Effect of miR-181b on the biological characteristics and clinical drug resistance of small cell lung cancer by targeting gene ACE2

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Research

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Abstract

Objective To clarify the effect of miR-181b on the biological function of SCLC, to detect and verify its downstream target gene ACE2, and to explore the effect of clinical resistance on SCLC in order to find new specific diagnostic markers and therapeutic targets.

Methods 1. Collect blood samples from 30 SCLC patients and 30 normal persons in our department from 2017 to 2019 to detect the expression level of miR-181b; 2. Detect the expression level of miR-181b in SCLC cells by RT-PCR, and Screening of downstream target genes used by gene chip, verification with luciferase and Western Blot; 3. Collect the general data of 30 SCLC patients and 30 healthy people (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; 4. In the SCLC cell line and the SCLC mouse model constructed, different concentrations of EC chemotherapy and ACEI drugs were administered to detect the sensitivity of drug resistance and non-drug resistance.

Results 1. The expression level of miR-181b in SCLC patients was lower than normal people; 2. The expression level of miR-181b in SCLC cell lines was lower than normal cells; ACE2 was verified as a downstream target of miR-181b by gene chip screening; 3. miR-181b is low-expressed in SCLC patients, first-line chemotherapy can promote its recovery, but cannot repair to normal levels, and miR-181b has a certain effect on SCLC chemotherapy sensitivity; 4. miR-181b can enhance the drug sensitivity of SCLC drug-resistant cells, the application of ACEI should increase the risk of SCLC drug-resistant.

Conclusion 1. miR-181b has a low expression level in SCLC, and it can directly target the gene ACE2 to affect the biological characteristics of SCLC; 2. miR-181b is lowly expressed in SCLC patients, and first-line chemotherapy can promote it recovery, but cannot repair to normal levels, and ACEI drugs can increase the risk of SCLC drug-resistant.

Introduction

Small cell lung cancer (SCLC) is characterized by short tumor cell doubling time, early metastasis, and high recurrence. The incidence rate has been increasing in recent years. 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 less than 3 months are highly resistant (1, 2).

miRNA (micro RNA, small RNA) is a type of non-coding RNA about 22 nt long. It can regulate gene expression by binding to specific mRNA or regulating 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 that it has important significance in the occurrence and development of malignant tumors, but there was 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 SCLC treatment helps.

Materials And Methods

1. General Information

The peripheral blood samples of 30 patients with SCLC confirmed by pathology from the Department of Thoracic Medicine and Oncology Department of Hebei Provincial People's Hospital from December 2017 to December 2019 were collected and tested for miR-181b expression levels.

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

According to the instructions of Trizol (purchased from Shanghai Pufei), each group of RNA was extracted, and the primers of miR-181b and internal reference U6 were designed and synthesized by Guangzhou Ruipu. According to the instructions of the real-time fluorescence quantitative universal kit, cDNA synthesis, 20μl reverse transcription reaction system includes 2.0μl total RNA, 1.2μl reverse transcription primers, 10.0μl reverse transcription buffer, 0.2μl reverse transcriptase and 6.6μl DEPC water. Reaction conditions: 30 minutes at 26°C; 30 minutes at 42°C; 10 minutes at 85°C. The microRNA real-time fluorescence quantitative reaction system includes 0.08μl, 0.4μl Taq DNA polymerase (2.5U/μl), 2μl DNA template, 7.44μlddH2O for each of the upstream and downstream primers; the amplification conditions are denaturation at 95℃ for 3 minutes, 95℃ for 12 seconds, 62℃30 There are 40 cycles in seconds, 72℃ and 30 seconds. After the test, 3 repeats are set for each sample, and the average value is taken as the test result. The Ct value is used to indicate the expression level of the target gene mRNA. According to the dissolution curve of SYBR Green1 and the electrophoresis diagram of PCR products, it is judged that the system is free from pollution and non-specific amplification.

Relative quantitative analysis $F=2^{-\Delta\Delta Ct}$; ΔCt = target gene Ct value-internal reference gene Ct value; -ΔΔCt = NC group ΔCt average value-each sample ΔCt value; F value reflects the relative expression level of each target gene.

3. Downstream target gene screening

According to the product instructions, determine the suitability of the chip based on the original sample. According to the instructions of the kit, extract the total RNA samples of NCI-H446 cells, analyze them by Agilent 2100 RNA quality detector, and use gene chip 3undefinedIVT expression kit to prepare amplified RNA. After reverse transcription, cDNA is synthesized by single-stranded synthesis, and then double-stranded synthesis to obtain double-stranded DNA template, and then reversed in vitro to obtain biotinylated aRNA (amplified RNA). After purifying aRNA, it hybridizes with the chip probe, cleans and stains the chip, and scans to obtain images and original data.

4. Luciferase report analysis

NCI-H446 cells were seeded in 24-well plates at a density of 40-50%. The next day, transfected HEK 293T cells pmirGLO-ACE-2-3'UTR wild type (Wt) or pmirGLO-ACE-2-3'UTR mutant (Mut) (genefarma) with miR-181b mimics or miR-NC. The cells were then incubated in 37% 5% CO_2 for 2 days. Transfected harvested cells and used a dual Lu-ciferase reporter system (Promega Corpo-ration, Madison, WI, USA) for luciferase report analysis. Renin luciferase was used as an internal control to correct differences in transfection and harvest efficiency.

5. Western blot analysis

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

6. The effect of miR-181b on the biological function of SCLC cell line

1). Multiplication experiment

The cloning ability of tumor cells

Prepare a cell suspension in the logarithmic growth phase and count. Each experimental group was seeded with 400-1000 cells/well in a 6-well plate culture plate, and three replicate wells were set for each experimental group. Before the experiment was terminated, the cell clones were photographed under a fluorescence microscope. After washing and fixing, 500 μL of a 1:1 diluted crystal violet aqueous solution was added to each well to stain the cells for 1-5 minutes; ddH2O washed the cells several times and dried them. Digital cameras take pictures and organize them, and clone counts.

Cell cycle detection

Prepare a cell suspension, collect the cells in a 5 mL centrifuge tube, and set up three complex wells for each group; fix by centrifugation, washing, re-centrifugation, 75% ethanol fixation, centrifugation, washing, centrifugation, and de-fixation solution. According to 40 × PI mother liquor (2 mg/mL): 100 × RNase mother liquor (10 mg/mL): 1 × D-Hanks = 25: 10: 1000. Perform cell staining according to the ratio: add a certain volume of cell staining solution (0.6-1 mL) to resuspend according to the amount of cells, so that the cell pass rate is 300~800 Cell/s when it is on the machine. On-board testing and data analysis.

EDU detection

According to the instructions of the EDU kit (C10310 Ruibo), by detecting EDU infiltrating during DNA replication, new proliferating cells can be accurately detected with the help of fluorescence detection tools, and the percentage of cells in S phase can be quickly counted to analyze the cell proliferation.

2). Invasion experiment

Scratch test

According to the experimentally designed group, add about 3×10^4 infected cells to the wells; change the low-concentration serum medium the next day, use the scratcher to align the center of the lower end of the 96-well plate, and gently push up to form Scratches. Incubate at 37°C, 5% CO_2 incubator, and take pictures through the microscope to obtain pictures after migration 0 h, 8 (or 16) h and 24 h (or other time points).

Transwell testing

Transwell test was performed according to the instructions of Transwell kit (purchased from Corning) and MTS kit (purchased from Promega). And by calculating the average value and standard deviation of tumor cell metastasis of each group, the final p-value was obtained by T-Test analysis.

Invasion experiment

According to the instructions of the invasion kit (purchased from Corning), the invasion experiment was carried out, and a microscope was used to randomly select the field of view of each cell to take pictures, 4 100X photos and 9 200X photos. Count with 200X photos, and calculate the standard deviation of the number of invasion and metastasis cells in each group, and finally perform T-Test analysis to obtain the p value, and compare the difference in cell invasion ability between the experimental group and the control group.

7. Clinical data collection

Collected peripheral blood samples of 30 patients with SCLC confirmed by our department from December 2017 to December 2019. There were 22 males and 8 females, with a median age of 61.3 years (41-82 years); 16 smokers and 14 non-smokers. No chemical or radiotherapy was performed before admission. All patients had a functional score of >60 before chemotherapy, blood routine and renal function were within the normal range, and ECG was normal. All patients draw venous blood before chemotherapy. 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: 8 cases with limited period, 22 with extensive period, including 27 cases with lymph node metastasis and 3 cases without lymph node metastasis; There were 22 cases of metastasis and 8 cases of distant metastasis. The 30 normal

controls were all healthy and non-tumor; 18 males and 12 females, with a median age of 58 years (41 to 75 years); 5 smokers and 25 non-smokers (see table 1).

Table 1. General information about patients in control and experimental groups

Group（N）		Control Group	Experimental Group
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

8. SCLC cell line culture

Laboratory culture of SCLC (H446 cell line and H446 drug-resistant cell line) (purchased from the cell bank of the Chinese Academy of Sciences), using RPMI1640 medium containing 15% fetal bovine serum as the cell expansion medium, and cultured at 37°C and 5% CO_2 cells Cultivation in the box. The fresh medium was replaced in 48-hour cycles. Select the cells with good growth conditions and quickly fill the bottom of the culture flask. After centrifuging for 5min (1500rpm), slowly discard the supernatant along the direction of precipitation, and then add 1ml of the pre-prepared cell cryopreservation solution (medium: Fetal bovine serum: DMSO=5:4:1), and then repeatedly pipetting with a 1ml pipette to resuspend the pelleted cells. After being placed at 4℃ for 20min, transfer to a -20℃ refrigerator for 2h, then transfer to an ultra-low temperature refrigerator (-80℃), and store it for 48h. If it is to be stored for a long time, it must be transferred to a liquid nitrogen tank.

9. CCK8 detection of drug sensitivity of sensitive and resistant strains before and after overexpression

H446 series cells (H446, H446 overexpression strains) and H446 drug resistance series cells (H446, H446 overexpression strains) have a total of 4 types of cells, and each cell is divided into the following three subgroups: (1) blank control group (no cells) (Culture only); (2) Negative control group (no drug only cells); (3) Drug group (EC chemotherapy drugs), the drug group is divided into sub-groups according to the drug series concentration. Cells were inoculated into 96-well culture plates at 5×10^3 cells/well, and 3 parallel wells were set. After culturing the cells for 24h in the drug-containing medium, aspirate the medium, wash three times with PBS, add CCK8 reaction solution (100μl RPM1 1640 + 10μl CCK8), and incubate at 37℃, 5% CO_2 incubator for 1-4h. The instrument detects the optical density (D) value of each well at the wavelength of 450 nm, and calculates the cell survival rate. 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%. Repeat the experiment 3 times. According to the cell survival rate of each drug concentration, make a logarithmic curve to obtain the drug concentration (IC50) at 50% cell survival rate; and calculate the drug resistance index, drug resistance index = IC50 (H446 resistance) / IC50 (H446).

10. Use of ACEI drugs

Captopril was selected as miR-181b downstream target gene ACE2 inhibitor for sensitivity detection of drug-resistant and non-drug-resistant SCLC cells (same steps as 9).

11. Statistical analysis

The statistical analysis of this experimental data uses SPSS 21.0 for Windows software, and the results are expressed as mean ± standard deviation. The t test is used for comparison between two groups, and the analysis of variance is used for comparison between multiple groups, $p<0.05$. Indicates that the difference is statistically significant.

Results

1. miR-181b shows low expression abundance in NCI-H446 cell line

Using real-time quantitative qPCR method to detect the expression level of miR-181b in NCI-H446 cell line was significantly lower than the control group ($P <0.01$), the verification results are shown in Figure 1.

2. MiR-181b overexpression can promote the proliferation ability of NCI-H446 cell line

The results of cell clone detection showed that overexpression of miR-181b can induce NCI-H446 cells to be subcultured, and the clone generation of NCI-H446 cells increased from before expression (259 ± 5 mean ± standard deviation) to overexpression (334 ± 26 mean ± standard deviation), the difference between the two is statistically significant. As can be seen from Figure 2(A), cell cycle analysis showed that before and after miR-181b overexpression, in NCI-H446 cells, the number of G1 and S phase cells $P<0.05$, the number of S phase cells $P<0.05$, G- The number of cells in phase 2/M $P>0.05$ (Figure 2(B)). The results showed that the overexpression of miR-181b could increase the proportion of two cells in G1 phase, but there was no significant difference between the two types. EDU results showed that the proliferation rate of NCI-H446 cells in the miR-181b high expression group was significantly higher than that of the control Group ($P<0.05$) (Figure 2(C)).

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

The scratch test showed that miR-181b can inhibit the migration ability of NCI-H446 cells ($P<0.01$). Page 01). (Figure 3(A)) and Transwell show that NCI-H446 cells have weak migration ability before and after miR-181b overexpression, see Figure 3(B), we obtained the same results in the invasion test, see Figure 3(C).

4. 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), etc. passed ATF3, BCL2L11, CCND1, CCNE2, CDKN1A and other genes have an inhibitory

effect on Angiogenesis, Binding of tumor cell lines, Migration of tumor cell lines. (Figure 4). The effect correlation of the miR-181b-ACE2-ATF3 pathway can be obtained from this.

5. miR-181b-ACE2 pathway response verification test

RT-PCR was used to detect changes in the expression level of luciferase-labeled ACE2 gene after overexpression of miR-181b at different concentrations (1-fold, 10-fold, and 100-fold) (Figure 5). The results showed that after overexpression of miR-181b, ACE2 increased significantly (p=0.0029 years). Overexpression of ACE2 in turn promotes the expression of miR-181b (Figure 5 right).

6. 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, PPARA (Figure 6). They affect the cell growth cycle, biological characteristics and vascular growth-related factors.

7. Animal model verification

We divided the mice into three groups: blank control group (normal mouse), negative control group (H446 cells) and experimental group (mir-181b overexpressed H446 cells), we named it as negative control in Figure 7 Group "1" and experimental group "2". The tumor growth of the negative control group was significantly faster than that of the experimental group (Figure 7A), the tumor mass of the former was significantly higher than that of the control group (Figure 7A) and the latter (12.3±2.6gVS5.6±1.6gp=0.0023), and the blank control group It is empty by examining the biological characteristics of tumor cells EDU (Figure 7C) and cell proliferation experiments (Figure 7D). It can be seen that the negative control group is significantly better than the experimental group in terms of cell proliferation and proliferation. Luciferase-labeled ACE2 was overexpressed at different concentrations (1-fold, 10-fold, 100-fold) of miR-181b, and then detected by RT-PCR technology (Figure 7B). The results showed that ACE2 increased significantly after overexpression of miR-181b (p=0.021).

8. The clinical resistance of miR-181b and its target genes to SCLC

8.1 The expression level of miR-181b in healthy people and SCLC patients after chemotherapy

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, with a statistically significant difference (P<0.01) (as shown in Figure 8A), but after EC treatment, its expression level has increased, but it is still lower than the normal content (P <0.01). (See Table 2)

Table 2 The relative expression levels of miR-181b in each clinical group

Group	Quantity	miR-181b (ng/ml)
Control Group	30	9.32±3.45*
Pre Chemotherapy	30	0.19±0.03△
Post Chemotherapy	30	2.23±0.55

*: P<0.01, control group compared with before chemotherapy; △: P>0.05, control group compared with after chemotherapy

8.2 miR-181b expression level in SCLC patients with different chemotherapy cycles

After 1 cycle of chemotherapy, the expression level of miR-181b increased, but was lower than the normal level (P <0.01) (as shown in Figure 8B); after 6 times of chemotherapy, the content of each group showed a significant increase and decrease, including 3 4 There is a peak, and there is a significant difference between the groups before and after the comparison, and the content is increased (P <0.01). There is a statistical difference between groups 1 to 4 (P <0.01), indicating that the content is increasing with the progress of chemotherapy, but it has a downward trend since group 5, and there are obvious statistics between 5, 6 and 3, 4 Academic difference (P <0.01) (as shown in Fig8B). As the cycle of chemotherapy increases, its content may indicate that SCLC is resistant. (Table 3)

Table 3 miR-181b expression levels in different chemotherapy cycles

Chemotherapy cycle	Quantity	miR-181b (ng/ml)
1	30	1.90±0.34
2	30	2.88±0.24
3	30	4.23±0.45
4	30	3.98±0.66
5	30	2.22±0.45
6	30	2.19±0.24

8.3. The expression level of miR-181b in SCLC (H446 cell line and H446 drug-resistant cell line) By real-time quantitative qPCR technology, miR-181b was low in abundance in H446 cell line, and the content of miR-181b in H446 cell line was higher than that of H446 drug-resistant strains (P<0.01) (see Table 4).

Table 4 The expression levels of Mir-181b in different cell lines (*: P<0.01, compared with two strains)

Group	miR-181b（ng/ml）
Sensitive Strains	12.09±1.12
Drug-resistant Strain	2.57±0.90*

8.4. Sensitivity and resistance of SCLC cells to chemotherapy drugs after miR-181b transfection

The H446 cell line was over-expressed with miR-181b by lentivirus transfection, there was no significant difference between the two groups (P = 0.3741>0.05); the drug-resistant group had statistically significant difference before and after over-expression of miR-181b (P =0.01047<0.05); In addition, the over-expression of miR-181b in the drug-resistant group was statistically different from that in the sensitive group (P =0.00912<0.01VS P =0.0127<0.05) (Figure 9). After overexpression of miR-181b in the H446 drug-resistant cell line, the IC50 value of chemotherapeutic drugs decreased significantly (412.28±12.71 vs 269.55±10.21). (Table 5)

Table 5 IC50 values (± s) of chemotherapy drugs before and after miR-181b overexpression

Drug	H446		H446 Drug Resistance	
	Pre Overexpression	Post Overexpression	Pre Overexpression	Post Overexpression
EC	178.42±6.58	175.15±7.23*	412.28±12.71	269.55±10.21△

*: P>0.05; △: P<0.01.

8.5. Sensitivity detection of ACEI drugs to resistant and non-resistant SCLC cells

In SCLC cells in drug-resistant and non-drug-resistant groups, different results appeared after the addition of ACEI drugs before and after chemotherapy. For the drug-resistant group, the IC50 value of the drug after the addition of ACEI was higher than that before chemotherapy (p=0.0042), while for the non-drug group, the IC50 value of the drug was higher than that before chemotherapy (p =0.023), there is a significant statistical difference between the two groups (see Table 6); and its sensitivity can be seen that both groups showed a decreasing trend after applying ACEI (Figure 10).

Table 6 IC50 value of each group of chemotherapy drugs after adding SCEI cells to each group (± s)

Drug		H446		H446 Drug Resistance	
Add medicine	ACEI	Pre Overexpression	Post Overexpression	Pre Overexpression	Post Overexpression
EC		368.9±11.72	472.13±13.33*	192.25±10.02	174.2±9.63△

*: P0.01; △: P<0.01.

8.6 Results of adding ACEI and blank control in mouse SCLC model

HE staining of two groups of small mouse books showed that the mouse SCLC model was successfully constructed (see Figure 11). To show the activity of SCLC cells in each group, Ki-67 immunohistochemical staining was performed (see Figure 12). Ki-67 was 80% in the ACEI group and 65% in the non-group.

Discussion

miR-181b is down-regulated in human non-small cell lung cancer, glioma, prostate cancer, pancreatic cancer, gastric cancer, and myeloid leukemia tissues, while up-regulated in liver cancer, thyroid and breast cancer, pancreatic cancer, and head and neck tumor tissues, can be used as a biomarker for diagnosis and detection of tumors (6–8). These results suggest that the function of miR-181b may be unique, depending on the tumor type and cellular environment. Previous studies have shown that miR-181b is an important link between inflammation and malignant tumors. This is due to the transient expression of miR-181b to induce epigenetic switches, which can inhibit miR-181b target CYLD, thereby regulating NF-κB activity. The role. Therefore, miR-181b is part of the positive feedback loop for epigenetic conversion between inflammation and cancer, as shown in Fig. 13. Its target genes are Bcl-2, TIMP3, CYLD and CBX7, etc., which in turn affect the development and invasion of related tumors (9, 10).

The incidence of lung cancer ranks first among all cancers, and the mortality rate is also the highest. With the advent of targeted drugs, the treatment of NSCLC has made great progress, but the treatment of small cell lung cancer is still at a standstill. At present, the classic treatment plan of platinum plus etoposide combined with radiotherapy, although it has a certain effect on SCLC, is very prone to drug resistance, so it is urgent to study new treatment methods. At present, there are increasing researches on miRNA and tumor, and it is confirmed that there is a clear relationship between tumor and miRNA disorder. In this study, the real-time quantitative PCR method was used to detect the expression level of miR-181b in the peripheral blood of patients with SCLC. The results showed that the low expression level of miR-181b in the occurrence of SCLC may play a role in tumor suppressor genes, which is also the same as Cao et al (12) in the NSCLC study and Cinegaglia NC et al (13) in the study of lung adenocarcinoma on the results of miR-181b on other lung cancer types have similar conclusions.

The miR-181b and ACE genes are not on the same chromosome, and there is a mutual regulation relationship between them. A large number of studies have shown that angiotensin-converting enzyme is

involved in the pathogenesis of tumors. For example, Han et al. (14)found that upregulation of ACE in mice increased the risk of laryngeal cancer. Peddiredy, Vidyullatha and others(15) found that in India, the diversity of ACE and eNOS genes increased the risk of non-small cell lung cancer, and the expression of ACE in lung cancer tissues was lower than that of tissue adjacent to cancer tissues. It is just like Blánaid M Hicks and others(16) found that the use of ACEIs is associated with an increased risk of lung cancer, and this correlation is particularly significant in people who have used ACEIs for more than 5 years. In short, angiotensin-converting enzyme (ACE) is expressed in a variety of tumors, which can affect tumor proliferation, cell migration, angiogenesis and metastasis. However, after the application of ACE inhibitors, some tumors were suppressed, but the specific mechanism of action still needs further study. The miR-181b targeting ACE gene also increases the risk of small cell lung cancer.

The experiment also found that the expression level of miR-181b in plasma of SCLC patients changed with the extension of the treatment period. By detecting the level of miR-181b in peripheral blood after different chemotherapy cycles, it was found that in the 4 cycles before chemotherapy, the miR-181b level continued to rise with the increase in the number of chemotherapy, and reached a peak during 3 and 4 cycles, but its content was still low At the level of healthy people, as the number of chemotherapy increases, the probability of chemotherapy resistance increases, and with the increase in resistance, the level of miR-181b decreases after the fifth cycle. The results suggest that there is a concomitant relationship between miR-181b and SCLC resistance, that is, when the expression level of miR-181b decreases again, it may indicate the occurrence of SCLC resistance, and this is also the same as Wang Yuntao et al(17). The expression of miR-181b in lung cancer patients' tissues is low, and the corresponding clinical symptoms are consistent. Voortman J et al.(18) found in research 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 peripheral blood of SCLC patients may be Make it an effective indicator to predict the effect of chemotherapy. This is consistent with the results of this experiment.

The previous results showed that miR-181b was under-expressed in H446 cell line, while the expression levels in H446 cell line and corresponding drug-resistant cell line were detected, and the expression level of miR-181b in drug-resistant cell line was significantly lower In the H446 cell line, these results have similar results to clinical blood. In the overexpression experiment of miR-181b, we found that the expression level of miR-181b in two cell lines was regulated by overexpression of lentivirus transfection. After overexpression of its content, the H446 cell line was sensitive to drugs before and after transfection There was no statistically significant difference in sex, and the drug sensitivity of drug-resistant strains was statistically different before and after transfection. In addition, the over-expression of miR-181b in the drug-resistant group was statistically different from that in the sensitive group. Moreover, the IC50 value of the H446 drug-resistant cell line after transfection with lentivirus overexpressed miR-181b was significantly reduced. It can be inferred that the increased expression of miR-181b in SCLC may directly target the regulation of the activity of downstream substances, thereby affecting the inactivation or enhanced activity of signals such as certain proteins. These changes will lead to increased sensitivity of SCLC to chemotherapy drugs. It is also possible that the increase in miR-181b content will affect

upstream genes through a feedback mechanism, resulting in decreased activity of certain proteins. The specific mechanism is not yet clear, but all tend to think that changes in the expression level of miR-181b will regulate the activity of the target and cause changes in related functions.

MiR-181b also has different mechanisms in other tumor treatments and drug resistance. As Zhu et al.(19) found that in NSCLC-resistant A549/cisplatin (CDDP) cell lines, high expression of miR-181b can be reduced BCL2 protein levels, in turn, make it sensitive to CDDP-induced apoptosis. And to some extent participate in the formation of lung cancer cell line MDR. Studies have also found that increased expression of miR-181b can inactivate the Notch2/Hes1 signaling pathway and enhance the sensitivity to CDDP therapy. In addition, Mir-181b may be involved in the occurrence and development of tumors by regulating various targets such as histone metalloproteinase 3 (TIMP3) inhibitor, insulin-like growth factor 1 receptor (IGF-1R) and cAMP responsive element binding protein 1(CREB1).

ACE2 is the downstream target gene of miR-181b, and after administration of ACEI drugs, it was found that its family increased SCLC tumor resistance. This result is the same as Zosia Kmietowicz et al.(20) in its clinical application of ACEI drugs will increase The risk of lung cancer is similar. But Lever et al.(21) first proposed that ACEI may have anti-cancer effects. And elaborated that its anti-cancer effect mainly includes inhibiting tumor angiogenesis and extracellular matrix degradation, reducing cancer morbidity and mortality, inhibiting tumor growth, growth and metastasis, etc.(18)

Therefore, by regulating the expression of miR-181b, it has certain significance for the treatment of patients to increase the sensitivity of SCLC to chemotherapeutic drugs. However, there are many factors affecting SCLC resistance, and the specific regulatory mechanism still needs further experiments to discover its conduction pathways. Studies have shown that miRNA usually regulates more than 100 target genes to regulate tumor proliferation and metastasis(13). This study provides an experimental basis for further exploring the relationship between miR-181b and SCLC resistance, and the mechanism and pathway through which miR-181b affects SCLC resistance development still needs further research.

Conclusion

There is low expression of miR-181b level in SCLC, and it can directly target the gene ACE2 to affect the biological characteristics of SCLC; 2. miR-181b is low expressed in SCLC patients, and first-line chemotherapy can promote its recovery, but cannot return to normal levels, and ACEI drugs can increase the risk of SCLC drug-resistant.

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with

animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and material (Not applicable)

Conflicts of interest/Competing interests (include appropriate disclosures)

Article Title: Effect of miR-181b on the biological characteristics and clinical drug resistance of small cell lung cancer by targeting gene ACE2

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All authors of this manuscript have directly participated in planning, execution, and/or analysis of this study.

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I am one author signing on behalf of all co-authors of this manuscript, and attesting to the above.

Authors' Contributions

HL and JS participated in the design of experiment, the execution of experiment and the writing of the paper; SH was responsible for the collection and analysis of experimental data; QY was responsible for the polishing and modification of the paper; YW was responsible for the design of experiment, and guided the execution of experiment, data collection and analysis, writing and revising the paper. All authors read and approved the final manuscript.

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Figures

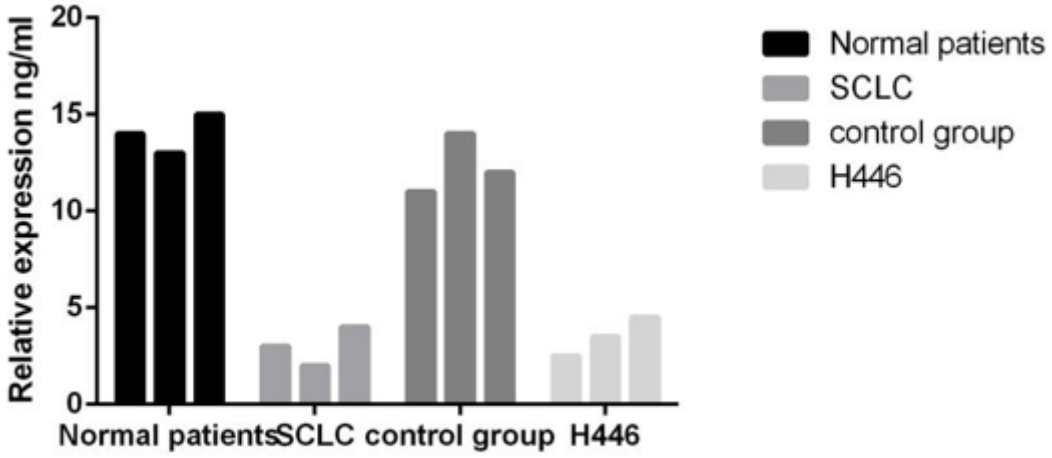

Figure 1

miR-181b expression levels in both groups Note: In the figure, the expression level of miR-181b in NCI-H446 cell line is significantly reduced compared with the internal control (P=0.0012).

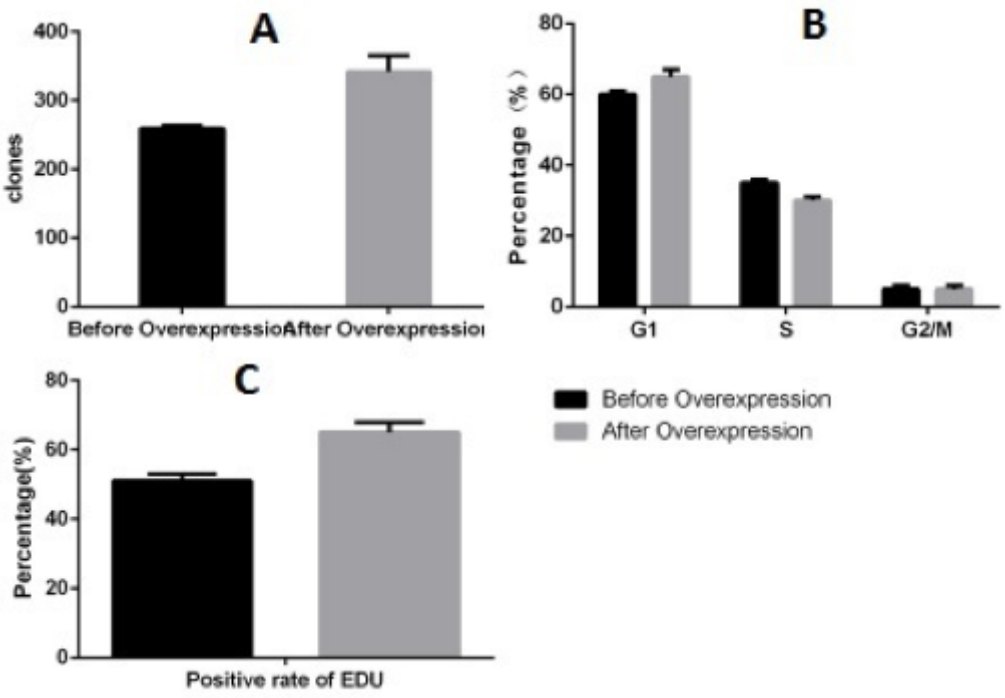

Figure 2

Tumor cell proliferative capacity: The above three figures are tumor cell clone detection (A), cell cycle detection (B) and EDU detection (C) before and after miR-181b overexpression

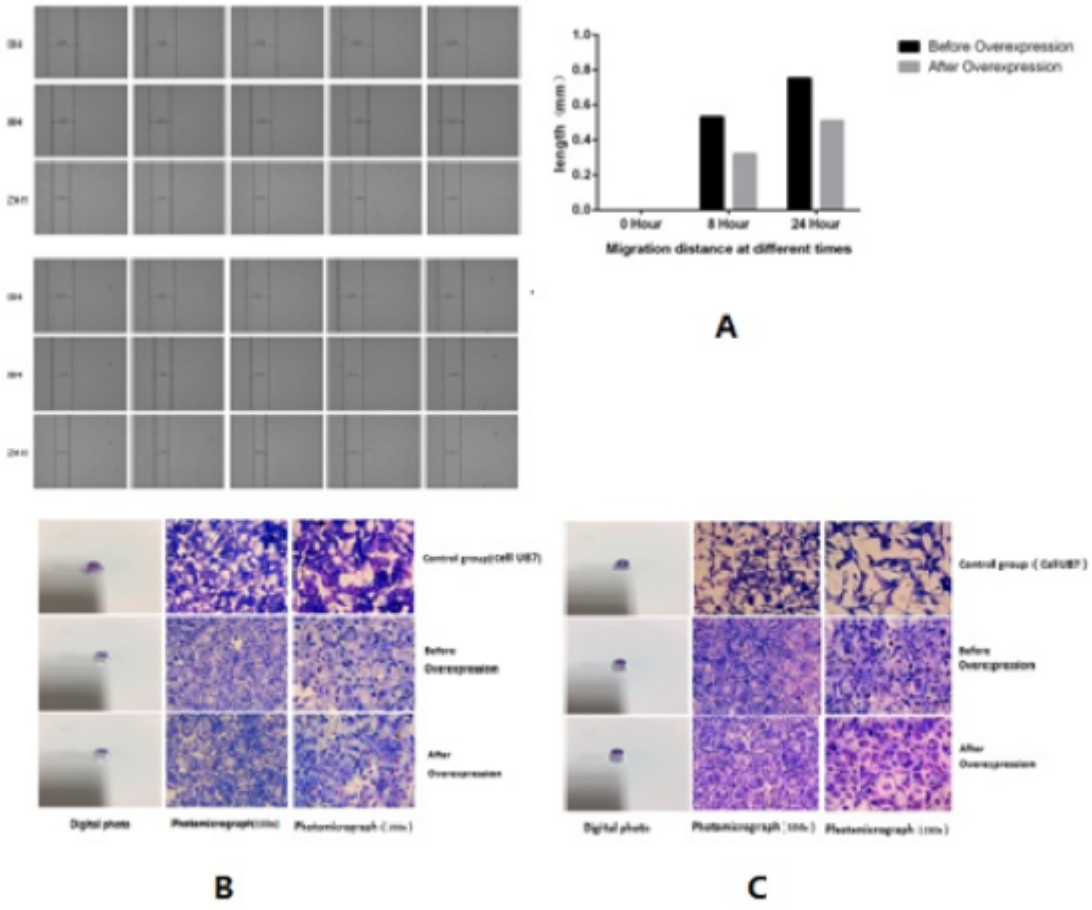

Figure 3

Tumor cell migration ability: Figure A shows miR-181b migration distance at different times before and after overexpression and analysis results. The first two images are visual images of the migration distance at different times under the microscope, the upper image is the migration image before expression, and the lower image is the migration image after expression. The figure below shows the results of data collection and analysis after processing by PS software. Panels B and C show the detection of transwell and migration ability before and after miR-181b overexpression (Giemsa staining).

ID	Consist...	Node Total	Regulator ...	Regulators	Target T...	Target Molecules...	Disease &...	Diseases & Functions	Known Regulat...
1	28.045	43	10	↓ACE2, ↑.....all 10	26	↓ATF3, ↓BCL.....all 26	7	Angiogenesis, Binding of tumor.....all 7	19% (13/70)
2	26.888	84	16	CHD4, Co.....all 16	61	↓ABCA1, ↑AK.....all 61	7	Apoptosis of embryonic cell l.....all 7	12% (13/112)
3	25.491	36	30	AFF, BMP2, ...all 30	5	↓ACVR1, ↓IL1B, ...all 5	1	Increased activation of alkal.....all 1	20% (6/30)
4	24.095	62	11	Apl, COL.....all 11	43	↓ABCA1, ↓AN.....all 43	8	Angiogenesis, Apoptosis of hem.....all 8	20% (18/88)
5	23.600	42	11	Alpha c.....all 11	25	↓ATF3, ↓CDK.....all 25	6	Apoptosis of lymphoid organ, C.....all 6	17% (11/66)
6	23.434	90	22	Betacat.....all 22	63	↓ACSL4, ↓AD.....all 63	5	Cell movement of breast cance.....all 5	27% (30/110)
7	23.307	92	19	↓AENT, ...all 19	70	↓ABCA1, ↓AC.....all 70	3	Cell movement of breast cance.....all 3	35% (20/57)
8	22.804	48	9	Alpha ca.....all 9	32	↓ABCA1, ↓AP.....all 32	7	Apoptosis of lymphoid organ, C.....all 7	10% (6/63)
9	22.203	71	11	COL18A1, ...all 11	50	↓ABCA1, ↑AK.....all 50	10	Apoptosis of lymphoid organ, ...all 10	17% (19/110)
10	21.292	94	26	BRCA1, C.....all 26	63	↓ABCA1, ↓AN.....all 63	5	Apoptosis of embryonic cell l.....all 5	17% (22/130)
11	20.977	47	9	CTBA, F3, ...all 9	27	↑CDH1, ↓CDK.....all 27	11	Apoptosis of lymphoid organ, ...all 11	22% (22/99)
12	20.616	33	13	CEBPA, Cg.....all 13	17	↓ABCA1, ↓BC.....all 17	3	Formation of glomerular cresc.....all 3	15% (6/39)
13	20.278	57	9	DACH1, IT.....all 9	38	↓ABCA1, ↓AMH, ...all 38	10	Apoptosis of lymphoid organ, ...all 10	19% (17/90)
14	19.800	37	8	CST5, FAT1, ...all 8	24	↓ANXA5, ↓BMP, ...all 24	5	Apoptosis of lymphoid organ, C.....all 5	20% (8/40)
15	19.290	118	18	COL18A1, ...all 18	90	↓ABCA1, ↓AB.....all 90	10	Apoptosis of lymphoid organ, ...all 10	17% (30/180)

Figure 4

Analysis table of regulatory effects

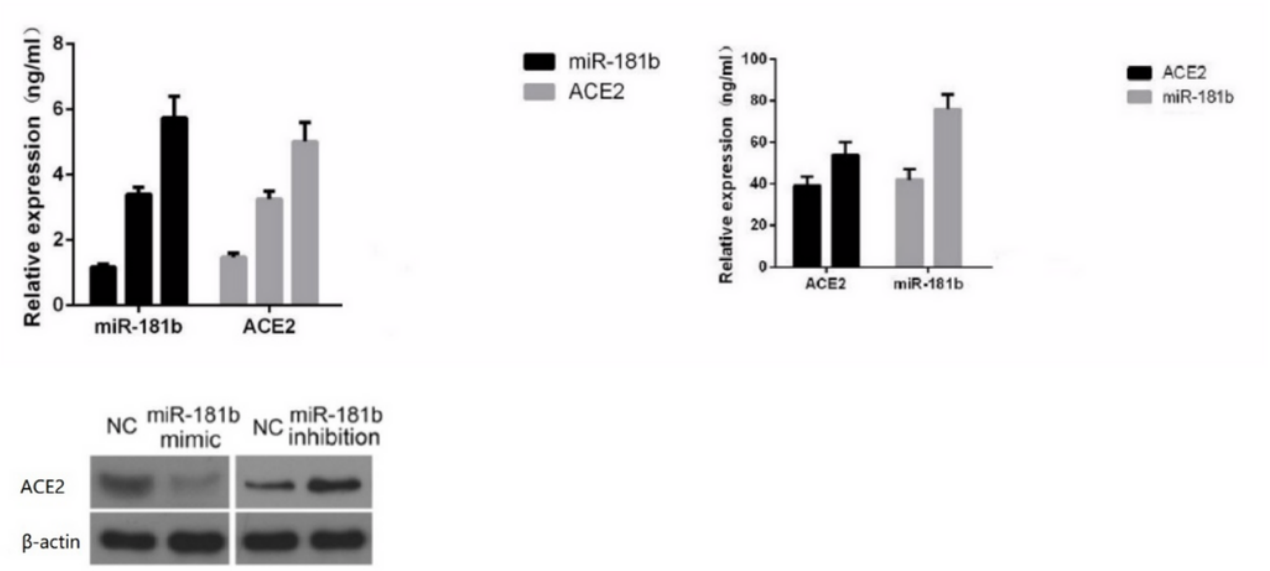

Figure 5

Relative expression levels of miR-181b and its target genes The expression levels of the downstream target gene ACE2 after overexpression of different concentrations of miR-181b.

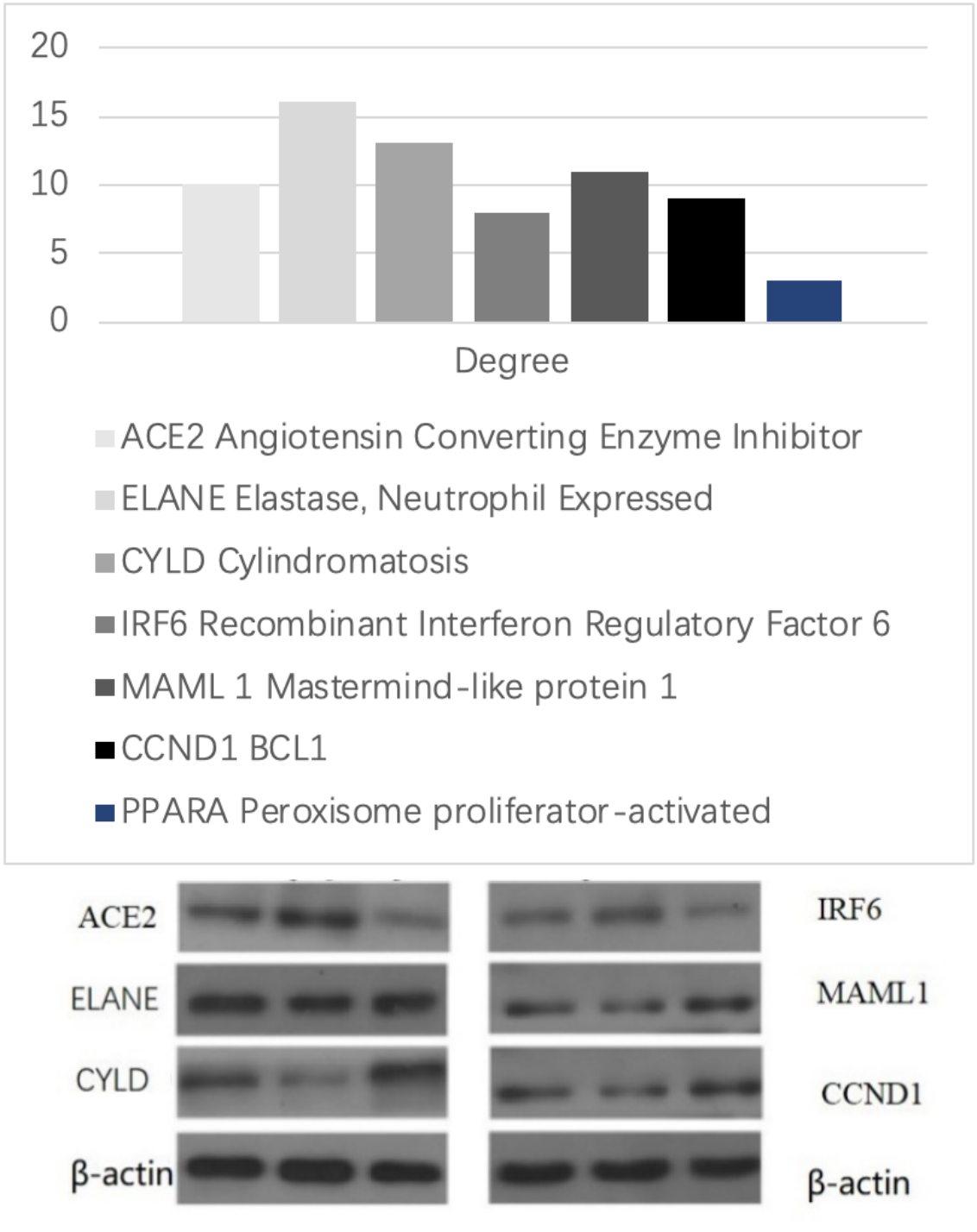

Figure 6

MiR-181b target gene protein electrophoresis results show the expression of downstream target genes such as ACE2 after miR-181b overexpression.

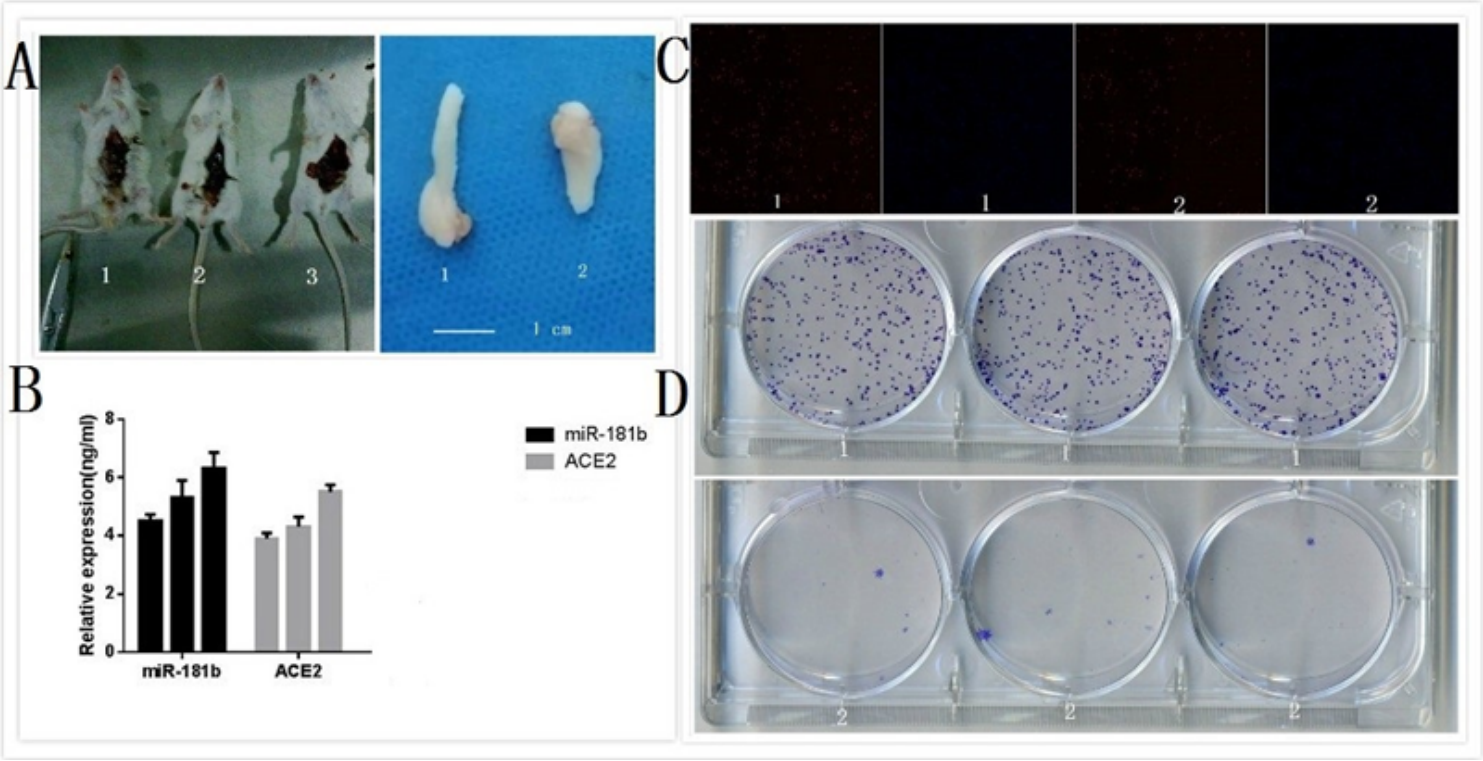

Figure 7

Description of various indicators of mice in each group. (A) Growth of mouse tumor tissue; (B) Relative expression level of miR-181b gene and its target gene; (C) EDU experiment; (D) Cell proliferation experiment.

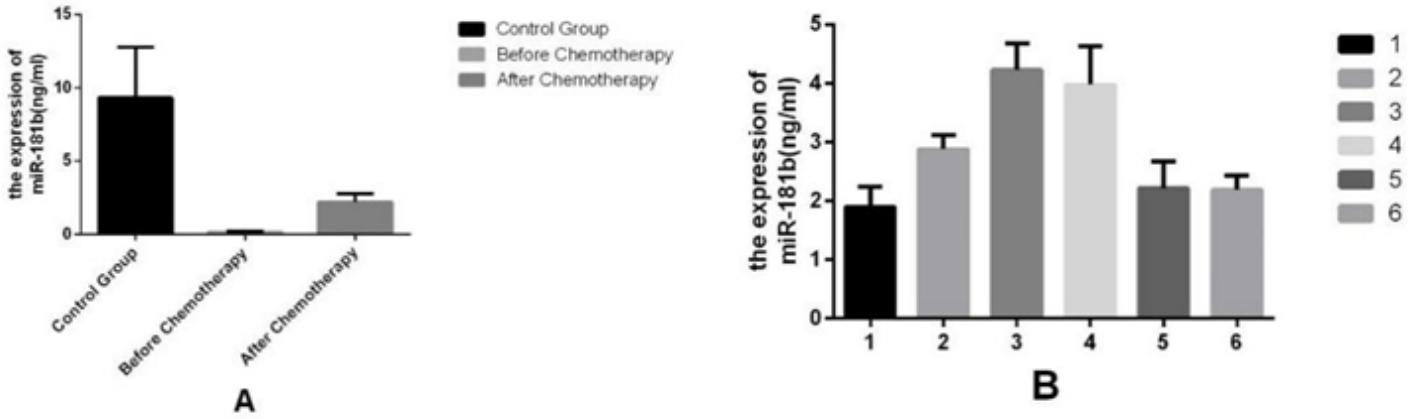

Figure 8

Expression levels of miR-181b before and after chemotherapy (A) and different chemotherapy cycles (B)

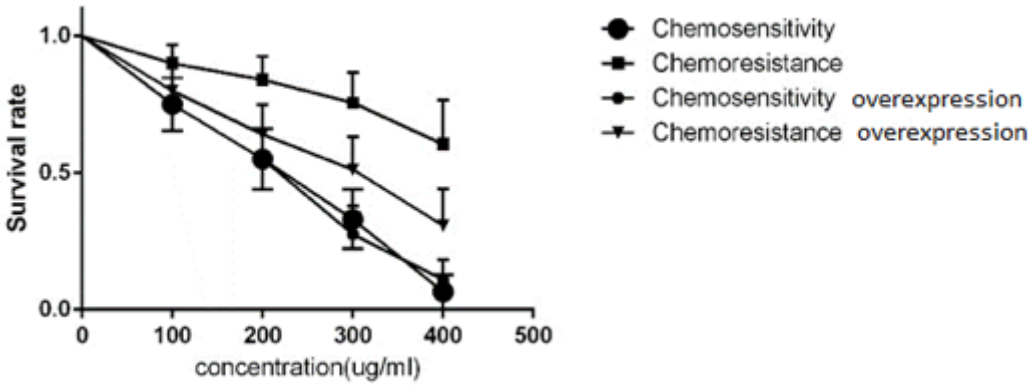

Figure 9

Drug sensitivity of each group of cells before and after miR-181b overexpression

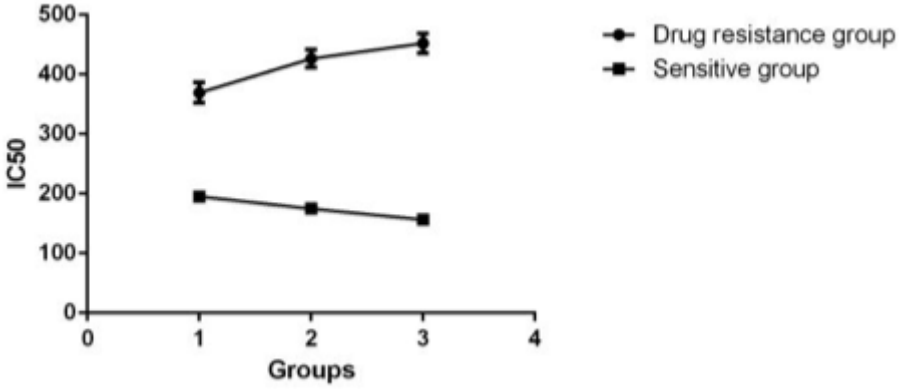

Figure 10

Sensitivity after applying chemotherapy drugs in drug-resistant and non-drug resistant groups

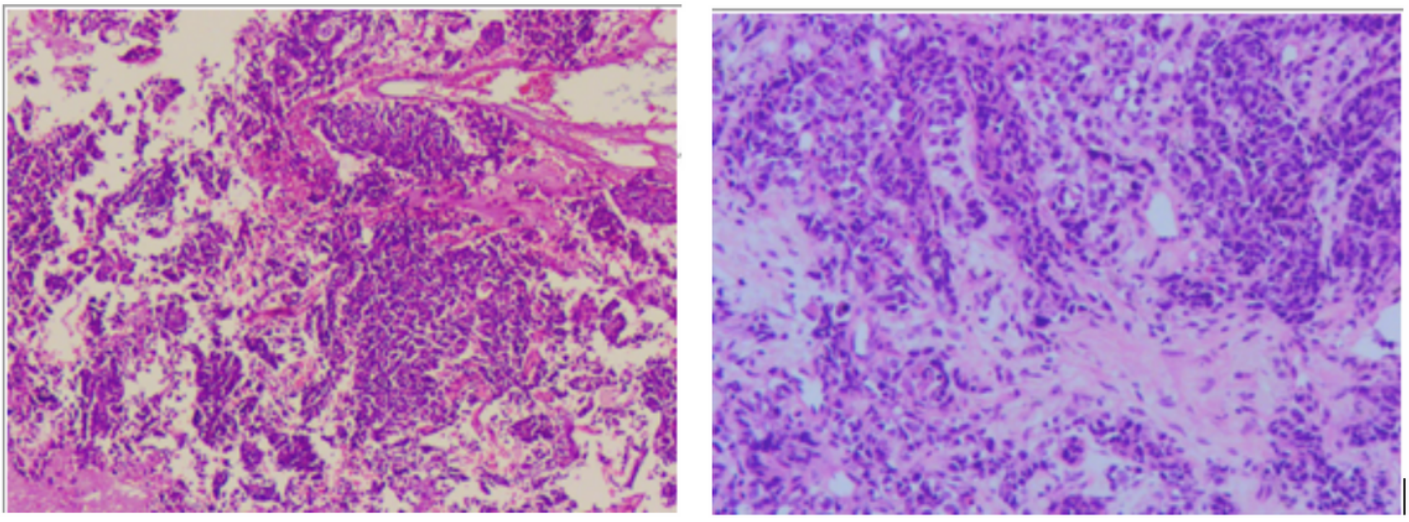

Figure 11

HE staining: the left picture shows the addition of ACEI drugs; the right picture shows the absence of ACEI drugs

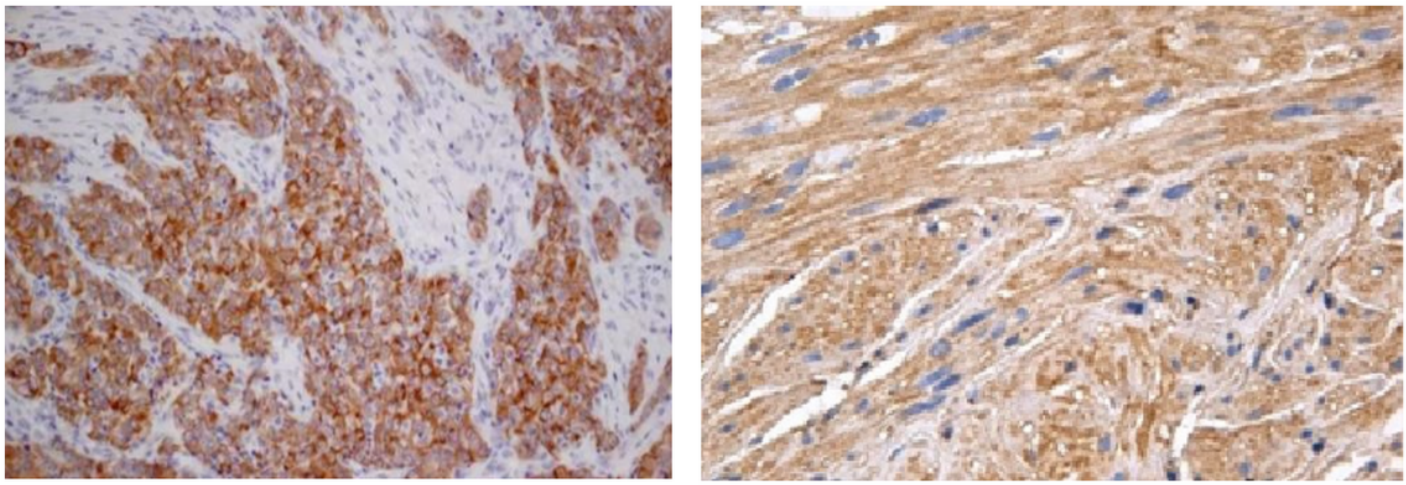

Figure 12

Immunohistochemical results: the left picture shows the addition of ACEI drugs; the right picture shows the absence of ACEI drugs

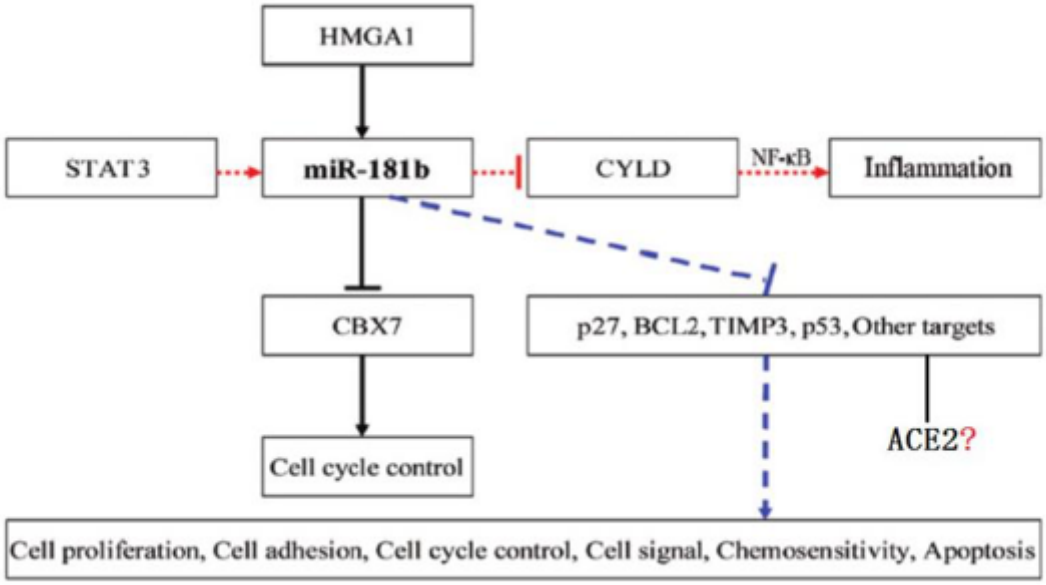

Figure 13

MiR-181b regulatory map