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MiR-875 can regulate the proliferation and apoptosis of non-small cell lung cancer cells *via* targeting SOCS2

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Abstract. – OBJECTIVE: To investigate the effect of miR-875 on the proliferation and apoptosis of non-small cell lung cancer (NSCLC) cancer cell line A549 and the related mechanism.

PATIENTS AND METHODS: 30 paired tumor tissue and the adjacent tissue were collected. Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) has been performed to detect the expression of miR-875 in NSCLC tissues and adjacent normal tissues. Moreover, suppressor of cytokine signaling 2 (SOCS2) has been predicted as a target of miR-875, and Dual-Luciferase reporter assay has been performed to confirm the targeting relationship; furthermore, the expression of SOCS2 in tumor tissue and the adjacent tissue were compared. Next, human NSCLC cell line A549 cells were cultured and transfected with miR-875 inhibitor with or without SOCS2 siRNA, and the proliferation and apoptosis of the cells were evaluated by Cell Counting Kit (CCK-8) and flow cytometry methods. Finally, the relative protein expression of Wnt and β-catenin were analyzed by Western blot analysis.

RESULTS: MiR-875 was significantly up-regulated in NSCLC tissues compared with the adjacent tissues. SOCS2 was confirmed as a target of miR-875, and the expression of SOCS2 was markedly decreased in NSCLC tissues. Moreover, the knockdown of miR-875 inhibited the proliferation and promoted the apoptosis of A549 cells, while transfection of SOCS2 siRNA can block miR-875 inhibitor-induced anti-proliferative effects. Finally, the transfection of miR-875 inhibitor decreased the expression of Wnt and β-catenin, and SOCS2 siRNA can reverse the effect.

CONCLUSIONS: MiR-875 may regulate the proliferation and apoptosis of NSCLC cells via targeting SOCS2, suggesting that miR-875 has the potential to become a therapeutic target for the treatment in NSCLC.

Key Words:
MiR-875, Proliferation, Apoptosis, SOCS2, NSCLC.

Introduction

Until now, cancer is one of the most serious threats to human health in the world. Cancers are classified by the type of cell that the tumor cells resemble like breast, prostate, lung, pancreas, colon, and so on. Among them, non-small cell lung cancer (NSCLC) remains a public-health issue on a global scale[1]. Even though surgical management, chemotherapy and radiotherapy are frequently used therapeutic modalities for the treatment of NSCLC, these managements cannot eliminate NSCLC[2-4]. In recent years, the molecule-targeted treatment of tumors found general acceptance with the molecule-targeted drugs extensively applied in clinical treatment[5,6]. As a new method of anti-tumor therapy, the molecule-targeted treatment of tumors has been demonstrated as effectiveness. Meanwhile, they have had some visible effects in clinical treatment. A deeper understanding of the molecular mechanism in NSCLC cannot only help researchers to find effective therapeutic targets, but also provide novel biomarkers for the risk assessment and early diagnosis of NSCLC.

MicroRNAs (miRNA) are small non-coding RNAs with the length of about 22 nucleotides. Previous studies[7,8] indicated that microRNAs may play a role as a negative regulator of genes by binding to the 3'- Untranslated Region (UTR) of its target mRNA and consequentially silencing the expression of its target gene. It has been widely established that miRNAs play critical roles in the regulation of multiple biological processes, including proliferation, differentiation, apoptosis, tumorigenesis, and metastasis, which may predict the overall prognosis in some types of cancers[9-13]. Previous reports[14] suggested that miR-875 is abnormally expressed in NSCLC; however, the

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potential target of miR-875 and the mechanism underlying how miR-875 participate in the pathogenesis of NSCLC cells remain obscure.

In the present work, we will focus on the roles of miR-875 in NSCLC and the related mechanism. We observed that miR-875 was up-regulated in NSCLC tissues compared with that in the adjacent tissue; moreover, the results of *in vitro* studies proved that the inhibition of miR-875 inhibited cell proliferation and promoted cell apoptosis in NSCLC. Our results indicated that miR-875 has the potential to become a therapeutic target for the treatment of NSCLC.

Material and Methods

Patients and Clinical Tissue Samples

A total number of 30 NSCLC tissue samples and adjacent tissue samples were obtained from NSCLC patients in the Jiujiang College Affiliated Hospital. All the collected cases were diagnosed as NSCLC pathologically without any preoperative radiotherapy and/or chemotherapy. This study was approved by the Ethics Committee of the Jiujiang College Affiliated Hospital. All samples were collected after the informed consent was obtained from each patient.

Cell Culture and Transfection

Human NSCLC cell line A549 cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; Invitrogen, Carlsbad, CA, USA) supplied with 10% of fetal bovine serum, (FBS; Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified incubator (5% CO_2). The cells were then transfected with miR-875 inhibitor, miR-875 inhibitor NC with or without SOCS2 siRNA following the manufacturer's protocols.

RNA Extraction and Quantitative Real Time-PCR (RT-qPCR)

Total RNA was first extracted from the cell and tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) based on the standard protocol and reverse transcribed into cDNA using the PrimeScript RT Master Mix (TaKaRa, Otsu, Shiga, Japan). Quantitative Real Time-PCR was performed to detect the expression levels of miR-875 and the genes using the SYBR premix Ex Taq (TaKaRa, Otsu, Shiga, Japan) on the ABI Biosystems. The relative expression level of miR-875 or SOCS2 were normalized by the $2^{-\Delta\Delta Ct}$ cycle threshold method to internal control U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The reactions were performed at 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. The sequences of the primers were: miR-875, forward 5'-CGAATGGGCCTAAGATCCCG-3', reverse 5'-GGAGCCCAGCACTTTGATCT-3'; SOCS2, forward 5'-CAGATGTGCAAGGATAAGCGG-3', reverse 5'-GCGGTTTGGTCAGATAAAGGTG-3'; GAPDH, forward 5'-GGTGAAGGTCGGAGTCAACGGA-3', reverse 5'-GTCATGGATGACCTTGGCCAGG-3'; U6, forward, 5'-CTCGCTTCGGCAGCACA-3', reverse 5'-AACGCTTCACGAATTTGCGT-3'.

Cell Proliferation Analysis

The effect of miR-875 on cell proliferation was measured by Cell Counting Kit (CCK-8) assay according to the manufacturer's instructions. Briefly, cells were washed with Phosphate-Buffered Saline (PBS) and harvested by trypsinization. Then, the cells were seeded onto 96 well plates and 10 µL of the CCK-8 solution was added to each well, and the plate was incubated for 1 to 4 hours. The absorbance was measured at 450 using a microplate reader.

Cell Apoptosis Analysis

At 48h post-transfection, A549 cells were washed with PBS, and Annexin V-fluorescein isothiocyanate (FITC) apoptosis kit (BD Biosciences, Franklin Lakes, NJ, USA) was used to quantify the apoptosis of A459 cells of the different groups by flow cytometry methods according to the manufacturer's protocol.

Western Blot

Total proteins were isolated from cells using protease inhibitor cocktail. The protein concentrations were determined by BCA (Bicinchoninic Acid) protein assay kit (Pierce, Waltham, MA, USA). Appropriate amounts of proteins were resolved by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gel, transferred onto polyvinylidene difluoride (PVDF) membrane, blocked in 5% non-fat dry milk in Tris-Buffered Saline (pH 7.4) containing 0.05% Tween 20, and blotted with the primary antibody. GAPDH served as the internal control. Chemiluminescence signals were detected incubated with horseradish peroxidase (HRP) and secondary antibodies. The relative expressions of the proteins were evaluated through the gray value ratio of each protein and GAPDH.

Dual-Luciferase Reporter Assay

Wild-type SOCS2 3'UTR (SOCS2-3'UTR) containing miR-875 binding site and mutant SOCS2 3'UTR (SOCS2-MUT) were cloned into the p-MIR-reporter plasmid (Thermo Fisher Scientific, Waltham, MA, USA) and transfected into 293 cells with miR-875 mimics or NC with Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) for 48 h. The activities of the Luciferases were detected by a kit (Beyotime Shanghai, China).

Statistical Analysis

All experiments were repeated at least three times. Statistical analyses were performed using IBM SPSS Statistic v17.0 (IBM, Armonk, NY, USA). All data were expressed as mean ± standard deviation. The comparison between the two groups was analyzed by the *t*-test, and the comparison among multiple groups was analyzed by the analysis of variance (ANOVA) with Tukey's post-hoc tests. $p<0.05$ was defined as statistically significant.

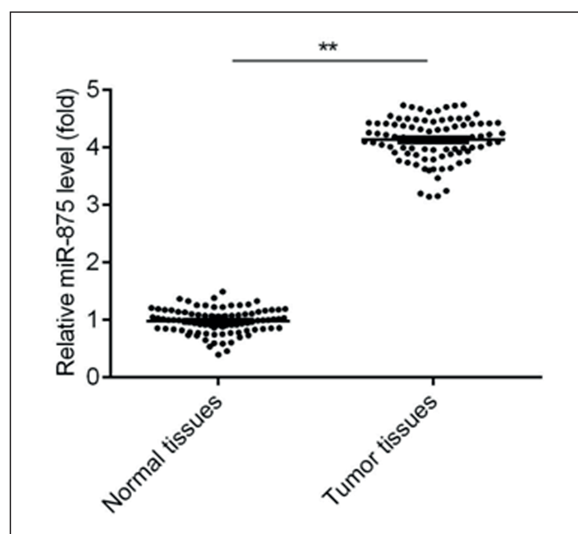

Figure 1. MiR-875 is upregulated in NSCLC tissue samples. RT-qPCR analysis of miR-875 mRNA expression in human NSCLC tissue and normal controls. The results were expressed relative to the value of normal controls that were assigned as a value of 1. The line indicated the mean expression values. $^{**}p<0.01$.

Results

MiR-875 Is Increased in Human NSCLC Tissues

The levels of miR-875 in all 30 pairs of human NSCLC tissues and adjacent normal tissues were detected. RT-PCR data showed that the level of miR-875 was substantially increased in NSCLC tissues in comparison with their paired normal tissues (Figure 1), and the difference was statistically significant ($p<0.01$).

SOCS2 is a Direct Target of MiR-875

Suppressor of cytokine signaling 2 (SOCS2) has been predicted as a target of miR-875 by online bioinformatic method (Targetscan). Next, we performed Dual-Luciferase reporter analysis to confirm the targeting relationship. First, we cloned the segment of SOCS2 3'UTR containing the putative binding site for miR-875 and the mutated segment in a Firefly Luciferase reporter vector to obtain p-SOCS2-wt or p-SOCS2-mut (Figure 2A). Then, 293T cells were transfected with miR-875 mimics and the reporter vectors, and the activities of the Luciferases were measured. It was observed that when co-transfected in 293T cells, miR-875 significantly repressed the Luciferase activity of p-SOCS2-wt, whereas a reversal of Luciferase expression was observed with p-SOCS2-mut deleted in the miR-875-binding sites (Figure 2B). Our data provided evidence that miR-875 could directly bind to SOCS2 3'UTR and thereby reduce mRNA and protein level of SOCS2.

To further investigate the relationship between miR-875 and SOCS2 in NSCLC, the expression of SOCS2 in tissue samples were examined by RT-qPCR methods. We discovered that SOCS2 was significantly decreased in human NSCLC tissues, both at mRNA and protein levels (Figure 3, $p<0.01$). These data suggested an inverse correlation in the expression level between miR-875 and its target SOCS2 in NSCLC.

MiR-875 Inhibitor Inhibited the Expression of SOCS2 in A549 Cells

Since an inverse correlation between miR-875 and SOCS2 expression was observed in human NSCLC tissues, we transfected A549 cells with miR-875 inhibitor and analyzed whether the expression of SOCS2 was enhanced by the miR-875 inhibitor. As indicated in Figure 4, miR-875 inhibitor caused a significant increase in SOCS2 expression, both at mRNA and protein levels ($p<0.01$).

MiR-875 Inhibitor Suppresses Proliferation of Human A549 Cells via Targeting SOCS2

Next, A549 cells were cultured; miR-875 inhibitor or NC was transfected into A459 cells, and CCK-8 assay was performed to assess the effect

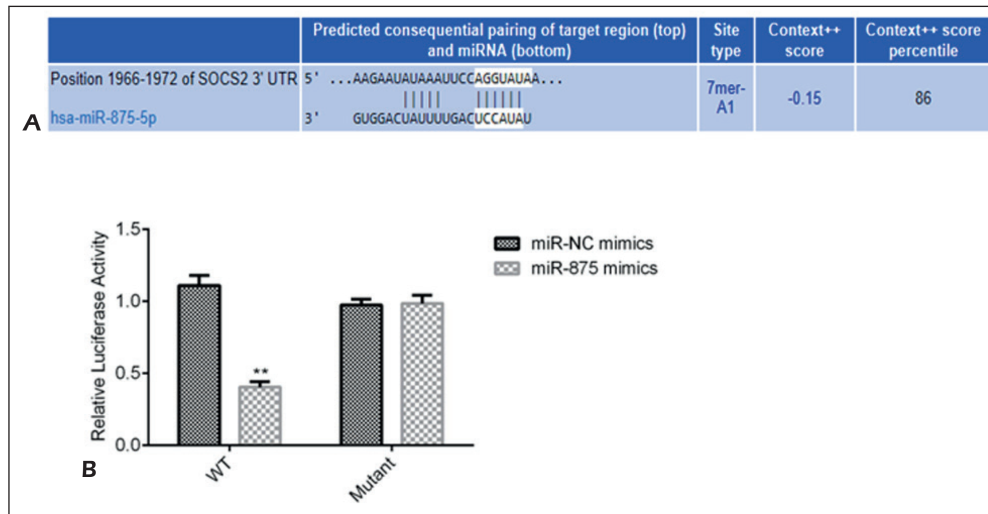

Figure 2. SOCS2 is a direct target of miR-875. A, The sequence alignment showed the relative position of the miR-875 binding site in the 3′ UTR of SOCS2 and the mutated nucleotides are indicated. The sequences were used to construct Luciferase reporter plasmids. B, Each reporter construct (p-SOCS2-wt or p-SOCS2-mut) was co-transfected with either miR-875 mimic or NC in 293T cells and Dual-Luciferase assay was measured 24 h later. The Luciferase activity was normalized to Renilla and presented as relative to NC (arbitrarily set at 1). Data are presented as means ± SD of three independent experiments. $^{**}p<0.01$.

of miR-875 on cell proliferation. As shown in Figure 5, miR-875 inhibitor led to a slight decrease in cell proliferation at 12 and 24 h post-transfection and a significant reduction in proliferation at 48h post-transfection ($p<0.01$); on the other hand, the co-transfection of the miR-875 inhibitor and SOCS2 siRNA can partially block miR-875 inhibitor-induced anti-proliferation effects.

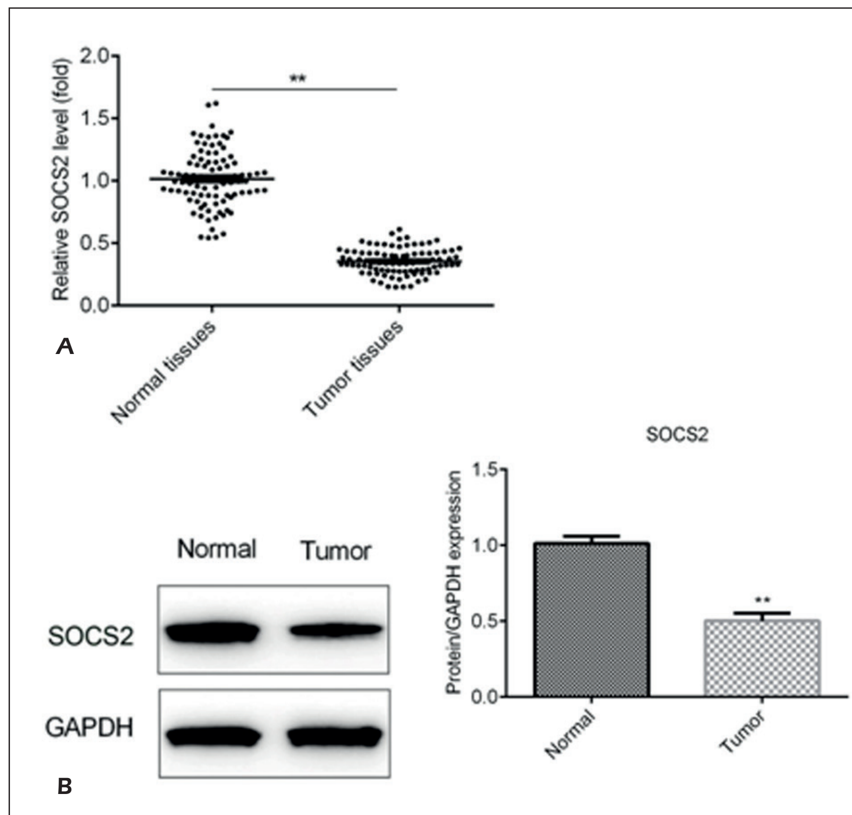

Figure 3. SOCS2 is down-regulated in NSCLC tissue samples. A, RT-qPCR analysis of SOCS2 mRNA expression in human NSCLC tissue and normal controls. The results were expressed relative to the value of normal controls that were assigned as a value of 1. The line indicated the mean expression values. $^{**}p<0.01$. B, Representative Western blot showed the expression of SOCS2 protein in human NSCLC cancer tissue and normal controls. GAPDH was used as the loading control.

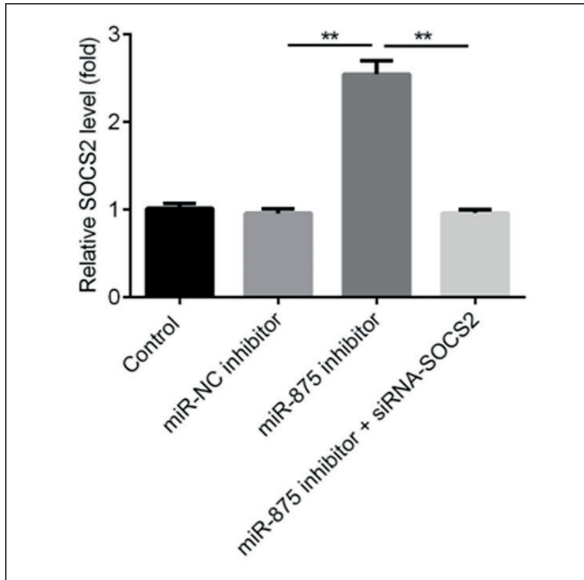

Figure 4. MiR-875 inhibitor inhibited the expression of SOCS2 in A549 cells. Representative Western blot showed the expression of SOCS2 in each cell group (control, NC, miR-875 inhibitor, siRNA). GAPDH was used as the loading control.

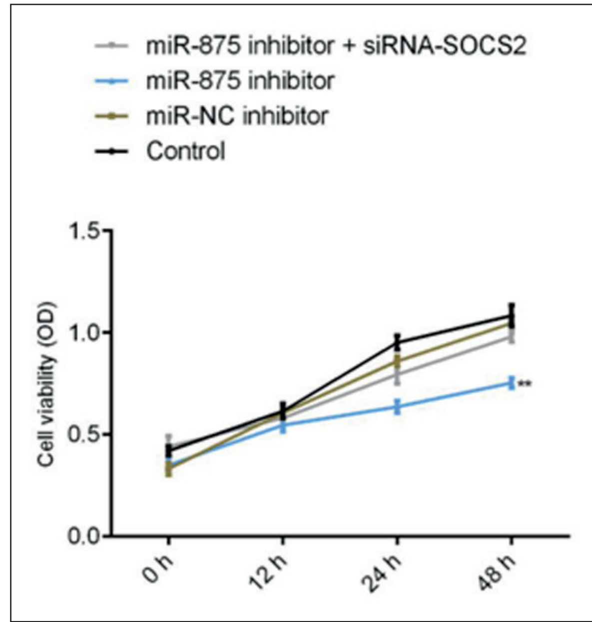

Figure 5. MiR-875 inhibitor suppresses the proliferation of A549 cells. CCK-8 assay was used to examine the cell proliferation in each group (control, NC, miR-875 inhibitor, miR-875 inhibitor + SOCS2 siRNA). All data shown are mean ± SD from three separate experiments. $^{**}p<0.01$.

MiR-875 Inhibitor Promotes the Apoptosis of Human A549 Cells via Targeting SOCS2

Moreover, the effects of the miR-875 inhibitor on cell apoptosis were examined by flow cytometry methods. As shown in Figure 6, miR-875 inhibitor led to significant increase in the apoptosis of the cells 48 h post-transfection ($p<0.01$); meanwhile, co-transfection of miR-875 inhibitor and SOCS2 siRNA can partially block miR-875 inhibitor-induced pro-apoptotic effects.

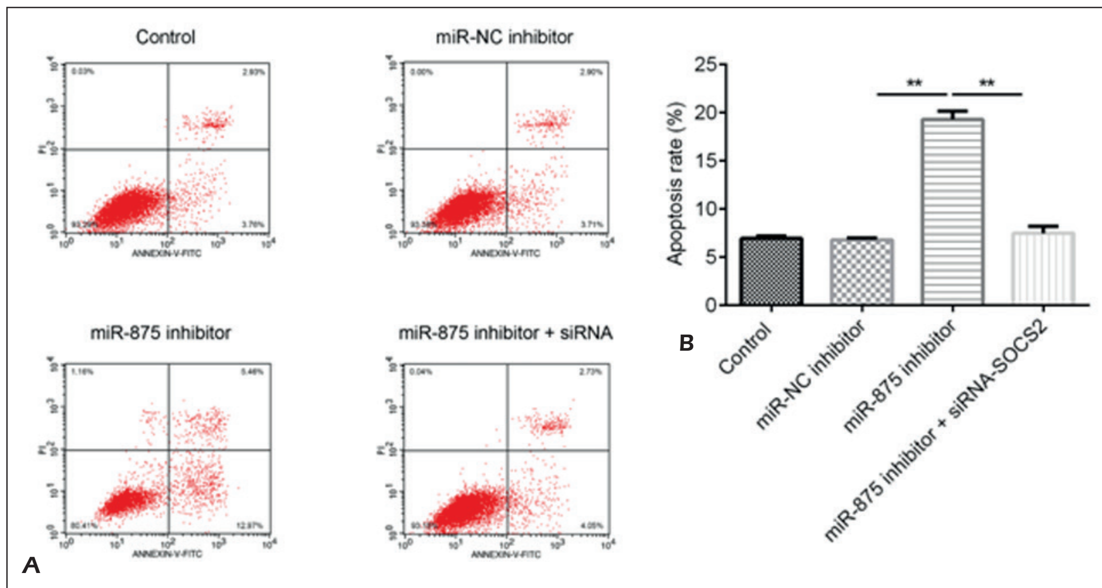

Figure 6. MiR-875 inhibitor promotes the apoptosis of A549 cells. Flow cytometry assay was used to examine the cell apoptosis in each group (control, NC, miR-875 inhibitor, miR-875 inhibitor + SOCS2 siRNA). All data shown are mean ± SD from three separate experiments. $^{**}p<0.01$.

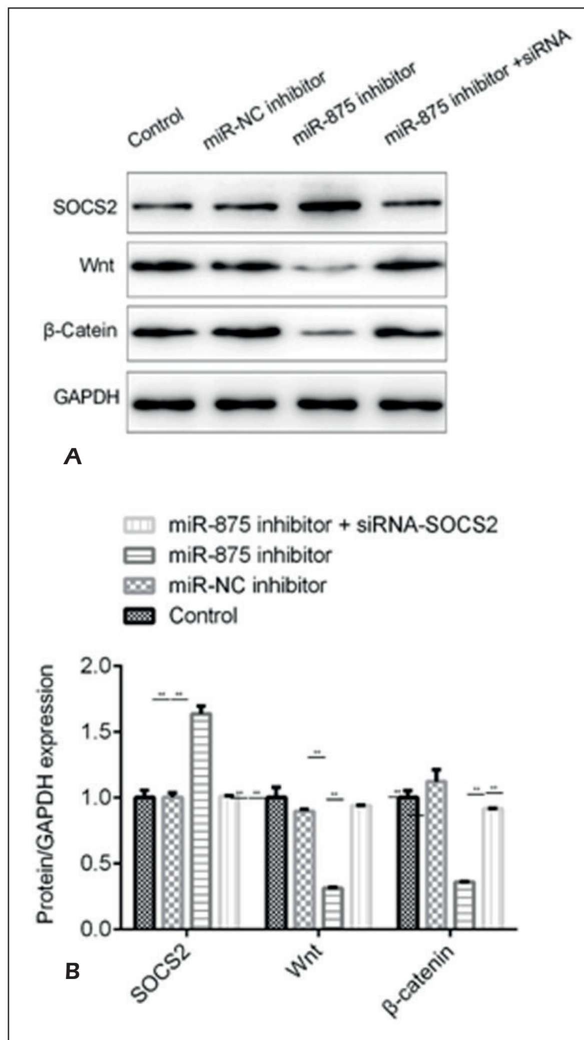

Figure 7. MiR-875 inhibitor inhibits the Wnt/β-catenin signaling pathway. Representative Western blot showed the expression of SOCS2, Wnt and β-catenin in each cell group (control, NC, miR-875 inhibitor, miR-875 inhibitor + SOCS2 siRNA). GAPDH was used as the loading control.

MiR-875 Inhibitor Inhibits the Wnt/β-Catenin Signaling Pathway via Targeting SOCS2

Finally, we analyzed whether miR-875 could inhibit the Wnt/β-catenin signaling pathway. As shown in Figure 7, miR-875 inhibitor led to a decrease in the protein expression of Wnt and β-catenin; meanwhile, the co-transfection of miR-875 inhibitor and SOCS2 siRNA can re-activate the Wnt/β-catenin signaling pathway compared with miR-875 inhibitor transfected cells ($p<0.01$).

Discussion

In this study, the roles of miR-875 in NSCLC have been investigated. We observed that miR-875 can regulate the proliferation and apoptosis of non-small cell lung cancer cells via targeting SOCS2, suggesting that miR-875 may serve as an oncomiR in NSCLC.

MiRNAs show great potential for the diagnosis and therapy for NSCLC[15-17]. However, the reports on the roles of miR-875 in NSCLC were limited. In the present work, we observed that miR-875 was significantly up-regulated in NSCLC. Combining the previous studies with our own, we confirmed that the level of miR-875 was significantly elevated in human NSCLC tissues in comparison with paired normal controls. These data suggested that miR-875 could function as an oncogene in NSCLC.

To clarify the roles of miR-875 in NSCLC pathogenesis, we performed *in vitro* studies in human NSCLC A549 cells. Cell proliferation and apoptosis are essential events accounting for tumor progression. First, our data showed that miR-875 inhibitor led to reduced proliferation and survival of A549 cells. Next, we proved that miR-875 inhibitor can promote the apoptosis of A549 cells *in vitro*. Notably, the transfection of miR-875 inhibitor also suppressed the Wnt/β-catenin signaling pathway, which has been known to regulate the carcinogenesis[15,18,19]. Our results indicated that the down-regulation of miR-875 could inhibit the proliferation and apoptosis of NSCLC cancer cells, probably by inhibiting the Wnt/β-catenin signaling pathway.

Bioinformatics analysis identified suppressor of cytokine signaling 2 (SOCS2) as one of the direct targets of miR-875. SOCS2 belongs to the SOCS protein family, and it is a transcription factor that regulates the differentiation and fate of the cells in adults[20], and recently, the roles of SOCS2 in tumorigenesis have also been discussed. In the case of lung cancer, SOCS2 has been reported as a tumor suppressor[21]; in the present work, we observed the lower level of SOCS2 in human NSCLC tissues, which was consistent with the previous studies. Therefore, we speculated that miR-875 could negatively regulate SOCS2 expression in NSCLC and exert its oncogenic effects. To confirm our speculation, we transfected A549 cells with miR-875 inhibitor with or with our SOCS2 siRNA and performed a series of experiments. Our data first confirmed the direct binding of miR-875 to SOCS2 3'UTR through Dual-Luciferase assay, and then showed that miR-875 inhibitor-induced enhanced

expression of SOCS2, suggesting that miR-875 inversely regulated SOCS2. Next, the co-transfection of miR-875 inhibitor and SOCS2 can partially block miR-875 inhibitor-induced anti-tumor effects. These results suggested that miR-875 may exert its carcinogenic behaviors by down-regulating the expression of SOCS2.

Conclusions

The present investigation demonstrated that miR-875 can regulate the proliferation and apoptosis of NSCLC cells, by negatively regulating its target SOCS2. Our results imply that miR-875 may play an important role in NSCLC pathogenesis and hold great promise for a novel therapeutic strategy for NSCLC patients.

Conflict of Interests

The authors declare that they have no conflict of interest.

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