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miR-135a Confers Resistance to Gefitinib in Non-Small Cell Lung Cancer Cells by Upregulation of RAC1

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The EGFR tyrosine kinase inhibitor gefitinib is used in therapy for non-small cell lung cancer (NSCLC). However, the therapeutic efficacy of gefitinib is known to be impeded by mutations of EGFR. The aim of the present study was to reveal the role of miR-135a in gefitinib resistance of NSCLC cells. Human NSCLC cell lines, NCI-H1650 and NCI-H1975, were transfected with miR-135a mimic/inhibitor or miR-135a inhibitor plus pEX-RAC1 (a RAC1-expressing vector). The effects of miR-135a and RAC1 expression on cell viability, apoptosis, migration, and invasion were then detected. The transfected cells were exposed to 0–20 μM gefitinib, and cell viability was then detected at 48 h posttreatment. Western blot analysis was performed to detect the expression changes of main factors in the PI3K/AKT pathway. miR-135a overexpression promoted viability, migration, and invasion, but inhibited apoptosis of NCI-H1650 and NCI-H1975 cells. Cell viability was significantly reduced by gefitinib, and the LC_{50} values of gefitinib in NCI-H1650 and NCI-H1795 cells were 0.845 and 0.667 μM, respectively. miR-135a overexpression could increase cell viability even under high concentrations of gefitinib. Rac1 was not predicted as a target of miR-135a, while miR-135a could upregulate the expression of RAC1. miR-135a promoted cell growth and metastasis and activated the PI3K/AKT signaling pathway via a RAC1-dependent manner. To conclude, this study demonstrated that miR-135a confers NSCLC cell resistance to gefitinib via upregulation of RAC1. Therapies designed to downregulate miR-135a may help NSCLC patients to overcome gefitinib resistance.

Key words: miR-135a; Drug resistance; Gefitinib; Non-small cell lung cancer (NSCLC); RAC1; PI3K/AKT signaling pathway

INTRODUCTION

Lung cancer has remained as the leading type of cancer worldwide in terms of high incidence and mortality rate[1,2]. Based on pathological features, lung cancer consists of two main types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), with NSCLC dominating over 80% of all lung cancer cases[3]. NSCLC is further classified into three subtypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma[4]. Patients with advanced or metastatic stage (III-b or IV) NSCLC are often treated with systemic chemotherapy, but response and survival rates continue to be modest[5].

The epidermal growth factor receptor (EGFR), a member of the human epidermal growth factor receptor (HER) family of receptor tyrosine kinases, is an important regulator of cell progression, division, and differentiation[6,7]. The EGFR-directed tyrosine kinase inhibitor (TKI) gefitinib is the approved therapy for NSCLC, harboring activating mutations in the EGFR kinase[7–9]. Unfortunately, the therapeutic efficacy of gefitinib is known to be impeded by mutations of EGFR[10]. However, insertions in exon 20 and T790M missense mutation are thought to be early genetic events that confer gefitinib resistance in NSCLC cells[11]. The T790M mutation in EGFR kinase causes gefitinib resistance by increasing the affinity for adenosine triphosphate (ATP)[12].

The phenomenon of gefitinib resistance has called for intense efforts in search of novel, alternative therapeutic options[10]. In this regard, microRNAs (miRNAs) have gained increasing attention in the implications of gefitinib-resistant NSCLC. For instance, overexpression of miR-30a-5p overcame gefitinib resistance through regulating the PI3K/AKT signaling pathway in NSCLC cells[13]. miR-200c enhanced sensitivity of drug-resistant

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NSCLC to gefitinib by suppression of the PI3K/AKT signaling pathway and inhibited cell migration via targeting zinc finger E-box binding homeobox 1 (ZEB1)[14].

The miR-135 family, including miR-135a and miR-135b, is highly conserved among mammals[15]. A previous study reported that serum miR-135a level was downregulated in NSCLC patients and was associated with poor prognosis[16]. Yan et al. revealed that miR-135a promoted gastric cancer cell resistance to oxaliplatin[17]. Zhou et al. demonstrated that overexpression of miR-135a sensitized lung cancer cell lines to cisplatin[18]. However, the role of miR-135a in gefitinib resistance of NSCLC cells has not yet been revealed.

In the present study, the expressions of miR-135a in two NSCLC cell lines (NCI-H1650 and NCI-H1975) were overexpressed or suppressed by transfection with the mimic/inhibitor of miR-135a. The effects of miR-135a expression on cell viability, apoptosis, migration, and invasion were monitored. In addition, the effects of miR-135a expression on gefitinib-induced decrease in cell viability were detected. The findings of this study indicated that therapies designed to downregulate miR-135a may help NSCLC patients to overcome gefitinib resistance.

MATERIALS AND METHODS

Cell Culture and Treatment

Two human NSCLC cell lines (NCI-H1650 and NCl-H1975) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, P.R. China). The two cell lines were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 100 μg/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Cergy Pontoise, France). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The medium was routinely changed 2–3 days after seeding.

For gefitinib treatment, cells were treated with 0.1, 1, 5, 10, and 20 μM gefitinib for 48 h, which was obtained from AstraZeneca (Macclesfield, UK).

Plasmid Construction and Transfection

miR-135a mimic, miR-135a inhibitor, and the negative controls (mimic NC and inhibitor NC) were synthesized by GenePharma (Shanghai, P.R. China). For RAC1 overexpression, the full-length sequence of RAC1 was constructed in pEX (GenePharma) and was referred as pEX-RAC1. All transfections were performed with Lipofectamine 2000 (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instruction. The final concentrations of miR-135a mimic, miR-135a inhibitor, mimic NC, inhibitor NC, and pEX-RAC1 vector used were 50 nM, 200 nM, 100 nM, 100 nM, and 2 μg/ml, respectively. Stable transfected cells were selected by 0.5 mg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA). After approximately 4 weeks, G418-resistant cell clones were established.

Cell Viability Assay

Cell viability was assayed using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Gaithersburg, MD, USA). The miR-transfected cells were seeded into 96-well plates at a density of 5×10^3 cells/well. After 48 h of incubation at 37°C with or without gefitinib treatment, 10 μl of CCK-8 was added to the culture medium, and the cultures were then incubated for 4 h at 37°C. The absorbance at 450 nm was detected by a microplate reader (iMark; Bio-Rad, Hercules, CA, USA).

Apoptosis Assay

Cell apoptosis was detected using a commercial Annexin-V-FITC Apoptosis Detection Kit, which was purchased from Beyotime (Shanghai, P.R. China). After transfection with miR-135a mimic or inhibitor, the cells were then collected and washed twice with PBS. The cells were resuspended in 200 μl of binding buffer containing 10 μl of annexin V-FITC and 5 μl of propidium iodide (PI). The samples were incubated in the dark at room temperature, and then 300 μl of PBS was added. Annexin V^+ cells, as well as both annexin V^+ and PI^+ cells, were immediately detected by a FACScan (Beckman Coulter, Fullerton, CA, USA). The data were analyzed using FlowJo software (TreeStar, San Carlos, CA, USA).

Transwell Assay

Cell migration was determined using a modified Boyden chamber (Costar-Corning, New York, NY, USA) with an 8.0-μm pore polycarbonate filter insert in 24-well plates. The miR-transfected cells were resuspended in 200 μl of serum-free medium and were seeded on the upper compartment of the chamber. The lower compartment was filled with 600 μl of complete medium. After 36 h of incubation at 37°C, nontraversed cells were removed from the surface of the filter with a cotton swab. Traversed cells on the lower side of the filter were stained with crystal violet (Beyotime) and counted under a microscope (BH-2; Olympus, Tokyo, Japan).

Cell invasion assay was performed similarly, except the filter inserts were precoated with Matrigel (BD Biosciences, Bedford, MA, USA).

qRT-PCR

Total RNA was extracted from cells using TRIzol reagent (Life Technologies Corp.) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNAs using PrimeScript Reverse

Transcriptase (Takara, Dalian, P.R. China). qRT-PCR was performed on QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Carlsbad, CA, CA) with each reaction system containing 20 ng of cDNAs and the specific primers for miR-135a (forward: 5′-AGC ATA ATA CAG CAG GCA CAG AC-3′, reverse: 5′-AAA GGT TGT TCT CCA CTC TCT CAC-3′) or RAC1 (forward: 5′-GTA AAA CCT GCC TGC TCA TC-3′, reverse: 5′-GCT TCA TCA AAC ACT GTC TTG-3′). GAPDH (forward: 5′-ATT CCA TGG CAC CGT CAA GGC T-3′, reverse: 5′-TCA GGT CCA CCA CTG ACA CGT T-3′) and U6 (forward: 5′-CTC GCT TCG GCA GCA CA-3′,

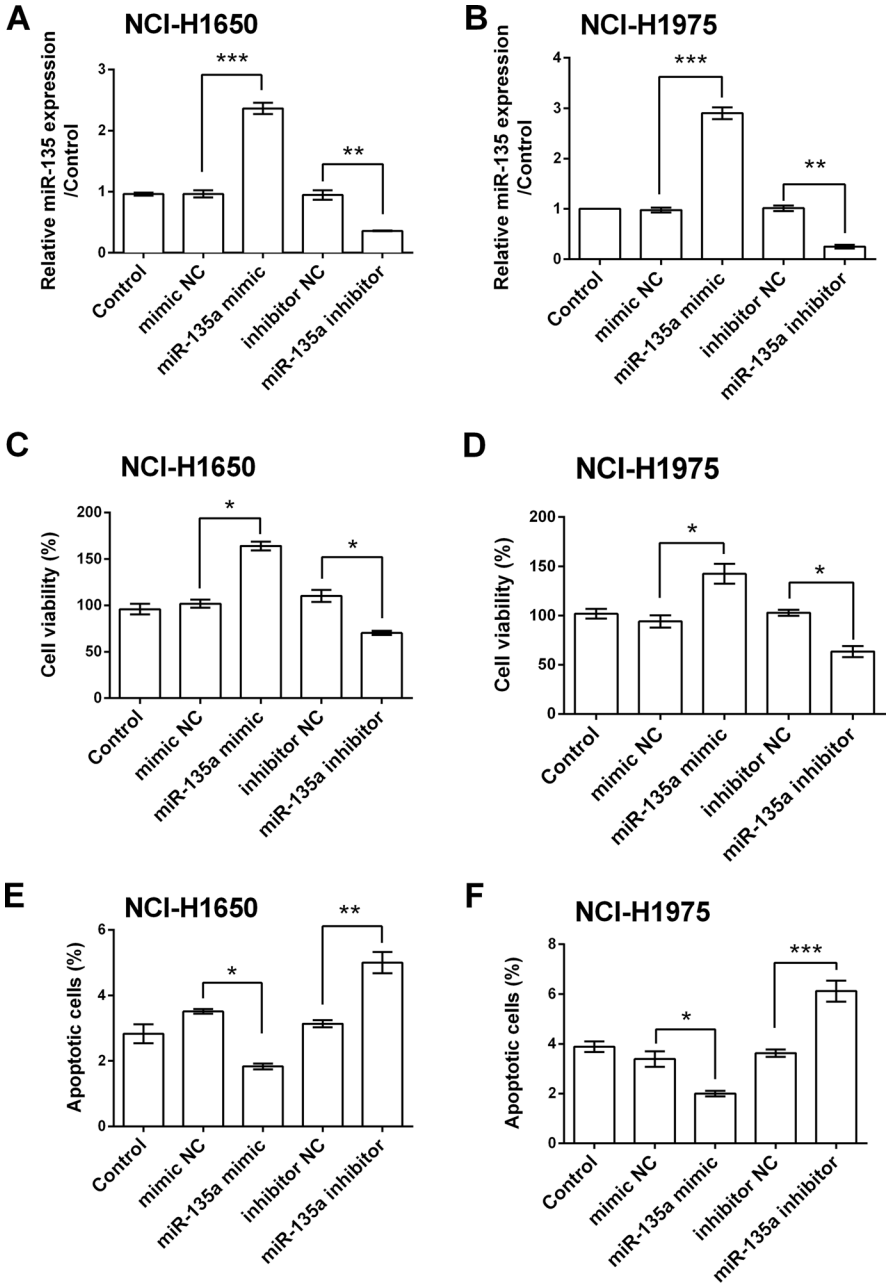

Figure 1. miR-135a promotes non-small cell lung cancer (NSCLC) cell growth. NCI-H1650 and NCI-H1975 cells were transfected with miR-135a mimic or miR-135a inhibitor. (A, B) The expression of miR-135a in the miR-transfected cells was monitored by qRT-PCR. (C, D) Viability of cells was detected by Cell Counting Kit-8 (CCK-8) analysis. (E, F) Apoptotic cell rate was measured by flow cytometry detection. Data are presented as the mean±standard deviation (SD) from three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ [one-way analysis of variance (ANOVA)].

reverse: 5′-AAC GCT TCA CGA ATT TGC GT-3′) were used as the internal control for RAC1 and miR-135a expression, respectively. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method[19].

Western Blot

Protein samples of cells were lysed by radioimmunoprecipitation assay (Beyotime) and quantified by BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The protein samples were separated by 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were first blocked in 5% skim milk at room temperature for 2 h and then incubated in the specific primary antibodies at 4°C overnight for the detection of B7-H4 (1:2,000; ab130151; Abcam, Cambridge, MA, USA), RAC1 (1:500; ab97732; Abcam), p-PI3K (1:1,000; #4228; Cell Signaling Technology, Danvers, MA, USA), PI3K (1:1,000; #4257; Cell Signaling Technology), p-AKT (1:1,000; #13038; Cell Signaling Technology), AKT (1:1,000; #4685; Cell Signaling Technology), and GAPDH (1:1,000; #2118; Cell Signaling Technology). The membranes were then incubated with anti-mouse (1:2,000; ab6789; Abcam), anti-rabbit (1:2,000; ab6721; Abcam), and anti-goat (1:5,000; ab6741; Abcam) IgG at room temperature for 1 h. ECL Plus Western Blotting Substrate (Pierce) was used to develop positive bands on the membrane.

Statistical Analysis

All experiments were repeated three times, and results are presented as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS 19.0 statistical software (IBM, New York, NY, US). The statistical difference between groups was calculated using one-way analysis of variance (ANOVA) or two-way ANOVA. A value of $p<0.05$ was considered to indicate a statistically significant result.

RESULTS

miR-135a Promotes NSCLC Cells Growth

To detect the functional impacts of miR-135a on NSCLC cells, the expression of miR-135a in NCI-H1650 and NCI-H1795 cells was first altered by transfection

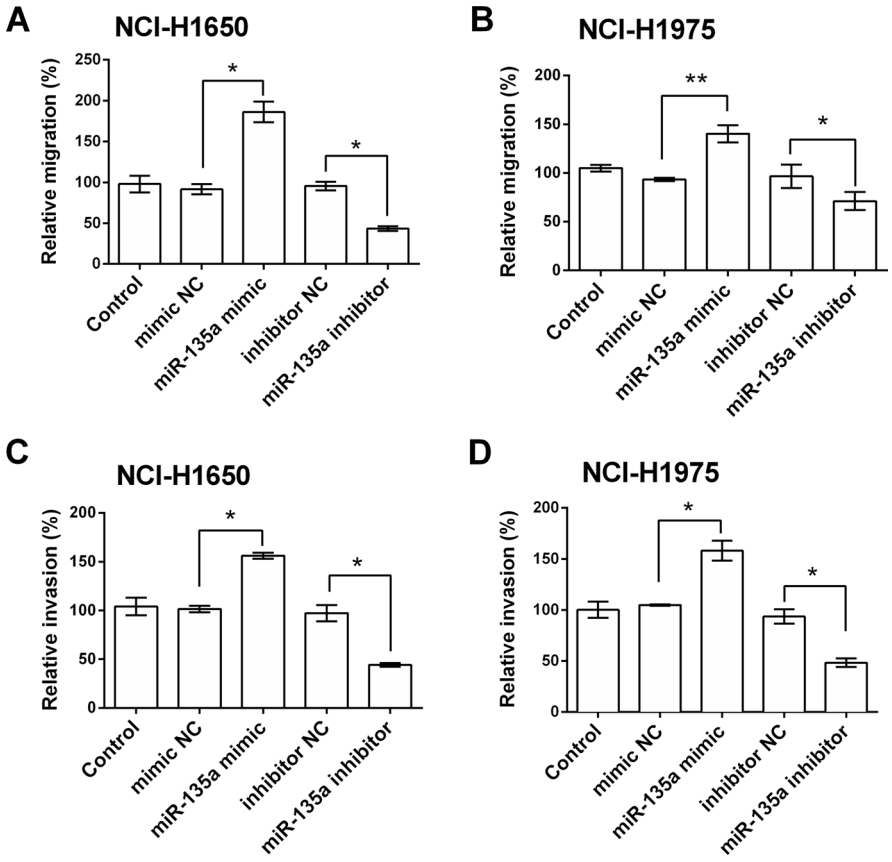

Figure 2. miR-135a promotes NSCLC cell metastasis. NCI-H1650 and NCI-H1975 cells were transfected with miR-135a mimic or miR-135a inhibitor. (A, B) Relative migration and (C, D) invasion of miR-transfected cells were analyzed by Transwell assay. Data are presented as the mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$ (one-way ANOVA).

with miR-135a mimic or inhibitor. qRT-PCR analysis results showed that the expression of miR-135a was significantly upregulated after transfection with miR-135a mimic when compared to the mimic NC group ($p<0.001$); in contrast, miR-135a level was downregulated by transfection with miR-135a inhibitor when compared to inhibitor NC ($p<0.01$) (Fig. 1A and B). These data suggested that the expression of miR-135a in NCI-H1650 and NCI-H1795 cells was successfully overexpressed and suppressed by transfection. CCK-8 assay results showed that viability of NCI-H1650 and NCI-H1795 cells was increased after miR-135a mimic transfection, whereas it was reduced by miR-135a inhibitor (all $p<0.05$) (Fig. 1C and D). Results from flow cytometry detection showed that apoptotic cell rate was decreased by miR-135a mimic ($p<0.05$) and was increased by miR-135a inhibitor ($p<0.01$ or $p<0.001$) (Fig. 1E and F). These data provide the preliminary evidence that miR-135a promoted cell viability but suppressed apoptosis for NSCLC cells.

miR-135a Promotes NSCLC Cell Metastasis

We next detected whether miR-135a expression was implicated in the metastasis of NSCLC cells by detection of cell migration, invasion, and metastasis-related protein expressions. Transwell assay results showed that the migratory and invasive capacities of NCI-H1650 and NCI-H1975 cells were promoted by transfection with miR-135a mimic and were decreased by transfection with miR-135a inhibitor ($p<0.05$ or $p<0.01$) (Fig. 2A–D). These data suggested that miR-135a might have the ability to improve metastasis of NSCLC cells.

miR-135a Confers Resistance to Gefitinib in NSCLC Cells

Studies have suggested that B7-H4 was a prognostic indicator of poor survival and different clinical pathological features in NSCLC[20–22]. In the present work, Western blotting analysis results showed that the expression level of B7-H4 proteins in NCI-H1650 and NCI-H1795 cells was upregulated by miR-135a mimic and was downregulated by miR-135a inhibitor (Fig. 3A and B). This finding further confirmed our aforementioned speculation that miR-135a exerted tumor-promoting activity in NSCLC.

We detected whether miR-135a expression has any functional impacts on NSCLC resistance to gefitinib. By performing CCK-8 assay, we found that cell viability was reduced by gefitinib in a dose-dependent manner (Fig. 3C and D). Gefitinib with a concentration of 20 μM proposed the strongest inhibitory effects on cell viability. The LC_{50} values of gefitinib in NCI-H1650 and NCI-H1795 cells were 0.845 and 0.667 μM, respectively. More important, cell viability was significantly increased by the addition of miR-135a mimic ($p<0.01$ or $p<0.001$) and was significantly decreased by the

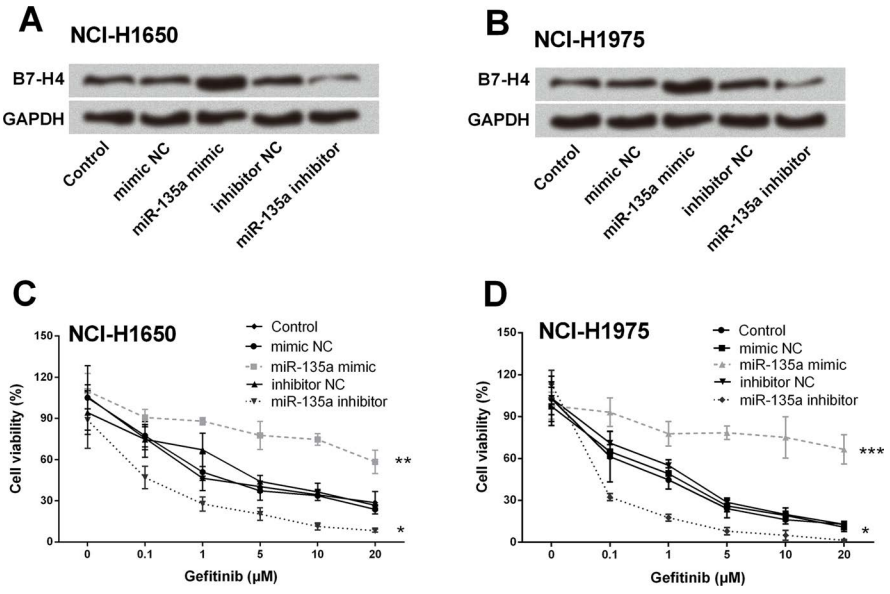

Figure 3. miR-135a confers resistance to gefitinib in NSCLC cells. NCI-H1650 and NCI-H1975 cells were transfected with miR-135a mimic or miR-135a inhibitor. (A, B) The expression of B7-H4 in the miR-transfected cells were detected by Western blot analysis. GAPDH acted as an internal control. (C, D) The viability of cells that were treated with 0–20 μM gefitinib and transfected with miR-134a mimic/inhibitor was detected by CCK-8 analysis. Data are presented as the mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (two-way ANOVA).

addition of miR-135a inhibitor ($p<0.05$). Based on these data, we inferred that miR-135a might confer resistance to gefitinib in NSCLC cells.

miR-135a Positively Regulates RAC1 in NSCLC Cells

Considering that RAC1 inhibition has been reported as a therapeutic target for gefitinib-resistant NSCLC[23], we detected the cross-regulation between miR-135a and RAC1 to detect whether RAC1 was involved in miR-135a-mediated NSCLC resistance. By screening in three online databases, including TargetScan (http://www.targetscan.org/vert_71/), microRNA.org (http://www.microrna.org), and starBase v2.0 (http://starbase.sysu.edu.cn/), Rac1 was not predicted as a direct target of miR-135a. qRT-PCR and Western blot analyses results showed that both the mRNA and protein levels of RAC1 in NCI-H1650 and NCI-H1975 cells were upregulated by miR-135a mimic ($p<0.01$ or $p<0.001$) and were downregulated by miR-135a inhibitor ($p<0.05$) (Fig. 4A–D). Based on these data, we speculated that RAC1 might be a downstream gene of miR-135, which was positively regulated by miR-135.

miR-135a Promotes NSCLC Cell Growth via Upregulation of RAC1

To explore whether RAC1 was involved in miR-135a-mediated NSCLC growth, miR-135a inhibitor and a RAC1-expressing vector (pEX-RAC1) were cotransfected into NCI-H1650 and NCI-H1975 cells. Western blot analysis results showed that miR-135a inhibitor-induced downregulation of RAC1 was recovered by the addition of pEX-RAC1 (Fig. 5A and B), indicating the success of transfection. Figure 5C–F shows that miR-135a inhibitor-induced decrease in cell viability and miR-135a inhibitor-induced increase in cell apoptosis were all abolished by the addition of pEX-RAC1 ($p<0.05$, $p<0.01$, or $p< 0.001$). These data gave us a hint that miR-135a-promoted NSCLC cell growth might be via upregulation of RAC1.

miR-135a Promotes Metastasis of NSCLC Cells via Upregulation of RAC1

We investigated whether RAC1 was involved in miR-135a-mediated NSCLC metastasis by detection of cell migration and invasion. As shown in Figure 6A–D, the migratory and invasive capacities of NCI-H1650

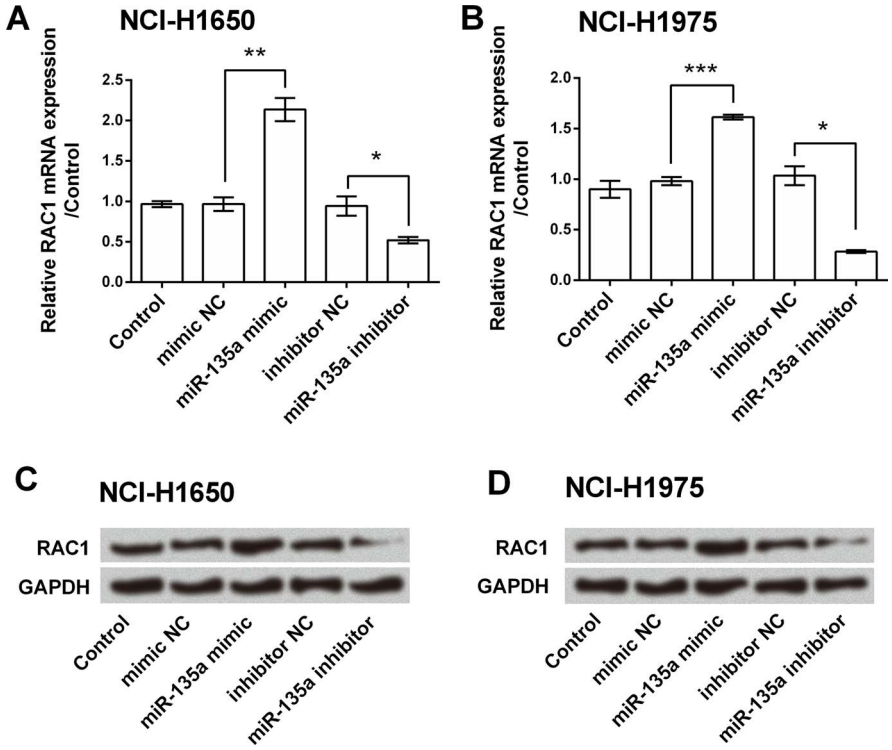

Figure 4. miR-135a positively regulates RAC1 in NSCLC cells. NCI-H1650 and NCI-H1975 cells were transfected with miR-135a mimic or miR-135a inhibitor. (A, B) The mRNA level of RAC1 in the miR-transfected cells was assessed by qRT-PCR. Data are presented as the mean±SD from three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (one-way ANOVA). (C, D) The protein level of RAC1 in the miR-transfected cells was assessed by Western blotting. GAPDH acted as an internal control.

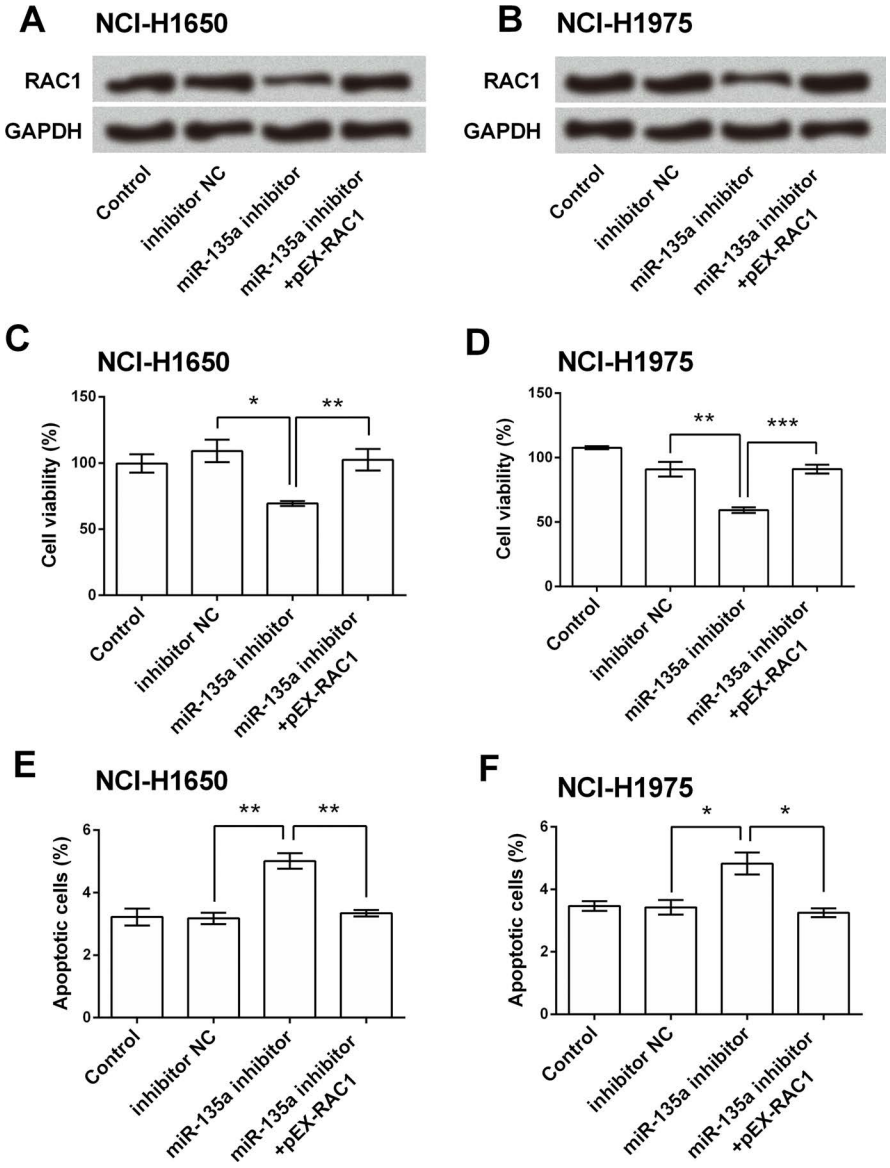

Figure 5. miR-135a promotes NSCLC cell growth via upregulation of RAC1. NCI-H1650 and NCI-H1975 cells were transfected with miR-135a inhibitor, or miR-135a inhibitor plus pEX-RAC1. (A, B) The protein level of RAC1 in the transfected cells was assessed by Western blotting. GAPDH acted as an internal control. (C, D) Cell viability was detected by CCK-8 assay. (E, F) Apoptotic cell rate was measured by flow cytometry detection. Data are presented as the mean ± SD from three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (one-way ANOVA).

and NCI-H1975 cells were decreased by the miR-135a inhibitor ($p<0.05$) and were reversed by the addition of pEX-RAC1 ($p<0.01$). These data indicated that miR-135a also could promote metastasis of NSCLC cells via upregulation of RAC1.

miR-135a Activates the PI3K/AKT Pathway in NSCLC Cells via Upregulation of RAC1

Furthermore, we focused on the PI3K/AKT signaling pathway to reveal the underlying mechanism(s) by which miR-135a mediated NSCLC cell growth and metastasis. Western blot analysis results showed that p-PI3K and p-AKT were remarkably upregulated by miR-135a mimic and were downregulated by miR-135a inhibitor (Fig. 7A and B). In addition, downregulation of p-PI3K and p-AKT induced by miR-135a inhibitor was reversed by the addition of pEX-RAC1. Total levels of PI3K and AKT were not altered by miR-135a mimic, miR-135a inhibitor, or miR-135a inhibitor plus pEX-RAC1. Therefore, we inferred that miR-135a activates the PI3K/AKT pathway in NSCLC cells via upregulation of RAC1.

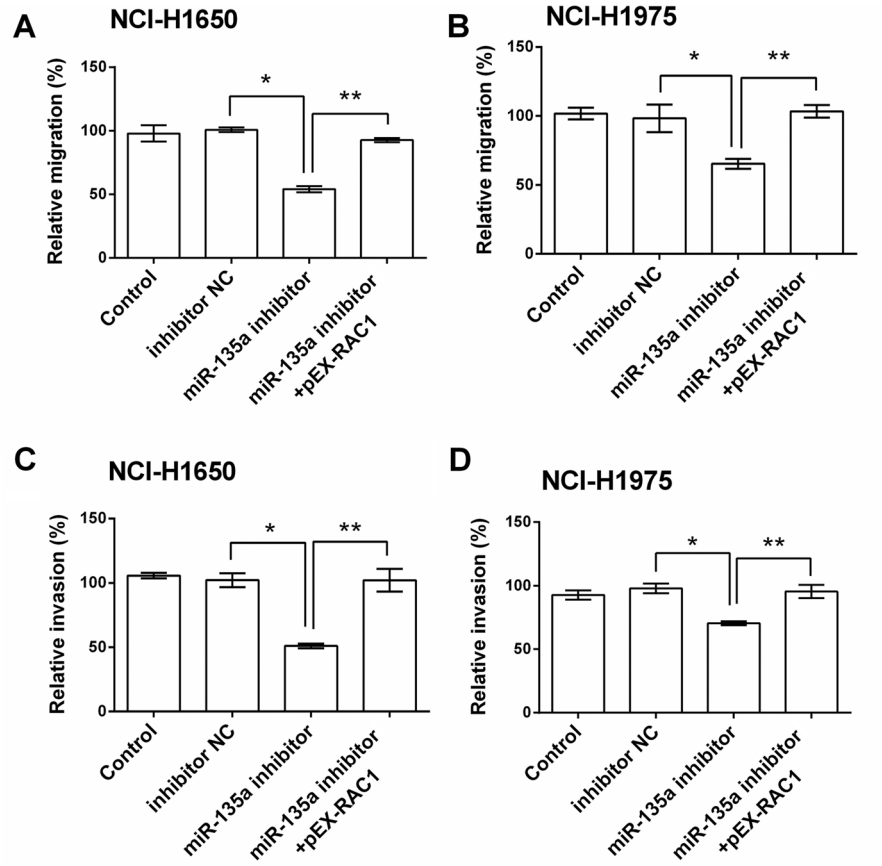

Figure 6. miR-135a promotes metastasis of NSCLC cells via upregulation of RAC1. NCI-H1650 and NCI-H1975 cells were transfected with miR-135a inhibitor, or miR-135a inhibitor plus pEX-RAC1. (A, B) Relative migration and (C, D) invasion of transfected cells were analyzed by Transwell assay. Data are presented as the mean ± SD from three independent experiments. $*p<0.05$, $**p<0.01$ (one-way ANOVA).

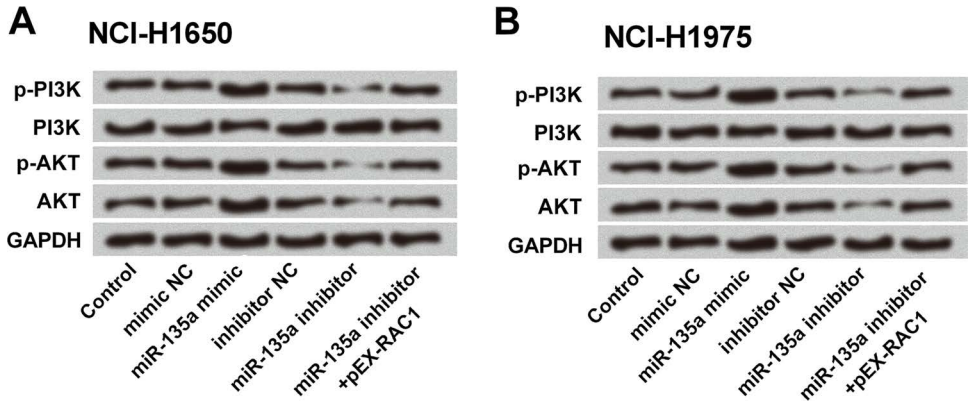

Figure 7. miR-135a activates the PI3K/AKT pathway in NSCLC cells via upregulation of RAC1. NCI-H1650 and NCI-H1975 cells were transfected with miR-135a inhibitor, or miR-135a inhibitor plus pEX-RAC1. (A, B) The expressions of main proteins in the PI3K/AKT signaling pathway were detected by Western blotting analysis. GAPDH served as an internal control.

DISCUSSION

Emerging evidence suggests that the antitumor activity of EGFR-TKIs in resistant NSCLC cell lines can be enhanced by combined therapy with miRNA interventions[10]. In our study, we found that miR-135a could promote NSCLC cell growth and metastasis. Knockdown of miR-135a enhanced the drug sensitivity of NSCLC cells to gefitinib in vitro. These findings indicated that therapies designed to downregulate miR-135a may help NSCLC patients to overcome gefitinib resistance.

In recent years, miR-135a has been widely studied due to its controversial role in cancers. Some studies suggested that miR-135a acts as a tumor suppressor and has a pivotal role in inhibiting tumor cell growth, metastasis, angiogenesis, as well as enhancing drug sensitivity[24–26]. For example, Fukagawa et al. demonstrated that a high amount of miR-135a-3p in serum samples was associated with favorable clinical prognosis, and overexpression of miR-135a-3p in ovarian cancer cells induced drug sensitivity to cisplatin and paclitaxel and suppressed cell proliferation and xenograft tumor growth[26]. Another study by Zhou et al. indicated that enforced miR-135a expression sensitized lung cancer A549/CDDP cells to cisplatin-induced apoptosis[18]. In contrast, some evidence has indicated that miR-135a possesses potent tumor-promoting activities[17,27–29]. For example, Zeng and his colleagues reported that miR-135a was upregulated in hepatocellular carcinoma (HCC) cell lines and tissues, and miR-135a overexpression promoted HCC cell migration and invasion[27]. Holleman et al. provided both in vitro and in vivo evidence that upregulation of miR-135a is associated with paclitaxel resistance[29]. These controversial results may reflect the diverse roles of miR-135a in different forms of cancer[29].

In terms of NSCLC, the amount of miR-135a in serum samples has been proposed as a potential biomarker, as the serum miR-135a level was downregulated in NSCLC patients and was associated with poor prognosis[16]. In a study by Shi et al., miR-135a was suggested as a tumor suppressor in NSCLC A549 cells by suppression of cell migration and invasion[30]. However, Zhao and his coworkers demonstrated that miR-135a inhibition protects A549 cells from LPS-induced apoptosis, indicating the tumor-promoting function of miR-135a on NSCLC[31]. In the current study, the promoting functions of miR-135a on NCI-H1650 and NCI-H1975 cell growth and metastasis were observed. Our findings were consistent with the study of Zhao et al. and were in contrast to Shi et al., suggesting that miR-135a might be an oncogene in NSCLC. The results of this study demonstrated that miR-135a confers resistance to gefitinib on NSCLC cells, indicating that knockdown of miR-135a has potential for overcoming gefitinib resistance.

Rac1 is a Rho family GTPase that is a part of the larger Ras superfamily of small GTPases[32]. Since its discovery, many functions of Rac1 in various cell types have been reported, such as regulation of cell proliferation, differentiation, nuclear transport, neuronal axon guidance, migration, morphogenesis, phagocytosis, actin cytoskeleton dynamics, and membrane vesicle trafficking[32,33]. In a recent study, RAC1 has been reported as an attractive target to inhibit tumor progression in NSCLC patients to overcome resistance to EGFR-TKI[23]. Zhu et al. demonstrated that miR-512-3p inhibited tumor cell adhesion, migration, and invasion in a RAC1-dependent manner of NSCLC A549 and H1299 cell lines[34]. In the current study, we found that RAC1 was positively regulated by miR-135a, indicating that miR-135a could interact with RAC1 in NSCLC cells. In addition, our data suggested that miR-135a confers NSCLC cell resistance to gefitinib in a RAC1-dependent manner, as miR-135a suppression could not inhibit NCI-H1650 and NCI-H1975 cell growth and metastasis when RAC1 was overexpressed. However, how miR-135a upregulated the expression of RAC1 is still unknown.

Some pathways are activated in the downstream of EGFR signaling, including the PI3K/AKT pathway[23]. Abnormal activation of the PI3K/AKT pathway is a frequent event in NSCLC[35]. Constitutive activation of the PI3K/AKT signaling pathway could be the result of genetic aberrations in any components of the PI3K/AKT pathway, its negative regulators, interconnected pathways, and RTK signaling resulting in abnormal growth and cell proliferation[36]. Our results indicated that the PI3K/AKT pathway was blocked by miR-135a suppression as PI3K and AKT were dephosphorylated. Interestingly, miR-135a suppression did not dephosphorylate PI3K and AKT when RAC1 was overexpressed. Therefore, we speculated that miR-135a activated the PI3K/AKT signaling pathway through RAC1 activity.

In conclusion, this study demonstrated that miR-135a promoted cell growth and metastasis in NSCLC cells and confers NSCLC cell resistance to gefitinib. The tumor-promoting activities of miR-135a in NSCLC cells might be via the regulation of RAC1 and the PI3K/AKT signaling pathway. This study provided the first evidence that miR-135a downregulation has the potential for overcoming the resistance to gefitinib in NSCLC patients.

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