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MicroRNA-134 regulates lung cancer cell H69 growth and apoptosis by targeting WWOX gene and suppressing the ERK1/2 signaling pathway

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ABSTRACT

MicroRNAs have been shown to act as crucial modulators during carcinogenesis. Recent studies have implied that miR-134 expression associated with epithelial-to-mesenchymal transition phenotype and invasive potential of NSCLC cells. Our study investigated the pathogenic implications of miR-134 in small cell lung cancer (SCLC). Overexpression or inhibition MiR-134 expression by miR-134 mimics or miR-134 inhibitors (anti-miR-134) in SCLC cell lines was detected using qRT-PCR. Lactate dehydrogenase (LDH) assay, MTT assays and flow cytometry were performed in order to clarify the growth and apoptosis of SCLC cells which had been transfected with miR-134 mimics or anti-miR-134. WWOX expression in H69 cells was detected by qRT-PCR and western blot, respectively. The results showed that overexpression miR-134 was significantly promoting SCLC cells growth and inhibit its apoptosis. In addition, reduced miR-134 expression was significantly correlated with cell growth inhibition and apoptosis promotion. Furthermore, transfection of miR-134 mimics into the SCLC cells markedly down-regulated the level of WWOX, whereas, anti-miR-134 up-regulated WWOX expression. We also found that overexpression WWOX attenuate miR-134 induced H69 cells growth, and promote cell apoptosis. Moreover, miR-134 promoted cell proliferation and inhibit apoptosis via the activation of ERK1/2 pathway. These findings suggest that miR-134 may be an ideal diagnostic and prognostic marker, and may be attributed to the molecular therapy of SCLC.

1. Introduction

Lung cancer, the most frequently occurring malignant cancer, is the leading cause of cancer-related deaths worldwide [1,2]. The prognosis for lung cancer patients is closely related to the disease stage of the patients at the time of diagnosis, and the 5-year survival rate of patients with clinical stage II–IV disease is approximately 5%–40% which far less than patients with clinical stage I disease [3]. Reports indicated that approximately 15% of lung cancer is small cell lung cancer (SCLC) [4]. Despite SCLC is highly responsive to both chemotherapy and/or irradiation, SCLC is always characterized by inexorable disease progression [5]. To improve the survival rate of patients with this disease, more efforts should be made to understand tumor biology and found novel therapeutic strategies.

MicroRNAs (miRNAs) have been report play important role in numerous developmental processes and tumorigenesis, including the progression of lung cancer. MiRNAs, a class of small, non-protein-coding RNAs, have emerged as post-transcriptional regulators which can negatively regulated the level of target genes [6,7], and then controlling many cellular biological functions such as differentiation, proliferation and apoptosis [8]. It is now well established that abnormal expression of miRNAs is associated with types of cancers, and some of miRNAs function as oncogenes or tumor suppressor genes [8]. Accumulated evidence has clearly shown the potential and important role of miR-134 in non-small cell lung cancer cells via inhibiting epithelial to mesenchymal

Abbreviations: SCLC, small cell lung cancer; Anti-miR-134, miR-134 inhibitors; LDH, lactate dehydrogenase assay; miRNAs, microRNAs; WWOX, WW domain-containing oxidoreductase.

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transition [9]. Numerous studies have documented the role of miR-134 in lung cancer that MiR-134 could increases the cell survival by inducing G1 arrest and downregulates MRP1/ABCC1 protein in drug-resistant small cell lung cancer cells [10]. However, to our best knowledge, there have been no reports about the mechanism of MiR-134 in lung cancer. Liu et al. showed that overexpression of miR-134 *in vitro* could induce oncogenicity and metastasis in head and neck carcinoma via targeting WWOX gene [11].

WW domain-containing oxidoreductase (WWOX), includes nine exons and encodes a 46-kDa protein, is located at the chromosome region 16q23 [12–14]. There has some evidence demonstrated that WWOX is a tumor suppressor [15,16] and WWOX overexpression promotes cancer cell apoptosis and suppresses tumorigenicity [17,18]. Another study has also demonstrated that the inactivation of WWOX contributes to the development of a wide type of cancers [19–21]. WWOX can regulation of the extracellular matrix [22], modulation of cell apoptosis [23,24] and cell bioenergetics [25] which all contributed to suppress tumor progression. Previous studies have reported that WWOX expressions were reduced or absent in the majority of lung cancer tissues [26] because of genetic or epigenetic alterations including the deletion and mutation of WWOX exons 6–8 in human non-small cell lung cancer [27,28].

Evidence suggested that MiR-134 may play important role in lung cancer, however, the full function and mechanism of MiR-134 in SCLC cell lines is still largely unexplained; As WWOX has been proposed to play roles in lung cancer, we aimed to investigate the interaction between MiR-134 and WWOX in SCLC cell lines. By overexpression MiR-134 in different SCLC cell lines, we found that MiR-134 promotes lung cancer cell growth and inhibits apoptosis by targeting WWOX gene. Inhibition MiR-134 expression or overexpression WWOX significantly inhibited cell proliferation and induced apoptosis of lung cancer cells. More importantly, we then identified that MiR-134 was capable of inhibiting ERK1/2 pathway activation. Taken together, our data support the suggestion that MiR-134 is a potential target for lung cancer prevention and therapy.

2. Materials and methods

2.1. Oligonucleotides

Human miR-134 mimics and negative control mimics (NC) were obtained from GenePharma (Shanghai, China), miR-134 inhibitors (anti-miR-134) or negative control (anti-NC) was synthesized by Ribobio (Guangzhou, China).

2.2. Cell culture and transfection

SCLC cell lines (U2020, U-1906 and H69) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, GIBCO BRL, Grand Island, NY, USA) supplemented with 10% FBS, 0.1 mg/mL streptomycin and 100 U/mL penicillin at 37 °C with 5% CO_2 in an incubator (Life Technologies, Baltimore, MD, USA). MiR-134 overexpression lung cancer cells were constructed according to the method described previously [9]. SCLC cells were transfected with miR-134 mimics and negative control mimics (NC) using Lipofectamine RNAiMAX (Life Technologies) at a final concentration of 75 nM according to the manufacturer's protocol. SCLC cells were assayed 72 h after transfection.

2.3. Lactate dehydrogenase (LDH) assay

Cell viability was measured by LDH assay [29]. In brief, LDH activity was determined using a commercial available LDH assay Kit (P Sigma St Louis, MO, USA). SCLC cells were cultured in 24-well cell culture plates in DMEM at 37 °C with 5% CO_2 in an incubator. 48 h after transfection, cell culture medium of each well was removed and further preceded for enzymatic analysis followed the manufacturer's instructions. From the data of cytotoxicity studies, the effects of MiR-134 on the cell viability were determined.

2.4. MTT assay

SCLC cell proliferation was determined by MTT methods followed the described method [30]. SCLC cells (5×10^5) were cultured in 96-well cell culture plates in DMEM at 37 °C with 5% CO_2. 48 h after transfection, MTT was added to each well at a final concentration 5 mg/ml. After a 4 h incubation, the crystals were dissolved in 200 μl DMSO at room temperature. The optical density (OD) was detected at 490 nm by using a microplate reader (Dynatech MR 400). Data were calculated using averaged results.

2.5. Flow cytometric analysis of apoptosis

Annexin V-FITC/PI kit (BD PharMingen, San Diego, CA) was used for the detection of SCLC cell apoptosis according to the manufacturer's instructions. Briefly, SCLC cells were trypsinized and 5×10^5 cells were washed with PBS. A549 cells were then processed for labeling with Annexin V/FITC and propidium iodide (PI), and analyzed by flow cytometry.

2.6. MiR-134 inhibition

MiR-134 inhibition in lung cancer cells was followed the method described previously [31]. SCLC cells were cultured in 24-well cell culture plates and transfected with 5 nmol/L of anti-miR-134 or negative control (anti-NC) (Ribobio, Guangzhou, China), using siPORT NeoFX Transfection Agent (Ambion) followed by the manufacturer's protocol. After transfection, cells were cultured for 48 h and then further analyzed.

2.7. Recombinant adenovirus construction and transfection

The WWOX adenovirus was constructed using the Adenovator-CMV5(CuO)-IRES-GFP transfer vector (Qbiogene, Carlsbad, CA, USA) as described preciously with minor modifications [32,33]. Briefly, total RNA was isolated from the A549 cells, and cDNA was reverse transcribed by the PrimeScript RT reagent kit (Takara, Shiga, Japan). The double-stranded cDNAs were amplification using two primers [32]: WWOX forward 5′-GCCAGGTGCCTCCACAGTCAGCC-3′ and WWOX reverse 5′-TGTGTGTGCCCA-TCCGCTCTGAGCTCCAC-3′. H69 cells were transfected with Ad-WWOX or Ad-GFP, and the efficiency of transfection was assessed by visualization of GFP-expressing cells.

2.8. Real-time quantitative PCR

Real-time quantitative PCR was performed as described earlier [31]. Total RNA was obtained by using TRIzol Reagent (Invitrogen) according to manufacturer's instructions. The cDNA was synthesized using TaqMan miRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative RT-PCR (qRT-PCR) reaction was done by using TaqMan Universal PCR Master Mix and TaqMan miRNA specific PCR-primers (Applied Biosystems). The reaction conditions was as follows: 95 °C, 10 min;

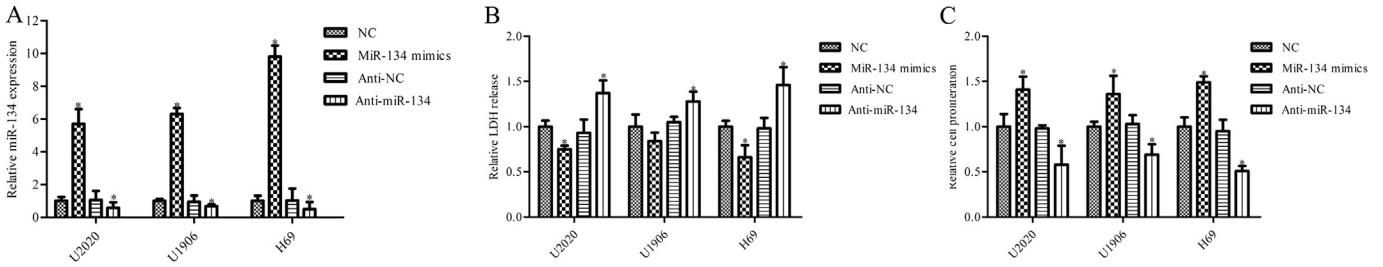

Fig. 1. MiR-134 promotes cell viability and proliferation of human lung cancer cells. (**A**) The miR-134 levels in the panel of SCLC cell lines were determined by qRT-PCR. (**B**) The cytotoxic effect of miR-134 on U2020, U-1906 and H69 cells was determined by the LDH release assay. (**C**) The proliferation of U2020, U-1906 and H69 cells was assayed by MTT method. miR-134 mimics and anti-miR-134 were used to alter miR-134 levels in cultured SCLC cell lines U2020, U-1906 and H69. All experiments were repeated at least 3 times. Values are presented as mean ± SEM. *P < 0.05 vs. NC.

followed by 38 cycles at 95 °C, 30 s; 95 °C, 50 s; 72 °C, 10 min. To determine the level of WWOX, the following primers were used: human WWOX Sense, Antisense, Expression levels of the miRNA was expressed relative to that of U6 or β-actin (control) and analyzed using the $2^{-\Delta\Delta CT}$ method [34].

2.9. Western blot analysis

Protein extraction and western blot analysis were done according to the described method (5). The following primary and secondary antibody were used: Rabbit anti-wwox polyclonal antibody, Rabbit anti-pERK1/2 polyclonal antibody, Rabbit anti-ERK1/2 polyclonal antibody, mouse anti-β-actin monoclonal antibody, HRP-conjugated rabbit anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG. These antibodies were all obtained from Abcam (Cambridge, MA, USA).

2.10. Statistical analysis

The data of all *in vitro* assays were analyzed using SPSS 13.0 statistical software and expressed as mean ± SEM. one-way ANOVA and Student t test were used to compare the values of the test and control groups. P < 0.05 was considered statistically significant.

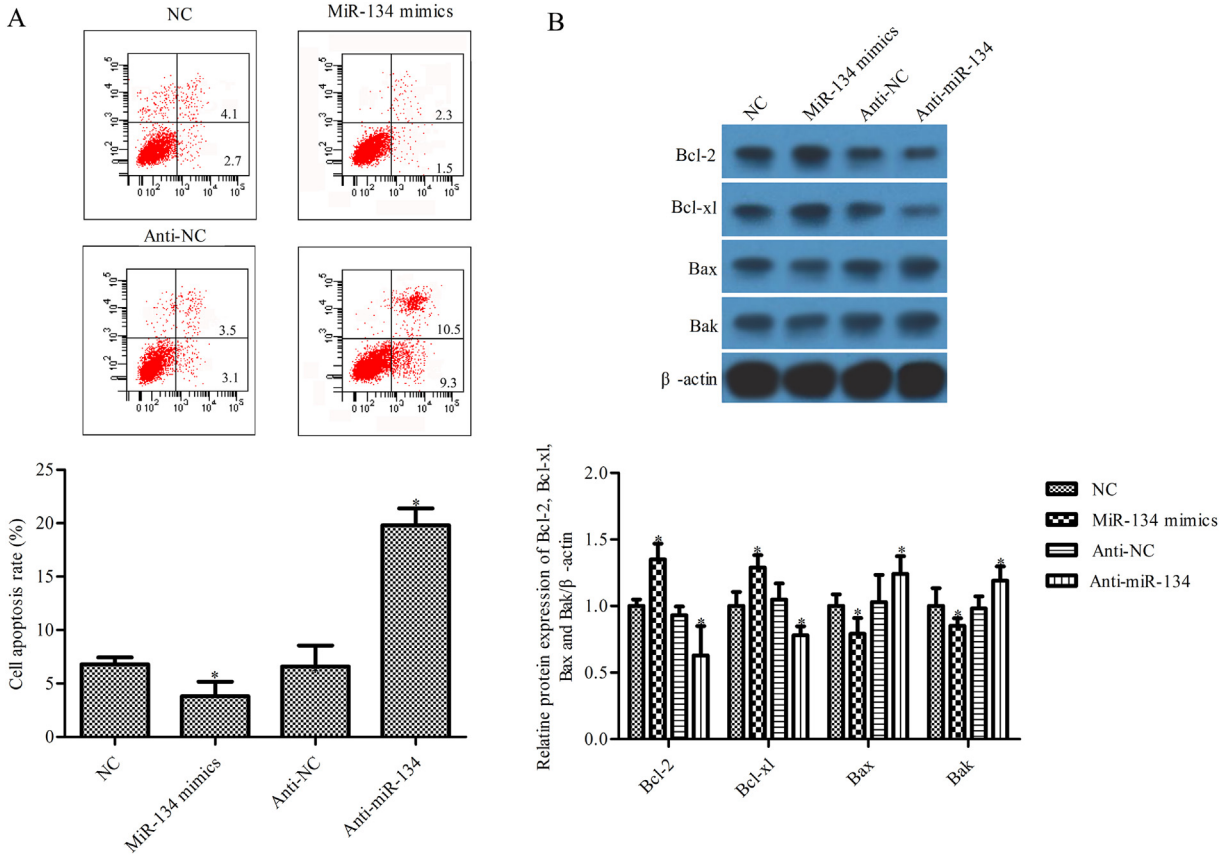

Fig. 2. MiR-134 inhibits H69 cell apoptosis. (**A**) The apoptosis of H69 cells was determined by flow cytometric analysis. (**B**) Western blotting was used to measure the expression of apoptosis related gene Bcl-2, Bcl-xl, Bax and Bak. All experiments were repeated at least 3 times. Values are presented as mean ± SEM. *P < 0.05 vs. NC.

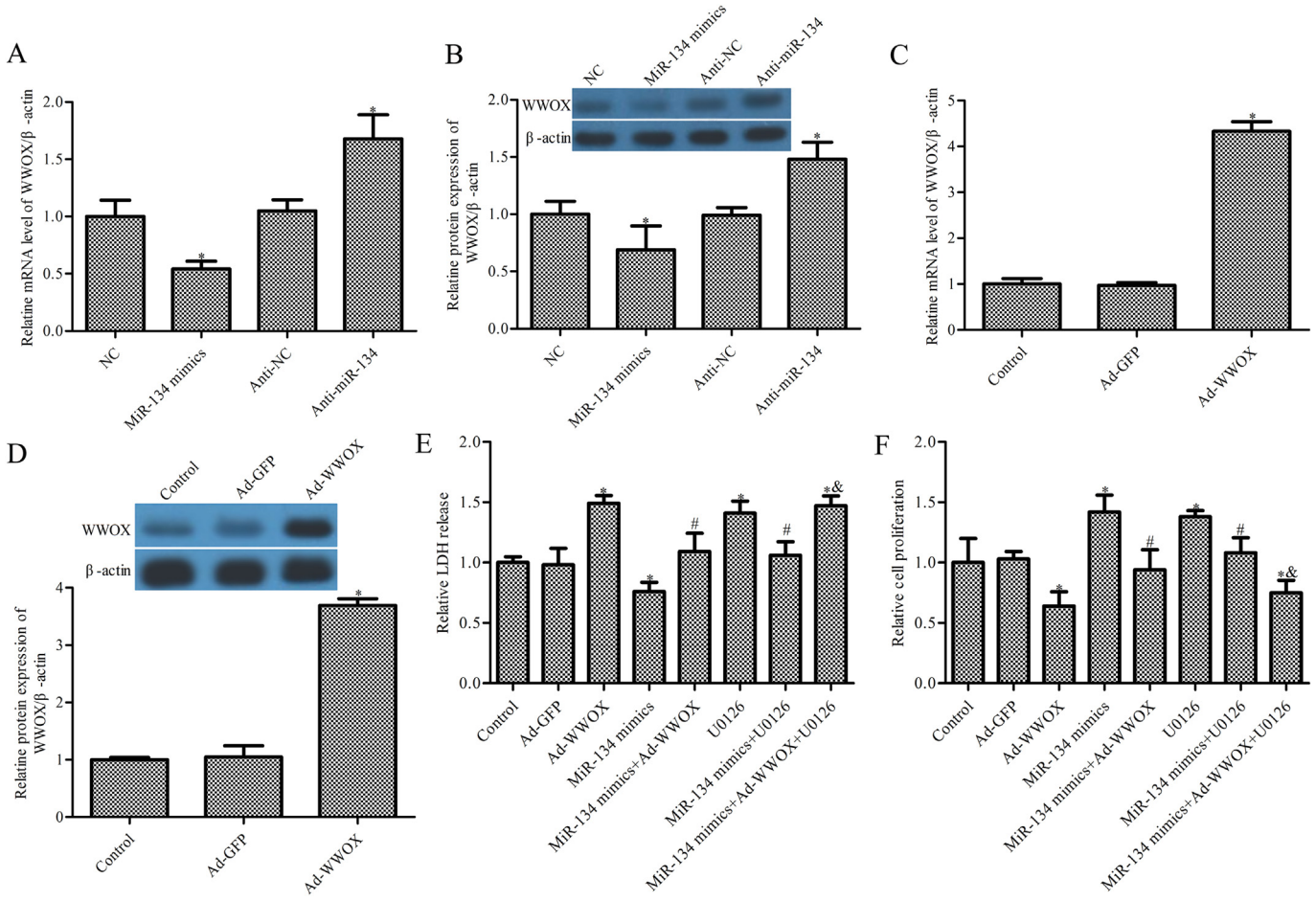

Fig. 3. MiR-134 down-regulated WWOX mRNA and protein expression in H69 cells. (**A**) The level of WWOX mRNA after miR-134 mimics and anti-miR-134 treatment of H69 cells. (**B**) WWOX protein expression in H69 cells. WWOX protein expression was measured by western blot method. Cells were treated with miR-134 mimics or anti-miR-134. (**C**–**D**) Overexpression of WWOX mRNA (**C**) and protein (**D**) was detected by qRT-PCR and western blot. (**E**–**F**) H69 cell viability and proliferation was determined by LDH assay and MTT assay, respectively. Cells were treated by miR-134 mimics, anti-miR-134, WWOX vector or ERK1/2 inhibitor U0126. All experiments were repeated at least 3 times. Values are presented as mean ± SEM. *P < 0.05 vs. NC or control. *P < 0.05 vs. NC or control, #P < 0.05 vs. MiR-134 mimics group, &P < 0.05 vs. MiR-134 mimics + Ad-WWOX group.

3. Results

3.1. Epigenetic regulation of MiR-134 in lung cancer cells

To assess the contributory role of miR-134 in regulating SCLC cell growth, miR-134 mimics and anti-miR-134 were used to alter miR-134 levels in cultured SCLC cell lines (U2020, U-1906 and H69). The expression of MiR-134 was measured by qRT-PCR. As shown in Fig. 1A, compared with negative control mimics (NC) group, ectopic transfection of miR-134 mimics for 72 h led to a dramatic increase in all three SCLC cell lines, and when these cells treated with anti-miR-134, the miR-134 expression in the three SCLC cell lines is markedly reduced ($P < 0.05$).

3.2. Growth of MiR-134-infected lung cancer cells in vitro

The cytotoxic effect of miR-134 overexpression on U2020, U-1906 and H69 cells was determined by the LDH release assay. The results indicated that a decrease in U2020, U-1906 and H69 cell death was observed in miR-134 mimics treatment compared with the NC group. And, the cell death was significant increase after anti-miR-134 treatment compared with the negative control (anti-NC) group (Fig. 1B). U2020, U-1906 and H69 cell proliferation was assayed by MTT method, and the results clearly showed that up-regulated miR-134 level by miR-134 mimics substantially increased the proliferation of SCLC cells, whereas down-regulated miR-134 via anti-miR-134 effectively reduced the cell proliferation compared with the anti-NC group (Fig. 1C). These data indicated that increased miR-134 levels promote SCLC cell growth, while decreased miR-134 levels inhibit cell growth. Since H69 cells were more sensitive to miR-134 ectopic transfection, thus, we selected this cell line for further studies.

3.3. Analysis of apoptotic pathways in MiR-134-reexpressing cells

To explore whether miR-134 could affect H69 cell apoptosis, flow cytometric analysis of H69 cells double-stained with Annexin V and PI was used. As shown in Fig. 2A, transfection of miR-134 mimics could significantly reduce H69 cell apoptosis, whereas transfection of anti-miR-134 could enhance H69 cell apoptosis ($P < 0.05$). In addition, either NC or anti-NC had no significant difference compared with the control group. To further confirm that miR-134 played important role in H69 cell apoptosis, we also examined the expression of apoptosis related gene Bcl-2, Bcl-xl, Bax and Bak using western blotting. Consistent with the results of flow cytometric analysis, the anti-apoptosis protein Bcl-2 and Bcl-xl

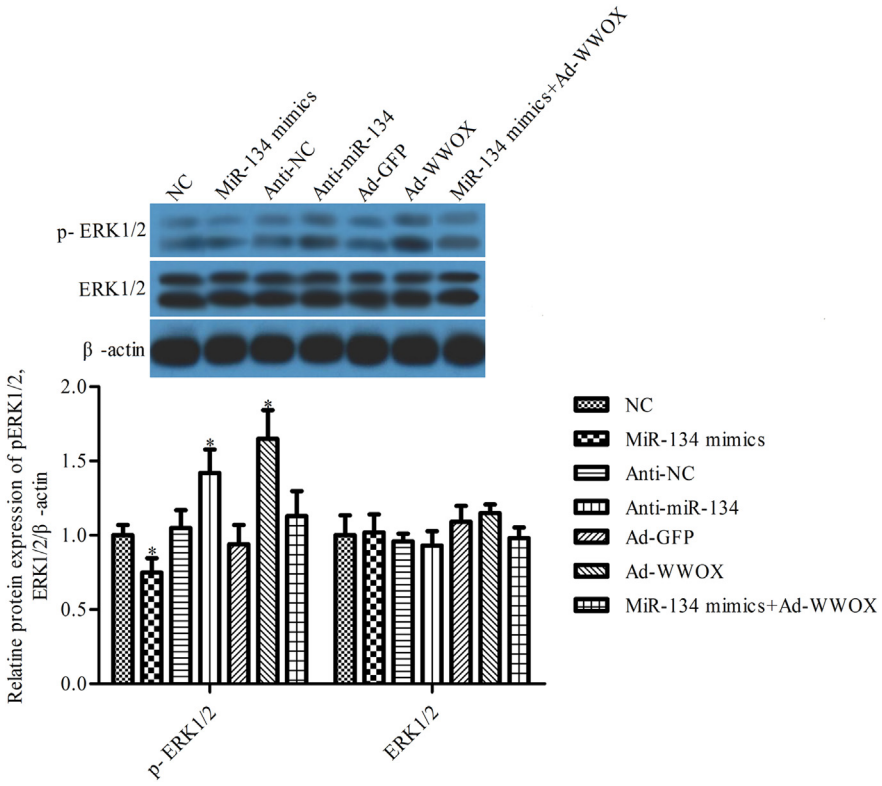

Fig. 4. Effects of miR-134 on the activity of the ERK pathway. The expression of p-ERK and ERK1/2 was detected by western blot assay. The expression of p-ERK was markedly decreased in miR-134 mimics group, whereas the anti-miR-134 markedly increased pERK expression. WWOX ablates the effect of miR-134 on the expression of pERK. All experiments were repeated at least 3 times. Values are presented as mean ± SEM. *P < 0.05 vs. NC.

were remarkably increased after transfection of miR-134 mimics and significantly decreased after transfection of anti-miR-134 (Fig. 2B, P < 0.05). And, the pro-apoptosis protein Bax and Bak were remarkably decreased after transfection of miR-134 mimics, whereas significantly increased after transfection of anti-miR-134 (Fig. 2B, P < 0.05).

3.4. WWOX is involved in MiR-134 induced cell growth in H69 cells

Prediction module (TargetScan v6.2; http://www.targetscan.org) and some reports showed that the WWOX tumor suppressor gene is a direct target of miR-134 [11]. WWOX is highly effective in preventing growth of lung cancer *in vitro* and *in vivo*. Thus we first measured that the effects of miR-134 mimics and anti-miR-134 on WWOX expression. Down-regulation of WWOX mRNA and protein expression was observed in H69 cells with ectopic miR-134 expression (Fig. 3A and B). The results also showed that MiR-134 inhibition significant up-regulated the expression of WWOX in H69 cells (Fig. 3A and B). To further confirm the function of WWOX in miR-134 induced H69 cell growth, transfection of a GFP-tagged WWOX plasmid and a control GFP plasmid was performed. Overexpression of WWOX mRNA and protein was seen in H69 cells (Fig. 3C and D). The overexpression of WWOX decreased the proliferation and increased the apoptosis of H69 (Fig. 3E–F). To ascertain that miR-134 was able to negatively regulate WWOX for H69 cells growth, the high expression of WWOX was shown to decrease the H69 cells growth which enhanced by miR-134 up-regulation (Fig. 3E–F). These results suggested that miR-134 regulate the growth of H69 cells by targeting WWOX.

3.5. The effects of MiR-134 is associated with ERK1/2 pathway

Emerging studies have revealed that tumor suppressor WWOX could activate ERK Pathway [35]. The ERK signaling substrate phosphorylation is involved in cell survival, proliferation, angiogenesis, invasion, motility and differentiation [36–38]. We next determined the expression of ERK and pERK which may be involved in the growth promotion and apoptosis inhibition of miR-134 in lung cancer cells. The results indicated that miR-134 mimics significantly inhibit the level of pERK, whereas the anti-miR-134 markedly increased pERK expression. WWOX overexpression markedly up-regulate the level of pERK compared with the Ad-GFP group. Moreover, overexpression of WWOX ablates the effect of miR-134 on the expression of pERK (Fig. 4). We also found that the inhibitor of ERK1/2 U0126 strikingly suppressed H69 cell survival and proliferation (Fig. 3E–F). U0126 treatment significant inhibited MiR-134 induced-H69 cell viability and proliferation ($p < 0.05$). Moreover, U0126 treatment decreased MiR-134 mimics and Ad-WWOX induced cell survival and proliferation ($p < 0.05$). These results suggested that miR-134 regulate the growth of H69 cells is associated with the ERK pathway.

4. Discussion

It is now well documented that miRNA expression is associated with tumor progression and prognosis, including lung cancer [39]. The dysregulation of many miRNAs were correlated with the therapeutic response [40] and survival [41] of patient with lung cancer. The miR-134 gene has been implicated in several pathological and physiological processes. According to the published estimates, miR-134 may be act as a tumor suppressor in many kinds

of cancers, such as non-small cell lung cancer [9]. Moreover, miR-134 down-regulated the expression of resistance associated protein MRP1/ABCC1 in H69AR cells [42]. Also, overexpression of miR-134 promotes cell proliferation and inhibits apoptosis in lung cancer cell lines [43]. Thus, understanding the roles and mechanism of miR-134 in SCLC might provide significative information for the treatment of SCLC. In the present study, we focused on the roles of miR-134 in SCLC.

Evidence showed that miR-134 expression is associated with drug-resistant of small cell lung cancer cells through down-regulation of MRP1/ABCC1 at the protein level and miR-134 increases the cell survival by inducing G1 arrest in drug-resistant subline H69AR cells [42]. To further determine the role of miR-134 in SCLC cell growth and apoptosis, we transfected miR-134 mimics and anti-miR-134 in cultured SCLC cell lines (U2020, U-1906 and H69). We examined miR-134 expression in SCLC cell lines through quantitative RT-PCR assay, and the results indicate that miR-134 expression is significantly upregulated in these three types of cells transfected with miR-134 mimics, whereas markedly reduced in cells transfected with anti-miR-134. miRNAs are now known to play important roles in the regulation of gene expression for developmental timing, cell proliferation and apoptosis [42]. In line with these findings, we also demonstrated that miR-134 overexpression promotes U2020, U-1906 and H69 cell survival and proliferation, whereas anti-miR-134 strikingly inhibited these effects.

Overexpression of miR-134 in glioblastoma cells significant promoted these cells apoptosis *in vitro* [44]. Xiaoying Zhang et al. showed that overexpression of miR-134 in A549 and Calu-3 cells can inhibit cell apoptosis *in vitro* [43]. Our results showed that overexpression of miR-134 in H69 cell significantly inhibited cell apoptosis, while miR-134 inhibitor markedly promotes these cell apoptosis. It is reported that cell apoptosis process was regulated by the Bcl-2 protein family [45]. Our findings also showed that miR-134 overexpression remarkably suppressed the level of pro-apoptosis gene Bax and Bak, and obviously promote the level of anti-apoptosis gene Bcl-2 and Bcl-xl (Fig. 2).

Further experiments demonstrated that the overexpression of miR-134 markedly reduced the mRNA and protein level of WWOX. WWOX gene is a tumor suppressor, and evidence suggested that WWOX expressions were reduced or absent in the majority of lung cancer tissues [26]. In the present study, down-regulation of WWOX mRNA and protein expression was observed in H69 cells with ectopic miR-134 expression, and miR-134 inhibitor of over-expression WWOX both up-regulated the level of WWOX. We also showed that the effects of miR-134 on the growth of H69 cells were dependent on the expression of WWOX. Taken as a whole, we provide evidence that miR-134 promote lung cancer cell H69 growth by targeting tumor suppressor WWOX gene. Our data also imply that overexpression of miR-134 can also modulate apoptosis in H69 cells via regulating the expression of Bcl-2, Bcl-xl, Bax and Bak. Moreover, our study also showed that miR-134 modulates the proliferation and apoptosis of H69 cells by targeting WWOX gene and suppressing the ERK1/2 signaling pathway. These findings support that an anti-miR-134 strategy may be serve as a potential intervention for SCLC.

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