cell lung cancer (NSCLC), with the latter accounting for 80% of lung cancer cases[4-6]. Surgical resection is the most effective strategy for the treatment of NSCLC in the early stage, alongside postoperative chemotherapy, radiotherapy or target therapy[7,8]. Nevertheless, surgery is not available for NSCLC patients in advanced stage or those with distant metastases[9,10]. Molecular target therapy is a hot topic in the research of NSCLC. Cancer target drugs, such as erlotinib or gefitinib, have been applied in clinical treatment for a long period. Their adverse events, however, are unavoidable[10,11]. Meanwhile, drug resistance is also an obstacle in cancer treatment[11]. Therefore, it is urgent to develop novel therapeutic targets and biological hallmarks that contribute to improving the clinical outcomes of NSCLC[12,13].

MiRNAs are non-coding RNAs with 19-25 nucleotides long. They are widely expressed in eukaryotes[14,15]. Through recognizing and binding to the 3'UTR of target mRNAs, miRNAs post-transcriptionally regulate gene expressions and functions[16,17]. Over 33% proteins are regulated by miRNAs. Notably, abnormally expressed miRNAs are involved in tumor development, drug resistance and tumor recurrence[18]. Previous studies[19,20] have shown that miR-572 participates in the development of malignant tumors.

KLF2 is an essential gene involved in lung development in the late stage, suggesting that KLF2 deficiency or loss-of-function is closely related to pulmonary diseases[21,22]. Basically, KLF2 is lowly expressed in cancer samples, serving as a potential tumor suppressor[23,24]. In this paper, the role of miR-572 in the progression of NSCLC and its possible mechanism were explored.

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Patients and Methods

NSCLC Patients and Samples

NSCLC and paracancerous samples were collected from 46 NSCLC patients undergoing radical resection. None of them received pre-operative chemotherapy or radiotherapy. This investigation was approved by the Ethics Committee of Jining No. 1 People's Hospital and conducted after informed consent was obtained from all participants in accordance with the Declaration of Helsinki. In addition, tumor staging was assessed based on the guideline proposed by the Union for International Cancer Control (UICC). Tumor tissues diagnosed with NSCLC were confirmed by the pathologist in our hospital. Other kinds of tumors that migrated to the lung were excluded.

Cell Culture

Human lung cancer cell lines (A549, H1299, PC-9, H358, SPC-A1) and one bronchial epithelial cell line (BEAS-2B) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Cell passage was conducted until cells were grown to 80-90% confluence.

Transfection

Transfection plasmids were purchased from GenePharma, Shanghai, China. Cells were cultured to 70% confluence and transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) for 48 h for *in vitro* experiments.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, the absorbance value at 490 nm of each sample was recorded using the Cell Counting Kit (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell Assay

A total of 200 μL of suspension (2×10^5 cells/ml) was applied in the upper layer of a transwell chamber (Millipore, Billerica, MA, USA) that was inserted in a 24-well plate with 460 μL of medium containing 10% FBS in the bottom. After 48-h incubation, bottom cells were reacted with 15-min methanol, 20-min crystal violet and captured using a microscope. At last, migratory cells were counted in 5 random fields per sample.

Wound Healing Assay

Cells were inoculated in 6-well plates and grown to 90% confluence. After an artificial wound was made in cell monolayer, the original medium was replaced with a medium containing 1% FBS. 24 hours later, wound closure was captured.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

RNAs extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were reversely transcribed into cDNAs using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. Primers used in this study were listed below: MiR-572: forward: 5'-ACCCGGUGGCGGCUCGCUG-3' and reverse: 5'-CUCGGCACAAAUCUUCAGAGC-3', U6: forward: 5'-CTCGCTTCGGCAGCACA-3' and reverse: 5'-AACGCTTCACGAATTTGCGT-3', KLF2: forward: 5'-ACTCACACCTGCAGCTACGC-3' and reverse: 5'-AGTGGTAGGGCTTCTCACCTGT-3', and GAPDH: forward: 5'-CAAGGTCATCCATGACAACTTTG-3' and reverse: 5'-GTCCACCACCCTGTTGCTGTAG-3'.

Western Blot

Cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. Membranes were reacted with primary and secondary antibodies for indicated time. Ultimately, band exposure and analyses were finally conducted.

Luciferase Assay

Cells inoculated in 24-well plates were co-transfected with NC mimic/miR-572 mimic and KLF2-WT/KLF2-MUT, respectively. 48 hours later, cells were lysed for measuring luciferase activity.

Statistical Analysis

SPSS 22.0 (SPSS IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as

mean ± standard deviation. Differences between groups were analyzed by the *t*-test. Chi-square test was used for analyzing the relationship between miR-572 level and clinical characteristics of NSCLC patients. Pearson correlation test was applied for assessing the correlation between expression levels of miR-572 and KLF2 in NSCLC samples. Survival analysis was conducted by Kaplan-Meier methods. $p<0.05$ represented that the difference was statistically significant.

Results

MiR-572 Was Upregulated In NSCLC Samples

Compared with that in bronchial epithelial cells, miR-572 was upregulated in lung cancer cells (Figure 1A). Similarly, NSCLC samples expressed higher abundance of miR-572 than controls (Figure 1B). Included 46 NSCLC patients were classified into two groups based on the median level of miR-572. Then, the relationship between miR-572 level and clinical features of NSCLC was analyzed. As shown in data, miR-572 level was closely linked to rates of lymphatic metastasis and distant metastasis in NSCLC but unrelated to age, sex and pathological staging in NSCLC patients (Table I). Kaplan-Meier curves revealed a poor prognosis in NSCLC patients expressing high level of miR-572 (Figure 1C).

Knockdown of MiR-572 Suppressed Proliferative and Migratory Abilities In NSCLC

MiR-572 inhibitor was constructed, and its transfection efficacy in A549 and SPC-A1 cells was tested (Figure 1D). After knockdown of miR-572, viability in A549 and SPC-A1 cells was markedly decreased (Figure 2A). Transwell assay results showed a lower migratory rate in A549 and SPC-A1 cells with miR-572 knockdown (Fig-

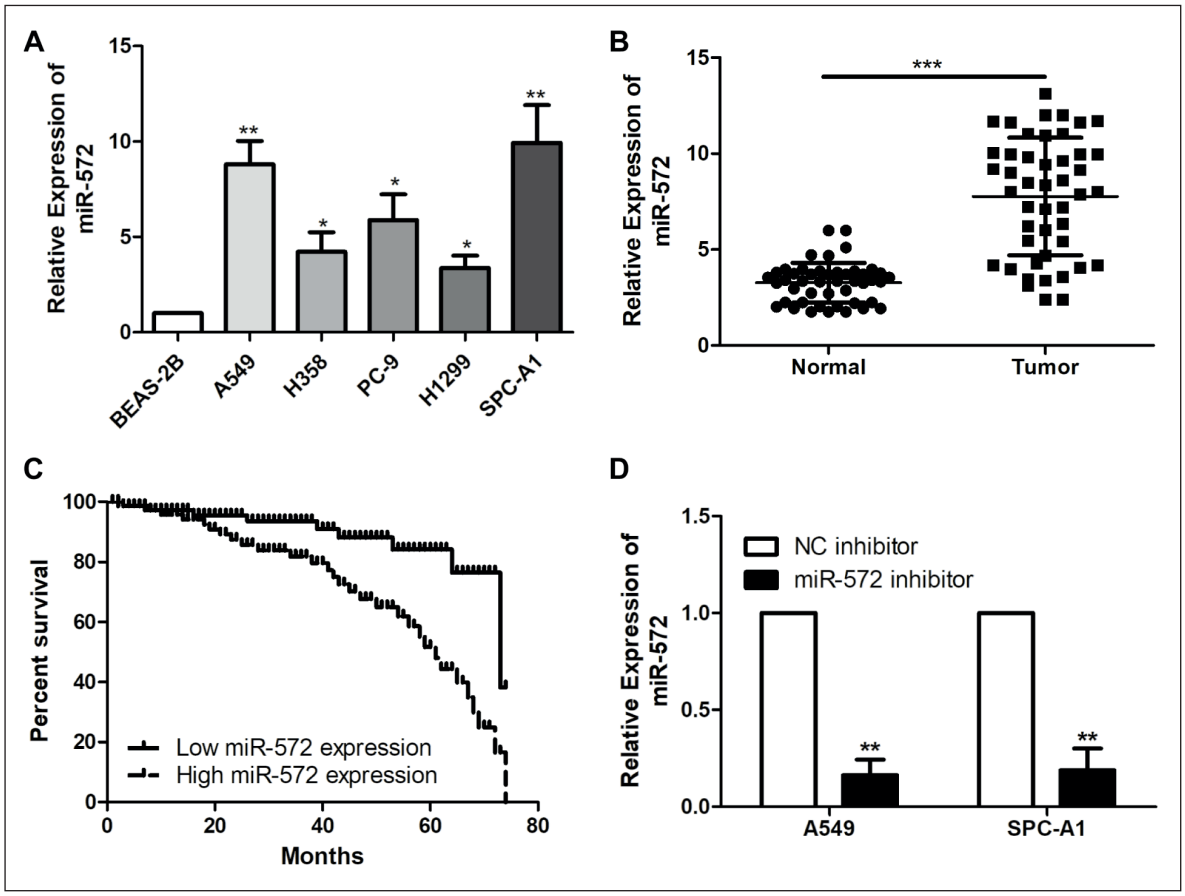

Figure 1. MiR-572 was upregulated in NSCLC samples. **A,** MiR-572 level in bronchial epithelial cells and lung cancer cells. **B,** MiR-572 level in NSCLC samples and paracancerous samples. **C,** Survival in NSCLC patients expressing a high or low level of miR-572. **D,** Transfection efficacy of miR-572 inhibitor. Data were expressed as mean±SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table I. Association of miR-572 expression with clinicopathologic characteristics of non-small cell lung cancer.

Parameters	No. of cases	miR-572 expression		*p-value*
		Low (%)	High (%)	
Age (years)				0.979
<60	18	11	7	
≥60	28	17	11	
Gender				0.331
Male	22	15	7	
Female	24	13	11	
T stage				0.067
T1-T2	28	20	8	
T3-T4	18	8	10	
Lymph node metastasis				**0.001**
No	30	24	6	
Yes	16	4	12	
Distance metastasis				**0.009**
No	33	24	9	
Yes	13	4	9	

ure 2B). Besides, wound healing percentage was decreased after knockdown of miR-572, indicating the suppressed migratory ability in NSCLC (Figure 2C).

Interaction Between MiR-572 and KLF2 In NSCLC

Protein level of KLF2 was found to be upregulated by knockdown of miR-572 in A549 and SPC-A1 cells (Figure 3A). Subsequently, si-KLF2 was constructed and transfection with it effectively silenced KLF2 level (Figure 3B). As expected, miR-572 was upregulated after transfection with si-KLF2 (Figure 3C). Bioinformatics prediction revealed binding sequences in the 3'UTR of miR-572 and KLF2 (Figure 3D). Luciferase assay showed that overexpression of miR-572 markedly decreased luciferase activity in wild-type KLF2 vector. However, luciferase activity in mutant-type KLF2 was not influenced by miR-572, demonstrating the binding between miR-572 and KLF2 (Figure 3E). Pearson correlation test obtained a negative correlation between expression levels of miR-572 and KLF2 in NSCLC samples (Figure 3F).

KLF2 Was Responsible for NSCLC Development Regulated By MiR-572

The involvement of KLF2 in the malignant development of NSCLC was explored. Of note, the downregulated miR-572 in A549 and SPC-A1 cells transfected with miR-572 inhibitor was partially elevated by co-silence of KLF2 (Figure 4A). Besides, the decreased migratory cell number in lung cancer cells with miR-572 knockdown was partially reversed by silenced KLF2 (Figure 4B).

Discussion

NSCLC has been a severe killer in the world[1-3]. NSCLC patients tend to be younger because of pollution and smoking[4,5]. Specific screening and diagnostic approaches of NSCLC in the early stage are still limited. A great number of NSCLC patients are diagnosed in the middle or advanced stage and thus lose the surgical opportunity. The 5-year survival of NSCLC is only 15%[6-8]. Great efforts have been made in improving imaging examinations and cancer screening[9,10]. It is necessary to further analyze molecular mechanisms underlying the occurrence, development and recurrence of NSCLC[11-13].

MiRNAs have been identified to be involved in the pathological development of NSCLC[14,15]. Differentially expressed miRNAs between cancer cells and normal cells may be attributed to the cancer-associated locus and genetic polymorphisms[14,15]. Thousands of miRNAs have been predicted in human genome, accounting for 3% in total genes[16]. They are vital regulators in cell phenotypes and disease development. Determination of miRNA expression pattern is beneficial to cancer screening, diagnosis and treatment[17,18]. It is reported that miR-572 influences the development of colorectal cancer and nasopharyngeal cancer[19,20]. Therefore, the objective of this study was firstly to elucidate the oncogenic role of miR-

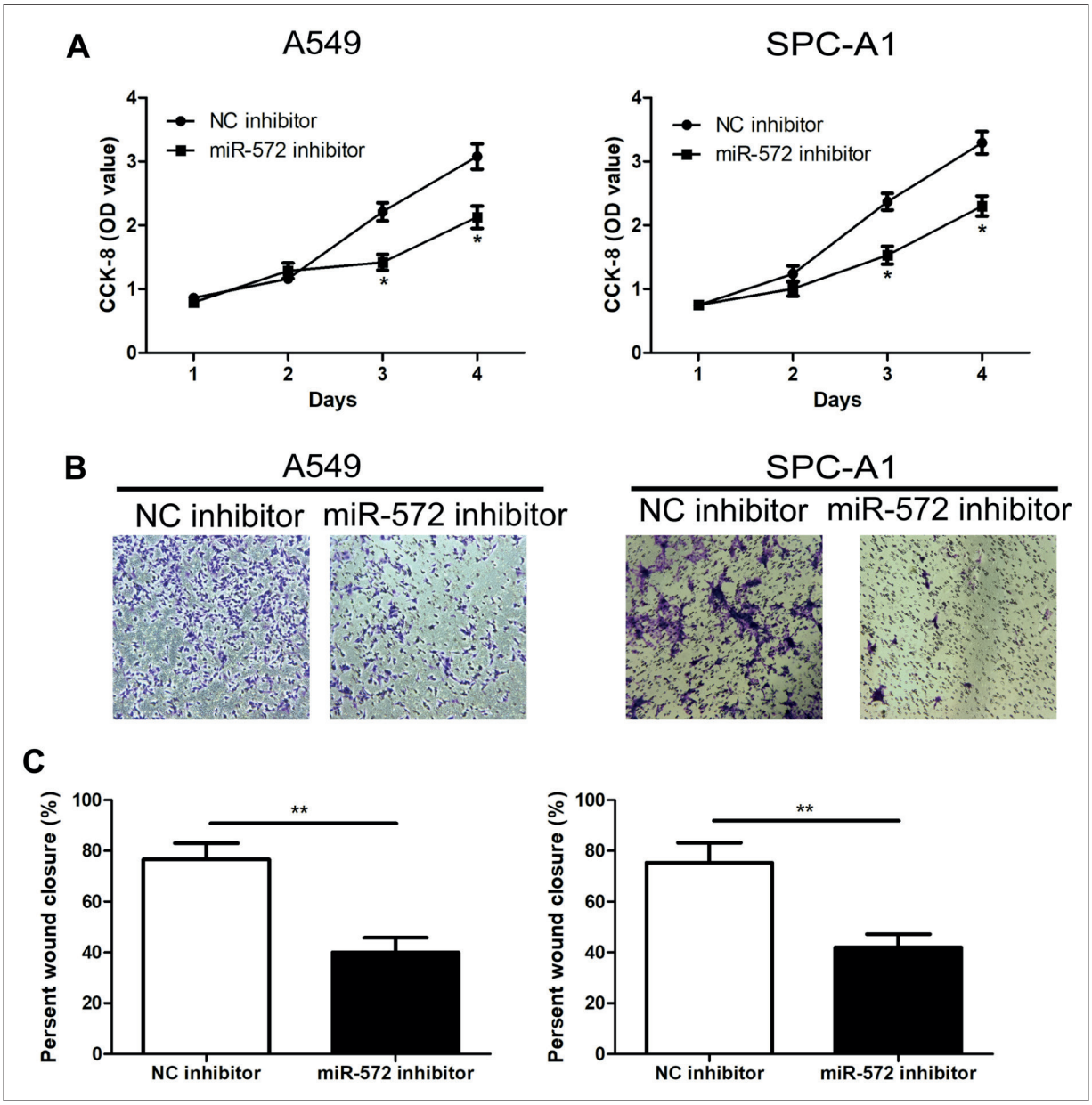

Figure 2. Knockdown of miR-572 suppressed proliferative and migratory abilities in NSCLC. **A,** Viability in A549 and SPC-A1 cells transfected with NC inhibitor or miR-572 inhibitor. **B,** Migration in A549 and SPC-A1 cells transfected with NC inhibitor or miR-572 inhibitor (magnification: 40×). **C,** Wound healing percentage in A549 and SPC-A1 cells transfected with NC inhibitor or miR-572 inhibitor (magnification: 40×). Data were expressed as mean±SD. *p<0.05.

572 in the progression of NSCLC, as well as the specific mechanism of miR-572 regulating KLF2. In this paper, it was found that miR-572 was up-regulated in NSCLC samples, and its level was remarkably linked to metastasis and prognosis in NSCLC patients. It was believed that miR-572 was a promising hallmark in predicting the clinical outcomes of NSCLC. A549 and SPC-A1 cells expressed the highest abundance of miR-572 in the five tested lung cancer cell lines, and they were utilized for the following experiments. *In vitro* experiments demonstrated that miR-572 promoted proliferative and migratory abilities in NSCLC.

Differentially expressed miRNAs have been detected in cancers[14,15]. Every single miRNA

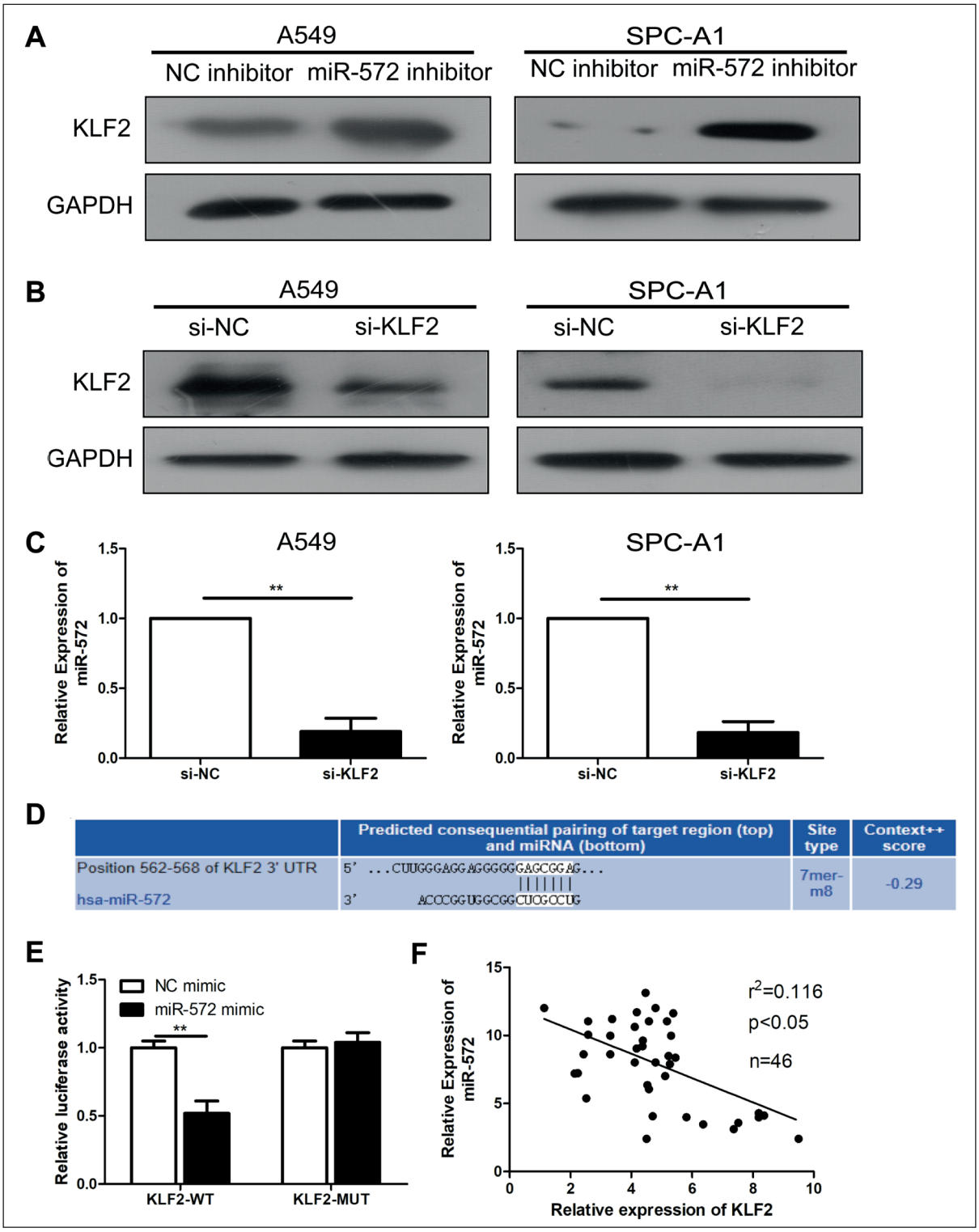

Figure 3. Interaction between miR-572 and KLF2 in NSCLC. **A,** Protein level of KLF2 in A549 and SPC-A1 cells transfected with NC inhibitor or miR-572 inhibitor. **B,** Protein level of KLF2 in A549 and SPC-A1 cells transfected with si-NC or si-KLF2. **C,** MiR-572 level in A549 and SPC-A1 cells transfected with si-NC or si-KLF2. **D,** Binding sequences in the 3'UTR of miR-572 and KLF2. **E,** Luciferase activity in cells co-transfected with NC mimic/miR-572 mimic and KLF2-WT/KLF2-MUT. **F,** A negative correlation between expression levels of miR-572 and KLF2 in NSCLC samples. Data were expressed as mean±SD. ***p*<0.01.

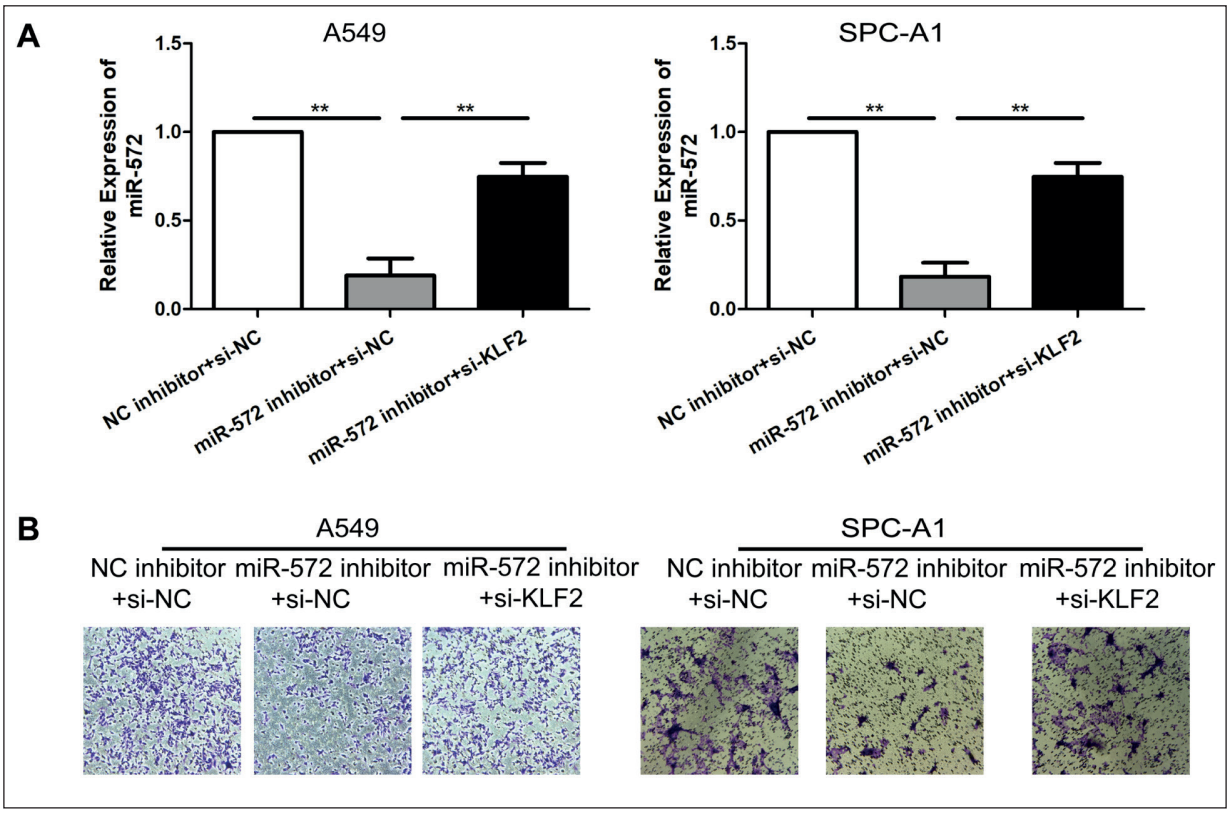

Figure 4. KLF2 was responsible for NSCLC development regulated by miR-572. **A,** MiR-572 level in A549 and SPC-A1 cells transfected with NC inhibitor+si-NC, miR-572 inhibitor+si-NC or miR-572 inhibitor+si-KLF2. **B,** Migration in A549 and SPC-A1 cells transfected with NC inhibitor+si-NC, miR-572 inhibitor+si-NC or miR-572 inhibitor+si-KLF2 (magnification: 40×). Data were expressed as mean±SD. $^{**}p<0.01$.

controls hundreds of targets and multiple genetic pathways[25,26]. It was discovered in this study that KLF2 was the target gene of miR-572, and KLF2 level was negatively correlated with miR-572 level in NSCLC samples. The findings of this study revealed that miR-572 was up-regulated in NSCLC tissues and cell lines. Besides, the higher level of miR-572 represented more lymph node metastasis and distance metastasis of NSCLC patients. *In vitro* experiments verified that knockdown of miR-572 inhibited the malignant development of NSCLC cells. Notably, miR-572 was capable of reversing regulatory effects of miR-572 on proliferative and migratory abilities in NSCLC, so it was proposed that miR-572 stimulated the malignant development of NSCLC through negatively regulating KLF2. However, there are still several limitations in this current study. Limited sample size and lack of in vivo animal experiments weakened the evidence level of this study. Also, the potential signaling pathway involved in the regulation of miR-572/KLF2 in NSCLC still remains to be explored. In our future research, we plan to conduct the tumor formation assay in nude mice to investigate the effects of miR-572/KLF2 in tumorgenesis in vivo. We will also perform the bioinformatics analyses together with the molecular biological experiments to further study how miR-572/KLF2 works in the regulation of NSCLC development.

Conclusions

MiR-572 is closely linked to metastasis and prognosis in NSCLC patients, and it promotes the malignant development of NSCLC *via* targeting KLF2.

Conflicts of Interest

The authors declare no conflicts of interest.

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