Oncology Research, Vol. 27, pp. 1015–1023
Printed in the USA.

0965-0407/19 $90.00 + .00
DOI: https://doi.org/10.3727/096504018X15247341491655
E-ISSN 1555-3906
www.cognizantcommunication.com

miR-185 Inhibits the Proliferation and Invasion of Non-Small Cell Lung Cancer by Targeting KLF7

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MicroRNAs (miRNAs) are short endogenous noncoding RNAs that frequently play vital roles in many cancer types. Herein we demonstrated that miR-185 was remarkably downregulated in NSCLC tissues compared with adjacent normal tissues. A lower level of miR-185 was associated with lymph node metastasis. Functional assays showed that upregulation of miR-185 inhibited the proliferation, colony formation, and invasion capacities of NSCLC cells in vitro. Furthermore, we found that miR-185 suppressed the epithelial–mesenchymal transition (EMT) process. Bioinformatics analysis and luciferase reporter gene assays revealed that Kruppel-like factor 7 (KLF7) was the target of miR-185. Overexpression of miR-185 reduced the expression of KLF7 in NSCLC cells. Upregulation of KLF7 partly neutralized the inhibitory effects of miR-185 on the proliferation and invasion of NSCLC. Additionally, we confirmed that miR-185 suppressed the tumor growth of NSCLC A549 cells in vivo. Taken together, these results demonstrate that miR-185 acts as a suppressor by targeting KLF7 in NSCLC.

Key words: miR-185; Non-small cell lung cancer (NSCLC); KLF7; Metastasis

INTRODUCTION

Lung cancer is responsible for the most cancer-associated deaths worldwide, which makes it a major medical issue. The current therapeutic options are largely ineffective, resulting in the survival rate remaining unsatisfactory[1]. Non-small cell lung cancer (NSCLC), which represents approximately 85% of lung cancer cases, presents a particularly challenging cancer type due to its late detection and low sensitivity to chemotherapy and radiotherapy[2]. In addition, 50%–60% of patients with NSCLC develop metastasis during the course of treatment, and up to 30% of patients present with metastasis at the time of diagnosis. In order to improve the low survival rate, it is critical to better understand the process of lung cancer metastasis[3].

MicroRNAs (miRNAs), a class of small noncoding RNAs (~22 nucleotides), primarily function through binding with the 3′-untranslated region (3′-UTR) of target mRNAs, which results in inhibition of their translation or degradation[4]. The dysregulation of miRNAs has been demonstrated in malignant tumors, including NSCLC[5]. miR-17-5p, miR-137, miR-130, and miR-218 function as tumor suppressors[6]. However, other miRNAs, including miR-21, miR-544a, and miR-196a, function as oncogenes in some kinds of tumors[7]. miR-185-5p is commonly found to be dysregulated in many types of cancers, including liver cancer, breast cancer, and kidney cancer[8]. Recent studies suggested that miR-185-5p plays a significant function in the drug resistance of cancer cells[9]. Nevertheless, the underlying biological mechanism of miR-185 in regulating the progression of NSCLC still needs to be investigated.

In this study, we demonstrated that miR-185 was remarkably downregulated in NSCLC tissues and cells. Overexpression of miR-185 inhibited the growth and invasion of NSCLC in vitro as well as suppressed the tumor growth in vivo. Furthermore, we proved that miR-185 suppressed the progression of NSCLC cell by regulating KLF7.

MATERIALS AND METHODS

NSCLC Tissues

Thirty-seven cases of NSCLC tissues and adjacent normal tissues were obtained from the Affiliated Hongqi Hospital of Mudanjiang Medical University. All patients were diagnosed with NSCLC by two professional

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pathologists. No patient had received preoperative adjuvant therapy. Written informed consent was obtained from all participants. The tissue samples were frozen in liquid nitrogen and stored at −80°C before analysis. This research was approved by the ethics committee of the Affiliated Hongqi Hospital of Mudanjiang Medical University and performed in accord with the Helsinki Declaration.

Cell Culture

Four human NSCLC cell lines (A549, H1299, H1975, and HCC827) and the normal bronchial epithelial cell line BEAS-2B were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, P.R. China). All cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Carlsbad, CA, USA) containing 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin (Thermo Fisher Scientific) at 37ºC and 5% CO_2.

Cell Transfection

miR-185 mimic, miR-185 inhibitor, or miR-NC (negative control) was constructed and purchased from RIBOBIO (Guangzhou, Guangdong, P.R. China). miR-185 mimic or miR-185 inhibitor was transfected into cells using Invitrogen™ Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA). The full-length sequence of KLF7 was cloned into pcDNA3.1 (Sigma-Aldrich, St. Louis, MO, USA) to construct pcDNA3.1-KLF7 overexpression plasmid.

qRT-PCR Assay

Total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). RNA (1 μg) was reversed to DNA by PrimeScript RT Master Mix (TAKARA, Dalian, Liaoning, P.R. China). qRT-PCR was performed using IQ™ SYBR Green Supermix and the iQ5 real-time detection system (Bio-Rad Laboratories, Hercules, CA, USA). The primers used for PCR were as follows: GAPDH: 5′-GGAGCGAGATCCCTCCAAAAT-3′ (forward), 5′-GGCTGTTGTCATACTTCTCATGG-3′ (reverse); KLF7: 5′-AGACATGCCTTGAATTGGAACG-3′ (forward), 5′-GGGGTCTAAGCGACGGAAG-3′ (reverse); N-cadherin: 5′-TCAGGCTGTGGACATAGAAACC-3′ (forward), 5′-GCTGTAAACGACTCTGGCACT-3′ (reverse); E-cadherin: 5′-AAAGGCCCATTTCCTAAAA ACCT-3′ (forward), 5′-TGCGTTCTCTATCCAGAGG CT-3′ (reverse). The mRNA levels were normalized to GAPDH and calculated using the $2^{-\Delta\Delta CT}$ method.

Cell Proliferation and Colony Forming Assay

Cell vitality was measured using MTT assay. miR-185 mimic or miR-185 inhibitor-transfected cells (2×10^3) were seeded into 96-well plates. Cells were cultured for 1, 2, 3, and 4 days, respectively. Then 10 μl of MTT (5 mg/ml) solution was added into the plates, and cells were incubated for 4 h. The absorbance (OD) was detected at 450 nm using a microplate reader. For colony formation assay, cells (1×10^3) were seeded into 12-well plates and cultured for 14 days. Then cell colonies were fixed and stained using 1% crystal violet solution for 15 min.

Cell Invasion Assay

Cell invasion was determined using a Transwell chamber (BD Biosciences, Franklin Lakes, NJ, USA). miR-185 mimic or miR-185 inhibitor-transfected cells (1×10^6) were resuspended and were seeded into the upper chamber. RPMI-1640 containing 20% FBS was added into the lower chamber. After 48 h, the invaded cells were fixed using methanol and stained using 1% crystal violet. The number of invaded cells was calculated under a microscope[10].

Western Blot Assay

Total proteins were extracted using RIPA (Thermo Fisher Scientific). Proteins (30 μg) were separated using 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Braunschweig, Germany). Then the membrane was blocked using 5% skimmed milk and incubated with KLF7 antibody, E-cadherin, N-cadherin, or GAPDH (all Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. After that, the PVDF membrane was incubated with goat anti-rabbit IgG (Santa Cruz Biotechnology). Protein bands were detected using the ECL system (Millipore)[11].

Luciferase Reporter Gene Assay

The wild-type (WT) 3′-UTR of KLF7 containing binding sites with miR-185 or mutant-type (MUT) 3′-UTR of KLF7 was inserted into pmiR-GLO vector. A549 cells were cotransfected with miR-185 mimic and WT 3′-UTR of KLF7 or MUT 3′-UTR of KLF7 using Lipofectamine 2000 reagent according to the manufacturers' instruction. Luciferase activity was detected and analyzed by Dual-Luciferase Reporter system (Promega, Madison, WI, USA).

Xenograft Model

miR-185 stably transfected or control A549 (1×10^6) cells were subcutaneously inoculated into nude mice ($n=6$ for each group). Tumor volume was measured every week, and tumor volume was calculated as the following formula: length × width^2/2. Tumor weights were also measured. After 4 weeks, all the mice were sacrificed, and the tumor tissues were isolated for Western blot analysis. All experiment protocols were approved

by the Institutional Animal Care and Use Committee of The Affiliated Hongqi Hospital of Mudanjiang Medical University.

Statistical Analysis

Statistical analysis was performed with SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Results were presented as mean ± SD. Differences in the results of two groups were evaluated using either two-tailed Student's *t*-test or one-way ANOVA followed by post hoc Dunnett's test. A value of $p < 0.05$ was considered as statistical significance.

RESULTS

miR-185 Is Downregulated in NSCLC Tissues and Cells

To identify the potential dysregulation of miRNAs that were aberrantly expressed in NSCLC, the levels of miRNAs in control normal and NSCLC tissues using GEO data set GSE29248 were analyzed. The heat map generated using the differential miRNAs showed that miR-185 was remarkably downregulated (fold change = −3.65) in NSCLC tissues (Fig. 1A). Next, the levels of miR-185 in 37 cases of NSCLC tissues and the adjacent normal tissues were detected using qRT-PCR assay. As shown in Figure 1B, miR-185 was significantly downregulated in NSCLC tissue compared with the adjacent tissue. Additionally, we found that the lower level of miR-185 was associated with the lymph node metastasis in patients with NSCLC (Fig. 1C). The levels of miR-185 in NSCLC cell lines, including A549, H1299, H1975, and HCC827, as well as the normal lung epithelial cell, BEAS-2B, were determined. The results indicated that miR-185 was downexpressed in NSCLC cell lines compared with BEAS-2B cells (Fig. 1D). Thus, these results demonstrated that miR-185 was remarkably downregulated in NSCLC tissues and cells.

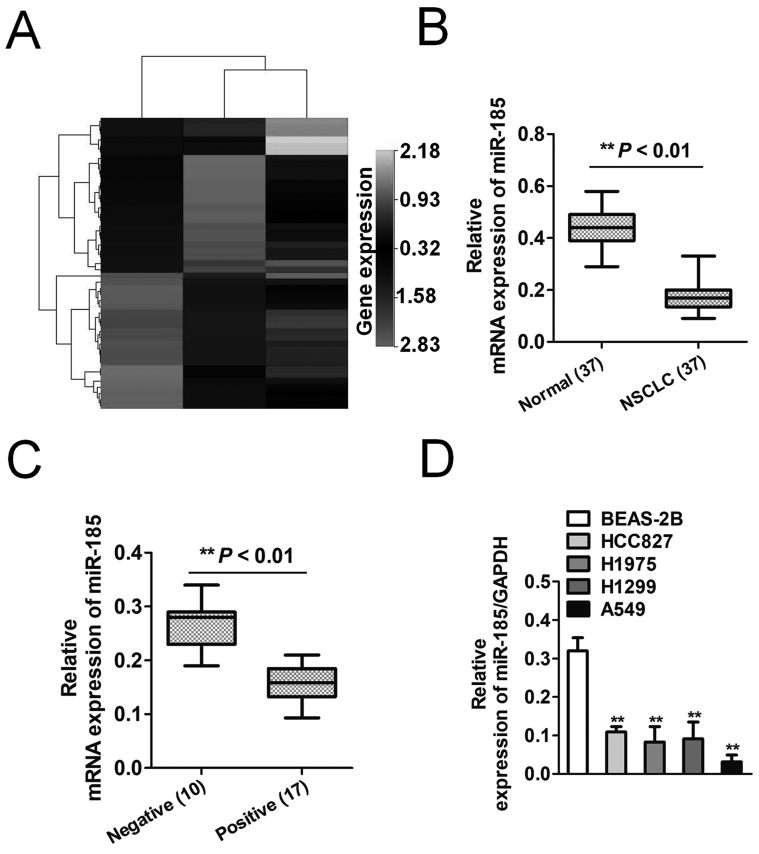

Figure 1. miR-185 is remarkably downregulated in NSCLC. (A) Microarray analysis of miRNA expression in NSCLC tissues and corresponding normal tissues. (B) The levels of miR-185 were examined in 37 pairs of non-small cell lung cancer (NSCLC) tissues and adjacent normal tissues using qRT-PCR analysis. $^{**}p < 0.01$ compared to normal. (C) The relationship between miR-185 level and lymph node metastasis is shown. $^{**}p < 0.01$ compared to negative. (D) The level of miR-185 was determined in NSCLC cell lines (A549, H1299, H1975, and HCC827), and the normal bronchial epithelial cell line, BEAS-2B, using qRT-PCR analysis. $^{**}p < 0.01$ compared to BEAS-2B. Values are shown as mean ± SD.

miR-185 Inhibits the Growth and Invasion of NSCLC Cell In Vitro

To explore the effects of miR-185 on the aggressiveness behaviors of NSCLC, the gain-of-function and loss-of-function assays were performed. miR-185 mimic or miR-185 inhibitor was transfetced into A549 cell to increase the level of miR-185 or decrease the level of miR-185 (Fig. 2A). The proliferation of miR-185 or miR-185 inhibitor-transfected A549 cells was examined using MTT assay. As shown in Figure 2B, the proliferation of A549 was significantly inhibited by miR-185, whereas it was increased by miR-185 inhibitor transfection. The colony-forming assay showed that overexpression of

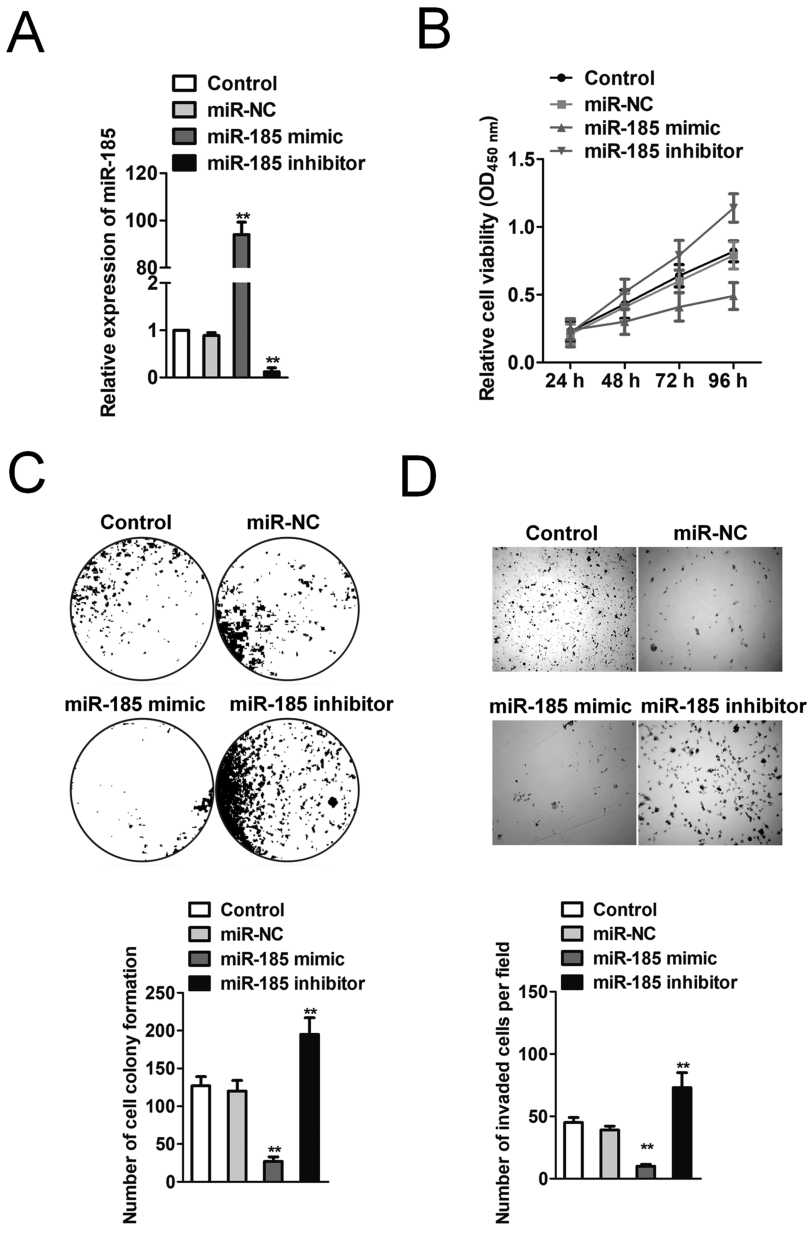

Figure 2. miR-185 inhibits the growth and invasion of NSCLC. (A) The expression of miR-185 was detected after A549 cells were transfected with miR-NC, miR-185 mimic, or miR-185 inhibitor. (B) MTT assays were adopted to assess cell growth after transfection with miR-185 mimic or miR-185 inhibitor. (C) Cell colony formation assays were used to assess cell growth after cells were transfected with miR-185 mimic or miR-185 inhibitor. (D) Cell invasion assays were adopted to assess cell invasion capability after cells were transfected with miR-185 mimic or miR-185 inhibitor. $^{**}p < 0.01$ compared to control. Values are shown as mean ± SD.

miR-185 decreased the colony formation of A549 cells, while downregulation of miR-185 promoted the colony formation of A549 cells in vitro (Fig. 2C). Furthermore, Transwell invasion assay showed that overexpression of miR-185 inhibited the invasion ability of A549 cells when compared to the control; however, miR-185 inhibitor had opposite effects on the invasion of A549 cells (Fig. 2D). Subsequently, we detected the EMT-related markers (E-cadherin and N-cadherin) to evaluate the effects of miR-185 on the EMT process of NSCLC cells. We found that the expression of E-cadherin was increased by miR-185 mimic in A549 cells, and the expression of N-cadherin was inhibited by miR-185 mimic in A549 cells (Fig. 3A and B). Thus, these results showed that miR-185 inhibited the malignant behaviors of NSCLC in vitro.

KLF7 Is the Target of miR-185 in NSCLC

By miRNA target-predicted databases (TargetScan and miRanda), KLF7 was identified as a possible target of miR-185. The WT 3′-UTR of KLF7 containing the putative miR-185 binding sites or MUT 3′-UTR of KLF7 was inserted into pmiR-GLO vector (Fig. 4A). A549 cells were cotransfected with miR-185 mimic in combination with luciferase reporter vector containing WT 3′-UTR of KLF7 or MUT 3′-UTR of KLF7. The results showed that the luciferase activity was significantly reduced in cells that were cotransfected with miR-185 and WT 3′-UTR of KLF7; however, no significant inhibition was observed in cells that were cotransfected with miR-185 and MUT 3′-UTR of KLF7 (Fig. 4B). In addition, the mRNA level and protein expression of KLF7 were inhibited in A549 cells that were transfected with miR-185, whereas the level of KLF7 was increased by miR-185 inhibitor (Fig. 4C and D). Thus, these results demonstrated that KLF7 was the direct target of miR-185 and was negatively regulated by miR-185 in NSCLC cells.

miR-185 Inhibits the Proliferation and Invasion of NSCLC Cells by Regulating KLF7

Next, the mRNA level of KLF7 in 37 cases of NSCLC tissues and adjacent normal tissues was further analyzed.

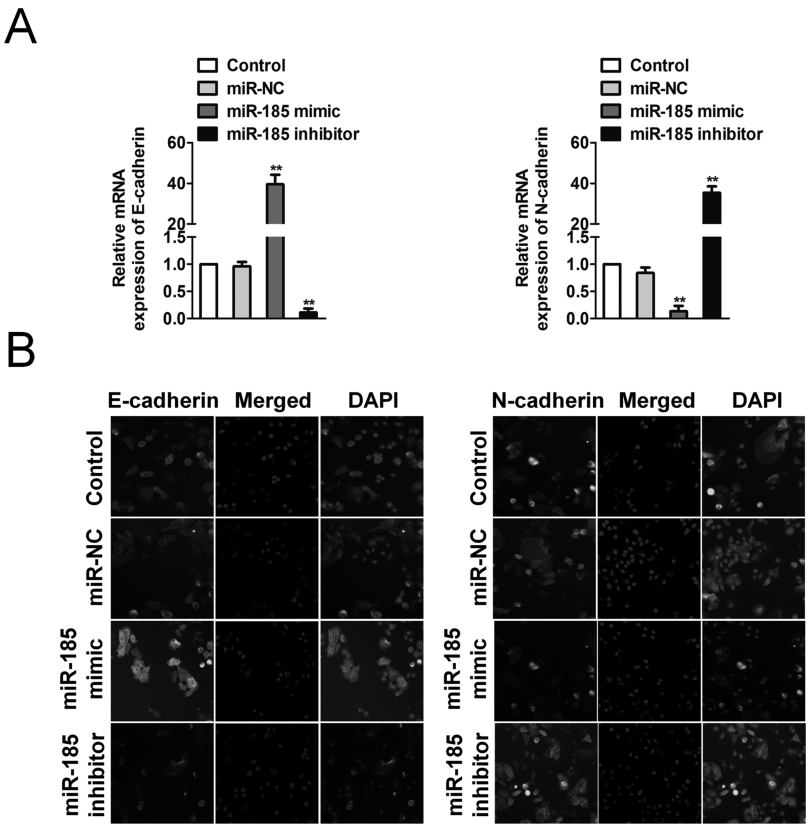

Figure 3. Effect of miR-185 on epithelial–mesenchymal transition (EMT)-related markers in A549 cells. (A) The mRNA levels of E-cadherin and N-cadherin were examined using qRT-PCR assays after A549 cells were transfected with miR-185 or miR-185 inhibitor. (B) The expressions of E-cadheirn and N-cadherin were examined using immunofluorescence staining after A549 cells were transfected with miR-NC or miR-185 inhibitor. $^{**}p < 0.01$ compared to control. Values are shown as mean ± SD.

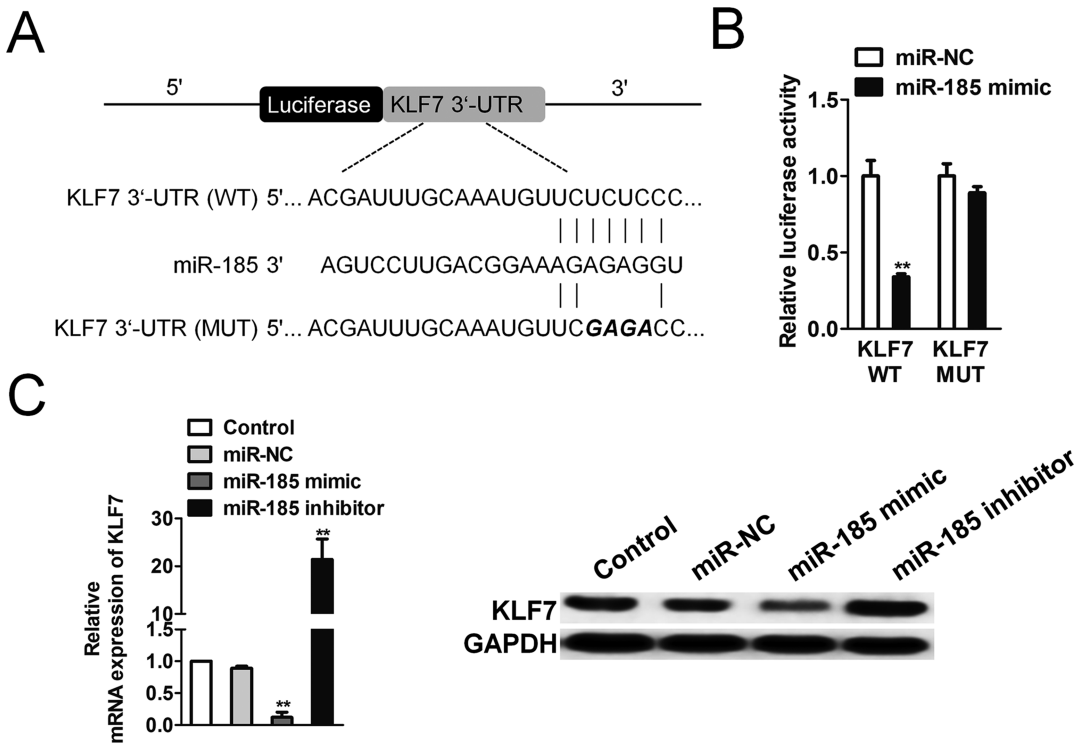

Figure 4. Kruppel-like factor 7 (KLF7) is the target of miR-185 in NSCLC. (A) KLF7 was a possible target of miR-185. The sequence of wild-type (WT) 3′-UTR of KLF7 containing the binding sites or mutant-type (MUT) 3′-UTR of KLF7 was inserted into the pmiR-GLO vector. (B) A549 cells were cotransfected with miR-185 mimic in combination with pmiRGLO-KLF7-3′UTR-WT or pmiRGLO-KLF7-3'UTR-MUT luciferase activity vector. The relative luciferase activity was detected. $^{**}p<0.01$ compared to miR-NC. (C) The mRNA level and protein expression of KLF7 were examined using qRT-PCR and Western blotting. $^{**}p<0.01$ compared to control. Values are shown as mean ± SD.

As shown in Figure 5A, KLF7 was remarkably overexpressed in NSCLC tissues in comparison with the normal tissues. The levels of KLF7 were also higher in NSCLC cells compared to that in BEAS-2B cells (Fig. 5B). The Spearman correlation analysis revealed that the level of miR-185 was negatively associated with the expression of KLF7 in NSCLC tissues (Fig. 5C). To investigate whether miR-185 regulated the proliferation and invasion of NSCLC cells by inhibiting the expression of KLF7, we performed the MTT and invasion assays. First, A549 cells were cotransfected with pcDNA3.1-KLF7 plasmid and miR-185 (Fig. 5D). Then the MTT results showed that downexpression of miR-185 inhibited the proliferation of A549 cells, and overexpression of KLF7 rescued the growth of A549 cells that were inhibited by miR-185 (Fig. 5E). Consistently, the invasion ability was suppressed in miR-185 alone-transfected A549 cells and was rescued in A549 cells that were cotransfected with pcDNA3.1-KLF7 plasmid and miR-185 (Fig. 5F). Taken together, these findings suggested that miR-185 inhibited the proliferation and invasion of NSCLC cells by regulating KLF7.

miR-185 Inhibits the Growth of NSCLC Cell In Vivo

To further investigate the potential role of miR-185 in the tumor growth of NSCLC cells in vivo, stably transfected miR-185 or control cells were subcutaneously injected into nude mice to construct the xenograft tumor model. The results showed that the tumor size and tumor volume in mice that were inoculated with miR-185-transfetced A549 cells were inhibited compared with the control group (Fig. 6A). The tumor weight was also reduced in the miR-185 group in comparison with the control group (Fig. 6B). Also, qRT-PCR analysis results showed that the level of KLF7 was lower in the miR-185 group when compared with the control group (Fig. 6C). These results showed that miR-185 inhibited the growth in NSCLC cells in vivo.

DISCUSSION

A recent report indicated that miRNAs play crucial roles in a series of biological processes including tumorigenesis[12]. Abnormal expression of miR-185 has been proven to affect tumor progression. miR-185 has been

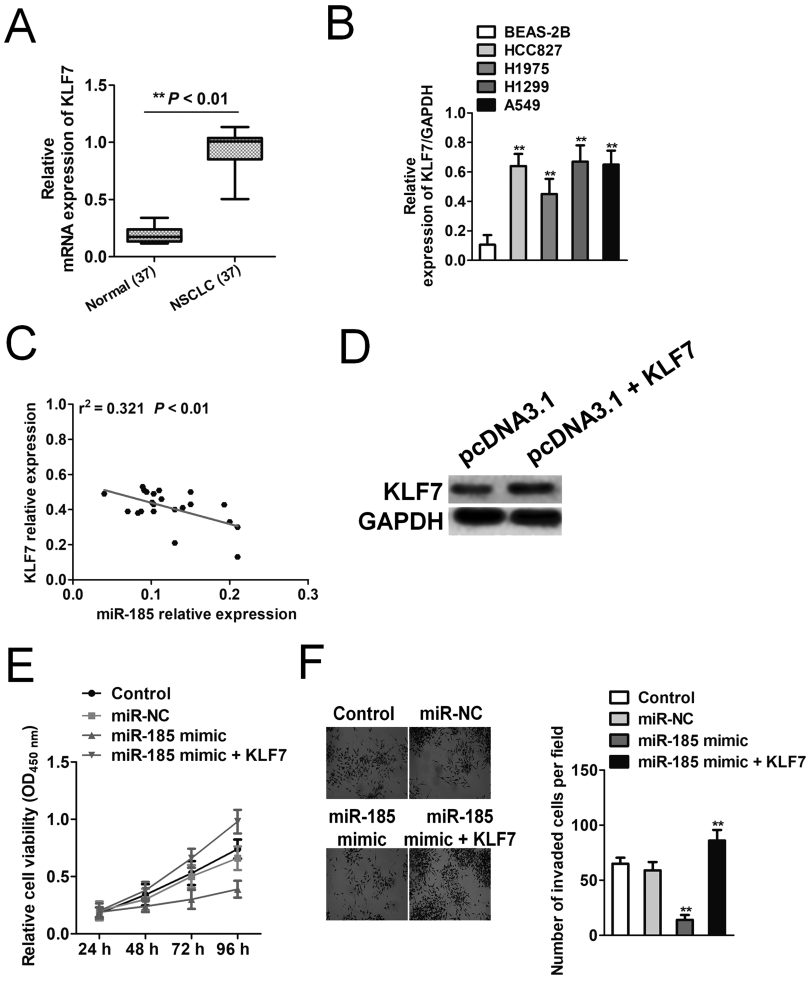

Figure 5. miR-185 restrains the proliferation and invasion by regulating KLF7 in NSCLC. (A) The expression of KLF7 was examined in NSCLC tissues and adjacent normal tissues using qRT-PCR analysis. $^{**}p<0.01$ compared to normal. (B) The expression of KLF7 was examined in NSCLC cell lines (A549, H1299, H1975, and HCC827) and the normal bronchial epithelial cell line BEAS-2B using qRT-PCR analysis. $^{**}p<0.01$ compared to BEAS-2B. (C) Spearman correlation analysis revealed that miR-185 level was negatively associated with KLF7 level in NSCLC tissues. (D) A549 cells were cotransfected with pcDNA3.1-KLF7, and the expression of KL7 was detected using Western blotting assay. (E) MTT assay was used to measure A549 cell growth after transfection with miR-185 mimic or miR-185 mimic plus pcDNA3.1-KLF7. (F) Cell invasion assay was used to evaluate cell growth after transfection with miR-185 mimic or miR-185 mimic plus pcDNA3.1-KLF7. $^{**}p<0.01$ compared to control. Values are shown as mean ± SD.

found to be abnormally expressed in multiple tumor types and plays significant roles on the proliferation, apoptosis, drug resistance, and metastasis of cancer cells. The current study showed that miR-185 was remarkably downregulated in NSCLC tissues when compared to normal tissues, and the lower level of miR-185 was related with lymph node metastasis of NSCLC patients. Furthermore, by gain-and-loss function assays, we demonstrated that downregulation of miR-185 inhibited the proliferation and invasion capacities of NSCLC cell in vitro. In vivo, overexpression of miR-185 also suppressed the tumor growth of NSCLC A549 cells. These above results indicated that miR-185 inhibited the progression of NSCLC.

Furthermore, by performing bioinformatics analysis and luciferase reporter gene assays, we found that KLF7 was the target of miR-185. In a previous investigation, KLF7 was found to function as an oncogene in some cancer types[13]. KLF7, also referred to as ubiquitous Krüppel-like factor, is expressed at low levels in numerous human tissues[14]. The loss of KLF4 and subsequent downregulation of SMAD family member 7 (Smad7) activates the oncogenic transforming growth factor-β (TGF-β) signaling and improves the development of hepatocellular carcinoma (HCC)[15]. In addition, KLF5 promotes the metastasis of bladder cancer cells by upregulating the expression of FYN proto-oncogene[13]. This study identified that

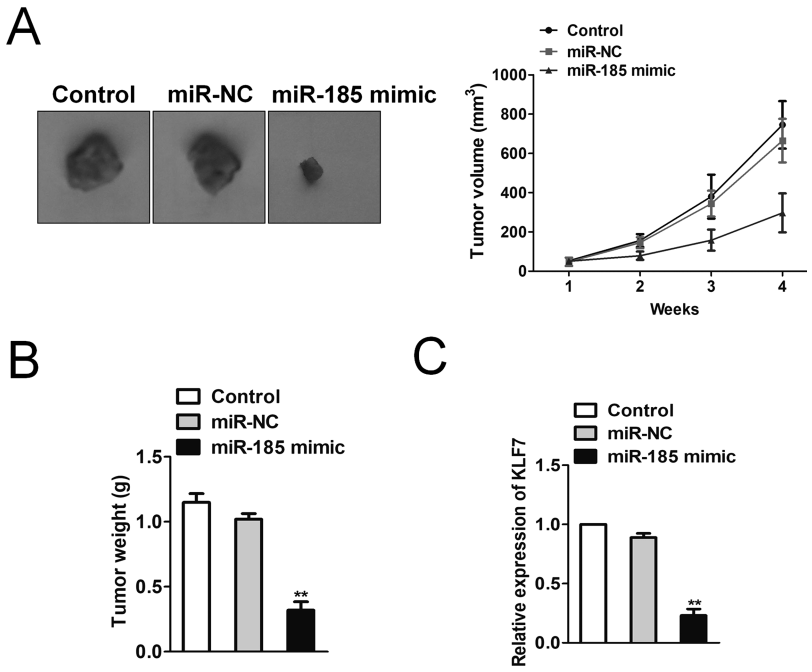

Figure 6. miR-185 inhibits the growth of NSCLC in vivo. (A) Stably transfected miR-185 or control cells were subcutaneously inoculated into nude mice. The tumor volume was significantly lower in the miR-185 group than in the control group. (B) Tumor weight was significantly reduced in the miR-185 group compared with the control group. (C) The mRNA levels of KLF7 in the tumor tissues that were formed by miR-185-transfected A549 or control cells were examined using qRT-PCR analysis. $^{**}p<0.01$. Values are shown as mean ± SD.

KLF7 was the target of miR-185, and overexpression of miR-185 restrained the expression of KLF7 in NSCLC. Overexpression of KLF7 reversed the inhibitory effect of miR-185 on the growth and invasion of NSCLC A549 cells. Thus, these results indicated that miR-185 regulated the progression of NSCLC by inhibiting KLF7 expression.

In conclusion, we demonstrated that miR-185 was downregulated in NSCLC. miR-185 suppressed the progression of NSCLC A549 cells in vitro and in vivo. Moreover, miR-185 inhibited NSCLC cell proliferation and invasion by inhibiting KLF7 expression. Thus, all these results indicated that miR-185 served as a suppressor in NSCLC.

ACKNOWLEDGMENT: The study was supported by the Health and Family Planning Commission of Heilongjiang Province 2017-340, 2013-199, 2010-283 Planning from Mudanjiang Medical University 2011-28. The authors declare no conflicts of interest.

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