Epidermal growth factor receptor expression affects proliferation and apoptosis in non-small cell lung cancer cells via the extracellular signal-regulated kinase/microRNA 200a signaling pathway

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Abstract. The present study assessed the function of epidermal growth factor receptor (EGFR) and its molecular targets in non-small cell lung cancer. The results of the present study demonstrated that EGFR protein and mRNA expression in the normal adjacent tissue specimens was decreased compared with that in the lung cancer tissue samples. Compared with the BEAS-2B normal bronchial epithelial cells, EGFR and phosphorylated (p)-extracellular signal-regulated kinase (ERK) protein expression in the SW-900 and A549 lung cancer cells was increased and microRNA (miR)200a expression in the SW-900 and A549 cells was inhibited compared with the BEAS-2B cells. Downregulating miR200a expression significantly suppressed proliferation and promoted apoptosis and caspase (CASP)3 and CASP9 function in the A549 cells and significantly inhibited EGFR and p-ERK protein expression in the A549 cells, compared with the BEAS-2B cells. The results of the present study indicated that downregulating miR200a significantly suppressed proliferation and promoted apoptosis in A549 cells via the regulation of the EGFR and ERK 1/2 signaling pathways.

Introduction

Lung cancer is a malignant tumor that threatens human health and life, and exhibited the highest global occurrence rate and lethality among all types of cancer in 2012 (1). Its morbidity is increasing in multiple countries (1). At present, the treatment options for patients with lung cancer include surgery, radiotherapy, and chemotherapy. In non-small cell lung cancer (NSCLC), the typical first choice of therapy is operative treatment but, once NSCLC has been definitively diagnosed, surgery is not possible in 75% of patients since lesions have already developed, or the state of their health following surgery would potentially be poorer compared with that prior to surgery (2). Therefore, the patient may opt for chemotherapy. Though chemotherapy may decrease the recurrence rate of NSCLC in patients, only ~30% of patients with NSCLC undergo effective treatment with platinum-based first-line chemotherapy, and chemotherapy resistance is common (3). Furthermore, the 5-year survival rate for patients with NSCLC is only 15% (4). Previous research has indicated that, although novel chemotherapeutics have improved the curative effect in patients with transfer NSCLC, the associated median survival time remains only 8-9 months (5). Therefore, it is crucial to understand the occurrence and development of lung cancer, identify the molecular mechanisms underlying metastasis, and develop effective treatments for patients with malignant lung tumors.

The epidermal growth factor receptor (EGFR) family, also known as the Erb-b2 receptor tyrosine kinases (ERBB), includes four main members: EGFR, ERBB2, ERBB3 and ERBB4. The EGFR family members are allosteric enzymes located at the cell surface (3). The members may be divided into three areas: A ligand-coupling domain that protrudes out of the cytomembrane, an intramembrane tyrosine kinase-activated functional domain and a transmembrane domain. The EGFR family is associated with tumorigenesis and tumor development (6). The coding products of the EGFR family exhibit phosphatase functions, and participate in cytoskeletal protein recombination, thereby facilitating signal transduction, proliferation, apoptosis regulation and malignant transformation in cells (7). Increased EGFR expression has been reported in multiple tumors, including colon, esophageal, breast, lung, ovarian, cervical and pancreatic cancer, and glioma (8). Abnormal EGFR expression is associated with malignant cell proliferation, adhesion, vascularization, metastasis and radiosensitivity (8). EGFR represents one of the most promising therapeutic targets in the study of antineoplastic molecules (9). In previous years, anti-EGFR molecular targeted drugs have received increasing attention (10). A series

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of antineoplastic drugs targeting EGFR have previously been studied. Cetuximab and gefitinib have undergone clinical trials and acquired improved curative effects (10,11). There are >10 EGFR-targeting inhibitors, and they have acquired antineoplastic curative effects by being used to treat different types of human tumor (2). Therefore, the present study evaluated the function of EGFR and its molecular targets in NSCLC.

Materials and methods

Lung cancer specimens. From July 2015 to January 2016, 12 patients (male, 56.5±6.2 years age) with lung cancer who underwent surgery were enrolled in the present study at the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China). Normal adjacent tissues specimens were used as controls. The study was approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University and all patients provided written informed consent.

Immunohistochemical analysis. Cancer tissue samples were fixed with 4% paraformaldehyde for 24 h at room temperature, paraffin-embedded, and tumor tissue was cut into 4-μm-thick tissue samples. The sections were deparaffinized, then washed with PBS, and immersed in 3% hydrogen peroxide to block endogenous peroxidase activity at room temperature for 10 min. Sections were incubated with anti-ERGF (cat. no. sc-367974; 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature. Sections were incubated with goat anti-rabbit IgG, horseradish peroxidase-conjugated secondary antibody (cat. no. 7074; 1:500; Cell Signaling Technology, Inc.) for 1 h at room temperature. Tissue samples were observed with a Nikon E200 light microscope (Nikon Corporation, Tokyo, Japan) at magnification, x20.

Cell culture. The bronchial epithelioid cell line BEAS-2B, and the human lung cancer SW-900 and A549 cell lines were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 50 U/ml penicillin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 50 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA) at 37˚C in a humidified atmosphere of 5% CO_2.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the lung cancer tissue samples or BEAS-2B, SW-900 and A549 cell lines using TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RT was performed at 94˚C for 5 min and 42˚C for 30 min with the PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). The RT-qPCR exponential phase occurred for 40 cycles to permit quantitative comparison of the complementary DNAs amplified from identical reactions using SYBR Premix Taq (Takara Biotechnology Co., Ltd.). The primer sequences used were as follows: EGFR forward, 5'-TGGAGCTACGGGGTGACCGT-3' and reverse, 5'-GGTTCAGAGGCTGATTGTGAT-3'; GAPDH forward, 5'-ACCTGACCTGCCGTCTAGAA-3' and reverse, 5'-TCC ACCACCCTGTTGCTGTA-3'; microRNA (miR)200a forward, 5'-GCTCACCCTTGCAGGTCTCC-3' and reverse, 5'-CCC GAAACCCAGCCGCATC-3'; U6 forward, 5'-CGCTTCGGC AGCACATATACTA-3' and reverse, 5'-CGCTTCACGAAT TTGCGTGTCA-3'. Each cycle was as follows: 94˚C for 5 min, followed by 30 cycles of 94˚C for 10 sec, 60˚C for 30 sec and 72˚C for 30 sec. Data analysis was performed using the $2^{-\Delta\Delta Cq}$ method (12). Experiments were repeated in triplicate.

miR transfection. si-miR200a (5'-ACAUCGUUACCAGACAGU GUUA-3') and miR-negative control (NC; 5'-CAGAUUUUG UGUAGUACAA-3') were designed and synthesized by Zimmer Company (Shanghai, China). Anti-miR200a (100 ng) and miR-NC (100 ng) were transfected into the A549 cells ($1x10^5$) using Lipofectamine 2000® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 6 h in DMEM, and then the medium was changed to DMEM, according to the manufacturer's protocol.

MTT assay. Following transfection with anti-miR200a and miR-NC for 48 h, A549 cells ($1x10^3$) were seeded onto a 96-well plate and cultured in DMEM. Subsequently, 20 μl MTT (5 μg/ml) was added to each well and the cells were cultured for 4 h at 37˚C. The medium was then discarded and 150 μl dimethyl sulfoxide was added (Invitrogen; Thermo Fisher Scientific, Inc.). Absorbance was measured using a multi-well spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) at 490 nm.

Flow cytometry analysis and apoptosis assay. Following transfection with anti-miR200a and miR-NC for 48 h, the A549 cells ($1x10^6$) were seeded onto a 6-well plate and cultured in DMEM. An Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection kit (BD Biosciences, San Jose, CA, USA) was used according to the manufacturer's protocol to detect apoptosis. Annexin V-FITC (10 μl) was added to the cells for 15 min. Subsequently, a further 5 μl Annexin V-FITC was added to the cells for 5 min. The apoptosis rate was assessed using a FACSCalibur™ flow cytometer (BD Biosciences) Data was analyzed using FlowJo 7.6.1 (FlowJo LLC, Ashland, OR, USA).

Analysis of caspase (CASP)3/9 activity. Following transfection with anti-miR200a and miR-NC for 48 h, the A549 cells were seeded onto a 6-well plate ($1x10^6$) and cultured in DMEM. Ac-DEVD-pNA from a Caspase 3 Activity Assay kit, or Ac-LEHD-pNA from a Caspase 9 Activity Assay kit (both from Beyotime Institute of Biotechnology, Haimen, China) were added to each well and the cells were cultured for 1 h at 37˚C. CASP3/9 activity was measured using a multi-well spectrophotometer (BioTek Instruments, Inc.) at 405 nm.

Western blot analysis. Following transfection with anti-miR200a and miR-NC for 48 h, the A549 cells were seeded onto a 6-well plate. Total protein from the A549 cells was subsequently extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with protease inhibitors (Sigma-Aldrich; Merck KGaA). Protein content was quantified using the bicinchoninic acid method (Beyotime Institute of Biotechnology). Protein (50 μg/lane) was fractionated using SDS-PAGE on a 10% gel and subsequently transferred to polyvinylidene fluoride (PVDF) membranes

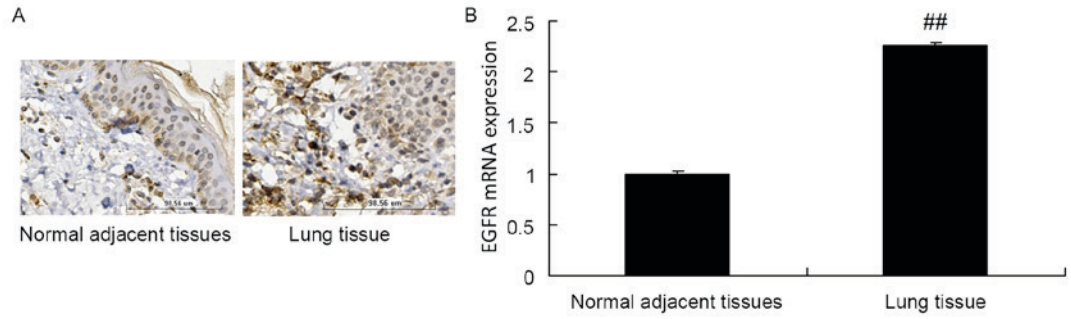

Figure 1. EGFR expression is upregulated in A549 cells. (A) EGFR protein expression in lung cancer and normal adjacent tissue specimens, as assessed using immunohistochemical analysis. Magnification, x10. (B) EGFR mRNA expression in lung cancer and normal adjacent tissue specimens, as assessed using reverse transcription-quantitative polymerase chain reaction. ##P<0.01 vs. normal adjacent tissue specimens. EGFR, epidermal growth factor receptor.

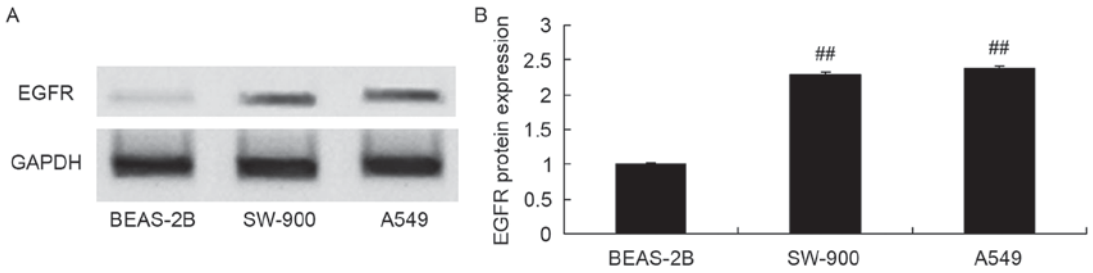

Figure 2. Expression of EGFR protein in A549 cells. EGFR protein expression, as assessed using (A) western blot analysis and (B) statistical analysis of western blotting results. ##P<0.01 vs. the BEAS-2B cell group. EGFR, epidermal growth factor receptor.

(EMD Millipore, Billerica, MA, USA). The PVDF membranes were blocked using 0.5% non-fat milk in TBS containing 0.1% Tween-20 at 37˚C for 1 h. The membranes were incubated with anti-ERGF (cat. no. sc-367974; 1:500, Santa Cruz Biotechnology, Inc.), anti-phosphorylated (p)-extracellular signal-regulated kinase (ERK; cat. no. sc-23759-R; 1:500, Santa Cruz Biotechnology, Inc.) and anti-GAPDH (sc-25778, 1:2,000, Santa Cruz Biotechnology, Inc.) antibodies overnight at 4˚C. The membrane was washed three times for 10 min each, with TBS containing 0.1% Tween at room temperature and incubated with goat anti-rabbit IgG, horseradish peroxidase-conjugated secondary antibody (cat. no. 7074; 1:5,000, Cell Signaling Technology, Inc.) for 1 h at room temperature. Protein expression was visualized using an enhanced chemiluminescent kit (GE Healthcare, Chicago, IL, USA) and analyzed using Bio-Rad Laboratories Quantity One software 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data were presented as the mean ± standard error using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Statistical analysis was performed using the Student's t-test or, when comparing multiple groups, one-way analysis of variance with Bonferroni's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

EGFR expression is upregulated in lung cancer tissue. The protein expression of EGFR in normal adjacent tissue specimens was decreased compared with that in lung cancer tissue samples (Fig. 1A). Furthermore, the RT-qPCR results of the present study revealed that the mRNA expression of EGFR in normal adjacent tissue specimens was decreased compared with that in lung cancer tissue samples (Fig. 1B).

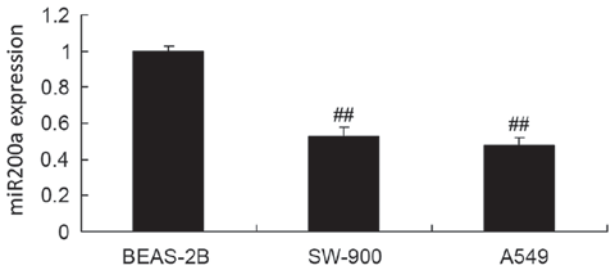

Figure 3. Expression of miR200a in A549 cells. ##P<0.01 vs. the BEAS-2B cell group. miR, microRNA.

Expression of EGFR protein in A549 cells. EGFR protein expression was measured in the BEAS-2B, SW-900 and A549 cell lines using western blot analysis. EGFR protein expression in the lung cancer cell lines SW-900 and A549 was increased compared with that in the bronchial epithelioid cell line BEAS-2B (Fig. 2).

Expression of miR200a in A549 cells. RT-qPCR was used to assess miR200a expression in the BEAS-2B, SW-900 and A549 cell lines. The expression of miR200a in the BEAS-2B cells was significantly decreased compared with that in the SW-900 and A549 cells (Fig. 3).

Expression of ERK protein in A549 cells. p-ERK protein expression was measured in the BEAS-2B, SW-900 and A549 cells using western blot analysis. Protein expression of

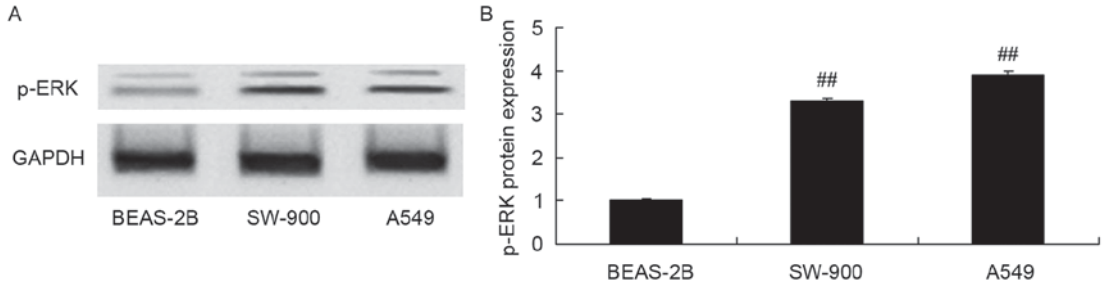

Figure 4. Expression of ERK protein in A549 cells. p-ERK protein expression, as assessed using (A) western blot analysis and (B) statistical analysis of western blotting results. ##P<0.01 vs. the BEAS-2B cell group. ERK, extracellular signal-regulated kinase; p-, phosphorylated.

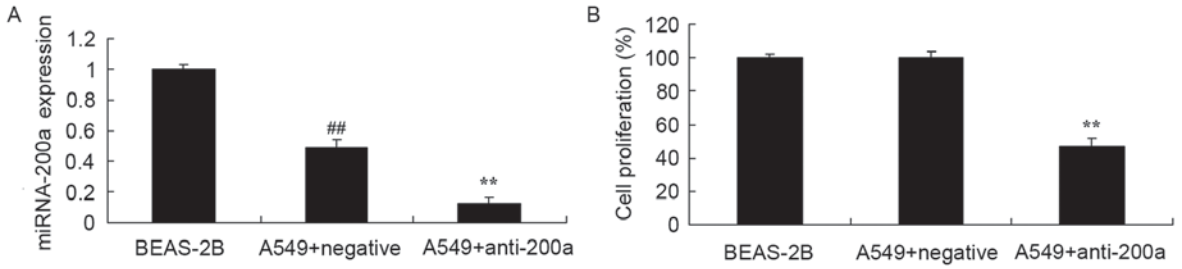

Figure 5. Effect of downregulating miR200a expression on the proliferation of A549 cells. Effect of downregulating miR200a on (A) miR200a expression and (B) the proliferation of A549 cells. ##P<0.01 vs. the BEAS-2B cell group; **P<0.01 vs. the miR-negative control-transfected A549 cells. miR/miRNA, microRNA.

p-ERK in SW-900 and A549 cells was significantly increased compared with that in BEAS-2B cells (Fig. 4).

Effect of downregulating miR200a expression on A549 cell proliferation. To evaluate the effect of downregulating miR200a expression on A549 cell proliferation, anti-miR200a and miR-NC were transfected into A549 cells. miR200a expression in A549 cells was significantly decreased compared with that in BEAS-2B cells, and miR200a expression in A549 cells transfected with anti-miR200a was significantly suppressed compared with that in A549 cells transfected with miR-NC (Fig. 5A). Proliferation was significantly suppressed in A549 cells in which miR200a was downregulated compared with that in miR-NC-treated A549 cells (Fig. 5B).

Effect of downregulating miR200a expression on A549 cell apoptosis. Flow cytometry analysis was used to assess the effect of downregulating miR200a on the apoptosis of A549 cells. The apoptosis rate of A549 cells in which miR200a was downregulated was significantly promoted compared with that of miR-NC-treated A549 cells (Fig. 6).

Effect of downregulating miR200a expression on CASP3/9 activity in A549 cells. The present study evaluated the effect of downregulating miR200a expression on CASP3/9 activity in A549 cells. CASP3 and CASP9 activity was significantly increased in A549 cells treated with anti-miR200a compared with that in miR-NC-treated A549 cells (Fig. 7).

Effect of downregulating miR200a expression on EGFR protein expression in A549 cells. The present study assessed the effect of downregulating miR200a expression on the protein expression of EGFR in A549 cells. EGFR protein expression

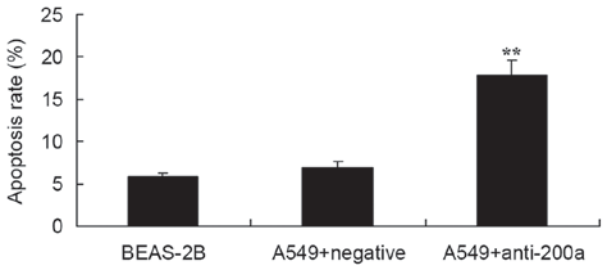

Figure 6. Effect of downregulating miR200a expression on the apoptosis of A549 cells. **P<0.01 vs. the miR-negative control-transfected A549 cells. miR, microRNA.

was significantly inhibited in A549 cells in which miR200a was downregulated compared with that in miR-NC-treated A549 cells (Fig. 8).

Effect of downregulating miR200a expression on the protein expression of ERK in A549 cells. To assess the effect of downregulating miR200a expression on the protein expression of ERK in A549 cells, p-ERK protein expression in SW-900 and A549 cells was measured using western blot analysis. The protein expression of p-ERK was significantly suppressed in A549 cells in which miR200a was downregulated compared with that in miR-NC-treated A549 cells (Fig. 9).

Discussion

Lung cancer was the most common malignant tumor globally in 2012 (13). There are ~1.8 million incident cases (14), and lung cancer-associated mortality occurred in >1.5 million people globally, with the majority of these mortalities being

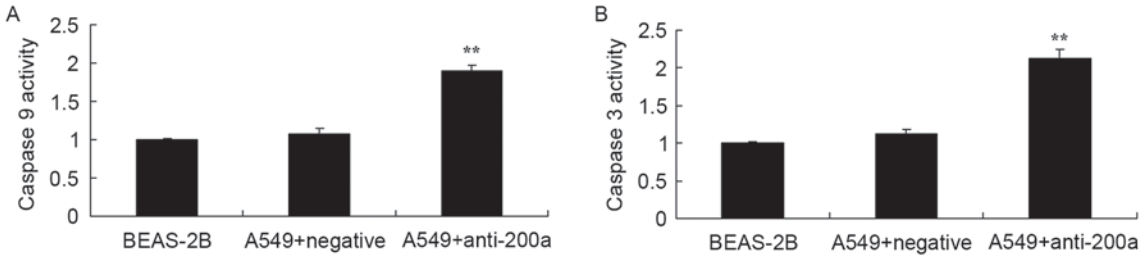

Figure 7. Effect of downregulating miR200a expression on CASP3/9 activity in A549 cells. (A) CASP3 and (B) CASP9 activity in A549 cells. **P<0.01 vs. the miR-negative control-transfected A549 cells. miR, microRNA; CASP, caspase.

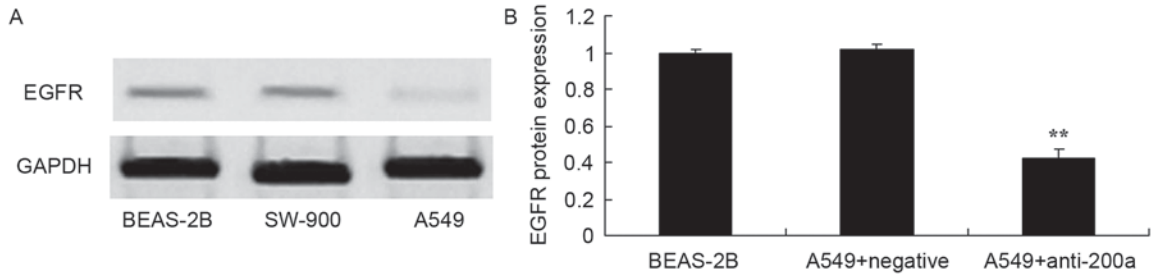

Figure 8. Effect of downregulating miR200a expression on the protein expression of EGFR in A549 cells. EGFR protein expression, as assessed using (A) western blot analysis and (B) statistical analysis. **P<0.01 vs. the miR-negative control-transfected A549 cells. miR, microRNA; EGFR, epidermal growth factor receptor.

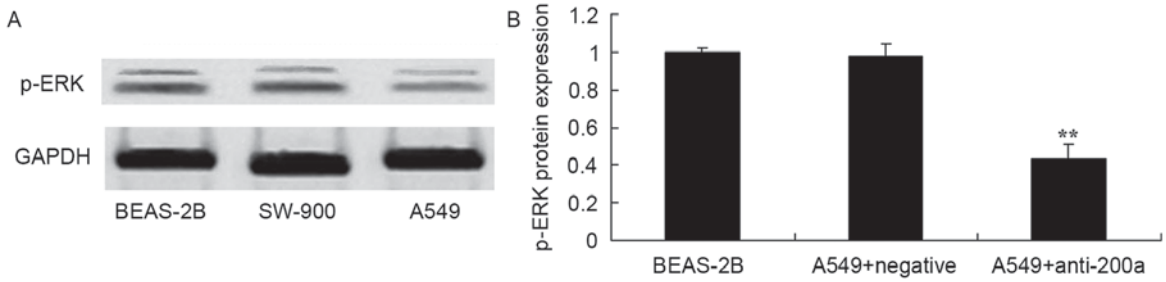

Figure 9. Effect of downregulating miR200a expression on the protein expression of ERK in A549 cells. p-ERK protein expression, as assessed using (A) western blot analysis and (B) statistical analysis. **P<0.01 vs. the miR-negative control-transfected A549 cells. miR, microRNA; ERK, extracellular signal-regulated kinase; p-, phosphorylated.

male. NSCLC accounts for >80% of patients with lung cancer. Numerous patients exhibit NSCLC in the middle or advanced stage at the time of diagnosis. By this stage, the 5-year survival rate has decreased (13). Therefore, diagnosing NSCLC early is crucial. At present, imagological examination is a major diagnostic tool in oncology, but increased expense and risks of radiation exposure, among other factors, render it inappropriate for large-scale screening, so it is crucial to be able to accurately screen using inspection, decreased expenses and trauma (15). The importance of miRs has become apparent in oncology (16). Furthermore, as the molecular diagnosis marker of NSCLC, EGFR has received increased attention (15). The present study revealed that mRNA expression of EGFR in normal adjacent tissue specimens was decreased compared with that in lung cancer tissue samples.

miRs are single-stranded, highly conserved, noncoding nucleotide molecules that are 19-25 nt in length. By degrading or inhibiting mRNA targets, miRs participate in multiple important biological processes, including growth, differentiation, proliferation, apoptosis, hormone secretion and neoplasia in animal and plant cells (14). miRs are also associated with sensitivity to tumor drugs. By downregulating miR200C expression, sensitivity to adriamycin is decreased in breast cancer cells (17). In acute promyelocytic leukemia, increased expression of miR125b is involved in the action of current therapeutics (18). In colorectal cancer, upregulating miR224 may increase resistance to methylamine (19). A previous study revealed that NSCLC tissues often possess a deficiency of mixed miR128b (20). miR128B may regulate EGFR gene and protein expression (20). The present study demonstrated that the expression of miR200a in BEAS-2B cells was increased compared with that in SW-900 and A549 cells.

EGFR has attracted attention since it was discovered in 1962 (8). EGFR may combine with ligands to phosphorylate the isogeneous second area of the SRC proto-oncogene protein, induce normal mitosis, and promote homeostasis, cell differentiation and migration; abnormal expression is associated with the proliferation, adhesion, vascularization and

metastasis of malignant cells (21). EGFR has gained attention in the study of antineoplastic molecular targeted therapy. The present study demonstrated that downregulating miR200a significantly inhibited EGFR protein expression in A549 cells. Zhen *et al* (22) reported that overexpressing miR200a significantly downregulated EGFR expression in NSCLC cells.

ERK is a protein kinase that is located in the terminal position in the signaling pathway of mitogen-activated protein kinase (MAPK), and forms p-ERK via phosphorylation (23). p-ERK may influence the transcriptional expression of associated genes, is associated with cell proliferation and serves a crucial function in malignant transformation. Among the multiple components of the MAPK signaling pathway, ERK is crucial (24). ERK helps to regulate cell proliferation and serves a function in multiple physiological and pathological processes, including cell differentiation, period circular regulation and intercellular functional synchronization (24). A previous study revealed that the ERK1/2 signaling pathway is activated in NSCLC; ERK1/2 exhibited increased expression and phosphorylation (25). On entering the nucleus, p-ERK1/2 affects the expression of jun proto-oncogene, nuclear factor κB subunit 1, ELK1, fos proto-oncogene, MYC proto-oncogene, and transcription factors, phosphorylates multiple substrates of nuclear transcription factors, regulates the transcription of associated genes, and serves a key function in malignant transformation (26). The results of the present study suggested that downregulating miR200a significantly suppressed p-ERK protein expression in A549 cells. Liu *et al* (27) demonstrated that miR200a expression was associated with the neurotrophic receptor tyrosine kinase 2/ERK/protein kinase B signaling pathway in mice exposed to chronic, unpredictable, moderate stress.

To conclude, the present study suggested that downregulating miR200a significantly suppressed proliferation and promoted apoptosis in A549 cells *in vitro*, partly through the regulation of the EGFR and ERK1/2 signaling pathways, and thereby may facilitate the development of aggressive tumors. The results of the present study suggested that miR200a/EGFR/ERK1/2-based prevention and therapeutics in patients with NSCLC may prove clinically beneficial.

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