Original Article

miR-185 acts as a tumor suppressor by targeting AKT1 in non-small cell lung cancer cells

Shuai Li, Yulian Ma, Xinfang Hou, Ying Liu, Ke Li, Shuning Xu, Jufeng Wang

Department of Internal Medicine, Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, Zhengzhou, Henan, China

Received June 7, 2015; Accepted July 21, 2015; Epub September 1, 2015; Published September 15, 2015

Abstract: Increasing evidence has shown that microRNAs play critical roles in the initiation and progression of non-small cell lung cancer (NSCLC). miR-185 is deregulated in various cancers, whereas its functional mechanism in NSCLC is still unclear. Here, we confirmed that the expression of miR-185 was significantly down-regulated in NSCLC tissues and cell lines. miR-185 over-expression caused significant suppression of in vitro cell proliferation, migration and invasion, and in vivo tumor growth. We subsequently identified that AKT1 was a target gene of miR-185. Re-expression of AKT1 could partially rescue the inhibitory effects of miR-185 on the capacity of NSCLC cell proliferation and motility. Collectively, we conclude that miR-185 has a critical function by blocking AKT1 in NSCLC cells, and it may be a novel therapeutic agent for miRNA based NSCLC therapy.

Keywords: miR-185, AKT1, non-small cell lung cancer

Introduction

Lung cancer is the most common malignant tumors in humans and the leading cause of cancer-related deaths in the world [1]. Non-small cell lung cancer (NSCLC) accounts for at least 80% of all lung cancers [2]. Despite diagnosis and clinical treatment strategies have been significantly improved, poor prognosis still exists in many NSCLC patients with a low 5-year overall survival rate and a high recurrence rate [3, 4]. Therefore, it is utterly important to understand the precise molecular mechanisms of the tumorigenesis of NSCLC. Recently, a large number of studies have shown that microRNAs (miRNAs) play an essential role in NSCLC pathogenesis, which provides new insights into the therapeutic strategy against this disease [5, 6].

MiRNAs are a family of small (approximately 22 nucleotides in length), single-stranded, endogenous non-coding RNAs which regulate gene expression by causing mRNA degradation or repressing translation through binding to complementary sites in 3'-untranslated regions (3'-UTRs) [7-9]. MiRNAs are implicated in a wide range of important physiological processes [10-12]. Emerging evidence demonstrates that miRNAs act as novel oncogenes or tumor suppressors, and aberrant miRNA expression has been observed in various types of human cancers including NSCLC [13-15]. Recently, miR-185 has been reported to be down-regulated in gastric cancer, breast cancer, glioma, clear cell renal cell carcinoma and hepatocellular carcinoma [16-20]. However, the functional mechanisms of miR-185 in NSCLC are largely unknown.

In the current study, we confirmed that miR-185 was significantly down-regulated in NSCLC tissues and cell lines. Over-expression of miR-185 suppressed NSCLC cell growth, migration and invasion in vitro, and tumor growth in nude mice. AKT1 was recognized as a direct and functional target of miR-185. Over-expression of AKT1 could partially rescue the inhibitory effects of miR-185. Our results suggest that miR-185 and its downstream target gene AKT1 may be useful for treatment of NSCLC in the future.

Materials and methods

Tissue samples

Twelve paired NSCLC tissues and adjacent non-tumor tissues used in this study were collected...
miR-185 acts as a tumor suppressor by targeting AKT1 in NSCLC

from patients who underwent surgical resection at Affiliated Cancer Hospital of Zhengzhou University. Tissue samples were immediately snap-frozen and stored in liquid nitrogen until total RNAs or proteins were extracted. Informed consent was obtained from all patients, and the research was approved by the Ethics Committee of Zhengzhou University.

Cell lines and cell culture

All cell lines (A549, H460, H1299 and 16HBE) were obtained from the Cell Resource Center, Shanghai Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences. All cells were maintained in RPMI-1640 (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), and incubated in a humidified atmosphere at 37°C under 5% CO₂.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from tissues or cells was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Total RNA was transcribed into cDNA using Prime Script RT reagent Kit (Takara, Dalian, China). Total miRNA was transcribed into cDNA using One Step Prime Script miRNA cDNA Synthesis Kit (Takara). qRT-PCR was carried out using a SYBR Premix Ex Taq II (TAKARA, Dalian, China) on ABI 7500 HT system (Applied Biosystems, Foster City, CA, USA). The relative genes expression was calculated the 2ΔΔCt method. β-actin or U6 was used as endogenous control [21].

Construction of plasmids

miR-185 mimic and negative control were purchased from RiboBio (Guangzhou, China). The 3'UTR segment of the human AKT1 (containing the predicted miR-185 binding site) was PCR-amplified from genomic DNA and inserted into the pGL3 vectors (Promega, Madison, WI, USA). The corresponding sequences carrying the miR-185 binding site were mutated by the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and inserted into the pGL3 vectors. For AKT1 over-expression, open reading frame cDNA for AKT1 was inserted into pcDNA3.1 vector (Invitrogen). Transfection was done using the Lipofectamine 20000 (Invitrogen) according to the manufacturer’s protocol.

MTT assay

Cell viability was detected by the MTT assay. After being seeded onto the 96-well culture plates for 24 h, H1299 and A549 cells were transfected with miR-185 mimic or negative control. 1, 2, 3 and 4 days after transient transfection, cells were incubated with 20 μl MTT (5 mg/ml) at 37°C for another 4 h. The media was discarded and 150 μl DMSO was added to the cells. Optical density values were examined at 490 nm wavelength with a microplate reader (Bio-Rad, Richmond, CA, USA).

Tumor xenografts

All animal experiments were performed according to the protocols approved by The Animal Care and Use Committee of Fudan University, China. BALB/c nu/nu female mice (4-week old) were purchased from Experimental Animal Center of Fudan University. 2.5 × 10⁵ H1299 cells treated with miR-185 mimic or negative control were injected subcutaneously into the mice. Tumor growth was monitored by measuring maximum (a) and minimum (b) length of the tumor every 5 days. Tumor volume (V) was calculated as follows: V = ½ ab². After 30 days, all mice were sacrificed, and tumors were collected and weighed.

Wound-healing and transwell invasion assays

For wound-healing assay, 1 × 10⁶ cells were seeded in a six well plate, cultured for 12 h and transfected with miR-185 mimic or negative control. Upon reaching the appropriate confluence, the confluent monolayer was scratched with a 200 μl pipette tip, washed with PBS twice and cultured again for up to 24 h with serum-free medium. Images of the wound closure were taken at 0 and 24 h under a microscope and the width of the wound gaps were validated with Image J software and normalized to 0 h wounds [22]. For the invasion assay, 5 × 10⁴ cells in serum-free RPMI-1640 were placed into the upper chamber of each insert with Matrigel (BD Biosciences, SanJose, CA, USA). RPMI-1640 with 10% FBS was added in the lower chamber to act as the nutritional attractant. After 48 h, the cells that had invaded through the membrane were fixed with 20% methanol and stained with 0.1% crystal violet for 30 min, imaged, and counted using a microscope.
miR-185 acts as a tumor suppressor by targeting AKT1 in NSCLC

Luciferase assay

293 T cells were cultured in 24-well plates. After 24 h, the wide-type or mutated AKT1 3’UTR constructs was co-transfected with miR-185 mimic or negative control and pRL-TK vector using Lipofectamine 2000 (Invitrogen). Firefly and renilla luciferase activities of the cell lysates were detected at 48 h post-transfection using the Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase served as an internal control. The relative luciferase activity was a ratio of firefly and renilla luciferase activity for each sample.

Western blotting

Cells were collected and lysed with cell lysis buffer containing protease inhibitors. Protein concentrations were measured using the BCA method (Thermo Scientific, Rockford, IL, USA). Equal amounts of proteins was resolved on SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk, incubated overnight at 4°C with AKT1 antibody (1:500, Abcam, Cambridge, UK) and GAPDH (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then with the secondary antibody. Signal was visualized with enhanced chemiluminescence (Millipore) [23].

Statistical analysis

All statistical analyses were analyzed using SPSS 16.0 software. Quantitative data are expressed as mean ± standard deviation. Differences between two groups were made using the Student’s t-test. P values less than 0.05 were considered statistically significant.

Results

The expression levels of miR-185 in NSCLC tissues and cell lines

To understand the relationship between miR-185 and NSCLC, we first examined miR-185 expression in NSCLC tissues and their matched non-tumor tissues using qRT-PCR. The results showed that miR-185 was down-regulated in NSCLC samples compared with the non-tumor counterpart (Figure 1A). We further confirmed the miR-185 level in three human NSCLC cell lines including H460, A549 and H1299. As shown in Figure 1B, all three human NSCLC cell lines expressed much lower levels of miR-185 compared to those in the normal lung bronchus epithelial cell line 16HBE. The low miR-185 expression in NSCLC strongly suggests that miR-185 might be involved in the pathogenesis and development of NSCLC.
miR-185 acts as a tumor suppressor by targeting AKT1 in NSCLC

To investigate the role of miR-185 in NSCLC cells, we first evaluated the effects of miR-185 on NSCLC proliferation using a gain-of-function approach. H1299 and A549 cells were transfected with miR-185 mimic or negative control, and over-expression of miR-185 was confirmed by qRT-PCR (Figure 2A). miR-185 over-expression in both cell lines resulted in decreased cell viability at 96 h post-transfection, as detected by MTT assays (Figure 2B). To further evaluate the effect of miR-185 on tumorigenicity, miR-185-transfected H1299 cells or controls were subcutaneously injected into the nude mice. After 30 days, xenografted tumor volume in miR-185 over-expressing cells was significantly smaller than that in the controls (Figure 2C). Similarly, the tumors in the control group grew much faster and had much heavier tumor weights than those in miR-185-transfected group (Figure 2D). Collectively, these data demonstrate that miR-185 over-expression suppresses NSCLC cell proliferation in vitro and in vivo.
miR-185 acts as a tumor suppressor by targeting AKT1 in NSCLC

Next, we assessed the effects of miR-185 on the migratory and invasive ability of NSCLC cells. The wound healing assay showed that the migratory ability of miR-185-transfected H1299 and A549 cells was much lower than that of the control cells (Figure 3A). Consistent with the migration result, transwell invasion assay demonstrated that cell invasive ability was markedly inhibited in miR-185 mimic treated cells as compared with control cells (Figure 3B). These results suggest that miR-185 remarkably reduces the migratory and invasive ability of NSCLC cells.

**Ectopic expression of miR-185 represses NSCLC cell migration and invasion**

**miR-185 directly targets the AKT1 3′UTR**

Bioinformatics analyses of AKT1 3′UTR revealed one putative binding site for miR-185. To study the potential interaction, AKT1 3′UTR and its corresponding mutant counterparts were cloned into pGL3 luciferase reporter vectors, and co-transfected into 293 T cells with miR-185 mimic or negative control (Figure 4A). As indicated in Figure 4B, miR-185 decreased the luciferase activity of the Luc-AKT1 3′UTR compared to the control vector, whereas the mutant counterpart blocked this decrease. To further investigate whether AKT1 is targeted by miR-185, miR-185 mimic or negative control was transfected into H1299 and A549 cells,
miR-185 acts as a tumor suppressor by targeting AKT1 in NSCLC

and AKT1 expression levels were analyzed by western blotting and qRT-PCR. The results indicated that AKT1 protein and mRNA expression levels were significantly lower in miR-185-transfected cells than those in the controls (Figure 4C and 4D). These data suggest that miR-185 negatively regulates AKT1 expression in NSCLC cells.

Ectopic expression of AKT1 without 3'UTR restores the miR-185-induced effects

Since miR-185 suppressed NSCLC cell proliferation, migration and invasion, and inhibited AKT1 expression, we predicted that miR-185-mediated proliferative and metastatic inhibition was ascribable to AKT1 gene. Thus, we constructed AKT1 over-expressing vector (lacking its 3'UTR), and co-transfected into H1299 cells along with miR-185 mimic. AKT1 protein expression was confirmed by western blotting (Figure 5A). In addition, we found that decreased cell viability, migration and invasion via miR-185 mimic were restored by the AKT1 over-expression (Figure 5B-D). Taken together, the cancer suppressor role of miR-185 in NSCLC is at least partially by inhibiting its target gene AKT1.

Discussion

In the current study, we studied the possible role of miR-185 in NSCLC. We found that miR-185 was commonly downregulated in NSCLC specimens and cell lines, compared with adjacent non-cancerous tissues and the normal lung bronchus epithelial cell line, respectively. Furthermore, over-expression of miR-185 sig-
miR-185 acts as a tumor suppressor by targeting AKT1 in NSCLC

miR-185, located on 22q11.21, has been reported to be dysregulated in several malignant tumors. Tang et al. found that miR-185 was downregulated in glioma, and its overexpression inhibited glioma cell invasion by targeting CDC42 and RhoA [18]. Xiang and colleagues found that miR-185 was significantly downregulated in the cisplatin-resistant ovarian cell lines SKOV3/DDP and A2780/DDP, compared with their sensitive parent line SKOV3 and A2780, respectively. Overexpression of miR-185 increased cisplatin sensitivity of SKOV3/DDP and A2780/DDP cells by inhibiting proliferation and promoting apoptosis through

**Figure 5.** Ectopic expression of AKT1 without 3′UTR restores the miR-185-induced effects. (A) H1299 cells were transfected with negative control oligonucleotides, miR-185 mimic or miR-185 plus AKT1 (without 3′UTR). After 48 h, the protein expression of AKT1 was determined by western blotting. MTT assay (B), scratch wound healing assay (C) and transwell invasion assay (D) were used to assess cell proliferation, migration and invasion. Each sample was analyzed in triplicate. *P < 0.05, **P < 0.01.

nificantly suppressed *in vitro* cell proliferation, migration and invasion, and *in vivo* tumor growth by directly targeting AKT1.
miR-185 acts as a tumor suppressor by targeting AKT1 in NSCLC

Suppressing DNMT1 directly [24]. In triple-negative breast cancer, miR-185 was found to be strongly downregulated in cancer tissues and cell lines and that its expression levels were associated with lymph node metastasis, clinical stage, overall survival, and relapse-free survival. Ectopic expression of miR-185 inhibited TNBC cell proliferation in vitro and in vivo by directly targeting DNMT1 and E2F6 [25]. miR-185 was also reported to inhibit HCC cell proliferation and invasion in vitro and prevented tumor growth in SCID mice [20]. In NSCLC, previous studies have found that miR-185 suppressed cell growth and induce a G1 cell cycle arrest in H1299 cells [26], however, the functional mechanism of miR-185 in NSCLC cells are not fully understood. In the present study, we confirmed that miR-185 is downregulated in NSCLC tissues and cell lines, and functions as a tumor suppressor role in the development of cancer cells.

Aberrant PI3K/AKT pathway activation is found in a variety of cancers, including lung cancer, resulting in the development and progression of these malignancies [27, 28]. AKT1/protein kinase B α is the most extensively investigated member of the serine/threonine protein kinase subfamily and is usually referred as AKT [29]. Sun and colleagues showed that AKT1 kinase activity is elevated in prostate, breast cancers and ovarian carcinomas, and its constitutive activation is required for oncogenic transformation in mouse NIH3T3 cells [30]. Linnerth-Petrik et al. found that Akt1 ablation significantly delays initiation of lung tumor growth in a mouse model [27]. Thus, AKT1 may be a valuable therapeutic target for suppressing oncogenesis. In this study, we showed that miR-185 suppresses AKT1 expression by directly targeting its 3'UTR in NSCLC cells, and re-introduction of AKT1 remarkably attenuated the tumor suppressive effect of miR-185 on NSCLC cells.

In conclusion, we found that miR-185 functions as a tumor suppressor in NSCLC cells through repressing AKT1 expression. This is the first study to demonstrate that the miR-185/AKT1 axis regulates the proliferation, migration and invasion of NSCLC cells. The current findings facilitate our understanding of the molecular pathogenesis of NSCLC and support the potential of miR-185 as an effective novel therapeutic target for treatment of the disease.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jufeng Wang, Department of Internal Medicine, Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, Zhengzhou, Henan, China. E-mail: benbensss1@163.com

References

miR-185 acts as a tumor suppressor by targeting AKT1 in NSCLC


