

# MicroRNA-365 inhibits the progression of lung adenocarcinoma through targeting ETS1 and inactivating AKT/mTOR pathway

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**Abstract.** – **OBJECTIVE:** MicroRNAs (miRNAs) act as important regulators in human cancers by regulating the gene expression. The dysregulation of miR-365 has been investigated in many cancers. However, the function of miR-365 remains unknown in lung adenocarcinoma. Therefore, the regulatory mechanism of miR-365 was explored in lung adenocarcinoma.

**PATIENTS AND METHODS:** The expression of miR-365 was detected in cell lines and 67 lung adenocarcinoma tissues using qRT-PCR. The Kaplan-Meier analysis was used to determine the association between miR-365 expressions and the survival rate in patients with lung adenocarcinoma. Transwell assay was then performed to investigate the effect of miR-365 on invasion and migration of lung adenocarcinoma cells.

**RESULTS:** Downregulation of miR-365 and upregulation of ETS1 were identified in lung adenocarcinoma. Furthermore, miR-365 reversely regulated ETS1 expression in lung adenocarcinoma. Functionally, the overexpression of miR-365 inhibited proliferation, migration, and invasion of lung adenocarcinoma cells. However, the upregulation of ETS1 lessened the inhibitory effect of miR-365 in lung adenocarcinoma. In addition, miR-365 inhibited EMT and inactivated AKT/mTOR pathway in lung adenocarcinoma.

**CONCLUSIONS:** MiR-365 inhibits the progression of lung adenocarcinoma by targeting ETS1 and inactivating the AKT/mTOR pathway.

*Key Words:*

Lung adenocarcinoma, MiR-365, ETS1, EMT, AKT/mTOR pathway.

## Introduction

Lung cancer is a malignant tumor with the fastest growth in morbidity and mortality, threatening people's health and life<sup>1</sup>. In the past 50 years, the incidence and mortality of lung cancer have increased significantly in many countries<sup>2</sup>.

Among them, non-small cell lung cancer (NSCLC) accounts for 85%. About half of NSCLC cases are diagnosed as lung adenocarcinoma<sup>3</sup>. There are many factors that cause lung cancer, including alcohol, smoking, and genetic factors<sup>4</sup>. Most patients with lung adenocarcinoma can develop distant metastases in advanced stage. Therefore, the prognosis of patients with lung adenocarcinoma remains poor, and the 5-year overall survival (OS) is about 13-58.3%<sup>5</sup>. Lung adenocarcinoma is characterized by high invasiveness and early metastasis<sup>6</sup>. Therefore, it is necessary to further analyze the molecular mechanism of lung adenocarcinoma progression and design effective diagnostic and therapeutic strategies for lung adenocarcinoma patients.

Various miRNAs have been reported to be involved in many important processes in life, including early development, cell proliferation, apoptosis, cell death, fat metabolism, and cell differentiation<sup>7</sup>. In addition, the role of miRNAs in human cancer has been identified, including lung adenocarcinoma<sup>8</sup>. MiR-29c functioned as a tumor inhibitor *via* mediating VEGFA in lung adenocarcinoma<sup>9</sup>. Conversely, miR-590 accelerated the migration and invasion of lung adenocarcinoma cells by downregulating OLFM4<sup>10</sup>. MiR-365 plays different effect in human diseases, and promoted the development of cutaneous squamous cell carcinoma<sup>11</sup>. Chen et al<sup>12</sup> reported that miR-365 restrained the proliferation of cholangiocarcinoma cells and induced apoptosis by targeting E2F2. Although the expression of miR-365 has been reported to be reduced in non-invasive lung cancer<sup>13</sup>, its function in lung adenocarcinoma remains unclear.

ETS1 is a transcription factor belonging to the ETS family. The expression of ETS1 has been reported to be associated with tumor angiogenesis, lymph node metastasis, and patient sur-

vival in esophageal squamous cell carcinoma<sup>14</sup>. In addition, the upregulation of proto-oncogene ETS1 has been identified in pre-invasive breast cancer<sup>15</sup>. Furthermore, ETS1 has been reported to be carcinogenic in human clear cell renal cell carcinoma<sup>16</sup>. Xu et al<sup>17</sup> proposed that miR-129 blocked epithelial-mesenchymal transition (EMT) in prostate cancer by mediating the ETS1/AKT/mTOR pathway. EMT and AKT/mTOR pathways are well-known to be involved in tumorigenesis<sup>18,19</sup>. Therefore, this study explored the relationship between miR-365 and the EMT/AKT/mTOR pathway. At the same time, the interaction between miR-365 and ETS1 was also analyzed in lung adenocarcinoma. This study will help us better understand the pathogenesis of lung adenocarcinoma.

## Patients and Methods

### *Clinical Tissues*

Lung adenocarcinoma samples used in this experiment and their clinical and follow-up information were provided by the Affiliated Hospital of Qingdao University. All 67 patients with lung adenocarcinoma did not receive any treatment before surgery. Participants provided informed consents. The experiment was approved by the Human Ethics Committee of the Affiliated Hospital of Qingdao University. This study was conducted in accordance with the Helsinki Declaration.

### *Cell Culture and Transfection*

Human lung adenocarcinoma cell lines NCI-H1975, NCI-H2228 and normal lung epithelial cell BEAS-2B were purchased from Zhongke Quality Inspection Biotechnology (Beijing, China). The culture conditions of these cells included Roswell Park Memorial Institute-1640 (RPMI-1640) medium with 10% fetal bovine serum (FBS HyClone, South Logan, UT, USA), 5% CO<sub>2</sub> and 37°C. MiR-365 mimics, miR-365 inhibitors, negative control (NC), and ETS1 plasmid (GenePharma, Shanghai, China) were transferred to NCI-H1975 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), respectively.

### *Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)*

The total RNA in frozen NCI-H1975 cells was extracted using TRizol reagent (TaKaRa, Otsu, Shiga, USA). First-Strand complementary deoxyribose nucleic acid (cDNA) Synthesis kit (Prome-

ga, Madison, WI, USA) was added to the mixture of the reverse transcription reaction system to obtain cDNA solution. The mixture of qRT-PCR standard reaction system was then added to SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR solution was placed on ABI7300 real-time PCR machine (Applied Bio-systems, Foster City, CA, USA) for PCR amplification reaction. The reaction conditions were: pre-denaturation at 93°C for 2 minutes followed by 1 minute at 93°C, 1 minute at 55°C, 1 minute at 72°C for a total of 40 cycles, and finally extended at 72°C for 7 minutes. U6 and  $\beta$ -actin were used as controls for miR-365 and ETS1, respectively. The mRNA expression was analyzed by the  $2^{-\Delta\Delta Ct}$  method. The primers used were: miR-365 forward: 5'-CGT AAT GCC CCT AAA AAT-3' and reverse, 5'-GTG CAG GGT CCG AGG T-3'; U6-forward: 5'-GCT TCG GCA GCA CAT ATA CTA AAA T-3' and reverse, 5'-CGC TTC ACG AAT TTG CGT GTC AT-3'; ETS1-forward: 5'-GCT AGC ATG GCA CTG AAG GGG CTG GGG-3' and reverse, 5'-GCG GCC GCC ATT ATG AAT GAA ATT CTT TG-3';  $\beta$ -actin forward: 5'-AGA AGG CTG GGG CTC ATT TG-3', and reverse, 5'-AGG GGC CAT CCA CAG TCT TC-3'.

### *Western Blot Analysis*

Radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) was used to extract proteins. Then, protein was separated by 10% concentrated sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) protein loading buffer for electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Next, the protein membrane was immediately placed in wash solution and rinsed for 1-2 minutes to wash away the membrane transfer liquid. The primary antibodies (Vimentin, N-cadherin, E-cadherin, AKT, p-AKT, mTOR, p-mTOR, ETS1, and  $\beta$ -actin) were added to incubate the protein overnight at 4°C. The diluted secondary antibody was added and incubated for 1 h at room temperature. Finally, the proteins were detected using enhanced chemiluminescence (ECL) reagent (Millipore, Billerica, MA, USA).

### *Cell Counting Kit-8 (CCK-8) Assay*

NCI-H1975 cell suspension was prepared in 96-well plates. The plates were pre-incubated in an incubator for 24 h (at 37°C, 5% CO<sub>2</sub>). NCI-H1975 cells were incubated for 24, 48, 72, and 96 h, respectively. Next, we incubated the

cells with CCK-8 solution for 4 h. Finally, the absorbance at 450 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

### Transwell Assay

The upper chamber of the transwell chamber (8- $\mu$ m pore size membranes) was coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) for cell invasion. Matrigel was polymerized into a gel at 37°C for 30 min. NCI-H1975 cell suspension ( $2 \times 10^4$ , 100  $\mu$ L) was added to the upper chamber. RPMI-1640 medium containing 20% FBS (600  $\mu$ L) was added to the lower chamber. After 24 h of routine incubation, the transwell chamber was fixed in methanol for 30 minutes and stained with 0.1% crystal violet for 20 min. The number of invading cells was observed under a microscope of 200 times. The transwell cell migration assay was performed without Matrigel. Other process is similar to the transwell invasion assay.

### Dual-Luciferase Assay

The 3'-UTR of WT-ETS1 or Mut-ETS1 was inserted into the PGL4.23 Luciferase vector (Genomeditech, Shanghai, China). Next, the Luciferase vector and miR-365 mimics were transfected into NCI-H1975 cells and incubated for 48 h. Luciferase activity was measured using a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

### Statistical Analysis

Data are shown as mean $\pm$ standard deviation (SD).  $p < 0.05$  was considered to be a significant difference. One-way analysis of variance (ANO-

VA) test followed by the post-hoc test (Least Significant Difference) was performed for the comparison between multiple groups. Survival curves were plotted using Kaplan-Meier. Survival differences were compared using the log-rank test.

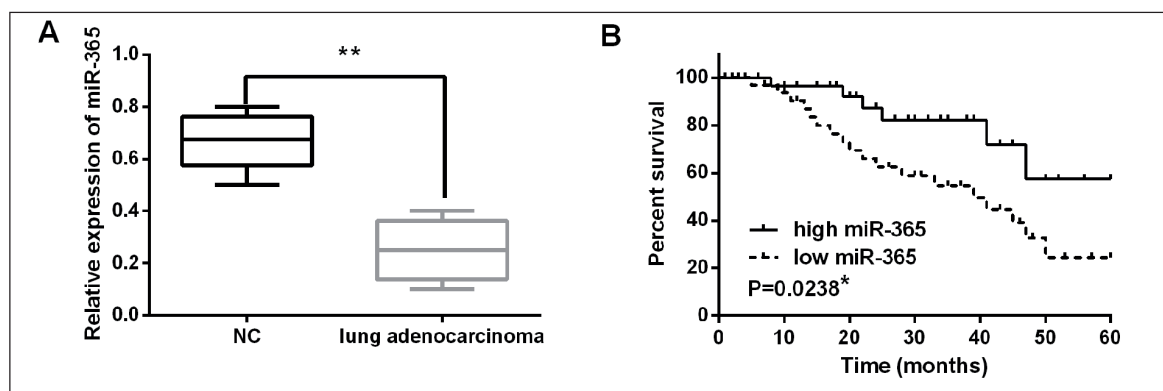
## Results

### Decreased MiR-365 Expression Participated in Lung Adenocarcinoma Progression

MiR-365 expression was first examined in lung adenocarcinoma tissues. Downregulation of miR-365 was detected in lung adenocarcinoma tissues compared to normal tissues ( $p < 0.01$ , Figure 1A). Furthermore, the downregulation of miR-365 was closely correlated with lymph node metastasis or TNM stage in patients with lung adenocarcinoma ( $p < 0.05$ , Table I). In addition, the shorter overall survival in lung adenocarcinoma patients was associated with low miR-365 expression ( $p < 0.05$ , Figure 1B). These results suggest that miR-365 may regulate the occurrence and prognosis of lung adenocarcinoma.

### MiR-365 Inhibited Cell Proliferation, Migration, and Invasion in Lung Adenocarcinoma

Next, miR-365 expression was detected in NCI-H1975, NCI-H2228, and BEAS-2B cells. Similarly, miR-365 expression was reduced in NCI-H1975 and NCI-H2228 cell lines compared to BEAS-2B cells ( $p < 0.01$ , Figure 2A). To further explore the function of miR-365 in the progression of lung adenocarcinoma, miR-365 mimics



**Figure 1.** MiR-365 expression was decreased in lung adenocarcinoma. **A**, MiR-365 expressions were observed in lung adenocarcinoma tissues. **B**, Worse prognosis in lung adenocarcinoma patients was related to low miR-365 expression. \* $p < 0.05$ , \*\* $p < 0.01$ .

**Table 1.** The relationship between miR-365 expression and clinic-pathological characteristics of lung adenocarcinoma patients.

Characteristics	Cases (n = 67)	miR-365		p-value
		High (n = 23)	Low (n = 44)	
Age (years)				0.45
≥ 60	30	9	21	
< 60	37	14	23	
Gender				0.44
Male	40	15	25	
Female	27	8	19	
TNM stage				0.011*
I+II	18	6	12	
III+IV	49	17	32	
Lymph node metastasis				0.008*
No	52	18	34	
Yes	15	5	10	
Differentiation				1.55
Well	28	12	16	
Moderate-poor	39	11	28	

Statistical analyses were performed by the  $\chi^2$ -test. \* $p < 0.05$  was considered significant.

or inhibitors were transfected into NCI-H1975 cells. MiR-365 mimics or inhibitor significantly regulated its expression in NCI-H1975 cells ( $p < 0.01$ , Figure 2B). Functionally, CCK-8 assay suggested that miR-365 mimics restrained cell proliferation in NCI-H1975 cells ( $p < 0.01$ , Figure 2C). MiR-365 inhibitor promoted proliferation of NCI-H1975 cells ( $p < 0.05$ , Figure 2D). In addition, transwell assay suggested that cell migration was suppressed by miR-365 overexpression in NCI-H1975 cells, but was promoted by the knockdown of miR-365 ( $p < 0.01$ , Figure 2E). The same effect of miR-365 on cell invasion was also observed in NCI-H1975 cells ( $p < 0.01$ , Figure 2F). Taken together, miR-365 plays an inhibitory role in the progression of lung adenocarcinoma.

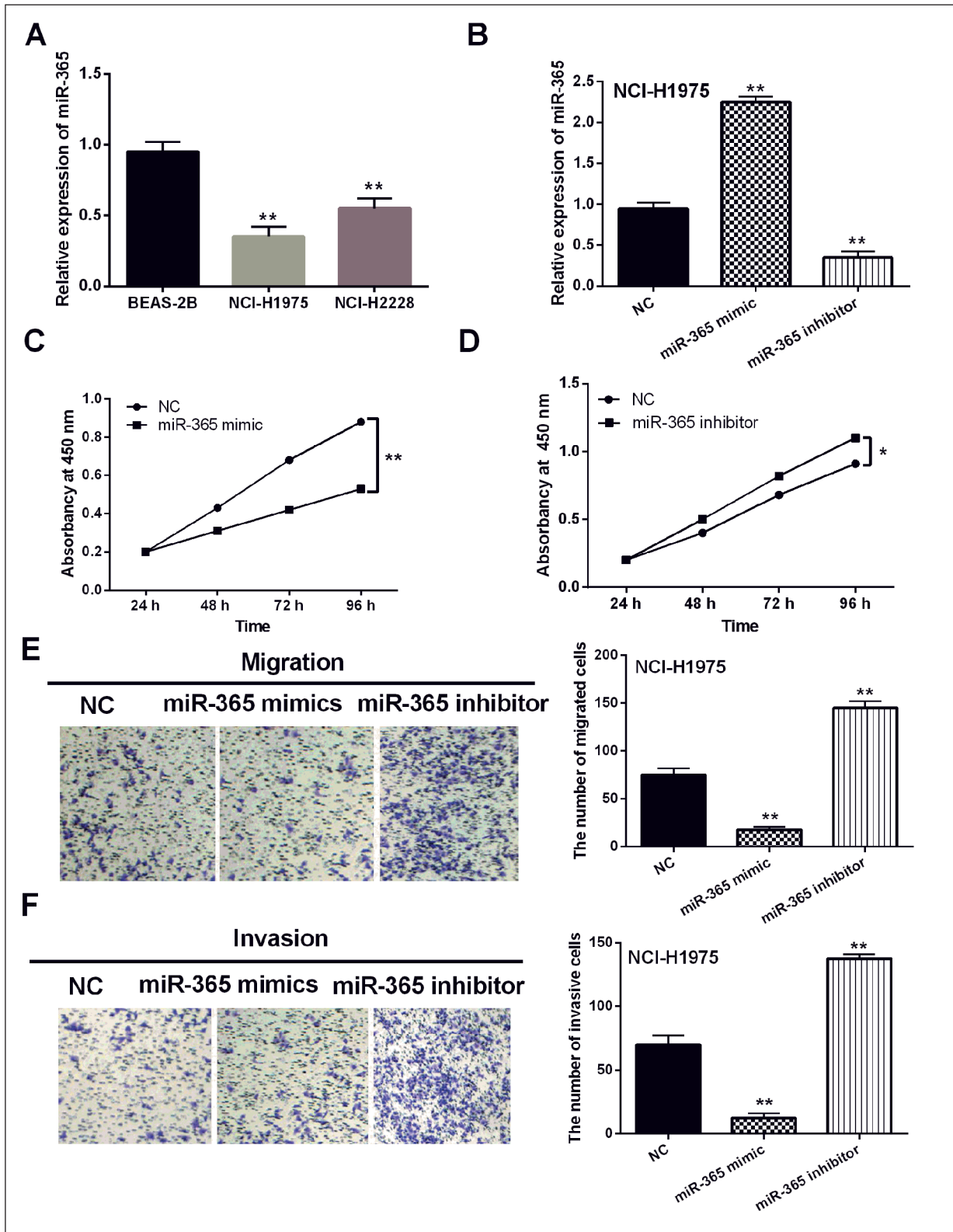
#### **ETS1 Was a Direct Target of MiR-365**

To further illustrate how miR-365 inhibits the development of lung adenocarcinoma, the Target-Scan (<http://www.targetscan.org/>) database was used to identify the target genes for miR-365. Among these predicted target genes, we paid more attention on ETS1. The role of ETS1 has been widely investigated in human cancers. The binding site between miR-365 and ETS1 is shown in Figure 3A. Luciferase reporter assay suggested that the overexpression of miR-365 reduced the Luciferase activity of Wt-ETS1, but did not affect Mut-ETS1 Luciferase activity ( $p < 0.01$ , Figure 3B). Furthermore, miR-365 negatively regulated the expression of ETS1 in lung adenocarcinoma

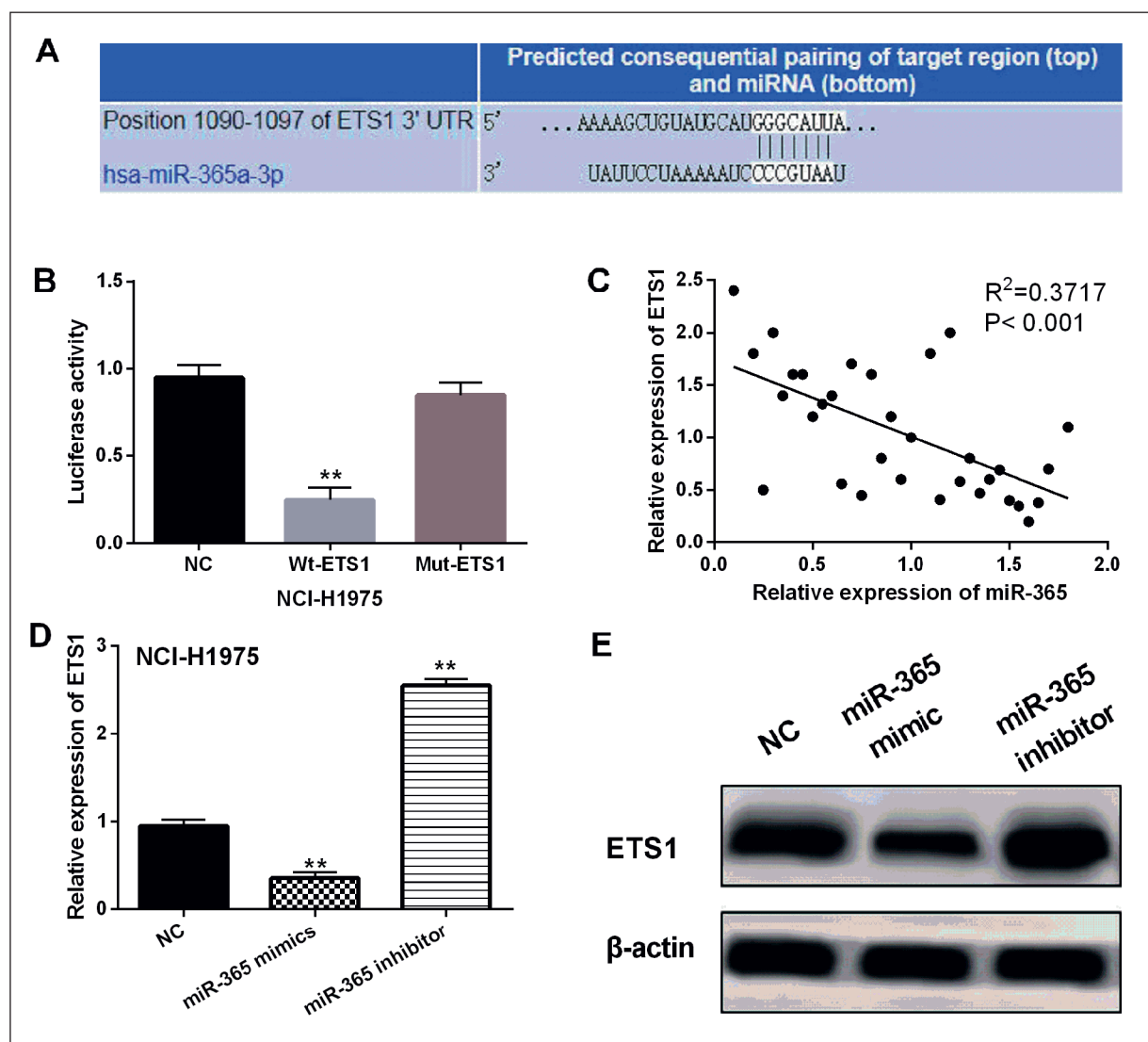
( $p < 0.001$ ,  $R^2 = 0.3717$ ; Figure 3C). In addition, miR-365 mimics reduced the expression of ETS1 in NCI-H1975 cells. In contrast, miR-365 inhibitor promoted ETS1 expression ( $p < 0.01$ , Figure 3D, 3E). Therefore, miR-365 was considered to directly target ETS1 and negatively regulate the expression of ETS1 in lung adenocarcinoma.

#### **Upregulation of ETS1 Impaired the Inhibitory Effect of MiR-365 in Lung Adenocarcinoma**

Next, ETS1 expressions were observed using qRT-PCR. We found that ETS1 was upregulated in lung adenocarcinoma tissues compared to normal tissues ( $p < 0.01$ , Figure 4A). In addition, high expression of ETS1 was associated with shorter overall survival ( $p = 0.0299$ , Figure 4B), predicting poor prognosis in patients with lung adenocarcinoma. Therefore, it is suspected that ETS1 is involved in the pathogenesis of lung adenocarcinoma. To elucidate the interaction between miR-365 and ETS1, NCI-H1975 cells containing miR-365 mimics and ETS1 vector were constructed. ETS1 vector restored the decreased expression of ETS1 induced by miR-365 mimics ( $p < 0.01$ , Figure 4C). Functionally, upregulation of ETS1 restored miR-365 mediated the inhibition of cell proliferation ( $p < 0.01$ , Figure 4D). The same results were also identified for cell migration and invasion in NCI-H1975 cells ( $p < 0.01$ , Figures 4E, 4F). Briefly, the upregulation of ETS1 attenuated the inhibitory effect of miR-365 in lung adenocarcinoma.



**Figure 2.** MiR-365 inhibited cell invasion, migration, and proliferation in lung adenocarcinoma. **A**, MiR-365 expression was observed in NCI-H1975, NCI-H2228, and BEAS-2B cells. **B**, MiR-365 expression in NCI-H1975 cells containing its mimics or inhibitor. **C-F**, MiR-365 regulated cell proliferation, migration, and invasion (magnification, 200×). \* $p < 0.05$ , \*\* $p < 0.01$ .

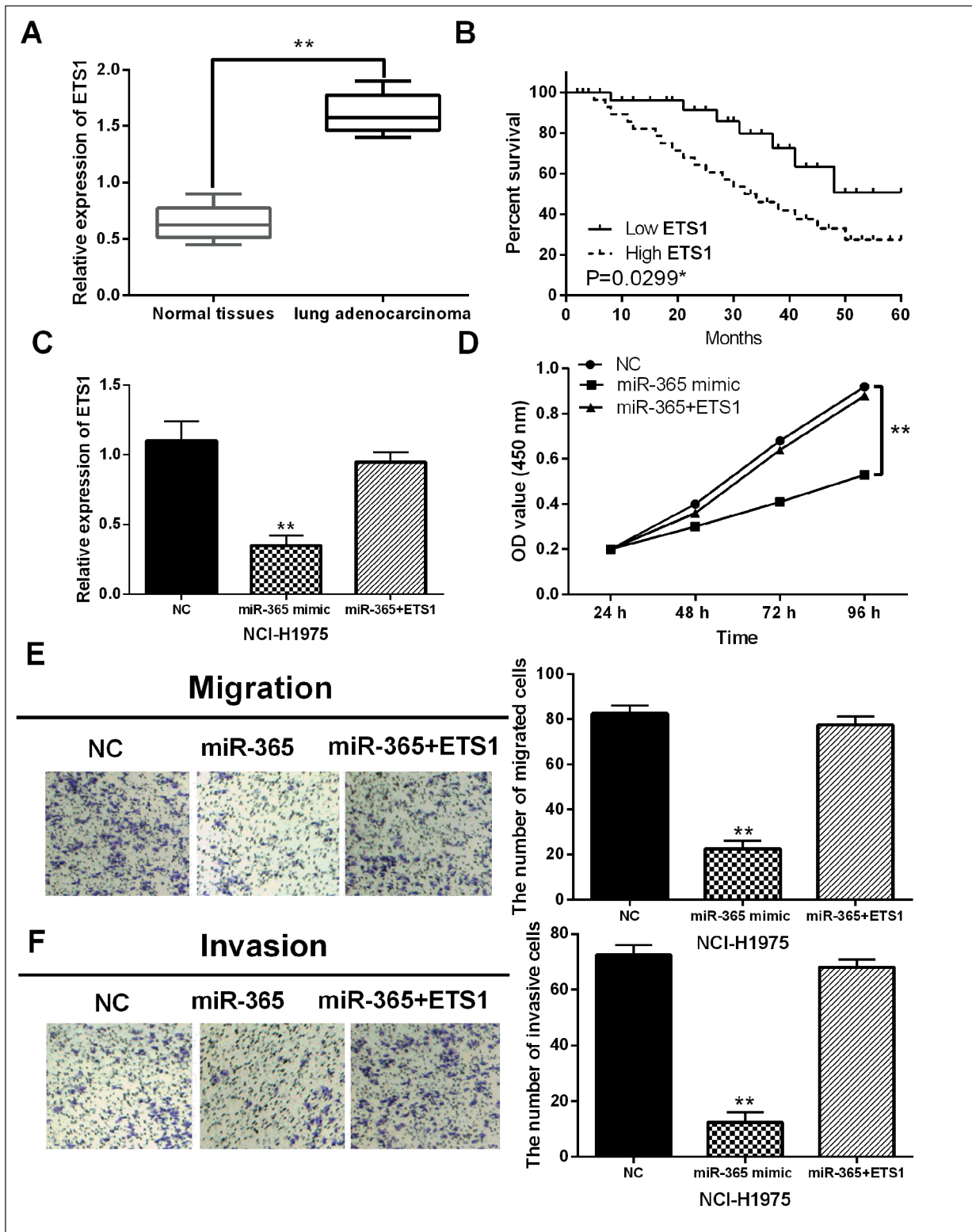


**Figure 3.** MiR-365 directly targets ETS1. **A**, The binding sites between miR-365 and ETS1. **B**, Luciferase reporter assay. **C**, MiR-365 was negatively correlated with ETS1 in lung adenocarcinoma tissues. **D**, **E**, MiR-365 regulated ETS1 expression in NCI-H1975 cells. **\*\*** $p < 0.01$ .

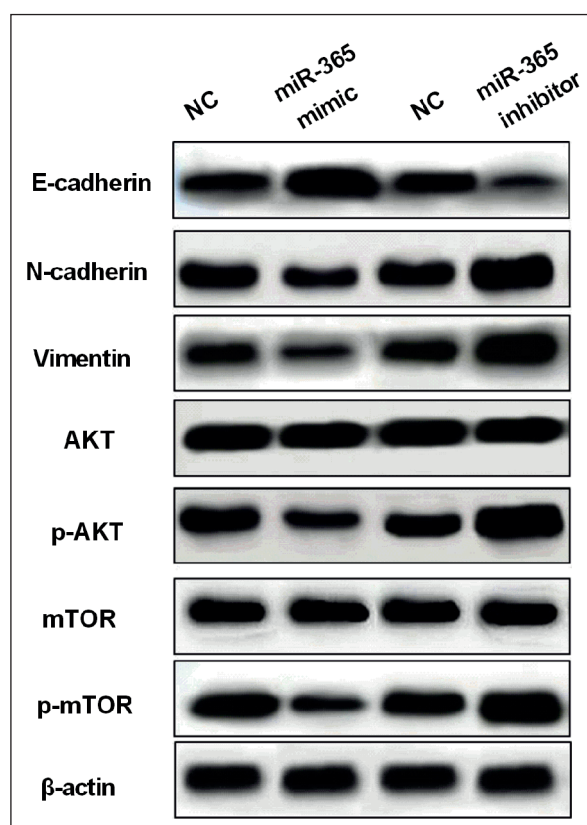
### **MiR-365 Inhibited EMT and Inactivated AKT/mTOR Pathway in Lung Adenocarcinoma**

Last but not least, the effect of miR-365 on EMT and AKT/mTOR pathway was analyzed in lung adenocarcinoma. Western blot assay suggested that miR-365 regulated the expressions of Vimentin, N-cadherin, and E-cadherin. It showed that the overexpression of miR-365 enhanced the E-cadherin expression level and suppressed the expressions of Vimentin and N-cadherin in NCI-H1975 cells (Figure 5). However, the downregulation of miR-365 showed opposite effects on their ex-

pression (Figure 5). The AKT/mTOR pathway has been implicated in the pathogenesis of lung cancer. Therefore, in this study, we investigate how miR-365 regulates the AKT/mTOR pathway. The results indicated that miR-365 mimics suppressed the expressions of p-AKT and p-mTOR, whereas miR-365 inhibitor enhanced the expression levels of p-AKT and p-mTOR (Figure 5). However, miR-365 had no effect on the expressions of AKT and mTOR (Figure 5). Taken together, miR-365 inhibited EMT and inactivated AKT/mTOR pathway in lung adenocarcinoma, thereby inhibiting the progression of lung adenocarcinoma.



**Figure 4.** Upregulation of ETS1 abolished the suppressive effect of miR-365 in lung adenocarcinoma. **A**, ETS1 expression was observed in lung adenocarcinoma tissues. **B**, Good prognosis in lung adenocarcinoma patients was related to low ETS1 expression. **C**, ETS1 expressions were observed in NCI-H1975 cells with miR-365 mimics and ETS1 vector. **D**, **E**, **F**, Cell proliferation, migration, and invasion were identified in NCI-H1975 cells containing miR-365 mimics and ETS1 vector (magnification, 200×). \* $p < 0.05$ , \*\* $p < 0.01$ .



**Figure 5.** MiR-365 inactivated EMT/AKT/mTOR pathway in lung adenocarcinoma. MiR-365 regulated protein expressions of Vimentin, N-cadherin, E-cadherin, AKT, mTOR, p-AKT, and p-mTOR in NCI-H1975 cells.

## Discussion

MiR-365 was downregulated in different cancers, such as breast cancer, gastric cancer, and colon cancer<sup>20-22</sup>. Here, the downregulation of miR-365 was also found in lung adenocarcinoma. Furthermore, miR-365 inhibited cell proliferation, migration, and invasion in lung adenocarcinoma. Similarly, miR-365 has been reported to inhibit proliferation, migration, and invasion of ovarian cancer cells<sup>23</sup>. Chen et al<sup>24</sup> also found the prognostic significance and anti-proliferation effect of miR-365 in hepatocellular carcinoma. In the current work, low miR-365 expression was found to be associated with shorter overall survival in lung adenocarcinoma patients. More importantly, miR-365 has been reported to play inhibitory role in NSCLC by targeting TTF-1<sup>25</sup>. In our study, miR-365 directly targets ETS1 to participate in the progression of lung adenocarcinoma.

MiR-365 is involved in different cancers by targeting some genes, such as BAX, Cyclin D1, and HDAC4<sup>26-28</sup>. To the best of our knowledge, the relationship between miR-365 and ETS1 has not been reported. Therefore, we investigated the molecular mechanism of miR-365 and ETS1 in lung adenocarcinoma. It was found that the upregulation of ETS1 attenuated the inhibitory effect of miR-365 in lung adenocarcinoma. Recently, many researches demonstrated that ETS1 is involved in the development of human cancer. Of note, ETS1 controlled cell invasion and growth in breast cancer<sup>29</sup>. In addition, ETS1 has been reported to promote proliferation, migration, and invasion of lung adenocarcinoma cells<sup>30</sup>. The same results of ETS1 were also detected in this study. MiR-365 was found to inhibit the progression of lung adenocarcinoma by targeting ETS1, which has not been investigated previously.

In addition, we investigated how miR-365 regulates EMT and AKT/mTOR pathway in lung adenocarcinoma. In our study, the relationship between miR-365 and EMT or AKT/mTOR pathway was first reported in lung adenocarcinoma. In lung adenocarcinoma, miR-218 has been proposed to suppress EMT by targeting Robo1<sup>31</sup>. Here, miR-365 also inhibited EMT in lung adenocarcinoma to regulate cell metastasis. Besides that, it is well known that the AKT/mTOR pathway is closely related to cell survival in human cancer<sup>32</sup>. Furthermore, ROR1 silencing inhibited tumor cell proliferation in lung adenocarcinoma *via* AKT/mTOR signaling pathway<sup>33</sup>. In this investigation, the overexpression of miR-365 also inactivated the AKT/mTOR signaling pathway, thereby inhibiting cell viability. Briefly, miR-365 exerts an inhibitory effect on the development of lung adenocarcinoma by inactivating EMT and AKT/mTOR pathway.

## Conclusions

We first revealed that miR-365 was downregulated in lung adenocarcinoma and inhibited cell viability and metastasis by downregulating ETS1. In addition, miR-365 also inhibited EMT and AKT/mTOR pathway, acting as a tumor suppressor in lung adenocarcinoma. This study may provide potential therapeutic targets for patients with lung adenocarcinoma.



### Conflict of Interest

The Authors declare that they have no conflict of interests.

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