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Effect of microRNA-181b on the biological characteristics and clinical drug resistance of small-cell lung cancer by targeting angiotensin converting enzyme 2

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Abstract

Objective: To clarify the effect of miR-181b on the biological function of small-cell lung cancer (SCLC) and explore the effect of clinical resistance on SCLC.

Methods: Blood samples were collected from 30 SCLC patients and 30 non-SCLC patients in our department from 2017 to 2019 to detect the expression level of miR-181b. The expression level of miR-181b was detected in SCLC cells by RT-PCR, and screening of downstream target genes by gene chip, verification with luciferase, and Western blotting. In addition, collect the general data of 30 SCLC patients and 30 non-SCLC patients (control group), the patients were diagnosed by pathology and undergoing EC protocol in the Department of Thoracic Surgery and Oncology of our hospital to detect the expression level of mir-181b in different periods. Furthermore, in the SCLC cell line, EC chemotherapy was administered to detect the sensitivity of drug resistance and nondrug resistance.

Results: miR-181b in SCLC patients was lower than in normal people as well as the drug-sensitive cell line. ACE2 was verified as a downstream target of miR-181b by gene chip screening. First-line chemotherapy can promote the recovery of miR-181b, but cannot repair to normal levels. miR-181b can enhance the drug sensitivity of SCLC drug-resistant cells.

Conclusion: miR-181b directly targets ACE2 to affect the biological characteristics of SCLC. The expression level of miR-181b is highly related to the drug resistance of SCLC, which suggests that miR-181b could be a potential biomarker candidate for treatment efficacy of SCLC.

KEYWORDS
ACE2, drug resistance, microRNA (miR)-181b, small cell lung cancer

INTRODUCTION

Small cell lung cancer (SCLC) is characterized by short tumor cell doubling time, early metastasis, and high recurrence. Chemotherapy is the main treatment for SCLC. Most patients are very sensitive to chemotherapy drugs, but they are prone to relapse after chemotherapy. At present, it is believed that chemotherapy resistance is the key factor that causes SCLC to relapse. The effectiveness of second-line chemotherapy in patients with relapsed SCLC depends mainly on the length of remission after first-line chemotherapy.

Patients with a remission period of <3 months are highly resistant.[1,2]

microRNA (small RNA, miRNA) is a type of noncoding RNA about 22 nt long. It can regulate gene expression by binding to specific messenger RNA (mRNA) or regulating the protein translation process of specific mRNA, and is widely involved in cell growth and development, differentiation, proliferation, and a variety of important biological processes such as apoptosis and tumor formation.[3,4] As a member of the mRNA family, miR-181b has been confirmed in recent years as having important significance in

the occurrence and development of malignant tumors, but there has been little research on the biological characteristics of SCLC and clinical treatment resistance.[5] Here, we study the expression level and biological characteristics of miR-181b in SCLC patients and cell lines, screen and clarify their downstream target genes, explore the effect of miR-181b downstream target genes on SCLC resistance, and hope to benifit the treatment of SCLC.

MATERIALS AND METHODS

Human tissue samples

This study was approved by the Ethics Committee of the Hebei Provincial People's Hospital (He Bei, China). Written informed consent was obtained from all patients. A total of 60 peripheral blood samples of 30 SCLC patients (from December 2017 to December 2019) diagnosed by pathology and 30 healthy people were collected at the Department of Thoracic Medicine and Oncology Department, Hebei Provincial People's Hospital.

Cell culture and RNA/plasmid transfection

The human SCLC cell line (NCI-H446 cell line and NCI-H446 drug-resistant cell line) were purchased from the cell bank of the Chinese Academy of Sciences (Beijing, China). The human normal bronchial cell line HBE cells were obtained from the American Type Culture Collection. The authenticity of all cell lines was confirmed by short tandem repeat (STR) DNA profiling analysis. Cells were cultured in RPMI1640 medium (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified atmosphere at $37°C$ with 5% CO_2.

All miR-181b mimics, inhibitors, and corresponding negative controls (miR-NC, miR-NC2) used for transfection were purchased from GenePharma Co., Ltd (Shanghai, China). Transfection was conducted using Lipofectamine 3000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions.

RT-qPCR to detect the expression level of miR-181b in SCLC

Trizol (Pufei, Shanghai, China) was used to isolate the total RNA from serum, and the collected RNA was stored in RNase-free tubes at $-80°C$ before use. The primers of miR-181b and internal reference U6 were designed and synthesized by Guangzhou Ruipu (Guangzhu, China). cDNA was synthesized using the PrimeScript RT reagent kit (Takara, Kusatsu, Japan), and the stem-loop RT primer method was applied in miRNA reverse transcription. qRT-PCR was performed using the SYBR-Green Master mix (Takara) in a CFX96 touch system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). U6 was used as an endogenous control for mRNA or miRNA detection, and the fold change was calculated with the relative quantification method ($2^{-\Delta\Delta Ct}$).

Clone formation and EdU detection for cell proliferation

Each group of NCI-H446 cells was seeded into a six-well plate with a density of 1×10^3 per well with three replicates. The clones were incubated in a humidified atmosphere at $37°C$ with 5% CO_2 for 2 weeks. Subsequently, 4% formaldehyde solution was used to fix the clones, then crystal violet dye solution was added for 5 min to stain the clones. The clones were calculated under an inverted fluorescence microscope from Leica (Wetzlar, Germany).

The fraction of DNA-replicating cells, which represents cell proliferation status, was assessed using an ethynyldeoxyuridine (EdU) detection kit (RiboBio, Guangzhou, China). The EdU incorporation rate was calculated as the ratio of the number of EdU-incorporated cells to the number of Hoechst 33342-staining cells. At least 500 cells were counted for every group.

CCK-8 assay

The EC (epirubicin and cyclophosphamide) were used to determine the effects of miR-181b on SCLC cells drug resistance. NCI-H446 and NCI-H446 resistance cells were seeded into 96-well plates at a density of 5×10^3 cells/well, with or without transfection with miR-181b. The EC drugs were subsequently treated. After culturing the cells for 24 h in the drug-containing medium, the medium was aspirated, washed three times with PBS, CCK8 reaction solution (100 μL RPM1 1640 plus 10 μL CCK8) was added, and the mixture incubated at $37°C$ in a 5% CO_2 incubator for 4 h. The optical density (D) value of each well at a wavelength of 450 nm was detected by imark microplate reader (Bio-Rad, Hercules, CA, USA). Survival rate (%) = [(average D value of each drug concentration − average D value of blank well)/(average D value of negative control group without drug − average D value of blank control group)] × 100%. The experiment was repeated three times. According to the cell survival rate of each drug concentration, a logarithmic curve was calculated to obtain the drug concentration (IC_{50}) at 50% cell survival rate; and the drug resistance index, drug resistance index = IC_{50}(H446 resistance)/IC50 (H446), was calculated.

Cell cycle detection

Each group of NCI-H446 cells was harvested and fixed by ice-cold ethanol (70%) for 30 min. The cells were centrifuged and resuspended 0.1% Triton X-100 PBS solution. Subsequently, cells were stained by propidium iodide (PI) for 30–35 min. BD FACS verse flow cytometry was used to

analyze the cell cycle population. Data were analyzed using Modfit software.

Luciferase report analysis

NCI-H446 cells were grown in a 48-well plate with 200 μL of complete medium with density of 40%–50%. Luciferase activity was detected 48 h post-transfection using the dual-luciferase reporter assay system (Promega, Corporation, Madison, WI, USA). pRL-TK (Promega) expressing Renilla luciferase served as an internal control to correct variances in transfection and harvest efficiency. To verify the target genes of miR-181b, NCI-H446 cells were transfected with 10 nM NC or miR-181b mimics, with pmirGLO-ACE-2-3′UTR wild type (Wt) or pmirGLO-ACE-2-3′UTR mutant (Mut) (genefarma) transfection.

Downstream target gene screening

A total RNA of NCI-H446 cells were isolated and analyzed by an Agilent 2100 RNA quality detector and a gene chip 3'-IVT expression kit was conducted to prepared amplified RNA (aRNA). After reverse transcription, cDNA was synthesized by single-stranded synthesis and then double-stranded synthesis to obtain a double-stranded DNA template, and then reversed in vitro to obtain biotinylated amplified RNA (aRNA). After purifying the aRNA, it was hybridized with the chip probe, then the chip was cleaned and stained, and scanned to obtain images and original data.

Western blot

Proteins were collected in cold RIPA buffer. Samples were collected and the protein concentration was measured using BCA protein A determination (Beyotime Biotechnology, Shanghai, China). The proteins were then separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked in TBST-0.1% (0.1% Tween-20, Tris-base buffer) with 5% skim milk, then incubated with primary antibodies (including ACE2, ELANE, IL17C, IRF6, MAML1, CYLD, ATF3, BCL2, CCND1, and β-actin (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The next day, the membrane was washed three times with TBST-0.1% buffer. The secondary antibodies (Microwell) were incubated. ECL reagent (Thermo Scientific, Waltham, MA, USA) was used to visualize the signal on the membrane.

Wound-healing assay

The wound-healing assay was used to assess the effect of miR-181b on cell migration. Cells were seeded into 96-well plates with a density of 3×10^4 cells/well and cultured until confluent. The monolayer cells were scratched with a sterile 10-μL pipette tip followed by washing with PBS to remove the floating cells. miR-181b mimics or NC mimics were treated for 8, 16, and 24 h. Those that migrated to the wounded region were counted.

Migration assay

Transwell chambers (8 μm; Corning, NY, USA) were used. Briefly, 1×10^6 cells in the serum-free medium (100 μL) were seeded into the upper chambers and miR-181b mimics were added. The lower chamber was supplemented with 650 μL of RPMI medium containing 10% FBS and the same concentration of miR-181b mimics as in the upper compartment. After incubation at 37°C for 24 h, cells were fixed with 90% ethanol, stained with crystal violet, and then visualized using a Leica microscope (Wetzlar, Germany).

TABLE 1 General information about patients in the control and experimental groups

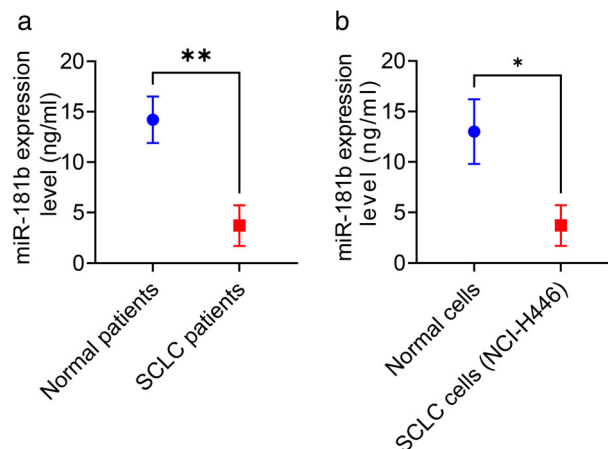| Group	Control group (N)	Experimental group (N)
Gender		
Male	18	22
Female	12	8
Age (year)		
≤55	22	3
>55	8	27
Stage		
Circumscriptum	–	9
Metastatic	–	21
Smoking		
Yes	5	16
No	25	14

FIGURE 1 miR-181b expression is downregulated in SCLC patients and SCLC cells. (a) miR-181b expression is downregulated in the peripheral blood of 30 SCLC patients. *$P < 0.05$ versus peripheral blood samples from normal patients. (b) miR-181b expression is downregulated in the SCLC cell line. The expression of miR-181b was detected by RT-qPCR

Clinical data collection

The peripheral blood samples of 30 patients with SCLC diagnosed by our department from December 2017 to December 2019 were collected. There were 22 males and eight females, with a median age of 61.3 years (41–82 years). Among them were 16 smokers and 14 nonsmokers. No previous chemical or radiotherapy had been performed. All patients had a functional score over 60 before chemotherapy. The blood routine and renal function were within the normal range, with normal electrocardiograph (ECG). According to the TNM staging revised by the International Anti-Cancer Union (UICC) and the American Joint Committee on Cancer (AJCC) 7th edition in 2009, eight cases with limited period, 22 of 30 are with extensive period, 27 cases had lymph node metastasis, and three cases were without lymph node metastasis. There were 22 cases of metastasis and eight cases of distant metastasis. The 30 normal controls were all healthy, 18 males and 12 females, with a median age of 58 years (41–75 years), five smokers and 25 nonsmokers (Table 1).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). The t test was used for comparison between two groups, and the analysis of variance was used for comparison between multiple groups. $P < 0.05$ indicates that the difference is statistically significant. All data are shown in three independent experiments. SPSS software was used for statistical processing.

RESULTS

miR-181b is downregulated in SCLC patients as well as SCLC cell lines

The expression level of miR-181b in both SCLC patients and SCLC cell lines was detected by RT-qPCR analysis. The results show significant differences between normal people and SCLC patients for miR-181b expression level. In addition, in SCLC cell line NCI-H446, the expression level of
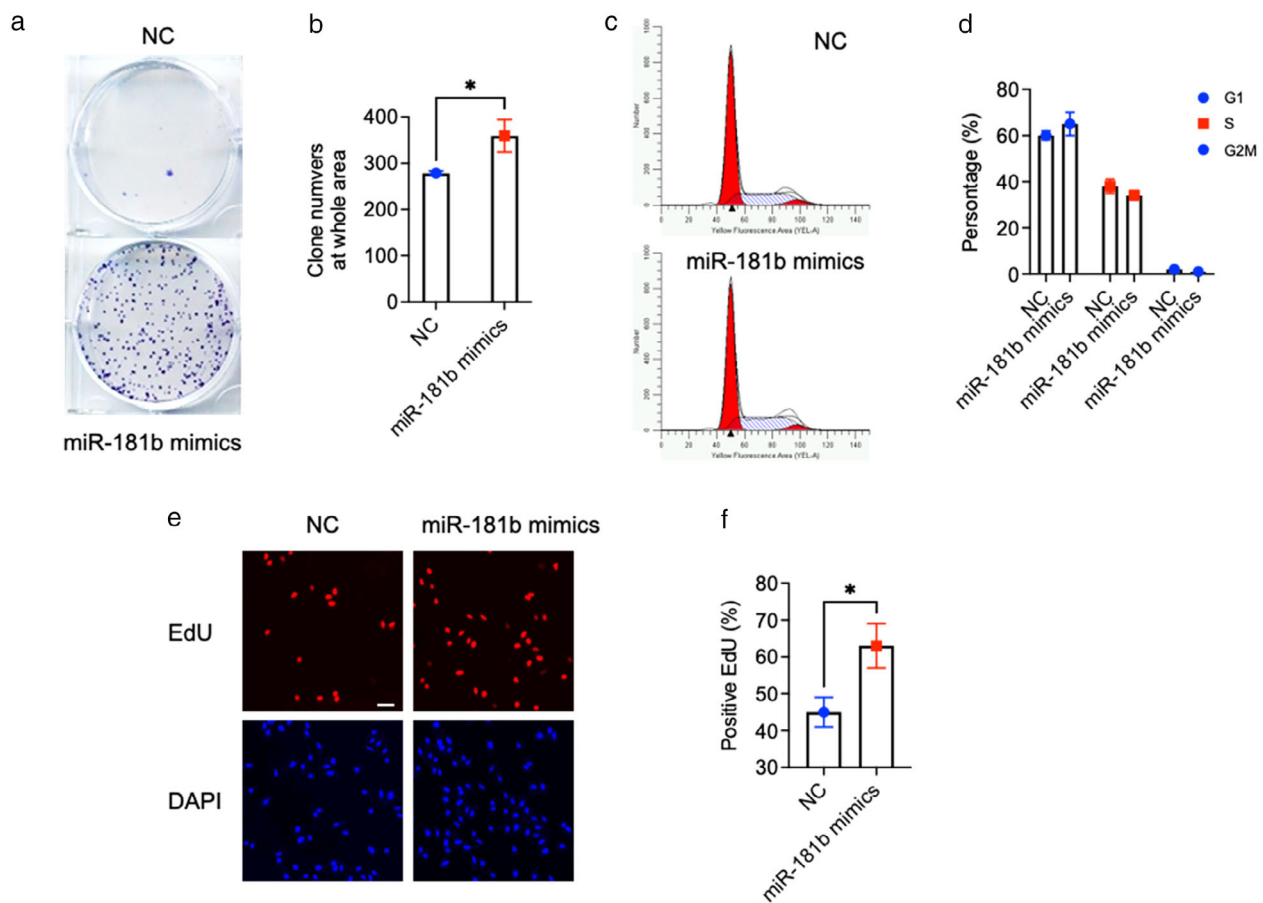

FIGURE 2 Overexpression of miR-181b significantly enhanced the proliferation of SCLC cells. (a) Plate clone formation results of miR-181b-overexpressed HCI-H446 cells. The bar plot (b) indicates the statistics character of (a). (c) The plots show the results from PI-staining flow cytometry assays, illustrating the effects of miR-181b on SCLC cell cycle arrests. (d) Statistical analysis of (c). (e) Immunofluorescence for EdU detection. Red indicates EdU-positive cells and blue indicates 4',6-diamidino-2-phenylindole (DAPI) which marks the nuclei. (f) Statistical analysis of (e). Data are expressed as mean ± SD for at least three independent experiments

miR-181b was still downregulated compared with human normal bronchial cell line HBE cells.

miR-181b overexpression can promote the proliferation ability of the NCI-H446 cell line

We examined whether miR-181b could alter the proliferation profile of SCLC cells (Figure 1). In the plate clone results, overexpression of miR-181b significantly increased the clone numbers of NCI-H446 cells (334 ± 26 clones vs 259 ± 5 clones; Figure 2a), indicating that miR-181b may play a role in the promotion of the proliferation of SCLC cells. PI staining for cell cycle detection was conducted to detect the potential effects of miR-181b on SCLC cells cycle. However, no significance was observed on cell cycle distribution after miR-181b overexpression in NCI-H446 cells. To verify the promotion on proliferation of miR-181b, an EdU assay was conducted. The results shown in Figure 2b illustrate that the EdU-positive cells were significantly higher in the miR-181b group than in the control group, which further demonstrates the exacerbating proliferation effects of miR-181b.

miR-181b can inhibit the migration ability of the NCI-H446 cell line

Tumor metastasis at early stage is one of the most significant hallmarks of SLCL. Hence, migration and invasion assays were conducted to identify the effects of miR-181b on those phenotypes. As illustrated in Figure 3a, miR-181b can inhibit the migration ability of NCI-H446 cells through antagonizing the coalescence of cells wound.
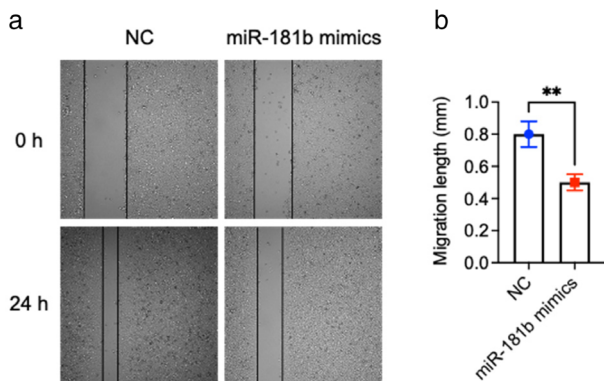
Gene chip targeting of miR-181b downstream regulatory genes

Based on the IPA (Integrated Online Integrated Analysis Software www.ingenuity.com) regulatory effect analysis, we found that the regulators ACE2, ELANE, IL17C, IRF6, MAML1, miR-155-5p (miRNAs w/seed UAAUGCU), ATF3, BCL2L11, CCND1, CCNE2, CDKN1A, and other genes have an inhibitory effect on angiogenesis, binding of
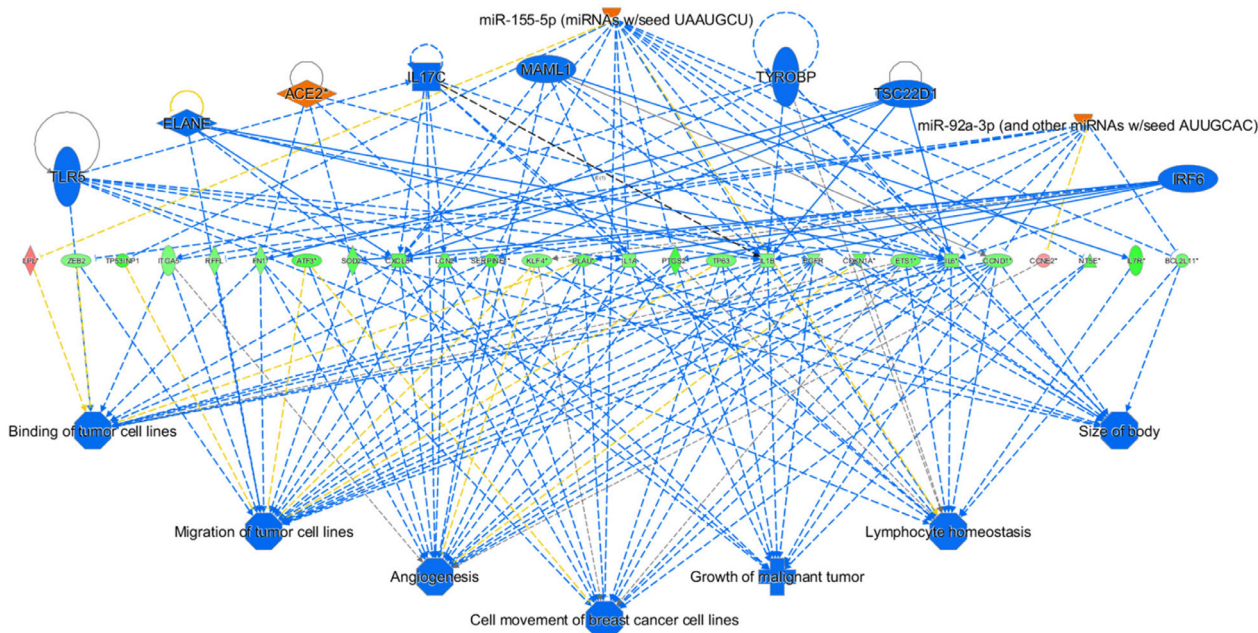

FIGURE 3 miR-181b inhibited the migration ability of the NCI-H446 cell line. (a) Migration of NCI-H446 cells with or without miR-181b overexpression was determined using a wound-healing assay. (b) Percentages of NCI-H446 cells that migrated to the wound area following miR-181b overexpression relative to those of the control cells. Data are expressed as mean ± SD for at least three independent experiments

FIGURE 4 Analysis of the regulatory effects of miR-181b by bioinformatic methods

tumor cell lines, and migration of tumor cell lines (Figure 4).

Western blot verification of downstream proteins

We screened for several target protein genes that changed most significantly in downstream target regions. We know that the proteins regulated by the target genes include ACE2, ELANE, CYLD, IRF6, MAML1, CCND1, and PPARA (Figure 6). They affect the cell growth cycle, biological characteristics, and vascular growth-related factors.
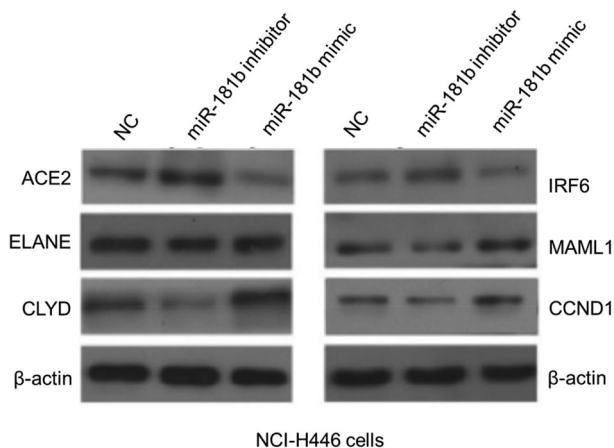

FIGURE 5 miR-181b is involved in ACE2-related molecule pathways. Western blot results show the expression of ACE2, ELANE, CLYD, IRF6, MAML1, and CCND1 after miR-181b inhibitor and mimic transcription in NCI-H466 cells. Data are expressed for at least three independent experiments

Expression of miR-181b is regulated by chemotherapy in clinic

The expression level of miR-181b was detected in healthy people as well as SCLC patients before and post chemotherapy. Interestingly, comparing the content of miR-181b in the blood samples of each group, miR-181b in the peripheral blood of untreated SCLC patients was significantly reduced compared with the normal control group ($P < 0.01$) (Figure 5a). However, after EC (epirubicin and cyclophosphamide) chemotherapy, the miR-181b was significantly upregulated compared with patients without EC treatment. In particular, although increased after EC chemotherapy, the level of miR-181b was still lower than that of healthy people ($P < 0.01$).

Since a significance difference in miR-181b expression was found after EC treatment, it is unclear how miR-181b changed in different cycles of EC chemotherapy. Hence, we detected the peripheral level of miR-181b in SCLC patients after different cycles of chemotherapy. As illustrated in Figure 5b, the expression of miR-181b significantly increased in the first three cycles of EC treatment, while after four cycles of chemotherapy the miR-181b decreased and stabilized at a low level (around 2 ng/mL).

Expression of miR-181b is regulated in chemotherapeutic drug-resistance SCLC cells

The expression of miR-181b was evaluated in resistant SCLC cells (H446R) as well as its parental cells. As illustrated in Figure 6a, the expression of miR-181b was potently lower in H446R cells. To further explain the level of miR-181b with chemotherapy resistance in SCLC, we evaluated the expression level of miR-181b in drug-resistant H446R
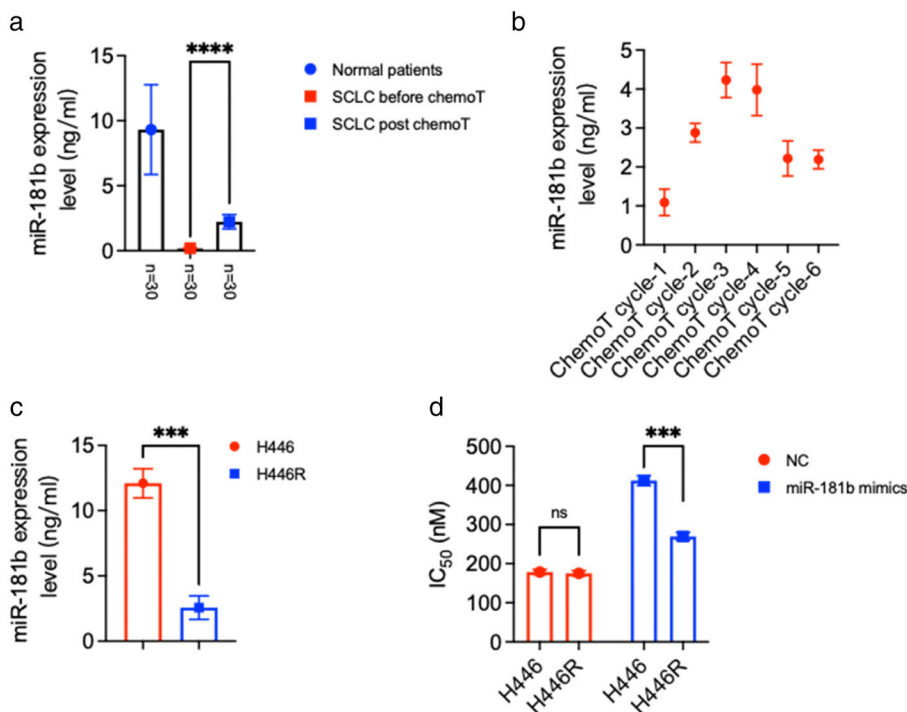

FIGURE 6 Expression of miR-181b is regulated by chemotherapy in clinic as well as cell line level correlated with the drug resistance in SCLC. (a) The expression level of miR-181b in normal patients, SCLC untreated patients, and SCLC patients after chemotherapy. (b) The expression level of miR-18b in different chemotherapy cycles. (c) The expression level of miR-181b in parental H446 and resistance NCI-H446 cells. (d) The sensitivity of H446 and H446R cells to the EC drug after miR-181b overexpression. Data are expressed as mean ± SD for at least three independent experiments

cells. As shown in Figure 6c, in H446R cells, miR-181b significantly decreased compared with parental H446 cells. To further evaluate the effects of miR-181b on SCLC cell drug resistance, we established an miR-181b-overexpression model in both parental as well as resistant H446 cells, and evaluated the cell viability under EC treatment. As illustrated in Figure 6d, no significant difference was observed after overexpression of miR-181b in parental H446 cells. Interestingly, overexpression of miR-181b significantly decreased the cell viability after EC drug treatment, indicated the resistance regulatory effects of miR-181b.

DISCUSSION

SCLC is a kind of lung carcinoma that has a tendency to disseminate early, 80%–85% of patients present with advanced or extensive disease (ES-SCLC) when diagnosed.[6] Although chemotherapy has provided SCLC patients with considerable survival benefits for a long time, the majority of patients relapse and rarely survive for over 2 years.[7] Despite consistent overall response rates of ≥50%, until recently median survival times and 2-year survivals only ranged between 7–10 months and 10%–20%, respectively.[8] Several chemotherapy agents possess activity against SCLC, both as single agents and in combination, but etoposide-platinum has emerged as the preferred first-line regimen. On relapse, many patients remain candidates for additional therapy. However, the sensitivity of relapsed SCLC to further therapies is markedly reduced and dependent on the level and duration of response to the initial treatment.[9] Hence, there is an urgent need to investigate novel biomarkers to indicate the efficacy of chemotherapy as well as the progress of SLCL during the course of treatment.

In recent decades, small nucleotide molecules called mRNAs have come into the limelight by regulating a series of cancer biology processes including cell cycle, metastasis, angiogenesis, metabolism, and apoptosis.[10] For example, it is documented that miR-181b is downregulated in human non-small-cell lung cancer, glioma, prostate cancer, pancreatic cancer, gastric cancer, and myeloid leukemia tissues, while it is upregulated in liver cancer, thyroid and breast cancer, pancreatic cancer, and head and neck tumor tissues, for which miR-181b could be a potential biomarker for diagnosis as well as prognosis of such malignancies in clinic.[11–13] In addition, the different expression of miR-181b in different cancers indicates its diverse mechanisms in cancer proliferation, metastasis, as well as drug resistance. Previously, studies have indicated that miR-181b is an important regulatory factor between inflammation and cancer.[14,15] In the present study, we evaluated the crucial role of miR-181b in SCLC proliferation, migration, and drug resistance.

First, the expression level of miR-181b in the peripheral blood of patients with SCLC was evaluated. The results show that the low expression level of miR-181b in SCLC may play a role in tumor suppressor genes, as also found by Cao et al.[16] in a non-small-cell lung cancer (NSCLC) study and Cinegaglia et al.[17] in a study of lung adenocarcinoma on the results of miR-

181b on other lung cancer types. Our results also indicate that miR-181b has the efficacy to promote the proliferation but inhibit the migration of SCLC in the cell line model. However, although miR-181b shows a pro-viability characteristic, cell cycle detection did not show any difference after miR-181b overexpression. The potential targets of miR-181b were evaluated by bioinformatic technology, which identified that ACE2 is the one of the candidate targets of miR-181b, and the ACE2-related cell signaling molecules were verified by Western blot.

To further understand the role of miR-181b in SCLC drug resistance, we detected the level of miR-181b in peripheral blood after different chemotherapy cycles, which indicated that in the first four cycles of chemotherapy the miR-181b level continued to rise, reaching a peak between the third and fourth cycles, but decreasing after the fifth cycle. These results suggest that there is a concomitant relationship between miR-181b and SCLC resistance, which agrees with the previous study by Wang et al.[18] Voortman et al.[19] found that low expression of a single miRNA predicts poor survival or high recurrence of lung cancer, including miR-181, miR-221, etc. Therefore, changes in the content of miR-181b in the peripheral blood of SCLC patients may make this an effective indicator to predict the effect of chemotherapy, which is also consistent with our in vitro studies.

Multiple roles of miR-181b have been studied in different cancers on drug resistance. For example, in NSCLC-resistant A549/cisplatin (CDDP) cell lines, high expression of miR-181b can be reduced by the inhibition of BCL2 protein levels, in turn, make it sensitive to CDDP-induced apoptosis.[20] Moreover, upregulation of miR-181b can inactivate the Notch2/Hes1 signaling pathway and enhance sensitivity to CDDP therapy. In addition, miR-181b may be involved in the occurrence and development of tumors by regulating targets such as histone metalloproteinase 3 (TIMP3) inhibitor,[21,22] insulin-like growth factor 1 receptor (IGF-1R), and cAMP responsive element binding protein 1(CREB1).[23,24] Our present study adds to these findings and further indicates the important role of miR-181b in lung cancer.

As a potential target of miR-181b, angiotensin-converting enzyme has been found to be involved in the pathogenesis of a majority of tumors. For example, upregulation of ACE in mice could increase the risk of laryngeal cancer.[25] Moreover, the diversity of ACE and eNOS genes increased the risk of NSCLC in India, and the expression of ACE in lung cancer tissues was lower than that of tissue adjacent to cancer tissues.[26] Administration of ACEIs, including captopril, is associated with an increased risk of lung cancer, particularly among those who have used ACEIs for over 5 years.[27] The present study provides some clues that miR-181b targeting ACE gene may also increases the risk of small cell lung cancer.

This study provides an experimental basis for further exploring the relationship between miR-181b and SCLC resistance, but the mechanism and pathway through which miR-181b affects SCLC resistance development still need further research.

miR-181b has a low expression level in SCLC, and it can directly target the gene ACE2 to affect the biological

characteristics of SCLC. In addition, miR-181b is low expressed in SCLC patients, and first-line chemotherapy can promote its recovery, but cannot make it return to normal levels, which suggests a significant role of miR-181b in indicating the process during SCLC treatment.

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CONFLICT OF INTEREST
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT
Data are available upon request to the correspondence author.

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