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MiR-126-3p suppresses the growth, migration and invasion of NSCLC via targeting CCR1

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Abstract. – OBJECTIVE: MicroRNA (miRNA) plays vital roles in the development of different cancers. In the current work, we explored the function of miR-126-3p in the growth and metastasis of non-small-cell lung cancer (NSCLC) cell *in vitro* and *in vivo*.

PATIENTS AND METHODS: The expressions of miR-126-3p in NSCLC cell lines were assessed using the quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), colony formation, wound healing and transwell invasion were applied to reveal the role of miR-126b-3p on NSCLC cell growth, migration and invasion. The expressions of epithelial-mesenchymal transition (EMT) associated markers (E-cadherin and N-cadherin) were assessed by immunofluorescence staining. The Xenograft model and lung metastasis model were applied to explore the impact of miR-126-3p on the growth and aggressiveness of NSCLC cell *in vivo*.

RESULTS: MiR-126-3p was significantly down-regulated in NSCLC cell lines and tissues. The up-regulation of miR-126-3p inhibited the growth, colony formation, migration and invasion of NSCLC cell. Furthermore, the xenograft model indicated that miR-126-3p suppressed NSCLC cell growth and lung metastasis by targeting chemokine (C-C motif) receptor 1 (CCR1). In addition, we demonstrated that the over-expression of CCR1 rescued the inhibitory effects of miR-126-3p on NSCLC cells growth, migration and invasion. Finally, knocked-down of CCR1 was able to mimic the inhibitory effects of miR-126-3p on the progression of NSCLC cell.

CONCLUSIONS: These findings indicate that miR-126-3p plays an important role in the growth, migration and invasion of NSCLC by targeting CCR1.

Key Words:
NSCLC, MiR-126-3p, CCR1, Migration, Invasion.

Introduction

Non-small-cell lung cancer (NSCLC) is one of the most common malignant cancers and remains the leading cause of cancer-related death worldwide[1-4]. Metastasis is one of the major causes of NSCLC-associated death. The epithelial-mesenchymal transition (EMT) process is an important event that is involved in tumor metastasis[5]. In cancer cell EMT process, the epithelial cell loses the epithelial phenotype (down-regulation of epithelial marker, E-cadherin), and acquires the mesenchymal phenotype (up-regulation of mesenchymal marker, N-cadherin and extracellular matrix (ECM) disruption). MicroRNAs (miRNAs), which belong to a kind of small non-coding RNAs, regulate the levels of target proteins in post-transcriptionally dependent manner[6,7]. The complicated regulatory network of miRNAs not only regulates the expression of target protein via one miRNA, but also allows the combination of multiple miRNAs to regulate target genes[8,9]. Recent investigations demonstrate that miRNAs are closely related with cancer development, including the growth, metastasis and angiogenesis of several cancers[10]. MiRNAs regulate the level of cancer-related genes, and serve as an oncogene or suppressor gene in the tumorigenesis and development of cancer[11]. Previous in-

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vestigations[12-14] have demonstrated that numerous miRNAs participate in the progression of NSCLC, including miR-101-3p, has-miR-9-5p and miR-19a. MiR-126, which originates from a common precursor structure located at chromosome 9q34.3, is down-expressed in various types of cancers and acts as cancer suppressor[15]. In gastric carcinoma, miR-126 is remarkably down-expressed and the up-regulation of miR-126 inhibits the growth of SGC-7901 cells by inducing cell cycle arrest in the G0/G1 phase[16]. In addition, miR-126 inhibits the progression of esophageal cancer by regulating vascular endothelial growth factor A (VEGF-A)[17]. All these results prove that miR-126 is dysregulated in tumor and exerts vital roles in the progression of cancer. In NSCLC, miR-126-5p decreases the enzymatic activity of MDH1, mitochondrial respiration and causes cell death in NSCLC cell lines[18]. However, the potential function of miR-26-3p in the growth and aggressiveness of NSCLC remain unknown. In this work, we found that miR-126-3p was down-regulated in NSCLC. Up-regulation of miR-126-3p inhibits the growth and aggressiveness of NSCLC cell H1975 and A549. In addition, we demonstrated that chemokine (C-C motif) receptor 1 (CCR1), the direct target gene of miR-126-3p, exerts crucial roles in the progression of NSCLC cell regulated by miR-126-3p.

Materials and Methods

Cell Culture and NSCLC Tissues

The NSCLC cell lines (A549, H1975, HCC827 and H1299) and SV40-immortalized non-tumorigenic human bronchial epithelial cells BEAS-2B were obtained from Nanjing Biotechnology co. Ltd (Nanjing, Jiangsu, China). The cell was maintained at 37°C in an atmosphere of 5% CO^2 in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 μg/mL streptomycin and 100 U/mL penicillin (Beyotime, Shanghai, China). 31 pairs of human NSCLC tissues and corresponding adjacent normal tissues were obtained from patients with NSCLC, who received surgery at the First Affiliated Hospital of Guiyang University of Chinese Medicine. No patients received treatment before clinical surgery. Ethical approval was obtained from the Ethics Committee of the First Affiliated Hospital of Guiyang University of Chinese Medicine. The study conforms to the Code of Ethics of the World Medical Association (Declaration of Helsinki) printed in the British Medical Journal (18 July 1964).

Cell Transfections

MiR-126-3p mimics and its corresponding negative control (miR-ctr) were purchased from GenePharma (Shanghai, China). H1975 or A549 cell was transfected with miR-126-3p or miR-ctr using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). The full-length open reading frame of CCR1 was cloned into pcDNA3.1 (+) (GenePharma, Shanghai, China) to generate CCR1 expression vector (pcDNA-CCR1). Co-transfection of pcDNA-CCR1 and miR-126-3p was conducted using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Immunoblotting Assay

Total proteins were prepared from cells using the radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) buffer. 25 μg proteins were separated using 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto polyvinylidene difluoride (PVDF) membranes. After membranes were blocked using Tris-Buffered Saline and Tween (TBST). The membranes were incubated with primary antibodies against CCR1 (Proteintech, Wuhan, Hubei, China) or GAPDH (Proteintech, Wuhan, Hubei, China). Next, the cell was incubated with horseradish peroxidase (HRP) conjugated IgGs (1:10000, Proteintech, Wuhan, Hubei, China) and target band was assessed using the enhanced chemiluminescence (ECL) system (Millipore, Billerica, MA, USA).

MTT Assay

The cell was cultured in 96-well plate for 1, 2, 3, or 4 days. After that, 5 mg/ml of methyl thiazolyl tetrazolium solution (MTT; Sigma-Aldrich, St. Louis, MO, USA) was added into 96 well plates and the cells were cultured in an incubator for 4 h. Then, the cell supernatant was removed and dimethylsulfoxide (DMSO; 200 μl) was added into 96 well plates. Finally, the optical density (OD) was measured at 490 nm.

Wound Healing Assay

Cells (1×105 per well) were seeded into 6 well plates for 24 h. The cells were starved in fetal bovine serum (FBS)-free culture medium for 12 h and a wound was made by scratching the cell monolayer using a 100 μL pipette tip. Next, the cell

debris was removed and the cells were incubated with complete medium. The wound was photographed at 0 h and 24 h19.

Invasion Assay

Cells (1×105 per well) were cultured into the upper chamber of transwell containing membrane (8 μm pore size) that was pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The complete medium containing 20% fetal bovine serum (FBS) was added into the lower chamber. After 24 h, the invaded cells on the lower chamber were stained by 1% crystal violet and the number of the invaded cell was analyzed.

Colony Formation Assay

Cells (1 × 103 per well) were seeded into 25 mm culture dish, and cultured for 4 weeks. Finally, colonies were stained using 1% crystal violet and cell colonies were counted.

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

Total RNA was extracted from cells using TRIzol (TaKaRa, Otsu, Shiga, Japan). 1 μg of RNA was reverse-transcripted to complementary DNA (cDNA) using PrimeScript RT Reagent Kit (TaKaRa, Otsu, Shiga, Japan). To quantify the level of miR-126-3p, the stem-loop quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was conducted using the TaqMan® miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) on the ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). U6 was the endogenous control. qRT-PCR analysis of CCR1 was conducted using SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan) on the ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the endogenous control. The primers were as follows (sense and antisense): GAPDH: AGGTCGGTGTGAACGGATTTG and GGGGTCGTTGATGGCAACA; CCR1: GACTATGACACGACCACAGAGT and CCAACCAGGCCAATGACAAATA; MiR-126-3p: ACACTCCAGCTGGG TCGTACCGTGAGTAAT and CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCGCATTAT; U6: CTCGCTTCGGCAGCACA and AACGCTTCACGAATTTGCGT.

Immunofluorescence Staining

The cell was perforated with 10% Triton X-100 (Sangon, Shanghai, China). Then, the cells were incubated with primary antibodies overnight at 4°C. Next, the cells were incubated with goat anti-rabbit IgG secondary antibody Alexa Fluor 488 (Beyotime, Shanghai, China) for 2 h. Cell nucleus was stained using 4',6-diamidino-2-phenylindole (DAPI; Beyotime, Shanghai, China). Image of immunofluorescence staining was taken using a microscope.

Luciferase Reporter Assay

The wild-type (wt) or mutant-type (mut) 3'-untranslated region (3'-UTR) of CCR1 gene was cloned into the pGL3 Luciferase reporter vector. The pGL3-CCR1-3'-UTR and the Renilla plasmid were cotransfected into miR-126-3p mimics or miR-ctr transfected 293T cell using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After 24 h, the Luciferase activity was assessed using the Luciferase assay system (Promega, Madison, WI, USA).

Tumorigenicity

Female nude mice were bought from the Shanghai Slake Laboratory Animal co. LTD (Shanghai, China). Animal experiments were conducted in accordance with standard operating procedures approved by the Committee on the Use and Care of Animals at Guizhou Medical University. Our animal experiments were conducted in accordance with Institutional Guidelines and the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996). 100 μl miR-126-3p or miR-ctr transfected A549 cells (1 × 107) were subcutaneously into nude mice. The tumor was measured each week and calculated as the following formula: 0.5 × Length × Width2. The animals were euthanized and the tumors were utilized for qRT-PCR analysis.

Experimental Metastasis Assay

Female nude mice were bought from Shanghai Slake Laboratory Animal co. LTD (Shanghai, China). 100 μl miR-ctr or miR-126-3p transfected A549 cells (1 × 105) were injected into nude mice through lateral tail vein. After four weeks, nude mice were sacrificed and lung tissues were applied for hematoxylin-eosin (H&E) staining. The number of lung metastatic nodules was determined under a dissecting microscope.

Statistical Analysis

Data are presented as the mean ± SD of three repeated experiments. Differences in the results of the two groups were evaluated using either two-tailed Student's *t*-test or one-way ANOVA

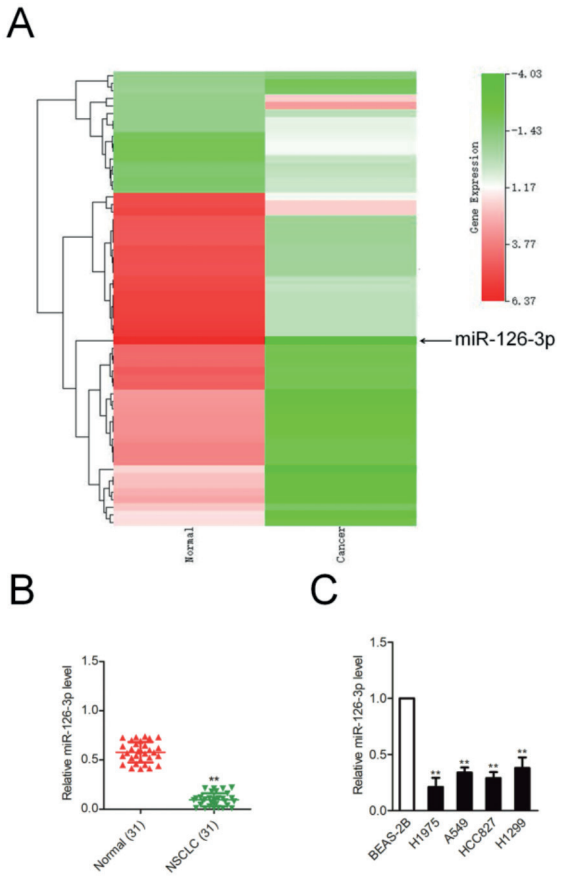

Figure 1. MiR-126-3p was down-regulated in NSCLC. A, Microarray analysis of miRNA expression in NSCLC tissues corresponding normal tissues. B, The levels of miR-126-3p in NSCLC tissues and control normal tissues were detected using qRT-PCR assay. $^{**}p < 0.01$ compared to normal. C, qRT-PCR was adopted for the analysis of the levels of miR-126-3p in a panel of NSCLC cell lines. U6 was used as loading control. $^{**}p < 0.01$ compared to BEAS-2B.

followed by post-hoc Dunnett's test. *p*-value of < 0.05 was considered statistically significant.

Results

The Level of MiR-126-3p Is Down-regulated in NSCLC

To identify the miRNAs which were dysregulation in NSCLC, the expression of miRNAs between primary NSCLC vs. metastatic NSCLC tissues was compared by GEO data set GSE114711. The heat map generated with differential genes using the GEO data set revealed that miR-126-3p was remarkably down-expressed ($p < 0.01$) in NSCLC (Figure 1A). To investigate the biological function of the altered miR-126-3p expression in NSCLC, we evaluated miR-126-3p expression in 31 NSCLC tissues and 31 normal tissues using qRT-PCR. As shown in Figure 1B, miR-126-3p was significantly down-regulated in the NSCLC tissues when compared to normal tissues. Similarly, the level of miR-126-3p was generally lower in NSCLC cell lines than that in BEAS-2B cell (Figure 1C). Thus, the findings suggest that miR-126-3p is down-expressed in NSCLC.

Upregulation of MiR-126-3p Inhibits the Growth of NSCLC Cell in vitro

To explore the effect of miR-126-3p in the growth of NSCLC *in vitro*, NSCLC cell H1975 and A549 was transfected with miR-126-3p to increase the level of miR-126-3p. The qRT-PCR result indicated that the level of miR-126-3p was markedly increased in H1975 and A549 cells that were transfected with miR-126-3p (Figure 2A). Next, we detected the impact of miR-126-3p overexpression on the proliferation of H1975 and A549 cell and the findings suggested that up-regulation of miR-126-3p remarkably inhibited the proliferation of NSCLC cell *in vitro* (Figure 2B). The colony formation assay indicated that miR-126-3p over-expressing suppressed the colony formation of H1975 and A549 cell (Figure 2C). All these observations indicate that miR-126-3p serves as an anti-oncogene in NSCLC.

Upregulation of MiR-126-3p Decreases the Aggressiveness of NSCLC Cell

Then, we explored the morphology changes of H1975 and A549 cell after miR-126-3p transfection. As shown in Figure 3A, NSCLC cells were changed from mesenchymal morphology to epithelial morphology once was transfected with miR-126-3p. Immunofluorescence staining using E-cadherin or N-cadherin future confirmed the morphological alterations (Figure 3B), which suggests that miR-126-3p induced mesenchymal-epithelial transition (MET) of H1975 and A549 cell, as the expression of the mesenchymal marker, N-cadherin was decreased whereas the expression of E-cadherin was increased after H1975 and A549 cell was transfected with miR-126-3p mimics. EMT progress always accompanies alteration of the invasion and migration of cancer cell. Hence, we assessed the impact of miR-126-3p on the aggressiveness of NSCLC cell *in vitro*. As shown in Figure 3C-3D, up-regulation of miR-126-3p dramatically suppressed the migration and invasion abilities of H1975 and A549 cell *in vitro*.

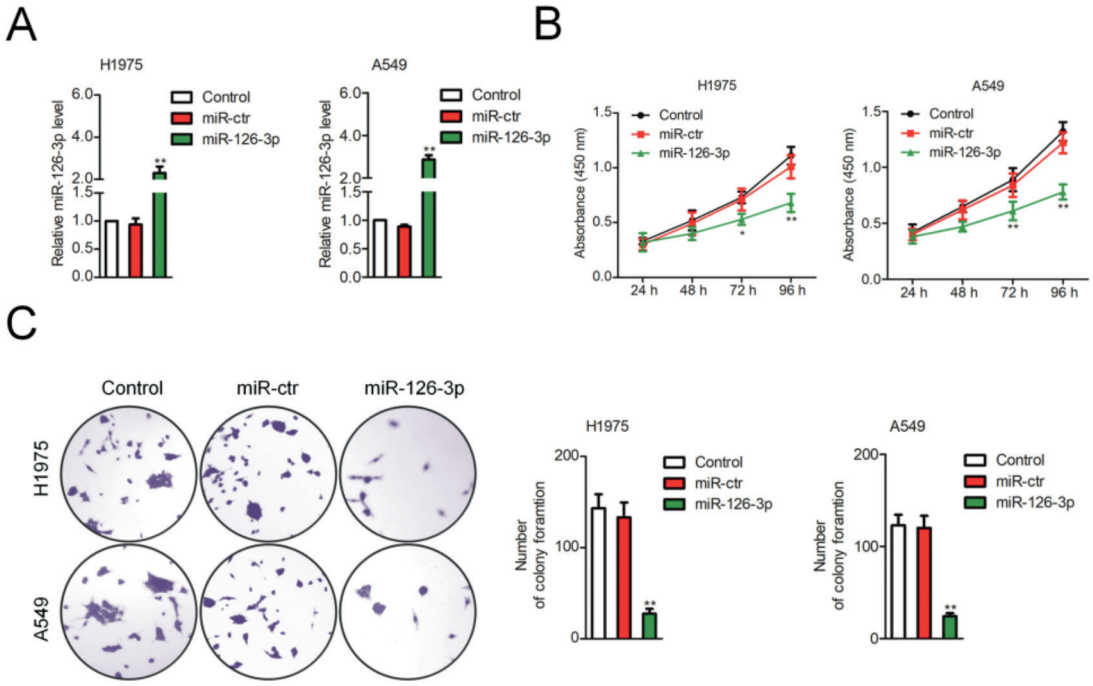

Figure 2. Overexpression of miR-126-3p inhibits the proliferation and colony formation of H1975 and A549 cell *in vitro*. ***A,*** H1975 and A549 cell was transfected with miR-126-3p mimics or miR-ctr. The level of miR-126-3p was measured using qRT-PCR assay. ***B,*** MTT assays were conducted using H1975 and A549 cell that was transfected miR-126-3p. ***C,*** The colony formation assay was carried out using miR-ctr or miR-126-3p mimics transfected H1975 and A549 cells. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control.

Upregulation of MiR-126-3p Suppresses the Growth and Metastasis of A549 Cell in vivo

In vitro, up-regulation of miR-126-3p inhibited the growth and aggressiveness of H1975 and A549 cell. Whether miR-126-3p suppressed the growth and metastasis of NSCLC cell *in vivo* needed further explored. As shown in Figure 4A-B, up-regulation of miR-126-3p inhibited the tumor growth of A549 cell, and the volume of tumor that was formed by miR-126-3p over-expressing cells was markedly smaller than the tumor that was formed by miR-ctr transfected A549 cell. The levels of miR-126-3p in the collected tumors were also assessed using qRT-PCR assay, and the result indicated that the level of miR-126-3p was up-regulated in tumor derived from miR-126-3p over-expressing group (Figure 4C), which confirmed that miR-126-3p restrained the growth of NSCLC cell *in vivo*. Next, we explored the effect of miR-126-3p on the metastasis of NSCLC cell *in vivo* using experimental metastasis test. MiR-ctr or miR-126-3p transfected A549 cells were injected into nude mice via a lateral tail vein. After four weeks, nude mice were sacrificed and metastasis foci were mainly found in the lungs. As shown in Figure 4D, injection of miR-ctr transfected A549 cell produced numerous lung metastasis foci while the up-regulation of miR-126-3p markedly inhibited the lung metastasis of A549 cell. These findings demonstrate that miR-126-3p suppresses the tumor growth and metastasis of NSCLC cell *in vivo*.

CCR1 Is the Target of MiR-126-3p

The bioinformatics online analysis tool Targetscan (http://www.targetscan.org/vert_72/) and miRBase (http://starbase.sysu.edu.cn/index.php) were selected to predict the target genes of miR-126-3p, and we found that CCR1 was the target gene of miR-126-3p (Figure 5A). To analyze whether CCR1 was the direct target of miR-126-3p, wild-type (wt) or mutant-type (mut) 3'-UTR of CCR1 was inserted to the downstream of the Luciferase reporter vector. The Luciferase activity assay indicated that the up-regulation of miR-126-3p decreased Luciferase activity in A549 cell that was transfected with wt 3'-UTR of CCR1

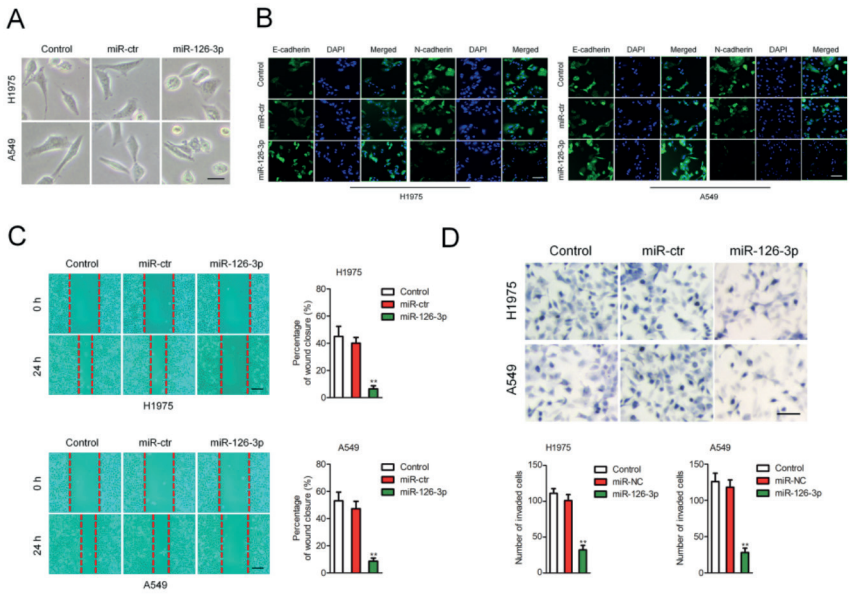

Figure 3. Overexpression of miR-126-3p inhibits the migration and invasion of H1975 and A549 cell *in vitro*. ***A,*** Cell morphology was captured with optical microscope after cell was transfected with miR-126-3p or miR-ctr for 48 h. ***B,*** Representative confocal images of immunofluorescence staining. Green, E-cadherin or N-cadherin. Blue, DAPI for nucleus. ***C,*** H1975 and A549 cell was transfected with miR-126-3p mimics or miR-ctr. Then, the migration of H1975 and A549 cell was determined using the wound healing assay. ***D,*** H1975 and A549 cell was transfected with miR-126-3p mimics or miR-ctr. Then, the invasion of H1975 and A549 cell was determined using the transwell invasion assay. $^{**}p < 0.01$ compared to control.

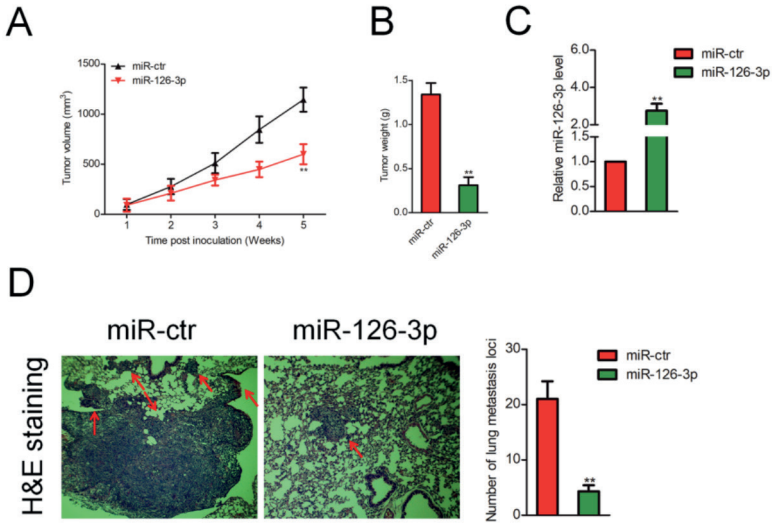

Figure 4. Overexpression of miR-126-3p inhibits the tumor growth and metastasis of A549 cells *in vivo*. A, A549 cells were implanted into nude mice. The volume of tumor was measured once a week. B, The tumors tissues were collected from nude mice at the end of experiment, and the tumor weight is shown, n = 6. C, The levels of miR-126-3p in tumors were detected using qRT-PCR assays. D, Representative pictures of H&E stained using lungs from nude mice (left panel). The numbers of lung metastasis were quantified and showed by each data point (right panel). **p < 0.01 compared to miR-ctr.

whereas had no significant inhibitory effect on the Luciferase activity of A549 cell that was transfected with mut CCR1 (Figure 5B). Furthermore, the expression of CCR1 was markedly decreased when miR-126-3p mimics was transfected into A549 cell (Figure 5C). Consistently, the

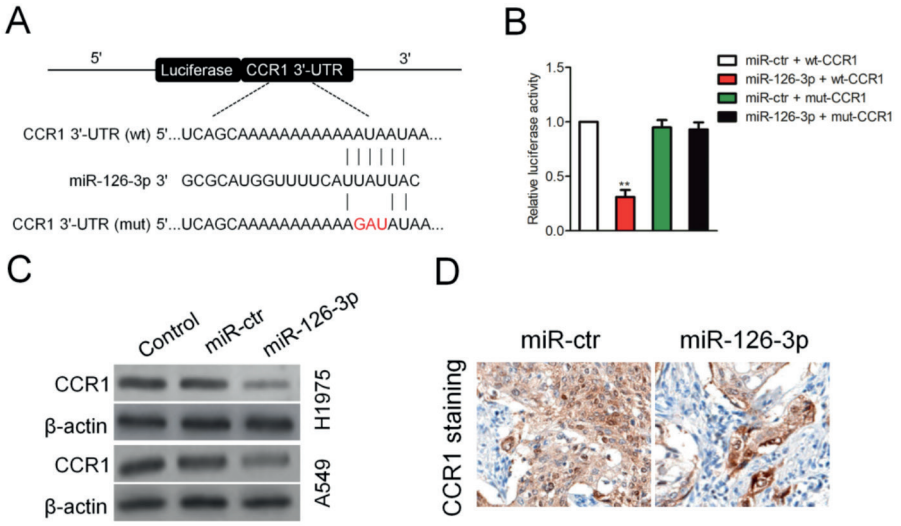

Figure 5. CCR1 is a direct target of miR-126-3p. ***A,*** Predicted miR-126-3p target sequences in 3'-UTR of CCR1. ***B,*** The pGL3-CCR1-3'-UTR vector and miR-126-3p were transfected into A549, then the cells were seeded in 96-well culture plates. The relative Luciferase activity is defined as the value of activity of Luciferase gene folded over internal control. $^{**}p < 0.01$ compared to miR-ctr + wt-CCR1. ***C,*** The expression level of CCR1 after overexpressing miR-126-3p was measured by qRT-PCR and Western blot. ***D,*** The expression of CCR1 was measured by IHC.

immunohistochemistry (IHC) assay using tumor that was formed by miR-126-3p transfected A549 cell or miR-ctr transfected A549 cell suggested that CCR1 was down-regulated when miR-126-3p was up-regulated (Figure 5D). These findings demonstrate that CCR1 is the direct target of miR-126-3p.

The Inhibitory Impact of MiR-126-3p on NSCLC Cell Is Neutralized by Overexpression of CCR1

To investigate whether miR-126-3p restrained the growth, mobility and invasion of NSCLC cell by regulating CCR1, A549 cell was cotransfected with CCR1 and miR-126-3p. The qRT-PCR and immunoblotting assay indicated that the expression of CCR1 was not decreased by miR-126-3p in A549 cell that was cotransfected miR-126-3p and CCR1 (Figure 6A-6B). Next, proliferation assay and colony formation assay indicated that co-transfection of miR-126-3p and CCR1 increased A549 cell growth and colony formation when compared with the cell that was transfected with miR-126-3p alone, which confirms that miR-126-3p inhibited A549 cell growth by down-regulating CCR1 and the inhibitory effect of miR-126-3p on A549 cell growth could be recused by overexpression of CCR1 (Figure 6C-6D). Consistently, miR-126-3p and CCR1 cotransfected into A549 cell promoted migration and invasion when compared with cell that was transfected with miR-126-3p alone, which confirms that miR-126-3p inhibited the migration and invasion of A549 cell by down-regulating CCR1 and the inhibitory effect of miR-126-3p on A549 cell aggressiveness could be recused by overexpression of CCR1 (Figure 6E-6E).

Down expression of CCR1 Mimics the Inhibitory Impact of MiR-126-3p on A549 cell

The up-regulation of miR-126-3p suppressed the growth and aggressiveness of A549 cell by regulating CCR1, which indicated that CCR1 might play important roles in NSCLC progression. Nevertheless, the function of CCR1 in NSCLC cell growth and whether CCR1 knocked-down could mimic the inhibitory impacts of miR-126-3p on NSCLC cell was still not well investigated. Two different short hairpin RNAs (shRNA) targeting CCR1 were transfected into A549 cell to decrease the level of CCR1 (Figure 7A). Then, the effects of CCR1 knocked-down on the growth and colony formation of A549 cells were analyzed. As shown in Figure 7B-7C, the down-expression of CCR1 in A549 suppressed growth and colony formation *in vitro*. Consistently, CCR1 knockdown

had similar impacts on the migration and invasion of A549 cell as the overexpression of miR-126-3p (Figure 7D-7E). All results suggest the probably oncogenic effect of CCR1 in NSCLC

Discussion

Cancer cell metastasis is a complicated process, which consists of invasion, intravasation migration, extravasation and forms metastatic foci[20]. Numerous miRNAs have been demonstrated to participate in each step of tumor metastasis[20-22]. Investigating the precise molecular mechanism of the NSCLC metastasis is very important for developing the effective therapy options for NSCLC. Our study proved that miR-126-3p regulated the growth and aggressiveness of NSCLC cell by targeting CCR1. First, to explore the dysregulation pattern of miRNAs in NSCLC, we used an online miRNA array dataset to analyze the different miRNAs between NSCLC tissues and cor-

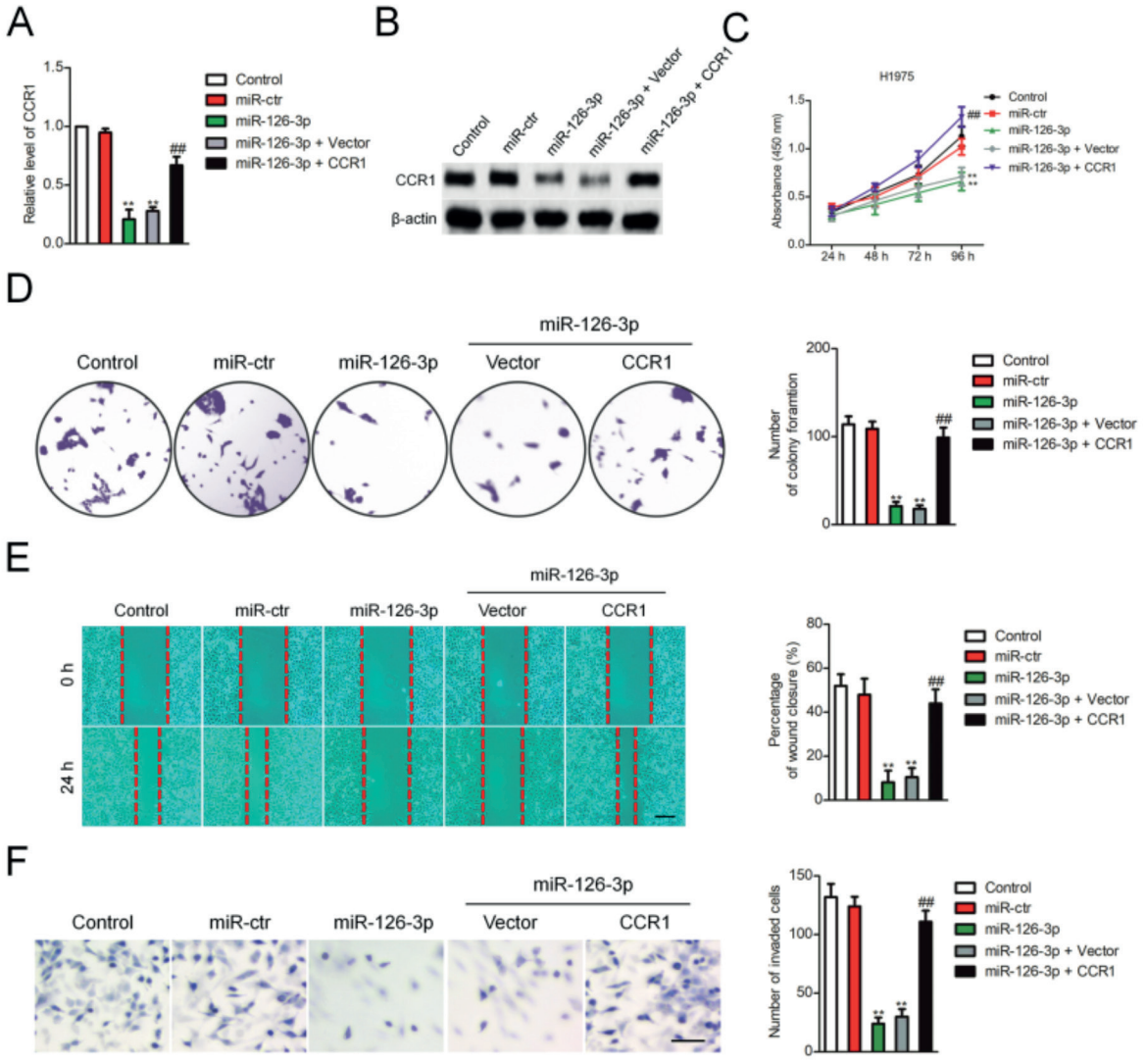

Figure 6. The inhibitory effect of miR-126-3p on A549 cell is rescued by overexpression of CCR1. ***A,*** The expression level of CCR1 was detected by qRT-PCR. ***B,*** The expression level of CCR1 was detected by Western blot. ***C,*** Cell proliferation activity was measured by MTT assay as described. ***D,*** A549 cells were transfected with miR-126-3p alone or co-transfected with miR-126-3p and CCR1. Colony formation assay was conducted. ***E,*** The migration of A549 cell was measured by the wound healing assay. ***F,*** Cell invasion ability was measured by the transwell invasion assay. $^{**}p < 0.01$ compared to control, $^{\#\#}p < 0.01$ compared to miR-126-3p.

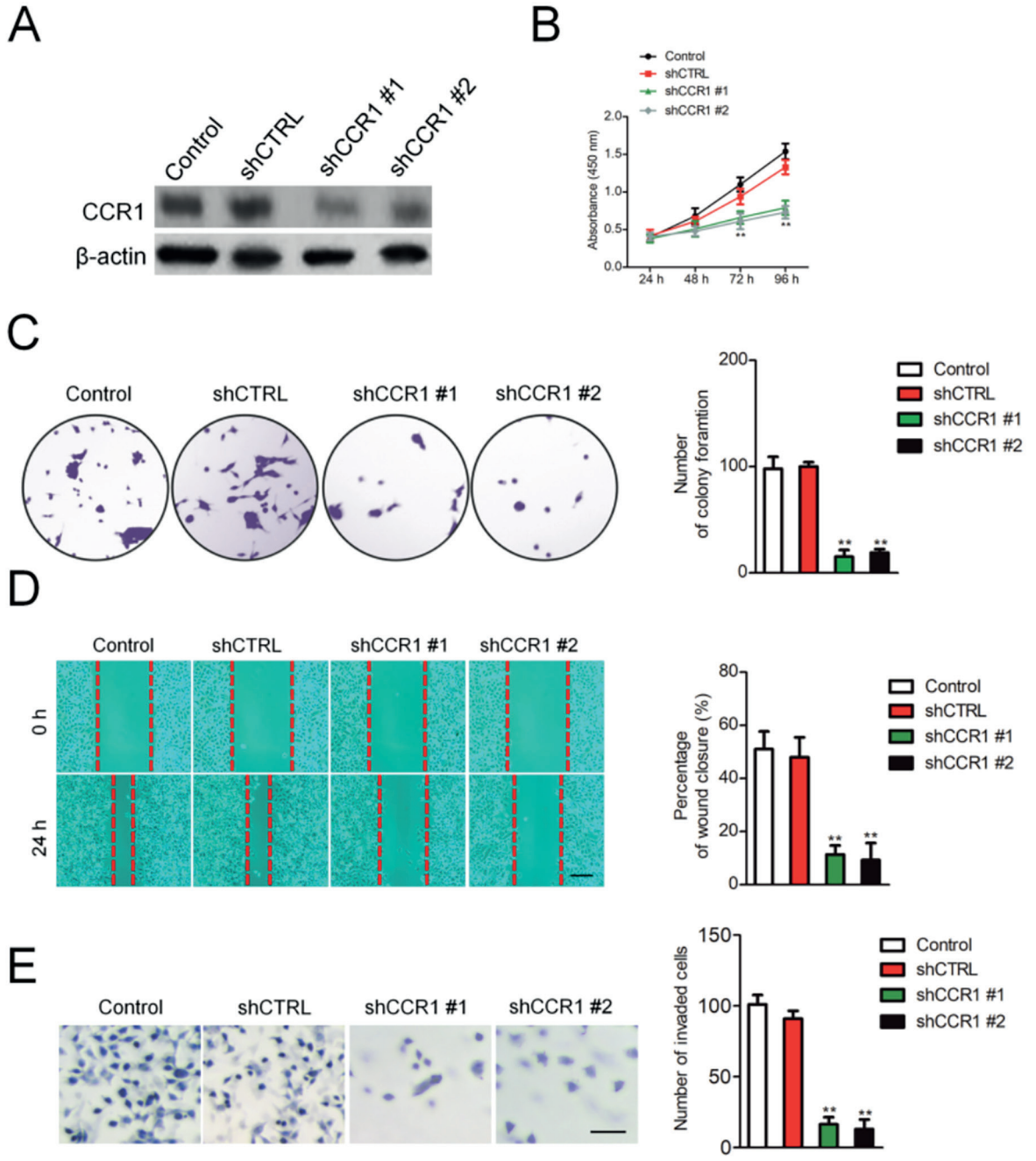

Figure 7. Down-regulation of CCR1 mimics the effects of miR-126-3p on the proliferation, migration and invasion of NSCLC cell. ***A,*** A549 cell was transfected with shCCR1 or shCTRL, and the expression of CCR1 was detected by qRT-PCR and Western blot. ***B,*** The proliferation of A549 was measured by MTT assay as described. ***C,*** Colony formation assay. The quantitative analysis of colonies is shown. ***D,*** The migration of A549 cell that was transfected with shCTRL or shCCR1 was detected using wound healing assay. ***E,*** Cell invasion ability was measured by the transwell invasion assay. $^{**}p < 0.01$ compared to control.

responding normal tissues. Among the miRNAs detected, miR-126-3p was the most down-expressed in NSCLC when compared to normal tissues. Hence, we detected the function of miR-126-3p in NSCLC growth and metastasis. Previous studies report that miR-126-3p directly target some oncogenes or tumor suppressor, and the dysregulation of miR-126-3p leads to the alternation of these target genes, which play vital roles in the progression of cancers. Recently, the investiga-

tions about the functions of miR-126-3p in cancer development have been widely conducted. MiRNA-126-3p suppresses cell proliferation by targeting phosphoinositide-3-Kinase Regulatory Subunit 2 (PIK3R2) in Kaposi's sarcoma cells23. In ovarian cancer, miR-126-3p inhibits the proliferation and invasion of cancer cell via targeting Plexin B2 (PLXNB2)24. However, the potential roles of miR-126-3p in NSCLC have not been well explored. As the level of miR-126-3p is decreased in NSCLC, we speculated that miR-10a might be anti-oncogene in NSCLC progression. Then, the effects of miR-126-3p on NSCLC cell growth and metastasis were analyzed. The up-regulation of miR-126-3p suppressed the migration and invasion of NSCLC cell, as well as suppressed the EMT *in vitro*. Consistently, the up-regulation of miR-126-3p suppressed the lung metastasis and tumor growth of NSCLC cell *in vivo*. To reveal the underlying mechanism by which miR-126-3p regulates the aggressiveness of NSCLC cell, we further explored the potential target of miR-126-3p in NSCLC. Based on the bioinformatics analysis and Luciferase reporter assay, we validated the CCR1 gene was the target of miR-126-3p. The expression of CCR1 was significantly decreased in NSCLC cell that was transfected with miR-126-3p, which suggested that CCR1 might play a role in mediating miR-126-3p regulation on NSCLC growth and metastasis. The current work suggests that CCR1 is over-expressed in NSCLC and exerts a vital role in the migration and invasion of NSCLC cell. Although several important findings were revealed in this study, there were also some limits. First, as miR-126-3p plays an important role in the progression of NSCLC, its level in NSCLC tissues and its relationship with the prognosis of patients remained to be explored. In the experiment metastasis assay, to intuitively measure the impact of miR-126-3p on NSCLC cell metastasis *in vivo*, fluorescence labeling might be inserted into cells to construct the metastasis model. In addition, as an oncogene, how CCR1 plays its important roles in the migration invasion of NSCLC cell by interacting with the downstream signaling pathway has not yet been investigated, which are the issues we will investigate in our subsequent research.

Conclusions

We demonstrate that miR-126-3p is down-expressed in NSCLC. The up-regulation of miR-126-3p suppresses the growth, migration, invasion and metastasis of NSCLC cell. CCR1, as a target protein of miR-126-3p, exerts a crucial role in miR-126-3p regulating the process of NSCLC cell. These observations might help us to deeper understand the underlying mechanisms of miR-126-3p in regulating the progression of NSCLC, and provide a potential therapeutic target for combating the metastasis of NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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