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miR-16 regulates proliferation and invasion of lung cancer cells via the ERK/MAPK signaling pathway by targeted inhibition of MAPK kinase 1 (MEK1)

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Abstract

Objective: The ERK/MAPK signaling pathway regulates cell proliferation and invasion. MAPK kinase 1 (MEK1) is a protein kinase upstream of ERK that can activate the pathway. Expression of microRNA (miR)-16 in lung cancer tissues is decreased. The aim of this study was to determine roles of miR-16 in proliferation and invasion of lung cancer cells.

Methods: We used a luciferase reporter assay to determine a regulatory relationship between miR-16 and MEK1 and assessed expression of MEK1 in normal lung cells and lung cancer cell lines. Plate cloning, flow cytometry, and Transwell experiments demonstrated the proliferation and invasion ability of cells transfected with wild-type and mutant MEK1.

Results: We confirmed a regulatory relationship between miR-16 and *MEK1* mRNA. Expression of miR-16 was decreased and that of MEK1 and p-ERK1/2 were increased in lung cancer cell lines compared with normal cells. Transfection with miR-101 mimic or small interfering (si)-MEK1 significantly downregulated expression of MEK1 and p-ERK1/2 in Anip973 cells.

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Conclusions: Decreased miR-16 expression may play a role in upregulating expression of MEK1 and promoting proliferation and invasion of lung cancer cells. Overexpression of miR-16 downregulated the ERK/MAPK pathway by inhibiting MEK1 expression, attenuating clone formation and invasion, and inhibiting cell proliferation.

Keywords
MicroRNA-16, miR-16, MAPK kinase 1, MEK1, ERK/MAPK, lung cancer, invasion, proliferation

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Introduction

Lung cancer is one of the most common human malignant tumors. Lung cancer is the leading cause of morbidity and mortality in men and the second highest cause in women.[1,2] The disease poses a serious threat to human life and carries enormous social burden.[3]

The mitogen-activated protein kinase (MAPK) signaling pathway family includes four main conduction pathways: ERK, c-Jun N-terminal kinase (JNK), P38 MAPK, and ERK5/megalysin-activated protein kinase pathway (big MAP kinase 1, BMK1). The ERK-mediated MAPK signaling pathway is considered a classic MAPK signal transduction pathway. If the extracellular signal-stimulating or intracellular pathway transduction molecules are abnormal, overactivation of the ERK/MAPK signaling pathway may occur, which promotes cell proliferation and cycling; inhibits apoptosis and differentiation; enhances cell motility, migration, and invasion; and is closely associated with the occurrence, prognosis, and metastasis of a variety of tumors.[4–6] MAPK kinase 1 (MEK1) is a tyrosine/threonine (Tyr/Thr) double-specific protein kinase. It plays a role upstream of the ERK protein, specifically phosphorylates the ERK protein, and activates the ERK/MAPK signaling pathway, affecting the occurrence and development of tumors.[7,8]

MicroRNA, an endogenous single-stranded, non-coding small molecule RNA, which consists of 20 to 24 nucleotides, is involved in post-transcriptional regulation of gene expression by complementary binding to the 3′-UTR of target gene mRNA, affecting the stability of mRNA and leading to its degradation or inhibiting its translation. MicroRNAs can negatively regulate genes at the post-transcriptional level,[9] and thus participate in many organisms' regulation of learning functions, including cell proliferation, differentiation, or apoptosis. An increasing number of studies have shown that abnormalities in microRNAs play diverse roles in promoting or suppressing cancer.[10–12] The expression of microRNA (miR)-16 is significantly reduced in patients with lung cancer, indicating that it may act as a tumor suppressor gene in the pathogenesis of lung cancer.[13,14] In this study, lung cancer cells were used as the research object, and the predicted targets were verified by double luciferase reporter gene assay. We detected the proliferation and invasion ability of miR-16 cells by plate cloning experiment, Transwell experiment, and EdU staining, and detected the effect of miR-16 on the expression of predicted targets using western blots. The aim of the study was to

explore whether miR-16 plays a role in regulating MEK1 expression, ERK/MAPK pathway activity, and proliferation and invasion of lung cancer cells, to provide a theoretical basis for clinical prevention and treatment of lung cancer.

Materials and methods

Main reagents and materials

High-metastatic lung cancer cells (Anip973), low-metastatic lung cancer cells (AGZY83-a), and normal human lung cells (BEAS-2B) were purchased from Shanghai Bioleaf Biotechnology Co. Ltd. (Shanghai, China). RPMI-1640 and streptomycin were purchased from HyClone (Los Angeles, CA, USA). Fetal bovine serum (FBS) and LHC-8 culture medium were purchased from Gibco (Gaithersburg, MD, USA). The transfection agent FuGENE6 was purchased from Roche (Basel, Switzerland). The total RNA extraction reagent TRNzol Universal was purchased from Beijing Tiangen Biochemical Technology Co. Ltd. (Beijing, China). The fluorescent quantitative PCR reagent TransScript Green One-Step qRT-PCR SuperMix was purchased from Beijing TransGen Biotech (Beijing, China). The miR-16 mimic, miR-16 inhibitor, and miR-NC (negative control) were purchased from Guangzhou Ribobio (Guangzhou, China). Mouse anti-human MEK1, p-ERK1/2, and β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Transwell chambers were purchased from Corning (Corning, NY, USA). Matrigel glues were purchased from BD Biosciences (San Jose, CA, USA). Dual-luciferase activity assay kits were purchased from Promega (Madison, WI, USA), and pLUC luciferase gene reporter plasmids were purchased from Ambion (Austin, TX, USA). EdU cell proliferation flow detection reagents were purchased from Molecular Probes (Shanghai, China). BeyoECL Plus chemiluminescence reagents were purchased from Jiangsu Beyotime (Jiangsu, China).

Cell culture

The lung cancer cell lines (Anip973 and AGZY83-a) were cultured in RPMI-1640 medium containing 10% FBS and 1% streptomycin. BEAS-2B cells were cultured in LHC-8 medium containing 10% FBS and 1% streptomycin. All cells were cultured in a cell incubator containing 5% CO_2 at 37°C and sub-cultured at a ratio of 1:3. Cells that grew well in the logarithmic phase were tested.

Construction of a luciferase reporter gene vector and luciferase reporter gene experiment

The PCR product of the 3′-UTR full-length fragment of the *MEK1* gene or the fragment containing the mutant was ligated into the luciferase reporter vector pLUC and transformed into DH5α competent cells. The positive clones were preliminarily screened by PCR. The plasmids with correct sequencing were selected and designated as pLUC-MEK1-3′-UTR-wt (wild type) and pLUC-MEK1-3′-UTR-mut (mutant), respectively. Then, pLUC-MEK1-3′-UTR-wt or pLUC-MEK1-3′-UTR-mut was co-transfected with miR-16 mimic, miR-16 inhibitor, or miR-NC to HEK293T cells using FuGENE6. After a 48-hour culture, the cells were washed twice with PBS and completely lysed with the PLB lysate of the phospholipase B kit. Ten microliters of the lysate supernatant was added to a 96-well plate, and then an appropriate amount of Stop & Glo test solution (Promega) was added to detect dual luciferase activity at a wavelength of 560 nm.

Cell transfection and grouping

Anip973 cells were cultured *in vitro* and divided into four transfection groups: miR-NC, miR-16 mimic, small interfering (si)-NC, and si-MEK1. One day before transfection, groups of cells were spread on culture plates. On the day of transfection, when cell fusion was 50% to 70%, 5 μL of FuGENE6 reagent was diluted with 100 μL of serum-free minimal medium and double antibody and then gently and thoroughly mixed. The above four types of plasmids (miR-NC, miR-16 mimic, si-NC, and si-MEK1) were added into the corresponding Eppendorf tubes according to the groups, gently mixed, and incubated for 20 minutes at room temperature. The original culture medium was aspirated and the transfection complex was added to the cells and mixed well by shaking the culture plate. After a 6-hour incubation, the culture medium was changed to serum-containing conventional medium and double antibody, and the cells were cultured for 72 hours. Then, the cells were collected and cell proliferation, invasion, and related indices were detected.

Detection of gene expression by quantitative real-time PCR

RNAs were extracted using TRNzol Universal kits, and gene expression was detected with one-step quantitative real-time (qRT)-PCR and TransScript Green One-Step qRT-PCR SuperMix. The 20-μL qRT-PCR reaction system included template RNA (1 μg), 0.3 μM pre-primer, 0.3 μM post-primer, 10 μL of 2× TransStart Tip Green qPCRSuperMix, 0.4 μL of RT Enzyme Mix, 0.4 μL of Dye II, and deionized water. The qRT-PCR reaction conditions were as follows to detect gene expression in the Applied Biosystems QuantStudio 3 Real-time Quantitative PCR system (Thermo Fisher Scientific, Waltham, MA, USA): 45°C for 5 min for reverse transcription; 94°C for 30 s; and 94°C for 5 s and 60°C for 30 s × 40 cycles.

Western blot

The cells in all treatment groups were digested by trypsin after centrifugation and washed twice with PBS. An appropriate amount of RIPA lysate was added and fully lysed on ice, and then the protein supernatant was transferred to a new precooled Eppendorf tube to determine the protein concentration. Fifty micrograms of protein was loaded onto a 10% sodium dodecyl sulfate-PAGE gel. When the bromophenol blue reached the bottom of the gel, electrophoresis was ended, and membrane transfer was performed. The membranes were blocked with 5% skim milk powder at room temperature, followed by incubation with primary antibody (the dilution ratios of MEK1, p-ERK1/2, and β-actin were 1:4000, 1:2000, and 1:10,000, respectively) for 12 hours overnight at 4°C. The membranes were washed three times with PBST (PBS-Tween), horseradish peroxidase (HRP)-labeled secondary antibody (1:25,000 dilution) was added and incubated for 60 minutes at room temperature, and then the membranes were washed three times with PBST. An appropriate amount of BeyoECL Plus working solution was added dropwise to the ProBlot membrane to react for 2 to 3 minutes in the dark, exposed, developed, and fixed in a dark room. Finally, the membranes were scanned to save the data.

Plate cloning experiment

The cells of each transfected group were inoculated onto a 10-cm plate at a density of 100 cells/plate. After culturing for 2 weeks, the culture dish was removed and washed twice in PBS, and the cells were

fixed with 4% paraformaldehyde and stained with Giemsa. Then, the number of colonies with >10 cells was counted and the clone formation rate was calculated as follows: clone formation rate = number of clones/number of inoculated cells × 100%. Three parallel samples were established for each transfection group, and the mean value of the three parallel samples was obtained. The experiments were repeated three times.

Transwell assay for cell invasion

Six hundred microliters of complete medium containing serum and double antibody was added to 24-well plates. The Transwell chamber with Matrigel glue was placed in a 24-well plate, and 200 μL of cells in all transfection groups was resuspended in serum-free RPMI-1640 and added to the upper chamber (three parallel samples in each group). After a 48-hour culture, the filter membrane was removed and, after fixing in 4% paraformaldehyde and 0.1% crystal violet dye, observed under an inverted microscope. Five fields of view were obtained randomly to count the number of cells passing through the pores in each field of view and the average was calculated. The experiment was repeated three times. In a single experiment, three parallel samples were established in each transfection group, and the average of the three parallel samples was calculated.

Detection of cell proliferation by flow cytometry

Cells in all transfection groups were inoculated in 6-well plates (three parallel samples were established for each group). Cells were resuspended in complete medium and cell proliferation was detected by Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay kits (Thermo Fisher Scientific, Waltham, MA, USA). After incubation for 2 hours in 10 μM EdU (modified thymidine analogue), cells were cultured for 48 hours and collected by trypsin digestion. After centrifugal washing, immobilization, and permeabilization, Alexa Fluor 488-labeled reaction solution was added and incubated at room temperature in the dark for 30 minutes. After centrifugal washing, cell proliferation was detected on a Beckman Coulter CytoFLEX flow cytometer (Beckman Coulter Inc., Brea, CA, USA).

Statistical analysis

Statistical analysis was performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Measurement data are expressed as the mean ± standard deviation. The measurement data between groups were compared using a *t*-test, and $P < 0.05$ was considered a statistically significant difference.

Results

Targeted regulatory relationship between miR-16 and MEK1

Bioinformatics analysis showed that a targeted complementary binding site exists between miR-16 and the 3′-UTR of *MEK1* mRNA (Figure 1a). The dual luciferase reporter gene assay showed that transfection of miR-16 mimic altered relative luciferase activity in HEK293T cells transfected with pMIR-MEK1-3′-UTR-wt but not in HEK293T cells transfected with pMIR-MEK1 -3′-UTR-mut, demonstrating that a targeted regulatory relationship existed between miR-16 and MEK1 (Figure 1b).

Expression of miR-16 was decreased and that of MEK1 increased in lung cancer cells

The qRT-PCR results showed that the expression of *MEK1* mRNA was

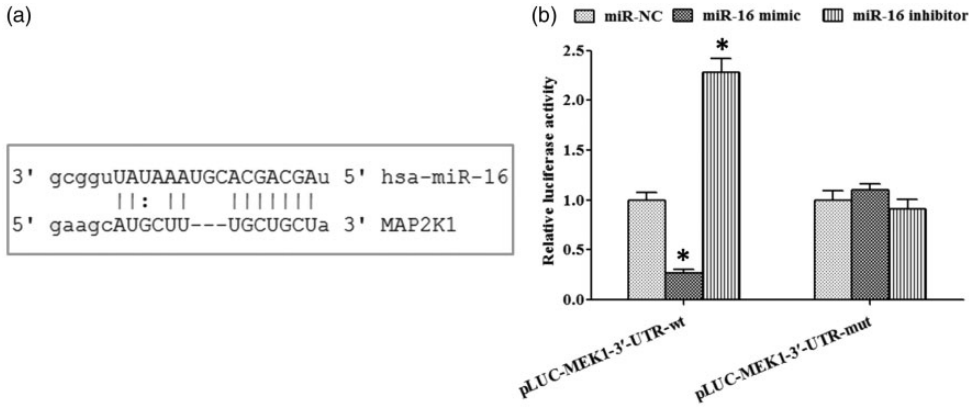

Figure 1. Targeted regulatory relationship between miR-16 and MEK1. (a) Action site between miR-16 and 3′-UTR of *MEK1* mRNA; (b) dual luciferase gene reporter assay. Values are mean ± standard deviation; *indicates a significant difference compared with miR-NC, $P < 0.05$. MEK1, MAPK kinase 1; miR-NC, negative control; pLUC-MEK1-3′-UTR-wt, luciferase reporter plasmid with wild-type MEK1 3′-untranslated region (UTR); pLUC-MEK1-3′-UTR-mut, luciferase reporter plasmid with mutant MEK1 3′-UTR.

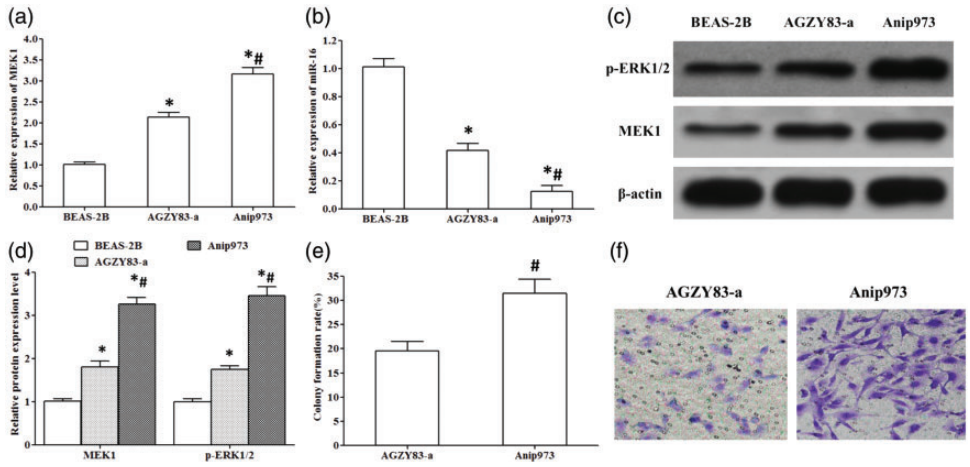

Figure 2. Decreased expression of miR-16 and increased expression of MEK1 in lung cancer cells. (a) Detection of expression of *MEK1* mRNA by qRT-PCR; (b) detection of miR-16 expression by qRT-PCR; (c) detection of MEK1 and p-ERK1/2 protein by western blot; (d) comparison of the level of protein expression; (e) detection of colony-forming ability by plate clone assay; (f) detection of cell invasion ability by Transwell assay. Values are mean ± standard deviation; * indicates a significant difference compared with BEAS-2B cells, $P < 0.05$; # indicates a significant difference between Anip973 cells and AGZY83-a cells, $P < 0.05$. MEK1, MAPK kinase 1; BEAS-2B, normal human lung cells; Anip973, high-metastatic lung cancer cells; AGZY83-a, low-metastatic lung cancer cells.

significantly increased ($P < 0.05$) in lung cancer cells compared with normal human lung cells (BEAS-2B), and that expression of *MEK1* mRNA was significantly higher ($P < 0.05$) in Anip973 cells than in AGZY83-a cells (Figure 2a). The expression of miR-16 was significantly decreased ($P < 0.05$) in lung cancer cells compared

with the BEAS-2B cell line and was significantly lower ($P < 0.05$) in Anip973 cells than in AGZY83-a cells (Figure 2b). Western blot analysis showed that the expression of MEK1 and p-ERK1/2 proteins was significantly higher ($P < 0.05$) in high-metastatic Anip973 cells than in low-metastatic AGZY83-a cells, whereas the expression of MEK1 and p-ERK1/2 proteins in AGZY83-a cells was significantly higher ($P < 0.05$) than that BEAS-2B cells (Figures 2c and 2d). The results of plate cloning experiments showed that clone formation was significantly greater ($P < 0.05$) in Anip973 cells than in AGZY83-a cells (Figure 2e). Transwell assays showed that the invasion ability of high-metastatic Anip973 cells was significantly higher ($P < 0.05$) than that of AGZY83-a cells (Figure 2f).

Elevated expression of miR-16 or decreased expression of MEK1 attenuated proliferation and invasion of lung cancer cells

Transfection of miR-16 mimic significantly downregulated ($P < 0.05$) the expression of MEK1 and p-ERK1/2 in Anip973 cells (Figures 3a-c), inhibited clone formation

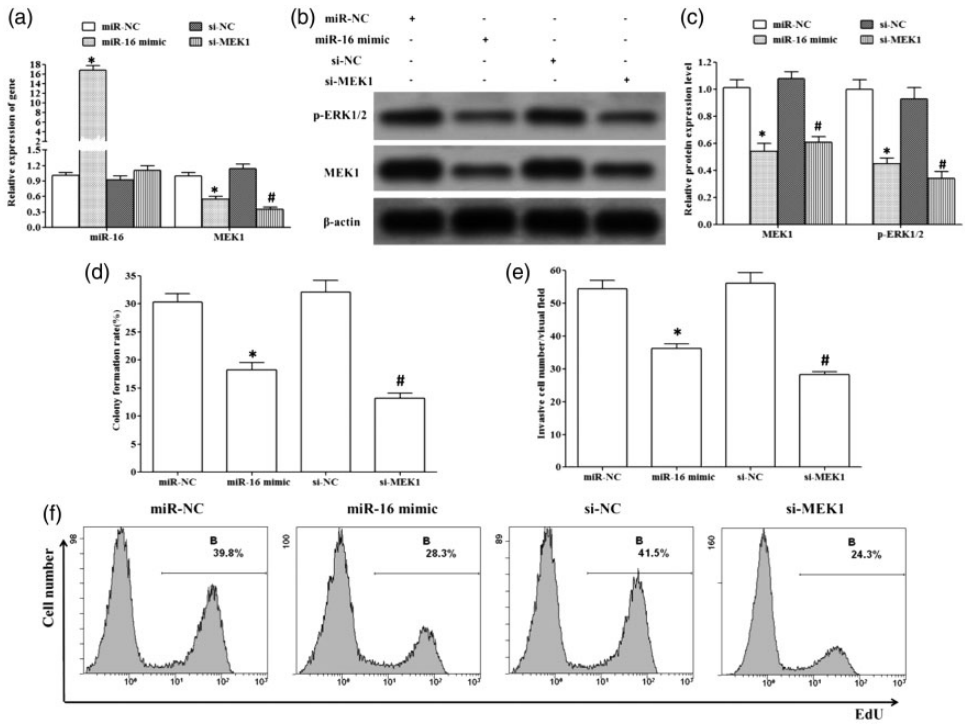

Figure 3. Elevated expression of miR-16 or decreased expression of MEK1 attenuates proliferation and invasion of lung cancer cells. (a) Detection of gene expression by quantitative real-time PCR; (b) detection of protein expression by western blot; (c) comparison of levels of protein expression; (d) detection of colony forming ability by the plate clone assay; (e) detection of cell invasion ability by Transwell assay; (f) detection of cell proliferation by EdU staining, where B is the cell proliferation value. Values are mean ± standard deviation; * indicates a significant difference between miR-16 mimic and miR-NC, $P < 0.05$; # indicates a significant difference between si-MEK1 and si-NC, $P < 0.05$. MEK1, MAPK kinase 1; NC, negative control; si, small interfering.

(Figure 3d), and attenuated invasion (Figure 3e) and proliferation (Figure 3f) of cells. Transfection of si-MEK1 downregulated the expression of MEK1 and p-ERK1/2 similarly to transfection of miR-16 mimic (Figures 3a-c) and significantly attenuated ($P < 0.05$) clone formation (Figure 3d), invasion (Figure 3e), and proliferation (Figure 3f) of cells.

Discussion

With industrial development, poor air quality, and an increasing number of smokers, the incidence of lung cancer is on a rising trend. The onset and progression of lung cancer is rapid, with a high degree of malignancy, high mortality, and low survival. Therefore, lung cancer is a serious threat to human life and health.[3] Although the survival of patients with lung cancer has improved with surgical treatment, radiotherapy, chemotherapy, immunotherapy, and other comprehensive approaches, survival and prognosis of patients remain poor, and the 5-year survival rate is only 15% to 20%. If distant metastases occur, the 5-year survival rate is reduced to <5%.[15,16] Therefore, identifying signal molecules with abnormal changes that underlie the pathogenesis of lung cancer is important for improving the early diagnosis of lung cancer, the effect of therapy, and the prognosis.

Abnormal expression of microRNAs can affect the cell cycle and growth pattern in normal and tumor tissues. In 2002, scientists discovered that miR-16 has a tumor suppressor-like effect in chronic lymphocytic leukemia,[17] and studies have shown that miR-16 is associated with malignant tumors including human glioma, colorectal cancer, and lung adenocarcinoma. Ke et al.[13] found that the expression of miR-16 was significantly decreased in tumor tissues of lung cancer patients. Overexpression of miR-16 in lung cancer cell lines A549 and Calu3 inhibited the proliferation and decreased migration and invasion of lung cancer cells by inhibiting the expression of the target gene *HDGF*. The expression of miR-16 is decreased in paclitaxel-resistant lung cancer cells, and elevated expression of miR-16 significantly inhibits the proliferation of lung cancer cells and induces G_0/G_1 cell cycle arrest.[14] Elevated expression of miR-16 may inhibit the survival of lung cancer cells, attenuate the ability of clonal formation and induce apoptosis by targeting the anti-apoptotic gene Bcl-2 and upregulating the expression of proapoptotic factors p27 and Bax.[18,19] A number of studies have found that expression of miR-16 in peripheral blood serum of patients with lung cancer is significantly reduced, and its expression level can be used as an independent predictor of survival and prognosis of patients: the 2-year survival rate (2.4%) of lung cancer patients with low expression of miR-16 in peripheral blood serum was significantly lower than that of patients with high expression (39.3%).[20,21] Thus, downregulated expression of miR-16 may be a detrimental factor in the development of lung cancer.

Expression of MEK1 and enhanced functional activity are related to the incidence of lung cancer.[22] The inhibition of MEK1 function can attenuate the malignant biological characteristics of lung cancer to some extent.[23,24] MEK1 also plays an important regulatory role in the process of epithelial-mesenchymal transition (EMT) by mediating the activation of the ERK/MAPK pathway[7,8] and is associated with the occurrence, progression, and poor prognosis of various tumors.[25–27] Song et al.[24] revealed that after MEK1 function and ERP/MAPK pathway activity were inhibited by the inhibitor CZ0775, the proliferation of lung cancer cells was significantly decreased and apoptosis significantly increased. Lu et al.[28] revealed that treatment with the MEK1-specific inhibitor

PD98059 significantly inhibited the transduction of ERP/MAPK pathway activity, inhibited the migration of NCI-H1299 lung cancer cells, and attenuated invasion. Together, these studies suggest that MEK1-mediated ERK/MAPK signal pathway transduction regulates the proliferation and invasion of lung cancer cells. The inhibition of MEK1 function by intervention measures impedes the proliferation and invasion of lung cancer cells. At present, studies on the biological effects of lung cancer cells regulated by miR-16 or MEK1 have been reported; however, whether there is a direct relationship between miR-16 and MEK1, or targeted regulation, has not previously been reported.

In the current study, bioinformatics analysis showed that a target binding site exists between miR-16 and the 3′-UTR of *MEK1* mRNA. Transfection of miR-16 mimic altered the relative luciferase activity in HEK293T cells transfected with pMIR-MEK1-3′-UTR-wt but not that in HEK293T cells transfected with pMIR-MEK1-3′-UTR-mut, confirming that a targeted regulatory relationship exists between miR-16 and MEK1. The invasive ability and colony-forming ability of Anip973 cells were significantly enhanced. Western blot results showed that the expression of MEK1 and p-ERK1/2 was significantly increased in lung cancer cells compared with normal human lung cells (BEAS-2B), whereas the expression of miR-16 was significantly decreased, and the expression of MEK1 and p-ERK1/2 in high-metastatic Anip973 cells was significantly lower than that in low-metastatic AGZY83-a cells. Such a regulatory effect is related not only to the pathogenesis but also to the metastasis of lung cancer cells. The lower the expression level of miR-16 in lung cancer cells, the stronger the metastatic ability. Transfection of miR-16 mimic decreased the expression of MEK1 in Anip973 cells, decreased the expression of p-ERK1/2, and attenuated the proliferation and invasion of cells. Transfection of si-MEK1 had a similar effect.

This research suggests that decreased expression of miR-16 may play a role in upregulating the expression of MEK1 and promoting the proliferation and invasion of lung cancer cells. The overexpression of miR-16 can reduce the activity of the ERK/MAPK pathway by targeted inhibition of MEK1 expression, attenuate clone formation and invasion of lung cancer, and inhibit cell proliferation. In the current study, we reported a regulatory effect of miR-16 on the proliferation and invasion of lung cancer cells by targeting MEK1 at the cellular level *in vitro*. It remains unclear whether this targeted regulatory effect exists in tumor tissues of patients with lung cancer, which will need to be assessed through collection of samples. In addition, it is unclear which type of target molecules downstream of the ERK/MAPK signal pathway are affected by miR-16 to achieve the observed regulation of proliferation and invasion of lung cancer. In the current study, we confirmed that miR-16 has a regulatory effect on the proliferation and invasion of lung cancer cells by targeting MEK1, providing a theoretical basis for early clinical diagnosis and prevention of lung cancer.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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