

Article

Effect of microRNA-145 on proliferation and apoptosis of human non-small cell lung cancer A549 cells by regulating mTOR signaling pathway[†]

Running title: miR-145 regulating mTOR pathway and NSCLC cells

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ABSTRACT

Our study aims to explore the impact of microRNA-145 (miR-145) on proliferation and apoptosis of non-small cell lung cancer (NSCLC) cells by modulating mTOR signaling pathway. Paired NSCLC and adjacent normal tissues were obtained from 50 NSCLC patients between January 2014 and January 2016. Human lung adenocarcinoma A549 cell line was assigned into the control, mimics, mimics-negative control (NC), inhibitor and inhibitor-NC groups. Quantitative RT-PCR and Western blot were applied to detect the expressions of miR-145 and mTOR. MTT, scratch test, Transwell and flow cytometry were carried out to analyze the effect of miR-145 on A549 cell proliferation, migration, invasion, apoptosis and cycle distribution and regulation of mTOR signaling pathway. MiR-145 expression in NSCLC tissues was lower than that in adjacent normal tissues, while mTOR expression was higher in NSCLC tissues than in adjacent normal tissues. Compared with the control and mimics groups, cell proliferation, migration and invasion abilities decreased in the mimics group; additionally, cell apoptosis rate increased and cells were mostly in G1 phase. MiR-145 inhibited the activation of mTOR signaling pathway. Compared with the control and inhibitor-NC groups, mTOR signaling pathway was activated and cell proliferation, migration and invasion abilities increased in the inhibitor group, and cell apoptosis rate decreased and cells were less in G1 phase. The study indicated that miR-145 expression was low in NSCLC and influenced NSCLC cells by negatively regulating mTOR signaling pathway. This article is protected by copyright. All rights reserved

Keywords: MicroRNA-145; mTOR; Non-small cell lung cancer; Cell proliferation; Cell apoptosis

INTRODUCTION

Lung cancer, one of the most frequently diagnosed cancers, is considered as the leading cause of cancer death worldwide, especially among males [Torre et al., 2015]. Over 1.5 million new lung cancer cases are diagnosed in the world annually [de Groot and Munden, 2012]. Epidemiological studies have clearly indicated cigarette smoking as the predominant cause of lung cancer, with over 85% cases are current or former smokers [de Groot and Munden, 2012, Nair et al., 2014]. It is classified as small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), with the latter type accounting for about 85% [Campayo et al., 2013]. NSCLC consists of three sub-types: squamous cell carcinoma, adenocarcinoma, and large cell carcinoma [Govindan et al., 2012]. Most of lung cancers are diagnosed at the advanced stage as the early stage is asymptomatic [DeSantis et al., 2014]. Surgical resection is the main treatment for most NSCLC patients at the early stage, but it has a high relapse rate (20%-70%, relying on the stage) after surgery [Campayo et al., 2013, Spits and Neefjes, 2016]. Chemotherapy, radiotherapy and target therapy are utilized in treating advanced NSCLC [Hsieh et al., 2016]. It is estimated that the overall 5-year survival rate for advanced NSCLC remains poor, about 15% [Kremer et al., 2016]. With the advances in the detection of genetic alterations, a new understanding of the molecular etiology of lung cancer has generated, which facilitates the diagnosis and treatment of NSCLC [Paleiron et al., 2016].

MicroRNAs (miRNAs) are small (20-24 nucleotides) non-coding RNAs which are generated from longer transcripts pre-miRNA [Spizzo et al., 2010]. Accumulating evidence has shown miRNAs' significant role in regulating biological processes, including developmental timing, cell proliferation, differentiation and death, by targeting downstream genes, either oncogenes or tumor suppressors [Xu et al., 2012, Zou et al., 2012, Zhao et al., 2013]. Therefore, miRNAs may be proposed as a new biomarker for efficient tumor treatment [Xia et al., 2015]. MiR-145 is known as a tumor suppressor in the way of inhibiting tumor cell growth, invasion, metastasis, tumorigenesis, and regulating cell apoptosis, cycle, and epithelial to mesenchymal transition [Li et al., 2015]. Its

expression is commonly down-regulated in various malignancies, including colon, breast and lung cancers [Spizzo et al., 2010, Zou et al., 2012]. The mammalian Target Of Rapamycin (mTOR), a serine/threonine protein kinase, is critically regulating basic cell behaviors, like cell proliferation, cell growth and cell cycle progression [Wang et al., 2010, Laplante and Sabatini, 2012]. It has proven to be one of the important targets in tumor therapy and its inhibitors indicate encouraging effects [Xu et al., 2012]. Recently, few studies have focused on the association between miR-145, mTOR signaling pathway and NSCLC. In this study, we investigated whether miR-145 had effect on NSCLC cell proliferation and apoptosis by regulating mTOR signaling pathway to find out new effective target for NSCLC treatment.

MATERIALS AND METHODS

STUDY SUBJECTS

Paired NSCLC and adjacent normal tissues were obtained from 50 patients who underwent NSCLC resection between January 2014 and January 2016 in the department of thoracic surgery at Fujian Provincial Cancer Hospital. All patients were pathologically diagnosed with primary NSCLC and their complete medical history and follow-up information were available. None of them had prior to surgery received radiotherapy, chemotherapy, or other anticancer therapy. According to the 7th edition of tumor, node, metastasis (TNM) classification in lung cancer by the International Union Against Cancer (UICC) and the American Joint Committee on Cancer [Wrona and Jassem, 2010], the pathological tumor types and clinicopathological stages were as follows: 31 cases were in clinical stage I, 9 cases in stage II, and 10 cases in stage III and IV; adenocarcinoma in 23 cases, squamous cell carcinoma in 23 cases, and poorly differentiated lung cancer in 4 cases; 27 cases in pathological stage I, 14 cases in stage II and 9 cases in stage III. Adjacent normal tissues, more than 5 cm away from NSCLC tissues, were stained using hematoxylin and eosin (HE) to confirm that there was no infiltration of NSCLC cells. The NSCLC and adjacent normal tissues were kept for

further study. The study was approved by the Ethical Committee of Fujian Provincial Cancer Hospital. Written informed consents were obtained from all study subjects or their legal guardians.

All the collection and use of tissue samples complied with the guidelines and principles of the Declaration of Helsinki.

CELL LINES AND CELL CULTURE

Normal endothelial cells BEAS-2B and human lung adenocarcinoma cell lines H1650, H1975, A549, and H292 were purchased from American type culture collection (ATCC). The cell lines were cultured in RPMI-1640 medium containing fetal bovine serum (FBS; 10%) (Gibco Company, Grand Island, NY, USA), 100 units/ml penicillin (Hyclon) and 100 mg/ml streptomycin (Hyclon), and incubated in an incubator with 5% CO₂ at 37°C. When the cells covered 80% of the microscopic fields, 0.25% trypsin was applied to digest and obtain passage cells for following experiments.

CELL TRANSFECTION AND GROUPING

The A549 cell line was selected and assigned into 5 groups: the control group (non-transfection), the mimics group (miR-145 over-expressed group), the mimic negative control (NC) group, the inhibitor group (miR-145 low-expressed group) and the inhibitor NC group. All oligonucleotide DNA sequences (Table 1) were synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China). At 24 h before transfection, A549 cells were maintained in the plate for routine incubation. At 1 h before transfection, medium was changed and 2 ml RPMI-1640 medium was added into each well. Transfection mixture was configured with Lipofectamine 2000 kit (Invitrogen Inc., Carlsbad, CA, USA). Serum-free medium without double antibodies was added to the control group, while serum-free without double antibodies medium containing corresponding oligonucleotide fragments (final concentration: 300 pmol per well) encapsulated in liposome (Invitrogen Inc., Carlsbad, CA, USA) was added to other four groups. The transfected cells were cultured in serum-free blocking

solution for 4 h, added with 10% FBS, and then incubated in an incubator with 5% CO₂ at 37°C.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

The miRNA was extracted from tissue samples and cells in five groups using miRNeasy Mini kit (Qiagen, Germany). From tissue samples and cells in five groups total RNA was isolated using Trizol (Invitrogen Inc., Carlsbad, CA, USA). Its concentration and purity were assessed by NanoDrop2000 (Thermo Fisher Scientific, California, USA), then stored at -80°C for further study. The cDNA of miR-145 and U6 was synthesized using specific stem-loop reverse transcription primer (0.15 μM). Primers for miR-145 and U6 were 5'-CTCAACTGGTGTCTGGAGTCGGCAATTCATTGAGAGGGATTC-3' and 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATATGGAAC-3', respectively. According to Gene sequence published in GenBank database, PCR reaction primers (Table 2) were designed using Primer 5.0 design software, and synthesized by Shanghai GenePharma Co., Ltd. PCR reaction was performed with ABI PRISM 7500 real-time PCR System (ABI Company, Oyster Bay, NY) and SYBR Green I fluorescence kit (Takara Biotechnology Ltd., Dalian, China). PCR reliability was evaluated by dissolution curve, with U6/β-actin as internal control. The curve threshold (CT) value (amplified kinetic curve infection point) was obtained. Relative expression of target gene was calculated via $2^{-\Delta\Delta Ct}$ method ($\Delta Ct = CT_{\text{target gene}} - CT_{\text{internal control}}$, $\Delta\Delta Ct = \Delta Ct_{\text{experiment group}} - \Delta Ct_{\text{control group}}$).

WESTERN BLOT

The extracted cell protein concentration was determined using BCA kit (Wuhan Boster Co., Ltd, Wuhan, China) instructions. The protein added with loading buffer (30 μg per well) was boiled for 10 min at 95°C. The protein was separated using sodium dodecyl sulfate polyacrylamide gel (Wuhan Boster Co., Ltd, Wuhan, China) electrophoresis (SDS-PAGE; 10%), with 80 v to 120 v,

followed by wet-transfer process (100 mv) utilizing polyvinylidene fluoride (PVDF) membrane for 45-70 min. Then the membranes were blocked with 5% albumin from bovine serum (BSA) at room temperature for 1 h, and then they were incubated overnight at 4°C with corresponding primary antibodies (1: 1000) (Abcam Inc., Cambridge, MA, USA). After rinsed by TBST (3 times/5 min), membranes were subsequently incubated for 1 h with secondary antibodies. Then membranes were washed again (3 times/5 min) and developed using chemiluminescence reagent. β -actin was used as an internal control. Bio-Rad Gel Doc EZ imager (GEL DOC EZ IMAGER, Bio-Rad, California, USA) was applied for developing. The gray value of target band was analyzed by Image J software.

MTT ASSAY

After diluted at a certain concentration, transfected A549 cells were inoculated in 96-well plates (about 5×10^3 /well). Then the cells were cultured in a CO₂ incubator at 37°C for 24 h, 48 h and 72 h. With 20 μ l MTT solution (Promega Corporation, Madison, WI, USA) added to each well, the cells continued to be cultured for 4 h. The optical density (OD) at 490 nm wavelength was measured using a multifunctional microplate reader. The cell survival rate was calculated as: $(OD_{\text{experiment well}} - OD_{\text{blank well}}) / (OD_{\text{control well}} - OD_{\text{blank well}}) \times 100\%$.

SCRATCH TEST

Cells in the 6-well plate were transfected and then incubated at 37°C. When all cells attached to plate bottom, a wound was scratched gently in each well with a sterilized gun head. The width of each wound was ensured to be consistent as far as possible. The image was captured and labeled to ensure photographing in the same field; the time was recorded as 0 h. After the cells were incubated for 24 h at 37°C, the culture medium was removed. The plate was washed with phosphate buffered saline (PBS) in triplicate to remove cell debris, then added with serum-free medium and photographed. The time was recorded as 24 h. The migration distance at 0 h and 24 h was observed

under a microscope; several fields were randomly selected and photographed. The scratch healing rate was calculated at 24 h; the scratch healing = (the width of the scratch at 0 h – the width of the scratch at 24 h) / the width of the scratch at 0 h $\times$ 100%. Three wells were set in each group.

TRANSWELL ASSAY

Each Transwell chamber (Chambers, German) was added with matrigel (3.9 mg/ml, 60-80 μ l) and cultured for 30 min at 37°C until the matrigel solidified. After added with pre-warmed medium, the cells were hydrated in a 37°C incubator for 2 h. Complete medium (0.5 ml) was added into each well. Transwell chambers were added with cell suspension (0.5 ml, 5×10^4 /ml) and cultured at 37°C for 24 h. The cells in the upper chamber were removed, washed with PBS, fixed with pre-cooled methanol and migrated to low chamber for 30 min. The cells were stained with 0.1% crystal violet solution for 10 min and photographed under the Olympus inverted microscope in 6 fixed fields. The number of cells that migrated to lower chambers was counted by click-counting software, followed by statistical analysis.

FLOW CYTOMETRY

After 24 h of transfection, the cells were digested with trypsin, centrifuged, collected, washed with cold PBS, and re-suspended in PBS buffer containing calcium for single cell suspension at a concentration of 1×10^6 cells/ml. A total of 100 μ l suspension was transformed into tubes at room temperature, added with propidium iodide (PI, 10 mg/ml) and RNase A (10 mg/ml), mixed, and cells were cultured for 30 min at 4°C. Then the cells (10^4 cells were obtained each time) were added with staining buffer (400 μ l), followed by subsequent analysis using flow cytometry (BD Bioscience) equipped with Cell Quest software. Annexin V positive cells were apoptotic cells. PI positive and Annexin V negative cells were necrotic cells. The apoptosis rate was calculated as the number of Annexin V⁺PI⁺ cells + the number of Annexin V⁺PI⁻ cells) / $10^4 \times 100\%$. Next, 100 μ l

cell suspension was added into tubes, and then added with 1 ml PI/TritonX-100 staining solution containing 0.2 mg RNase A, 20 µg PI, and 0.1% TritonX-100. Cells were cultured for 30 min at 4°C. Then flow cytometer was used to measure cell cycle.

STATISTICAL ANALYSIS

SPSS 18.0 software (IBM Corporation, NY, USA) was applied for statistical analysis. Measurement data were presented as mean $\pm$ standard deviation ($\bar{x} \pm s$), and those with normal distribution were analyzed with *t*-test for pairwise comparison. One-way analysis of variance was used for multi-group comparison. Categorical data were expressed as ratio and percentage and tested by Chi-square. $P < 0.05$ was considered as statistical significance.

RESULTS

MIR-145 EXPRESSION IN NSCLC AND ADJACENT NORMAL TISSUES

Among 50 paired tissue samples, miR-145 expression in NSCLC tissues was lower than that in corresponding adjacent normal tissues in 32 pairs, of which 22 pairs were significantly lower (decrease more than twice). The results suggested that miR-145 expression was related to clinical stages and pathological tumor types, and was significantly lower in NSCLC compared with adjacent normal tissues (all $P < 0.05$) (Figure 1).

MTOR EXPRESSION IN NSCLC AND ADJACENT NORMAL TISSUES AND ITS CORRELATION WITH MIR-145

Among 50 paired tissues samples, mTOR expression in NSCLS tissues was higher than that in corresponding adjacent normal tissues in 30 pairs, of which 19 pairs were significantly higher (increase more than twice) ($P < 0.05$). MTOR expression in NSCLC was significantly higher compared with that in adjacent normal tissues, which was an opposite trend to miR-145 expression

(Figure 2).

EXPRESSIONS OF MIR-145 AND MTOR IN NSCLC CELL LINES

It was found that miR-145 expression was lower in cell line A549 ($P < 0.05$), and significantly decreased in H1650, H1975 and H292 cell lines (all $P < 0.01$) compared with normal endothelial cells BEAS-2B. In contrast, mTOR expression was the lowest in normal endothelial cells BEAS-2B, higher in A549, and significantly increased in H1650, H1975, and H292 cell lines (all $P < 0.01$) (Figure 3).

EFFECT OF MIR-145 ON MTOR EXPRESSION IN EACH GROUP

Compared with the control group, mTOR expression exhibited no obvious changes in the mimics-NC and inhibitor-NC groups (all $P > 0.05$), but exhibited significant changes in the mimics group and inhibitor group (all $P < 0.05$). Compared with the control and mimics-NC groups, mTOR expression in the mimics group significantly decreased ($P < 0.05$). Besides, compared with the control and inhibitor-NC groups, mTOR expression in the inhibitor group significantly increased ($P < 0.05$) (Figure 4).

EFFECT OF MIR-145 ON NSCLC A549 CELL PROLIFERATION ABILITY

After cells were transfected and cultured for 24 h, 48 h and 72 h, the effect of miR-145 on A549 cell proliferation was detected. It suggested that compared with the control group, A549 cell proliferation exhibited not significant changes in the mimics-NC and inhibitor-NC groups (all $P > 0.05$), but exhibited significant changes in the mimics and inhibitor groups (all $P < 0.05$). Compared with the control and mimics-NC groups, A549 cell proliferation was significantly inhibited in the mimics group ($P < 0.05$). Compared with the control and inhibitor-NC groups, A549 cell proliferation significantly increased in the inhibitor group ($P < 0.05$). The results indicated that

miR-145 inhibited A549 cell proliferation (Figure 5).

EFFECT OF MIR-145 ON NSCLC CELL MIGRATION AND INVASION ABILITY

Compared with the control group, A549 cell migration and invasion ability exhibited no significant changes in the mimics-NC and inhibitor-NC groups (all $P > 0.05$), but exhibited significant changes in the mimics and inhibitor groups ($P < 0.05$). Besides, compared with the control and mimics-NC groups, A549 cell migration and invasion ability were significantly inhibited in the mimics group ($P < 0.05$). Compared with the control and inhibitor-NC groups, the rate of A549 cell migration and invasion significantly increased in the inhibitor group ($P < 0.05$). The results suggested that miR-145 could inhibit NSCLC A549 cell migration and invasion ability (Figure 6).

EFFECT OF MIR-145 ON CELL CYCLE DISTRIBUTION

After cells in each group were transfected and cultured for 24 h, cell cycle was analyzed. It revealed that compared with the control group, there were no significant changes examined in cell cycle in the mimics-NC and inhibitor-NC groups (all $P > 0.05$). Compared with the control and mimics-NC groups, the cells in the mimics group were mostly in G1 phase and significantly increased, but significantly decreased in S and G2 phases (all $P < 0.05$). Compared with the control and inhibitor-NC groups, the cells in the inhibitor group were less in G1 phase, but significantly increased in S and G2 phases ($P < 0.05$). It was suggested that miR-145 inhibited cell cycle from G1 phase to S phase (Table 3).

MIR-145 INVOLVED IN REGULATING CELL APOPTOSIS

The apoptosis rate was measured after cells were transfected and cultured for 24 h. It was found that compared with the control group, cell apoptosis rate exhibited no obvious difference in the mimics-NC and inhibitor-NC groups (all $P > 0.05$). Compared with the control and mimics-NC

groups, cell apoptosis rate significantly increased in the mimics group (all $P < 0.05$). Compared with the control and inhibitor-NC groups, cell apoptosis rate significantly decreased in the inhibitor group ($P < 0.05$). The results suggested that miR-145 promoted NSCLC cell apoptosis (Table 4).

EFFECT OF MIR-145 ON MTOR SIGNALING PATHWAY

Protein expressions of mTOR signaling pathway-related proteins were determined after the cells were transfected and cultured for 24 h. The results showed that compared with the control group, mTOR signaling pathway exhibited no significant difference in the mimics-NC and inhibitor-NC groups (all $P > 0.05$). Compared with the control and mimics-NC groups, mTOR signaling pathway was inhibited, and phosphorylation among proteins mTOR, p70S6K, S6, eIF4E, and 4E-BP1 significantly decreased in the mimics group (all $P < 0.05$). Compared with the control and inhibitor-NC groups, mTOR signaling pathway was activated and phosphorylation among proteins mTOR, p70S6K, S6, eIF4E, and 4E-BP1 significantly increased in the inhibitor group (all $P < 0.05$). The results suggested that miR-145 inhibited activation of mTOR signaling pathway (Figure 7).

DISCUSSION

NSCLC is the commonest cause of death among all malignant tumors in the world, and most of the patients have been diagnosed at the stage when operative treatments are not applicable [Ramlau et al., 2012]. The most effective treatment is recognized to be the combination of surgery, chemotherapy and radiotherapy, despite of a low overall 5-year survival rate [Kremer et al., 2016]. Researchers hope to find a better treatment to improve the survival rate of NSCLC, and molecular targeted therapy has become a hot spot with the continuous development of medical research. The present study made efforts to elucidate the impact of miR-145 on cell proliferation and apoptosis of NSCLC cells by regulating mTOR signaling pathway and finally made a conclusion that miR-145 expression was low in NSCLC and influenced NSCLC cells by negatively regulating mTOR

signaling pathway.

Firstly, the expression of miR-145 in NSCLC is lower than that in the adjacent tissues, while the expression of mTOR in NSCLC is higher than that in the adjacent tissues. Previous study has showed that miR-145 plays a significant role in cell apoptosis, neural development, proliferation and stem cell differentiation, and inhibits tumor growth and angiogenesis with low expression [Xu et al., 2012]; [Zou et al., 2012]. Besides, miR-145 is proven to inhibit proliferation, migration and invasion of NSCLC cells and decreased miR-145 expression is often found in NSCLC cells which is associated with poor survival [Ye et al., 2015]. Consistently, Study has found that miR-145 is down-regulated in NSCLC tissues, in which the down-regulation of miR-145 has connection with low differentiation and late clinical stage [Shen et al., 2015]. As a serine/threonine protein kinases, mTOR could make contributions to protein synthesis, cell growth and cell cycle progression [Wang et al., 2010]. Additionally, mTOR is certified to mediate a variety of cellular and molecular functions through the PI3K/Akt/mTOR signaling pathway, and play a key role in the occurrence and progression of the tumor by changing the expression of its target gene [Zhang et al., 2015]. Also, PI3K/Akt/mTOR signaling pathway could motivate PI3K, Akt and mTOR in many different cancers, and expression of mTOR increased in NSCLC, so that mTOR inhibitors could be a promising therapeutic strategy for NSCLC treatment [Bhatt et al., 2010, Johnson et al., 2010, Chen et al., 2015]. Moreover, overexpression of mTOR is thought as the poor prognostic factor in early stage NSCLC, which is consistent with our results [Gately et al., 2012].

In addition, our study has illustrated that miR-145 could inhibit mTOR, and the low expressions of mTOR, p70S6K, S6, eIF4E and 4E-BP1 could inhibit the proliferation of NSCLC cells and promote their apoptosis. Previous study has found that miR-145 is a tumor-suppressive miRNA in cells and could obviously inhibit proliferation, migration and invasion in vitro [Lu et al., 2013]. Also, a previous study proved that mTOR activation may promote gene expression by decreasing miR-143/miR-145 expression [Fang et al., 2012, Cioce et al., 2016]. Meanwhile, a study

illustrated that miRNAs serve as new downstream parts of the mTOR-signaling pathway, which is essential to tumor responses to mTOR inhibitors [Totary-Jain et al., 2013]. Both mTOR complexes mTORC1 and mTORC2 contain mTOR and mTORC1 has downstream regulation pathways including p70S6K (ribosomal p70S6 kinase), 4E-BP1, and mTORC1 activation could be regulated via PI3K/Akt/mTOR and LKB1/AMPK/mTOR signaling pathways [Wang et al., 2014]. Similarly, mTOR and 4E-BP1 is thought to increase significantly in some cancers, and mTOR/4E-BP1 pathway could be an important regulator of protein synthesis associated with cell differentiation, proliferation and survival [Zhou et al., 2004]. Meanwhile, overexpression of eIF4E or S6K1 could be found in some cancers [Totary-Jain et al., 2013]. Also, the inhibition of mTOR/p70S6K/ eIF4E/ S6 phosphorylation is able to prevent recurrence and p70S6K phosphorylation could be served as a molecular biomarker to predict recurrence of cancers and tumors [Kim et al., 2014]. Besides, the inhibition of mTOR could prevent the phosphorylation of P70S6K, 4E-BP1 and other proteins in connection with transcription and cell cycle, which could block the cell cycle from G phase to S phase of the synthesis of essential proteins [Vignot et al., 2005].

In conclusion, our study explored the relationship between miR-145 and mTOR signaling pathway in NSCLC, and collectively we found low expression of miR-145 in NSCLC, and miR-145 can influence the proliferation and apoptosis of NSCLC cells by negative regulation of mTOR signaling pathway, and therefore miR-145 is expected to become a novel biomarker of diagnosis and treatment of NSCLC. However, the inner mechanism of miR-145 and mTOR effect on NSCLC is still unclear; more researches need to be studied for further NSCLC treatment.

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CONFLICT OF INTEREST

Authors declare that they have no existing conflicts of interest concerning this article.

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Figure 1 MiR-145 expressions in NSCLC and adjacent normal tissues

Note: * refers to $P < 0.05$.

Figure 2 Relative mTOR expressions in NSCLC and adjacent normal tissues

Note: * refers to $P < 0.05$.

Figure 3 Expressions of miR-145 and mTOR in NSCLC cell lines.

Note: A: miR-145 expressions in cell lines detected by RT-qPCR; B: mTOR expressions in cell lines detected by RT-qPCR; C: mTOR expressions in cell lines detected by Western blot assay; D: gray value analysis; * refers to $P < 0.05$ when compared with BEAS-2B; ** refers to $P < 0.01$ when compared with BEAS-2B.

Figure 4 Effect of miR-145 on mTOR expressions in each group

Note: A: the results of RT-qPCR indicated that miR-145 was involved in regulating mRNA expressions of mTOR; B: the results of Western blot indicated that miR-145 influenced mTOR protein expressions; C: gray value analysis; * refers to $P < 0.05$ when compared with the control group; & refers to $P < 0.05$ when compared with mimics-NC group; # refers to $P < 0.05$ when compared with the inhibitor-NC group.

Figure 5 Effect of miR-145 on lung cancer A549 cell proliferation

Note: A: the optical density (OD) of each group at different time points detected by MTT; B: the comparison of the cell survival rate in each group at different time points; * refers to $P < 0.05$ when compared with the control group; & refers to $P < 0.05$ when compared with the mimics-NC group; # refers to $P < 0.05$ when compared with the inhibitor-NC group.

Figure 6 Effect of miR-145 on NSCLC cell migration and invasion

Note: A: the effect of miR-145 on A549 cell invasion ability; B: the results of Transwell invasion indicated that miR-145 influenced A549 cell invasion ability; C: the statistical analysis of Scratch test; D: the statistical analysis of Transwell assay; *refers to $P < 0.05$ when compared with the control group; & refers to $P < 0.05$ when compared with the mimics-NC group; # refers to $P < 0.05$ when compared with the inhibitor-NC group.

Figure 7 Effect of miR-145 on mTOR signaling pathway

Note: A: the results of Western blot indicated that miR-145 inhibited mTOR signaling pathway; B: gray analysis; *refers to $P < 0.05$ when compared with the control group; & refers to $P < 0.05$ when compared with the mimics-NC group; # refers to $P < 0.05$ when compared with the inhibitor-NC group.

Table 1 Oligonucleotides sequences

Oligonucleotides	Sequence (5'-3')
miR-145 mimics	TCCCTAAGGACCCTTTTGACCTG
mimics-NC	GUCCAGUUUUUCCCAGGAAUCCCU
miR-145 inhibitor	GTCCAGTTTTCCCAGGAATCCC
inhibitor-NC	ACUACUGAGUGACAGUAGA

Note: NC, negative control.

Table 2 qRT-PCR primer sequences

Gene	Sequence (5'-3')
miR-145	Forward: CGGCGTCCAGTTTTCCCAGG
	Reverse: GTGCAGGGTCCGAGGT
U6	Forward: CGCTTCGGCAGCACATATACTAAAAT
	Reverse: CGCTTCACGAATTTGCGTGTCAT
mTOR	Forward: ACAGCCCAGGCCGCATTGTC
	Reverse: TCCAGGGACTCCGTCAGGCG
β -actin	Forward: GGA CTTCGAGCAAGAGATGG
	Reverse: AGCACTGTGTTGGCGTACAG

Note: qRT-PCR, quantitative real-time polymerase chain reaction.

Table 3 Cell cycle distribution after transfection in the control, mimics, mimics-NC, inhibitor and inhibitor-NC groups (%)

Phase	Control	Mimics	Mimics-NC	Inhibitor	Inhibitor-NC
G0/G1	63.16 ± 1.42	70.87 ± 2.03* ^{&}	63.01 ± 1.37	56.04 ± 1.18* [#]	63.02 ± 1.46
S	25.18 ± 1.23	23.06 ± 1.15* ^{&}	25.13 ± 1.21	27.13 ± 1.29* [#]	25.39 ± 1.25
G2/M	11.66 ± 2.19	6.07 ± 1.91* ^{&}	11.86 ± 2.36	16.83 ± 1.44* [#]	11.59 ± 1.73

Note: * refers to $P < 0.05$ compared with the control group; & refers to $P < 0.05$ compared with the mimics-NC group; # refers to $P < 0.05$ compared with the inhibitor-NC group; NC, negative control.

Table 4 Cell apoptosis rate after transfection in the control, mimics, mimics-NC, inhibitor and inhibitor-NC groups (%)

Apoptosis	Control	Mimics	Mimics-NC	Inhibitor	Inhibitor-NC
Early apoptosis	3.63 ± 0.17	14.73 ± 4.81 ^{*&}	3.54 ± 0.05	0.69 ± 0.02 ^{*#}	3.81 ± 0.34
Late apoptosis	9.10 ± 0.56	21.52 ± 2.73 ^{*&}	8.93 ± 0.23	3.52 ± 0.11 ^{*#}	9.29 ± 0.62

Note: * refers to $P < 0.05$ compared with the control group; & refers to $P < 0.05$ compared with the mimics-NC group; # refers to $P < 0.05$ compared with the inhibitor-NC group; NC, negative control.

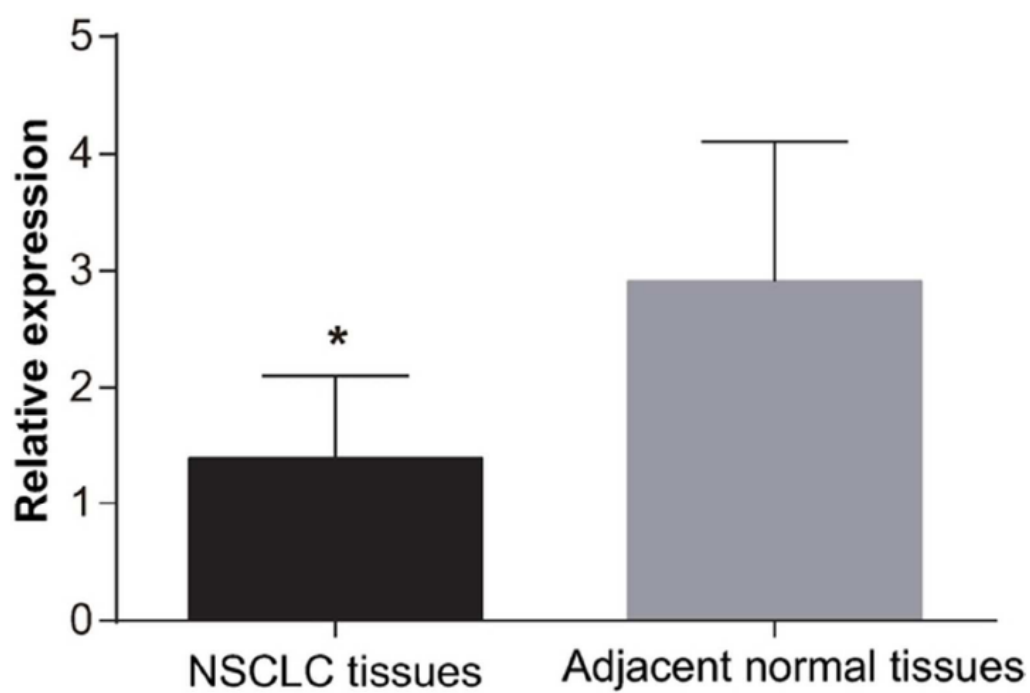


Figure 1

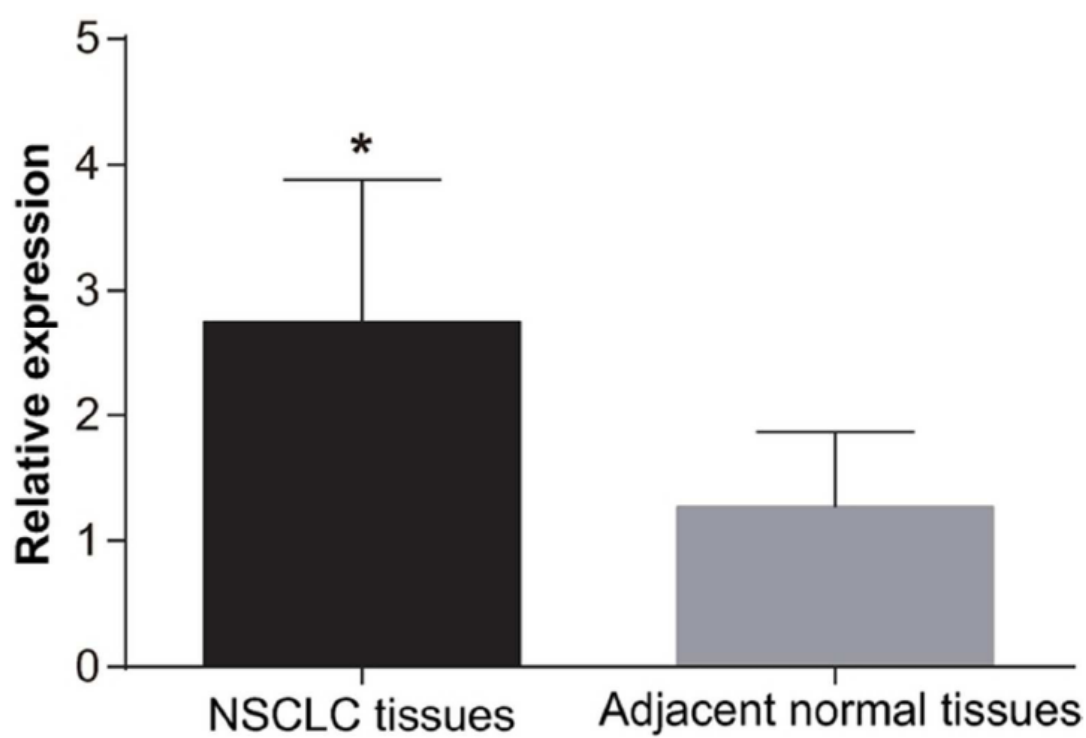


Figure 2

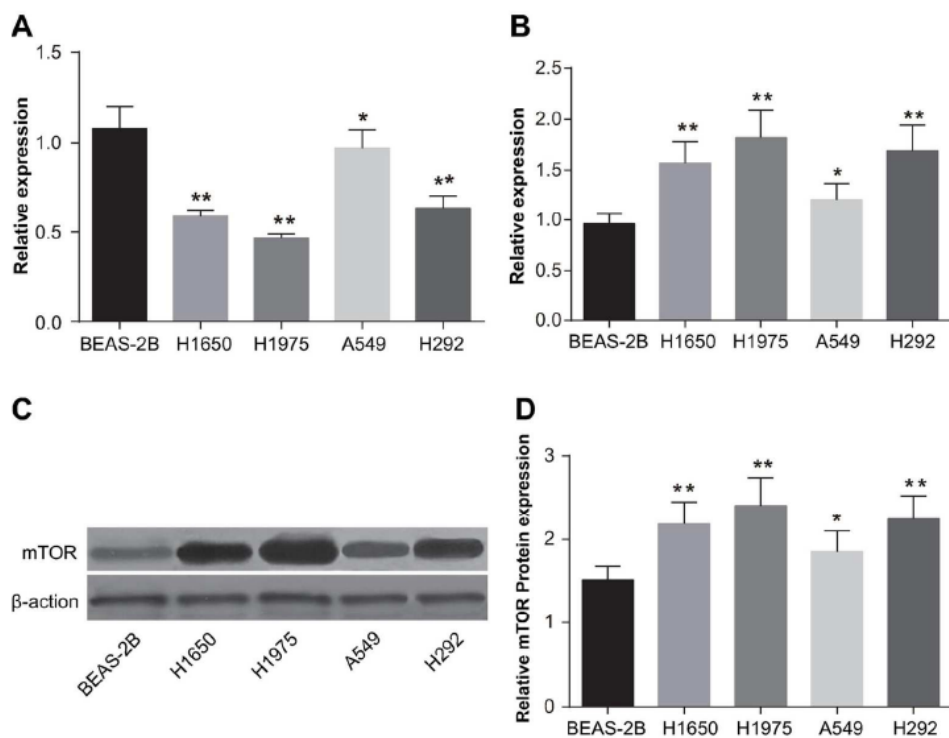


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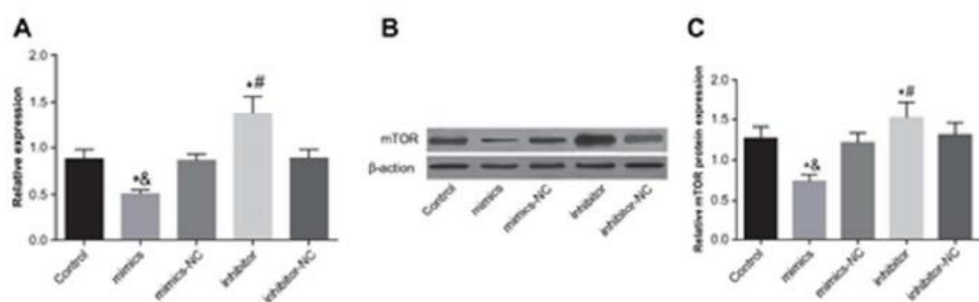


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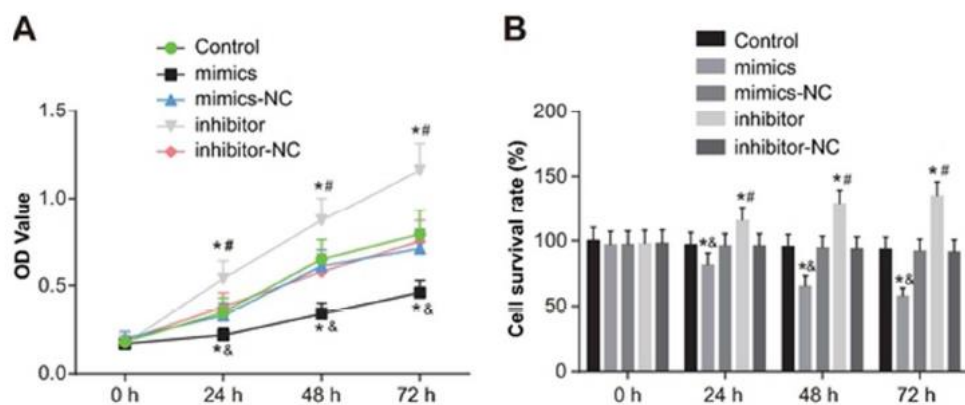


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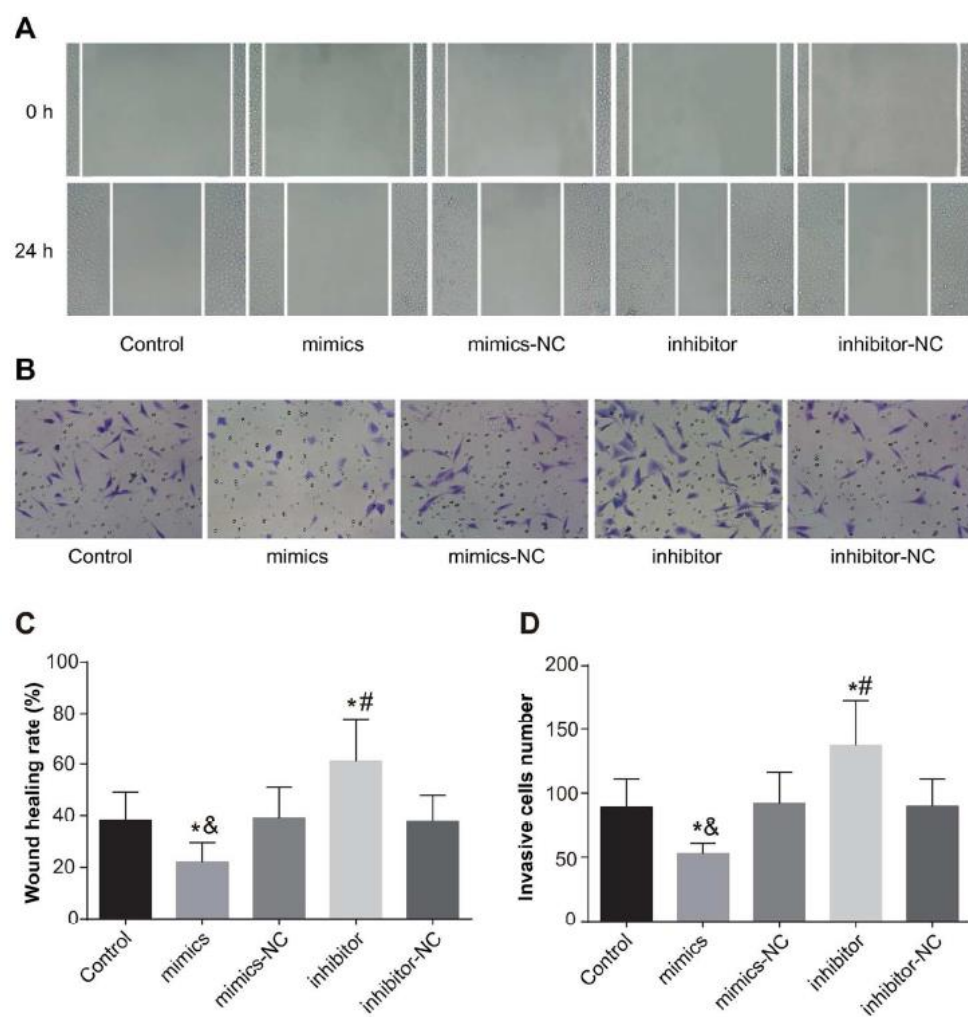


Figure 6

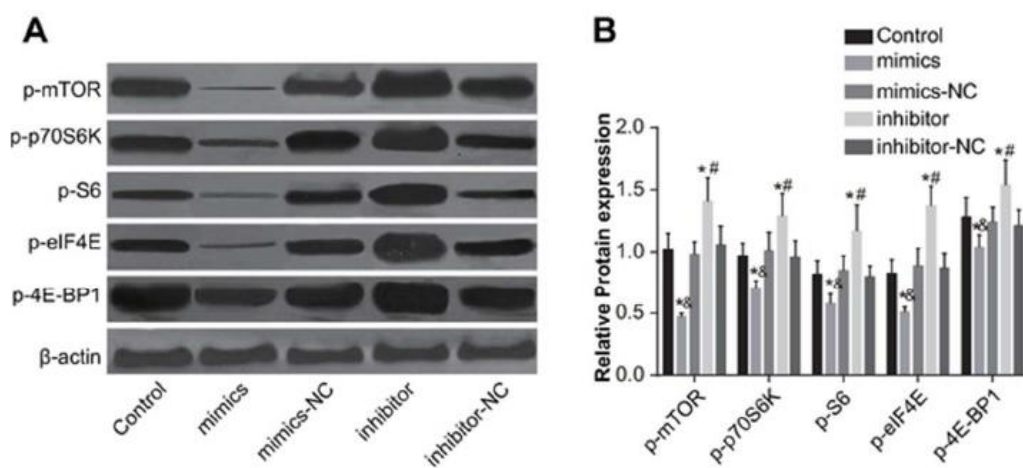


Figure 7