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ORIGINAL ARTICLE

The effects of miR-375 expression in NSCLC via the 14-3-3ζ/ERK/MYC pathway

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Abstract

Objective There are several reports that suggest a significant role played by microRNAs (miRNAs) in cell invasion, metastasis, differentiation, and apoptosis in lung cancers. miR-375 is one such miRNA that has been detected in a variety of tumors, but its specific activity in non-small cell lung cancer (NSCLC) remains unclear.

Methods In this study, we regulated the expression of miR-375, to evaluate its influence on the 14-3-3ζ/ERK/MYC pathway in NSCLC.

Results The results of our experiments suggest that miR-375 and 14-3-3ζ are highly expressed in NSCLC, and the over-expression of miR-375 increases the invasive, metastatic, and proliferative ability and decreases the apoptotic ability of NSCLC cells. In addition, protein expression levels of 14-3-3ζ, p-ERK, and MYC increased following the overexpression of miR-375.

Conclusion Overall, our findings indicate that miR-375 increases the malignant potential of NSCLC via the 14-3-3ζ/ERK/MYC pathway.

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Key words: NSCLC; miR-375; 14-3-3ζ; ERK; MYC

Lung cancer remains the leading cause of cancer death and is the most frequently diagnosed cancer in men and women [1–2]. Moreover, the morbidity and mortality rates of lung cancer have steadily increased over the last few decades [1]. Lung cancer is broadly classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for 75%–80% of all lung cancers and is further divided into large cell carcinoma, adenocarcinoma and squamous cell carcinoma of which, adenocarcinoma is the most common type of lung cancer [3]. MicroRNAs (miRNAs) are a class of small endogenous noncoding RNA molecules, 19–22 nucleotides long, which act as negative regulators of target gene expression in the post-transcriptional phase to modulate a variety of cell processes [4–5]. miRNAs can regulate cellular processes such as proliferation, growth, apoptosis and differentiation [5–6]. miRNAs are believed to be oncogenes when they are highly expressed in cancers [7–8]. Among all miRNAs, miR-375 specifically can affect cell proliferation, apoptosis and survival [9–10].

The 14-3-3 protein is widely expressed in eukaryotic cells. Proteins can mediate a variety of signaling pathways in diverse biological processes. They can also bind to either phosphoserine/phosphothreonine in specific sequences of target proteins to mediate their function [11–13]. The 14-3-3ζ subtype combines with other signal molecules to participate in signal transduction, apoptosis, cell cycle regulation, and malignant transformation [11–14]. The 14-3-3 protein has seven subtypes (α/β, γ, σ, ε, ζ, η, and θ/τ) of which the positive expression of the zeta subtype has been identified in non-small cell lung cancer [15–17]. Our study primarily explored the effect of miR-375 on NSCLC, through the 14-3-3ζ/ERK/MYC pathway.

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Materials and methods

Cell and tissues

Two lung cancer cell lines (H520, A549) and a normal lung cell line (BEAS-2B) were obtained and cryopreserved in the laboratory. Lung cancer cells were cultured in RPMI-1640 medium and normal cells were cultured in LHC-9 medium, both of which were supplemented with 10% fetal bovine serum (ExCell Bio, Shangai). Trypsin-EDTA (Solarbio, Beijing) was used to digest the parietal cells. The sample was obtained from the Qingdao Municipal Hospital. The participants in this study signed a written consent and the study was approved by the Ethics Committee of the Qingdao University Affiliated Hospital.

Cell Transfection

The lentiviral cell line which overexpressed miR-375, (Lv-miR-375-A549) and the lentiviral cell line without the miRNA (Lv-NC-A549) were obtained from Jikai gene. A viral inoculum, at MOI=20, was transfected into the A549 cell line and cultured at 37oC, in 5% CO_2, for eight hours. Viruses were then isolated and cultured further.

cDNA Synthesis and qRT-PCR

The ISO plus RNA Kit (Takarabio, Japan) was used to extract total RNA from the cells. Reverse transcription was performed to synthesize cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takarabio). SYBR Premix Ex Taq RR420A (Takarabio) was used for fluorescent quantitative PCR using the CFX96 Touch Real-Time PCR Detection System (BioRad, CA, USA). The primers used in the experiment were purchased from Shanghai Sangon Biotech. The endogenous target gene mRNA was based on beta-actin, and the miRNA, RNA U6, was used as the endogenous reference.

The primers designed were as follows:

Forward primer: 5 '-CTCGCTTCGGCAGCACA-3';

Reverse primer: 5 '-AACGCTTCACGAATTTGCGT-3'

For miR - 375:

Forward primer: 5 '-CGCGCTTTGTTCGTTCGGCTC-3';

Reverse primer: 5 '-ATCCAGTGCAGGGTCCGAGG-3'

Transwell assay

Invasion assay: The complete medium dilution matrix with a ratio of 1:9 (Corelle, Cambridge, MA, USA) on the indoor shop on the three-hole room, was placed in an incubator for 4 hours. Following solidification of the Matrigel, the three kinds of cells in the 50 000 / three holes were inoculated into the small room, the room on the train medium without the bovine serum, and the room under the medium with 15% fetal bovine serum. After 48 hours, the cells were extracted, and the number of cells that had migrated through were fixed with methanol, stained with crystal violet, photographed, and counted. The extracted cells were re-inoculated, and the above steps were repeated.

Mobility test: three cells were inoculated into three small rooms with 50 000 / orifice and were incubated for 24 hours according to the above methods. Then the steps in the invasion experiment were carried out.

Wound healing assay

The same number of cells were inoculated in a 6-well plate. When the cells reached confluency, a scratch (wound) was made in the cell layer with a sterile pipette tip. Unadhered cells were washed off with phosphate buffered saline (PBS). The wound was photographed at 0 and 48 hours and its size was measured.

Cell proliferation assay

Cell proliferation was evaluated using CCK-8 (Multi Sciences, China). Briefly, 5×10^3 cells/well were plated in 96 well plates and 100 μL of culture medium was added to each well. After 24 h, the CCK-8 × stock solution was mixed with the culture medium at a ratio of 1:9. Then, 100 μL of the above mixture was added to each well and after 4 h, absorbance was measured using a microplate reader (Bio-Rad, CA, USA).

Flow cytometry

Three types of cells, including A549, Lv-NC-A549, and Lv-mir-375-A549, were collected and flow cytometry was performed using the Annexin V-APC/7-AAD Kit (Multi Sciences) according to the manufacturer's instructions.

Western blot analysis

The cells were washed twice with PBS, scraped off with a cell scraper, and centrifuged at 5000 *xg* at 4 °C, for 5 min; the supernatant was discarded. RIPA buffer was then added to the cell pellet. Phosphatase and protease inhibitors were also added to the cell pellet. This was followed by incubation on ice for 15 min, vortexing and lysing on ice for 15 min and centrifugation at 12,000 *xg* for 10 min. The supernatant, sample buffer and SDS-loading buffer were placed in a boiling water bath for 10 minutes. The proteins were separated by SDS-PAGE and transferred onto a PVDF membrane (Millipore, USA). The membrane was blocked with 5% skimmed milk and was then incubated with primary antibodies (Anti 14-3-3 antibody, 1:1000 dilution; Anti MYC antibody, 1:1000 dilution; Anti ERK antibody, 1:1000 dilution) (Cell Signaling Technology, USA) overnight at 4 °C. After washing, secondary antibody (1:1000) (Abcam, USA) was added and the membrane was placed on a low speed shaker for 2 h. Chemiluminescent signals were detected using enhanced chemiluminescent substrate reagents

(ECL).

Statistical analysis

All experimental data were averaged at least three times. The Bonferroni correction test was used to compare the two groups. The difference between groups was analyzed by One-way ANOVA test and a P value of < 0.05 was considered as statistically significant.

Results

MiR-375 and 14-3-3ζ are upregulated in NSCLC tissues and in the A549 cell line

The expression levels of miR-375 and 14-3-3ζ were detected using RT-PCR in 10 NSCLC tissue samples, normal lung tissues, and in the A549, H520, and BEAS-2B cell lines. The results showed that the expression levels of miR-375 and 14-3-3ζ in NSCLC tissues were higher than those in normal lung tissues and the expression levels of miR-375 and 14-3-3ζ were higher in A549 cells compared to those in H520 and BEAS-2B cells (P <0.05) (Fig. 1a and 1b). These data suggest that miR-375 and 14-3-3ζ might be potential prognostic markers for NSCLC.

The level of miR-375 was upregulated in A549 cells

Considering the increase of miR-375 in A549 cells, we presumed that miR-375 might be an oncogene in NSCLC. Therefore, we decided to overexpress miR-375 in A549 cells through lentiviral transfection. The transfection success rate exceeded 80% and therefore could be used for further experiments (Fig. 1c). We then investigated whether miR-375 was successfully upregulated using qRT-PCR. The results confirmed that exogenous miR-375 was successfully upregulated in A549 cells (P < 0.05) (Fig. 1d). The expression of 14-3-3 ζ subtype was also increased along with the upregulation of miR-375 (P < 0.05) (Fig. 1d).

MiR-375 promotes the migration and invasion of A549 cells

Previous experimental results have shown that miR-375 is highly expressed in NSCLC tissues (Fig. 1a and 1b). However, the influence of miRNA-375 on the invasion and metastasis of NSCLC remains to be explored. We first established a cell line, stably expressing miR-375 through lentiviral transfection. Next, we investigated the changes in cell invasion and metastasis following stable transfection of miR-375, using transwell and wound healing assays. The transwell assay showed an increase in the invasion and migration capability of cells stably expressing miR-375 compared to control cells (P <0.05)

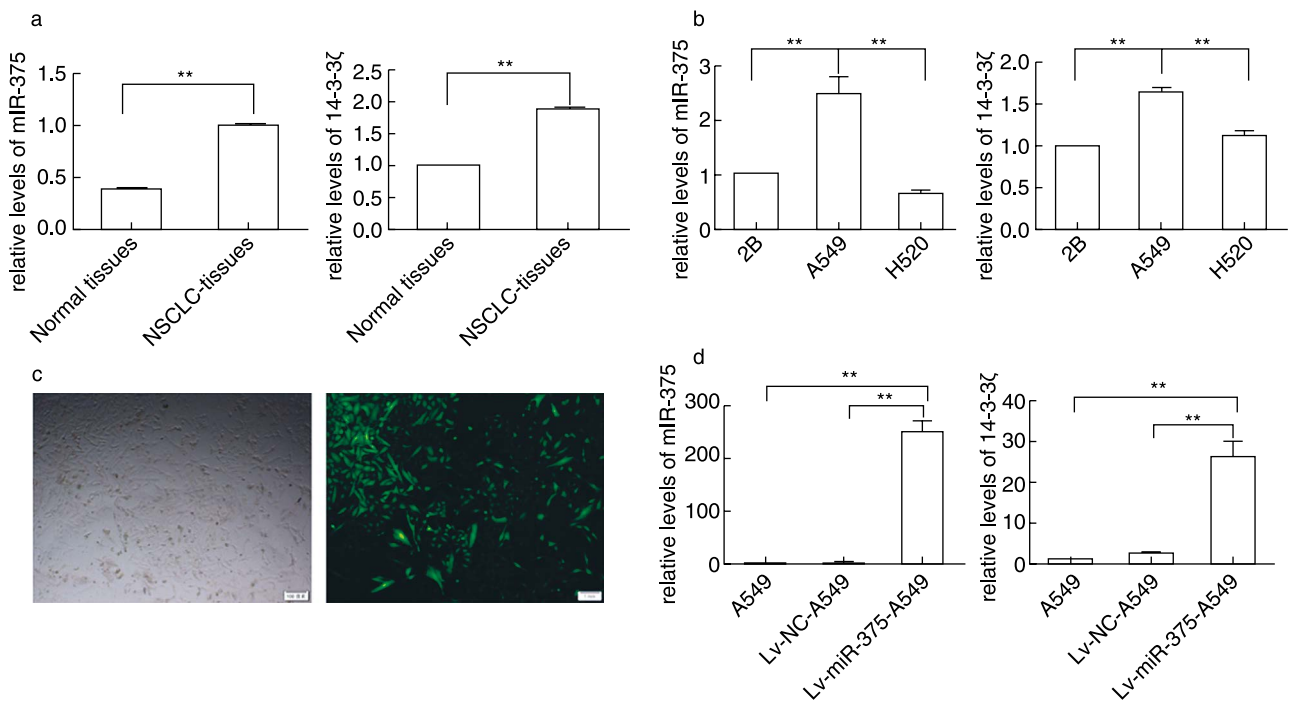

Fig. 1 Upregulated expression levels of miR-375 and 14-3-3ζ in tissues and cell lines and successful overexpression of miR-375. (a) Expression of miR-375 and 14-3-3ζ in normal tissues and NSCLC tissues; (b) The expression of miR-375 and 14-3-3ζ in normal and NSCLC cells; (c) Effect of lentiviral transfection on A549 cells; (d) Expression of miR-375 and 14-3-3ζ in A549, Lv-NC-A549, and Lv-miR-375-A549 cells. $^{**}P$ < 0.01

(Fig. 2b and 2c). Similarly, the wound healing assay also showed increased migratory ability in cells stably expressing miR-375 ($P < 0.05$) (Fig. 2a). Collectively, these data indicate that miR-375 has a positive effect on the invasion and metastasis of human NSCLC.

MiR-375 promotes the proliferation of A549 cells

Next, we investigated whether miR-375 regulated the proliferation of A549 cells. CCK-8 reagent was added 24, 48, and 72 h after the inoculation of A549 cells, and cell proliferation was evaluated after 4 h. The results showed that compared to the control and empty vector groups, the proliferative ability of A549 cells was significantly increased with the overexpression of miR-375 ($P < 0.05$) (Fig. 3a). This data confirms that miR-375 promotes the proliferation of A549 cells.

MiR-375 inhibits the apoptosis of A549 cells

We investigated the apoptotic ability following overexpression of miR-375, using flow cytometry. Under similar conditions, the apoptotic rate in cells overexpressing miR-375 was 12.1%, while the apoptotic rates in the control and empty vector transfected groups were 27.57% and 26.55%, respectively. The apoptotic rate in the cells with miR-375 overexpression was >10% than those in the other two groups ($P < 0.05$) (Fig. 3b). This demonstrates that miR-375 inhibits the apoptosis of A549 cells.

MiR-375 can regulate the 14-3-3 /ERK/MYC signaling pathway in NSCLC

The 14-3-3ζ/ERK/MYC signaling pathway plays an important role in cell proliferation, invasion and metastasis. Therefore, we investigated whether this signaling pathway is affected by miR-375 expression in NSCLC. As expected, we found that with the overexpression of miR-375, the expression of 14-3-3ζ and MYC was increased ($P < 0.05$) (Fig. 4a, 4b and 4c), and while the expression of total ERK did not change significantly ($P > 0.05$) (Fig. 4a and 4d), the expression of p-ERK was significantly increased ($P < 0.05$) (Fig. 4a

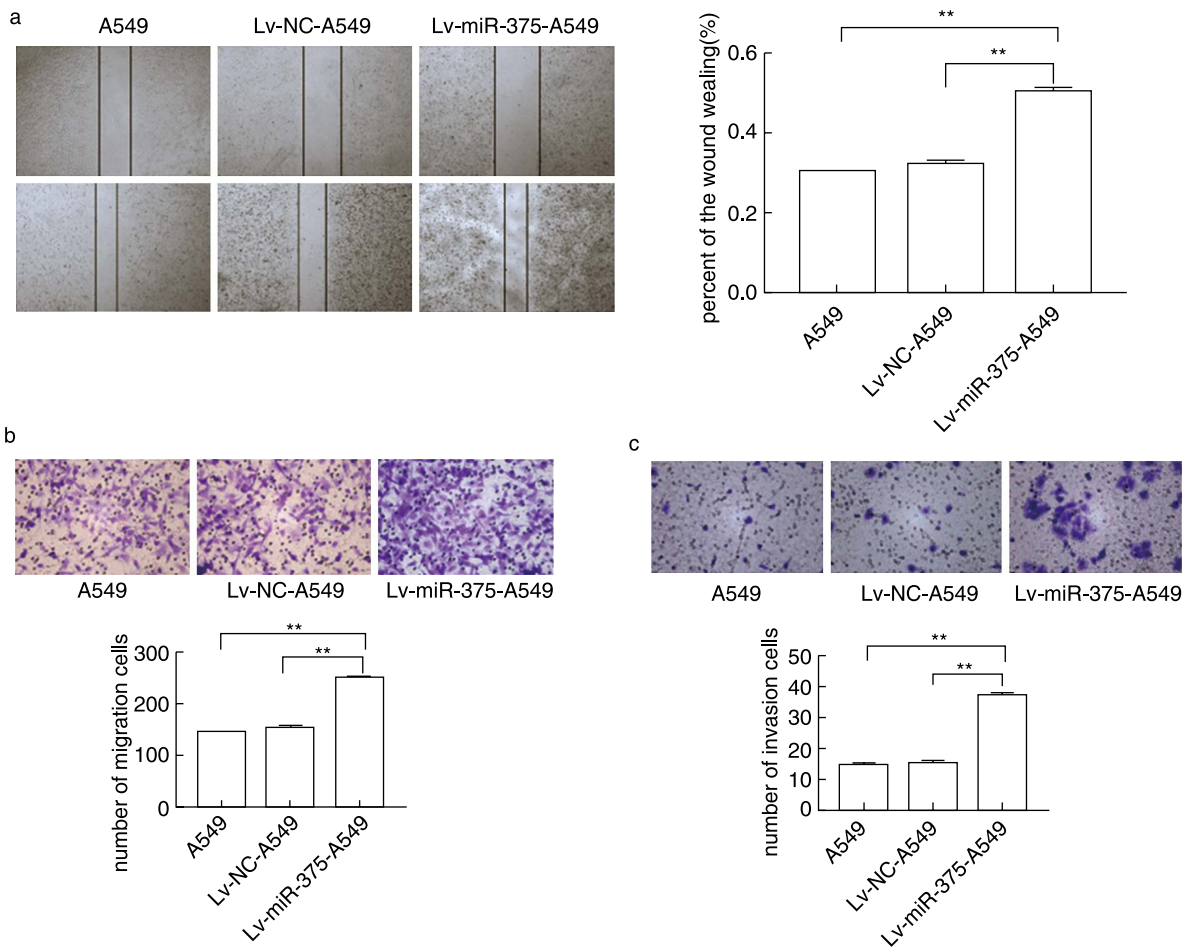

Fig. 2 The effects of miR-375 on cell invasion and migration. (a) Image and data analysis of wound healing assay; (b) Image and data analysis of transwell migration assay; (c) Image and data analysis of transwell invasion experiment. $^{**}P < 0.01$

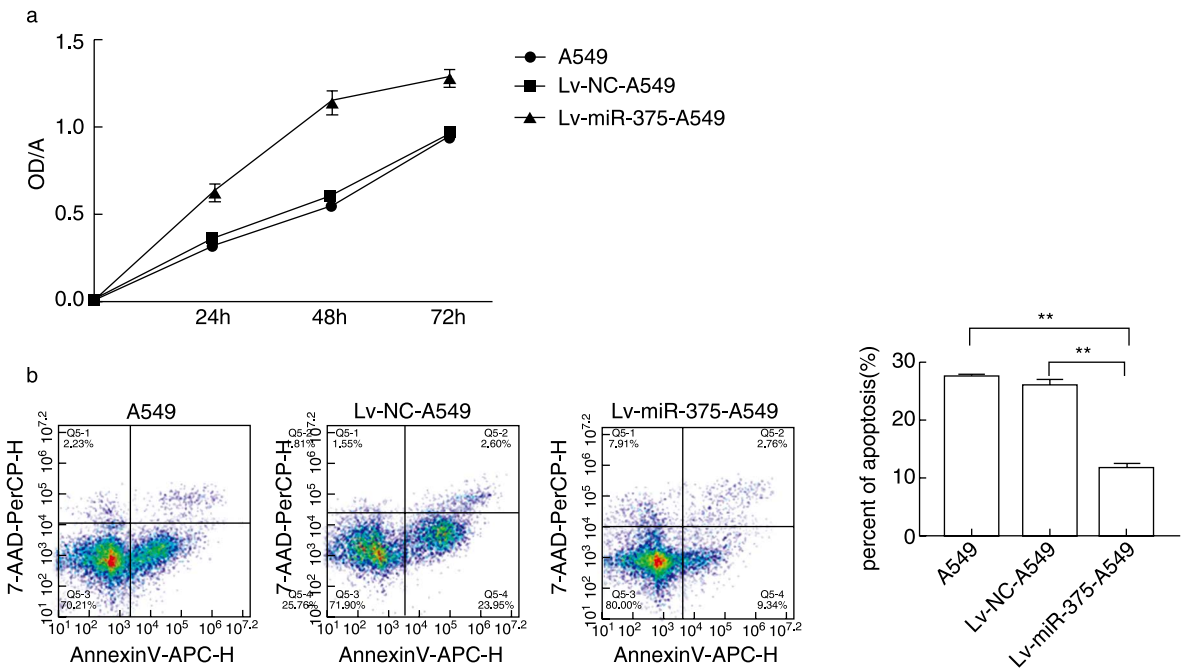

Fig. 3 The effect of miR-375 on cell proliferation and apoptosis. (a) Data analysis of apoptosis assay; (b) Flow cytometry results - image and data analysis. $^{**}P < 0.01$

and 4e). This analysis indicates that the 14-3-3ζ/ERK/MYC signaling pathway is regulated by miR-375, which might explain the tumor promoting effect of miR-375 in NSCLC.

Discussion

Inhibiting metastatic progression in order to prolong the survival of patients with NSCLC remains a challenge. Existing evidence demonstrates that the regulation of miRNAs in NSCLC affects the regulation of various processes of tumorigenesis and cancer progression [18]. Previous studies have shown that more than half of the miRNAs are located in cancer-related genomic regions, suggesting that the dysregulation of miRNAs possibly plays a key role in tumor occurrence and progression19. Understanding the molecular mechanism of these miRNAs might lead to the identification of novel diagnostic and therapeutic targets for NSCLC. Research suggests that miR-375 plays an important role in many diseases [20–22]. It is undebatable that miR-375 is an important cancer-related miRNA. However, the specific mechanism of action of miR-375 in NSCLC remained unclear. Therefore, our study was focused on elucidating the role of miR-375 in NSCLC.

In the present study, our experiments demonstrate that miR-375 is highly expressed in the NSCLC cell line, A549. Overexpression of miR-375 in these cells resulted in increased invasion, proliferation, and metastasis, and a decrease in apoptosis. Hence, miR-375 was hypothesized as an oncogene in NSCLC. This observation is consistent with the findings from other studies which also have demonstrated increased expression of miR-375 in NSCLC, prostate carcinoma, and breast cancer [23–26]. In contrast, there are other evidences which suggest that miR-375 is often poorly expressed in many types of cancers and can, in fact, act as a tumor suppressor through the regulation of many important target oncogenes [22, 27–30]. However, based on our results and supporting literature, we believe that the expression and role of miR-375 varies in different tumors, which might enable it to play both an oncogenic as well as tumor suppressor role depending on the tumor type. Several studies are now investigating the potential value of miR-375 as a prognostic biomarker, but the specific role of miR-375 in cancer remains to be elucidated.

We found that the mRNA and protein expression of 14-3-3ζ was upregulated when miR-375 was highly expressed, indicating that miR-375 might promote the expression of 14-3-3ζ. Members of the 14-3-3 family of proteins include seven mammalian isoforms (α/β, γ, σ, ε, ζ, η, and θ/τ) and have acidic polypeptides of approximately 28–33 kDa size. To date, only six members of the 14-3-3 protein family (α/β, γ, σ, ε, θ/τ, and ζ) and particularly the 14-3-3ζ subtype has been found to be positively expressed in NSCLC15. This subtype forms complexes with Hsp27

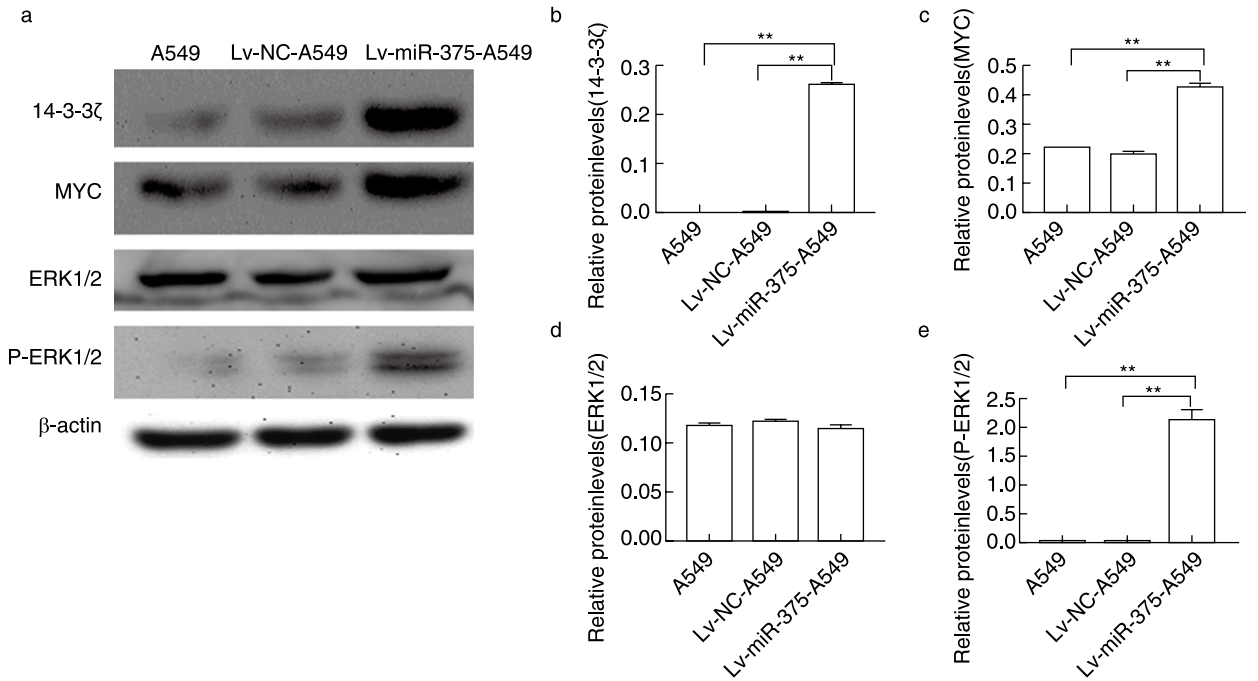

Fig. 4 14-3-3ζ/ERK/MYC signaling pathway is regulated by miR-375. (a) Protein expression of 14-3-3ζ/MYC/ERK in A549, Lv-NC-A549, and Lv-miR-375-A549; (b) Expression of 14-3-3ζ in the three groups of cells; (c) Expression of MYC in the three groups of cells; (d) Expression of ERK1/2 in the three groups of cells; (e) Expression of p-ERK1/2 in the three groups of cells. $^{**}P < 0.01$

or β-catenin leading to increased epithelial-mesenchymal transition, metastasis, and angiogenesis. Hence, it has been implicated as a prognostic and therapeutic target [16–17, 31–32]. Our experimental results indicate that the expression of 14-3-3ζ can be modulated by miR-375, but we have not conducted luciferase experiments to verify whether 14-3-3ζ is a direct target of miR-375. However this association has previously been demonstrated in another study [33].

Extracellular signal-regulated kinase (ERK1/2) is a serine-threonine kinase that is positively regulated by MEK1/2-mediated phosphorylation. The ERK pathway is a classical, mitogen-activated protein kinase (MAPK) signaling cascade and ERK1/2 regulates cell proliferation, growth, apoptosis, and differentiation [34–35]. In our experiments, we overexpressed miR-375 and consequently, the expression of 14-3-3ζ protein also increased. Considering that, the altered expression of 14-3-3ζ has been linked to ERK/MYC signaling pathway [36–38], we also evaluated the expression of ERK and MYC. According to the results of western blot analysis, we found that miR-375 affects the ERK/MYC pathway through the regulation of 14-3-3ζ expression.

In summary, our results suggest that miR-375 regulates tumorigenesis and malignant progression of NSCLC via 14-3-3ζ/ERK/MYC signaling pathway. Therefore, miR-375 could be a potential molecular target in the treatment of NSCLC. Further studies are required to evaluate the potential of utilizing miR-375 as a therapeutic target for NSCLC.

Conclusion

Our findings suggest that miR-375 promotes the malignancy of non-small cell lung cancer through the 14-3-3 ζ /ERK/MYC pathway.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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