Abnormal activation of the EGFR signaling pathway mediates the downregulation of miR-145 through the ERK1/2 in non-small cell lung cancer

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Abstract. The expression of miR-145 with tumor suppressor function is decreased in lung cancer cells. Epidermal growth factor receptor (EGFR) signaling pathway is abnormally activated in lung cancer cells. It is not clear whether the EGFR signaling pathway is involved in the regulation of miR-145 expression in lung cancer. In the present study, we found that the reduction of miR-145 was associated with EGFR abnormal activation in lung cancer cells. AG1478, an inhibitor of EGFR, may restore the expression of miR-145, indicating that EGFR activation is involved in the downregulation of miR-145 in lung cancer cells. Then, the application of STAT3, AKT and ERK1/2 inhibitors and siRNA against these signaling molecules indicated that ERK1/2 or AKT instead of STAT3 was involved in the process of miR-145 downregulation by EGFR. It was confirmed that AKT through activation of the ERK1/2 signaling molecules mediated the effect of EGFR on miR-145. Furthermore, we found that EGFR downregulated miR-145 through ERK1/2 in lung cancer cells. These findings establish EGFR and miR-145 links in lung cancer cells and therefore contribute to a better understanding of the role of EGFR in lung cancer cells, and provide clues for in-depth study of miR-145 expression and a possible direction for the further increase of miR-145 in lung cancer cells.

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Introduction

In recent years, several studies have indicated that aberrant miRNA levels occur in multiple human malignancies including lung cancer (1-3). It has been proposed that some miRNAs are downregulated during tumorigenesis and seem to function as tumor suppressors, while others are upregulated and may control proto-oncogenic signals (4,5). miR-145 with tumor suppression gene function has been described in lung cancer (6). Decreased levels of miR-145 expression that can control many tumor-associated targets such as c-Myc, STAT1, YES, lose their tumor suppression function and contribute to tumorigenesis (7,8). Although miR-145 may potentially alter complex cellular processes, the molecular mechanisms of downregulated miR-145 levels in lung cancer cells remain largely unknown.

Epidermal growth factor receptor (EGFR) is a 170 kDa type I transmembrane growth factor receptor with tyrosine kinase (TK) activity and belongs to the HER/erbB family of receptor TKs, which contains HER1 (EGFR), HER2, HER3 and HER4 (9). With binding to its specific ligands (such as EGF), homodimerization and/or heterodimerization with other family members activates the TK, and subsequently leads to autophosphorylation of the cytoplasmic domain of the receptor and enables it to interact with adaptor molecules, which couple the receptors to downstream signaling pathways. It was reported that all lung carcinomas almost closely related to EGFR ectopic expression, including overexpression of EGFR protein, EGFR TK domain mutations or EGFR gene copy amplification, any of which may result in overactivation of EGFR signaling pathway (10). Previous studies have shown that activation of the EGFR signaling pathway interacts with miRNAs to promote tumor formation (11). Due to the EGFR signaling pathway activation and the downregulation of miR-145 in lung cancer cells, we inferred that there is some connection between them. To date, there is no evidence to support that the activation of EGFR plays a regulatory role in miR-145 in lung cancer cells.

In the present study, we identified that EGFR signaling pathway negatively regulated the expression of miR-145 by ERK1/2 in human lung cancer cell lines. These findings may

indicate that the EGFR with abnormal activation affects the expression of miRNAs through its downstream signaling molecules, involved in the occurrence and development of lung cancer.

Materials and methods

Cell culture, reagents and treatments. Normal human lung epithelial cell line (BEAS-2B), human lung adenocarcinoma cell line (H1650) with EGFR mutation (del L747_E749 and A750P), human lung adenocarcinoma cell line (H1975) with EGFR mutation (L858R), human lung adenocarcinoma cell lines (A549 and H292) with wild EGFR were obtained from American Type Culture Collection (Manassas, VA, USA). All cell culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Briefly, the cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum under standard conditions (37˚C in a humidified atmosphere containing 5% CO_2)

To evaluate the effect of AG1478 on the EGFR signaling pathway and miR-145 expression levels, cells were serum starved for 24 h, incubated in the presence or absence of AG1478 (5 μM; Calbiochem) for 2 h, and then for an additional 24 h in the presence or absence of EGF (20 ng/ml; Promega). U0126 (Cell Signaling Technology, Inc., Beverly, MA, USA), a pharmacological MEK1/2 inhibitor, was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and stored at -20˚C until used (10 μM). LY194002, PI3K kinase inhibitor and AG490, JAK2 inhibitor, were purchased from Calbiochem and dissolved in DMSO at a recommend concentration and stored at -20˚C.

Western blot analysis. Cells were washed once with PBS and then lysed in buffer containing 10 mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, pH 8.0, 10 ml/l Triton X-100, 1 mM DTT and 0.1 mM phenylmethanesulfonyl fluoride (PMSF). After 30 min on ice, lysates were collected and clarified by centrifugation at 15,000 x g for 5 min at 4˚C. Protein concentrations were measured by BCA protein detection kit (Pierce, Rockford, IL, USA). Equal amounts of protein (10-30 μg/lane) from whole-cell lysates were separated by gel electrophoresis on 10% gels, transferred to nitrocellulose membranes and were probed with specific primary antibody [phospho-EGFR (Tyr1068) rabbit mAb; phospho-AKT (Ser473) rabbit mAb; phospho-ERK1/2 (Thr202/Tyr204) rabbit mAb; phospho-STAT3 (Tyr705) rabbit mAb; EGFR rabbit mAb; ATK rabbit mAb; ERK1/2 rabbit mAb; STAT3 rabbit mAb; Cell Signaling Technology, Inc.] and then with the appropriate HRP-conjugated secondary antibodies. Proteins were detected using the enhanced chemiluminescence detection kit (Thermal Science, Rockford, IL, USA). For loading control, the membrane was probed with a monoclonal antibody for GAPDH (Kangchen Biotechnology, Shanghai).

RNA isolation. Total RNA was isolated from the cultured cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions and quantified by a spectrophotometer. To assess the purity of RNA, optical density (OD) was measured at 260 and 280 nm for determination of OD_{260}/OD_{280} ratio. The RNAs with >1.8 OD ratios were used in this study. Relative abundance and integrity of 18s and 28s ribosomal bands were assessed with formaldehyde denaturing agarose gel. Those RNAs that exhibited intact 18s and 28s ribosomal bands with 1:1.5 relative abundance ratios were used in the present study.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. Expression of mature miRNAs was examined by qRT-PCR analysis using a Real-time PCR Universal Reagent kit according to the manufacturer's instructions (GenePharma, Co., Ltd., Shanghai). In brief, two-step qRT-PCR procedure was performed. Reverse transcription was performed in 20 μl volume, starting with 10 ng of total RNA. The reaction mixture was initially heated to 25˚C for 30 min, 42˚C for 45 min, 85˚C for 10 min and finally to 4˚C for 5 min. In the PCR step, PCR products were amplified from cDNA samples using specific miRNA primers and all assays were performed in triplicate on the MX3000P Real-time PCR Instrument (Stratagene, USA). The primers used were: miR-145, 5'-ATC GTC CAG TTT TCC CAG G-3' (forward), and 5'-CGC CTC CAC ACA CTC ACC-3' (reverse); U6 snRNA, 5'-ATT GGA ACG ATA CAG AGA AGA TT-3' (forward), and 5'-GGA ACG CTT CAC GAA TTT G-3' (reverse). The assay tubes were initially heated to 95˚C for 3 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. The expression levels of candidate miRNAs were evaluated by the comparative CT method and were normalized using U6 snRNA as the endogenous control. Relative quantitative expression levels of miRNAs were determined by the $2^{-\Delta\Delta CT}$ method.

Transient transfection of small interfering RNA (siRNA). The human cell lines were seeded in a 6-well plate and cultured to 70% confluence. siRNA oligonucleotides targeting STAT3, AKT, ERK1/2 and negative control were purchased from GenePharma. The siRNA sequences were: STAT3 siRNA sense, 5'-GCA GCA GCU GAA CAA CAU GTT-3' and antisense, 5'-CAU GUU GUU CAG CUG CUG CTT-3'; AKT siRNA sense, 5'-UGC CCU UCU ACA ACC AGG ATT-3' and antisense, 5'-UCC UGG UUG UAG AAG GGC ATT-3'; ERK1 siRNA sense, 5'-GAC CGG AUG UUA ACC UUU ATT-3' and antisense, 5'-UAA AGG UUA ACA UCC GGU CTT-3'; ERK2 siRNA sense, 5'-CAC CAA CCA UCG AGC AAA UGT T-3' and antisense, 5'-CAU UUG CUC GAU GGU UGG UGT T-3' and negative control sense, 5'-UUC UCC GAA CGU GUC ACG UTT-3' and antisense, 5'-ACG UGA CAC GUU CGG AGA ATT-3'. Cells were transfected with siRNA (100 nM) by using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. The selective silencing of STAT3, AKT, ERK1/2 was confirmed by western blot analysis.

Statistical analysis. All experiments were repeated a minimum of three times. Error bars indicate standard errors of mean. Microsoft Excel or Instat software (GraphPad Prism4; San Diego, CA, USA) was used to analyze the data. A Student's t-test or one-way analysis of variance (ANOVA) was used for parametric data. Correlation analysis was made by using Pearson's correlation coefficient. $P<0.05$ was considered to indicate a statistically significant difference.

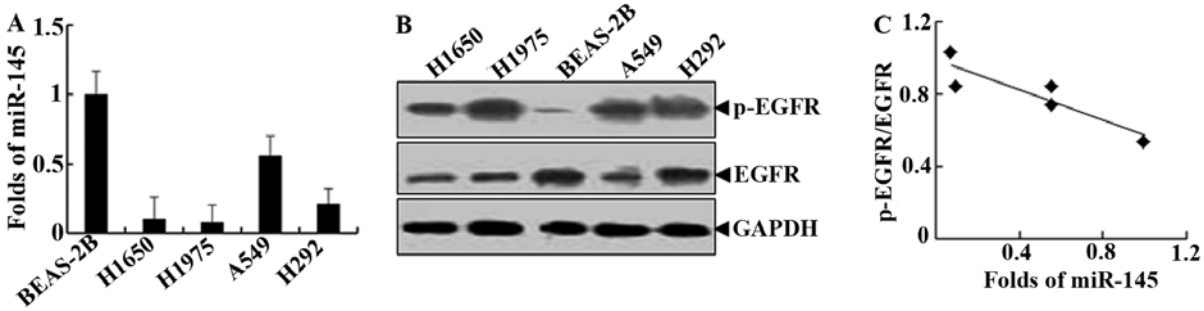

Figure 1. The relationships between the downregulation of miR-145 and the activation of EGFR were detected in lung cancer cells. miR-145 expression levels were analyzed by qRT-PCR. miR-145 expression was normalized by U6 RNA. miR-145 in BEAS-2B was defined as 1. (B) The activation of the EGFR was determined by western blotting in BEAS-2B and lung cancer cells. (C) Correlation between miR-145 expression and p-EGFR levels (Pearson's correlation, r=-0.926, P<0.05). The miR-145 data were from panel (A), and the pi-EGFR data were obtained by quantitatively analyzing the results shown in panel (B).

Results

The EGFR signaling pathway is associated with the downregulation of miR-145 in lung cancer cells. The levels of miR-145 in human normal lung bronchial epithelial cells (BEAS-2B) and lung cancer cells (H1975, H1650, A549 and H292) were determined by qRT-PCR. The results showed that the levels of miR-145 in four lung cancer cell lines were significantly lower than in normal lung epithelial cells (P=0.025; Fig. 1A). Although there were no significant changes in EGFR protein between human normal lung bronchial epithelial cells and lung cancer cells, EGFR activity in the form of pi-EGFR expression in lung cancer cells was higher than in normal lung epithelial cells (P=0.032; Fig. 1B). The quantitative comparison of miR-145 and p-EGFR levels showed a significant negative correlation between these two factors (Pearson's correlation, r=-0.926, P<0.05). These results suggest that the activated EGFR signaling pathway may be functionally associated with miR-145 downregulation.

Activation of the EGFR signaling pathway downregulates the expression of miR-145 and inhibition of the EGFR signaling pathway restores the expression of miR-145 in lung cancer cells. To further investigate the effect of EGFR status on miR-145 expression, EGFR-mutant H1975 cells, EGFR wild-type A549 cells and human normal lung BEAS-2B cells were first starved for 24 h with serum free culture and then treated with AG1478 (a specific inhibitor of EGFR) in the presence or absence of EGF (12,13). The results showed that there was no change in the total EGFR protein. In the three cells, the levels of p-EGFR protein were significantly increased in the cells treated with EGF, compared with the respective control group. The AG1478 completely blocked the activation of EGF to EGFR (Fig. 2A, C and E). The levels of miR-145 were significantly decreased in the treatment of EGF. The AG1478 may restore the downregulation of EGF to miR-145 in A549, H1975 and BEAS-2B cells (Fig. 2B, D and F).

AG490, LY294002 and U0126 upregulate the levels of miR-145 in lung cancer cells. Due to the mutation of EGFR in H1975 cells, the EGFR signaling pathway is highly activated (14). This was confirmed by detecting the EGFR downstream signaling molecules STAT3, ERK1/2 and AKT. STAT3, ERK1/2 and AKT activity was inhibited when AG1478 blocked EGFR phosphorylation in H1975 cells (Fig. 3A). However, it was not clear whether these signaling molecules were involved in the process of miR-145 downregulation by EGF-EGFR. Therefore, H1975 cells were treated with the three signaling molecule inhibitors AG490, LY294002 and U0126 (15-17). The data obtained indicated that the levels of miR-145 were upregulated after the inhibitors blocked the corresponding signal molecules (Fig. 3B-G).

STAT3 signaling molecules are not involved in the regulation of miR-145 downregulated by EGFR in H1975 cells. Although the AG490, a relatively specific chemical inhibitor for STAT3, may not inhibit the phosphorylation of AKT, it reduces the levels of p-ERK1/2 (18). Thus, we hypothesized that the levels of miR-145 in lung cancer cells may be restored by the non-specific inhibition of AG490. In order to determine whether STAT3 signaling molecules are involved in the regulation of miR-145 expression, the siRNA against STAT3 was used to treat H1975 cells. The results showed that the levels of miR-145 presented almost no change while STAT3 expression and its phosphorylation levels were suppressed; there was also no effect on p-ERK1/2 (Fig. 4). These observations suggest that STAT3 signaling molecules were not involved in the downregulation of miR-145 by the activation of EGFR in lung cancer cells.

AKT signaling molecules are involved in the regulation of miR-145 expression through the activation of ERK1/2. LY294002 is a potent inhibitor of phosphoinositide 3-kinases (PI3Ks), an upstream molecule of AKT (19). In H1975 cells treated with LY294002, the activation of AKT and ERK1/2 was effectively suppressed, but LY294002 did not inhibit the STAT3 activity (Fig. 5A). At the same time, the miR-145 levels were increased by LY294002 in H1975 cells. Therefore, we hypothesized that the levels of miR-145 in lung cancer cells may be restored by the pi-ERK1/2 inhibition of LY294002. To explore the role of AKT in the regulation of miR-145, the siRNA against AKT was used to treat H1975 cells. The data demonstrated that ERK1/2 phosphorylation levels were blocked after the specific inhibition of the activation of AKT signaling molecules (Fig. 5B). We detected that miR-145 expression was upregulated in the AKT siRNA group (Fig. 5C). This indicated that the miR-145 levels were regulated by AKT via the activation of ERK1/2.

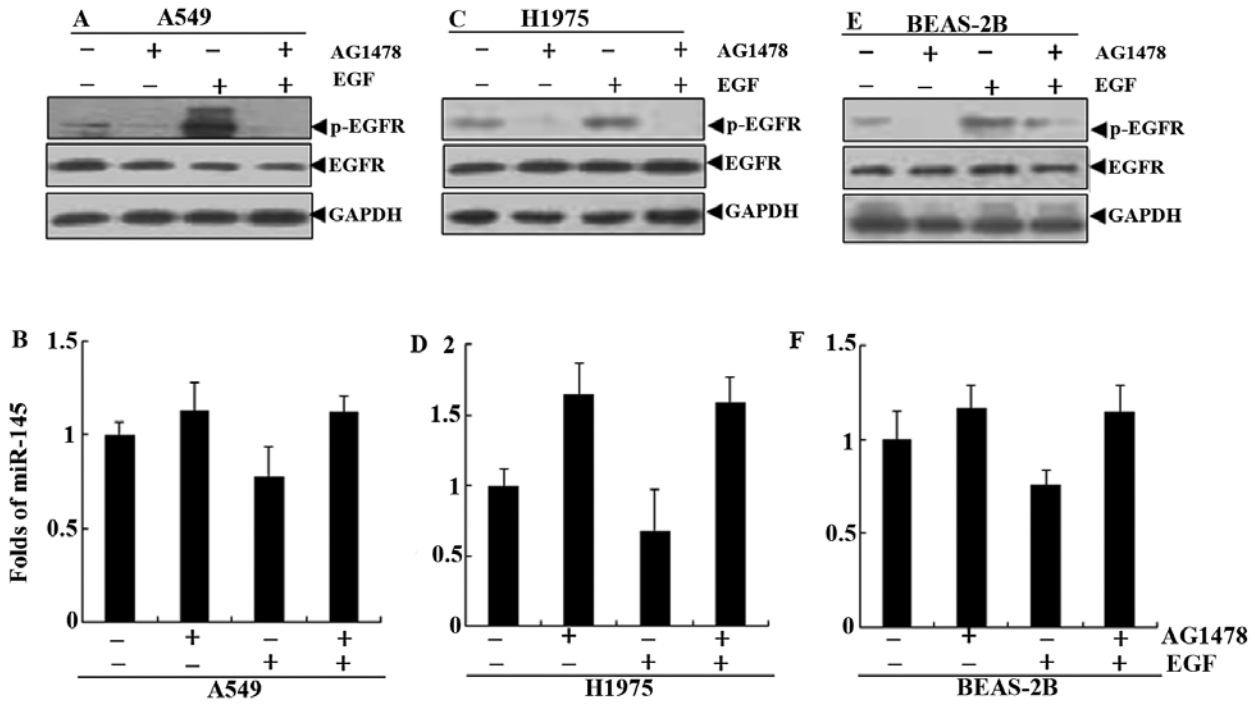

Figure 2. AG1478 blocks the effects of miR-145 downregulated by EGF. BEAS-2B, A549 and H1975 were cultured with serum-free RPMI-1640 for 24 h, and the cells were then treated with or without 20 ng/ml EGF for 24 h after using 5 μM AG1478 for 2 h. (A, C and E) Specified protein was detected by western blotting. (B, D and F) The levels of miR-145 were detected by qRT-PCR. The relative expression miR-145 was defined as 1 in respective control cells. miR-145 expression levels were expressed values relative to untreated cells in the absence of EGF. Data were means ± SD from 3 independent experiments.

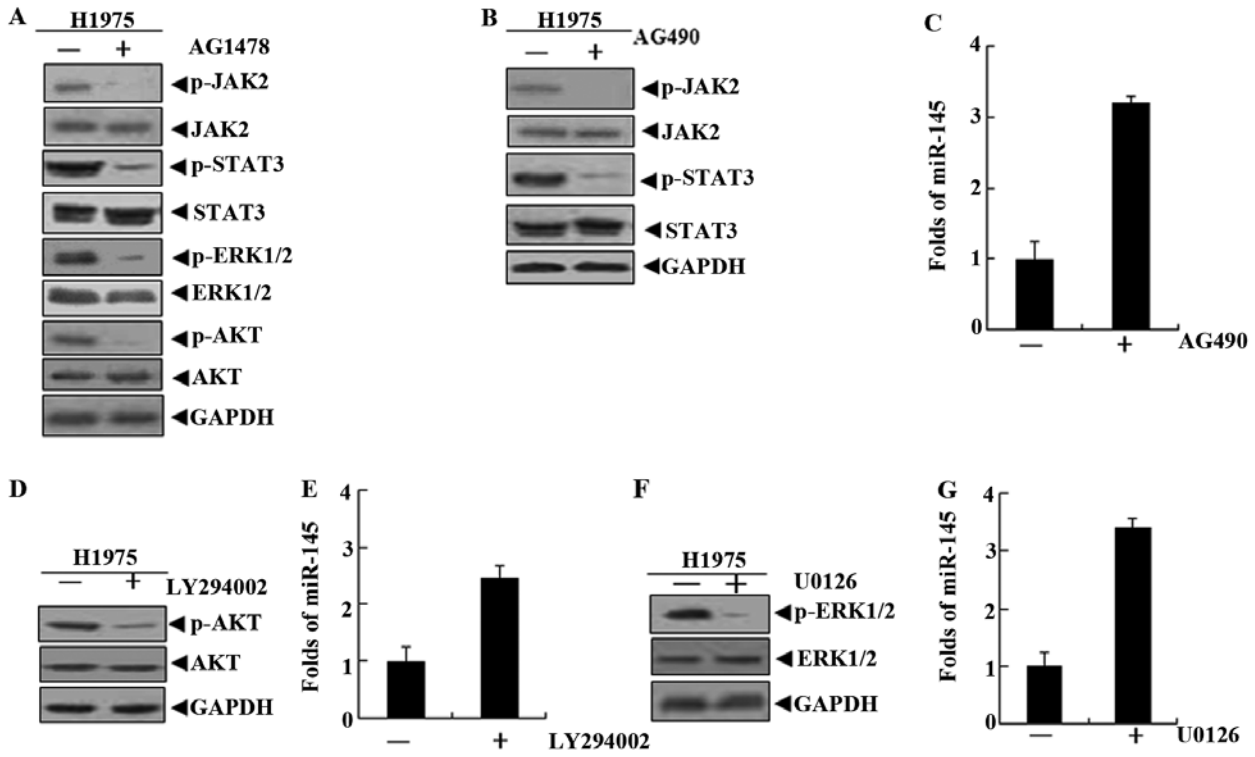

Figure 3. STAT3, AKT and ERK1/2 which are downstream signaling molecules of EGFR affect the expression of miR-145 in lung cancer cells. (A) H1975 cells were treated with 5 μM AG1478 for 24 h. Specified protein was detected by western blotting. H1975 cells were treated with 10 μM AG490 for 24 h. (B) The activation levels of JAK2 and STAT3 were analyzed by western blotting. (C) The miR-145 expression levels were detected by qRT-PCR. H1975 cells were treated with 10 μM LY294002 for 24 h. (D) The activation levels of AKT were analyzed by western blotting. (E) The miR-145 expression levels were detected by qRT-PCR. H1975 cells were treated with 10 μM U0126 for 24 h. (F) The activation levels of ERK1/2 were analyzed by western blotting. (G) The miR-145 expression levels were detected by qRT-PCR.

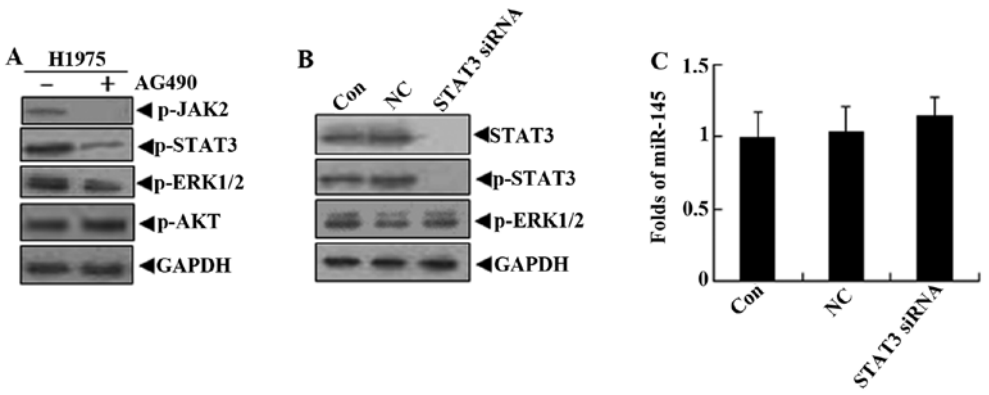

Figure 4. STAT3 signaling molecules are not involved in the regulation of miR-145 levels in lung cancer cells. (A) H1975 cells were treated with 10 μM AG490 for 24 h. JAK2, STAT3 and ERK1/2 activation levels were analyzed by western blotting. H1975 cells were transfected with siRNA against STAT3 for 48 h and the levels of p-STAT3 and p-ERK1/2 were analyzed by western blotting (B) and the levels of miR-145 were detected by qRT-PCR (C).

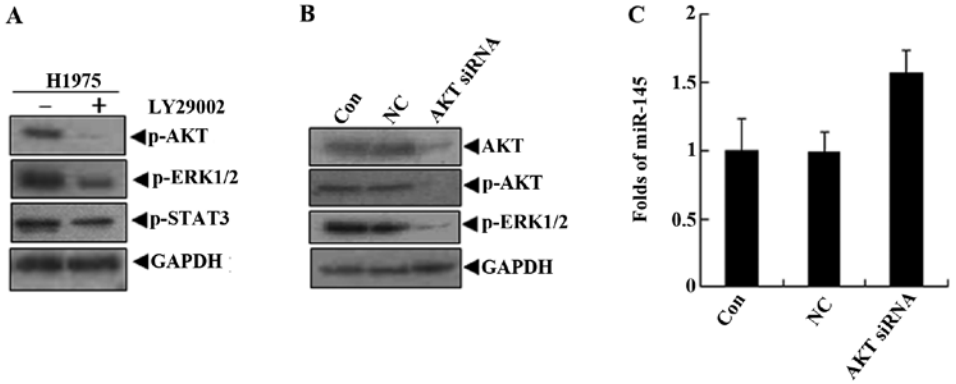

Figure 5. AKT signaling molecules affect the levels of miR-145 through ERK1/2 activated in lung cancer cells. (A) H1975 cells were treated with 10 μM LY294002 for 24 h. AKT and ERK1/2 activation levels were analyzed by western blotting. H1975 cells were transfected with siRNA against AKT for 48 h and the levels of p-AKT and p-ERK1/2 were analyzed by western blotting (B) and the levels of miR-145 were detected by qRT-PCR (C).

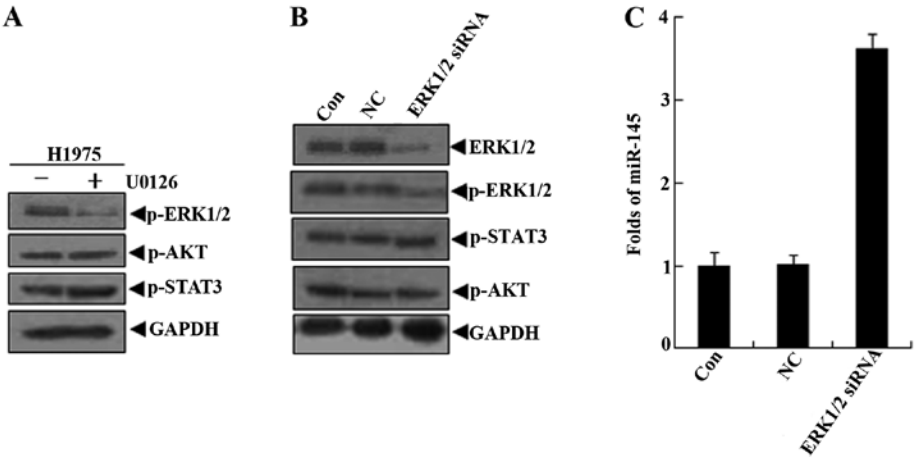

Figure 6. ERK1/2 signaling molecules contribute to the levels of miR-145 in lung cancer cells. H1975 cells were treated with 10 μM U0126 for 24 h. (A) STAT3, AKT and ERK1/2 activation levels were analyzed by western blotting. H1975 cells were transfected with siRNA against ERK1/2 for 48 h and the levels of p-STAT3, p-AKT and p-ERK1/2 were analyzed by western blotting (B) and the levels of miR-145 were detected by qRT-PCR (C).

ERK1/2 is involved in the downregulation of miR-145 expression by EGFR. U0126, is a highly selective inhibitor of both MEK1 and MEK2, a type of MAPK/ERK kinase (20,21). In H1975 cells treated with U0126 for 24 h, western blot results confirmed that U0126 only inhibited the activation of ERK1/2, and there were no inhibitory effects on the AKT and STAT3 signaling molecules (Fig. 6A). Similarly, in applications for ERK1/2 siRNA in H1975 cells, no effect on the activation of

AKT and STAT3 was observed, while the ERK1/2 activity was specifically silenced (Fig. 6B). The miR-145 levels were upregulated, compared with the control group and negative control group (Fig. 6C). These findings suggested that ERK1/2 mediated the downregulation of miR-145 in the EGF-EGFR signaling pathway in lung cancer cells.

Discussion

It has been confirmed that miR-145 has a tumor suppressor function (22,23). miR-145 had a lower level of expression in lung, breast, prostate and colorectal cancer cells (24-26). However, it is unclear why miR-145 is downregulated in these tumor cells. The EGFR signaling pathway is activated and treated as the target molecule in lung cancer cells. Therefore, we chose the lung cancer cells to explore how the EGFR signaling pathway may be involved in the molecular mechanism of miR-145 downregulation. The results showed that the EGFR signaling pathway mediated the downregulation of miR-145 through ERK1/2 signaling molecules in lung cancer cells.

There are generally two forms of the activation of EGFR in lung cancer cells. First, it is the excessive activation of wild-type EGFR; second, it is more important that the mutation of EGFR protein in ATP binding sites causes its conformational change, which is easier to bind ATP (9,27). Therefore, we chose the wild-type EGFR (A549 and H292), the mutant EGFR (H1650 and H1975) and normal lung bronchial epithelial cells (BEAS-2B) to investigate the link between the activation of EGFR and the downregulation of miR-145. The results indicated that the wild-type and mutant EGFR in lung cancer cells were activated compared to normal EGFR in lung bronchial epithelial cells under conventional culture conditions, which was consistent with the expected results. The levels of miR-145 in lung cancer cells were not the same, but they were less than in normal lung bronchial epithelial cells. To determine the relationship between the activation of the EGFR and the levels of miR-145 expression in lung cancer cells, the correlation of the both was analyzed. Finally, statistical results indicated that the activation of EGFR was negatively correlated with the levels of miR-145 in lung cancer cells.

In order to verify the existence of negative contact between them, EGF, a physiological ligand of EGFR, stimulated the A549, H1975 and BEAS-2B cells. The results showed that EGF may significantly reduce the levels of miR-145 in lung cancer cells or in normal cells, while it activated the EGFR in corresponding cells. In addition, AG1478, a potent and selective inhibitor of EGFR, may block EGF downregulation of the levels of miR-145 in these cells. We observed that the activation of EGFR and the expression of miR-145 had indeed a negative correlation through the activation and inhibition of EGFR, indicating that the activation of EGFR may reduce the levels of miR-145 in lung cancer cells.

EGFR is a membrane protein. It is obvious that it may downregulate the expression of miR-145 by the associated signaling molecules. STAT3, AKT and ERK1/2 are the downstream signaling molecules of EGFR (28-30). AG1478 may inhibit the activation of STAT3, AKT and ERK1/2 in H1975 cells. Next, we applied STAT3, AKT and ERK1/2 signaling molecule inhibitors (AG490, LY294002 and U0126) to explore their role in the downregulation of miR-145. Notably, the results showed that three inhibitors may restore the expression of miR-145, indicating that these signaling molecules were involved in the regulation of miR-145 expression. We first considered whether there was interaction among them. Western blot results showed that AG490 or LY294002 not only inhibited the activation of STAT3 or AKT, but also blocked ERK1/2 phosphorylation. U0126 only inhibited the activation of ERK1/2 and had no effect on the phosphorylation levels of STAT3 and AKT. These data suggest that the signal molecule inhibitors may suppress the activation of ERK1/2. Thus, we assume that EGFR mediates the downregulation of miR-145 through the activation of ERK1/2 in lung cancer cells.

To confirm this conjecture, siRNAs for STAT3, AKT and ERK1/2 were used. siRNAs against STAT3 did not increase the levels of miR-145 and there was no effect on ERK1/2 phosphorylation. This showed that the activation of STAT3 was not involved in the process of the downregulation of miR-145 and it also suggested that AG490 may be a non-specific inhibitor for ERK1/2. siRNAs for AKT significantly increased the levels of miR-145 and inhibited the activation of ERK1/2. This indicated that AKT may be an upstream molecule of ERK1/2. It is possible that LY294002 and siRNAs for AKT blocked the activity of ERK1/2. siRNAs against ERK1/2 only significantly elevated the miR-145 and had no effect on the activation of STAT3 and AKT. This strongly supported that EGFR mediated the expression of miR-145 through the activation of ERK1/2 in lung cancer cells. Regarding the downregulation of miR-145 by ERK1/2, this requires further in-depth study.

Collectively, we found that the EGFR activation was negatively correlated with the downregulation of miR-145 in lung cancer cells. The further signal molecules studied revealed that EGFR mediated the downregulation of miR-145 through ERK1/2 activation. These data provide a molecular mechanism which elaborated the downregulation of miR-145 in lung cancer cells. Moreover, it also provides a possible direction for the intervention of miR-145 expression in lung cancer cells.

Acknowledgements

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