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Role of miR-579-3p in the development of squamous cell lung carcinoma and the regulatory mechanisms

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Abstract. – OBJECTIVE: The aim of this study was to explore the role of microRNA-579-3p (miR-579-3p) in the development of squamous cell lung carcinoma (SCLC). Our findings might provide new insights into the treatment of SCLC.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine the expression level of miR-579-3p in 30 pairs of CRC tissues and para-cancerous tissues. The relation between miR-579-3p expression and clinical features of SCLC patients was analyzed. MiR-579-3p expression in SCLC cells was further verified by qRT-PCR as well. In addition, the effects of miR-579-3p on the migration and invasion of SK-MES-1 cells were examined through the transwell assay.

RESULTS: QRT-PCR results revealed for the first time that miR-579-3p was significantly down-regulated in SCLC tissues. This indicated that miR-579-3p was possibly involved in the development of SCLC. According to bioinformatics prediction websites and the luciferase activity assay, macrophage scavenger receptor 1 (MSR1) was predicted and verified as the target gene of miR-579-3p. Transfection of miR-579-3p mimics significantly reduced the protein expression level of MSR1 in cells, eventually inhibiting the proliferation, invasion, and migration of SCLC cells.

CONCLUSIONS: The miR-579-3p/MSR1 axis might be a novel regulatory pathway of apoptosis, which could be used as potential therapeutic sites in SCLC.

Key Words:

Lung cancer, Squamous cell lung carcinoma (SCLC), MicroRNA-579-3p (MiR-579-3p), Macrophage scavenger receptor 1 (MSR1).

Introduction

Lung cancer is the most common malignant tumor seriously threatening human health. Early clinical symptoms of lung cancer are not significant, and there is a lack of effective screening indexes and diagnostic methods. Therefore, the morbidity and mortality rates of lung cancer rank 1st among all the malignant tumors in the world. Statistics have shown that lung cancer accounts for 13% of all new cases of cancer[1]. Non-small cell lung cancer (NSCLC) accounts for approximately 80-85% of all lung cancers, with a 5-year survival rate of about 17%[2]. The histological subtypes of NSCLC mainly include adenocarcinoma (AC) and squamous cell lung carcinoma (SCLC). It is well-known that SCLC accounts for more than 30% of NSCLC patients and is frequently occurring in the smoking crowd[3]. Currently, the treatment of NSCLC is based on the clinical stage. However, lung cancer has extremely complex biological characteristics. Meanwhile, significant differences are observed in clinical manifestations, therapeutic methods, and prognosis among different histological types of lung cancer. With the rapid development of gene detection technique and targeted molecular therapy, the importance of SCLC as an "independent" type of lung cancer has gradually been recognized[4-6]. However, due to the lack of early specific symptoms, most patients have already been in the middle-advanced stage when first diagnosed, losing the opportunity of operation. In recent years, with the deepening of scientific research and the success of clinical practice, staged achievements have been obtained in the occurrence and development, in the effective screening indexes, and in the targeted drug therapy of lung cancer. However, there are still limited scientific researches and clinical achievements of SCLC. Therefore, improving the early diagnostic efficiency of SCLC patients is of fundamental significance in improving the prognosis of patients.

Micro-ribonucleic acids (miRNAs) are a kind of endogenous, highly-conserved small non-cod-

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ing RNA molecules with about 19-24 nucleotides in length[7]. Since miRNAs were first discovered in *Caenorhabditis elegans* in 1993, they have been found in many species of plants and animals[8,9]. MiRNAs can bind to the 3' untranslated regions (3'-UTR) of target genes, eventually leading to mRNA degradation or protein translation inhibition. Furthermore, they can regulate genes at the post-transcriptional level[7]. It has been found that about 30% of mRNAs in the human body are regulated by miRNAs. Meanwhile, one single miRNA molecule can simultaneously regulate hundreds of mRNA molecules. Ultimately, they widely participate in various pathophysiological processes, including cell proliferation, differentiation, apoptosis, angiogenesis, and metabolism[10,11]. In addition, miRNAs can often serve as key regulators of cancer-related signaling pathways[12,13]. Therefore, changes in miRNA levels are associated with the occurrence and development of various human malignant tumors.

MiR-579-3p, as a member of the miRNA family, has shown strong regulatory potential. Recently, several studies[14-16] have revealed the certain regulatory ability of miR-579-3p. However, the exact role of miR-579-3p in tumors still needs further exploration. The aim of this study was to explore the role and mechanism of miR-579-3p in SCLC.

Patients and Methods

Tissue Samples and Cell Lines

SCLC tissues were collected from 30 patients who underwent surgical treatment in our hospital from December 2016 to October 2017. At the same time, paired paraneoplastic tissues were obtained from patients. Collected tissues were quickly frozen in liquid nitrogen after isolation. No radiotherapy or chemotherapy was performed in any patient before surgery. Meanwhile, the pathological diagnosis was SCLC, while paraneoplastic histopathology was negative. After all, the Declaration of Helsinki should be mentioned and respected. This investigation was approved by the Ethics Committee of Ganzhou People's Hospital. Signed written informed consents were obtained from all participants before the study.

SCLC cell lines (SK-MES-1, NCL-H520, and NCL-H226) and normal lung tissue epithelial cell line (BEAS-2B) were cultured in Modified Eagle's medium (MEM) (added with 1% GlutaMAX and 1% sodium pyruvate) containing 10% of fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator with 5% CO_2 at 37°C under saturated humidity. The cell passage was performed when appropriate. Subsequently, cells in the logarithmic growth phase were collected for experiments.

Target Gene Prediction

The target gene of miR-579-3p was predicted using bioinformatics prediction websites Targetscan (http://www.targetscan.org/vert_71/) and miRanda (http://www.microrna.org/).

Cell Transfection

SK-MES-1 cells were first inoculated into 6-well plates and cultured until 50-70% of cell density. 10 μL of miRNA-NC and miRNA-mimics were added into each well in NC group and mimics group for transfection, respectively. Lv-MSR1 (macrophage scavenger receptor 1) was added in mimics + MSR1 group while transfected with mimics.

Dual Luciferase Reporter Gene Assay

SK-MES-1 cells were first inoculated into 24-well plates. When 60% of cell reached fusion after 24 h, transient transfection was conducted according to relevant instructions. MiR-579-3p mimics/NC and pmirGLO-MSR1 plasmid (wt)/(mut) were co-transfected, followed by culture for another 36 h. The luciferase activity was analyzed in accordance with the instructions of the dual luciferase activity assay kit by a multi-functional microplate reader.

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

Total RNA was extracted from cells according to the instructions of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA was then reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using miRNA reverse transcription kit. After obtained cDNA was diluted at 1:50, the expression of miR-579-3p was detected *via* real-time fluorescence quantitative PCR using BIO-RADCFX96 system (Bio-Rad, Hercules, CA, USA). U6 was used as an internal reference. The multiple relations between groups were calculated according to the formula $RQ=2^{-\Delta\Delta CT}$. Primer sequences used in this study were as follows: miR-579-3p, F: 5'-GCACGGAACTTCCCTTGACGTC-3', R: 5'-GCTCTAGGGATCGTCGCCGAA-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Western Blot (WB) Analysis

Total protein was extracted from cells using protein extraction kit. Subsequently, the concentration of extracted protein was determined by the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). 50 μg of protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 10%) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) through semi-dry transfer method. After sealing with 5% skim milk powder for 2 h, the membranes were washed with Tris-Buffered Saline and Tween-20 (TBST) for 3 times. Then, the membranes were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were incubated again with the corresponding secondary antibody at room temperature for 2 h, followed by washing again with TBST. Finally, immune-reactive bands were observed using the gel imaging system.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) Assay

SK-MES-1 cells were first inoculated into 96-well plates and cultured for 24 h. After transfection, the cells were incubated at 37°C under 5% CO_2 for 48 h. 20 μL of MTT (5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added into each well, followed by incubation under the above condition for 4 h. The supernatant was then discarded, and 150 μL of dimethyl sulfoxide was added into each well to dissolve formazan crystals. Finally, optical density at 490 nm was determined using a microplate reader.

Transwell Assay

Migration assay: after hydration pretreatment, cell suspension containing 2×10^5 cells was added into the upper transwell chamber. Meanwhile, the medium containing 10% of FBS was added to the lower transwell chamber, followed by incubation at 37°C under 5% CO_2 and saturated humidity for 24 h. After that, the chamber was taken out, and non-migrating cells in the upper chamber were wiped clean with cotton swabs. Then, the cells were fixed, stained and observed under a microscope. Finally, the number of cells passing through the membrane was counted in 5 randomly-selected fields (200×).

Invasion assay: the transwell chamber was coated with matrix membrane, and the membrane was coated with Matrigel. After the Matrigel was coagulated, the experiment was performed. The remaining operations were the same as those of the cell migration assay.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for all the statistical analyses. Experimental data were presented as mean ± SD ($\bar{x} \pm s$). One-way ANOVA was used to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). The *t*-test was performed to compare the difference between the two groups. $p<0.05$ was considered statistically significant. All experiments were repeated 3 times.

Results

MiR-579-3p Expression in SCLC Tissues and Cells

QRT-PCR results (Figure 1A) indicated that the expression level of miR-579-3p in SCLC tissues was significantly lower than that of para-cancer tissues. Meanwhile, qRT-PCR results showed that the expression levels of miR-579-3p in three SCLC cell lines decreased markedly as well. The down-regulation of miR-579-3p was the most significant in SK-MES-1 cells (Figure 1B). Thus, SK-MES-1 cells were chosen for subsequent investigations.

Target Gene Prediction Results

Target prediction websites predicted MSR1 and miR-579-3p had binding sites at positions 1104-1111. Then, we synthesized mutant MSR1 by changing the base of the binding site (Figure 2A). Transfection efficiency was verified by the qRT-PCR assay. As shown in Figure 2B, miR-579-3p mimics could significantly increase the expression level of miR-579-3p in SK-MES-1 cells. Luciferase reporter gene assay indicated that miR-579-3p could significantly change the luciferase activity in wild-type MSR1. However, it had no significant effects on mutant-type MSR1 (Figure 2C).

MiR-579-3p Decreased the Expression Level of MSR1

The WB assay demonstrated that up-regulation of miR-579-3p expression in SK-MES-1 cells significantly reduced the protein expres-

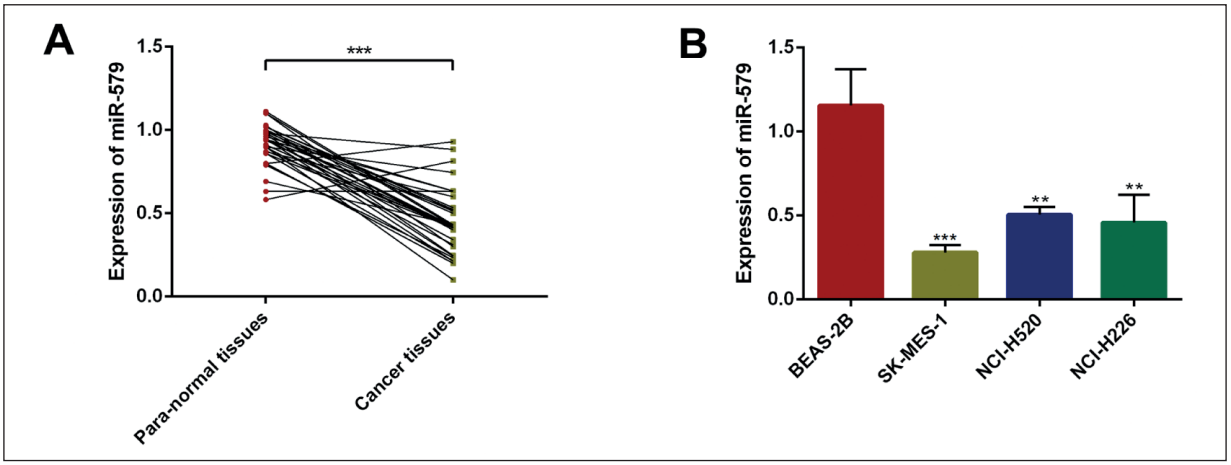

Figure 1. Expressions of miR-579-3p in SCLC tissues and cells. **A,** Difference in the expression of miR-579-3p between SCLC tissues and para-cancer tissues (***p<0.001). **B,** Expression of miR-579-3p in different cell lines (**p<0.01, ***p<0.001 compared with BEAS-2B cells).

sion level of MSR1. This was consistent with the mechanism by which miRNA regulated the expression of target genes after transcription. At the same time, WB results confirmed the negative regulation of miR-539-3p on MIRS1 (Figure 3A).

MiR-579-3p Inhibited Cell Proliferation

The MTT assay showed that the proliferation curve of SK-MES-1 cells with high expression of miR-539-3p was significantly lower than that of control group. After restoring the expression of MSR1 in cells, the proliferative ability could be restored to the level of control group (Figure 3B).

MiR-579-3p Inhibited Cell Invasion and Migration

The effects of miR-579-3p on the migration and invasion of SK-MES-1 cells were examined through the transwell assay. As shown in Figure 4, we found that miR-579-3p significantly reduced the invasion and migration abilities of

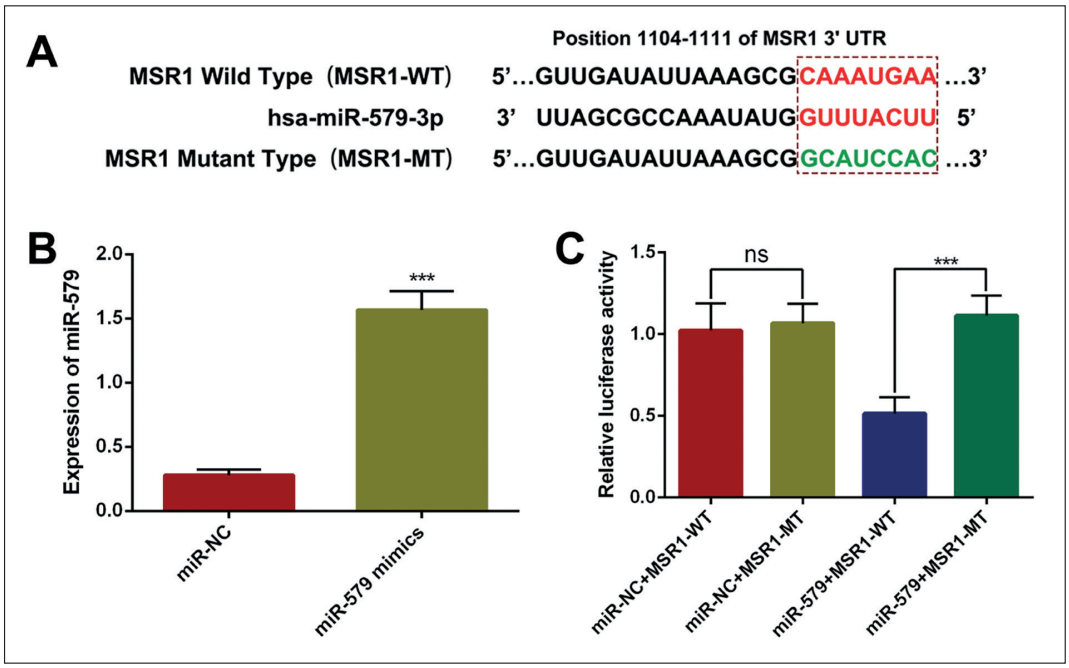

Figure 2. MSR1 was a direct and functional target of miR-579-3p. **A,** Diagram of putative miR-579-3p binding sites of MSR1. **B,** Transfection efficiency detected by qRT-PCR (***p< 0.001). **C,** Relative activities of luciferase reporters (***p< 0.001).

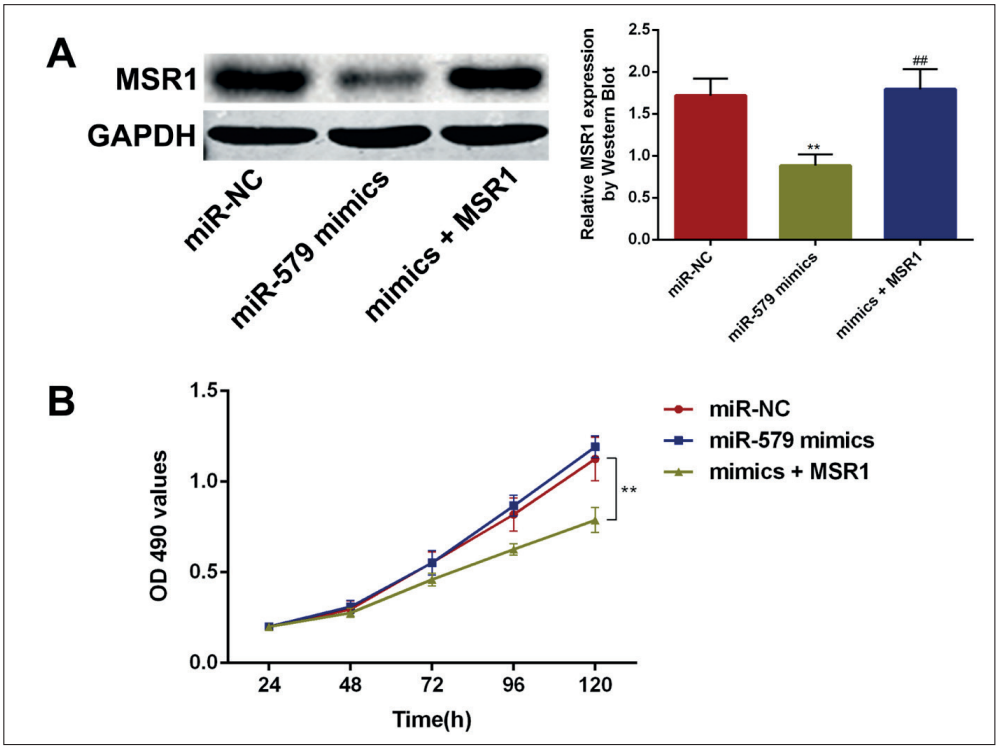

Figure 3. A, MiR-579-3p decreased the expression level of MSR1 detected by Western blot (**p<0.001 *vs.* NC group; $^{\#\#}p$<0.01 *vs.* mimics group). **B,** Cell proliferation detected by CCK-8 assay (**p<0.01).

SK-MES-1 cells. However, when the expression of MSR1 was restored, the metastatic ability of SK-MES-1 cells recovered.

Discussion

Among the common pathological types of lung cancer, the morbidity rate of SCLC is second only to AC, showing a significantly increasing trend in recent years. The mechanisms of the occurrence and development of NSCLC have been gradually confirmed by researchers in clinical practice. However, these studies[17-19] have mainly focused on lung AC cells represented by A549. Meanwhile, there are few studies on SCLC. Therefore, it is extremely urgent to deeply explore the molecular mechanism of SCLC. Currently, the key roles of miRNAs in the occurrence and clinical treatment of tumors have been emphasized. Moreover, their effects on tumor proliferation, migration and invasion, cell cycle and targeted drugs have been confirmed as well. Generally,

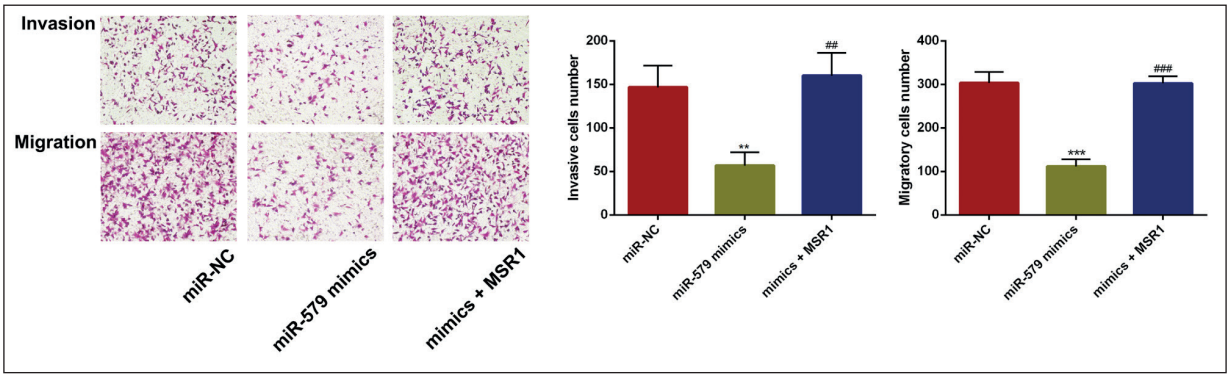

Figure 4. MiR-579-3p/MSR12 axis inhibited the invasion and migration of SCLC cells (Magnification × 200). (**p<0.01, ***p<0.001 *vs.* NC group; $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 *vs.* Mimics group).

miRNAs play important roles as tumor-inhibiting factors through down-regulating the expression of oncogenes or up-regulating the expression of tumor suppressor genes. At the same time, studies have demonstrated that miRNAs can simultaneously regulate multiple signaling pathways and participate in signal transduction pathways.

The macrophage scavenger receptor 1 (MSR1) gene is located at 8p22, which encodes type A MSR protein. MSR1 also mediates the expression of recognition receptors with phagocytic function on the surface of the macrophage membrane, thereby mediating the production of inflammation and affecting cellular biological behaviors. Scholars[20-22] have also shown that MSR1 gene exerts a certain association with diseases, such as prostate cancer and atherosclerosis.

In this work, the expression of miR-579-3p in tumor tissues and para-carcinoma normal tissues of 30 SCLC patients was detected. Results indicated for the first time that miR-579-3p was significantly down-regulated in SCLC. This indicated that miR-579-3p was possibly involved in the occurrence and development of SCLC. Subsequent bioinformatics software and luciferase reporter gene assay predicted and verified that the target gene of miR-579-3p was MSR1. The transfection of miR-579-3p mimics significantly reduced the protein expression level of MSR1 in cells, eventually inhibiting the proliferation, invasion, and migration of SCLC cells.

Conclusions

These results indicated that, the functions of miR-579-3p, have great potential to become new target and direction for treatment of malignant SCLC. Our findings provided new clues also for clarifying the pathogenesis of this tumor.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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