Original Article
MicroRNA-187 inhibits PTEN expression and promotes cell proliferation in non-small-cell lung cancer

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Abstract: The recent discovery of microRNAs (miRNAs) has provided a novel mechanism for the tumorigenesis. In the present study, we explored the expression and function of miR-187 in non-small-cell lung cancer (NSCLC). Quantitative real-time PCR analysis showed that miR-187 was up-regulated in NSCLC specimens and cultured cancer cells. In vitro studies demonstrated that overexpression of miR-187 mimics enhanced cell proliferation and invasion. Mechanistically, the targets of miR-187 were predicted by bioinformatics tools. Luciferase reporter assays and western blot further revealed that the phosphatase and tensin homolog (PTEN) gene, a tumor suppressor, was suppressed by miR-187. As a result, overexpression of miR-187 mimics led to an enhanced activation of AKT signaling. In agreement, restoration of PTEN expression or inhibition of AKT activity by its antagonist in lung cancer cells largely attenuated the oncogenic roles of miR-187. Therefore, our results suggest that miR-187 promotes NSCLC progression by directly targeting PTEN and may serve as a potential therapeutic target for cancer therapy.

Keywords: Non-small-cell lung cancer, cell proliferation, miR-187, PTEN

Introduction

Non-small-cell lung cancer (NSCLC) is the leading cause of cancer-related deaths and with poor prognosis worldwide [1]. Intensive studies have demonstrated the molecular mechanisms of several tumor suppressors and oncogenes in NSCLC development, including PTEN, p53, FoxO1, Wnt/β-Catenin and EGFR signaling [2-7]. However, the roles of non-coding RNAs medicated post-transcriptional regulation that contributed to the tumorigenesis of NSCLC remains to be determined.

MicroRNAs (miRNAs), a class of small and non-coding RNAs, suppress mRNA translation or promote mRNA cleavage by base pairing with a seed region in the 3'-untranslated region (3'-UTR) of target genes [8, 9]. It has been shown that dysregulation of several miRNAs is involved in the NSCLC proliferation, invasion, metastasis, and drug resistance [10-12], suggesting that a better understanding of the mechanisms underlying miRNA function may offer new targets for cancer treatment.

The expression and roles of miR-187 has been revealed in several types of human cancers. For instance, single-nucleotide polymorphisms inside miR-187 target sites influence breast cancer susceptibility [13]. Further studies showed that miR-187 expression in breast cancer leads to a more aggressive, invasive phenotype and acts as an independent predictor of outcome [14]. Besides, miR-187 was overexpressed in thyroid tumors, compared with hyperplastic nodules in the surgical samples [15]. Moreover, ectopic expression of miR-187 in ovarian cancer cells promoted cell proliferation through targeting disabled homolog-2 (DAB2) [16]. Interestingly, a recent study found that the eight-miRNA signature, including miR-187, is an independent prognostic marker of overall survival of lung adenocarcinoma patients [17]. However, until now, the expression and biological function of miR-187 in NSCLC remains unknown.

In the present study, we investigated the expression of miR-187 in 35 pairs of primary NSCLC tissues along with their matched adja-
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cent normal tissues, and explored its function in NSCLC. Our results suggest that miR-187 has a potentially important role on the initiation and/or progression of NSCLC.

Materials and methods

Human tissue samples

35 parried of primary NSCLC tissues and adjacent non-tumor normal specimens were obtained from patients in the Second Hospital of Jilin University from 2011 to 2013 with informed consent and agreement. The study protocol was reviewed and approved by the Institutional Review Board of the Second Hospital of Jilin University. All tissue samples were from untreated patients undergoing surgery and were snap frozen in liquid nitrogen and then stored at -80°C.

miR-187 mimics and inhibitors

The human miR-187 duplex mimic, miR-187 inhibitor and negative control miRNA duplex (miR-NC) and were designed and synthesised by GenePharma Inc. (Shanghai, China). miR-187 inhibitor is chemically modified, single-stranded nucleic acids designed to specifically bind to and inhibit endogenous miR-187 molecules.

Cell culture and transfection

Non-small-cell lung cancer cell lines (A549, H1299 and H460), human bronchial epithelial (HBE) cell line was provided by the Institute of Biochemistry and Cell Biology of Chinese Academy of Science, China. Cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen) and maintained at 37°C in a humidified atmosphere with 5% CO₂. All transfections were performed using Lipofectamine 2000 reagents (Invitrogen) according to the manufacturer’s instructions.

BrdU incorporation, cell invasion assays

A cell proliferation enzyme-linked immunosorbent assay (BrdU kit; Beyotime) was used to analyze the incorporation of BrdU during DNA synthesis following the manufacturer’s protocols. Absorbance was measured at 450 nm in the Spectra Max 190 ELISA reader (Molecular Devices, Sunnyvale, CA). Invasion assays were conducted using a specialized Chemicon invasion chamber which included a 24-well tissue culture plate with 12 cell culture inserts (Millipore, Bedford, MA, USA).

Real-time PCR analysis

Total RNA from tissues and cells was extracted using the TRIzol Kit (Invitrogen) according to the manufacturer’s instructions. Quantitative real-time PCR was performed using SYBR Premix Ex Taq reagents (Takara, Shiga, Japan). Relative expression levels of miR-187 were calculated using the 2^-ΔΔCt method with U6 as the endogenous reference gene.

Western blot

Cells and tissues were harvested and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM 2-Mercaptoethanol, 2% w/v SDS, 10% glycerol). After centrifugation at 10,000×g for 10 min at 4°C, proteins in the supernatants were quantified and separated by 10% SDS PAGE. Immunoblot was performed using primary antibodies targeting PTEN and AKT (Abcam, Cambridge, Massachusetts, USA). Protein levels were normalized to total GAPDH, using a rabbit anti-GAPDH antibody (Abcam). The proteins were visualized by an ECL chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK).

miRNA target prediction

Putative miR-187 target genes were screened by the combined use of miRBase (http://microrna.sanger.ac.uk), miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) and TargetScan (http://genes.mit.edu/targetscan) target prediction programs.

Plasmid construction and luciferase reporter assays

The 3'-untranslated region (3'-UTR) of human PTEN gene was cloned into the pmirGlo plasmid (Promega, WI, USA) between the XhoI and XbaI sites. The pmirGlo plasmids containing mutated PTEN 3'-UTR were constructed using the KOD-plus mutagenesis kit (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. For luciferase reporter assays, the miRNA mimics (25 nM) and plasmid (100 ng) were co-transfected into A549 cells. Transfection efficiency was normalized by co-transfecting Simian virus 40 (SV40) plasmids (Promega). Luciferase values were measured using the...
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Figure 1. Up-regulation of miR-187 in NSCLC tissues and cell lines. (A, B) Relative expression levels of miR-187 in 35 cases of human tissues (A) and cell lines (B). miR-187 expression was analyzed by quantitative real-time PCR and normalized to the endogenous control U6.

Figure 2. The effect of miR-187 overexpression on NSCLC growth in vitro. (A, B) Cell proliferation (A), invasion (B) assays in A549 and H460 cells expressing miR-187 mimics or negative control (NC). (C) Colony formation and quantification of colonies as function of miR-187 over-expression.

Dual-Luciferase Reporter Assay System (Promega).

Statistical analysis

Data were expressed as mean ± standard error of the mean (SE). Analysis was conducted with GraphPad Prism version 6.01 (GraphPad Software). Student’s t test was used to analyze the significance between two groups. Significance between two groups was analyzed using the unpaired two-tailed t test (*P<0.05, **P<0.01, ***P<0.001).

Results

miR-187 is up-regulated in NSCLC tissues

To determine the expression and significance of miR-187 in NSCLC carcinogenesis, quantitative real-time PCR analysis was conducted in 35 cases of NSCLC and adjacent normal tis-
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As shown in the Figure 1A, miR-187 was significantly up-regulated in NSCLC tissues (Figure 1A). In addition, the increased expression of miR-187 was also observed in human NSCLC cell lines including A549, H1299 and H460, compared with bronchial epithelial (HBE) cells. These results suggest that the up-regulation of miR-187 may affect with the tumorigenicity of NSCLC.

miR-187 promotes NSCLC cell proliferation and invasion

Next, to evaluate the biological roles of miR-187 in NSCLC cell lines, its mimic and the negative control (NC) were transfected into A549 and H460 cells separately. As a result, forced overexpression of miR-187 mimics enhanced cell proliferation and invasion abilities (Figure 2A, 2B). Colony formation rate was also increased after overexpression of miR-187 (Figure 2C), suggesting that miR-187 can enhance the growth of NSCLC cells in vitro.

Identification of PTEN as a novel target of miR-187

Previous study has shown that miR-187 could inhibit DAB2 expression in ovarian cancer cells [16]. However, mRNA and protein levels of DAB2 remained unaffected in NSCLC cells overexpressing miR-187 (Figure 3A, 3B). Therefore, potential targets of miR-187 were screened by bioinformatics analyses. Three prediction algorithms (PicTar, miRWalk and Target Scan) were used to identify the putative target genes of miR-187. Among them, the tumor suppressor gene PTEN was chosen for further experiments, since it harbored a miR-187 binding site in its 3'-untranslated region (3'-UTR) (Figure 3C).

To test this hypothesis, the 3'-UTR fragment, containing the potential binding site (wild-type...
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or mutant) (Figure 3C), was cloned and inserted into the pGL3 vector immediately downstream of the luciferase reporter. As shown in Figure 3D, overexpression of miR-187 mimics led to a reduction of luciferase activity when the reporter construct contained the wild-type 3'-UTR (Figure 3D). In contrast, mutation of the potential binding site largely abolished the suppressive effect of miR-187 mimics (Figure 3D), suggesting that miR-187 could bind to the seed sequence to inhibit PTEN expression. In agreement, our western blot experiments showed that endogenous protein levels of PTEN were substantially down-regulated by miR-187 mimics in A549 and H460 cells (Figure 3E, 3F).

It has been well-established that PTEN could oppose phosphoinositide 3-kinase (PI3K) function, leading to inactivation of AKT signaling [18]. Therefore, phosphorylated AKT was assessed in these cells. In agreement, transfection of miR-187 mimics led to a significant activation of AKT, as shown by its phosphorylation at the Serine 143 (S143) (Figure 4A, 4B).

**Restoration of PTEN expression or inhibition of AKT signaling reversed the oncogenic roles of miR-187**

Given the regulatory roles of miR-187 on the PTEN-AKT signaling pathway, we further verify

Figure 5. Restoration of PTEN expression reversed the oncogenic roles of miR-187. (A) Representative protein levels of PTEN in A549 cells. Cells were pre-transfected with miR-187 mimics or negative control (NC) for 24 hr, and then transfected with PTEN expression plasmid for another 24 hr. (B-D) Cell proliferation (B), invasion (C) and quantification of colonies (D) were determined in A549 cells.

Figure 6. Inhibition of AKT signaling reversed the oncogenic roles of miR-187. (A-C) Cell proliferation (A), invasion (B) and quantification of colonies (C) assays were determined in A549 cells. Cells were pre-transfected with miR-187 mimics or negative control (NC) for 24 hr, and then treated with LY294002 (20 nM) or vehicle control (Ctrl) for another 12 hr.
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Firstly, A549 cells were transfected with PTEN expression plasmids to restore its expression (Figure 5A). As shown in Figure 5B-D, re-introduction of PTEN reversed the proliferative roles of miR-187 (Figure 5B-D). Next, we treated A549 cells with LY294002, a morpholine-containing chemical compound that is a potent inhibitor of AKT signaling [19]. Consistently, the oncogenic roles of miR-187 in the cell proliferation and invasion were also blocked by LY294002 treatment (Figure 6A-C), underlining the specific importance of the PTEN-AKT pathway for miR-187 action in the NSCLC tumorigenesis.

Inhibition of miR-187 suppresses NSCLC growth

Finally, we determined whether inhibition of miR-187 could suppress NSCLC growth. Inhibitors of miR-187 and negative controls (NC) were transfected into A549 cells, followed by cell proliferation and invasion analysis. As a result, suppression of miR-187 significantly inhibited cell proliferation and invasion (Figure 7A, 7B). Besides, up-regulation of PTEN and inactivation of AKT signaling was observed in both cells (Figure 7C). Therefore, our results suggest that down-regulation of miR-187 could reduce NSCLC progression in vitro.

Discussion

In this study, we provide the first integrated investigation of the function of miR-187 in NSCLC at both clinical and cellular levels. Firstly, miR-187 expression was increased in NSCLC tissues, compared with adjacent normal tissues. Secondly, ectopic overexpression of miR-187 mimics promoted, whereas its inhibitor suppressed NSCLC cell proliferation and invasion. Moreover, luciferase reporter and western blot assays showed that miR-187 has a direct contact with PTEN 3'-UTR, to down-regulate PTEN expression. It has to be noted that miR-187 was down-regulated in clear cell renal cell carcinoma and inhibited cell growth and migration through targeting B7-H3 [20]. Therefore, miR-187 could act as either an oncogene or a tumor suppressor, which might rely on cellular context.

PTEN, which dephosphorylates PI(3,4,5)P3, an important activator of Akt, is a tumor suppressor gene mutated in many human cancers [21]. Bronchioalveolar epithelium-specific null mutation of PTEN promotes lung carcinogenesis in mice [22]. Besides, loss of PTEN expression is observed in most NSCLC patients [23]. However, the mechanism by which PTEN expression is regulated in NSCLC remains poorly understood. It has been shown that genetic alterations, including LOH or inactivating mutation of PTEN, and promoter methylation are rare in NSCLC [24, 25], suggesting that other suppressive mechanisms may play important roles. Interestingly, recent studies have shown that PTEN could be down-regulated by several miRNAs in NSCLC, including miR-21, miR-92b, miR-205 and miR-214 [26-30]. Therefore, together with these reports, our data highlight an important role of miRNAs in the regulation of PTEN expression. Understanding of these regulatory mechanisms might have therapeutic benefits against NSCLC.

Figure 7. Suppression of miR-187 inhibits NSCLC cell proliferation. (A, B) Cell proliferation (A) and invasion (B) assays were determined in A549 cells transfected with miR-187 inhibitors or negative controls (NC). (C) Representative protein levels of PTEN and phosphorylated AKT were analyzed in A549 cells.
This study has some limitations. Firstly, whether other important molecules are involved in the roles of miR-187 should be determined. Besides, whether this complicated network involving miR-187 and PTEN-AKT regulates tumorigenesis in vivo remains unexplored. Further studies are still needed to clarify these issues.

In summary, this study provides a miR-187-mediated mechanism for the frequent down-regulation in NSCLC pathogenesis, causing uncontrolled proliferation and invasion. Further investigation will help to clarify whether therapeutic tools targeting this pathway can be developed for lung cancer treatment and whether this pathway provides a prognostic biomarker for lung cancer patient survival.

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Disclosure of conflict of interest

None.

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