# MicroRNA-27a-3p regulates the proliferation and chemotaxis of pulmonary macrophages in non-small cell lung carcinoma tissues through CXCL2

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**Abstract.** The present study aimed to investigate microRNA (miRNA)-27a-3p expression in the pulmonary macrophages and peripheral blood of patients with early non-small cell lung carcinoma (NSCLC) and its regulatory effect on the infiltration of pulmonary macrophages into cancer tissues and invasion of NSCLC cells. Blood specimens were withdrawn from 36 patients with NSCLC and 29 healthy subjects. NSCLC tissues and cancer-adjacent tissues were both obtained from patients with NSCLC; furthermore, certain tissue samples were used to extract macrophages. The levels of miRNA-27a-3p and C-X-C motif ligand chemokine 2 (CXCL2) mRNA were detected by reverse transcription-quantitative PCR and the levels of CXCL2 protein were measured by ELISA and western blot analysis. A dual-luciferase reporter assay was performed to determine the interactions between miRNA and mRNA. An MTT assay was employed to examine the viability of transfected cells and macrophages and a Transwell assay was performed to assess chemotaxis. The differential expression of miRNA-27a-3p in NSCLC tissues, pulmonary macrophages and peripheral blood indicated that miRNA-27a-3p exerted different roles in these specimens. CXCL2 was upregulated in NSCLC tissues at both transcriptional and translational levels. In addition, the untranslated region of CXCL2 was confirmed to be directly targeted by miRNA-27a-3p prior to its transcriptional activation. Furthermore, miRNA-27a-3p regulated CXCL2 expression, thereby affecting the proliferation of human pulmonary macrophages. The present study highlights that miRNA-27a-3p expression in the pulmonary macrophages and peripheral blood of patients with NSCLC is downregulated, while its target gene CXCL2 is upregulated. miRNA-27a-3p may regulate the viability and chemotaxis of macrophages in tumor tissues of patients with NSCLC through CXCL2 and is expected to become a genetic marker of this disease.

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*Key words:* non-small cell lung carcinoma, miR-27a-3p, CXCL2, macrophage

## Introduction

The occurrence and death rates of lung cancer (LC) remain the highest among all malignant neoplasms (1,2), with ~6 million patients dying of LC each year in China (3). LC may be classified as non-small cell LC (NSCLC) or SCLC based on its histological characteristics. Among all LC cases, NSCLC accounts for 80-85% and its 5-year overall survival is only 16% (4). Being an insidious disease, LC is commonly diagnosed at an advanced stage.

Overexpression of microRNA (miRNA)-27a is observed in numerous types of cancer, such as pancreatic cancer (5), breast cancer (BC) (6), ovarian cancer (OC) (7), esophageal cancer (8) and renal cell carcinoma (RCC) (9), and it is associated with the biological behaviors of tumors cells. High levels of miRNA-27a are also related to the survival rates and clinical outcome of patients with BC (10). miRNA-27a-3p overexpression may contribute to the invasiveness and metastasis of RCC cells and oral squamous cell carcinoma stem cells (11,12). However, certain studies have demonstrated that miRNA-27a-3p is downregulated in hepatocellular carcinoma (HCC) and esophageal squamous cell carcinoma, and may exhibit inhibitory effects on these tumor types (13,14). At present, the abnormal expression and functional role of miRNA-27a-3p in tumor cells are still controversial.

Chronic inflammation is one of the physiological causes of LC and inflammatory response is one of the important characteristics of early NSCLC (15). It has been indicated that upregulation of COX-2 has a prominent role in NSCLC initiation (16). In addition, there are various cytokines participating in tumor processes (17). C-X-C motif chemokine ligand 2 (CXCL2) is involved in chronic inflammatory responses by recruiting, maturating and activating immune cells and secretory proteins with small molecular weight (18). Certain studies

have confirmed that CXCL2 exhibits strong chemotactic activity, promotes inflammatory injury, induces angiogenesis and increases cancer cell proliferation (19,20). Other studies have also indicated that CXCL2 is abnormally expressed in numerous types of tumor and immune cells (21-25). However, the regulatory relationship between CXCL2 and miRNA-27a-3p remains to be clarified.

In the present study, both miRNA-27a-3p and CXCL2 levels were detected in the macrophages and peripheral blood of patients with early NSCLC, and the regulatory relationship between them was predicted and verified. In addition, the functional roles of miRNA-27a-3p and CXCL2 in regulating the proliferation of human pulmonary macrophage cell lines were studied and the underlying molecular mechanisms were explored.

## Materials and methods

*Subjects*. A total of 36 patients with NSCLC treated at the Central Hospital Affiliated to Shandong First Medical University (Jinan, China) between June 2016 and September 2017 were enrolled into an observation group. Furthermore, 29 healthy subjects who underwent medical examinations during the same period of time were enrolled into a control group. Fasting peripheral blood was withdrawn from all subjects and kept in EDTA anticoagulant tubes at -20˚C. Lung tumor tissues were freshly obtained from the patients with NSCLC and their tumor-adjacent tissues were used as controls. The tissues were frozen in liquid nitrogen prior to use. Pulmonary macrophages were isolated from a proportion of the tissues using a tissue macrophage extraction kit (cat. no. JH0217; Beijing Baiao Laibo Technology Co., Ltd.). Among the patients with NSCLC, 21 were males and 15 were females (age range, 38-62 years; mean age, 52.3 years). Among the healthy subjects, 18 were males and 11 were females (age range, 36-65 years; mean age, 51.8 years) (Table I). All patients were having the first onset and diagnosed with early NSCLC (stages 1-2), and had no history of hormone therapy, chemotherapy, radiotherapy or Traditional Chinese Medicine administration. Ethical approval for the present study was obtained from the Ethics Committee of Shandong University (no. H18026). All subjects or their family members provided written informed consent.

*Isolation of pulmonary macrophages*. Pulmonary macrophages were isolated from a proportion of the tissues using a tissue macrophage extraction kit (JH0217; Beijing Baiao Laibo Technology Co., Ltd.). In brief, lung tissues were ground into powder in frozen form and washed with cold PBS prior to filtration with a mesh (50 $\mu$m). The resulting cell suspension was mixed with the solution from the tissue macrophage extraction kit (volume ratio, 1:1) prior to centrifugation at room temperature and 4,000 x g for 20 min. The white cloudy cell layer between the upper layer and middle layer was aspirated and then dispensed into a 15-ml centrifuge tube. After washing the extracted cells with 5 volumes of PBS for 3 times (centrifugation conditions: 37˚C, 2,500 x g and 5 min), the pellet was resuspended with F12/DMEM medium plus 10% FBS (Thermo Fisher Scientific, Inc.). Pulmonary macrophages were thereby isolated, and their identity was verified according to a previous report (26).

*Reverse transcription-quantitative PCR (RT-qPCR)*. An RNA extraction kit (Tiangen Biotech, Co., Ltd.) was employed to isolate total RNA from tissues and macrophages. The integrity of total RNA was detected by gel electrophoresis, while the purity of total RNA was examined by calculating the RNA absorbance at 260/280 nm ratio with a spectrophotometer (NanoDrop OneC; Thermo Fisher Scientific, Inc.). Total RNA (1 $\mu$g) was used for RT (cat. no. KR107; TIANScript II cDNA First Strand Synthesis Kit; Tiangen Biotech, Co., Ltd.) according to the manufacturer's protocol and the template cDNA was stored in a freezer (-20˚C). The primer sequences were as follows: MiRNA-27a-3p forward, 5'-CGCCGTTCACAGTGGCTAAG-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'; and U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The RT-qPCR reaction mixture (20 $\mu$l) consisted of qPCR mixture [10 $\mu$l; SuperReal PreMix (SYBR Green); cat. no. FP204; Tiangen Biotech, Co., Ltd.], forward and reverse primers (0.5 $\mu$l each), cDNA (2 $\mu$l) and $ddH_2O$ (7 $\mu$l). According to the manufacturer's instructions, the reaction conditions comprised an initial denaturation step for 5 min at 95˚C, followed by 46 cycles of 10 sec at 95˚C, 25 sec at 56˚C and 30 sec at 72˚C. The results were analyzed by the $2^{-\Delta\Delta Cq}$ method (27) and the ratio of miRNA-27a-3p to U6 was calculated.

For the analysis of CXCL2, the primer sequences were as follows: CXCL2 forward, 5'-CTGCTGCTCCTGCTCCTG-3' and reverse, 5'-TGAGACAAGCTTTCTGCCCA-3'; and GAPDH forward, 5'-AGGAGCGAGACCCCACTAACAT-3' and reverse, 5'-GTGATGGCATGGACTGTGGT-3'. The qPCR reaction mixture [20 $\mu$l; SuperReal PreMix (SYBR Green); cat. no. FP204; Tiangen Biotech, Co., Ltd.] consisted of qPCR mixture (10 $\mu$l), upstream and downstream primers (0.5 $\mu$l each), cDNA (2 $\mu$l) and $ddH_2O$ (7 $\mu$l). According to the manufacturer's instructions, the reaction conditions comprised an initial denaturation for 5 min at 95˚C, followed by 46 cycles of 20 sec at 95˚C, 20 sec at 55˚C and 30 sec at 72˚C. The results were analyzed by the $2^{-\Delta\Delta Cq}$ method (27) and the ratio of CXCL2 vs. GAPDH was calculated.

*Cell transfection*. Cells ($3x10^5$) were grown in 24-well plates with F12/DMEM medium plus 10% FBS. Upon attaining 70% confluency, the cells were subjected to transfection. In the first and second vials, 0.5 $\mu$g plasmids/agomiR (designed/customized by Sangon Biotech Co., Ltd.) and 1 $\mu$l Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) were added into 50 $\mu$l Opti Mem medium (Thermo Fisher Scientific, Inc.) separately. Following a 5-min incubation, the two vials were combined and incubated again at room temperature for 20 min. Subsequently, the cells were exposed to the mixture at 37˚C for 6 h and the medium was replaced with 10% FBS-containing F12/DMEM. Following a 48-h incubation, the cells were harvested for subsequent analyses. The sequence for hsa-agomiRNA-27a-3p was 5'-UUCACAGUGGCUAAGUUCCGCGCGGAACUUAGCCACUGUGAA-3' and that for agomiR-negative control (NC) was 5'-UUUGUACUACACAAAAGUACUGCAGUACUUUUGUGUAGUACAAA-3'.

*Bioinformatics analysis*. The functional roles of miRNAs can be assessed by bioinformatic prediction tools. In this experiment,

Table I. Clinicopathological parameters of the patients of the present study.

| Group | N | Sex | | Age, years | BMI, kg/m$^2$ | Number of cases at stage | | |
|---|---|---|---|---|---|---|---|---|
| | | Male | Female | | | IIb | IIIa | IIIb |
| NSCLC | 36 | 21 | 15 | 52.30±9.23 | 21.43±3.82 | 5 | 20 | 11 |
| Control | 29 | 18 | 11 | 51.8±10.06 | 22.76±2.97 | 3 | 16 | 10 |

BMI, body mass index; NSCLC, non-small cell lung cancer.

miRwalk3.0 (http://mirwalk.umm.uni-heidelberg.de/) was employed to identify the downstream target of miRNA-27a-3p.

*Dual-luciferase reporter (DLR) assay*. Based on the bioinformatics prediction data obtained using miRwalk3.0 (http://mirwalk.umm.uni-heidelberg.de/), the mutant and wild-type (WT) miRNA-27a-3p seed regions in the 3' untranslated region of CXCL2 were identified and synthesized chemically by Sangon Biotech Co., Ltd. Their two ends were first joined by *Hind*III (D6389; Beyotime Institute of Biotechnology) and *Spe*I (RK21113; ABclonal Technology Co., Ltd.) restriction sites and subsequently cloned into the luciferase reporter vector pMIR-REPORT (Ambion; Thermo Fisher Scientific, Inc.). AgomiRNA-27a-3p (100 nM; Sangon Biotech Co., Ltd.) was co-transfected with the plasmid containing mutant or WT 3' untranslated region sequences (0.8 $\mu$g) into 293T cells (Cell Bank of the Chinese Academy of Sciences). Furthermore, pMIR-REPORT empty vector and agomiR-27a-3p were transfected into 293T cells as a negative control group. Following a 24-h incubation, a DLR assay kit (cat. no. E1980; Promega Corporation) was used to lyse the transfected cells. The resulting luminescence intensities were recorded using a GloMax 20/20 luminometer (Promega Corporation). Renilla luciferase activity was employed as a standard reference.

*Western blot analysis*. The tissue (100 mg) was homogenized and lysed with 600 $\mu$l ice-cold RIPA buffer (Beyotime Institute of Biotechnology) for 30 min. After centrifugation (8,000 x g, 10 min, 4˚C), the total protein content was determined by a BCA assay kit (cat. no. P0011; Beyotime Institute of Biotechnology). The protein samples were mixed with 5X SDS loading buffer and then heated in a water bath for 5 min. Following 10% SDS-PAGE, the separated protein samples (20 $\mu$g) were transferred onto PVDF membranes (MilliporeSigma) at 100 V for 2 h and then blocked with 5% non-fat milk (cat. no. P0216; Beyotime Institute of Biotechnology) at room temperature for 1 h. Subsequently, the membranes were exposed to β-actin (cat. no. ab129348; 1:5,000 dilution; Abcam) or rabbit anti-human CXCL2 (cat. no. ab9841; 1:1,000 dilution; Abcam) polyclonal primary antibodies at 4˚C for 24 h. After rinsing 3 times with PBS-Tween-20 (0.1%) for 5 min each, the membranes were exposed to goat anti-rabbit HRP-conjugated secondary antibody (cat. no. ab6721; 1:10,000 dilution; Abcam) at 37˚C for 1 h and then rinsed again with PBS-Tween-20 (3 times, 5 min each). Finally, the protein blots were visualized with an ECL substrate kit (cat. no. ab133406; Abcam) and quantified by Image lab V3.0 software (Bio-Rad Laboratories, Inc.). The protein level of CXCL2 was normalized to that of β-actin.

*ELISA*. A human MIP2 ELISA kit (CXCL2; Abcam) was employed to detect the serum and extracellular levels of CXCL2. The samples (10 $\mu$l liquid samples and 40 $\mu$l diluent) and standards (50 $\mu$l) were loaded into the microplate wells, while for the blank group, wells were left empty. HRP-labelled conjugate (100 $\mu$l) was added to the sample and standard wells, followed by incubation at 37˚C for 1 h. After rinsing the plates 5 times, the substrates A and B (50 $\mu$l each) were placed into all wells, which were then incubated at 37˚C for 15 min. Finally, stop solution (50 $\mu$l) was pipetted into each well and the optical density (450 nm) was recorded using a microplate reader (Thermo Fisher Scientific, Inc.) within 15 min.

*MTT assay*. The transfected cells (2x10$^3$ cells/well) were grown in 96-well plates. After 1, 2 and 3 days of transfection, MTT (5 g/l, 20 $\mu$l) was placed into the designated wells, followed by incubation at 37˚C for 4 h. The medium was discarded and replaced with DMSO (150 $\mu$l) to solubilize the formazan crystals. Finally, the optical density (490 nm) was recorded using a microplate reader (Thermo Fisher Scientific, Inc.) and a cell viability curve was constructed. This experiment was performed in triplicate.

*Transwell assay*. Macrophages (cat. no. GNR 9; Cell Bank of the Chinese Academy of Sciences) were precultured with serum-free DMEM for 12 h to reduce the influence of serum and then seeded into Transwell inserts (cat. no. CLS3412; Corning, Inc.) at a density of 2x10$^5$/well. The chambers were placed into 24-well plates (bottom wells) containing 500 $\mu$l supernatants of cells transfected with agomiRNA-27a-3p or agomiR-NC and then incubated at 37˚C for 24 h. Finally, the Transwell inserts containing fixed cells were soaked in 0.1% crystal violet solution at room temperature for staining for 15 min (the cells on the lower side of the membrane were stained and those on the upper side were removed) prior to observation and counting under a microscope.

*Statistical analysis*. Statistical analyses were performed using SPSS 18.0 (SPSS, Inc.). All values were expressed as the mean ± standard deviation. After performing normality tests, differences between two groups were compared using an unpaired Student's t-test. Comparison among multiple groups was performed using one-way ANOVA. In case of heterogeneity of variance, Dunnett's T3 or Tamhane's T2 method was employed, while for homogeneity of variance,

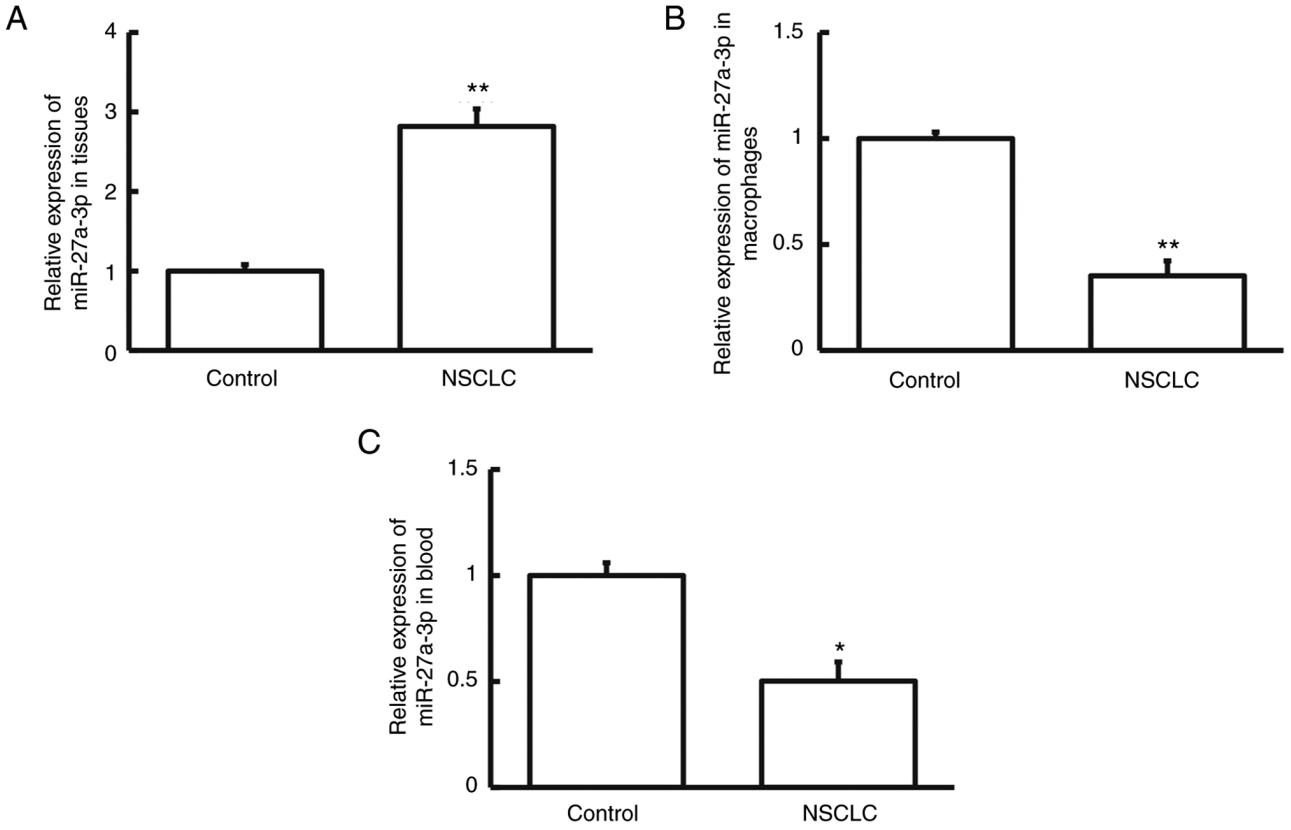


Figure 1. Relative expression of miR-27a-3p in (A) tumor-adjacent (control) and tumor tissues and (B) macrophages isolated from tumor-adjacent (control) and tumor tissues from patients with NSCLC and (C) peripheral blood from healthy subjects (control) and patients with NSCLC. $^{*}$P<0.05 and $^{**}$P<0.01 compared with control group. NSCLC, non-small cell lung cancer; miR, microRNA.

Student-Newman-Keuls and least-significant difference tests were performed. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Differential expression of miRNA-27a-3p in NSCLC tissues, pulmonary macrophages and blood specimens*. To measure the levels of miRNA-27a-3p, RT-qPCR was performed. The results indicated that miRNA-27a-3p levels were markedly higher in NSCLC tissue than in cancer-adjacent tissue (Fig. 1A; P<0.01). However, miRNA-27a-3p levels in the pulmonary macrophages isolated from NSCLC tumor tissues were significantly lower than in those isolated from cancer-adjacent tissues (Fig. 1B; P<0.01). Similarly, the peripheral blood level of miRNA-27a-3p in patients with NSCLC was decreased compared to that in healthy subjects (Fig. 1C; P<0.05). The differential expression of miRNA-27a-3p in NSCLC tissues, pulmonary macrophages and peripheral blood suggests that miRNA-27a-3p exerts different roles in these specimens.

*CXCL2 is upregulated in NSCLC tissues and peripheral blood at both transcriptional and translational levels*. To examine the levels of CXCL2, RT-qPCR and western blot analyses were performed. As presented in Fig. 2A and B, CXCL2 expression levels in the pulmonary macrophages isolated from NSCLC tissue were markedly higher than in those isolated from cancer-adjacent tissue (P<0.05). As indicated in Fig. 2C and D, the peripheral blood levels of CXCL2 in NSCLC were significantly higher in patients with NSCLC than in control subjects (P<0.05). These findings indicate that CXCL2 is upregulated in NSCLC tissues and peripheral blood at both transcriptional and translational levels.

*The untranslated region of CXCL2 is targeted by miRNA-27a-3p*. On the basis of a bioinformatics prediction using miRwalk3.0, CXCL2 was identified as a downstream target of miRNA-27a-3p. The sequences of the agomiRNA-27a-3p seed region on CXCL2 were synthesized (Fig. 3A). The results of the DLR assay indicated that the fluorescence intensity of pMIR-REPORT-WT and agomiRNA-27a-3p co-transfected cells was markedly reduced compared to that of the NC group (P<0.05; Fig. 3B). However, the fluorescence intensity of pMIR-REPORT-mutant and agomiRNA-27a-3p co-transfected cells was relatively similar compared to that of the NC group (P>0.05; Fig. 3B). This observation suggests that the untranslated region of CXCL2 is targeted by miRNA-27a-3p prior to its transcriptional activation.

*miRNA-27a-3p regulates CXCL2 expression that affects the proliferation of human pulmonary macrophages*. The level of miRNA-27a-3p was much higher in agomiRNA-27a-3p-transfected human pulmonary macrophages than in agomiR-NC-transfected macrophages (P<0.01;

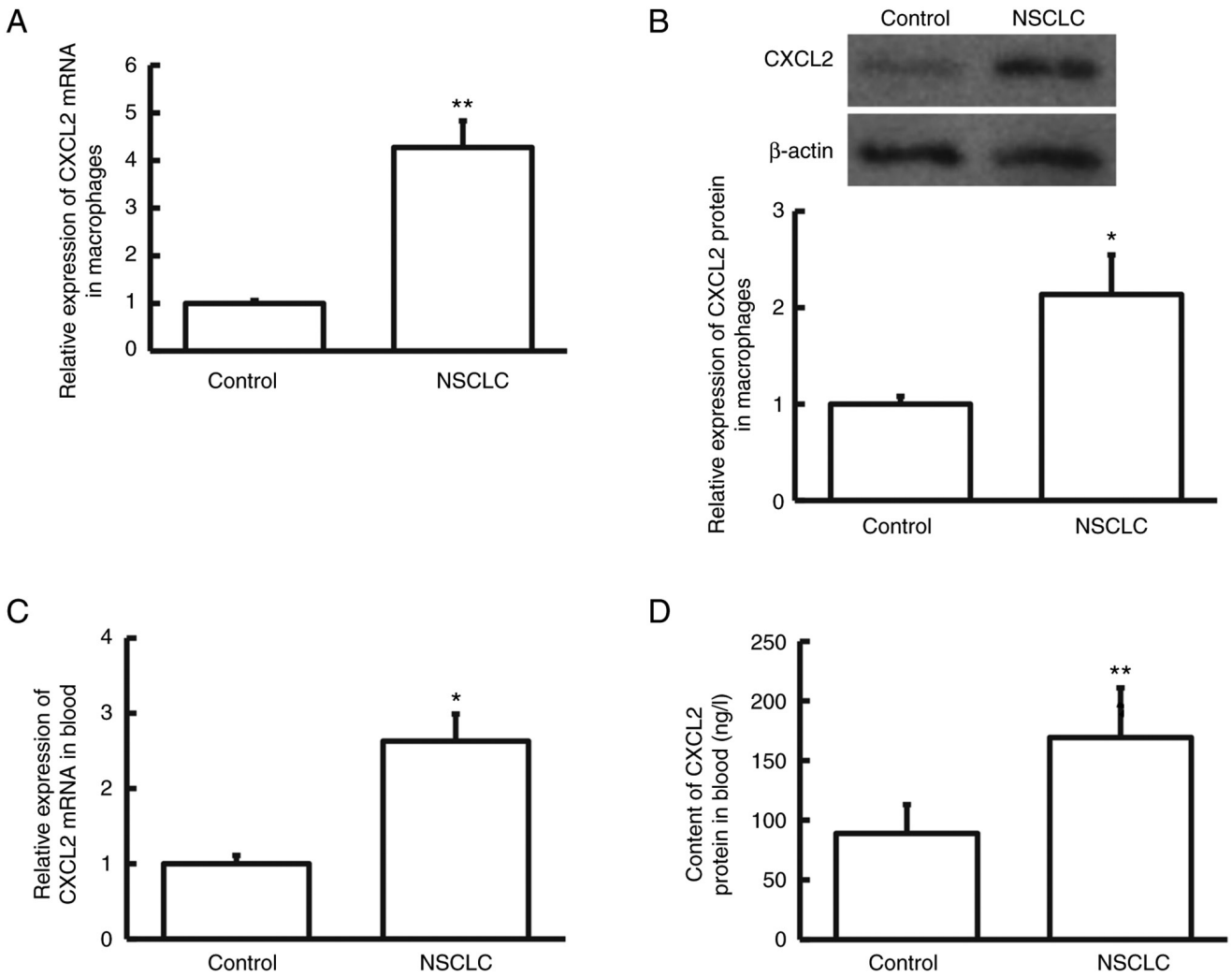


Figure 2. Expression of CXCL2 in pulmonary macrophages and peripheral blood. Relative expression of CXCL2 (A) mRNA and (B) protein in pulmonary macrophages isolated from tumor-adjacent tissues (control) and NSCLC tissues. (C) Relative expression of CXCL2 mRNA and (D) serum content of CXCL2 protein (ng/l) in peripheral blood from healthy subjects (control) and patients with NSCLC. *P<0.05 and **P<0.01 compared with control group. NSCLC, non-small cell lung cancer; CXCL2, C-X-C motif ligand chemokine 2.

Fig. 4A). To test the effect on the viability of macrophages, an MTT assay was performed. The result demonstrated that agomiRNA-27a-3p-transfected human pulmonary macrophages were less viable than agomiR-NC-transfected macrophages at 48 and 72 h (P<0.05; Fig. 4B). The protein and mRNA levels of CXCL2 in agomiRNA-27a-3p-transfected human pulmonary macrophages were relatively lower compared to those in agomiR-NC-transfected macrophages (P<0.05; Fig. 4C and D). Furthermore, the CXCL2 content in the supernatant of agomiRNA-27a-3p-transfected human pulmonary macrophages was markedly decreased compared with that in agomiR-NC-transfected macrophages (P<0.05; Fig. 4E). After treatment of human neutrophils with agomiRNA-27a-3p- or agomiR-NC-transfected human pulmonary macrophage supernatants, Transwell data revealed that the number of neutrophils with chemotaxis was markedly lower among the agomiRNA-27a-3p-transfected macrophages than among agomiR-NC-transfected macrophages (P<0.05; Fig. 4F). These findings demonstrate that miRNA-27a-3p regulates CXCL2 expression that affects the proliferation of human pulmonary macrophages.

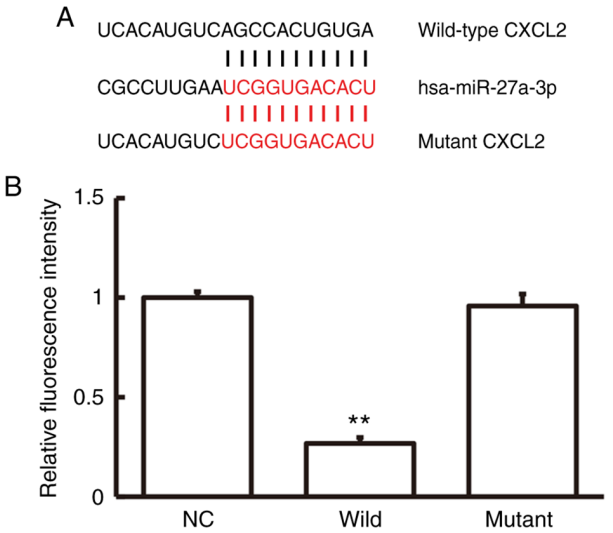


Figure 3. Direct interaction between miR-27a-3p and CXCL2 mRNA. (A) Predicted wild-type and mutant binding sites in CXCL2 mRNA. (B) Fluorescence intensity tested in dual-luciferase reporter assay. **P<0.01 compared with NC group. CXCL2, C-X-C motif ligand chemokine 2; miR, microRNA; NC, negative control.

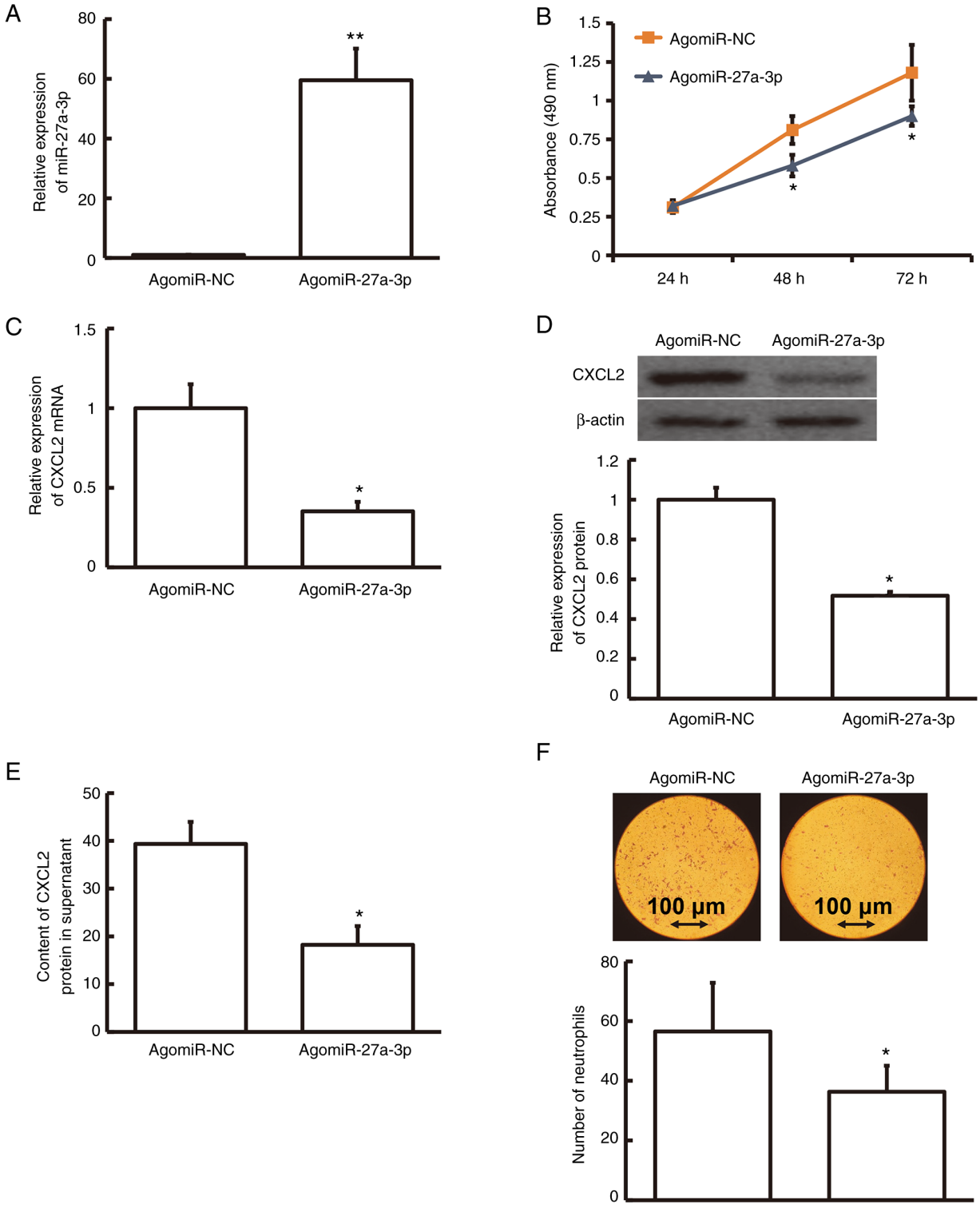


Figure 4. Effect of miR-27a-3p overexpression on the proliferation of macrophages and CXCL2 expression. (A) Relative expression of miR-27a-3p in macrophages transfected with agomiR-NC or agomiR-27a-3p. (B) Proliferation of macrophages transfected with agomiR-NC or agomiR-27a-3p. (C) Relative expression of CXCL2 mRNA in macrophages transfected with agomiR-NC or agomiR-27a-3p. (D) Relative expression of CXCL2 protein in macrophages transfected with agomiR-NC or agomiR-27a-3p. (E) Content of CXCL2 protein in culture supernatant of macrophages transfected with agomiR-NC or agomiR-27a-3p. (F) Chemotaxis of human neutrophils induced by supernatant of macrophages transfected with agomiR-NC or agomiR-27a-3p (scale bars, 100 $\mu$m). $^{*}$P<0.05 and $^{**}$P<0.01 compared with agomiR-NC group. CXCL2, C-X-C motif ligand chemokine 2; miR, microRNA; NC, negative control; agomiR, miR agonist.

## Discussion

Approximately 80-85% of all LC cases are NSCLC and the five-year overall survival rate is only 16% (28). It is particularly crucial to explore the molecular mechanisms of LC occurrence and to find novel specific diagnostic and therapeutic targets. The mechanisms of miRNA-27a-3p action may be complex and several downstream targets have been

identified. It has been reported that miRNA-27a-3p overexpression may downregulate the protein level of BTG2 and subsequently activate c-myc via the ERK/MEK/Ras pathway, thus inhibiting the apoptosis of gastric cancer cells (29). In addition, miRNA-27a-3p is downregulated in HCC tissue and reduces the expression of dual specificity phosphatase 16, thus inhibiting the growth, invasion and migration of HCC cells (13). miRNA-27a-3p is upregulated in colorectal cancer and promotes colorectal cancer proliferation by modulating β-catenin/Wnt signal transduction (30). miRNA-27a is also overexpressed in cervical cancer and promotes the malignant behavior of cervical tumor cells by downregulating the levels of sprouty2, prohibitin and forkhead box O1 (31). However, miRNA-27a has an antitumor effect in certain other tumor types. For instance, the C allele in rs895819, a genetic polymorphism of miRNA-27a, was indicated to reduce the risk of BC in Iranians, thereby protecting against this disease (32). miRNA-27a exerts a tumor-suppressor effect on acute leukemia by regulating the expression of 14-3-θ, a family member of anti-apoptotic proteins (33).

In the present study, it was observed that miRNA-27a-3p was upregulated in NSCLC tissues and downregulated in pulmonary macrophages and peripheral blood. Thus, it may be speculated that differential miRNA-27a-3p expression exerted different roles in patients with early-stage NSCLC. Considering the key roles of inflammation in early NSCLC, the expression of miRNA-27a-3p and CXCL2 in macrophages was examined. Bioinformatics prediction suggested that CXCL2 was directly targeted by miRNA-27a-3p; thus, the expression of CXCL2 was measured in the corresponding samples. The present findings highlighted that the expression trend of miRNA-27a-3p was contrary to that of CXCL2, which was in good compliance with the negative regulation of microRNA on mRNA. Furthermore, the DLR assay demonstrated that miRNA-27a-3p directly targeted CXCL2 mRNA. Due to the low expression of miRNA-27a-3p in macrophages, miRNA-27a-3p was overexpressed in macrophages by plasmid transfection. It was observed that the proliferation rate of macrophages decreased significantly, suggesting that miRNA-27a-3p exerts a regulatory effect on macrophage immune responses in patients with early NSCLC.

CXCL2 is a member of a chemokine family that regulates inflammatory response and injury repair and also participates in important physiological functions, such as cytoskeleton reconstruction, cell migration and immune response (34). CXCL2 is a proto-oncogene that may involve in the interaction between tumor and immune cells, and its overexpression has non-negligible roles in angiogenesis, tumor formation and metastasis. In esophageal cancer, CXCL2 gene knockout may significantly inhibit cisplatin-induced apoptosis, mainly by delaying the activation of caspase. In addition, the serum level of CXCL2 in patients with esophageal cancer is noticeably increased and the degree of increment is positively related to tumor size, TNM staging and the degree of lymph node diffusion (35). CXCL2 overexpression is also observed in other tumor types, such as OC, endometrial cancer, bladder cancer, HCC and gastrointestinal stromal tumor (36-39). Numerous chemokines are also involved in tumorigenesis and malignant transformation. For instance, chemokine-mediated angiogenesis is involved in the malignant transformation of tumors (40). The expression levels of CXCL1, CXCL2 and CXCL3 are significantly increased in human melanoma; knockout of these three genes in mouse cells significantly reduced the angiogenesis and growth rate of melanoma (41). In a tumorigenic experiment of esophageal cancer cells in mice, temozolomide effectively inhibited the growth rate of the tumor and improved the survival rate of mice. *In vitro* experiments indicated that temozolomide significantly reduced the expression of CXCL2 (42), suggesting that CXCL2 has an essential role in tumor progression. CXCL2 is overexpressed in OC tissues and overall survival analysis revealed that CXCL2 is related to metastasis and unfavorable survival of patients with OC (43). Another study suggested that the serum level of CXCL2 in patients with HCC is higher than that in patients with benign liver tumor or that in healthy subjects, and its overexpression is associated with TNM stage, tumor size, vascular embolism and Edmondson grade (44).

In the present study, it was first observed that the expression of miR-27a-3p was upregulated in tumor tissues and downregulated in pulmonary macrophages and blood of patients with early NSCLC. It may be speculated that these differences have a role in the processes of early NSCLC. Considering the key role of inflammation in early NSCLC, the study then focused on macrophages. After overexpression of miR-27a-3p, not only a significant downregulation of CXCL2 expression in macrophages was observed, but also a significant reduction of CXCL2 released into the cell supernatant. As CXCL2 is a chemokine, the supernatant of cells transfected with agomiR-27a-3p, or agomiR-NC as the control, was used to induce the chemotaxis of human neutrophils. The results were consistent with the speculations. It was indicated that in early NSCLC, the expression of miR-27a-3p in macrophages is decreased, resulting in increased release of CXCL2, so as to recruit more immune cells in the early stage of LC and participate in early tumor immunity.

In the present study, miRNA-27a-3p overexpression not only significantly decreased CXCL2 expression in macrophages, but also significantly reduced the content of CXCL2 released into the cell supernatant. As CXCL2 is a chemokine, the supernatants of cells transfected by agomiRNA-27a-3p or agomiR-NC were used to induce human neutrophil chemotaxis and the results were consistent with the expected outcomes. Macrophages transfected with agomiRNA-27a-3p had significantly inhibited chemotaxis of neutrophils. It may be speculated that in early NSCLC, the downregulation of miRNA-27a-3p in macrophages leads to an increase in CXCL2 release in order to recruit more immune cells in patients with early-stage LC to activate early tumor immunity. However, the present study has certain limitations. It was only speculated that miRNA-27a has the potential to become a biomarker. Due to individual differences and the common characteristics of miRNA, it is difficult for a single miRNA to become an independent marker. In the present study, the clinicopathological data and miRNA-27a levels exhibited no significant correlation. Therefore, no receiver operating characteristic curve was drawn.

In conclusion, the present study demonstrated that the abnormal expression patterns of miRNA-27a-3p and CXCL2 in the peripheral blood and macrophages of patients with early NSCLC may have a prospect for clinical diagnosis and may

be utilized as an outstanding auxiliary for determining the prognosis of patients with NSCLC. However, direct evidence for the interaction between miRNA-27a-3p and CXCL2 in patients with NSCLC is required in order to provide a solid foundation for the diagnosis, treatment and prevention of this disease.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

CZ and RZ contributed to the design of the study. CZ, BL, FK and SZ performed the experiments. CZ and BL analyzed the data. CZ, BL and RZ interpreted results and prepared the manuscript. FK and SZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

All procedures performed in the current study were approved by the Ethics Committee of Shandong University (no. H18026). Written informed consent was obtained from all patients or their families.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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