ORIGINAL RESEARCH

MiR-936 Targets GPR78 and Regulates Chemotherapy Resistance in Non-Small Cell Lung Cancer by Activating the Galphaq Rho GTPase Pathway

Xiuming Huang, MM; Yanyan Wang, MM; Minbiao Chen, MM; Gao Li, MM

ABSTRACT

Objective • We aimed to explore the mechanism of microRNA-936 (miR-936) targeting G protein coupled receptor 78 (GPR78) regulating chemoresistance of non-small cell lung cancer (NSCLC) by activating the Galphaq Rho GTPase pathway.

Methods • We added cisplatin to DMEM medium of HCC827/cisplatin cells and adjusted the final concentration to 1 μg/mL. Cells were divided into the control group and the miR-936 transfection group. Tissue samples were divided into the normal tissue group and the NSCLC tissue group. The mRNA expression of miR-936 in tissue samples was analyzed via reverse transcription polymerase chain reaction (RT-PCR). Cell migration and invasion were detected by wound healing assay. Cell counting kit 8 (CCK-8) was used to detect the cell viability 1, 2 and 3 days after cisplatin induction. The toxicity of cisplatin was analyzed by flow cytometry. The targeting relationship between miR-936 and GPR78 was detected by luciferase reporter gene assay. The regulation of miR-936 on GPR78/Rho GTPase was analyzed by Western blot.

Results • The expression of miR-936 in NSCLC was lower than in normal tissues ($P < .05$). The number of cell migrations and invasions in the miR-936 transfection group was lower than in the control group ($P < .05$). The cell viability in the miR-936 transfection group was lower than in the control group on the 1st, 2nd and 3rd day ($P < .05$). With the increase in cisplatin concentration, the apoptosis rate of cells increased in a dependent manner ($P < .05$). Compared with GPR78 Mut, overexpression of miR-936 inhibited the luciferase activity of GPR78 WT 3'- UTR ($P < .05$). The expression of GPR78, RhoA, Rac1 and ABCB1 protein in the miR-936 transfection group was lower than in the control group ($P < .05$). The expression of GPR78 protein in the inhibitor+miR-936 transfection group was lower than in the inhibitor+control group ($P < .05$).

Conclusion • miR-936 targets GPR78 and improves the sensitivity of NSCLC cells to cisplatin via the Galphaq Rho GTPase pathway. (*Altern Ther Health Med.* 2023;29(2):58-63)

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INTRODUCTION

Lung cancer is a common cancer with more than 1.6 million deaths worldwide every year.[1] Non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancer cases with a high mortality rate.[2] In recent decades, great progress has been made in the prevention and detection of NSCLC. Cisplatin is the first-line chemotherapy drug in NSCLC;[3] however, the curative effect varies from patient to patient. The development of drug resistance is still the chemotherapy challenge in NSCLC. Therefore, it is urgent to explore new ways to improve the sensitivity of cisplatin in the treatment of NSCLC.

microRNA (miRNA) is a classic non-coding small RNA that plays a key regulatory role in different pathological and physiological processes via post-transcriptional mechanism by binding to the 3'- untranslated area (3'-UTR) of the target gene.[4] And miR can act as a tumor oncogene or suppressor gene by regulating the target gene, which is usually dysfunctional in cancer.[5] G protein-coupled receptor (GPR) is one of the largest and most diverse membrane protein families in the mammalian genome. These receptors contain 7 transmembrane helices with an extracellular N-terminal and an intracellular C-terminal.[6] The activated GPR transduces extracellular stimuli to send intracellular signals through the interaction between its intracellular domain and heterotrimer G protein.[7] Up to now, 18 different human G

proteins have been identified to be coupled with GPR.[8] Rho family GTP enzymes, including RhoA, Rac1 and Cdc42, are a small (approximately 21kDa) signal G protein family.[9] Rho protein acts as a switch by alternating between the inactive guanosine diphosphate binding state and the active guanosine triphosphate binding state.[10] This process can be accelerated by a large family of Rho guanine nucleotide exchange factors. GPR can activate RHO GTpases by coupling Rho-GEF with its heterotrimeric guanine nucleotide binding protein.[11]

This single transduction pathway involves many physiological functions, including cell migration and drug resistance. There is currently a lack of studies on miRNAs or GPRs and tumors, especially in NSCLC. In our study, the NSCLC cell line HCC827 was used as the research object, and the mechanism of miR-936 targeting GPR78 to regulate chemotherapy resistance of NSCLC by activating Galphaq Rho GTPase pathway was discussed.

MATERIALS AND METHODS

Cell Culture and Tissue Samples

Human NSCLC cell line HCC827 was purchased from the American Type Culture Collection (Manassas, Virginia, USA). All cryopreserved cells were resuscitated and cultured in DMEM medium containing 10% fetal bovine serum (FBS) (Shanghai Biyuntian Company, China), penicillin and streptomycin (Shanghai Biyuntian Company, China) and placed in an incubator at 37°C, 5% CO_2 concentration and 100% humidity. In addition, cisplatin was added to DMEM medium (Shanghai Biyuntian Company, China) of HCC827/cisplatin cells and the final concentration was adjusted to 1 μg/mL. When the cells were in the logarithmic phase, passage and follow-up experiments were carried out. Tissues from a total of 25 patients with NSCLC and matched normal tissues were obtained from the Cancer Center of Central Hospital. All patients were diagnosed as having NSCLC for the first time without chemotherapy before or after surgery. Informed consent was signed by all patients, and the study was approved by the hospital ethics committee.

Cell Transfection

The synthesized precursor hsa-miR-936 was cloned into lentiviral vector pLKO.1-GFP to generate hsa-miR-936 lentiviral construct. The 3' UTR fragment of GPR78 3'UTR was cloned into psiCHECK-2 double luciferase reporter gene construct (Suzhou Hongxun Biotechnology Co., Ltd, China). HCC827 cells were infected by lentivirus, and stable cell lines were obtained via puromycin screening.

Experimental Group

HCC827/ cisplatin cells were divided into the empty transfection group (control group) and the miR-936 simulated transfection group (miR-936 transfection group). A549/DPP cells in logarithmic phase were inoculated into 96-well plates with 1.5×10^4 cells per well and a total volume of 100 μL per well and cultured in an incubator at 37°C for 24 h. The tissue samples in the study were divided into the normal tissue group and the NSCLC tissue group.

Real-time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was obtained from tissue samples using TRIzol reagent (Invitrogen, Waltham, Massachusetts, USA) according to the manufacturer's instructions. The concentration of isolated total RNA was measured by NanoDrop ND-1000 spectrophotometer (Agilent, Santa Clara, California, USA). Genomic DNA was removed and RT was performed using PrimeScript™ RT Reagent kit with gDNA Eraser. According to the manufacturer's suggestion, SYBR Green PCR kit (all China Dalian Bao Biotechnology Co., Ltd.) was used for qRT-PCR. The 7900 real-time PCR system (applied biological system; Thermo Fisher Scientific Company, Waltham, Massachusetts, USA) was used for this process, and β-actin was used as internal reference.

Transwell and Scratch Healing

Transwell (Thermo Fisher Scientific) was used to detect the migration and invasion of NSCLC cells. Transwell measurement was carried out by Invariance Chamber according to the manufacturer's instructions. 1×10^5 HCC827 were inoculated into the upper chamber coated with matrix glue (for invasion test) or without matrix glue (for migration test) and tumor supernatant, while the lower chamber was inoculated with ectomycorrhizal (ECM) fungi containing 10% FBS and incubated for 48 hours. Invasive and migrating cells on the lower surface were stained with 0.1% crystal violet and counted in 5 random fields under the microscope.

Cell Viability Determination

Cell viability was measured by Cell Counting Kit-8 (Biyuntian Bio, Shanghai, China) according to the manufacturer's instructions. The cells were placed in a 96-well plate and after cisplatin treatment or transfection, CCK-8 reagent was added to each well to incubate the cells for 2 hours. Absorbance at 450 nm was detected by microplate reader (Thermo Fisher Scientific) to evaluate cell viability.

Apoptosis Analysis

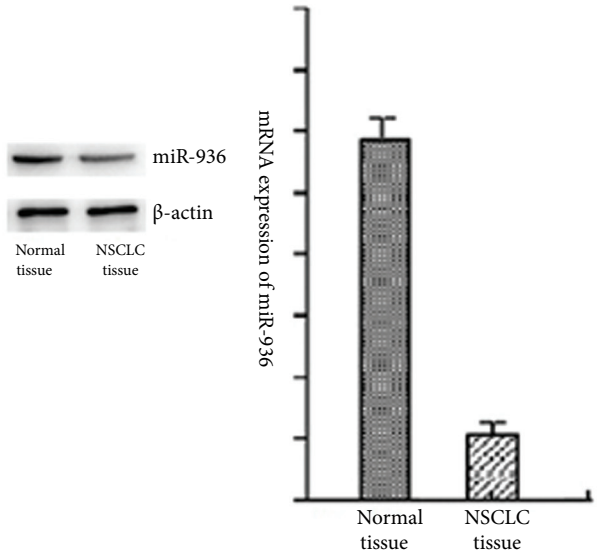
Apoptosis was measured by flow cytometry using the Annexin V-FITC apoptosis kit (Thermo Fisher Scientific). After 24 hours of cisplatin treatment, cells were collected, and 1×10^5 cells were resuspended in 200 μL binding buffer. Next, the cells were incubated with 5 μL Annexin V-FITC and propidium iodide for 10 minutes in the absence of light. The stained cells were examined by flow cytometry (Buildings, San Jose, California), and the apoptosis rate was expressed by the percentage of cells in the upper and the lower right quadrants.

Detection of Luciferase Reporter Gene

GPR78 mutation and wild-type (WT) 3'-UTR fragment were cloned into psiCHECK-2 reporter gene. HEK293T cells were inoculated in 24-well plates and co-transfected with mutant or WT 3'-UTR luciferase reporter gene and pLKO.1-GFP-miR-936.

According to the protocol, 24 h later cell lysate was obtained, and the firefly/renilla luciferase ratio was measured

Figure 1. RT-PCR analysis



Abbreviations: RT-PCR = reverse transcription polymerase chain reaction

Table 1. Expression Analysis of miR-936 mRNA in Tissue Samples ($x \pm s$; n = 24)

Groups	miR-936
Normal tissue	5.98 ± 0.38
NSCLC tissue	1.06 ± 0.08
t value	21.862
P value	<.001

Abbreviation: NSCLC, non-small cell lung cancer.

Figure 2. Transwell test

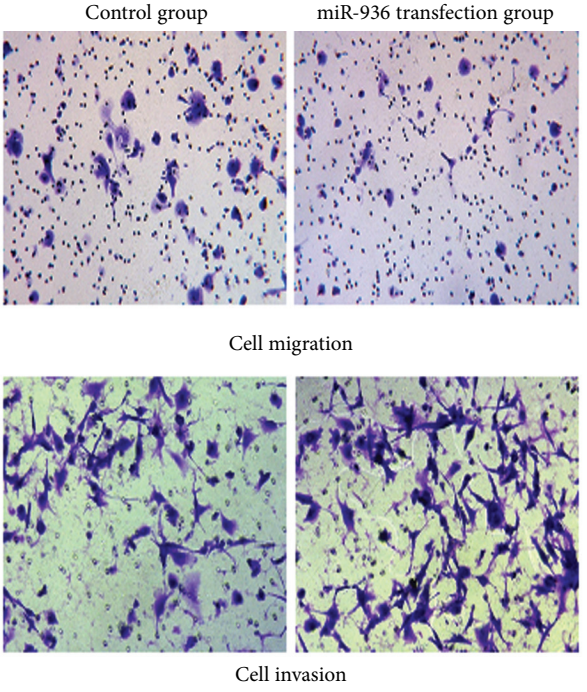


Table 2. Detection of Cell Migration and Invasion ($x \pm s$; n = 24)

Groups	Cell migration	Cell invasion
Control	163.27 ± 36.55	148.45 ± 25.79
miR-936 transfection	85.38 ± 16.29	96.32 ± 18.33
t value	13.026	9.275
P value	<.001	<.001

with the double luciferase reporter gene detection kit (Promega™, Madison, Wisconsin, USA).

Western Blot

A549/cis and H1299/cis cells were lysed in cold protein extraction buffer (Solarbio, Norton, Virginia USA) and centrifuged to separate total protein. The protein was quantified by dioctanoic acid detection kit (Beyotime Biotechnology, Jiansu, China), and denatured by boiling water bath with loading buffer (Beyotime) for 10 minutes. Next, 20 μg of protein sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfected into polyvinylidene fluoride membrane (Millipore, Beary, Massachusetts, USA). Blocking buffer (5%) was used to block nonspecific sites. The membrane interacted with primary antibody purchased from Abcam (Cambridge, UK) overnight at 4°C, including anti-GPR78(1:1000 dilution), anti-RhoA (1:1000 dilution), anti-Rac1(1:1000 dilution), anti-ABCB1(1:1000 dilution) or anti-β-actin (1:1000 dilution). β-actin was used as internal control. Protein bands were developed by enhancing chemiluminescence reagents (Thermo Fisher Technology). The relative protein abundance was analyzed by Quantity One 1-D Analysis Software (Hercules, California USA).

Statistical Analysis

IBM® SPSS statistical software version 17.0 (Chicago, Illinois USA) was used to analyze the data. *t* test of 2 independent samples was used to compare the differences between the 2 groups. $P < .05$ meant the difference was statistically significant.

RESULTS

The Expression of miR-936 Was Downregulated in NSCLC

The mRNA expression of miR-936 in tissue samples was analyzed by RT-PCR. The expression of miR-936 in NSCLC was lower than in normal tissues ($P < .05$). The data showed that the progress of NSCLC may be related to the expression of miR-936 (see Figure 1; Table 1).

Overexpression of miR-936 Inhibited Migration and Invasion of NSCLC Cells

The migration and invasion of cells were detected by Transwell assay. Compared with the control group, the number of cell migrations and invasions in the miR-936 transfection group decreased ($P < .05$), indicating that the overexpression of miR-936 inhibited the migration and invasion of NSCLC cells. (see Figure 2; Table 2)

Table 3. Cell Viability Detected by CCK-8 ($x \pm s$; n = 24; %)

Groups	Day 1	Day 2	Day 3
Control	86.19 ± 13.46	73.58 ± 16.25	58.43 ± 13.49
miR-936 transfection	64.28 ± 15.81	48.34 ± 11.41	32.65 ± 8.33
t value	14.203	11.371	12.519
P value	<.001	<.001	<.001

Abbreviations: CCK-8, cell counting kit 8.

Table 4. Analysis of Cell Apoptosis ($x \pm s$; n = 24)

Groups	**6** μM	**10** μM
Control	25.35 ± 6.66	34.27 ± 9.21
miR-936 transfection	41.67 ± 12.39	67.35 ± 14.28
t value	13.145	9.374
P value	<.001	<.001

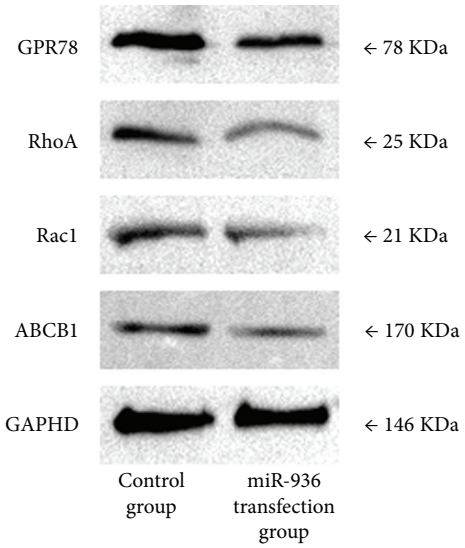
Table 5. Detection of Luciferase Reporter Gene ($x \pm s$, n = 24)

Groups	GPR78-Wt	GPR78-Mut
Control	4.94 ± 0.15	4.98 ± 0.18
miR-936 transfection	1.02 ± 0.01	1.93 ± 0.16
t value	11.822	9.306
P value	<.001	<.001

Table 6 Expression of GPR78, RhoA, Rac1 and ABCB1 protein ($x \pm s$, n = 24)

Groups	GPR78	RhoA	Rac1	ABCB1
Control group	2.94 ± 0.16	1.86 ± 0.14	4.96 ± 0.38	3.93 ± 0.26
miR-936 transfection group	1.04 ± 0.02	1.03 ± 0.02	1.05 ± 0.06	1.08 ± 0.12
t value	8.552	11.739	10.414	11.662
P value	<.001	<.001	<.001	<.001

Figure 3. Western blot analysis



Analysis of Cisplatin-Induced Drug Resistance

CCK-8 was used to detect the cell viability on the 1st, 2nd and 3rd day after cisplatin induction. The results showed that cell viability in the miR-936 transfection group was lower than in the control group on the 1st, 2nd and 3rd day ($P < .05$), indicating that miR-936 overexpression increased the drug sensitivity of cells to cisplatin (see Table 3).

Analysis of Apoptosis

Flow cytometry was used to analyze the toxic effects of different concentrations of cisplatin on cells. The results showed that with an increase in cisplatin concentration, the apoptosis rate of cells increased in a dependent manner, and the apoptosis rate in the miR-936 transfection group was higher than in the control group ($P < .05$). The results showed that the overexpression of miR-936 significantly increased the toxicity of cisplatin to cells and drug sensitivity (see Table 4).

miR-936 Was the GPR78 Target

The targeting relationship between miR-936 and GPR78 was detected by luciferase reporter gene. The results showed that the overexpression of miR-936 significantly inhibited the luciferase activity of GPR78 WT 3'-UTR compared with GPR78-Mut ($P < .05$), indicating that miR-936 directly regulated GPR78 in a targeted manner (see Table 5).

Western blot analysis

The regulatory effect of miR-936 on GPR78/Rho GTPase was analyzed by Western blot. The expression of GPR78, RhoA, Rac1 and ABCB1 protein in the miR-936 transfected group was lower than in the control group ($P < .05$) (see Figure 3; Table 6).

Galphaq mediated activation of RhoA and Rac1 induced by GPR78

Western blot analysis showed that the protein expression of Galphaq, RhoA, Rac1 and GPR78 in the inhibitor control group was lower than that in the control group ($P < .05$), but there was no difference in GPR78 protein expression ($P > .05$). Compared with miR-936 transfection group, the protein expression of RhoA, Rac1 and GPR78 in inhibitor+miR-936 in the transfection group showed no difference, but the protein expression of Galphaq decreased ($P > .05$). The protein expression of GPR78 in the inhibitor+miR-936 transfection group was lower than in the inhibitor+control group ($P < .05$), which indicated that Galphaq mediated the activation of RhoA and Rac1 induced by GPR78 (see Figure 4; Table 7).

DISCUSSION

Lung cancer is the most common malignant tumor, and its morbidity and mortality are the highest among all malignant tumors. NSCLC is a kind of lung cancer, accounting for approximately 85% of all newly diagnosed lung cancers. At present, there are many treatments for NSCLC, such as surgery, radiotherapy and chemotherapy, immunotherapy, etc.[12,13]

Figure 4. Western blot analysis.

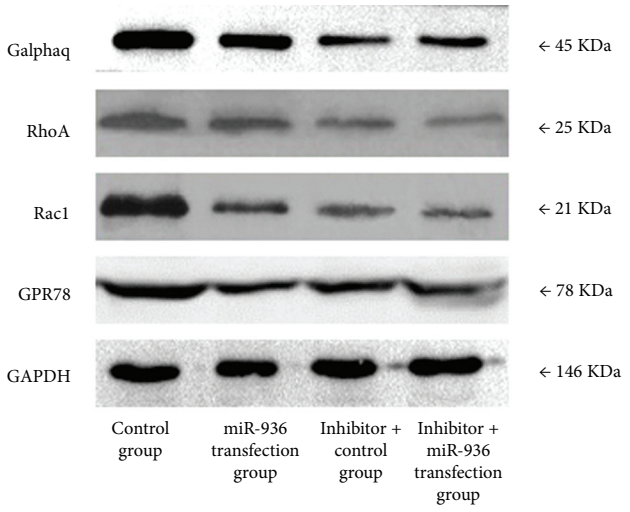


Table 7. Expression of Galphaq, RhoA, Rac1 and GPR78 ($x \pm s$; n = 24)

Groups	Galphaq	RhoA	Rac1	GPR78
Control	2.86 ± 0.14	2.92 ± 0.14	4.95 ± 0.16	3.89 ± 0.15
miR-936 transfection	2.85 ± 0.12	1.03 ± 0.02	1.05 ± 0.06	1.02 ± 0.03
Inhibitor+control	1.02 ± 0.03	1.04 ± 0.02	1.06 ± 0.04	3.93 ± 0.15
Inhibitor +miR-936 transfection	1.03 ± 0.02	1.01 ± 0.01	1.07 ± 0.03	1.02 ± 0.01
F value	21.045	19.223	40.528	23.445
P value	<.001	<.001	<.001	<.001

Because the early symptoms of NSCLC are not obvious and relatively hidden, most patients are diagnosed with advanced tumors and lose the chance to undergo radical surgery.[14] Chemotherapy is one of the primary methods for treating advanced NSCLC; however, due to drug resistance, the clinical efficacy of chemotherapeutic drugs is greatly reduced.

At present, cisplatin is one of the most effective and widely used anti-tumor drugs.[15] It has been used to treat a variety of solid malignant tumors, but long-term cisplatin use leads to drug resistance in tumor cells and weakens its clinical efficacy.[16] Patients with NSCLC are sensitive to cisplatin in the initial stage, but resistant to cisplatin in the later stage, leading to failure of chemotherapy.

An increasing number of studies show that miR is abnormally expressed in human malignant tumors. miR-936 induces cell cycle arrest and inhibits tumor cell proliferation by targeting CKS1. In addition, the overexpression of miR-936 improves the drug sensitivity of NSCLC cells to cisplatin, indicating that the discovery that miR-936 is sensitive to the anticancer effect of cisplatin in NSCLC is of great significance. Previous studies have shown that downregulated miRNA is usually described as a biomarker or therapeutic target of NSCLC.[17] For example, it has recently been reported that miR-194 acts as a tumor suppressor in NSCLC, and the expression of miR-375 is also decreased. Overexpression of miR-375 can reduce the proliferation, movement and invasion of NSCLC cells by targeting IGF1R. Previous studies have reported that miR-936 is downregulated in glioma and induced cell cycle arrest by targeting CKS1. Another report showed that miR-936 can directly target E2F2 to inhibit the invasion and proliferation of NSCLC. Finding miRNA target genes is the basis of understanding its carcinogenic regulatory mechanism and effective molecular therapeutic targets.

G Protein Coupled Receptors (GPCRs) are the general name of a large class of membrane protein receptors. The common feature of these receptors is that there are 7 transmembranes in their 3-dimensional structure α Helix, and the C-terminal of the peptide chain and the inner ring (the 3rd inner ring) connecting the 5th and 6th transmembrane helices (counting from the N-terminal of the peptide chain) contain the binding site of G protein (guanylic acid binding protein). In this study, we paid attention to the regulation of GPR78 by miR-936 and the expression of downstream signal proteins. An miRNA can target a variety of mRNAs to regulate cell proliferation, differentiation, apoptosis and other biological functions.[18,19]

In the study of cisplatin-induced drug-resistant cells, when we overexpressed miR-936 with lentivirus, it was found that the expression of GPR78, RhoA and Rac1 in the cells was significantly lower than in the control group. By measuring the cell viability and apoptosis rate, our research showed that the overexpression of miR-936 was helpful to cisplatin-induced viability inhibition and apoptosis, indicating that miR-936 inhibits cisplatin resistance in NSCLC cells. It is reported that ABCB1 is one of the main pharmacogenetic predictors of cisplatin-based chemotherapy in NSCLC. In this study, we found that expression of ABCB1 decreased after miR-936 overexpression. It was revealed that miR-936 inhibited drug resistance by reducing the expression and function of ABCB1, which was consistent with previous studies. That is, the consumption of chromosome helicase/ATPaseDNA binding protein 1-like gene downregulated the expression of ABCB1 to reduce the drug resistance of cisplatin-resistant NSCLC cells.

To understand the mechanism of miR-936 as a tumor suppressor of lung squamous cell carcinoma (LSCC), we conducted luciferase reporter gene assay to test whether miR-936 can directly interact with 3'-UTR of GPR78. The results showed that in HEK293T cells, compared with mutant 3'-UTR, the overexpression of miR-936 significantly inhibited the luciferase activity of GPR78WT3'-UTR. We found that miR-936 directly inhibited the expression of GPR78 by binding its 3'-UTR. GPR78 is an orphan GPR, located in the region of chromosome 4p, which has been shown to be related to schizophrenia and bipolar disorder. Recently, it was confirmed that GPR78 was highly expressed in lung cancer cells, and knock-down of GPR78 significantly inhibited cell migration and metastasis. We downregulated the expression of GPR78 in NSCLC by transfection of miR-936, and found that the downregulation of GPR78 significantly increased cisplatin drug sensitivity, because cell viability decreased significantly after the addition of cisplatin. Previous reports showed that Galphaq was the main Gα protein

involved in the activation of GTPases in the Rho family. Similar to our previous findings, we found that the inhibition of GPR78 expression by HCC827 can significantly reduce the activation of RhoA and Rac1. To study whether Galphaq/RhoGTPase was involved in the regulation of miR-936/GPR78, we co-expressed GPR78 and the RGS domain of GRK2 (GRK2-RGS). The results showed that after blocking the activity of Galphaq, the expression of RhoA and Rac1 regulated by miR-936/GPR78 was restricted by GRK2-RGS.

We also studied the molecular mechanism of GPR78 regulating cell migration. It was found that down-regulation of GPR78 inhibited the expression of RhoA and Rac1. Previous studies have shown that RhoA and Rac1 regulate the elongation of cell morphology and cancer cell stress.[20] Our research showed that the activation of RhoA and Rac1 induced by GPR78 was mediated by the Gαq signaling pathway, because blocking the Gαq signaling pathway inhibited the activation of RhoA and Rac1. According to reports, GPR78 is a Gs-coupled receptor that regulates the level of cell cAMP.[21] It is known that several GPCRs regulate tumor progression and improve the sensitivity of chemotherapy drugs by coupling with the Gα protein signaling pathway.[22,23] For example, GPR56 is involved in the progression of pancreatic cancer and glioblastoma by coupling with Gα12/13 and Gαq/11, CD97 promotes the progression of thyroid cancer by coupling with Gα12/13 and GPR116 regulates the chemosensitivity of breast cancer by activating the Gαq-RhoGTPase pathway. Therefore, our research showed that GPR78 could combine with the classical Gαq protein to mediate the metastasis of cancer cells and chemotherapy resistance in lung cancer.

In a word, our study proved that miR-936/GPR78 is expressed in lung cancer cells and plays an important role in regulating the migration and drug resistance of tumor cells. It was proven that miR-936 targeted GPR78 and improved the sensitivity of NSCLC cells to cisplatin via the Galphaq Rho GTPase pathway. The results showed that the miR-936/GPR78/Galphaq Rho GTPase axis can be used as a new biomarker and a therapeutic target for NSCLC.

Study Limitations

This study was limited by the fact that it did not use gene cloning technology to explore the complete upstream and downstream signal transduction pathways of the Galphaq Rho GTPase pathway. This limitation will be addressed in subsequent studies.

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AUTHOR CONTRIBUTIONS

Xiuming Huang and Yanyan Wang contributed equally to this paper and should be regarded as co-first authors. Minbiao Chen is the lead contact.

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