



## MiR-654-3p targets SRC to suppress tumor growth in non-small cell lung cancer

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### ABSTRACT

Around the world, cancer-related death is primarily caused by lung cancer all the time. MiR-654-3p plays an outstanding role in the development of cancer, but the mechanism of miR-654-3p in non-small cell lung cancer (NSCLC) is uncertain. For this purpose, a quantitative real-time polymerase chain reaction (qRT-PCR) was carried out to detect the expression of miR-654-3p and SRC mRNA. Western blot was used to estimate the level of SRC protein. The mimics enhanced miR-654-3p, while inhibitors knocked it down. Functional experiments were performed to evaluate the proliferation and migration capacities of cells. Flow cytometry assay was utilized to measure apoptosis rates and cell cycles of cells. TargetScan bioinformatics database was queried to identify the probable target gene for miR-654-3p. Dual-fluorescence assay was implemented to verify whether miR-654-3p targets SRC. Subcutaneous tumorigenesis was used to estimate the function of miR-654-3p in vivo. Results showed that low expression of miR-654-3p was found in NSCLC tissues and cells. Up-regulated miR-654-3p suppressed cell proliferation and migration, promoted apoptosis, and blocked cells in the G1 phase, while down-regulated miR-654-3p created the opposite results. Dual-fluorescence assay confirmed that miR-654-3p was directly bound to SRC. Compared with the control group, the effects of miR-654-3p were neutralized in the group, which was co-transfected with miR-654-3p mimics and SRC over-expression plasmids. In vivo, the tumor volume in the LV-miR-654-3p group was smaller than that in the control group. It was concluded that miR-654-3p acts in an anti-cancer role and suppresses tumor progression via regulating SRC, which lays a theoretical foundation for targeted therapy of NSCLC. MiR-654-3p is expected to be a new miRNA-based therapeutic target.

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### Introduction

According to Global Cancer Statistics 2020, lung cancer continues to be the leading cause of cancer-related death in the world (1). Regardless of the conspicuous progress that has been received in early diagnosis and therapeutic strategies of lung cancer in the past 20 years, there is still a long way to go. Because of advanced stage, metastasis, drug resistance, recurrence and other reasons, the clinical outcome and survival status of lung cancer remain poor, and the five-year net survival of lung cancer was disappointing, 10%-20% in most countries (2,3). NSCLC represents 85% of lung cancer cases (4), so it is crucial to explore the developmental mechanism of NSCLC.

Recently, microRNAs (miRNAs), composed of 20-24 nucleotide molecules, are mainly involved in the regulation of mRNAs degradation and protein expression levels through binding to the 3'-untranslated region (UTR) of mRNAs (5). They take part in a number of biological characteristics of lung cancer, such as cell proliferation, apoptosis, migration, invasion and cell cycle regulation (6). They also have been regarded as potential biomarkers for NSCLC. For instance, miR-218 decreased capacities of proliferation, invasion, and colony formation in lung cancer cells and repressed tumor growth in vivo (7). Patients with NSCLC who had low levels of miR-489-3p had

a poor prognosis (8). MiR-654-3p acted as an inhibitory role in nasopharyngeal carcinoma, liver cancer, thyroid cancer, ovarian cancer, colorectal cancer and spinal osteosarcoma (9-15). It could predict the prognosis of liver cancer (9). Although miR-654-3p has been found to be lowly expressed in NSCLC tissue and to inhibit tumor growth in previous studies (16), its specific mechanism is not completely clear. Therefore, exploring downstream biomolecular targets and possible signal pathways of miR-654-3p will be of great significance to the treatment of NSCLC.

### Materials and Methods

#### Cell lines

Human lung cancer cell lines (A549, H1299, SK-MES-1) and normal lung epithelial cells (BEAS-2B) were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China) and cultured in F-12K, RPMI-1640 and MEM medium (Procell, Wuhan, China) respectively, with 10% fetal bovine serum (Procell, Wuhan, China) and 100 U/ml penicillin and 100 µg/ml streptomycin (Procell, Wuhan, China) at 37°C with 5% CO<sub>2</sub>.

#### Cell transfection and infection

H1299 cells were transfected with miR-654-3p mimics (RiboBio Co., Ltd., Guangzhou, China), miR-654-3p

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inhibitors (RiboBio Co., Ltd., Guangzhou, China), and plasmids constructed with the gene encoding human SRC (Public Protein/Plasmid Library, Jiangsu, China) respectively. In contrast, the mimic control, inhibitor control, and SRC control plasmid served as the corresponding negative control. Transfection procedures were carried out with Lipo8000™ Transfection Reagent (Beyotime, Shanghai, China) under instructions.

In vivo, a Lentivirus vector over-expressing miR-654-3p called LV-miR-654-3p was applied to infect H1299 cells, against the negative control vector called LV-miR-control. Then, the cells were grown in the medium containing Puromycin with a concentration of 2 $\mu$ g/ml.

### Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was carried out to detect miR-654-3p and mRNA expression levels. The two-step qRT-PCR of miRNA was performed with miRcute Plus miRNA First-Strand cDNA Kit and miRcute Plus miRNA qPCR Kit (SYBR Green) (TIANGEN BIOTECH Co., Ltd., Beijing, China), while that of mRNA were implemented by using HiScript III RT SuperMix for qPCR (+gDNA wiper) and Taq Pro Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China). According to manufacturer protocol, the procedures were implemented at QuantStudio 6 Flex Real-Time PCR Software (Thermo Fisher Scientific, Inc., United States). The experimental data were analyzed by the relative quantitative method ( $2^{-\Delta\Delta Ct}$ ). Referencing standards were U6 for miRNA and GAPDH for mRNA. The forward primers of U6 and miR-654-3p were provided by TIANGEN BIOTECH Co., Ltd. (cat. no. CD201-0452 and CD201-0145; Beijing, China), and the primer sequences of SRC and GAPDH were designed as shown in Figure 1A.

### Western blot

The lysis of cells and tissue was accomplished by using the Radio Immunoprecipitation Assay (RIPA; Solarbio, Beijing, China). A BCA kit (Beyotime, Shanghai, China) was used to measure protein concentration. Proteins of equal mass were added to the page gel, separated by SDS-PAGE electrophoresis buffer (cat. no. P0014D; Beyotime, Shanghai, China), and transmitted to polyvinylidene fluoride membranes (PVDF; Millipore, USA). Then, membranes were blocked by QuickBlock™ Blocking Buffer (cat. no. P0252; Beyotime, Shanghai, China) and incubated overnight with primary antibodies at 4°C. The next day, membranes were incubated with horseradish peroxidase-labeled secondary antibody at room temperature for 1.5 h. Finally, the results were visualized with the ECL kit (cat. no. P0018FS; Beyotime, Shanghai, China). Primary antibodies were GAPDH Rabbit Monoclonal Antibody (1: 2,500; cat. no. AF1186; Beyotime, Shanghai, China) and anti-SRC recombinant rabbit monoclonal antibody (1:2, 500; cat. no. ET1702-03; Huabio, Hangzhou, China). GAPDH acted as a loading control. Data were analyzed with Image-J software.

### Cell proliferation assay

Cell proliferation ability was detected by using a Cell counting kit-8 (CCK8; Sigma-Aldrich LLC, USA). H1299 cells were seeded into 96-well plates at 3000 cells/well, transfected with miRNA mimics, inhibitors, SRC plasmids,

or the corresponding negative controls, and detected at 0, 24, 48 and 72 h after transfection. The optical density (OD) of cells was measured at 450 nm on a microplate reader (Thermo Fisher Scientific, Inc., United States).

### Colony formation assay

Cells were seeded into 6-well plates (400 cells/well) and were cultured for 12 days. After discarding the culture medium, the cells were fixed with 4% paraformaldehyde for 20 minutes, then stained with 0.1% crystal violet for 20 minutes at room temperature. Finally, we took pictures and counted the number of colonies. Data were analyzed with Image-J software.

### Cell migration assay

Transwell chambers were used to assess the migration abilities of cells. A total of  $4 \times 10^4$  cells were suspended in the upper chambers, while the lower chambers were filled with the medium containing 20% FBS as a chemo-attractant. And cells were incubated for 48 h at 37°C with 5% CO<sub>2</sub>. Then, the cells were fixed with 4% paraformaldehyde for 20 minutes, followed by staining with 0.1% crystal violet for 20 minutes at room temperature. Finally, cells in the upper chambers were gently scraped off with cotton swabs, while cells in the lower chambers were taken for pictures with an inverted microscope (x100; Leica, Wetzlar, Germany).

### Flow cytometry assay

Initially, trypsin solution without EDTA was used to collect cells. Secondly, cells were stained with Annexin V-APC/7-AAD Apoptosis Detection Kit (KeyGEN BIOTECH Co., Ltd., Jiangsu, China) and Cell Cycle Kit (Beyotime, Shanghai, China). Lastly, cells were detected on a flow cytometer (Agilent Technologies, Inc., United States), following the instructions of the manufacturer. Data were analyzed with NovoExpress1.5.6.

### MiRNA target prediction and differential expression analysis

The target genes of miR-654-3p were predicted by using TargetScan (<http://targetscan.org/>). The gene expression difference between cancer tissue and normal tissue was analyzed by Ualcan (<http://ualcan.path.uab.edu/>).

### Luciferase reporter assay

Wild-type (WT) or mutant (MUT) SRC 3'-UTR sequences were inserted into the vector pmirGLO, to form pmirGLO-SRC-wild-type (SRC-WT) and pmirGLO-SRC-mutant (SRC-MUT). H1299 cells were cultured in 24-well plates ( $8 \times 10^4$  cells/well), divided into four groups, and co-transfected respectively with SRC-WT plasmids+miR-654-3p mimics, SRC-WT plasmids+miR-654-3p mimic control, SRC-MUT plasmids+miR-654-3p mimics, SRC-MUT plasmids+miR-654-3p mimic control. The concentration of SRC-WT and SRC-MUT was 500 ng/well, and the mimics and mimic control were with a concentration of 10 pmol/well. Transfection procedures were accomplished by using Lipofectamine 2000 Reagent (Invitrogen, CA, USA). Forty-eight hours later, the luciferase activity values were detected with a Dual-Glo Luciferase Assay kit (Promega, USA), and the relative fluorescence intensity was calculated.

**Mice model**

Ten mice were randomly divided into two groups. They were from the Laboratory Animal Center of North Sichuan Medical College (Nanchong, China), between the ages of 4 and 6 weeks. A total of  $3 \times 10^6$  cells were suspended in 100  $\mu$ L PBS and injected subcutaneously to the right flanks of each mouse. The volume of the tumors was measured every four days and calculated with the formula:  $V = (\text{length} \times \text{width}^2)/2$ . The mice were sacrificed 36 days after injection. Tumor tissues were removed for Western blot and other experiments. All the animals and experiments were executed in accordance with the Guidelines for Care and Use of Laboratory Animals and were approved by the Ethics Committee of North Sichuan Medical College.

**Statistical analysis**

Data were presented as the mean  $\pm$  SD and analyzed with SPSS 23.0 software (IBM, Inc., USA). One-way analysis of variance (ANOVA), a least significant difference (LSD), Games-Howell, Independent-Samples T-test and Non-parametric test were used for statistical analysis. Pictures were made with GraphPad Prism 8.0 version (GraphPad Software, San Diego, CA, USA).  $P < 0.05$  was considered to indicate a statistically significant.

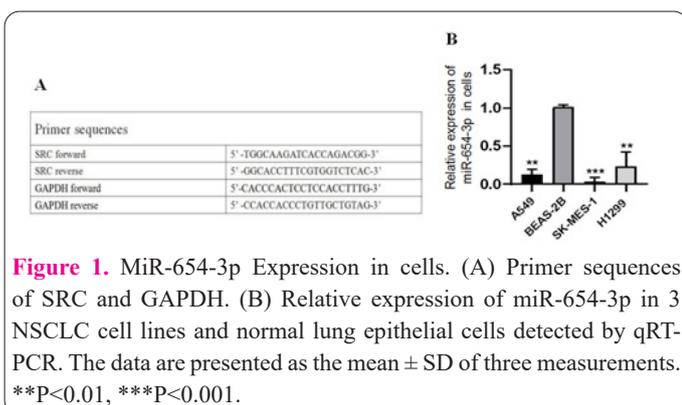
**Results**

**MiR-654-3p expression in NSCLC cell lines and BEAS-2B**

Based on previous research, the expression of miR-654-3p was lower in NSCLC tissue compared to normal lung tissue, and there was a negative correlation between miR-654-3p expression level and tumor size (16). Subsequently, we detected the expressions of miR-654-3p in NSCLC cell lines (A549, H1299, SK-MES-1) and normal lung epithelial cells (BEAS-2B). Compared with normal lung epithelial cells, the expressions of miR-654-3p decreased in the above NSCLC cell lines (Figure 1B,  $P < 0.01$ ).

**MiR-654-3p affected cell cycles, proliferation, migration and apoptosis**

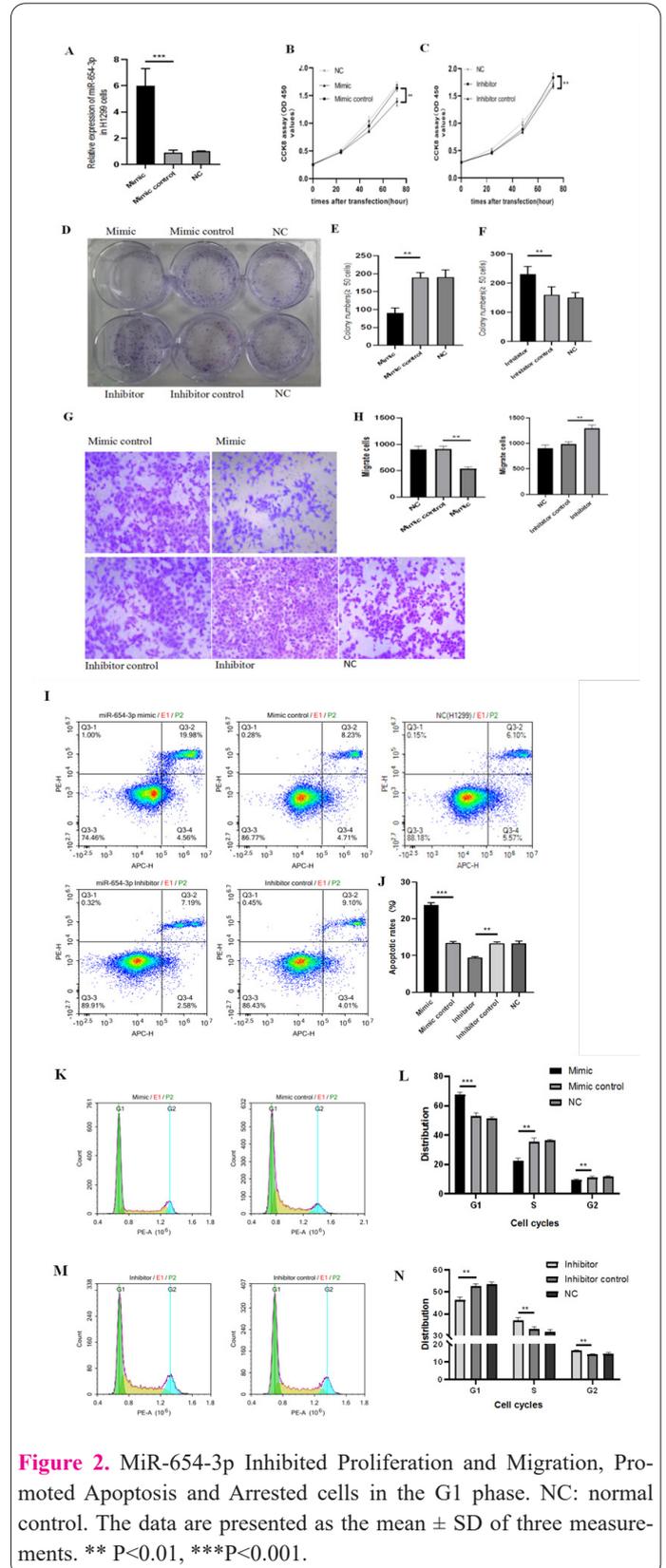
A series of biological behavior experiments were conducted on NSCLC cells to determine whether miR-654-3p participates in cell proliferation, colony formation, apoptosis, migration, and cell cycle. We used miR-654-3p mimics and inhibitors to regulate miR-654-3p expression in H1299 cells. The results were shown in Figures. Compared with the mimic control group, the average OD value of cells was lower in the mimic group (Figure 2A,  $P < 0.01$ ). Compared with the inhibitor control group, the average



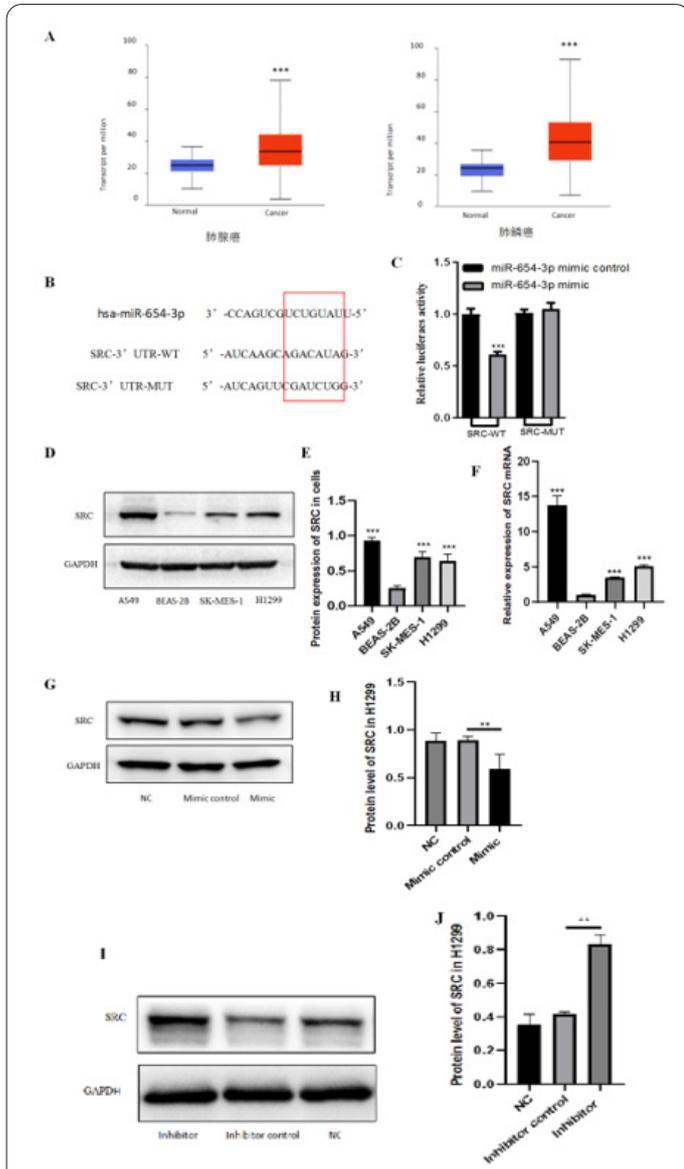
OD value of cells was higher in the inhibitor group (Figure 2B,  $P < 0.01$ ). The average number of colonies and cells migrating to the lower chambers were decreased in the mimic group (Figure 2C-F,  $P < 0.01$ ). Flow cytometry assay demonstrated the proportion of apoptotic cells and the percentage of cells in the G1 phase were increased in the mimic group (Figures 2G-J,  $P < 0.01$ ). However, the inhibitor group did the opposite effects (Figures 2C-J,  $P < 0.01$ ).

**MiR-654-3p acted by directly targeting SRC**

In order to investigate the mechanism of miR-654-3p,



**Figure 2.** MiR-654-3p Inhibited Proliferation and Migration, Promoted Apoptosis and Arrested cells in the G1 phase. NC: normal control. The data are presented as the mean  $\pm$  SD of three measurements. \*\*  $P < 0.01$ , \*\*\* $P < 0.001$ .



**Figure 3.** MiR-654-3p Acted by Directly Targeting SRC. (A) Predicted target binding sites of miR-654-3p and designed mutated sequences. (B) Expression of SRC in Lung adenocarcinoma and lung squamous cell carcinoma from Ualcan database. NC: normal control. The data are presented as the mean ± SD of three measurements. \*\* P<0.01, \*\*\*P<0.001.

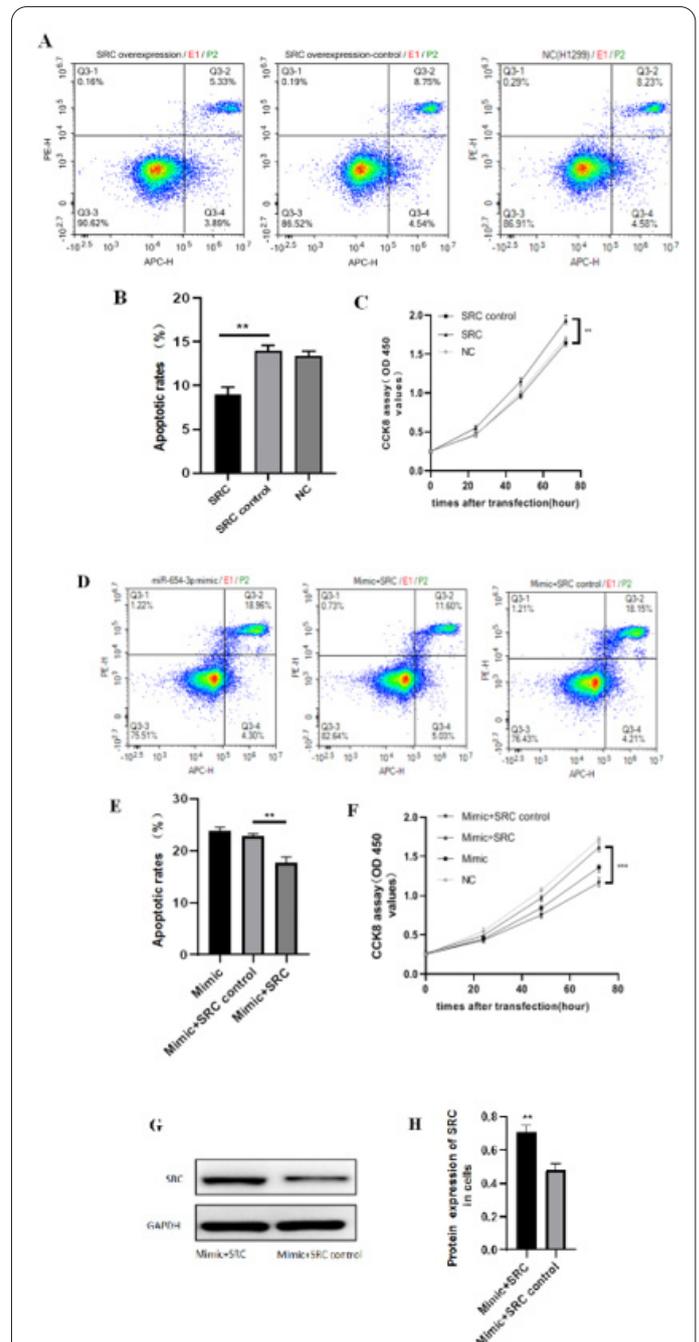
we used the TargetScan bioinformatics database to identify the probable target molecules of miR-654-3p (Figure 3A). Taking into account the results of previous studies and the Ualcan database, SRC was selected as the target gene for further investigation. SRC was a proto-oncogene that encodes a tyrosine kinase, and there was a greater expression of SRC mRNA in NSCLC tissue compared to normal lung tissue (Figure 3B, P<0.01), which was also related to the tumor stage. We used qRT-PCR and Western blot to detect the expression levels of SRC mRNA and SRC protein in cells. As shown in the Figures, the expression level of SRC was significantly increased at both RNA (Figure 3C, P<0.01) and protein levels (Figure 3D, 3E, P<0.01) in NSCLC cell lines, compared with that in normal lung epithelial cells. Dual-fluorescence assay was carried out to verify the relationship between miR-654-3p and SRC. As a result, compared with the other groups, the luciferase activity in the group co-transfected with SRC-WT plasmids and miR-654-3p mimics was weakened (Figure 3F, P<0.01). Additionally, the Western blot assay showed that

the level of SRC protein was decreased in cells transfected with miR-654-3p mimics (Figure 3G, 3H, P<0.01), while that was increased in cells transfected with miR-654-3p inhibitors (Figure 3I, 3J, P<0.01).

### Up-regulated SRC neutralized the effects of miR-654-3p

We transfected plasmids into H1299 cells to evaluate the functions of SRC. As shown in Figures, the proliferation ability of cells was elevated (Figure 4A, P<0.01) and the apoptotic proportion of cells was reduced (Figure 4B, 4C, P<0.01) in the group transfected with SRC over-expression plasmids, compared with that in SRC control group.

Then, in order to confirm the role of SRC in the miR-654-3p-associated anti-cancer mechanism and prove that SRC is the functional target gene of miR-654-3p, miR-



**Figure 4.** Up-regulated SRC Neutralized the Effect of miR-654-3p. NC: normal control. The data are presented as the mean ± SD of three measurements. \*\* P<0.01, \*\*\*P<0.001.

654-3p mimics and SRC plasmids were co-transfected into H1299 cells. Compared with the miR-654-3p mimic group, the proliferation ability of cells was enhanced (Figure 4D,  $P<0.01$ ) and the apoptotic proportion of cells was reduced (Figure 4E, 4F,  $P<0.01$ ) in the co-transfection with miR-654-3p mimics and SRC over-expression plasmids group. In addition, the Western blot assay revealed the SRC protein level of cells was elevated in the co-transfection with miR-654-3p mimics and SRC over-expression plasmids group (Figure 4G, 4H,  $P<0.01$ ).

### MiR-654-3p suppressed tumorigenesis in vivo

For examining the effects of miR-654-3p on the growth of NSCLC in vivo, the xenograft tumor model was established by subcutaneous injection of H1299 cells into the right flanks of mice. The ten mice were randomly divided into two groups: one was injected with cells infected by LV-miR-654-3p, and the other with cells infected by LV-miR-control.

Compared with the control group, the volume and mass of the tumor declined in the LV-miR-654-3p-infected group (Figure 5A, 5B,  $P<0.01$ ). Besides, Western blot assay showed that the SRC protein level of tumor tissue was lower in the LV-miR-654-3p-infected group (Figure 5C, 5D,  $P<0.05$ ).

### Discussion

Despite significant progress has been made in early diagnosis and therapeutic strategies for lung cancer in the past 20 years, lung cancer is still the dominant cause of cancer-related death and a severe threat to human health. Advanced stage, metastasis, drug resistance and recurrence are the major challenges in lung cancer treatment.

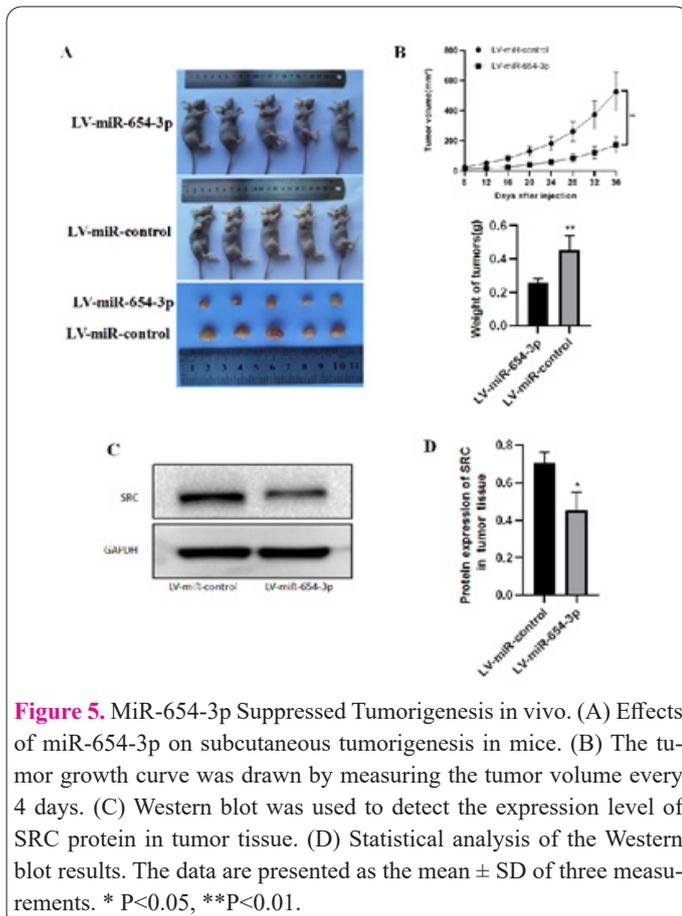
MiRNAs, composed of 20-24 nucleotide molecules, have become a hot research topic. Recently, increasing evidence has shown that miRNAs are critical regulators of gene expression and promising therapeutic targets for various malignancies (17,18). The targeted therapy, which is based on miRNAs, shows some promise from preclinical and clinical trials (19,20).

MiR-654-3p has been reported to act as a tumor suppressor in a variety of cancers, including nasopharyngeal carcinoma, colorectal carcinoma, lung cancer, liver cancer, thyroid carcinoma, ovarian carcinoma and osteosarcoma (9-16). Nevertheless, the role of miR-654-3p is still unclear in NSCLC. In particular, the effects of miR-654-3p on cell cycle, migration and cloning have not been reported in NSCLC.

In our study, we perfected the functional experiments related to biological behavior in H1299 cells. They indicated that increasing miR-654-3p levels inhibited cell cloning, proliferation, migration, arrested cells in the G1 phase, and induced cell apoptosis, whereas decreasing miR-654-3p levels created the opposite effects.

As is well known, the functions of miRNAs, such as promoting the ability of proliferation, migration and invasion in tumor cells (21), are mostly triggered by the specific binding between miRNA and 3'-UTR of the target mRNA. When they interact with each other, it may lead to the degradation of mRNA and a change in target protein levels (13). The post-transcriptional repression is considered the canonical mode of miRNA-mediated gene regulation (5). A single miRNA can target multiple downstream mRNAs and affect their expression levels (18). A single miRNA might have different functions in different cells, or even the effects are opposite. For instance, miR-654-3p binds to CREB1, affects the CREB1/PSEN1 axis and hinders the malignant progression in squamous cell carcinoma of the paranasal sinus (SNSCC) (9). In ovarian cancer cells, miR-654-3p can bind to AKT3 and inhibit cell proliferation, invasion and spheroid formation (13). In gastric cancer, miR-654-3p targets P21 to promote cell proliferation (22).

To better understand the work of miR-654-3p in NSCLC, we used the TargetScan website database to infer probable molecular targets. Considering the results of the Ualcan database and preceding studies, we selected SRC for the following experiments. SRC, a classic proto-oncogene that encodes a tyrosine kinase, was discovered in the 1970s. SRC was highly expressed in many malignant tumors, including NSCLC (23), and participates in various biological behaviors such as tumor cell proliferation, epithelial-to-mesenchymal transition, migration and invasion (24,25). SRC inhibitors have been developed for clinical treatment for many years. In our study, we observed that compared with normal lung epithelial cells (BEAS-2B), NSCLC cell lines expressed higher levels of SRC. This finding was consistent with the result reported in tissues by the Ualcan database and previous research. Dual-fluorescence assay confirmed that miR-654-3p is directly bound to the 3'-UTR of SRC mRNA. Through Western blot, we observed that the level of SRC protein decreased in H1299 cells transfected with miR-654-3p mimics, while it increased in the cells transfected with inhibitors. By co-transfecting miR-654-3p mimics and SRC over-expression plasmids into H1299 cells, we found that the effects of up-regulated miR-654-3p on inhibiting proliferation and pro-



**Figure 5.** MiR-654-3p Suppressed Tumorigenesis in vivo. (A) Effects of miR-654-3p on subcutaneous tumorigenesis in mice. (B) The tumor growth curve was drawn by measuring the tumor volume every 4 days. (C) Western blot was used to detect the expression level of SRC protein in tumor tissue. (D) Statistical analysis of the Western blot results. The data are presented as the mean  $\pm$  SD of three measurements. \*  $P<0.05$ , \*\* $P<0.01$ .

moting apoptosis in cells were reversed, as the level of SRC protein increased. These results indicated that SRC indeed interacts with miR-654-3p, and SRC is a functional target gene of miR-654-3p.

Furthermore, studies *in vivo* showed that up-regulated miR-654-3p reduced the level of SRC protein and decelerated the growth of xenografts. These findings were similar to those results *in vitro*.

Our research aims to evaluate the biological functions and explore the downstream targeted gene of miR-654-3p. we found and verified that SRC is a new functional target of miR-654-3p in NSCLC.

There are some deficiencies. We did not detect the levels of proteins related to cell cycles and apoptosis, and did not determine the expression of miR-654-3p in NSCLC patients' blood. Emerging evidence suggests that miRNAs in the blood can be used as potential and noninvasive biomarkers for the diagnosis and prognosis of some diseases, such as colorectal cancer (26), breast cancer (27), and lung cancer (28).

Therefore, in future research, apart from improving the detection of related proteins further to verify the effects on cell cycle and apoptosis, we also should collect patients' serum, and analyze the relationship between miR-654-3p and disease, so as to provide a theoretical basis for whether miR-654-3p is a potential biomarker.

To sum up, our study confirmed that miR-654-3p acted as an anti-cancer role in NSCLC, including inhibiting cell proliferation, arresting cells in the G1 phase, inhibiting migration and promoting apoptosis. MiR-654-3p suppressed tumor growth by targeting SRC in NSCLC. Our findings provide new insight into NSCLC development and a novel miRNA-based strategy for NSCLC treatment.

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