MicroRNA-133a downregulated EGFR expression in human non-small cell lung cancer cells via AKT/ERK signaling

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Abstract. MicroRNAs (miRNAs) may serve important roles in the regulation of human non-small cell lung cancer (NSCLC) cell growth and apoptosis. To the best of our knowledge, the present study was the first to explore the role of miRNA-133a/epidermal growth factor receptor (EGFR) in regulating NSCLC cell growth and apoptosis via the AKT/extracellular signal-regulated kinase (ERK) signaling pathway. It was determined that miRNA-133a expression was lower in NSCLC tissue than in the adjacent mucosa. Additionally, EGFR expression in the NSCLC tissue was higher compared with in the adjacent mucosa. Furthermore, the upregulation of miRNA-133a in NSCLC cells suppressed cell growth and induced apoptosis. Upregulating miRNA-133a also increased caspase-3 protein expression, while suppressing that of EGFR, phosphorylated (p)-AKT and p-ERK in NSCLC cells. Therefore, the results of the current study demonstrated that miRNA-133a downregulates EGFR expression in NSCLC via the AKT/ERK signaling pathway. These findings provide insights into the function of miRNA-133a in NSCLC, as well as into the molecular mechanisms underlying the occurrence of NSCLC.

Introduction

Human non-small cell lung cancer (NSCLC) is a malignant tumor with a high morbidity, and is the most common cause of cancer-associated mortality worldwide (1). With improvements in living standards and public health education, the morbidity of NSCLC has been reduced in recent years, but the morbidity for female patients with NSCLC remains the second highest among malignant tumors (2). Furthermore, the incidence of NSCLC is increasing for women. Therapeutic approaches for NSCLC are improving, but the prognosis and survival rate of NSCLC are unsatisfactory, which may be due to the lack of early diagnosis methods and treatment approaches for NSCLC, in addition to the complications associated with the disease (3). Therefore, it is necessary to explore the potential molecular mechanisms underlying the occurrence of NSCLC, in addition to novel approaches for the early diagnosis and treatment of NSCLC.

In recent years, the molecular diagnosis and targeted therapy of NSCLC have provided a new approach for the management of NSCLC (4). The use of individual genetic expression profiling and clinical staging of NSCLC in providing prognosis information has gradually become a focus for NSCLC research (5). The occurrence of NSCLC is associated with mutations, gene amplifications and epigenetic changes in tumor-associated genes (6). Epigenetic changes do not cause changes to the gene sequence, but can induce changes in the expression of tumor-associated genes, resulting in tumorigenesis and progression (6). Epigenetic research primarily focuses on the methylation of DNA, chromatin rearrangement and RNA editing changes. Additionally, miRNAs have become the focus of numerous studies in the field of cancer (7). It has been demonstrated that changes in miRNA expression are implicated in the occurrence of malignancies, at least to an extent (8).

miRNAs are small, non-coding RNAs that arise from transcriptional pri-miRNA via the activity of specific processing enzymes, including DROSHA and DICER (9). miRNA pairs with the mRNA of a target gene, resulting in mRNA degradation, or initiating gene silencing by preventing translation. Through interactions with a negative regulatory sequence in the 3′-non-coding region of specific mRNA targets, miRNA regulation is involved in a wide variety of normal and abnormal cell behaviors (10). miRNAs have potential as tumor biomarkers (11). Although the functions of a number of miRNAs have yet to be elucidated, miRNAs have been implicated in a wide range of physiological and developmental processes (10).

The present study aimed to explore the role of microRNA-133a signaling in the regulation of NSCLC

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development; to the best of our knowledge, the present study is the first to investigate this area of NSCLC research.

**Materials and methods**

**Tissue samples.** The present study was approved by the Institutional Medical Ethics Committee of The First Affiliated Hospital of the General Hospital of the Chinese People's Liberation Army (Beijing, China), and all participants (n=8; all male; 63±6 years old) provided informed, written consent. Human NSCLC and adjacent normal lung tissue samples (n=8) were obtained from The First Affiliated Hospital. NSCLC and adjacent normal tissues (>5 cm from tumor) were pathologically validated by a pathologist. None of the patients had received chemotherapy or radiotherapy prior to surgery.

Reverse transcription–quantitative polymerase chain reaction (RT-qPCR). Total RNA was prepared from the tissues or cells using TRIzol reagent (Biotech Scientific; PerkinElmer, Inc., Waltham, MA, USA), and 1-2 µg total RNA was used for reverse transcription to cDNA using One-Step RT-PCR Kit (Takara Biotechnology Co., Ltd., Dalian, China). RT-qPCR was performed using SYBR® Green Real-Time PCR Master mix (Takara Biotechnology Co., Ltd., Dalian, China) by the Stratagene Mx3000P Real-Time PCR system (Agilent Technologies, Inc., Santa Clara, CA, USA). The specific primer pairs used are as follows: miR-133a, forward, 5′-TTCTTGTAGACCTGTAAATG-3′ and reverse, 5′-AGC TACAGCTGGTTGAGGAG-3′; U6, forward, 5′-CTC GCT TGCCGACACA-3′ and reverse, 5′-AACGGTTCCAGAATT TGCGT-3′. The thermocycling conditions were as follows: 95°C for 5 min followed by 40 amplification cycles at 95°C for 15 sec and 60°C for 30 sec. The 2−ΔΔCq method (12) was used to calculate the relative change in RNA expression as a ratio.

Immunohistochemistry. Immunohistochemistry was performed to analyze EGFR expression. Sections (thickness, 4 µm) were prepared from paraffin-embedded NSCLC and adjacent tissue samples. Sections were prepared using citrate buffer (pH=6.0, Sangon Biotech Co., Ltd, Shanghai, China) to calculate the relative change in RNA expression as a ratio.

**Apoptosis assay.** H358 cells transfected with miRNA-133a mimics and negative plasmids was assayed 24, 48 h later using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis assay. Annexin V-FITC/PI (BD Biosciences, San Jose, CA, USA) was used to examine the rate of apoptosis using FlowJo software (version 7.6.1; FlowJo LLC, Ashland, OR, USA).

**Western blot analysis.** Transfected H358 cells were assayed at 24, 48 h using a western blot analysis. The cells were lysed with a RIPA buffer (Beutymite Institute of Biotechnology) containing a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol. Western blot analysis was measured using a Model 550 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).
were developed with an Amersham ECL Western Blotting Detection kit (GE Healthcare Life Sciences, Shanghai, China) and analyzed using Image Lab v.4.62 (Bio-Rad Laboratories, Inc.).

Statistical analysis. All values were expressed as the mean ± standard deviation using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Comparisons between the means of two groups were performed using Student's t-test, and with one-way analysis of variance followed by Tukey's post hoc test for multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

EGFR expression in NSCLC tissues and adjacent mucosa. As presented in Fig. 1A, the expression of EGFR mRNA in NSCLC tissues was higher than that in the adjacent mucosal tissues. Additionally, EGFR protein expression in the NSCLC tissues was higher than that in the adjacent mucosa (Fig. 1B).

miRNA-133a expression in NSCLC tissues and adjacent mucosa. The expression of miRNA-133a in NSCLC tissue was initially detected by RT-qPCR, which revealed that it was markedly lower than that in the adjacent mucosal tissue (Fig. 2).

NSCLC cell growth rate following miRNA-133a upregulation. To test the hypothesis that miRNA-133a may be involved in the alternative regulation of NSCLC growth, H358 cells were transfected with miRNA-133a mimics, and the expression of miRNA-133a was assessed via RT-qPCR. The miRNA-133a mimics significantly increased miRNA-133a expression in H358 cells, compared with in the control group (Fig. 3A). Furthermore, upregulated miRNA-133a significantly suppressed H358 cell growth, compared with that of the control group (Fig. 3B).

NSCLC apoptosis following miRNA-133a upregulation. NSCLC apoptosis was evaluated using an Annexin V-FITC/PI apoptosis assay, in order to assess the effect of upregulated miRNA-133a. The results showed that increased miRNA-133a significantly induced H358 cell apoptosis, as compared with the control group (Fig. 4).

Caspase-3 activity, protein expression and EGFR protein expression in NSCLC following miRNA-133a upregulation. To determine the levels of caspase-3 activity, protein expression and EGFR protein expression in NSCLC following the upregulation of miRNA-133a, a caspase-3 ELISA and western blot analysis were conducted. As depicted in Fig. 5, there were significant increases in caspase-3 activity, protein expression and EGFR protein expression in H358 cells following miRNA-133a elevation, as compared with those in the control group.

p-AKT, p-ERK and Bax protein expression in NSCLC following miRNA-133a upregulation. To investigate the association between p-AKT, p-ERK and Bax and miRNA-133a expression in H358 cells, p-AKT, p-ERK and Bax protein expressions was analyzed via western blotting. As presented in Fig. 6, miRNA-133a upregulation significantly suppressed p-AKT, p-ERK and Bax protein expression in H358 cells, as compared with in the control group.

Discussion

NSCLC is the most common cause of cancer-associated mortality. Although methods for the diagnosis and treatment of NSCLC have improved at a constant rate, the prognosis for patients with NSCLC remains poor (13). Clinical staging of NSCLC via pathological analysis is the recommended method for evaluating NSCLC prognosis and selecting the optimal treatment approach (14). With an increasing number
of in-depth studies of the effects of miRNA in tumors and other diseases, miRNA expression changes in NSCLC are gradually becoming a hotspot in this area of research. When classifying the subtype of a tumor, the miRNA expression profile of the tumor tissue can be more accurate than using mRNA (15). Studies have indicated that miRNAs are abnormally expressed in NSCLC, but there is no consensus on the miRNAs involved (8,15). A wide range of different alterations to miRNA expression have been identified (15). Potentially, miRNA analysis can be integrated into staging methods for the diagnosis and prognosis of NSCLC, and potentially allow individualized NSCLC treatment (16). In the present study, the expression of miRNA-133a in NSCLC tissue was markedly lower than that of the adjacent mucosa tissue. Furthermore, upregulating miRNA-133a significantly suppressed cell growth, induced apoptosis, and increase caspase-3 activity and protein expression in H358 cells. These data support that miRNA-133a is a tumor suppressor in lung cancer.

A total of 90% the identified EGFR gene mutations in NSCLC are located in exons 19-21 (17). EGFR can be expressed in epithelial, mesenchymal and neurogenic tissues and it serves a major role in regulating hyperplasia, growth and the differentiation of normal cells (17). EGFR also induces the growth activity to EGFR inhibitors (19). ERK activation mediated by Src can promote the epithelial-mesenchymal transition and promote their sensitivity to EGFR inhibitors (27). AKT and ERK pathways also interact to promote NSCLC cell proliferation and survival. Activating the AKT and ERK pathways simultaneously induces drug resistance in NSCLC cells (28). Mucin 1 mucoprotein may induce the ERK2 pathway to regulate cell growth and differentiation (28). In the present study, the upregulation of the expression of genes that promote tumorigenic cell behavior, including proliferation, invasion, metastasis, angiogenesis and dysplasia (18). The dimer formed upon the EGFR-N extracellular binding domain and ligand binding is the requirement for the phosphorylation of EGFR tyrosine residues and the activation of downstream signaling (19). In the present study, it was identified that upregulating miRNA-133a significantly suppressed the EGFR expression of H358 cells. Cui et al (20) identified that microRNA-133a regulated the proliferation of breast cancer through the EGFR/Akt signaling pathway. Song et al (21) demonstrated that miR-133a suppressed cervical cancer growth through the Akt and ERK signaling pathways. The data presented in the present study suggest that EGFR serves an important role in miRNA-133-induced lung cancer suppression.

Tumor cells commonly exhibit altered signal transduction and imbalanced cell growth, differentiation and apoptosis (22). PI3K/AKT signal transduction is an important intracellular signal transduction pathway that can induce the occurrence and development of numerous types of tumor by affecting cell cycle control, cell survival, metastasis, angiogenesis and chemotherapy resistance (23). Activation often occurs in the early stages of oncogenesis and tumor progression. The degree of signal pathway activation is an important indicator of the prognosis of patients with tumors (24). In the present study, the upregulation of miRNA-133a significantly suppressed the p-AKT protein expression in H358 cells. Cui et al (20) demonstrated that microRNA-133a regulated the proliferation of breast cancer cells through the EGFR/Akt signaling pathway. This is in accord with our novel finding that the regulation of Akt serves a role in the effect of miRNA-133a on lung cancer.

The ERK pathway is an important signal pathway in the occurrence and development of NSCLC, and is a vital target of anticancer treatment (25). Transcription factor AP-2β overexpression has been associated with the incidence of lung adenocarcinoma, which is associated with the regulation of the ERK pathway. Mitogen-inducible gene 6 expression downregulation inhibits NSCLC cell apoptosis, activates the ERK pathway and upregulates the anti-apoptotic protein Bcl-2 (26). ERK activation mediated by Src can promote the gefitinib resistance of NSCLC cells (26). Inhibition of the ERK pathway can prevent NSCLC cells from undergoing epithelial-mesenchymal transition and promote their sensitivity to EGFR inhibitors (27). AKT and ERK pathways also interact to promote NSCLC cell proliferation and survival. Activating the AKT and ERK pathways simultaneously induces drug resistance in NSCLC cells (28). Mucin 1 mucoprotein may induce the ERK2 pathway to regulate cell growth and differentiation (28). In the present study, the upregulation...
of miRNA-133a significantly inhibited p-ERK protein expression and induced the expression of Bax protein in H358 cells. Song et al (21) demonstrated that miR-133a suppresses cervical cancer growth through an effect on the AKT and ERK signaling pathways.

In conclusion, the present study has demonstrated that the upregulation of miRNA-133a significantly suppressed cell growth, induced apoptosis, and increased caspase-3 activity and protein expression via the EGFR/AKT/ERK signaling pathway in H358 NSCLC cells. Thus, targeting miRNA-133a may exhibit potential as a novel therapeutic strategy against NSCLC through the EGFR/AKT/ERK signaling pathway.

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Availability of data and materials
The analyzed data sets generated during the study are available from the corresponding author, on reasonable request.

Authors’ contributions
WZ designed the experiment. NNG, YNZ, SJL, SSL and JQY performed the experiment. WZ and JQY analyzed the data. WZ wrote the manuscript.

Ethics approval and consent to participate
The present study was approved by the Institutional Medical Ethics Committee of The First Affiliated Hospital of the Chinese People’s Liberation Army, and all participants provided informed, written consent.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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