miR-215 functions as a tumor suppressor and directly targets ZEB2 in human non-small cell lung cancer

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Abstract. MicroRNA-215 (miR-215) has previously been demonstrated to be dysregulated in a number of human malignancies and to be correlated with tumor progression. However, the expression and function of miR-215 in non-small cell lung cancer (NSCLC) has remained to be elucidated. Therefore, the present study aimed to investigate the effects of miR-215 in NSCLC tumorigenesis and development. Reverse transcription-quantitative polymerase chain reaction was used to evaluate miR-215 expression in NSCLC cell lines and primary tumor tissues. The association between miR-215 expression and certain clinicopathological factors was also determined, and the effects of miR-215 on the biological behavior of NSCLC cells were investigated. In addition, the potential regulatory function of miR-215 on zinc finger E-box-binding homeobox 2 (ZEB2) expression was examined. miR-215 expression was significantly downregulated in NSCLC cell lines and clinical specimens. Reduced miR-215 expression was significantly associated with lymph node metastasis and advanced TNM stage. Overexpression of miR-215 inhibited NSCLC cell proliferation, invasion and migration, and promoted cell apoptosis in vitro, and suppressed tumorigenicity in vivo. Furthermore, luciferase reporter assay analysis identified ZEB2 as a direct target of miR-215. These findings indicated that miR-215 may act as a tumor suppressor in NSCLC and may serve as a novel therapeutic agent for miR-based therapy.

Introduction

Lung cancer is one of the most common neoplasias, with ~1.5 million novel cases diagnosed every year, and the leading cause of cancer-associated mortality worldwide (1). Despite advances in clinical and experimental oncology, the prognosis of patients with lung cancer has remained unfavorable. Non-small cell lung cancer (NSCLC) accounts for ≥80% of all lung cancer, and its 5-year survival rate is ~15% (2,3). Similarly to other types of cancer, the development of NSCLC is a multistep process involving the accumulation of genetic and epigenetic changes (4). Previous studies have demonstrated diverse genetic alterations in NSCLC (5,6), but the molecular mechanisms underlying NSCLC carcinogenesis and progression are highly complex, and further identification of novel candidate molecules that participate in these processes is required for improving the diagnosis, prevention and treatment of this disease.

MicroRNAs (miRs) are a class of short (~22 nucleotides in length), endogenous, single-stranded, non-protein-coding RNAs that directly bind to the 3'-untranslated regions (3'-UTRs) of target mRNAs, resulting in mRNA degradation or translational suppression (7). It is well-known that miRs are involved in numerous biological processes, including cell growth, apoptosis, development, differentiation and endocrine homeostasis (8). A previous study also indicated that miRs are essential in the biology of human cancer, which may provide a novel and promising approach for the treatment of cancer (9). Dysregulation of miR expression has been frequently reported and closely associated with tumor initiation, promotion and progression. For example, miR-215 has been implicated in the pathogenesis of several human malignancies, is upregulated in cervical cancer (10), hepatocellular carcinoma (11), gastric cancer (12) and prostate cancer (13) and acts as a potential oncogene in these tumors. By contrast, miR-215 expression has been observed to be significantly reduced in esophageal adenocarcinoma (14), colon cancer (15) and renal cell carcinoma (RCC) (16), and in these cases it functions as a candidate tumor suppressor. However, to the best of our knowledge, the correlation between miR-215 expression and the clinicopathological characteristics of NSCLC has not previously been evaluated, and the biological roles of miR-215 and its direct functional targets in NSCLC remain poorly understood.

Epithelial-to-mesenchymal transition (EMT) has been recognized to be significant physiological process associated with cancer progression and metastasis (17). Zinc finger E-box-binding homeobox 2 (ZEB2), a key member of the ZEB family, induces EMT through repression of E-cadherin and promotes tumor development (18). High ZEB2 expression has been observed in diverse types of cancer, including...
NSCLC (19–23), where its upregulation is correlated with malignant character, chemotherapeutic resistance and poor patient survival. Notably, a number of miRs, including miR-132 (24), miR-144 (25) and miR-200c (26), participate in the regulation of ZEB2 activity in various tissues; however, the potential regulatory effect of miR-215 on ZEB2 expression in NSCLC has not been confirmed.

In the present study, the expression of miR-215 and its clinical significance in NSCLC were evaluated. The effects of miR-215 on NSCLC cell phenotype were also analyzed. Furthermore, the role of ZEB2 was investigated by luciferase reporter assay.

Materials and methods

Patients and clinical specimens. Paired NSCLC and adjacent non-cancerous lung tissues were obtained from 115 patients during curative resection of NSCLC in Zhongnan Hospital of Wuhan University (Wuhan, China) between January 2010 and December 2013. These tissues were flash-frozen in liquid nitrogen immediately after resection and stored at -80°C prior to use. None of the patients had received neoadjuvant chemotherapy or radiotherapy prior to surgery. Patient characteristics are presented in Table I. The present study was approved by the Research Ethics Committee of Zhongnan Hospital of Wuhan University, and written informed consent was obtained from each patient.

Cell lines and miR transfection. A total of four NSCLC cell lines (A549, H460, 95D and HCC827) and normal lung epithelial cells (NLECs) were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin G sodium, and 100 µg/ml streptomycin sulfate (Sigma-Aldrich Shanghai Trading Co, Ltd., Shanghai, China). All the cells were incubated at 37°C in a humidified atmosphere with 5% CO2.

For RNA transfection, 106 cells were seeded into each well of 24-well plate and incubated overnight. Subsequently, the cells were transfected with mature miR-215 mimics, miR-215 inhibitors (anti-miR-215), or negative control (mir-NC or anti-miR-NC) (Shanghai GenePharma Co, Ltd., Shanghai, China) at a concentration of 50 nM using Lipofectamine® 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was extracted from the cells and tissues using TRIzol reagent (Invitrogen Life Technologies). Complementary (c)DNA was reverse transcribed from the total RNA samples using specific miR primers from the TaqMan MicroRNA assay and reagents from the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems Life Technologies, Foster City, CA, USA). The primers for miR-215 and U6 were as follows: miR-215 forward, 5'-GGGTCCGAGGTATTCGCACT-3'; miR-215 reverse, 5'-CGATGACCTATGAATTGACAGACG-3'; U6 forward, 5'-GGGTCCGAGGTATTCGCACT-3'; U6 reverse, 5'-CGTTCACGAATTTCGTCATT-3'. Products were amplified by PCR using the TaqMan Universal PCR Master Mix kit (Applied Biosystems Life Technologies) and the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 74°C for 5 s. Small nucleolar RNA U6 was used as an internal standard for normalization. All the reactions were performed in triplicate, and the 2-ΔΔCT method (ΔΔCT = CTmiR-215 - CTU6) was used to quantify the relative quantity of miR-215.

Analysis of cell proliferation in vitro. The in vitro cell proliferation was measured using the MTT method. Briefly, cells were seeded into 96-well plates (2x104 cells/well) and incubated at 37°C following transfection. At various time-points (24, 48, 72 or 96 h), the culture medium was removed and replaced with fresh medium containing 0.5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA). The cells were then incubated for a further 4 h and resolved by dimethyl sulfoxide (Sigma-Aldrich). The absorbance was measured at 490 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Detection of apoptosis by flow cytometry. Apoptosis was detected by flow cytometric analysis. Briefly, the cells were washed and resuspended at a concentration of 1x106 cells/ml. The cells were then stained with Annexin V and propidium iodide, using an Annexin V apoptosis detection kit (Abcam, Shanghai, China). Following incubation at room temperature in the dark for 15 min, cell apoptosis was analyzed with a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA).

Transwell invasion assay. The invasion assay was performed using 24-well Transwell chambers (8 µm; Corning Life Sciences, Corning, NY, USA). Following transfection, tumor cells were resuspended in serum-free RPMI 1640 medium and 2x105 cells were seeded into the upper chambers covered with 1 mg/ml Matrigel (BD Biosciences, San Jose, CA, USA), while 0.5 ml RPMI 1640 containing 10% FBS was added to the bottom chambers. Following a 24-h incubation, the non-filtered cells were gently removed with a cotton swab. Filtered cells located on the lower side of the chamber were stained with 0.1% crystal violet (Sigma-Aldrich) and counted under a microscope (DP50; Olympus Corporation, Tokyo, Japan).

Scratch migration assay. The scratch migration assay was performed to evaluate the effect of miR-215 on NSCLC cell migration. When the cells transfected with miR-215 mimics, miR-215 inhibitors or NC reached confluence, a scratch in the cell monolayer was made with a cell scratch spatula. Following incubation of the cells under standard conditions for 24 h, images of the scratches were captured using a digital camera system coupled with a microscope (DP50; Olympus Corporation).

Target searches for miR-215. In order to identify potential mRNA targets of miR-215, database searches of microRNA target prediction engine TargetScan (http://www.targetscan.org) were conducted using the search term ‘miR-215’. The ZEB2 target was subsequently selected for further investigation as ZEB2 has been identified as an important oncogene in NSCLC (23) and a direct target of miR-215 in renal cell carcinoma (16).

Luciferase reporter assays. The pGL3-report luciferase vector (Sigma-Aldrich Shanghai Trading Co, Ltd.) was used for...
the construction of the pGL3-ZEB2 and pGL3-ZEB2-mut vectors. The pGL3-ZEB2-mut vector was constructed using ZEB2 that had undergone site-directed mutagenesis of the miR-215 target site using the Quik-Change site-directed mutagenesis kit (Agilent Technologies GmbH, Waldbronn, Germany). For the luciferase reporter assay, the cells were cultured in 24-well plates (10^5 cells/well) and transfected with the plasmids (100 ng/well) and miR-215 mimics using Lipofectamine 2000 (50 nM). At 24 h following transfection at 37˚C, luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA). The firefly luciferase activity was normalized to the Renilla luciferase activity for each transfected well.

Western blot analysis. Protein lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Kangcheng Biology Engineering Co., Ltd., Shanghai, China). Following blocking in 5% non-fat milk in 1X Tris-buffered saline (pH 7.4) containing 0.05% Tween-20, the membranes were incubated with purified rabbit anti-ZEB2 antisera (cat. no. LS-C160768; dilution, 1:1,000; LifeSpan BioSciences, Inc., Seattle, WA, USA) at 4˚C overnight. The following day, the membranes were washed with PBS and incubated with peroxidase-conjugated goat anti-rabbit IgG (cat. no. sc-2445; dilution, 1:4,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Immunodetection was conducted using chemiluminescence reagents (Pierce Biotechnology, Inc., Rockford, IL, USA) and exposed on X-ray film (Nikon Corporation, Tokyo, Japan). β-actin (cat. no. bs-0061R; Bioss, Inc., Woburn, MA, USA) was used as an internal reference for relative quantification.

Tumorigenicity in vivo. Tumor formation was studied by establishing a xenograft model. Commercial lentiviral vectors containing miR-215 (LV-miR-215; Shanghai GeneChem Co. Ltd., Shanghai, China) were used to infect NSCLC cells according to the manufacturer's instructions. An empty lentiviral construct served as a negative control (LV-NC). A total of 16 female BALB/c athymic nude mice (3-4 weeks old) were purchased from the Model Animal Research Center of Nanjing University (Jiangsu, China). NSCLC cells (1x10^6; 100 µl cell suspension) stably overexpressing miR-215 or NC were inoculated subcutaneously into the mice (n=8 per group).
Bidimensional tumor measurements were taken with vernier calipers every 4 days, and the tumor volume (mm$^3$) was calculated using the formula volume = (length x width$^2$)/2. Three weeks following inoculation, the mice were sacrificed by spinal dislocation and the tumors were weighed.

Statistical analysis. All data are presented as the mean ± standard deviation. Statistical analyses were performed using SPSS software, version 15.0 (SPSS Inc., Chicago, IL, USA). The differences between the groups were analyzed using Student's t-test or $\chi^2$ test. The associations between miR‑215 expression and ZEB2 protein levels were evaluated using Pearson's correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

miR‑215 expression is downregulated in NSCLC. RT-qPCR analysis was performed to detect miR‑215 expression in NSCLC tissues and cell lines. As presented in Fig. 1A, the results demonstrated that the expression levels of miR‑215 were significantly reduced in NSCLC specimens (8.2±1.9) compared with those in the corresponding adjacent non-cancerous tissues (19.2±4.0; P<0.001). The miR‑215 expression in the 4 NSCLC cell lines was also markedly downregulated, compared with that of the NLECs (Fig. 1B). Since among the 4 NSCLC cell lines the A549 cell line exhibited the lowest miR‑215 expression, while 95D cells expressed relatively high levels of miR‑215, these two cell lines were selected for miR‑215 mimics or miR‑215 inhibitor transfection and further analysis.

ZEB2 and miR‑215 expression are inversely correlated. ZEB2 protein levels were detected by using western blot analysis. The results demonstrated that the ZEB2 protein expression levels in the tumor samples were increased compared with those of the adjacent normal tissues (P<0.001; Fig. 1C). The ZEB2 protein expression levels in the NSCLC cells were also increased compared with those of the NLEC cells (Fig. 1D). In addition, a significant inverse correlation (R=−0.4216; P=0.0002) was observed between ZEB2 and miR‑215 protein expression levels in NSCLC tumor tissues (Fig. 1E).

miR‑215 expression is associated with certain clinicopathological features of NSCLC. The associations between miR‑215 expression and various clinicopathological parameters of NSCLC tissues are presented in Table I. The patients were divided into a high miR‑215 expression group and a low miR‑215 expression group, using the median miR‑215 expression value amongst all 115 NSCLC patients as a cut-off. As demonstrated in Table I, miR‑215 expression was significantly reduced in samples with lymph node metastasis (P=0.002) and advanced TNM stage (P<0.001). No significant differences were observed between miR‑215 expression and age, gender, smoking status, cell types, T stage or tumor differentiation.

miR‑215 influences the biological behaviors of NSCLC cells. To selectively overexpress or downregulate miR‑215, mature miR‑215 mimics or miR‑215 inhibitors were transfected into A549 or 95D cells. RT-qPCR analysis confirmed enhanced miR‑215 expression following miR‑215 mimics transfection and reduced miR‑215 expression following miR‑215
Figure 2. Effects of miR-215 mimics or inhibitors transfection on biological behaviors of non-small cell lung cancer A549 and 95D cells. (A) Reverse transcription-quantitative polymerase chain reaction analysis confirmed increased miR-215 expression in A549 cells transfected with miR-215 mimics, and reduced miR-215 expression in 95D cells transfected with miR-215 inhibitors. U6 RNA was used as an internal control. (B) MTT assay demonstrated that miR-215 reduced cell proliferation in vitro. (C) Cell apoptosis was detected by flow cytometric analysis following transfection with miR-215 mimics, miR-215 inhibitors or negative control. (D) Transwell invasion assay demonstrated that upregulation of miR-215 inhibited the invasive ability of A549 cells, while transfection of 95D cells with miR-215 inhibitors promoted cell invasion (magnification, x100). (E) Scratch migration assays confirmed the inhibitory effect of miR-215 on NSCLC cell migration. Data are presented as the mean ± standard deviation of experiments performed in triplicate. "P<0.01 vs. NC. NC, negative control; PI, propidium iodide.

Figure 3. ZEB2 is a direct target of miR-215. (A) miR-215-binding sites in the ZEB2 3'UTR region. ZEB2-mut indicates the ZEB2 3'UTR with a mutation in miR-215-binding sites. (B) Western blot analysis demonstrated that transfection of miR-215 reduced ZEB2 protein expression. (C) Relative luciferase assay comparing the pGL3-ZEB2 and pGL3-ZEB2-Mut vectors in A549 cells. Firefly luciferase activity was normalized to Renilla luciferase activity. Values are expressed as the mean ± standard deviation. "P<0.05 vs. control. ZEB2, zinc finger E-box-binding homeobox 2; mut, mutant; Hsa, Homo sapien.
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The results of the MTT assay also confirmed the inhibitory effect of miR-215 on NSCLC cell migration (Fig. 2E).

ZEB2 is the target gene of miR-215. Using the bioinformatics software TargetScan for target gene prediction, ZEB2 was identified as a potential target of miR-215. The predicted binding of miR-215 with the ZEB2 3’UTR is illustrated in Fig. 3A. To further confirm that ZEB2 is the direct target of miR-215 in NSCLC, miR-215 mimics were transfected into A549 cells, which significantly reduced ZEB2 protein expression levels in these cells (Fig. 3B). Subsequently, the pGL3-ZEB2 and pGL3-ZEB2-mut plasmids were created. The luciferase reporter assay demonstrated that transfection of miR-215 mimics induced a marked reduction in luciferase activity of pGL3-ZEB2 plasmid in A549 cells, without altering the luciferase activity of pGL3-ZEB2-mut (Fig. 3C). These data indicate that ZEB2 is a direct target of miR-215 in NSCLC.

Increased miR-215 expression suppresses xenograft tumor formation. To further evaluate the effects of miR-215 on tumor growth in vivo, A549 cells were engineered to stably overexpress miR-215 by lentiviral infection. These cells were injected subcutaneously into nude mice to form ectopic tumors. The cells transfected with negative lentiviral vector LV-NC were also inoculated. As indicated in Fig. 4A–C, the tumors formed from miR-215-overexpressing A549 cells were significantly smaller and exhibited reduced tumor weights compared with those of the control tumors. RT-qPCR analysis of the tumor tissues confirmed elevated miR-215 expression levels in miR-215-overexpressing tumors (Fig. 4D).

Discussion

The dysregulation of miRs has been demonstrated to be involved in tumorigenesis and progression in various types of tumor; however, their potential roles in NSCLC have remained to be elucidated. In the present study, reduced miR-215 expression was identified in NSCLC specimens and cells and was correlated with aggressive clinicopathological features. Overexpression of miR-215 significantly inhibited cell proliferation, invasion and migration, promoted cell apoptosis in vitro and suppressed tumorigenicity in vivo. In addition, ZEB2 was identified as a direct target of miR-215. To the best of our knowledge, the present study is the first to analyze the clinical significance and biological functions of miR-215 expression in NSCLC.

miR-215, an identified p53-induced miR, has been reported to be significant in the progression of cancer. Previous studies have confirmed that miR-215 is downregulated in esophageal adenocarcinoma (14), colon cancer (15) and RCC (16). Reduced miR-215 expression levels in colorectal cancer were found to be associated with increased tumor sizes and decreased disease-free survival times following radical surgery (27,28). Ectopic expression of miR-215 inhibited cell proliferation and triggered cell cycle arrest at G2 phase in HCT 116 colon cancer cells (29), and reduced cellular migration and invasion in an RCC cell line model (16). In contrast to the aforementioned antitumor properties, miR-215 also functions as an oncogene in several types of cancer. In cervical cancer, miR-215 expression of 95D cells with miR-215 inhibitors promoted cell invasion ability. The scratch migration assay also confirmed the inhibitory effect of miR-215 on NSCLC cell migration (Fig. 2E).
was significantly increased in the cancerous tissues of patients with lymph node metastasis, advanced ‘International Federation of Gynecology and Obstetrics’ tumor stage and poor survival (10). In gastric cancer, increased mir-215 expression was significantly correlated with tumor invasion and ‘Union for International Cancer Control’ stage (12). Previous studies have also demonstrated that miR-215 promotes the proliferation of hepatoma and gastric cancer cells (11,12). Anti-mir-215 markedly inhibited the tumor growth of hepatoma cells in nude mice (11). Taken together, these findings indicate that the role of mir-215 in human malignancies may be multifaceted, depending on the specific tissue involved. Currently, it is understood that miRs exert their oncogenic or tumor suppressor functions by regulating the expression of target genes (30). With regard to mir-215, several targets have been determined in previous studies, including protein tyrosine phosphatase receptor type T (1), thymidylate synthase (TS) (29), dihydrofolate reductase (29), retinoblastoma tumor suppressor gene 1 (RB1) (12), activated leukocyte cell adhesion molecule (ALCAM) (31) and activin receptor type 2B (32). ZEB2, as a tumor-promoting gene, has been demonstrated to be upregulated in various types of tumor, and identified as a target gene of a number of miRs. The significant role of ZEB2 has been highlighted in numerous previous studies, due to its function in inducing EMT and facilitating the metastasis of cancer cells. You et al (23) corroborated the contribution of ZEB2 to NSCLC cell migration and invas. White et al (16) demonstrated that mir-215 directly targets ZEB2 in RCC. Using the luciferase reporter assay, the present study demonstrated that ZEB2 was a direct target of miR-215 in NSCLC. However, there is not a ‘one-to-one’ connection between miRs and target mRNAs. An average miR may have ≥100 targets (33); and conversely, numerous miRs may converge on a single transcript target (34). ZEB2 is not the only miR-215 target dysregulated in NSCLC. Other functional targets of miR-215, including RB1 (35), TS (36), and ALCAM (37,38), also modulate NSCLC pathogenesis. Therefore, the potential regulatory circuitry affected by mir-215 is enormous, and the mechanisms underlying how mir-215 influences NSCLC progression require further clarification.

In conclusion, the results of the present study demonstrated that miRNA-215 was downregulated in NSCLC and correlated with aggressive clinicopathological features. The overexpression of miRNA-215 exhibited anti-tumor effects in vitro and in vivo. These findings indicate that miRNA-215 may be a potential novel target for gene therapy of NSCLC.

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