miR-143 suppresses the proliferation of NSCLC cells by inhibiting the epidermal growth factor receptor

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Abstract. MicroRNAs (miRs) regulate the proliferation and metastasis of numerous cancer cell types. It was previously reported that miR-143 levels were downregulated in non-small cell lung cancer (NSCLC) tissues and cell lines, and that the migration and invasion of NSCLC cells was inhibited upon suppression of cell proliferation and colony formation by the upregulation of miR-143. Epidermal growth factor receptor (EGFR), which is a vital factor in the promotion of cancer cell proliferation and has been investigated as a potential focus in cancer therapy, has been reported to be a possible target of miR-143. The present study aimed to investigate the role of miR-143 in NSCLC using NSCLC cell lines and primary cells from NSCLC patients. NSCLC cells were co-transfected with EGFR and miR-143, and the mRNA and protein expression of EGFR were analyzed. Furthermore, the activity of the transfected cancer cells with regard to colony formation, migration, invasion and apoptosis were evaluated. The levels of miR-143 were decreased in the NSCLC cell lines and primary cells from patients with NSCLC compared with the controls. Following transfection with miR-143, the ability of NSCLC cells to proliferate, form colonies, migrate and invade was inhibited. Similarly, knockdown of EGFR led to the suppression of NSCLC cell proliferation. The mRNA and protein expression levels of EGFR were significantly reduced following miR-143 overexpression, and the level of miR-143 was inversely correlated with that of EGFR in NSCLC cells. The results of the present study demonstrated that miR-143 was able to suppress NSCLC cell proliferation and invasion by inhibiting the effects of EGFR, suggesting that EGFR may be considered a potential target for NSCLC therapy.

Introduction

Lung cancer is the most common cause of cancer-associated mortality worldwide, and the incidence of lung cancer is rapidly increasing in developing countries (1). Lung cancer incidence rates are approximately twice as high in developed countries (61/100,000 males and 19/100,000 females) compared with developing countries (29/100,000 males and 10/100,000 females) (2,3). Of the types of lung cancer, non-small cell lung cancer (NSCLC) accounted for 80% of all lung cancer cases (4). The primary types of NSCLC are adenocarcinoma (including bronchioalveolar carcinoma), which accounts for 32-40% of NSCLC cases, squamous NSCLC, which accounts for 25-30% of cases, and large cell NSCLC, which account for 8-16% of cases (5). Despite recent advances in the diagnosis and chemotherapeutic and targeted treatment of NSCLC, including immunotherapy, such as epidermal growth factor receptor (EGFR) targeted treatment, insulin-like growth factor 1 receptor or EML4-ALK fusion protein interference (6), the overall survival rate of NSCLC patients remains low (5-year survival rate of 15%) and the recurrence rate of NSCLC remains high, even with early diagnosis (7). The poor prognosis of NSCLC may be due to the relatively limited understanding of its underlying etiology, in addition to the late presentation and heterogeneity of lung cancer (8). Therefore, elucidation of the molecular mechanisms underlying NSCLC tumor metastasis, including signaling pathways, associated receptors and/or binding sites, is urgently required.

MicroRNAs (miRs) have been revealed to function in the regulation of gene expression and have important roles in a wide range of physiological and pathological processes (9,10). The majority of miRs negatively regulate the expression of their target genes by binding to the 3'-untranslated regions (3'-UTRs) of mRNA, leading to mRNA degradation or translational suppression (11,12). In numerous biological processes, including cell proliferation, differentiation, migration and apoptosis, miRs are involved in regulating the expression of multiple target genes (13-15).

Aberrant miR expression has been associated with numerous types of cancer, and it has been suggested that miRs may act as inhibitors of oncogenes or as tumor suppressors (16-18). Various miRs, including miR-183, miR-21 and miR-155, have been shown to promote the development of NSCLC (19). Conversely, miR-99a, miR-340 and
miR-223, were reported to function as tumor suppressors in NSCLC (20,21). The survival rate of a cecal ligation and puncture-induced sepsis in a mouse model was shown to be improved by TLR3-activated mesenchymal stromal cells, whereas the effectiveness of this therapy was reduced by overexpression of miR-143 (22). Furthermore, miR-143 was demonstrated to upregulate the mRNA expression levels of absent in melanoma 2 and apoptosis-associated speck-like protein containing a CARD in a Jurkat cell line (23), and it was suggested that miR-143 may be used as a novel diagnostic biomarker for NSCLC (24). However, the function of miR-143 in NSCLC remains poorly understood. EGFR has been observed to be an important factor in the promotion of cancer cell proliferation (25). Numerous studies have been performed on EGFR as a targeted therapy for NSCLC (26). EGFR has been observed to be directly regulated by miR-128b (27). As a functional factor of cell proliferation, EGFR was considered to be a potential target of miR-143. Therefore, the present study aimed to investigate the role of miR-143 in NSCLC, particularly its ability to regulate the processes of cancer cell migration, invasion, proliferation and apoptosis. Furthermore, the EGFR was analyzed as a potential target of miR-143 in NSCLC cells.

Materials and methods

Patients and cell lines. NSCLC tissue samples, human NSCLC primary cells and matched normal tissues (normal tissue extracted adjacent to the NSCLC tissue in the same patient) were obtained from 35 patients at the Department of Respiratory Medicine of the China-Japan Friendship Hospital (Beijing, China) between January 2014 and January 2015. Written informed consent was obtained from all patients and the present study was approved by the Medical Ethics Committee of the China-Japan Friendship Hospital. The clinicopathological information for the patients is summarized in Table I. All tissue samples were stored in liquid nitrogen for subsequent processing. A549, H520 and H460 NSCLC cell lines, in addition to the 16HBE normal lung bronchus epithelial cell line, were obtained from the ATCC (Manassas, VA, USA). These cell lines differed in applications. The expression levels of miR-143 were investigated in the tissue samples, the 16HBE normal lung epithelial cell line and the three NSCLC cell lines (A549, H520 and H460). The suppressive effect of miR-143 on the proliferation, migration and invasion of NSCLC were studied in A549 cells. The potential target effects of EGFR were also studied in A549 cells.

RNA extraction and quantitative PCR (qPCR). The expression levels of miR-143 in 35 pairs of NSCLC tissue samples and their matched normal tissue samples were determined.

 Firstly, the tissues were sectioned into 2x2x2 mm sections and all the tissues were homogenized. Then, total RNA was extracted from the tissue homogenate using the mirVana™ miRNA Isolation kit (Ambion; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Thirdly, miR was extracted with a microRNA Fast Extraction kit (Spin column; Signalway Antibody Co., College Park, MD, USA) and detected with an All in One™ miRNA RT-qPCR Detection kit (GeneCopoeia, Inc., Rockville, MD, USA). The purity and quality of the RNA were verified by measuring the absorbance at 260 and 280 nm (A260 and A280) on the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). An A260/A280 ratio of ~2.0 was considered an acceptable quality.

 miR was extracted from the total RNA using a microRNA Fast Extraction kit (Spin-column; Signalway Antibody Co., College Park, MD, USA) and detected with an All-in-One™ miRNA qRT-PCR Detection kit (GeneCopoeia, Inc., Rockville, MD, USA) with SYBR Green reagents (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's protocol. The primers used to amplify miR-143 were purchased from Guangzhou RiboBio Co., Ltd (sequences unknown). Amplification was conducted with the following thermocycling conditions: 95°C for 20 sec followed by 50 cycles at 95°C for 10 sec and 55°C for 20 sec. Following EGFR mRNA amplification from the total RNA, the mRNA was reverse transcribed into cDNA using AMV reverse transcriptase (Promega Corporation, Madison, WI, USA). The expression level of EGFR mRNA was assessed using the SYBR Premix Ex Taq™ II (Tli RNase H Plus) kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol with the following primers (Sangon Biotech Co. Ltd., Shanghai, China): Forward, 5'-TGT TCCAACGAACTGGGCTAA-3' and reverse, 5'-TGGCTTTCGAGATGTGCT-3'. The PCR thermocycling conditions were as follows: 50°C for 10 sec and 95°C for 10 min, followed by 46 cycles at 95°C for 15 sec and 60°C for 1 min. EGFR and miR-143 expression were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used to detect GAPDH expression were as follows: GAPDH forward, 5'-AAT GAC CCC TTC ATT GAC-3', and reverse, 5'-TCCACGACGTACTCAAGGC-3'. The expression levels were quantified using the 2^{-ΔΔCq} method (28).

Cell survival assays. A549 cell proliferation was assessed using a BrdU Cell Proliferation ELISA kit (EMD Millipore, Billerica, MA, USA). Briefly, 6x10^4 A549 cells/well were cultured in 96-well plates in DMEM for 24 h at 37°C, then 10 ml BrdU reagent was added to each well and the plates were incubated for 1.5 h at 37°C. Absorbance at 450 nm was measured using a microplate reader.

Colony formation assay. In order to assess colony formation, 500 A549 cells/well were seeded into 6-well plates 24 h following transfection and cultured in DMEM for 2 weeks at 37°C. The culture medium was renewed after 3 days, following which the cells were fixed with methanol, stained with 0.5% crystal violet for 20 min and visible colonies were counted using a microscope (DM600; Leica Microsystems GmbH, Wetzlar, Germany). Triplicate wells were measured for each sample.

Materials and methods

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RNA extraction and quantitative PCR (qPCR). The expression levels of miR-143 in 35 pairs of NSCLC tissue samples and their matched normal tissue samples were determined.
Flow cytometry. Cell apoptosis was analyzed by flow cytometry and fluorescence-activated cell sorting was employed to determine the fraction of apoptotic cells. The NSCLC A549 cell lines were treated with 50 µM quercetin (Sigma-Aldrich) for 1, 2 or 4 days in order to trigger apoptosis, after which the cells (1x10⁶) were fixed with 80% ethanol, stained with propidium iodide solution and analyzed using the FACS Calibur™ flow cytometer with CellQuest software (version 7.5.3; BD Biosciences, San Jose, CA, USA). Another apoptosis assay was conducted by dual-laser flow cytometry. Briefly, following treatment with 50 µM quercetin, the cells were stained with 1 µg/ml Hoechst stain and 1 µg/ml 7-amino-actinomycin D. The co-stained cells were then analyzed with a FACS Calibur flow cytometer.

Migration and invasion. A549 cell migration and invasion were assessed using Transwell chambers. Briefly, A549 cells were transfected with miR-143 and then incubated for 24 h at 37°C in DMEM. The transfected cells (5x10⁴) were seeded into the upper chamber containing DMEM, following which DMEM supplemented with 10% FBS was added to the lower chamber to function as a chemoattractant. The chambers were incubated at 37°C in 5% CO₂ for 16 h, followed by removal of the cells remaining on the upper surface of the chamber. Cells that had migrated to the bottom surface were washed twice with cold phosphate-buffered saline, fixed in methanol and stained with 0.1% crystal violet. Stained cells were counted under a microscope. All assays were performed in triplicate.

Western blotting. The cells were treated with 50 µM quercetin for 2 days and subsequently lysed using lysis buffer (0.2 M ethylenediaminetetraacetic acid, 0.05 M Tris-HCl, pH 8.0, 5% Triton X-100) for 30 min on ice to obtain total protein. In order to validate the assays, A549 cells were treated with two different concentrations of quercetin (25 or 50 µM). Protein concentration was determined using a bicinchoninic acid assay. The proteins (2 µl/well) were separated by electrophoresis on a 10% sodium disulphide-polyacrylamide gel and transferred onto nitrocellulose membranes for incubation with rabbit monoclonal anti-EGFR (1:5,000; cat. no. EP38Y; Abcam, Cambridge, UK) and rabbit polyclonal anti-GAPDH (loading control; cat. no. ab9585; 1:2,500; Abcam) primary antibodies overnight at 4°C. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibodies (cat. no. ab9565; 1:2,500; Abcam) for 1 h at room temperature. Protein bands were detected with the WEST-ZOL (Plus Western Blot Detection System (Intron Biotechnology, Inc., Seongnam, Korea) and the relative intensity was calculated by normalizing to the GAPDH loading control using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). Experiments were performed in triplicate.

Plasmids and luciferase activity assays. miR-143, miR-143 inhibitor, control mimic and control inhibitors were obtained from Yoshimitsu Biotechnology Co., Ltd. EGFR short hairpin RNA (shRNA) was obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China) and used as an inhibitor of EGFR expression. For the expression of EGFR, the coding sequence of EGFR was amplified using the following primers: Forward, 5'-ACCTGCGTGAAGAAGTGTC-3', and reverse, 5'-CGT TACACACTTTGGCGCAAGG-3'. The coding sequence was subsequently cloned into PMD19T (Takara Bio, Inc.) by polymerase chain reaction (PCR), according to the manufacturer's instructions.

Luciferase activity assays were performed to assess the suppressive effect of miR-143 on EGFR expression in HEK293 cells, which was a model cell line specifically used to study the transfection of functional genes (ATCC). Briefly, to construct a luciferase expression vector containing part of the EGFR 3'-UTR, the wild-type (WT) 568-886 fragment of EGFR mRNA, encoding the potential miR-143 binding sites at position 2436-3121, was amplified using the following primers: Forward, 5'-ACCTGCGTGAAGAAGTGTC-3'; and reverse, 5'-CGT TACACACTTTGGCGCAAGG-3'. The PCR thermocycling conditions were as follows: 50°C for 10 sec and 90°C for 10 min, followed by 30 cycles at 90°C for 15 sec and 55°C for 1 min. Mutation of the EGFR 3'-UTR (Mut) was performed using the cobas® EGFR Mutation Test (Roche Molecular Diagnostics, Pleasanton, CA, USA). The resulting PCR fragment was inserted into the pGL3-Basic Vector (Promega Corporation, Madison, WI, USA) encoding the Bam HI/Sal I endonuclease restriction sites according to the manufacturer's protocol. The ligation was performed with DNA Ligase (Takara Bio, Inc.). Subsequently, 80% confluent HEK293 cells were co-transfected with 100 ng WT or Mut EGFR 3'-UTR and 80 nM miR-143 or control mimics using Lipofectamine 2000. A proportion of the cells were alternatively transfected with 80 ng PMD19T control vector in order to monitor the transfection efficiency. miR-NC was applied as a non-targeting negative control. Furthermore, all cells were transfected with pRL-SV40 (Promega Corporation) as a control for normalization. Cells were harvested 48 h after transfection for subsequent analyses. A549 cells were
transfected with miR-143, control mimic miR-143 inhibitor and control inhibitor (Shanghai GenePharma Co., Ltd., Shanghai, China). A549 cells were transfected with either EGFR-targeted shRNA or non-targeted shRNA. The mRNA and protein expression levels of EGFR were detected by qPCR and western blotting as previously described. The viability, migration and invasion of A549 cells were also detected as previously described.

Statistical analysis. Data are presented as the mean ± standard deviation. Student's t-test was performed, and χ² and Mann-Whitney U tests were applied to analyze the clinicopathological information of patients using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-143 is downregulated in NSCLC tissues and cell lines. The expression levels of miR-143 were measured in 35 NSCLC tissue samples and their matched normal tissue samples using qPCR. The expression levels of miR-143 were markedly decreased in NSCLC tissues compared with the normal control tissues (Fig. 1A). The expression levels of miR-143 in three NSCLC cell lines were similarly determined, and miR-143 was significantly downregulated in the NSCLC cell lines compared with the 16HBE normal lung bronchus epithelial cell line (Fig. 1B). These results suggest that the progression of NSCLC may be associated with downregulation of miR-143.

miR-143 suppresses NSCLC cell proliferation. miR-143 was transfected into A549 NSCLC cells to investigate its effect on NSCLC cell proliferation using the BrdU cell proliferation assay. Successful transfection of the cells with miR-143 mimic was confirmed by qPCR (Fig. 2A). Compared with the corresponding controls, A549 cell proliferation was markedly suppressed by miR-143 overexpression (Fig. 2B). In addition, flow cytometry demonstrated that miR-143 overexpression was able to induce the apoptosis of A549 cells (Fig. 2C). Furthermore, a colony formation assay was performed to assess whether varying the expression level of miR-143 results in A549 cell cycle arrest or cell death, since either of these would result in a reduction in colony number. The ability of A549 cells to form colonies was significantly inhibited following the overexpression of miR-143 (Fig. 2D). These results suggest that miR-143 is able to suppress NSCLC cell growth.

miR-143 suppresses NSCLC cell migration and invasion. Transwell assays were used to assess the migratory and invasive behaviour of A549 cells transfected with miR-143 or control mimics. Migration (Fig. 3A) and invasion (Fig. 3B) were markedly inhibited in A549 cells transfected with miR-143 compared with cells transfected with control mimics. These data suggest that NSCLC cell death and injury may be induced by miR-143.

EGFR is a potential target of miR-143. Luciferase activity assays were used to investigate the suppressive effect of miR-143 on EGFR expression in HEK293 cells. The luciferase activity of the WT EGFR was markedly inhibited in HEK293 cells transfected with miR-143 compared with the cells transfected with control mimics (Fig. 4A). miR-143 was used to determine the effects of miR-143 on EGFR. miR-143 inhibitor was used to determine the effects on EGFR following miR-143 inhibition. Control mimic was used as a negative control of miR-143 control. The length of the mimic was similar to miR-143 although lacked a known function. Control inhibitor was used as a negative control of miR-143 inhibitor. Its length was similar to miR-143 inhibitor but lacked a known function. Furthermore, the mRNA and protein expression levels of EGFR were significantly reduced in HEK293 cells transfected with miR-143, as compared with cells transfected with the control mimic (Fig. 4B-D). These results suggest that miR-143 overexpression induces a reduction in EGFR mRNA, leading to a reduction in the levels of EGFR protein. Furthermore, the inverse correlation between the levels of miR-143 and EGFR suggested that EGFR may be a target of miR-143.

EGFR inhibition and miR-143 overexpression exert identical effects. A549 cells were transfected with EGFR-targeted shRNA or non-targeted control to investigate whether shRNA EGFR silencing exerts similar effects on NSCLC cells to the effects associated with miR-143 overexpression. Downregulation of the protein and mRNA expression levels of EGFR...
following transfection of A549 cells with EGFR shRNA was confirmed by western blotting (Fig. 5A and B) and qPCR (Fig. 5C). Notably, the inhibition of EGFR by shRNA reduced the proliferation, migration and invasion of A549 cells (Fig. 5D-F). These results suggest that the inhibitory effects of EGFR-targeted shRNA on EGFR are similar to those of miR-143 overexpression.

Discussion

miRs have been shown to have fundamental roles in numerous biological processes, including carcinogenesis, angiogenesis, programmed cell death, cell proliferation, invasion, migration and differentiation (29,30). Previous studies have reported that miRs are associated with the onset and progression of various types of cancer, that miRs may act as tumor suppressors or oncogenes, and have implicated them in aberrations in the expression of key genes (28,31). The diverse roles described for miRs have been elucidated by analyzing their biological synthesis and functions (32). Each miR is capable of regulating the expression of numerous genes, thus allowing them to affect multiple cellular signaling pathways simultaneously (33).

Increasingly, previous studies have focused on the inhibitory effects of miRs on cancer and tumors; cyclooxygenase-2 activity was demonstrated to be suppressed by miR-143, preventing gastric cancer growth and inducing the apoptosis of gastric cancer cells (34). Furthermore, miR-145 levels were enhanced by histone deacetylase inhibitors in a Burkitt lymphoma cell line, and the levels of miR-143 were markedly downregulated in samples from patients with Burkitt lymphoma (35). Low and high levels of miR-143 in cancer
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cells and normal cells, respectively, were associated with the differential expression of reporters and tumor necrosis factor-α (36). In addition, a previous study demonstrated that the expression of DNA methyltransferase 3A was regulated by miR-143 in breast cancer cells (37), and it was reported that prostate cancer cell migration and invasion were enhanced following the loss of the tumor-suppressive miR-143/145 cluster due to alterations in the expression level of Golgi membrane protein 1 (38). Insulin activity, including insulin-mediated phosphorylation of Akt and insulin-stimulate glucose uptake, was shown to be inhibited by epicardial adipose tissue-type 2 diabetes-mediated release of activin A, which may have occurred due to miR-143 activation in cardiomyocytes (39). Furthermore, the expression of miR-143 was

Figure 4. EGFR is a potential target of miR-143. (A) The inhibitory effects of miR-143 on luciferase activity in HEK293 cells co-transfected with 100 nM WT or Mut EGFR 3′-untranslated region and miR-143 or negative control mimics. The (B) mRNA and (C and D) protein expression levels of EGFR in A549 cells transfected with miR-143 or control mimic and A549 cells transfected with miR-143 or control inhibitor, were detected by quantitative polymerase chain reaction and western blotting, respectively. GAPDH was used as an internal control. *P<0.05, **P<0.01 vs. the control. EGFR, epidermal growth factor receptor; miR-143, microRNA-143; WT, wild-type; Mut, mutant; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NC, normal control; shRNA, short hairpin RNA.

Figure 5. Similar effects were observed for EGFR inhibition and microRNA-143 overexpression. The (A) mRNA and (B and C) protein expression levels of EGFR were significantly reduced in the A549 cells transfected with EGFR shRNA compared with the cells transfected with the NC shRNA. The mRNA and protein expression levels of EGFR were detected by quantitative polymerase chain reaction and western blotting, respectively. The (D) viability, (E) migration and (F) invasion of A549 cells transfected with EGFR shRNA were significantly reduced, as compared with the cells transfected with the NC shRNA. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. the shRNA NC. EGFR, epidermal growth factor receptor; shRNA, short hairpin RNA; NC, normal control; OD, optical density.
affected by numerous factors associated with insulin sensitivity in a previous study (39).

EGFR, which is a transmembrane protein with cytoplasmic kinase activity, transduces important growth factor signaling from the extracellular environment to the interior of the cell (25). EGFR is expressed in >60% of NSCLCs and it has emerged as an important therapeutic target in the treatment of various types of tumor (26). Furthermore, it has been reported that NSCLC patients with EGFR mutations exhibit a better response to EGFR-tyrosine kinase inhibitor treatment, as compared with those without EGFR mutations (40).

The results of this study demonstrated that the level of EGFR was inversely correlated with that of miR-143 in NSCLC cells, suggesting that EGFR may be a downstream target of miR-143. Furthermore, miR-143-mediated suppression of EGFR may be a potential mechanism underlying the miR-143-mediated inhibition of NSCLC growth. The effects of inhibiting EGFR expression in HEK293 cells were similar to those observed for the overexpression of miR-143 in A549 NSCLC cells. In previous studies, upregulated EGFR expression was inversely associated with the levels of miR-143 in NSCLC tissues (41-45). miRs reduce the expression level of target genes by binding to mRNA; the mRNA may be degraded or its translation by the ribosome may be suppressed. Therefore, in the present study, the downregulated mRNA and protein expression levels of EGFR in NSCLC cells transfected with miR-143 may have been the result of miR-143-mediated downregulation of EGFR mRNA. The results of the present study suggested that miR-143 suppressed the growth, metastasis and invasion of NSCLC cell lines by downregulating the expression of EGFR.

In conclusion, the present study demonstrated that miR-143 was downregulated in NSCLC patient tissues and NSCLC cell lines. The overexpression of miR-143 in NSCLC cells was able to suppress tumor growth, potentially by inhibiting EGFR expression. The results of the present study provided some novel ideas for the future treatment of NSCLC.

References