MicroRNA-145 inhibits migration and induces apoptosis in human non-small cell lung cancer cells through regulation of the EGFR/PI3K/AKT signaling pathway

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Abstract. In the present study, the therapeutic effects and the underlying molecular mechanisms of microRNA (miR)-145 were investigated in non-small cell lung cancer (NSCLC) cells. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to examine miR-145 expression. An MTT assay and flow cytometry were used to investigate cell proliferation and apoptosis, respectively. The protein expression of Bax, epidermal growth factor receptor (EGFR), phosphatidylinositol 3-kinase (PI3K) and phosphorylated-protein kinase B (AKT) was examined by western blot analysis. miR-145 expression was downregulated in patients with NSCLC who were treated with chemotherapy. The downregulation of miR-145 in A549 cells reduced lactate dehydrogenase (LDH) expression, apoptosis, caspase-3/-9 levels and Bax protein expression, while it increased cell proliferation. Upregulation of miR-145 in A459 cells increased LDH, apoptosis, caspase-3/-9 levels and Bax protein expression, while it inhibited cell proliferation. The EGFR/PI3K/AKT signaling pathway was suppressed by miR-145 upregulation in A549 cells and induced by miR-145 downregulation. The EGFR inhibitor suppressed the EGFR/PI3K/AKT signaling pathway and increased the anticancer effects of miR-145 upregulation in A549 cells. The PI3K inhibitor suppressed the PI3K/AKT signaling pathway and reversed the anticancer effects of miR-145 upregulation in A549 cells. In conclusion, the present study demonstrated that miR-145 regulates the EGFR/PI3K/AKT signaling pathway in patients with NSCLC.

Introduction

Lung cancer is the primary type of malignant tumor that threatens human health; it accounts for the majority of cancer mortalities worldwide and 80% of cases have non-small cell lung cancer (NSCLC) pathology (1). Although improvements to diagnostic and treatment methods have greatly improved the rate of early diagnosis for NSCLC and provided guidance for personalized treatment, ~66% of patients are diagnosed when the disease has reached an advanced stage (2). Late diagnosis limits the effectiveness of first-line treatment to chemotherapy and the expected survival time for these patients is ~8 months. Research into the molecular phenotypes of lung cancer contribute to more accurate early diagnoses, advanced molecular classification and improved prognosis estimates (3). The molecular network underlying the development of lung cancer has been partially identified at the protein and gene level. Over the past 10 years, gene therapy based on the underlying molecular network of lung cancer development has advanced, however, the overall 5-year mortality rate for patients with lung cancer has not significantly improved (4).

Platinum-based chemotherapy regimens are the first-line treatment against NSCLC; however, the body may rapidly develop resistance to platinum drugs, which is a key obstacle in the clinical treatment of NSCLC (5). Platinum resistance is generally considered to be caused by multiple factors, including resistance-associated genetic changes, reduced drug accumulation, increased drug detoxication, enhanced apoptosis inhibition and DNA repair capacity, which involve multiple signaling pathways and numerous key factors (6). Recently, microRNAs (miRNAs) have been identified as a novel, alternative pathway for research into tumor drug resistance (7). Previous research has indicated that miRNAs are closely associated with the pathogenesis and drug resistance of tumors (7). The upregulation or downregulation of miRNA expression may directly cause the abnormal protein expression of target genes, which ultimately alter drugs for tumor cells through the cellular signaling pathways (8).

Research on the epidermal growth factor receptor (EGFR) signaling pathway tyrosine kinase inhibitor is the most thorough (9). At present, over 90% of all identified NSCLC EGFR gene mutations are located in the exons of chromosomes.

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EGFR is expressed in epithelial, mesenchymal and neurogenic tissues, which serve a vital role in regulating the proliferation, differentiation and growth of healthy cells. In addition, EGFR is closely associated with tumor cell growth, angiogenesis, tumor metastasis and cell apoptosis inhibition (10). A ligand binds with the N-terminal extracellular domain of EGFR to form a homodimer or heterodimer, which phosphorylates the tyrosine residue in the cell, thereby activating the downstream signaling pathways. These signaling pathways include the RAS/RAF/extracellular signal-regulated kinase/mitogen-activated protein kinase pathway, the phosphoinositide 3-kinase/protein kinase B (AKT) pathway and the signal transducer and activator of transcription 3/5 signal transduction pathway (11). Ligand binding ultimately results in a series of abnormal biological behaviors in tumor cells, including excessive proliferation and invasion, metastasis and angiogenesis (12).

Mutations within the EGFR are an important indicator of NSCLC progression within the Asian population (13). EGFR-tyrosine kinase inhibitors, including gefitinib and erlotinib, have a response rate of 60-80% when administered to NSCLC patients with EGFR mutation (14). Gefitinib has recently been demonstrated to be clinically effective in patients with NSCLC and brain metastasis, which indicates that EGFR mutations may lead to brain metastases. Therefore, it is important to investigate the association between EGFR mutations and NSCLC brain metastases (13).

The recent identification of a novel class of non-protein-coding miRNA, with no open reading frame, has confirmed that the genomic complexity of cancer far exceeds original expectations (15). miRNAs are a type of small endogenous non-coding RNA with no open reading frame, which are 20-25 nucleotides in length. They may regulate hundreds of target genes at a post-transcriptional level and therefore participate in numerous biological functions, including regulation of cell differentiation, proliferation and apoptosis (16). As miRNAs are regulatory factors of endogenous gene expression, they are also involved in the pathophysiological processes of a number of diseases through the regulation of multiple genes and their genetic networks (16). Previous studies have revealed that miRNAs may participate in tumor development or regression as either an oncogene or tumor-suppressor gene, respectively (8). Zhang et al revealed that miRNA-145 (miR-145), miR-20a, miR-21 and miR-223 in the plasma, are novel biomarkers for screening of early-stage NSCLC. However, the mechanism of miR-145-regulated cell death in NSCLC remains unknown. The aim of the present study was to evaluate the therapeutic effects and the underlying molecular mechanisms of miR-145 in NSCLC by chemotherapy.

Materials and methods

Cell lines and clinical specimens. The A549 human NSCLC cell line was purchased from the Cancer Research Institute of Hebei Medical University and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% (m/v) fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO₂.

The NSCLC samples (n=6) and normal healthy volunteers (n=6) samples were derived from patients undergoing surgical procedures at the Fourth Hospital of Hebei Medical University (Table I). Peripheral blood (10 ml) of NSCLC samples and normal healthy volunteers samples were centrifuged at 2,000 x g for 10 min at 4°C and serum was saved at -80°C. All human studies were approved by the Ethics Committee of Fourth Hospital of Hebei Medical University. All patients signed written informed consent forms prior to the study.

RNA extraction and quantitative RT-PCR. Total RNAs were isolated using TRIzol reagent (Thermo Fisher Scientific, Inc.). RT reactions were performed using 10 ng of total RNA samples and GeneChip WT (Takara Biomedical Technology Co., Ltd., Beijing, China). Quantitative RT-PCR was performed using the standard TaqMan MicroRNA assay protocol on a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) at 95°C for 15 min, followed by 45 cycles at 94°C for 15 sec, 55°C for 30 sec, and 70°C for 30 sec. The primer sequences were as follows: miR-145 forward, 5'-ATC GTC CAG TTT TCC AAG G-3' and reverse, 5'-CGC CTC CAC ACA CTACC C-3'; U6 forward, 5'-ATT GGA ACG ATAC AAG AGA AGA TT-3' and reverse, 5'-GGA AGC TTC AGC TAC GAAGATT-3' and reverse, 5'-GGA AGC TTC AGC TAC GAAGATT-3'. The fold change of miRNA-145 was calculated using the 2-ΔΔCq equation (17).

Cell transfection. The cells (1x10^5 cells/well) were plated in 6-well plates and transfected with either miR-145 mimics (100 nm) or miR-145 inhibitor (100 nm); both from Sangon Biotech, Co., Ltd., Shanghai, China) or PI3K inhibitor (1,3-dicaffeoylquinic acid; 10 µM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 48 h.

MTT assay and lactic dehydrogenase (LDH) activity. The cells (5x10^3 cells/well) were plated in 96-well plates and MTT (5 mg/ml) was added to the cells for 4 h at 37°C. Subsequently, the MTT solution was removed and DMSO solution was added into the cells for 20 min at 37°C. The absorbance was measured using a multi-well plate reader (Tecan Schweiz AG, Maennedorf, Switzerland) at 492 nm.

LDH activity was assessed using an LDH activity kit (Beyotime Institute of Biotechnology, Shanghai, China). The absorbance was measured using a multi-well plate reader (Tecan Schweiz AG) at 450 nm.

Transwell assays. For the cell migration Transwell assays, 24-well plates with 8.0-µm pore size polycarbonate membranes (5x10^4, 6.5 mm diameter; Corning Inc., Corning, NY, USA) were used. Serum-free medium (100 µl) was seeded into the upper chamber and 600 µl medium containing 10% FBS was added to the bottom chamber. Cell were cultured for 48 h and fixed with 4% paraformaldehyde and stained with 0.1% crystal
Table I. Basic knowledge of patients with NSCLC.

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<th>Variables</th>
<th>Volunteers (n=6)</th>
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NSCLC, non-small cell lung cancer.

Flow cytometry (FCM). The cells (1x10^6 cells/well) were plated in 6-well plates, collected and washed twice with PBS before being suspended with 500 µl of binding buffer (BD Biosciences, Franklin Lakes, NJ, USA). The cells were stained with 5 µl of Annexin V-fluorescein isothiocyanate and 5 µl of propidium iodide (PI) for 15 min at room temperature. A BD FACSCalibur flow cytometer (BD Biosciences) was used to detect cell apoptosis.

Western blot analysis and caspase-3/-9 activity levels. Cell were washed and splitted using RIPA lysis buffer (Beyotime Institute of Biotechnology) after centrifugation at 10,000 x g for 10 min at 4˚C. The protein concentration was calculated with a Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). Protein (50 µg) was separated on 8-10% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk at room temperature for 1 h and immunostained with primary antibodies: Bax (1:500; cat. no. sc-6236), EGFR (1:500; sc-71034), PI3K (1:500; cat. no. sc-7174), p-AKT (Ser473; 1:500; cat. no. sc-7985-R) and GAPDH (1:2,000; cat. no. sc-25778; all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C overnight. The membranes were then washed three times in TBST, and then incubated with anti-rabbit secondary antibody (1:2,000; cat. no. sc-2030; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. Subsequently, the membranes were detected with ECL Plus (GE Healthcare, Piscataway, NJ, USA) and quantitatively determined via densitometry with ImageJ software 2.1.4.7 (imagej.nih.gov/).

Protein (10 µg) was used to assess caspase-3 and caspase-9 activity levels using caspase-3/-9 activity kits (Beyotime Institute of Biotechnology). The absorbance was measured using a multi-well plate reader (Tecan Schweiz AG) at 405 nm.

Statistical analysis. The results were expressed as the mean ± standard deviation (n=3). Statistical significance of the results between each group was evaluated using one-way ANOVA followed by Tukey’s post test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-145 expression. Initially gene chip technology was used to measure the expression of miRNAs. It was observed that miR-145 expression was downregulated in the serum of patients with NSCLC compared with normal healthy volunteers (Fig. 1A and B). In addition, miR-145 expression was assessed using RT-PCR. The results revealed that miR-145 expression was downregulated in NSCLC patients compared to normal healthy volunteers (Fig. 1C).

Effect of miR-145 downregulation on cell growth in A549 cells. To confirm the potential role of miR-145 in patients with NSCLC, a miR-145 inhibitor was used to inhibit the expression of miR-145 in A549 cells (Fig. 2A). The miR-145 inhibitor significantly increased cell proliferation (Fig. 2B) and migration (Fig. 2F and G), and reduced LDH activity (Fig. 2C) and apoptosis (Fig. 2D and E) in A549 cells compared with the control group.

Effect of miR-145 downregulation on caspase-3/-9 activity levels and Bax protein expression in A549 cells. miR-145 downregulation resulted in significant inhibition of caspase-3/-9 activity levels and Bax protein expression in A549 cells compared with the control group (Fig. 3).

Effect of miR-145 upregulation on cell growth in A549 cells. miR-145 mimics were used to verify the effect of miR-145 upregulation on cell growth in A549 cells. The miR-145 mimics increased the expression of miR-145 in A549 cells (Fig. 4A). The miR-145 mimics also significantly inhibited cell proliferation (Fig. 4B) and migration (Fig. 4F and G) and induced LDH activity (Fig. 4C) and apoptosis (Fig. 4D and E) in A549 cells.

Effect of miR-145 upregulation on caspase-3/-9 activity levels and Bax protein expression in A549 cells. Caspase-3/-9 levels and Bax protein expression are common indicators of the apoptosis signaling pathway. It was analyzed whether miR-145 upregulation altered caspase-3/-9 activity levels and Bax protein expression in A549 cells. miR-145 upregulation caused a significant promotion in caspase-3/-9 activity levels and Bax protein expression in A549 cells compared with the control group (Fig. 5).

Effects of miR-145 on the EGFR/PI3K/AKT signaling pathway in A549 cells. The underlying mechanism of miR-145 on apoptosis was explored in A549 cells. Gene chip technology revealed that EGFR and PI3K expression were downregulated in A549 cells compared with the control group when miR-145 was overexpressed (Fig. 6A). In addition, miR-145 downregulation significantly induced the expression of miR-145 in A549 cells.
signaling pathway in A549 cells compared with the control group (Fig. 6B-E). The EGFR/PI3K/AKT signaling pathway was significantly suppressed in A549 cells when miR-145 was upregulated, compared with the control group (Fig. 7). These results further demonstrated that miR-145 may serve an important role in A549 cells in vitro.

**EGFR inhibitor suppresses the EGFR/PI3K/AKT signaling pathway in A549 cells following miR-145 upregulation.** The effect of EGFR on the EGFR/PI3K/AKT signaling pathway was investigated in A549 cells following miR-145 upregulation. The EGFR/PI3K/AKT signaling pathway was significantly suppressed in A549 cells treated with EGFR inhibitor (0.5 nM, 48 h; cat. no. AEE788) following miR-145 upregulation, compared with the miR-145 upregulation group (Fig. 8).

**EGFR inhibitor suppresses cell growth in A549 cells following miR-145 upregulation.** Significant inhibition of cell proliferation and migration was observed, as well as increases in LDH activity and apoptosis in A549 cells following treatment with miR-145 and the EGFR inhibitor, compared with the miR-145 upregulation group (Fig. 9).

**EGFR inhibitor suppresses caspase-3/9 activity levels and Bax protein expression in A549 cells following miR-145 upregulation.** A significant promotion in caspase-3/9 activity levels and Bax protein expression was observed in A549 cells following treatment with miR-145 and the EGFR inhibitor, compared with the miR-145 upregulation group (Fig. 10). These results indicated that EGFR is an important factor, which may enhance the effects of miR-145 on NSCLC chemotherapy.
Figure 3. Downregulation of miR-145 on caspase-3/-9 activity levels and Bax protein expression in A549 cells. Bax protein expression was determined using (A) western blot analysis and the results were (B) statistically analyzed. (C and D) Caspase-3/-9 activity levels in A549 cells. The results are expressed as the mean ± standard deviation (n=3). ##P<0.01 vs. the control group. miR, microRNA.

Figure 4. Upregulation of miR-145 on cell growth of A549 cells. (A) miR-145 expression, (B) cell proliferation, (C) LDH activity, (D and E) apoptosis and (F and G) migration in A549 cells. The results are expressed as the mean ± standard deviation (n=3). ##P<0.01 vs. the control group. miR, microRNA; LDH, lactate dehydrogenase.

Figure 5. Upregulation of miR-145 on caspase-3/-9 activity levels and Bax protein expression in A549 cells. Bax protein expression was determined using (A) western blot analysis and the results were (B) statistically analyzed. (C and D) Caspase-3/-9 activity levels in A549 cells. The results are expressed as the mean ± standard deviation (n=3). ##P<0.01 vs. the control group. miR, microRNA.
Figure 6. Downregulation of miR-145 on the EGFR/PI3K/AKT signaling pathway in A549 cells. (A) Common signaling pathways were evaluated using gene chip technology. (B) Western blot analysis was performed to determine (C) EGFR, (D) PI3K and (E) p-AKT protein expression in A549 cells. The results are expressed as the mean ± standard deviation (n=3). *P<0.01 vs. the control group. miR, microRNA; EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol 3-kinase; p-AKT, phosphorylated-protein kinase B.

Figure 7. Upregulation of miR-145 on the EGFR/PI3K/AKT signaling pathway in A549 cells. (A) Western blot analysis was performed to determine (B) EGFR, (C) PI3K and (D) p-AKT protein expression in A549 cells. The results are expressed as the mean ± standard deviation (n=3). **P<0.01 vs. the control group. miR, microRNA; EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol 3-kinase; p-AKT, phosphorylated-protein kinase B.

Figure 8. EGFR inhibitor suppresses the EGFR/PI3K/AKT signaling pathway in A549 cells following miR-145 upregulation. (A) Western blot analysis was performed to determine (B) EGFR, (C) PI3K and (D) p-AKT protein expression in A549 cells. The results are expressed as the mean ± standard deviation (n=3). ***P<0.01 vs. the control group. **P<0.01 vs. the miR-145-mimic group. miR, microRNA; EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol 3-kinase; p-AKT, phosphorylated-protein kinase B.
PI3K inhibitor suppresses the PI3K/AKT signaling pathway in A549 cells following miR-145 upregulation. The function of PI3K and the effect of miR-145 on the EGFR/PI3K/AKT signaling pathway was investigated in A549 cells. The PI3K inhibitor (1,3-dicaffeoylquinic acid; 10 µM, 48 h) significantly suppressed the PI3K/AKT signaling pathway in A549 cells following miR-145 upregulation, compared with the miR-145 upregulation group (Fig. 11).

PI3K inhibitor suppresses cell growth in A549 cells following miR-145 upregulation. The inhibition of PI3K significantly inhibited cell proliferation and migration, while it increased LDH activity and apoptosis in A549 cells following miR-145 upregulation, compared with the miR-145 upregulation group (Fig. 12).

PI3K inhibitor suppresses caspase-3/-9 activity levels and Bax protein expression in A549 cells following miR-145 upregulation. The inhibition of PI3K significantly increased the effects of miR-145 on caspase-3/-9 activity levels and Bax protein expression in A549 cells, compared with the miR-145 upregulation group (Fig. 13). The aforementioned results indicated that the effects of miR-145 occur through the inhibition of the PI3K/AKT signaling pathway in NSCLC.
At present, the principal methods of lung cancer classification and staging used within a clinical setting are primarily based on imaging diagnoses, and morphological changes in tissues and cells observed under a microscope (18). However, these diagnostic methods provide limited information and are insufficient for an accurate and in depth understanding of the molecular changes taking place during tumor genesis and development (1). Therefore, research into the molecular classification of lung cancer may contribute to more targeted treatments and accurate prognosis (4). The present study revealed that miR-145 expression was downregulated in patients with NSCLC compared with normal healthy volunteers, however there were only a total of six individuals in each group, which is a small sample size and represents a limitation of the study. Future studies should analyze data from larger groups to increase the validity of the results. The results of the present study demonstrated that the downregulation of miR-145 in A549 cells reduced LDH and apoptosis, increased cell proliferation, and inhibited caspase-3/-9 levels and Bax protein expression. Zhu et al (19) revealed that miR-122,
miR-145 and let-7b were underexpressed in castration-resistant prostate cancer. However, the present study only used A549 cells, which represents another limitation and in future studies it is recommended that additional cell lines are used.

In the majority of advanced NSCLC cases, the tumor pathology and EGFR status are determined using the primary tumor in the lung as opposed to the brain metastasis, as it is far easier to access and obtain (20). However, certain patients receive craniocerebral surgical resection or biopsy following the initial symptom of brain metastasis, which is able to positively confirm the diagnosis of NSCLC (21). When this occurs the brain metastasis sample may be utilized for EGFR detection. If there is consistency in the EGFR expression between the primary lesion and the brain metastasis, as determined by either diagnostic method, targeted therapies may be applied based on the results (22,23). Therefore, the authors consider that miR-145 upregulation suppressed the EGFR/PI3K/AKT signaling pathway in A549 cells. Cheng et al demonstrated that miRNA-145 downregulates mucin 5AC to alleviate airway remodeling through EGFR expression (24). p-EGFR may also participate in the effects of miR-145 in A549 cells, however, only EGFR protein expression was analyzed in present study. Further studies should analyze the function of p-EGFR protein expression and the effect of miR-145 on its expression in A549 cells.

The PI3K/AKT signaling pathway has been known for over 10 years. When PI3K is phosphorylated it triggers the production of the secondary messenger phosphatidylinositol trisphosphate (PIP3) on the plasma membrane (12). PIP3 then binds with a domain in the N-terminal of AKT, while AKT translocates from the cytoplasm to the cell membrane. Activated AKT either activates or inhibits downstream target proteins through their phosphorylation and thereby regulates cell proliferation, differentiation, apoptosis and migration (25). The results of the present study demonstrated that the EGFR inhibitor suppressed the EGFR/PI3K/AKT signaling pathway and increased the anticancer effects of miR-145 upregulation in A549 cells. Zhang et al (26) reported that synthetic miR-145 expression inhibits multiple myeloma cell growth through the PI3K/AKT signaling pathway. The present study only investigated the effect of the EGFR inhibitor on miR-145 upregulation in A549 cells, therefore future studies should also explore the effect of miR-145 inhibitors combined with EGFR inhibitors in A549 cells.

All members of the PI3K family are oncogenes, which are important kinases of inositol and phosphatidylinositol (27). PI3K consists of a regulatory subunit p85 and a catalytic subunit p110, which promotes the phosphorylation of the 3'hydroxyl on the inositol ring (28). AKT is a serine/threonine protein kinase with a molecular weight of 57 kDa; it is a homologue of the viral AKT oncogene in mammals (27). Activated AKT influences the active state of multiple downstream effector molecules, however these effects only occur after the PI3K/AKT signaling pathway is activated (27). AKT may inhibit cell apoptosis and activate effector molecules, including Bad, caspase-9, FKHR1 and nuclear factor-κB. AKT also participates in cell cycle regulation (it is clarified at present that AKT upregulates c-myc expression by increasing its transcription), promotes tumor angiogenesis (AKT may activate nitric oxide synthase and thereby stimulates the growth and proliferation of endothelial cells, increases vascular permeability and promotes angiogenesis following angiectasis, which provides sufficient nutrition for tumor cells) and enhances cell invasion and metastasis (11,29). In addition, it was revealed that the PI3K inhibitor suppresses the PI3K/AKT signaling pathway and reversed the anticancer effects of miR-145 upregulation in A549 cells. Boufraqech et al suggest that miR-145 suppresses thyroid cancer growth and metastasis through AKT3 expression (30). The PI3K/AKT signaling pathway regulates a number of anticancer signaling pathways, including mammalian target of rapamycin, glycogen synthase kinase-3 and Bad (31). Future studies should investigate the wide variety of anticancer signaling pathways that may be affected by miR-145 in NSCLC.

In summary, the present study revealed the potential role of miR-145 in patients with NSCLC. It was also demonstrated that the EGFR/PI3K/AKT signaling pathway was inhibited...
by miR-145, which ultimately inhibited tumor development. miR-145 has been revealed as a novel potential therapy for the targeted treatment of NSCLC.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors’ contributions

BL designed the experiment; CMD, YXL, JCP, NG and WWQ performed the experiment; BL and CMD analyzed the data; BL wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All human studies were approved by the Ethics Committee of the Ethics Committee of Fourth Hospital of Hebei Medical University.

Patient consent for publication

All patients signed written informed consent forms prior to the study.

Competing interests

The authors declare that they have no competing interests.

References


