MicroRNA-107 inhibits tumor growth and metastasis by targeting the BDNF-mediated PI3K/AKT pathway in human non-small lung cancer

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Received May 11, 2016; Accepted July 4, 2016

DOI: 10.3892/ijo.2016.3628

Abstract. Abnormal expression of microRNA-107 (miR-107) was found in non-small cell lung cancer (NSCLC). However, little is known about its role and molecular mechanism in NSCLC progression and metastasis. Therefore, the aims of this study were to clarify the potential role of miR-107 and molecular mechanism in NSCLC progression and metastasis. Quantitative real-time polymerase chain reaction assay showed that miR-107 expression levels were significantly decreased in NSCLC tissue and cell lines. Low miR-107 levels in tumor tissue correlated with advanced TNM stage and lymph node metastasis. Function assays showed that overexpression of miR-107 suppressed cell proliferation, migration and invasion in A549 cells in vitro, and inhibited NSCLC tumor growth in vivo. Further mechanism assays suggested the brain-derived neurotrophic factor (BDNF) was identified as a target gene of miR-107 in NSCLC cells. In addition, BDNF expression was upregulated, and inversely correlated with miR-107 in NSCLC tissues. Enforced overexpression of BDNF effectively reversed the tumor suppressive functions of miR-107 on NSCLC proliferation, migration and invasion. miR-107 overexpression or downregulation of BDNF was able to inhibit activation of PI3K/AKT signaling pathway. Taken together, our findings present the first evidence that miR-107 could suppress NSCLC metastasis by targeting BDNF and indirectly regulating PI3K/AKT signaling pathway, which may contribute to establish novel diagnostic markers and novel therapeutic targets for NSCLC.

Introduction

Lung cancer is one of the leading causes of cancer-related mortality worldwide, among which non-small cell lung cancer (NSCLC) accounts for ~85% of lung cancer (1). The population with NSCLC has grown fast over the past decades in China (2). Despite the considerable advances in medical and surgical treatment of NSCLC patients, the prognosis of NSCLC remains unsatisfactory and the 5-year survival rate of patients with NSCLC is <16% (3). Tumor metastasis is frequent, and a great challenge in the clinical treatment of NSCLC, and mostly responsible for the low 5-year survival rate (4). Therefore, there is an urgent need to find potential molecular mechanisms involved in NSCLC metastasis, which may contribute to establish novel diagnostic markers and novel therapeutic targets for NSCLC.

MicroRNAs (miRNAs) are a class of small non-protein-coding RNAs of ~22 nucleotides in size that negatively regulate mRNA stability and/or repress mRNA translation by binding to the 3’-untranslated region (3’-UTR) (5,6). miRNAs has been reported to play pivotal roles in a wide range of cellular processes including proliferation, cycle, differentiation, apoptosis and metastasis (7). miRNAs are dysregulated in many cancers and involve in the initiation and progression of various cancer types, and function either as oncomiRs or tumor suppressor miRNAs, based on the regulated tumor forms and their targeted genes (8,9). For NSCLC, numerous miRNAs have been identified to be involved in NSCLC procession and metastasis, and can act as potent therapeutic targets or diagnosis marker for NSCLC (10-12).

MicroRNA-107 (miR-107), located on chromosome 10, has been shown to be downregulated, and function as a tumor suppressor in several types of cancer, such as glioma (13), breast cancer (14), gastric cancer (15), cervical cancer (16) and renal clear cell carcinoma (17). Previous studies showed that the expression of miR-107 was reduced in NSCLC tissues, and overexpression of miR-107 was able to induce cell cycle arrest in human NSCLC cell lines (18), and increase cisplatin chemosensitivity of A549 non-small cell lung cancer cell line (19). However, the biological roles, especially with regard to migration and invasion, have not yet been thoroughly validated in NSCLC. Therefore, the aim of this study was to investigate the role of miR-107 on the procession and metastasis of NSCLC. In this study, we verified that miR-107 plays an inhibitory role in tumor growth and metastasis in NSCLC cells by targeting BDNF and indirectly regulating the PI3K/AKT signaling pathway.
Materials and methods

Cell lines and tissue samples. All of the NSCLC cell lines used in this study were obtained from the Cell Culture Center of the Shanghai Institute for Biological Sciences of Chinese Academy of Science (Shanghai, China), including four NSCLC cell lines (A549, H1299, SPCA1 and H358) and the normal lung cell line (BEAS-2B). The cells were grown in monolayer in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and maintained at 37°C in humidified air with 5% CO2.

A total of 30 tumor tissue specimens and corresponding adjacent normal lung tissues were obtained from patients who underwent curative resection for NSCLC at the First Hospital, Jilin University (Changchun, China) between August 2014 and September 2015. Relevant clinical data of NSCLC patients were collected and are listed in Table I. None of the patients received chemotherapy or radiotherapy before surgery. This study was approved by the Ethics Committee of the First Hospital, Jilin University. All patients signed a written consent for the use of their specimens and disease information.

miRNA, siRNA, plasmid construction, and transfection. miR-107 mimic (miR-107), and corresponding miRNA negative control (miR-NC) were synthesized by GenePharma (Shanghai, China). siRNAs against BDNF (si-BDNF) and corresponding scramble negative control (si-NC) were designed and synthesized by RiboBio (Guangzhou, China). The coding domain sequence of human BDNF mRNA was amplified by PCR, and inserted into pcDNA 3.0 vector (Invitrogen, Grand Island, NY, USA), named as pBDNF. Transfection was performed using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

RNA extraction and real-time PCR. Total RNA of the cultured cells and the tissues was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. The purity and concentration of total RNA were determined by a dual-beam ultraviolet spectrophotometer (Eppendorf, Hamburg, Germany). Then, a total of 3 µg of mRNA was reverse transcribed to single-stranded cDNAs using a PrimeScript® RT reagent kit (Takara Biotechnology Co., Ltd., Dalin, China). qRT-PCR for miR-107 and BDNF were performed using SYBR premix real-time PCR Reagent (Takara) under an ABI7900 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The primers for miR-107 and U6 were brought from Applied Biosystems. The primers for BDNF and β-actin used in this study were as described previously (20). U6 RNA was used to normalize the miR-107 RNA levels, and β-actin was used to normalize the level of BDNF mRNAs. The comparative 2−ΔΔCt method was employed for relative quantification.

Cell proliferation, migration and invasion assays. Cell proliferation was determined by Cell Counting Kit-8 assay (CCK-8, Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, transfected cells were seeded into 24-well plates at a density of 5x10^3 cells/well and cultured for 24-72 h. At indicated time (24, 48 and 72 h), 10 µl CCK8 solution were added to each well until visual color conversion occurred. The absorbance at 450 nm was read on a microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

To examine the migration ability of cells in vitro, a wound-healing assay was performed after transfection. Briefly, transfected cells (2x10^4 cells/well) were seeded into 24-well tissue culture plates. When cells were grown to a density of 70-80%, the linear wound of cellular monolayer were created by 200 µl pipette tips. The wound closure was observed and photographed at 0 and 24 h using an IX51 inverted light microscope (Olympus, Tokyo, Japan).

For Transwell migration assays, 5x10^4 cells were suspended in serum-free medium and seeded into upper Transwell chambers coated with Matrigel (BD Biosciences, Bedford, MA, USA), then 600 µl medium containing 10% FBS was added to the lower chamber. After incubation for 48 h in a humidified atmosphere of 5% CO2 at 37°C, cells that migrated onto the lower surface of the membrane were fixed with 100% methanol and stained with 0.1% crystal violet, while the non-invading cells on the upper membrane surface were removed with cotton swabs. Cells on the lower surface were photographed and counted at five randomly selected fields with a magnification of x200 by microscopy (Olympus).

MicroRNA target prediction and luciferase-reporter activity assay. miRNA targets were predicted using the algorithms TargetSan (https://www.targetscan.org) miRanda (http://www.microrna.org/) and PicTar (http://pictar.mdc-berlin.de/). The human BDNF 3’UTR oligonucleotides containing the wild-type (Wt) or mutant (Mut) binding site of miR-107 were cloned into the pGL3-control vector (Ambion, Austin, TX, USA) at the NheI and Xhol sites. For luciferase assays, cells were co-transfected with miR-107 mimic or miR-NC and Wt or Mut plasmid using Lipofectamine 2000 reagent. After 48-h transfection, luciferase activity was measured using the dual-luciferase assay system (Promega, Madison, WI, USA). Renilla-luciferase was used for normalization.

Western blotting. Total cellular and tissue proteins were extracted using RIPA lysis buffer containing proteinase inhibitor (Sigma, USA). Concentrations of total cellular protein were determined using a BCA assay kit (Pierce, Rockford, IL, USA). Equal amounts of proteins (25 µg/sample) were separated with 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad, Munich, Germany), followed by probing overnight at 4°C with antibodies against BDNF (1:1,000; Santa Cruz Biotechnology, CA, USA), AKT (1:1,000; Santa Cruz Biotechnology), p-AKT (1:1,000; Santa Cruz Biotechnology), PI3K (1:1,000; Santa Cruz Biotechnology) and p-PI3K (1:1,000; Cell Signaling Technology, CA, USA). The membranes were then incubated for 2 h at room temperature with the secondary HRP-conjugated antibodies (1:5,000; Cell Signaling Technology). Monoclonal mouse β-actin antibody (1:1000; Cell Signaling Technology) was used as an internal control. Proteins in the membrane were detected by the enhanced chemiluminescence system (ECL kit, Millipore, USA) and its band images were analyzed with the Bio-Rad ChemiDoc XRS system (Bio-Rad).
Xenograft tumor model. Young male athymic nude mice (6-week-old) were purchased from the Model Animal Research Center of Jilin University (Changchun, China). Nude mice were manipulated and cared for according to NIH Animal Care and Use Committee guidelines in the Experiment Animal Center of the Jilin University (Changchun, China).

Approximately 2x10^6 A549 cells stably carrying miR-107 or miR-NC was injected subcutaneously into the lower flanks of 8 nude mice. Tumor volumes were measured every 5 days from the sixth day post-injection onward for 30 days before the animals were sacrificed. Tumor volume was measured every week by measuring the length (L), width (w), and height (H) with calipers and using the formula: volume (v) = \( \frac{\pi}{6} \times L \times w \times H \). Thirty days after inoculation, mice were sacrificed. Tumor tissues were dissected, and the volume and weight were measured.

Statistical analysis. Statistical analysis was performed using the SPSS software package (SPSS Standard version 19.0, SPSS Inc., USA). Data are shown as mean ± standard deviation (SD) of at least three separate experiments. Statistical significance was analyzed using Student's t-test or one-way ANOVA. The relationship between miR-107 level and clinical and pathological variables was analysed using Pearson's \( \chi^2 \) test. The correlations between miR-107 expression and BDNF mRNA expression were analyzed using Pearson analysis. P-value <0.05 was considered as statistically significant.

Results

miR-107 is downregulated in both NSCLC cells and clinical specimens. To examine miR-107 expression levels, we first applied qRT-PCR technology and examined miR-107 expression in four NSCLC cell lines (A549, H1299, SPCA1 and H358) and a normal lung cell line (BEAS-2B). *P<0.05; **P<0.01 versus BEAS-2B. (B) Relative expression of miR-107 in a cohort of 30 human NSCLC tissues and adjacent normal tissues. The endogenous U6 RNA was used as the internal control. *P<0.01 versus normal.

miR-107 inhibits NSCLC cell growth in vitro and in vivo. To determine if miR-107 has the propensity to suppress tumori-
genesis, we introduced the miR-107 mimic into A549 cells, to alter the level of total miR-107 in A549 cells, which express the lowest level of miR-107 among four NSCLC cell lines (Fig. 1A). qRT-PCR confirmed the elevated level of miR-107 in the transfected A549 cells (Fig. 2A). Then cell proliferation was determined in A549 cells transfected with miR-107 mimic or miR-NC. The CCK-8 assay showed cell proliferation was obviously suppressed in A549 cells after manipulation of miR-107 mimic at 48- and 72-h time-points, while no significant difference was found at 24 h time-point (Fig. 2B). Besides, we explored the effect of miR-107 overexpression on in vivo growth of NSCLC tumors. The human A549 cells stably expressing miR-107 or miR-NC was implanted subcutaneously into nude mice to allow tumor formation. Tumors grew slower in the A549/miR-107 group than in the A549/miR-NC group (Fig. 2C). At day 30 post-injection, the mice were sacrificed, and tumor tissues were dissected, and weighed. A significant decrease in weight (Fig. 2D) was observed in mice injected with A549/miR-107 compared to the group injected with A549/NC.

**miR-107 inhibits NSCLC cell metastasis.** The above results showed that reduced expression of miR-107 was associated with lymph node metastasis (Table I), suggesting that it may regulate the metastasis process. To determine if this indeed is the case, we carried out wound-healing and Transwell invasion assays in A549 cells transfected with miR-107 mimic or miR-NC. The results showed that ectopic overexpression of miR-206 caused a suppression of cell migration (Fig. 3A and B) and invasion (Fig. 3C and D) capability in A549 cells. These results suggested that miR-107 inhibits NSCLC metastasis.

**BDNF is a direct target of miR-107.** Potential targets of miR-107 were predicted using three bioinformatic databases (TargetScan, miRanda and PicTar), BDNF was chosen as a target gene of miR-107, based on a putative target sequences at 299-305 bp of BDNF (Fig. 4A). To verify whether BDNF is a direct target of miR-107 in NSCLC cells, a human BDNF 3’UTR fragment containing the binding sites of miR-107 (Fig. 4A) or the mutant sites were cloned into the pGL3 vector, and miR-107 mimic or miR-NC were co-transfected into A549 cells and cultured for 48 h, then luciferase activities were measured. It was found that overexpression of miR-107 obviously suppressed the luciferase activity of wild-type BDNF 3’UTR, but the activity of the mutant-type BDNF 3’UTR was not changed (Fig. 4B), suggesting that BDNF is...
Figure 3. miR-107 inhibits NSCLC cell metastasis. (A) The wound-healing assay determined cell migration in A549 cells transfected with miR-107 or miR-NC. (B) Statistical results. (C) The Transwell invasion assay determined cell migration in A549 cells transfected with miR-107 or miR-NC. (D) Statistical results. *P<0.05, **P<0.01 versus miR-NC.

Figure 4. BDNF is a direct target of miR-107 in NSCLC cells. (A) The predicted binding sites for miR-107 in the 3'UTR of BDNF and the mutations in the binding sites are shown. (B) Relative luciferase activity in A549 cells co-transfection with wild-type or mutant-type 3'UTR BDNF reporter plasmids and miR-107 or miR-NC. Wt, wild-type; Mut, mutant-type. (C and D) BDNF mRNA expression (C) and protein expression (D) were measured in A549 cells transfected with miR-107 mimic or miR-NC. β-actin was used as the internal control. *P<0.05, **P<0.01 versus miR-NC.
a direct target of miR-107 in NSCLC cells. Then, qRT-PCR and western blot analysis confirmed that overexpression of miR-107 markedly inhibited BDNF expression on mRNA level (Fig. 4C) and protein level (Fig. 4D) in A549 cells. These results indicated that miR-107 can bind directly to BDNF and inhibits its expression.
miR-107 overexpression was able to promote cell proliferation (Fig. 6B-D). Therefore, our data clearly demonstrated that miR-107 inhibits NSCLC cell proliferation, migration, and invasion by targeting BDNF.

miR-107 inhibits the PI3K/AKT signaling. It has been shown that BDNF activation can trigger PI3K/AKT pathways (21), which regulates cell proliferation, apoptosis invasion, and inflammation in various cancers (22). Therefore, we investigated the possibility that miR-107 regulates this pathway by targeting BDNF. PI3K, p-PI3K, AKT and p-AKT protein expression was detected in A549 cells transfected with miR-107 mimic/miR-NC or si-BDNF/si-NC by western blotting. β-actin was used as the internal control.

Figure 7. miR-107 inhibits the PI3K/AKT signal pathway. PI3K, p-PI3K, AKT and p-AKT protein expression was detected in A549 cells transfected with miR-107 mimic/miR-NC or si-BDNF/si-NC by western blotting. β-actin was used as the internal control.

Inverse correlation between BDNF and miR-107 expression in NSCLC patients. We next examined the BDNF mRNA expression in tumor tissues and the corresponding adjacent normal lung tissues in a total of 30 patients with NSCLC by qRT-PCR (Fig. 5A). The data showed that BDNF expression was significantly increased in NSCLC tissues compared to the adjacent normal tissues. Pearson correlation analysis revealed that the expression of miR-107 was inversely correlated with BDNF in the 30 patients with NSCLC (r = -0.701; P < 0.001; Fig. 5B).

Overexpression of BDNF reverses the tumor suppressive effect of miR-107 in NSCLC. To evaluate if BDNF is responsible for the functional effects of miR-107 in NSCLC cells, we generated a BDNF overexpressing vector the pBDNF, and transfected it into miR-107 or miR-NC overexpressed A549 cells. The transfection efficiency was verified by western blot assay (Fig. 6A). Then, we carried out CCK8, wound healing, and Transwell invasion assays to evaluate the effect of BDNF overexpression on cell proliferation, migration and invasion in the above cells. BDNF overexpression reversed the inhibition effect on cell proliferation, migration and invasion in the above cells. BDNF overexpression reversed the inhibition effect on cell proliferation, migration and invasion in the above cells. These results supported the opinion of a previous study that low expression of miR-107 was significantly correlated with TNM stage, regional lymph node involvement, and tumor differentiation (23). We also showed that miR-107 significantly inhibited NSCLC proliferation, migration and invasion. These results suggested that miR-107 has a crucial role in NSCLC growth and metastasis.

Description

A large number of studies have indicated that miRNAs may play an important role in NSCLC initiation and development (10-12). We demonstrated that miR-107 is frequently downregulated in human NSCLC tissues and cancer cell lines, which is consistent with previous results (18). In addition, we found that downregulation of miR-107 was particularly significant in tumors with lymphatic metastasis, and advanced TNM stage. These results supported the opinion of a previous study that low expression of miR-107 was significantly correlated with TNM stage, regional lymph node involvement, and tumor differentiation (23). We also showed that miR-107 significantly inhibited NSCLC proliferation, migration and invasion. These results suggested that miR-107 has a crucial role in NSCLC growth and metastasis.

miR-107, located on chromosome 10, has been shown to be involved in various biological processes, including adipogenesis, hypoxia, angiogenesis and proliferation and cell cycle (24). miR-107 has been shown to function as a tumor suppressor in multiple cancer, such as glioma (13), breast cancer (14), gastric cancer (15), cervical cancer (16) and renal clear cell carcinoma (17). On the contrary, miR-107 was significantly upregulated in cancer tissues and cell lines, and miR-107 overexpression was able to promote cell proliferation in HepG2 cells, suggesting miR-107 as oncogene in liver cancer (25). Previously studies showed that the expression of miR-107 was reduced in NSCLC tissues (23), and overexpression of miR-107 was able to induce cell cycle arrest in human NSCLC cell lines (18), and increase cisplatin chemosensitivity of A549 (19). However, the potential roles and mechanism involved in NSCLC metastasis remains largely unknown. In this study, we showed that miR-107 inhibited NSCLC growth in vitro and in vivo, as well as NSCLC metastasis. Our results together with previous studies indicated that miR-107 function as a tumor suppressor miRNA in NSCLC.

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, has been shown to play an important role in the development and regeneration of the neurons (26). Binding of BDNF to its major receptor, tropomyosin-related receptor kinase B (TrkB) with high affinity and specificity (27), caused the activation of multiple downstream signaling pathway, such as PI3K/AKT, RAS/ERK, PLC/PKC, AMPK/ACC and JAK/STAT pathways (28). Recently studies have demonstrated that BDNF was able to promote tumorigenesis and progression in several human malignancies, such as clear cell renal cell carcinoma (29), breast (30), colon cancer (31), colorectal cancer (32), and neuroblastoma (33), suggesting that BDNF was closely associated with tumor progression. For NSCLC, It was reported that the expression of BDNF was upregulated in NSCLC tissues, and was associated with poor prognosis in
non-small cell lung cancer (34), and that BDNF facilitates tumorigenesis of NSCLC (35), and NSCLC metastasis (36). These studies suggested that BDNF functions as an oncogene in NSCLC. We confirmed that BDNF is a target of miR-107 in regulating NSCLC by luciferase activity assay, qRT-PCR and western blotting. In addition, we also found that BDNF expression was upregulated, and inversely correlated with miR-107 in NSCLC tissues. Enforced overexpression of BDNF effectively reversed the tumor suppressive functions of miR-107 on NSCLC proliferation, migration and invasion. Of note, we found that miR-107 overexpression or downregulation of BDNF was able to inhibit activation of PI3K/AKT signaling pathway. These results might suggest that miR-107 exerted its suppressive role in NSCLC by targeting BDNF and indirectly regulating PI3K/AKT signaling pathway.

In conclusion, this study showed that miR-107 was downregulated in NSCLC cell lines and tissues, and was associated with lymph node metastasis and TNM stage. Overexpression of miR-107 significantly decreased the proliferation, migration and invasion of NSCLC cells in vitro and suppressed tumor growth in vivo. We also identified a likely novel mechanism of miR-107 to suppress tumor growth and metastasis by inhibiting BDNF and indirectly regulating PI3K/AKT signaling pathway. Thus, miR-107 functions as a tumor suppressor in NSCLC by repressing BDNF. The identification of miR-107 and its target gene in non-small lung cell cancer may contribute to understanding the potential molecular mechanisms of NSCLC, and may have diagnostic as well as therapeutic value for non-small cell lung cancer.

Acknowledgements

This study was supported by The National Youth Science Foundation (81401883).

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