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MicroRNA-886-3P functions as a tumor suppressor in small cell lung cancer

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ABSTRACT
Small cell lung cancer (SCLC) is a highly aggressive disease and miRNAs may play an important role in modulating SCLC progression. We have previously screened 924 miRNAs and found that miR-886-3P was negatively associated with SCLC survival. In the current study, we further investigated the role of miR-886-3P mimic in regulating SCLC cell phenotypic alteration in vitro and xenograft tumor formation in vivo. We found that transfection of miR-886-3P mimic significantly inhibited SCLC cell proliferation, migration, and colony formation, and induced mesenchymal-epithelial transition (MET) by suppressing TGF-β1 synthesis in vitro. Furthermore, intra-tumor injection of miR-886-3P mimic lead to necrosis and suppression of tumor invasion to the surrounding tissue in the subcutaneous xenograft tumor, and intra-vein injection of miR-886-3P mimic suppressed xenograft lung cancer growth in vivo. These findings suggested that miR-886-3P functions as a tumor suppressor in SCLC and thus, it might be a potential therapeutic molecule in the treatment of lung cancer.

Introduction
Lung cancer is one of the most aggressive malignant cancers in the world. Small cell lung cancer (SCLC) accounts for approximately 15% of all newly diagnosed lung cancer cases. Based on whether a SCLC can be safely treated with definitive radiation fields, the American Veterans Administration Lung Study Group (VALG) defines SCLC stages as limited disease (LD) and extensive disease (ED). Platinum-based chemotherapy in combination with radiotherapy remains as the main therapeutic strategy for SCLC. However, despite extreme sensitivity to chemotherapy and radiotherapy, approximately 67% of newly diagnosed patients have ED, with median progression-free survival of 5.5 months and median overall survival of 6–12 months, and 5-year survival rates of 5–10%.

SCLC is often found early metastasis when it is diagnosed. Many factors may contribute to the aggressive phenotype of SCLC. In this context, it has been reported that miRNAs play an important role in phenotypic alteration of cancer cells through regulating epithelial-mesenchymal transition (EMT). EMT process involves distinct genetic and epigenetic alterations, and shifted expression of several biomarkers including E-cadherin, N-cadherin and vimentin. The EMT process has been described in different kinds of human malignant tumors including breast cancer, non-small cell lung cancer, colon cancer, and SCLC. TGF-β1 has been known to induce EMT in variety kinds of cells including lung cancer cells.

Due to limited therapeutic outcomes in SCLC, over the past few years, there has been a paradigm shift in cancer treatment from non-specific therapy to targeted therapies including immunotherapy and biological therapy. In this regard, several miRNAs have been reported to suppress or promote lung cancer cell survival or death. Consistent with these reports, we have previously reported that miR-886-3P was associated with survival of SCL. In the current study, we further investigated role of miR-886-3P in regulating small cell lung cancer cell proliferation, migration, colony formation, mesenchymal-epithelial transition (MET), and in vivo xenograft tumor formation and growth.

Materials and methods
Cell culture and transfection of exogenous mirnas
Human small cell lung cancer cell lines, NCI-H446 and NCI-H168, were obtained from Peking Union Medical University (Beijing, China) and maintained in RPMI 1640 medium (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, USA) and antibiotics at 37°C and 5% CO₂ atmosphere. Mimic of miR-886-3P and control miRNA were purchased from Ribobio (Guangzhou, China) and the plasmid pGL3-luciferase; cancer biology the plasmid was synthesized by Generay (Shanghai, China).

Transfection of miRNA mimic was carried out using Lipofectamine 2000 following the manufacturer’s instruction (Invitrogen, USA).

Immunoblotting
Cells were harvested with lysis buffer and briefly sonicated. Total protein concentration was measured with BAC Protein
Antibody for 1h at room temperature, blotting 4 weeks old (Beijing Vital Technologies, 15596 014) following the manufacturer’s protocol using SYBRP remix Ex Taq (Liaoning, China, RR420A). Two micrograms of total RNA were extracted using TRIzol Reagent (Invitrogen Life Technologies, 15596–026). Two micrograms of total RNA were converted to complementary DNA using Superscript reverse transcriptase (Invitrogen Life Technologies, 18064–014) following the manufacturer’s instructions. RT-PCR was performed in a 20µl reaction volume according to the manufacturer’s protocol using SYBRP remix Ex Taq (Liaoning, China, RR420A).

Statistical analysis
Statistical analysis was conducted using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Data are presented as the mean ± s.e.m. Differences in measured variables between experimental and control groups were assessed using the Student’s t-test. P values were calculated, and minimum statistical significance was accepted at P < 0.05, NS, not significant, *P < 0.05, **P < 0.01.

Results
**Overexpression of mir-886-3p resulted in phenotypic alteration of SCLC cells**
We have previously reported that miR-886-3p was downregulated in SCLC and thus, we hypothesized that lack of miR-886-3p may be associated with aggressive phenotype of small cell lung cancer. In the current study, therefore, exogenous mimic of miR-886-3p was transfected into the two cell lines of small cell lung cancer, NCI-H446 and NCI-H1688.
Phenotypes of the cells were then examined. As expected, expression of miR-886-3P significantly increased in the cells transfected with exogenous mimic of miR-886-3P both at mRNA (Figure 1A) and protein level (Figure 1B). Overexpression of miR-886-3P resulted in significant suppression of cell proliferation (Figure 1C), cell migration and invasion into Matrigel (Figure 1D) compared to the control cells (Figure 1E, migration in NCI-H446: 168 ± 25 vs 65 ± 10; Migration in NCI-H1668: 112 ± 20 vs 52 ± 22; invasion in NCI-I446: 195 ± 36 vs 79 ± 15; invasion in NCI-H1688 132 ± 19 vs 65 ± 14, *p < 0.05). In addition, ability of colony formation was also significantly reduced in the cells over-expressing miR-886-3P compared to the control cells (Figure 1F and G, NCI-H446: 148 ± 32 vs 31 ± 5; NCI-H1688: 521 ± 79 vs 209 ± 53, *p < 0.05).

**TGF-β1 was targeted by miR-886-3P**

By searching databases including [http://www.microrna.org/microrna/getTargets](http://www.microrna.org/microrna/getTargets), we found that TGF-β1 is a predicted target for miR-886-3P and thus effect of miR-886-3P mimic on TGF-β1 expression was assessed. It was demonstrated by the luciferase reporter assay that TGF-β1 promoter activity was significantly reduced in the cells transfected with miR-886-3P mimic compared to the cells transfected with negative control mimic (Figure 2A, NCI-H1688: 912 ± 149 of miR-886-3P mimic vs (2.12 ± 0.36)× 10^3 of control mimic; NCI-H446: (5.54 ± 0.45) × 10^3 of miR-886-3P mimic vs (1.42 ± 0.56)× 10^4 of control mimic, *p < 0.01). Furthermore, TGF-β1 protein was significantly reduced in the small cell lung cancer cells transfected with miR-886-3P mimic compared to the cells transfected with control mimic as evidenced by immunofluorescence staining (Figure 2B and 2C) and immunoblotting (Figure 2D).

**Microrna-886-3p resulted in mesenchymal-epithelial transition (MET) through suppressing TGF-β1**

TGF-β1 is known to induce epithelial-mesenchymal transition (EMT), one of the mechanisms by which cancer cell invades surrounding tissue. Since miR-886-3P targeting TGF-β1, we further examined if miR-886-3P could induce mesenchymal-epithelial transition (MET) through suppressing TGF-β1. As shown in Figure 3A, transfection of exogenous miR-886-3P mimic resulted in the morphological transition of the lung cancer cell lines from spindle-shape to round shape (Figure 3A). Next, miR-886-3P mimic-induced MET in small cell lung cancer cells was further examined by assessing the expression of biomarkers including E-cadherin, N-cadherin, and vimentin (Figure 3B and 3C). It was found that miR-886-3P significantly increased E-cadherin (NCI-H446: 0.49 ± 0.05 of miR-886-3P mimic vs 0.05 ± 0.01 of control mimic, *p < 0.05 compared to the cells transfected with Mimic-NC. **p < 0.01 compared to the cells transfected with Mimic-NC.***

Figure 1. In vitro suppression of SCLC cell proliferation, migration and invasion by exogenous miR-886-3P.

Small cell lung cancer cell lines (NCI-H1688 and NCI-H446) were transfected with miR-886-3P mimic (Mimic-886) or negative control mimic (Mimic-NC). Expression of miR-886-3P was then assessed by real-time PCR quantification (Panel A) or agarose electrophoresis analysis (Panel B). Panel C: Cell proliferation assessed by RTCA MP system. Green line: cells transfected with Mimic-NC; Red line: cells transfected with Mimic-886. Panel D: Representative image of crystal violet staining of migration and invasion of NCI-H446 and NCI-H1688 cells transfected with Mimic-NC or Mimic-886. Panel E: Quantification of cell migration and invasion expressed as Relative Cell Number of cells transfected with Mimic-886 versus the number of cells transfected with Mimic-NC. * p < 0.05 compared to the cells transfected with Mimic-NC. Panel F: Representative image of colony formation assay in the cells transfected with Mimic-NC or Mimic-886. Panel G: Quantification of the colonies expressed as number of colonies. * p < 0.05, ** p < 0.01 compared to the cells transfected with Mimic-NC.
control mimic; NCI-H1688: 0.12 ± 0.01 of miR-886-3P mimic vs 0.03 ± 0.01 of control mimic, \( p < 0.05 \), (Figure 3C), and in contrast, it significantly suppressed N-cadherin (NCI-H446: 0.02 ± 0.01 of miR-886-3P mimic vs 0.98 ± 0.06 of control mimic; NCI-H1688: 0.09 ± 0.01 of miR-886-3P mimic vs 0.75 ± 0.06 of control mimic, \( p < 0.05 \), (Figure 3C) and vimentin (NCI-H446: 0.03 ± 0.01 of miR-886-3P mimic vs 0.02 ± 0.01 of control mimic; NCI-H1688: 0.01 ± 0.01 of miR-886-3P mimic vs 0.82 ± 0.04 of control mimic, \( p < 0.05 \), (Figure 3C)), which was partially or completely blocked in the presence of TGF-ß1 as evidenced by immunoblotting (Figure 3B and 3C) or by confocal immunofluorescence staining (Figure 3D). Consistently, expression of miR-886-3P in the SCLCs resulted in significant up-regulation of E-cadherin (NCI-H446: 1.95 ± 0.21 of miR-886-3P mimic vs 1.49 ± 0.05 of control mimic; NCI-H1688: 1.04 ± 0.02 of miR-886-3P mimic vs 6.49 ± 0.87 of control mimic, \( p < 0.05 \), (Figure 3E)), but down-regulation of N-cadherin (NCI-H446: 4.88 ± 0.92 of miR-886-3P mimic vs 9.27 ± 0.29 of control mimic; NCI-H1688: 3.65 ± 0.21 of miR-886-3P mimic vs 5.62 ± 0.40 of control mimic, \( p < 0.05 \), (Figure 3E) and vimentin (NCI-H446: 6.55 ± 2.55 of miR-886-3P mimic vs 3.12 ± 0.06 of control mimic; NCI-H1688: 5.50 ± 1.03 of miR-886-3P mimic vs 9.32 ± 1.25 of control mimic, \( p < 0.05 \), (Figure 3E)).

**Effect of mir-886-3p mimic on subcutaneous tumor formation and growth**

To further determine whether miR-886-3P affect tumor growth in vivo, a xenograft tumor model was established by subcutaneously injecting NCI-H446 cells into the nude mice. As expected, xenograft tumors formed by the cells transfected with miR-886-3P mimic were significantly smaller and less weight in average compared to that formed by the cells transfected with control mimic (Figure 4A and 4B, 0.755 ± 0.219 × 10^3 mg of miR-886-3P mimic vs 1.42 ± 0.484 × 10^4 mg of control mimic, \( p < 0.05 \)). Stable expression of miR-886-3P in the xenograft tumor was confirmed by in situ hybridization (Figure 4C). Moreover, effect of miR-886-3P mimic on the expression of EMT/MET biomarkers in the xenograft tumor tissues was assessed by immunocytochemistry. As shown in Figure 4D, N-cadherin and vimentin were highly expressed in the tumors formed by the cells transfected with control mimic, while they were significantly suppressed in the tumors formed by the cells transfected with miR-886-3P mimic.

**Antitumor effect of mir-886-3p mimic delivered locally or systemically**

Next, in order to examine antitumor effect of miR-886-3P, following two different strategies were applied. First, NCI-H446 cells were injected subcutaneously and allowed to form xenograft tumors. The cholesterol conjugated miR-886-3P mimic or control mimic was then locally injected into the subcutaneous tumors. As shown in Figure 5A, intra-tumor injection of miR-886-3P mimic resulted in necrosis of the tumor tissue as well as suppression of intra-muscular invasion of the tumor. Consistent with the suppression of invasion, expressions of N-cadherin and TGF-ß1 were significantly reduced in the tumor injected with miR-886-3P mimic compared to that injected with control mimic (Figure 5B).
Second, luciferase-labeled NCI-H446 cells were injected into the tail veins and established a model of lung metastasis tumor. The cholesterol conjugated miR-886-3P mimic or control mimic was then systemically delivered by injecting into the tail veins. As shown in Figure 5C, the size of metastatic lung cancer was significantly smaller in the mice received cholesterol conjugated miR-886-3P mimic than in the mice injected with control mimic as evidenced by lung cancer luciferase imaging. Histological examination and immunohistochemistry further confirmed that systemic delivery of miR-886-3P mimic resulted in suppression of metastatic lung xenograft tumor growth although expression of TGF-ß1 was not significantly affected by the miR-886-3p mimic (Figure 5D).

**Discussion**

In the current study, we investigated the effect of miR-886-3P in suppressing proliferation, migration and formation of xenograft tumor by the small cell lung cancer (SCLC) cell lines. We found that transfection of exogenous miR-886-3P mimic resulted in alteration of SCLC cells from aggressive to non-aggressive phenotype, which includes reduced proliferation, migration, and colony formation. In addition, expression of TGF-ß1, N-cadherin, and vimentin was significantly suppressed, and in contrast, expression of E-cadherin was significantly increased in the cells overexpressing miR-886-3P. Furthermore, subcutaneous xenograft tumor formation and growth was significantly reduced in the SCLC cells containing exogenous miR-886-3P mimic compared to the cells...
transfected with control mimic. Intra-tumor or intra-venous injection of exogenous miR-886-3P mimic resulted in necrosis of the tumor tissue and suppression of the tumor invasion into the lung tissues. These findings suggested that lack of miR-886-3P may contribute the aggressive phenotype of small cell lung cancer.

Small cell lung cancer is a highly aggressive disease and most of the patients die of metastasis and recurrence. It has been reported that about 25% of small cell lung cancer patients have distant metastases at initial diagnosis. While multiple factors may be associated with early metastasis of SCLC, recent studies indicated that miRNA be one of the crucial factors that contribute to the aggressive phenotype of SCLC. Consistently, with limited number of cases, we have also previously reported that miR-886-3P might be associated with poor prognosis of SCL. In the current study, we further demonstrated that transfection of miR-886-3P mimic into the SCLC cell lines resulted in suppression of cell proliferation, migration and formation of subcutaneous xenograft tumor, suggesting miR-886-3P could modulate phenotype of small cell lung cancer.

Epithelial-mesenchymal transition (EMT) is a crucial step for a cancer cell migration and aggressive invasion to the surrounding tissue or metastasis distance organs. Accumulating evidence from in vitro studies has demonstrated that TGF-β1 plays an important role in EMT induction in variety kinds of cells including lung cancer cells. Blockade of TGF-β1 synthesis and its biological action in tumor and surrounding tissue, therefore, may result in suppression of EMT and consequent migration of the tumor cells. In support of this concept, the current study demonstrated that exogenous miR-886-3P mimic not only inhibited TGF-β1 production by the SCLC cell lines, but also

![Figure 4. Subcutaneous tumor formation and growth by the SCLC cells transfected with miR-886-3P mimic or control mimic. NCI-H446 cells were transfected with either Mimic-NC or Mimic-886 followed by subcutaneous injection into the nude mice as described in the methods. Panel A: Representative image of tumor formation and growth. Panel B: Comparison of tumor weight in the two groups (n=6 each group). * p < 0.05. Panel C: Representative images of in situ hybridization of human miR-886-3P (has-miR-886-3P) or U6 in the sections of subcutaneous tumors formed by the cells transfected with either Mimic-NC or Mimic-886. Panel D: Immunohistochemistry staining of N-cadherin and vimentin in the sections of subcutaneous tumors formed by the cells transfected with either Mimic-NC or Mimic-886.](image-url)
morphological alteration of SCLC cells from spindle-shape into round shape cells, a phenomenon of mesenchymal-epithelial transition (MET). Furthermore, miR-886-3P transfection in the SCLC cells leads to down-regulation of N-cadherin and vimentin, typical biomarkers of mesenchymal type and aggressive tumor cells; and in contrast, up-regulation of E-cadherin, a biomarker of epithelial cells. In addition, effect of miR-886-3P mimic on the alteration of EMT/MET biomarkers was partially blocked by the exogenous TGF-ß1. These findings indicated that TGF-ß1 play a role in the process of EMT and tumor cell migration, and that miR-886-3P could suppress EMT and tumor cell migration partially through regulating TGF-ß1 synthesis and its biological function on EMT of the SCLC cells.

Subcutaneous xenograft tumor formation in nude mice is often used as an in vivo model of tumor stud. Using this model, for the first time, we demonstrated that miR-886-3P mimic could inhibit subcutaneous xenograft tumor formation, growth, and invasion into the surrounding tissue, as well as significant suppression of N-cadherin and vimentin expression in the xenograft tumor tissue. In addition, local injection of miR-886-3P mimic into the subcutaneous xenograft tumor resulted in topical necrosis of the subcutaneous tumor and suppression of TGF-ß1 and N-cadherin expression. Furthermore, intravenous injection of miR-886-3P mimic could dramatically reduce the tumor size of metastatic xenograft lung cancer. These findings further suggested that miR-866-3P could be used as a therapeutically molecule that could be delivered either locally (intra-tumor injection) or systemically (intravenous injection).

Taken together, the current study demonstrated that exogenous miR-886-3P could lead to phenotypic alteration of small cell lung cancer cells from aggressive and mesenchymal phenotype into non-aggressive epithelial cell type as evidenced by down-regulation of N-cadherin and vimentin, and up-regulation of E-cadherin. Over-expression of miR-886-3P in the SCLC cell lines resulted in suppression of cell proliferation, migration, colony formation, and TGF-ß1 production. Injection of miR-886-3P mimic could suppress xenograft tumor formation, growth and invasion into the surrounding tissue. The findings of the current study suggested that miR-886-3P could be a biological therapeutic tool, which could be delivered either locally or systemically, in the treatment of small cell lung cancer.

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**References**


