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Original Article

MicroRNA-429 promotes the non-small cell lung cancer progression by targeting KLF4

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Abstract: MicroRNA (miR)-429 has been frequently reported to be involved in various tumors including lung cancer. However, the function role of miR-429 in non-small cell lung cancer (NSCLC) still remains elusive. In the study, we identified that miR-429 was up-regulated in the non-small cell lung cancer (NSCLC) patients comparing adjacent normal tissues and miR-429 expression levels were significantly associated with differentiation, lymphatic invasion, vascular infiltration and pathological stage in NSCLC patients. CCK-8 cell proliferation assays and western-blotting analysis results demonstrated that down-regulation of miR-429 inhibited the cell proliferation and cell cycle related protein expression in NSCLC cells. Furthermore, luciferase reporter assays confirmed that miR-429 directly binds to the 3'untranslated region of KLF4, and regulated the expression KLF4 at mRNA and protein levels. Thus, this study suggested that miR-429 promoted NSCLC cell proliferation by inhibiting KLF4 expression. The miR-429 may represent a potential therapeutic target for NSCLC treatment.

Keywords: Non-small cell lung cancer, miR-429, KLF4, cell proliferation

Introduction

Lung cancer is the leading cause of cancer related mortality in the world and non-small cell lung cancer (NSCLC) accounted for the most common type of lung cancer (~85%). Surgical resection remains the single most consistent and successful option for cure for patients diagnosed as having lung cancer and chemotherapy is beneficial for patients with locally advanced and metastatic disease [1, 2]. Although the advanced development of diagnosis and surgical treatment techniques, the 5-year survival rate for NSCLC patients remains a dismal rate of 15% [3]. Thus, it is important to investigate a novel targeted therapy for NSCLC patients.

MicroRNAs (miRNAs), a class of non-coding RNAs 18-25 nucleotides in length, function to lead to mRNA degradation or inhibit protein translation by directly binding to the 3'-untranslational region (UTR) of their target mRNAs [4, 5]. The down-regulation of miR-429 had been indicated to be involved in the development and progression in some tumors. Wu *et al* showed that miR-429 decreased the bladder cancer cell migratory and invasive abilities through reducing ZEB1 and β-catenin, restoring the E-cadherin expression and inactivation of MMP-2 [6]. Zhang *et al* found that miR-429 levels were negatively association with Bcl-2 in glioblastoma (GBM) and induced apoptosis of glioblastoma cell through regulating Bcl-2 expression [7]. Another reports showed that microRNA-429 suppressed cell proliferation, epithelial-mesenchymal transition, and metastasis by direct targeting of BMI1 and E2F3 in renal cell carcinoma [8] human. Tian *et al* found that MicroRNA-429 inhibited the migration and invasion of colon cancer cells by targeting PAK6/cofilin signaling [9]. In colorectal carcinoma, miR-429 was demonstrated to inhibit cells growth and invasion and regulated EMT-related marker genes by targeting Onecut2 [10].

In NSCLC, Xiao *et al* found that MiR-429 promoted the proliferation of non-small cell lung cancer cells via targeting DLC-1 [11]. Lang *et al* reported that microRNA-429 induced tumorigenesis of human non-small cell lung cancer cells and targeted multiple tumor suppressor genes including PTEN, RASSF8 and TIMP2 [12].

In present study, we identified that miR-429 expression was up-regulated in the non-small cell lung cancer (NSCLC) patients and miR-429 expression levels were significantly associated with differentiation, lymphatic invasion, vascular infiltration and pathological stage in NSCLC patients. Function assays demonstrated miR-429 promoted the cell proliferation. In addition, we found miR-429 directly binds to the 3'untranslated region of KLF4 and regulated the expression of KLF4. Thus, miR-429 may represent a potential therapeutic target for NSCLC treatment.

Materials and methods

Clinical patient samples

The 104 cases of NSCLC patient specimens were obtained from Department of Thoracic Surgery, Fujian Provincial Hospital from March 2011 to January 2015. All of the patients had no received radiation therapy or chemotherapy before the surgery. The NSCLC tissue samples were stored in liquid nitrogen after surgery at -80°C until RNA extraction. The tumors were classified according to World Health Organization classification. All patients signed informed consent for the collection and use of their tissues for this study. The methods were carried out in accordance with the approved guidelines by Fujian Provincial Hospital.

Cell culture and transfection

Five human NSCLC cell lines (SK-MES-1, H1299, H460, SPC-A1 and A549) and a normal lung epithelial cell line BEAS-2B were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China) and were used in this study. The cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere at 37°C with 5% CO_2.

Cell proliferation assay

The cells were transfected with miR-429 mimic or miR-NC and miR inhibitor or miR-NC were seeded into 96-well plates (5.0×10^3 cells per well). Cell proliferation was detected by Cell Counting Kit-8 assay (Dojindo, Japan). Cells were added with 10 μl CCK-8 and incubated for 2 h at 37°C. The absorbance of each well was read on a spectrophotometer at 450 nm. Three independent experiments were performed in quintuplicate.

Real-time quantitative polymerase chain reaction (RT-PCR) assays

Total RNA was isolated from the tissues and cells using TRIzol reagent (Invitrogen). TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.) was used to reverse RNA into DNA following the manufacturer's protocols. Real-time quantitative PCR was conducted using SYBR Green PCR kit (Takara, Dalian, China) on CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). GAPDH or U6 small nuclear RNA (snRNA) were used as an endogenous control for detecting miR-429 or KLF4 expression. The primer sequences were as follows: β-actin forward: 5'-CCT AGA AGC ATT TGC GGT GG-3' and reverse: 5'-GAG CTA CGA GCT GCC TGA CG-3'. MiR-429 forward: 5'-AAUACUGUCUGGUAAAACCGU-3', miR-429 reverse: 5'-CAAGAUCGGAUCUACGGGUUUU-3'. The threshold cycle (Ct) value was recorded. Each sample was measured in triplicate, and the relative expression of miR-377 to U6 and AEG-1 to β-actin was calculated using the $2^{-\Delta\Delta Ct}$ method.

Luciferase activity assays

A549 cells were cultured in 96-well plates at a concentration of 1×10^4 cells per well. Cell were transfected for 48 h, each well was added with 100 ng pMIR-REPORT plasmid containing 10 ng internal control vector pRL-SV40 (Promega, Madison, USA), miR-429 mimic and pmir-KLF4-WT or miR-429 mimic and pmir-KLF4-MUT using Lipofectamine 2000 transfection reagent (Invitrogen). Firefly and renilla luciferase activities were measured in cell lysates by the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity normalized to that of renilla for each assay.

Western blotting analysis

Western blot analysis was performed as described previously [13]. Briefly, Total protein was extracted by lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% deoxycholic acid, 0.02% sodium azide, 1% NP-40, 2.0 μg/ml aprotinin, 1 mM phenyl methyl sulfonyl fluoride). The total extracts were separatedby 10% SDS-polyacrylamide gels and tr-

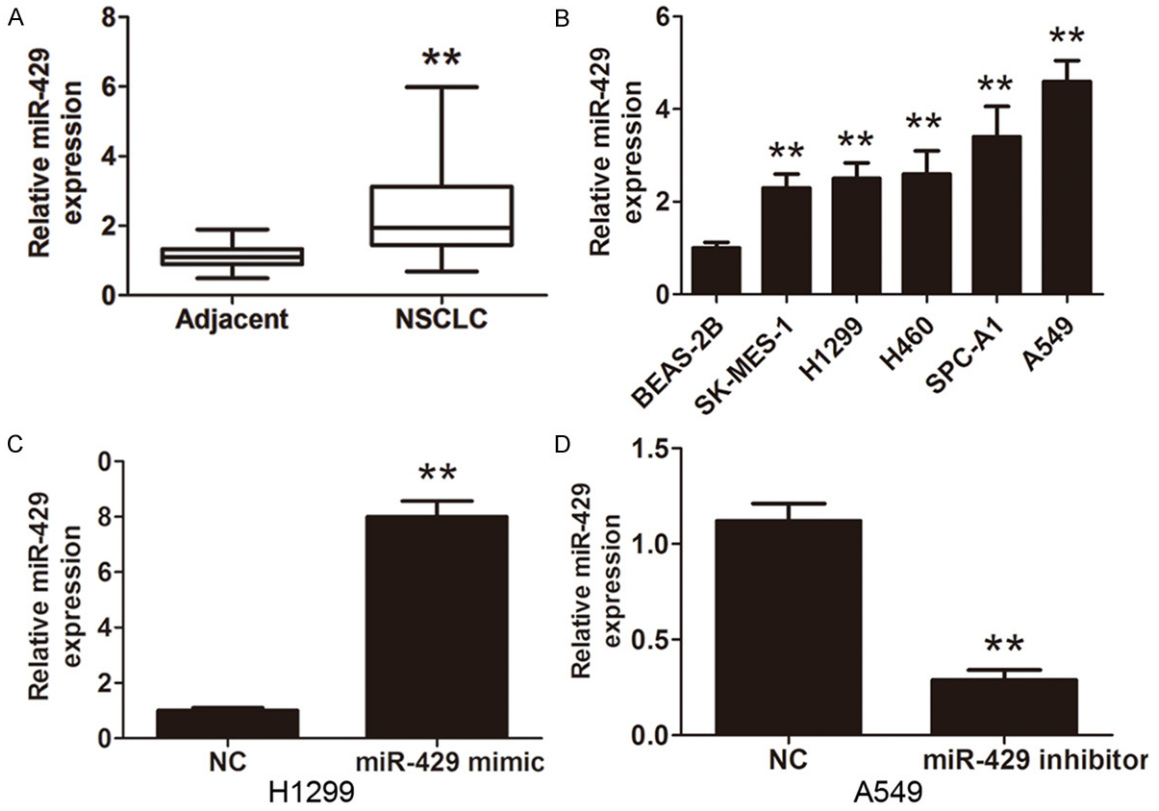

Figure 1. MiR-429 expression was upregulated in NSCLC tissues and cell lines. A. Relative expression of miR-429 in 104 NSCLC tissues compared with corresponding normal lung tissues. B. The miR-142-3p expression was significantly lower in NSCLC cell lines than that in normal lung epithelial cell line BEAS-2B. C. Relative expression of miR-429 in H1299 cell by transfecting miR-429 mimic. D. Relative expression of miR-429 in A549 cell by transfecting miR-429 inhibitor. Values are expressed as the mean ± standard deviation. **P<0.05.

ansferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were probed with a primary antibody against human GAPDH (dilution 1:1000, CST, USA), CyclinA1 (dilution 1:1000, Santa Cruz, USA), CDK1 (dilution 1:1000, Santa Cruz, USA), PCNA (dilution 1:1000, CST, USA), KLF4 (dilution 1:1000, Santa Cruz, USA), respectively. The membranes were incubated with (HRP)-conjugated secondary antibody (Santa Cruz). Bound antibody was detected using an enhanced chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.). The relative protein expression was analyzed by Image-Pro plus software 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Results

MiR-429 is up-regulated in NSCLC tissues

To explore the possible biological role of miR-429 in NSCLC development and progression, we examined the expression of miR-429 expression levels in 104 paired NSCLC tissues and corresponding adjacent non-cancerous tissues by qRT-PCR analysis. The results showed that miR-429 expression was significantly upregulated in NSCLC tissues compared with corresponding adjacent non-cancerous tissues (**Figure 1A**). To detect the correlation between the miR-429 expression levels and the clinicopathological characteristics, patients were divided into two groups: high miR-429 expression and low miR-429 expression. The results showed that miR-429 was significantly correlated with tumor differentiation level, lymphatic invasion, vascular infiltration and pathological stage in NSCLC patients (P<0.05, **Table 1**). To further evaluate the biological role of miR-429 in NSCLC, we examined miR-429 expression levels in several NSCLC cancer cell lines by qRT-PCR analysis. The results showed that miR-429 expression was significantly increased in five tumor cell lines (SK-MES-1, H1299,

Table 1. Relationship between clinicopathological features and miR-429 expression in lung cancer tissues

Variable	Number of Patients	MiR-429 expression		*P*-value
		Low (n=51)	High (n=53)	
Age (years)				0.079
≤60	56	23	33	
>60	48	28	20	
Gender				0.579
Male	70	33	37	
Female	34	18	16	
Smoking				0.277
Never	20	10	10	
Current	50	28	22	
Former	34	13	21	
Performance status				0.547
ECOG 0	42	21	21	
ECOG 1	37	20	17	
ECOG 2	25	10	15	
Differentiation				0.013**
Poor	56	20	36	
Moderate	30	19	11	
Well	18	12	6	
Lymphatic invasion				0.013**
Positive	30	9	21	
Negative	74	42	32	
Surgical margins				0.143
Free	86	45	41	
Not free	18	6	12	
Vascular infiltration				0.022**
Yes	29	9	20	
No	75	42	33	
Histology				0.086
SCC	52	20	32	
Adenocarcinoma	35	20	15	
Others	17	11	6	
Pathological stage				0.025**
I	64	38	26	
II	22	8	14	
IIIa	18	5	13	

**indicates that statistical significance of *P* value is less than 0.05.

H460, SPC-A1 and A549) compared with a normal lung epithelial cell line BEAS-2B (**Figure 1B**). Thus, our results demonstrated that miR-429 could play a key role in NSCLC development and progression.

MiR-429 promotes the NSCLC cells proliferation

We further investigated the effect of miR-429 on cell proliferation. We detected the expression of miR-429 by transfecting the miR-429 mimic or inhibitor into A549 cell. The results showed that miR-429 was significantly increased by transfecting with miR-429 mimic into 1299 cells and decreased by transfecting with miR-429 inhibitor compared with the miR-NC group into A549 cell (**Figure 1C** and **1D**). CCK8 cell proliferation showed that cell proliferation was inhibited by transfecting with miR-429 inhibitor to A549 cells, but was promoted by transfecting with miR-429 mimic into 1299 cells (**Figure 2A** and **2B**). Furthermore, we demonstrated that the cell proliferation related protein expression of Cyclin-A1, CDK2, PCNA was inhibited and p21 was increased by transfecting with miR-429 inhibitor into A549 cells. However, Cyclin-A1, CDK2, PCNA was increased and p21 was decreased by transfecting with miR-429 mimic into H1299 cells (**Figure 2C** and **2D**). Thus, these results showed that miR-429 promoted the NSCLC cells proliferation.

MiR-429 regulate the KLF4 expression in the NSCLC cells

We further investigated the biological mechanisms by which miR-429 promoted NSCLC cell tumorigensis. Online search for miR-429 targeting genes by miRanda (www.microrna.org) revealed that KLF4, a tumor suppressor could be a potential target of miR-429 (**Figure 3A**). Furthermore, we found that KLF4 mRNA and protein expression levels were increased by transfecting with miR-429 inhibitor into A549 cells, whereas, KLF4 mRNA and protein expression level was decreased by transfecting with miR-429 mimic into H1299 cells (**Figure 3B-E**). The luciferase activity in A549 cells was decreased in KLF4-WT group compared with the KLF4-MUT group by transfecting miR-429 mimic into the cells (**Figure 3F**). In addition, we also detected the expression level of KLF4 in NSCLC tissues. The results showed that KLF4 was down-regulated

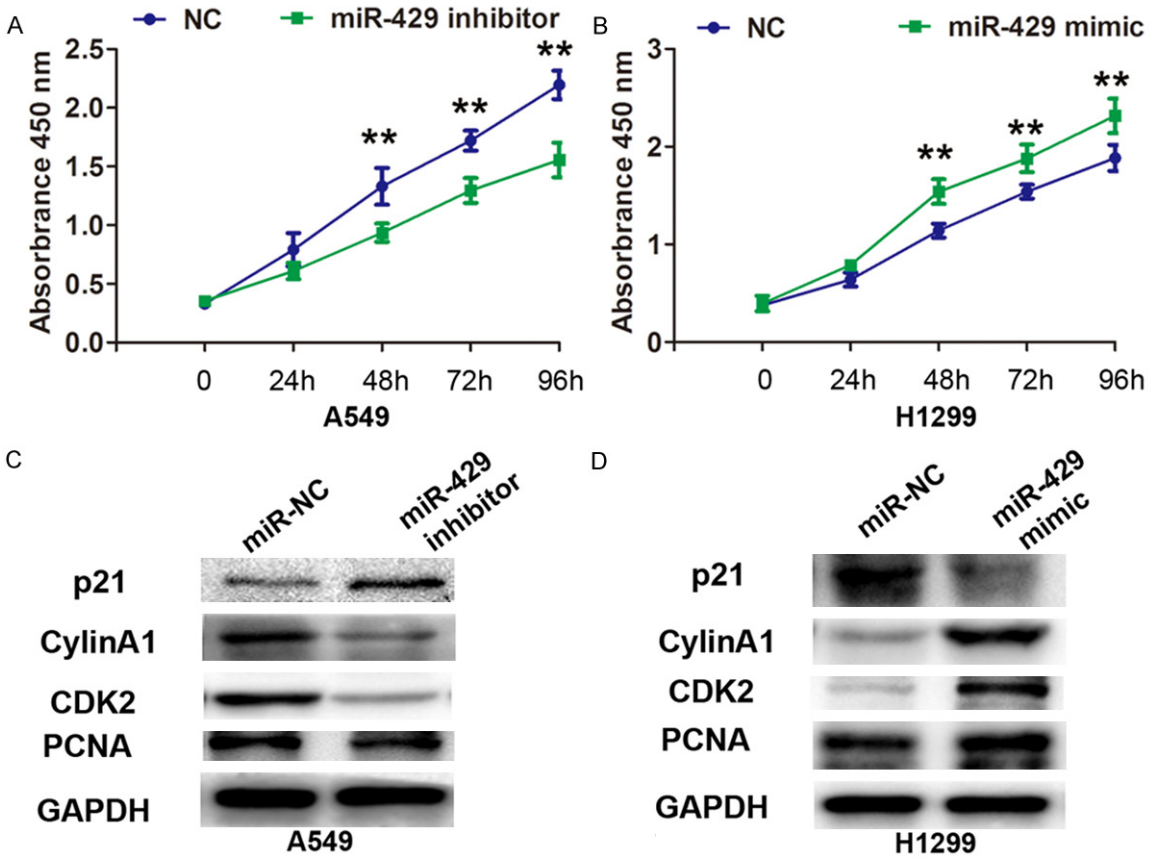

Figure 2. MiR-429 promoted the NSCLC cell proliferation. A. CCK8 cell proliferation ability was detected by transfecting miR-429 inhibitor into A549 cell. B. CCK8 cell proliferation ability was detected by transfecting miR-429 mimic into H1299 cell. C. Western-blotting analysis was performed the protein expression of P21, CyclinA1, CDK2, and PCNA by transfecting miR-429 inhibitor into A549 cell. D. Western-blotting analysis was performed the protein expression of P21, CyclinA1, CDK2, and PCNA by transfecting miR-429 mimic into H1299 cell. Values are expressed as the mean ± standard deviation. **P<0.05.

in NSCLC tissues than that in adjacent normal tissues (**Figure 3G**).

We next examined the cell proliferation by transfecting miR-429 mimic and si-KLF4 into A549 or H1299 cells. The results showed that cell proliferation was not significantly changed compared with miR-NC group, which demonstrated that si-KLF4 could reverse the miR-429 promoted cell proliferation ability in NCSCL (**Figure 4A** and **4B**). These results suggest that 3'UTR of KLF4 is a direct target of miR-429.

Discussion

During the past few decades, although diagnosis and treatment of NSCLC have developed rapidly, NSCLC remains the leading cause of cancer-related death worldwide. Accumulating evidence has indicated that endogenous miRNAs were involved in NSCLC development and progression [14, 15]. In the study, we identified that miR-429 was dramatically up-regulated in the non-small cell lung cancer (NSCLC) patients comparing adjacent normal tissues. Up-regulation of miR-429 expression promoted the cell proliferation in NSCLC cells. In previous study, MiR-429 was found to function as a tumor suppressor in some tumors, such as, MiR-429 was found to function as a tumor suppressor targeting FSCN1 in gastric cancer cells [16]. MiR-429 determined a poor outcome in pancreatic ductal adenocarcinoma patients and inhibited pancreatic ductal adenocarcinoma growth by targeting TBK1 [17]. MiR-429 inhibited oral squamous cell carcinoma growth by targeting ZEB1 [18]. Our results demonstrated that MiR-429 was found to function as a oncogene in NCSLC and the results was consistent with pre-

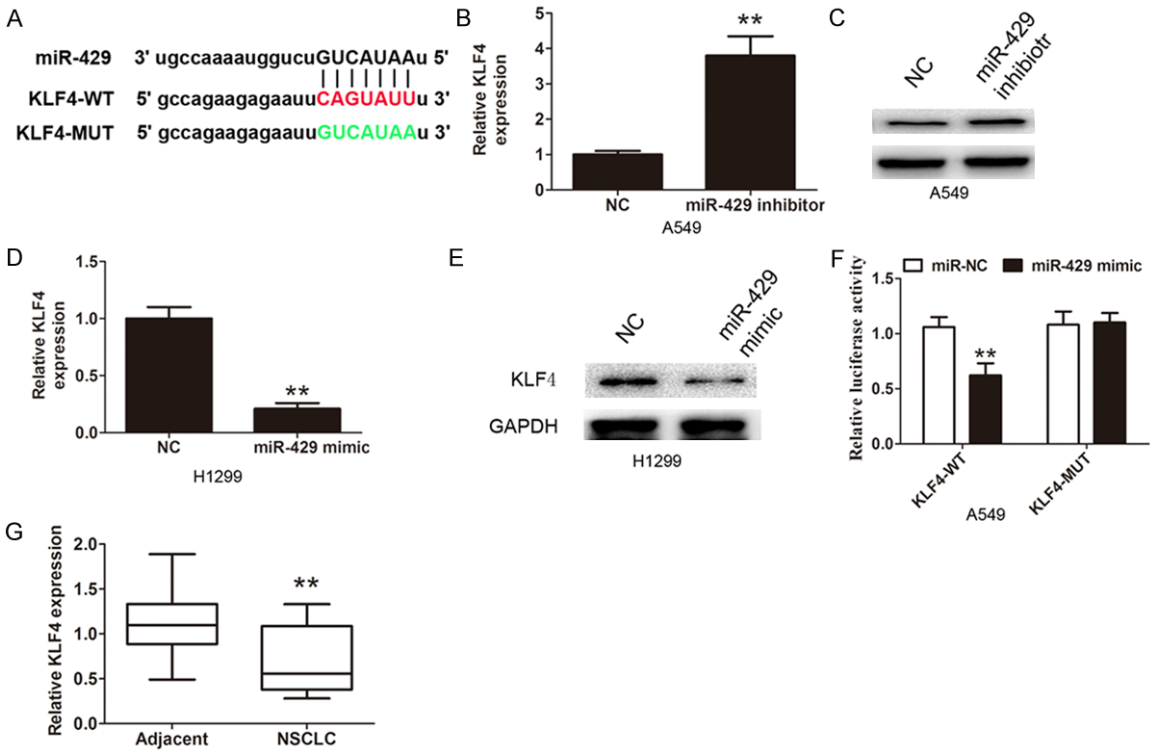

Figure 3. KLF4 is a direct target of miR-429. A. miR-429-binding sites in the KLF4 3'UTR region. KLF4-mut indicates the KLF4 3'UTR with a mutation in miR-429-binding sites. B and C. Relative mRNA and protein expression was detected by qRT-PCR and western-blotting assays after transfecting miR-429 inhibitor into A549 cell. D and E. Relative mRNA and protein expression was detected by qRT-PCR and western-blotting assays after transfecting miR-429 mimic into H1299 cell. F. Relative luciferase assay comparing the pmir-KLF4-Mut vectors in A549 cells. Firefly luciferase activity was normalized to Renilla luciferase activity. G. Relative expression of KLF4 in 104 NSCLC tissues compared with corresponding normal lung tissues. Values are expressed as the mean ± standard deviation. **P<0.05.

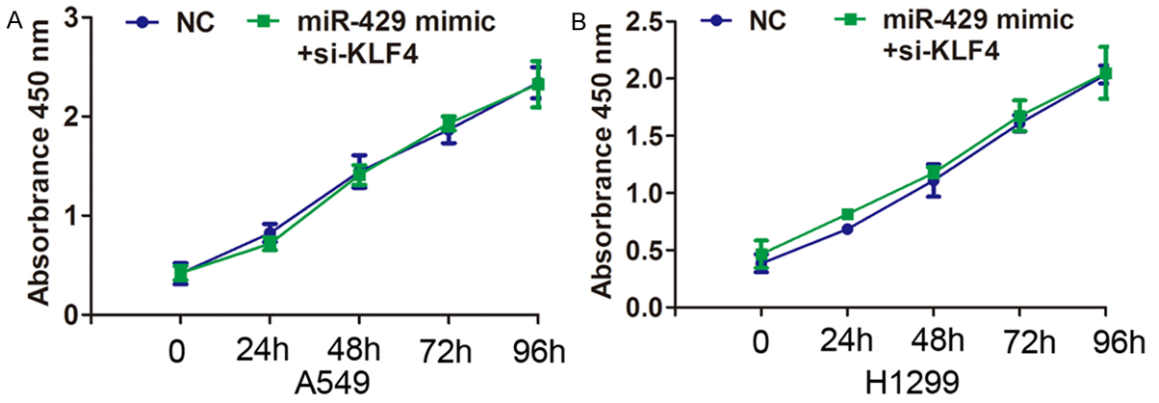

Figure 4. A. CCK8 cell proliferation ability was detected by transfecting miR-NC or miR-429 mimic+si-KLF4 into A549 cell. B. CCK8 cell proliferation ability was detected by transfecting miR-429 mimic+si-KLF4 into H1299 cell. Values are expressed as the mean ± standard deviation.

vious study in the NCSLC development and progression [11, 12].

Furthermore, luciferase reporter assays confirmed that miR-429 directly bound to the 3'untranslated region of KLF4, and qRT-PCR and western blotting analysis also showed that miR-429 suppressed the expression of KLF4 at the mRNA and protein level. KLF4 functions as a tumor suppressor in some tumors. Hu *et al*

reported that KLF4 downregulated hTERT expression and telomerase activity to inhibit lung carcinoma growth [19]. Zhou *et al* found that KLF4 inhibition of lung cancer cell invasion by suppression of SPARC expression [20]. Another study demonstrated that the enforced expression of KLF4 gene to lung cancer cells by ex vivo transfection or adenovector-mediated gene transfer suppressed tumor growth in vivo [21]. Our results found that co-transfection of miR-429 mimic and si-KLF4 reversed the effect of miR-429 promoted cell proliferation by CCK8 assays. Thus, these results indicated that miR-429 promoted cell proliferation of NSCLC cell lines by regulating KLF4.

In conclusion, our study demonstrated that miR-429 was up-regulated in NSCLC. Furthermore, we demonstrated that miR-429 promoted the proliferation ability of NSCLC cell lines and participated in the NSCLC process by targeting KLF4. Thus, miR-429 might be a new target for NSCLC therapy.

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Disclosure of conflict of interest

None.

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