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MiR-593-5p inhibited proliferation and migration of lung adenocarcinoma by targeting ICAM-1

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Abstract. – OBJECTIVE: The aim of this study was to investigate the role of microRNA-593-5p (miR-593-5p) in the development of lung adenocarcinoma (LA).

PATIENTS AND METHODS: The expression level of miR-593-5p in LA tissues and cell lines was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Luciferase reporter gene assay and Western blot were performed to evaluate the interaction between miR-593-5p and intercellular cell adhesion molecule-1 (ICAM-1). Furthermore, the effects of the miR-593-5p/ICAM-1 axis on A549 cells were determined by MTS, colony formation assay, and transwell assay, respectively.

RESULTS: MiR-593-5p was significantly downregulated in both clinical samples and cell lines. The bioinformatics analysis predicted that miR-593-5p could complementarily bind to the 3'-UTR of ICAM-1. Luciferase reporter gene assay confirmed that ICAM-1 was the direct target of miR-593-5p. Western blot results demonstrated that miR-593-5p could effectively reduce the protein expression of ICAM-1 in cells. *In vitro* experiments indicated that the proliferation and migration of A549 cells were significantly inhibited by miR-593-5p transfection. However, the overexpression of ICAM-1 could effectively reverse the inhibitory effects of miR-593-5p *in vitro*. These results indicated that the inhibitory effects of miR-593-5p on LA were achieved by regulating ICAM-1 expression.

CONCLUSIONS: MiR-593-5p/ICAM-1 axis might be a potential therapeutic target for the diagnosis and treatment of LA.

Key Words:
MicroRNA-593-5p (miR-593-5p), Lung adenocarcinoma (LA), Intercellular cell adhesion molecule-1 (ICAM-1).

Introduction

Lung cancer is one of the most common malignancies with the fastest increasing morbidity and mortality rates. Meanwhile, it is the main threat to the health and life of humans in the world. Non-small cell lung cancer (NSCLC) accounts for about 85-90% of all lung cancer patients[1]. Lung adenocarcinoma (LA) is the most common pathological type of NSCLC, which is also the main type among female and non-smoking male patients with lung cancer[2]. With the improvement of the medical level, the life quality of cancer patients has greatly ameliorated. However, due to the reason that early symptoms of lung cancer are not evident, many patients missed the best treatment time when diagnosed. At present, the 5-year survival rate of patients with LA is about 15-20%, and the overall survival still remains low[3,4]. Therefore, the exploration of more sensitive and specific diagnosis and treatment methods is particularly necessary.

Micro ribonucleic acids (miRNAs) are a class of non-coding RNAs with 21-25 nucleotides in length[5]. MiRNAs can downregulate the expression of the target genes by interfering in the translation of the target mRNAs[6]. MiRNAs participate in almost all basic signaling pathways *in vivo*. Meanwhile, they can regulate the expression of nearly 1/3 of human genes, including various important tumor-related genes[7]. The first miRNA was discovered in 1993[8]. Currently, miRNAs have been widely studied as research hotspots, especially in the field of cancer[9-12].

As a member of the miRNA family, miR-593-5p is abnormally expressed in many human malignant tumors, including gastric cancer[13], esophageal cancer[14], and tongue cancer[15]. However, few researches have focused on the relationship between miR-593-5p and LA. In this study, we first detected the expression of miR-593-5p in LA tissues and adjacent normal tissues. Subsequent-

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ly, we constructed miR-593-5p overexpression cells to explore the role of miR-593-5p in the occurrence and development of LA. Our findings might provide experimental and theoretical basis for early diagnosis and treatment of LA.

Patients and Methods

LA Cases and Cells

LA tissues and adjacent normal tissues were obtained from 42 patients who received surgery at the Second Affiliated Hospital of Soochow University. Preoperative chemotherapy or radiotherapy treatment was forbidden. The collected tissue samples were preserved in liquid nitrogen for use. After all, we respected the Declaration of Helsinki. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University. Signed written informed consents were obtained from all participants before the study.

The human LA cell line (A549) and normal human lung epithelial cell line (BEAS-2B) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) complemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator with 5% CO_2.

Luciferase Reporter Gene Assay

The binding sequence of miR-593-5p at the 3'-end of intercellular cell adhesion molecule-1 (ICAM-1) was confirmed on TargetScan, miRDB, and microRNA websites. The binding sequence was mutated. Subsequently, mutant ICAM-1 (MT-type) and non-mutant ICAM-1 (WT-type) were connected with the pGL3-Basic Luciferase reporter vector. Next, the PGL3-Basic vector with mutant ICAM-1 was transfected into A549 cells after lentivirus intervention on 24-well plates. The same treatment was performed on the pGL3-Basic vector connected with non-mutant ICAM-1 according to the instructions of the Luciferase Reporter Gene Assay Kit (Promega, Madison, WI, USA). Finally, the Luciferase activity was detected by a multi-function microplate reader.

Cell Transfection

MiR-593-5p mimics and LV-ICAM-1 were synthesized and transfected to A549 cells by using Lipofectamine RNAiMAX (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Cell Grouping

Three groups were established in this study, including: miR-NC group (A549 cells transfected with negative control), miR-593-5p mimics group (A549 cells transfected with miR-593-5p mimics), and mimics + ICAM-1 group (A549 cells transfected with miR-593-5p mimics and si-ICAM-1).

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

TRIzol was added in the cells to extract the total RNA under an RNase-free condition in strict accordance with RNA extraction kit (Invitrogen, Carlsbad, CA, USA). 2 μg of the total RNA was taken and diluted with 10.5 μL diethylpyrocarbonate (DEPC)-treated water (Beyotime, Shanghai, China). Subsequently, 1 μL reverse transcription primer oligo-dT (500 μg/mL) was added, followed by water bath at 70°C for 5 min and at 4°C for 5 min. Next, 4 μL 5 × Buffer, 2.5 μL deoxyribonucleoside triphosphate (dNTP) (10 mmol/L), 0.5 μL RNase inhibitor, and 0.5 μL Moloney murine leukemia virus (M-MLV) (200 U/μL) were added to make the final volume of 20 μL. After mixing, the mixture was centrifuged, followed by reverse transcription reaction. The reverse transcription conditions were as follows: 42°C for 60 min, 70°C for 15 min, and storage at 4°C. Specific qPCR conditions were as follows: 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min, for a total of 40 cycles. The relative expression level of miR-593-5p was calculated by the $2^{-\Delta\Delta Ct}$ method. U6 was used as an internal reference. The primer sequences used in this study were as follows: ICAM-1, F: 5'-CGGAGTCGCTCTCTCCTCGTC-3', R: 5'-CGTAGCGGATGTCCGCGAAGCGA-3'; miR-593-5p, F: 5'-GGCGTACAACATCTCTCGTG-3', R: 5'-AGTTGGAGTCGTCGGAAGTCAG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot Analysis (WB)

After the cells were transfected and cultured for 24 h, the medium was discarded. The cells were then washed with phosphate-buffered saline (PBS) and added with protein lysis buffer radioimmunoprecipitation assay (RIPA; Beyotime,

Shanghai, China). After centrifugation at 12000 rpm, the supernatant was collected. The concentration of the protein was determined by the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). Subsequently, the protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) *via* ice bath. After blocking with 5% BSA-Tris-Buffered Saline at room temperature for 1 h, the membranes were incubated with primary antibodies (ICAM-1, GAPDH) at 4°C overnight. On the next day, the membranes were incubated with the corresponding secondary antibody for 1 h and washed with Tris-Buffered Saline and Tween (TBST). Immunoreactive bands were exposed by the enhanced chemiluminescence (ECL) method.

Cell Proliferation

The cells in the logarithmic phase were collected and inoculated into 96-well plates at a density of 10,000 cells/well. After transfection for 24 h, the cell viability was determined by MTS colorimetry. Briefly, 15 μL MTS reagent (500 μg/mL) was added at 24, 48, 72, and 96 h, respectively, followed by culture for 2 h. Absorbance (A) at 570 nm was detected by a microplate spectrophotometer.

Colony Formation Assay

A large dish was prepared, and 10 mL medium and 800 cells were added to each well. The cells were cultured for 10-14 d under standard conditions, and the formation of colonies was observed. When there were macroscopic clones, the culture was terminated, and the medium was discarded. Thereafter, the cells were washed with PBS for 2 times and fixed with 4% paraformaldehyde (1 mL/well) for 15 min. Subsequently, the cells were rinsed slowly with running water, followed by staining with 1 mL crystal violet for 3 min. Finally, the number of formed colonies was counted and analyzed.

Cell Migration Assays

A total of 5×10^5 cells in the serum-free medium (500 μL) was added into the upper chamber. Meanwhile, the complete medium containing 10% serum (800 μL) was added into the lower chamber. After 24 h of incubation, the medium in the upper and lower chambers was discarded. The cells were washed with preheated PBS (PBS was gently pipetted to clean the lower surface of the filter membrane) for 2 times. Next, 4% paraformaldehyde (600 μL) was added to the lower chamber to immerse the lower surface of the filter membranes, followed by fixation for 15 min. Then, the transwell chambers were inverted to let the lower surface of the filter membrane face up and air-dried. A few drops of Giemsa staining solution were then dropped to the lower surface of the inverted transwell chamber for 10 min of staining. After washing with distilled water, un-migrated cells on the surface of the chambers were wiped off with a cotton ball. Finally, the migrating cells were observed under an inverted microscope, and the number of cells was counted.

Statistical Analysis

Prism 6.02 software (La Jolla, CA, USA) was used for all statistical analyses. The *t*-test was used to compare the differences between the two groups. One-way analysis of variance (ANOVA) was applied to compare the differences among different groups, followed by the post-hoc test (Least Significant Difference). All *p*-values were two-sided, and $p<0.05$ was considered statistically significant.

Results

MiR-593-5p Was Significantly Downregulated in Both LA Tissues and Cells

The expression level of miR-593-5p in 42 paired LA tissues, and normal tissues were detected by the qRT-PCR assay. The results showed that the expression level of miR-593-5p in LA tissues was significantly lower than that of adjacent normal tissues (Figure 1A). Next, we measured miR-593-5p expression in the human lung cancer cell line (A549) and normal human lung epithelial cells (BEAS-2B). As shown in Figure 1B, miR-593-5p was found lowly expressed in A549 cells, which was consistent with the results in clinical tissue samples.

ICAM-1 Was a Direct Target of MiR-593-5p in LA Cells

Subsequently, we explored the possible regulatory targets of miR-593-5p in LA. TargetScan, miRDB, and microRNA are commonly used online forecasting sites. Through prediction, we found that ICAM-1 had the binding site with miR-593-5p (Figure 2A). Therefore, ICAM-1 was screened to be our research object. Before, we

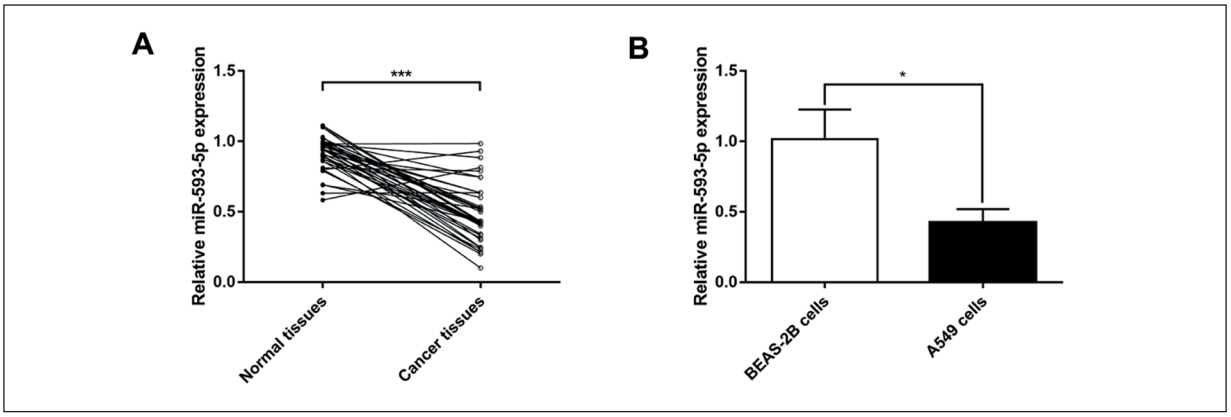

Figure 1. Expression of miR-593-5p in LA tissues and cells. **A,** Difference in the expression of miR-593-5p between LA tissues and the corresponding adjacent normal tissues. (***p<0.001). **B,** Difference in the expression of miR-593-5p between A549 cells and BEAS-2B cells. (*p<0.05).

verified the effect of miR-593-5p mimics transfection in A549 cells. QRT-PCR assay demonstrated that the transfection of miR-593-5p mimics significantly increased the expression of miR-593-5p in A549 cells (Figure 2B). Luciferase reporter gene assay (Figure 2C) indicated that miR-593-5p mimics decreased the Luciferase activity of the wild-type ICAM-1. However, it had no evident effect on mutant-type. This suggested that the expression of ICAM-1 could be regulated by miR-593-5p. To further confirm the effect of miR-593-5p on ICAM-1, we examined the expression changes of ICAM-1 in A549 cells after different treatments by Western blot. The results indicated that the expression level of ICAM-1 in A549 cells with high expression of miR-593-5p was significantly inhibited when compared with that of cells with low miR-593-5p expression. Meanwhile, the expression level of ICAM-1 increased again after the co-transfection of miR-593-5p and LV-ICAM-1 into A549 cells (Figure 2D).

MiR-593-5p Suppressed Proliferation of LA Cells

To explore the function of miR-593-5p on the proliferation of A549 cells, MTS and colony formation assay were performed. MTS results showed that the proliferation rates of A549 cells decreased remarkably by miR-593-5p mimics treatment (Figure 3A). Correspondingly, upregulating miR-593-5p significantly inhibited the tumorigenicity of A549 cells (Figure 3B). However,

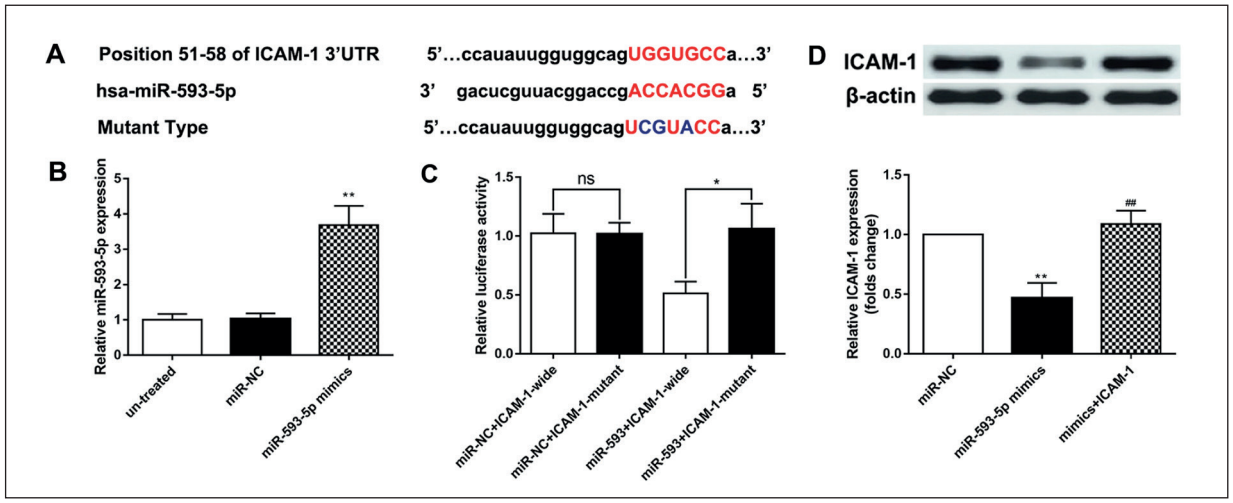

Figure 2. ICAM-1 is a direct and functional target of miR-593-5p. **A,** Diagram of putative miR-593-5p binding sites of ICAM-1. **B,** Transfection efficiency verified by qRT-PCR. (**p<0.01). **C,** Relative activities of Luciferase reporters. (*p<0.05). **D,** Protein expressions of ICAM-1 detected by Western blot. (**p<0.01 *vs.* NC group; $^{\#\#}p$<0.01 *vs.* Mimics group).

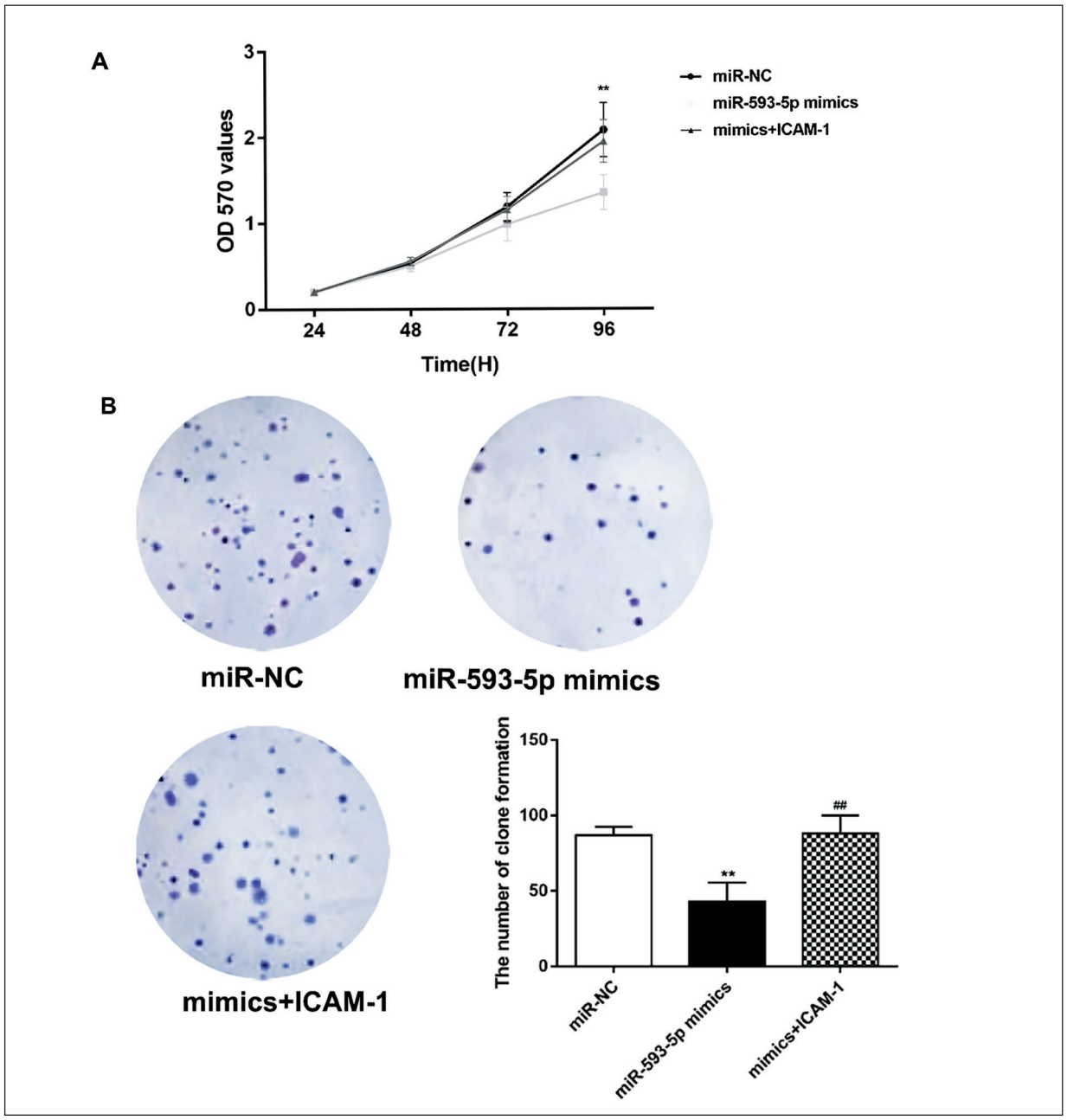

Figure 3. MiR-593-5p suppressed the proliferation of LA cells. **A,** The proliferation of LA cells detected by MTS assay after different treatments. (**p<0.01 *vs.* NC group). **B,** Assessment of colony formation **(× 40)**. (**p<0.01 *vs.* NC group; $^{\#\#}p$<0.01 *vs.* Mimics group).

when the expression of ICAM-1 was elevated at the same time, the proliferation and prognosis of A549 cells were resuscitated again.

MiR-593-5p Inhibited Migration of LA Cells

Migration can indirectly reflect the metastasis ability of tumor cells. In the present study, the transwell assay indicated that, compared with the miR-NC group, the overexpression of miR-593-5p in A549 cells significantly inhibited cell migration. However, no significant differences were observed in the migration ability of A549 cells between the miR-NC group and the mimics+ICAM-1 group (Figure 4).

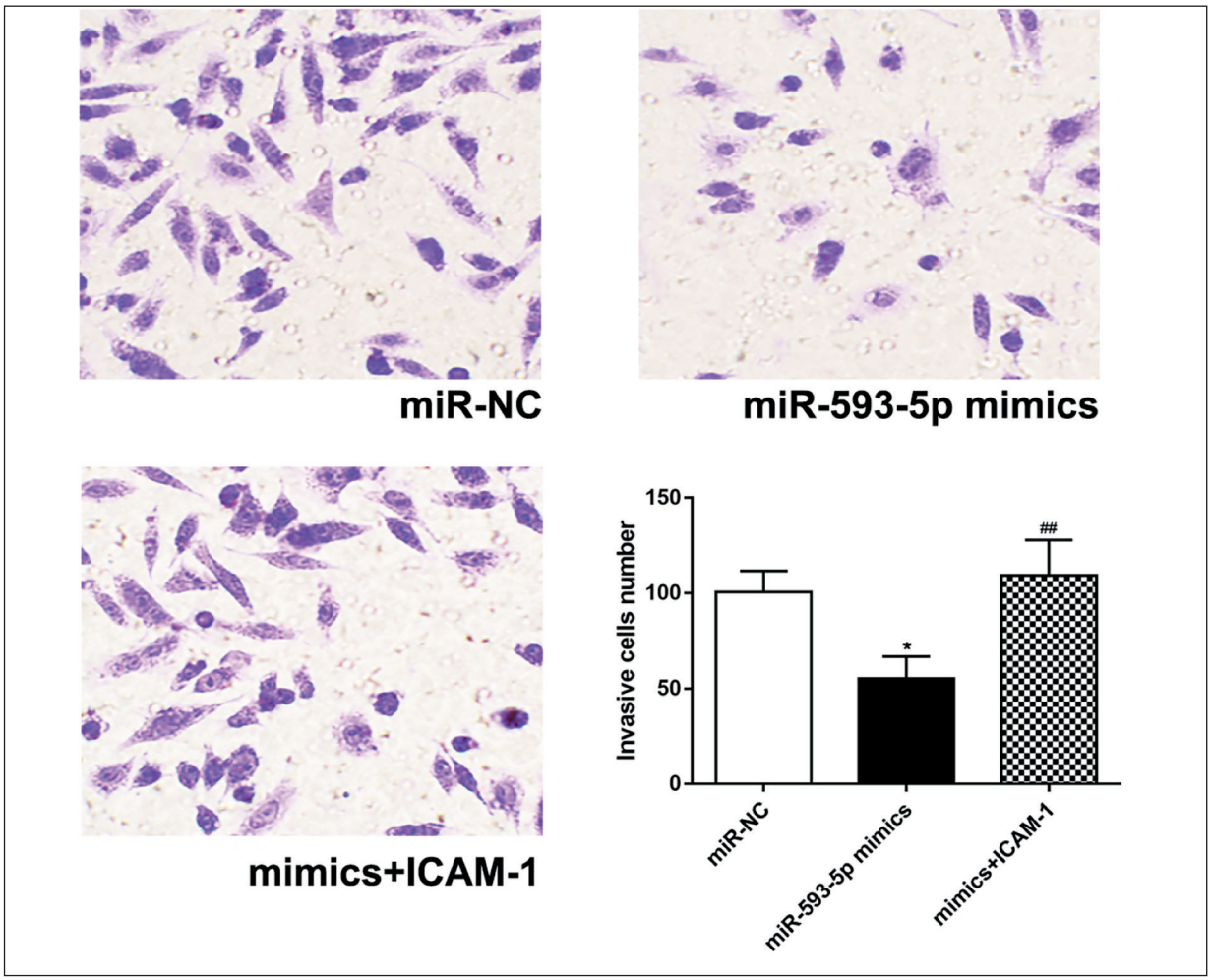

Figure 4. MiR-593-5p inhibited the migration of LA cells. The results were photographed and detected by microscope (× 200). (*p<0.05 *vs.* NC group; $^{\#\#}p$<0.01 *vs.* Mimics group).

Discussion

Currently, cancer is an insurmountable challenge all over the world. Among them, lung cancer is one of the most common malignant tumors with the fastest growth in morbidity and mortality. In recent years, lung cancer seriously threatens people's health and life quality. LA is an important subtype of lung cancer, whose incidence rate has increased year by year. LA shows poor sensitivity to chemoradiotherapy, leading to a low survival rate of patients[16]. Due to the increasing drug resistance in humans, it is urgent to fully understand the pathogenesis, development and progression of LA and to find out new effective diagnostic and therapeutic means. Various abnormally expressed miRNAs are detected in LA, which may affect the biological characteristics of LA cells[17-20].

MiRNAs can modulate the expression of nearly 1/3 human protein-related genes at the post-transcriptional level, mainly through the repression and degradation of mRNAs[21,22]. Eventually, they may regulate the growth, differentiation, invasion, and apoptosis of cells. Therefore, miRNAs have been confirmed to play important roles in the progression of tumors[23]. It has also been generally believed that carcinogenic miRNAs are overexpressed in tumors. Meanwhile, they exert relevant functions by enhancing the role of oncogenes or impairing the expression of tumor suppressor genes. On the contrary, miRNAs can be served as tumor suppressor genes to suppress the progression of malignant tumors.

In this study, miR-593-5p was found significantly downregulated in both LA tissues and

cell lines. Then, we focused on the exploration of targets for miR-593-5p in LA. The bioinformatics analysis predicted that miR-593-5p could complementarily bind to the 3'-UTR of ICAM-1. Dual-Luciferase reporter assay confirmed that ICAM-1 was the direct target of miR-593-5p. To further prove that ICAM-1 was a functional target for the biological role of miR-593-5p in LA, we examined the effect of miR-593-5p on the expression of ICAM-1 at the post-transcriptional level. Western blot results found that miR-593-5p could effectively reduce the protein expression of ICAM-1 in the cells.

Cell adhesion molecules (CAMs) play leading roles in physiological processes (including: maintenance of the normal cellular structure, immune regulation, and inflammatory response), tumor lymphatic metastasis, as well as blood circulation and metastasis[24,25]. They mainly include cadherin family, integrin family, selectin family, immunoglobulin superfamily, and hyalherin. ICAM-1 is a very important CAM in the immunoglobulin superfamily[26]. It can mediate the binding of cells to cells and extracellular matrix, thereby participating in the angiogenesis, invasion, and metastasis of tumor cells[27]. Highly expressed ICAM-1 reduces the adhesion between tumor cells, leading to the falling of tumor cells into the bloodstream. Excessive activation of highly expressed ICAM-1 increases the production of sICAM-1. Meanwhile, this competitively inhibits the immune recognition of cancer cells in the blood by ICAM-1/LFA-1 major histocompatibility complex 1 (MHC-1) antigen. Cancer cells may escape immune surveillance and easily metastasize through the blood circulation[28,29]. Therefore, ICAM-1 is highly expressed in many malignant tumors and is closely correlated with the development, progression, infiltration, and metastasis of tumors[30-34].

Conclusions

The upregulation of miR-593-5p significantly defeated the proliferation and migration of A549 cells. The overexpression of ICAM-1 could effectively reverse the inhibitory effects of miR-593-5p on cell proliferation and migration capacities. These results indicated that the inhibitory effect of miR-593-5p on LA was achieved by inhibiting ICAM-1. In conclusion, miR-593-5p/ICAM-1 axis might be a potential therapeutic strategy for the diagnosis and treatment of LA.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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