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Original Article

miR-106a promotes growth and metastasis of non-small cell lung cancer by targeting PTEN

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Abstract: MicroRNAs are a class of small non-coding RNAs that play essential roles in cancer development and progression. Recent studies suggested that abnormal expression of miRNAs occurs frequently in non-small cell lung cancer (NSCLC) tissues compared to adjacent normal tissues. In this study, we investigated the expression and the biological roles of miR-106a in non-small cell lung cancer. Our results showed that miR-106a was up-regulated in NSCLC tissues and cell lines. Inhibition of miR-106a in NSCLC cells substantially inhibited cell proliferation, migration, and invasion. Phosphatase and tensin homolog (PTEN) was identified as a direct target of miR-106a, and over-expression of miR-106a suppressed PTEN by direct binding to its 3'-untranslated region (3'-UTR). Furthermore, the presence of miR-106a was inversely correlated with PTEN in NSCLC tissues. Overall, this study suggested that miR-106a inhibited the growth and metastasis of NSCLC cells by decreasing PTEN expression. These data provide novel insights with potential therapeutic applications for the treatment of NSCLC.

Keywords: Non-small cell lung cancer, miR-106a, PTEN, proliferation, migration, invasion

Introduction

Lung cancer is the leading cause of cancer-related mortality, with 1.4 million deaths worldwide annually. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for more than 85% of all lung cancer cases [1, 2]. Despite great advances in chemotherapy and surgical techniques, the prognosis for NSCLC is still dismal, and the overall 5-year survival rate is about 15% [3]. The distant metastases are responsible for the failure of lung cancer therapy and the poor prognosis of lung cancer [4]. Therefore, it is urgent to further investigate the underlying mechanisms of NSCLC.

MicroRNAs (miRNAs) are small non-coding RNAs which regulate gene expression post-transcriptionally by binding to the 3'-untranslated region (3'-UTR) of target mRNAs, leading to the degradation of target mRNAs or inhibition of translation [5]. Recent studies revealed that miRNAs played significant roles in a wide range of physiological and pathological processes including tumorigenesis [6]. Increasing evidence implicates miRNAs in cancer progression, including tumor growth, differentiation, invasion, metastasis, and angiogenesis [7, 8]. It has been demonstrated that several miRNAs were dysregulated in non-small cell lung cancer tissues or cell lines. For example, miR-200c inhibited NSCLC cells migration, invasion, epithelial-mesenchymal transition through inhibition of ubiquitin specific peptidase 25 (USP25) expression [9]. MiR-132 blocked the migration and invasion of NSCLC cells through targeting the epithelial-mesenchymal transition regulator ZEB2 [10]. MiR-195 suppressed the proliferation, migration, and invasion of NSCLC cells through targeting hepatoma-derived growth factor (HDGF) [11]. miR-152 was down-regulated in NSCLC tissues and cell lines. Over-expression of miR-152 suppressed cell proliferation, migration and invasion through negatively regulation of fibroblast growth factor 2 (FGF2) [12].

Previous studies showed that miR-106a could act as a tumor suppressor or an oncogene in different cancers, which may be dependent on

cellular context. For example, miR-106a provides a tumor-suppressive effect via suppressing proliferation and inducing apoptosis in human glioma cells by targeting E2F1 independent of p53 status. Furthermore, miR-106a could inhibit glioma cell proliferation and glucose uptake by repressing SLC2A3 expression [13, 14]. Besides, recent investigation showed that miR-106a has an oncogenic role in pancreatic tumorigenesis by promoting cancer cell proliferation, epithelial-mesenchymal transition and invasion by targeting tissue inhibitors of metalloproteinase 2 (TIMP-2) [15]. In gastric cancer, miR-106a was significantly increased and down-regulated expression of miR-106a significantly inhibited gastric cancer cell proliferation and triggered apoptosis by targeting FAS [16]. However, the functional role and underlying mechanism of miR-106a involved in NSCLC remains unknown and demand further investigations.

Materials and methods

Clinical sample collection

Paired NSCLC and adjacent non-tumor tissues (located more than 5 cm away from the tumors) were obtained from 30 patients who underwent primary surgical resection of NSCLC at Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (Shanghai, China). None of the patients had received preoperative adjuvant therapy. These samples were snap-frozen in liquid nitrogen after resection. Prior patient consent and approval from the ethics committees of Shanghai Jiao Tong Uni-versity were obtained for the use of these clinical materials for research purposes.

Cell culture

The human lung cell lines A549, H1299, H460 and a normal bronchial epithelial cell line (16HBE) were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). All cell lines were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM) (HyClone) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin sodium, and 100 mg/ml streptomycin sulfate at 37°C in a humidified air atmosphere containing 5% CO_2. Cells were used when they were in the logarithmic growth phase.

RNA isolation and quantitative real-time PCR

Total RNA and miRNA were isolated using RNeasy Mini and miRNeasy Mini Kits (Qiagen) according to the manufacturer's protocol. The expression of miR-106a was determined by quantitative real-time PCR (qRT-PCR) using TaqMan MicroRNA Assay Kits (ABI) on a LightCycler 480 System II (Roche). PTEN primer: forward 5'-CCAGGACCAGAGGAAACCT-3', reverse 5'-GCTAGCCTCTGGATTTGA-3'. GAPDH primer: forward 5'-ATGTCGTGGAGTCTACTGGC-3', reverse 5'-TGACCTTGCCCACAGCCTTG-3'. The expression of PTEN was determined using SYGR green real-time PCR (Takara). The qRT-PCR data were normalized using the $2^{-\Delta\Delta Ct}$ method relative to GAPDH or U6.

Plasmid construction and cell transfection

miR-106a mimics and miR-106a inhibitors were obtained from Ribobio (Guangzhou, China). The 3'-UTR of PTEN which contains the potential binding sites of miR-106a was amplified using the following primer: forward 5'-CGAGCTCGGACGAACTGGTGTAATG-3', reverse 5'-CGACGCGTGTCCAGAGTCCAGCATAA-3'. The PCR fragment was inserted into pMir-Report vector (Ambion) within SacI and MluI restriction sites. Mutationwas performed with a fast mutation kit (NEB). Transfection was performed when cells were grown to 80% confluence, using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Luciferase activity assay

Luciferase activity assay was performed as previously described [17]. Briefly, HEK293 cells were cultured in 12-well plate (1×10^5 cells/well), and co-transfected with wild type or mutated 3'-UTRs of PTEN (WT and Mut respectively) luciferase reporter constructs and miR-106a mimics or control mimics with Lipofectamine 2000. 24 hours later, cell was harvested and luciferase activity was examined by Dual-Luciferase Reporter Assay Kit (Promega).

Cell viability assay

Cells were plated at 10^4 cells per well in 96-well plates with six replicate wells. After transfection as described previously, 20 μl of MTT (5 g/L, Sigma) was added into each well at each day of 4 consecutive days after treatment and

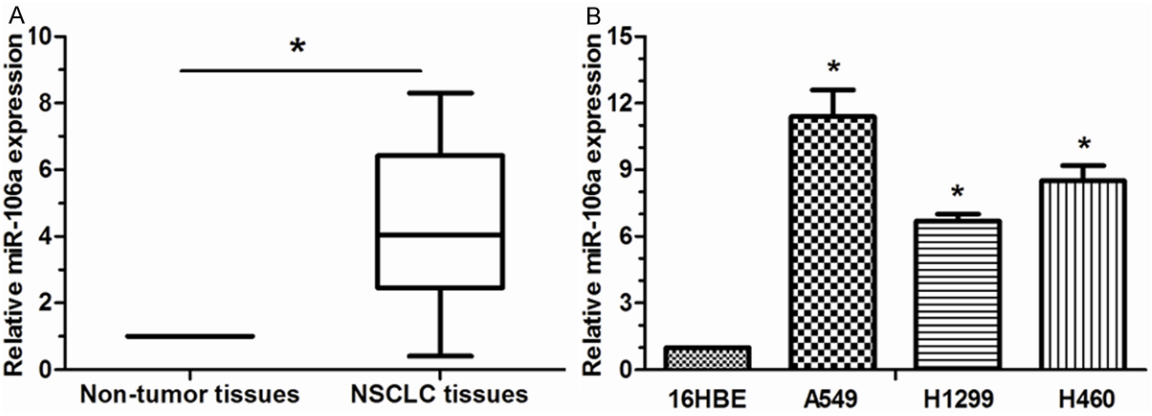

Figure 1. miR-106a was increased in NSCLC tissues and cell lines. A. miR-106a was significantly increased in NSCLC tissues compared with that in adjacent non-tumor tissues. B. miR-106a was significantly increased in three lung cancner cell lines, A549, H1299, and H460 compared with that in human bronchial epithelial cell line 16HBE cells. *$P < 0.05$.

the cells were incubated for additional 4 hours. The supernatant was then discarded. 200 μl of DMSO was added to each well to dissolve the precipitate. Optical density (OD) was measured at the wave length of 550 nm.

Colony formation assay

To assess colony formation, 24 hours after transfection, 500 cells were plated in 6-well plates and grown for 2 weeks; the culture medium was replaced every 4 days. Cells were fixed with methanol and stained with 0.5% crystal violet for 20 minutes; visible colonies were counted. Triplicate wells were measured for each group.

Migration and invasion assay

Migration and invasion assays were performed using transwell chambers. For migration assay, 5×10^4 cells were seeded into the upper chamber of transwells (BD Bioscience). For invasion assay, 1×10^5 cells were added into the upper chamber precoated with matrigel (BD Bioscience). In both assays, cells were maintained in medium without serum in the upper chamber, and medium containing 10% FBS was added to the lower chamber as chemoattractant. After 24 hours incubation, cells that did not migrate or invade through the membrane were wiped out. Then the membranes were fixed and stained with 0.5% crystal violet. Three random fields were counted per chamber using an inverted microscope (Olympus), and each experiment was repeated three times.

Western blotting

Cells were harvested and resuspended in PBS. After centrifugation at 2000 rpm for 5 minutes, the pellet was lysed in ice cold Lysis Buffer containing 1% Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) for 30 minutes. The supernatant was collected after 10 minutes of centrifugation at 12000 rpm, equaled by spectrophotometry, denatured with sample loading buffer for 10 minutes at 95°C and stored at 4°C for future use. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and incubated with primary antibodies overnight at 4°C followed by secondary antibodies for 1 hour at room temperature. Blots were visualized using an ECL detection system (Amersham) and analyzed by Kodak Digital Science 1D software (Eastman Kodak).

Statistical analysis

Statistical testing was conducted with the assistance of SPSS 17.0 software (IBM). All data were expressed as means ± SD. Student's t test and one-way analysis of variance (ANOVA) were used to analyze data. Results were considered significant when *P* value less than 0.05.

Results

miR-106a expression level was increased in NSCLC

In order to determine the expression levels of miR-106a in NSCLC tissues, the qRT-PCR

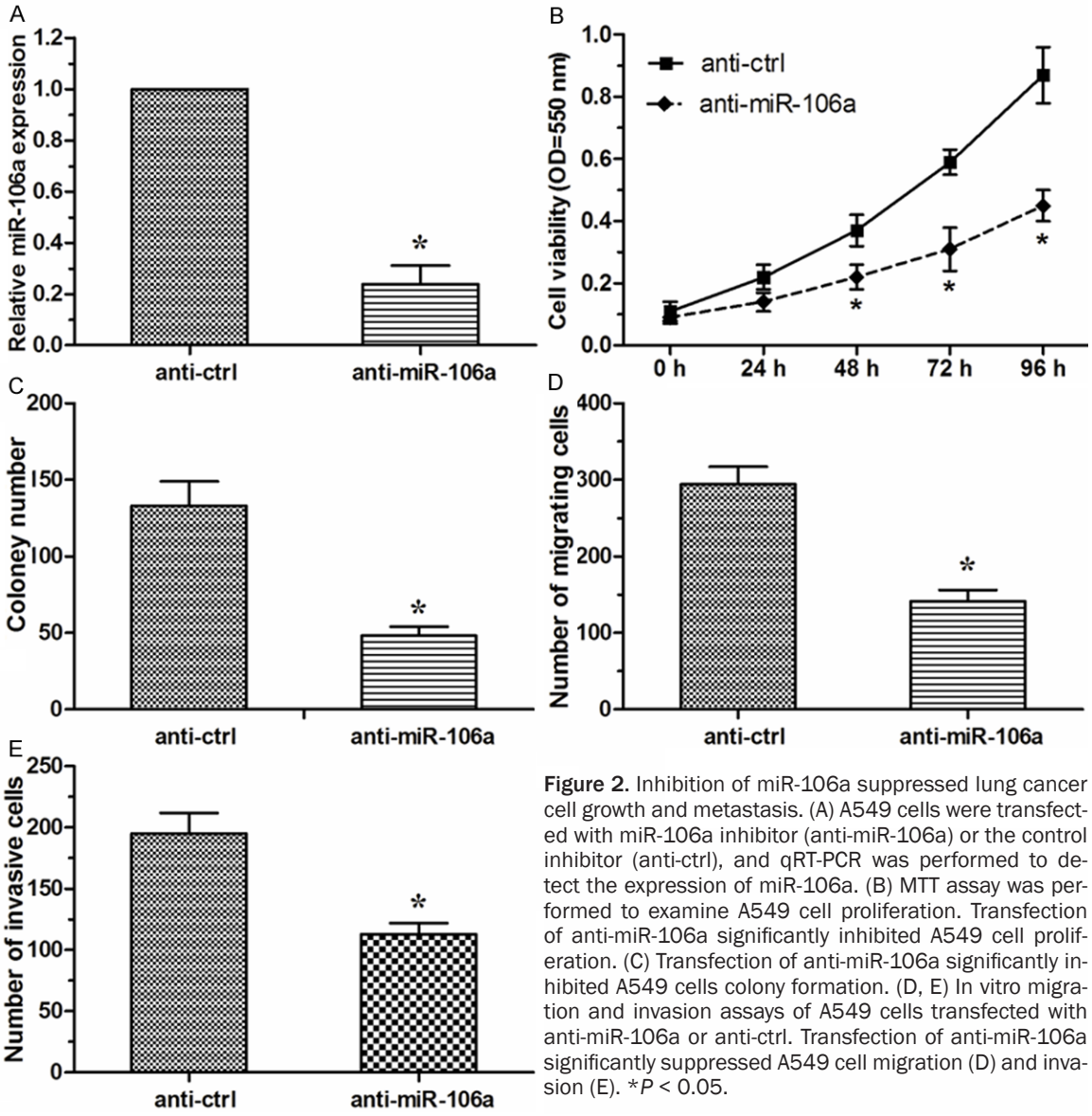

Figure 2. Inhibition of miR-106a suppressed lung cancer cell growth and metastasis. (A) A549 cells were transfected with miR-106a inhibitor (anti-miR-106a) or the control inhibitor (anti-ctrl), and qRT-PCR was performed to detect the expression of miR-106a. (B) MTT assay was performed to examine A549 cell proliferation. Transfection of anti-miR-106a significantly inhibited A549 cell proliferation. (C) Transfection of anti-miR-106a significantly inhibited A549 cells colony formation. (D, E) In vitro migration and invasion assays of A549 cells transfected with anti-miR-106a or anti-ctrl. Transfection of anti-miR-106a significantly suppressed A549 cell migration (D) and invasion (E). *$P < 0.05$.

detection was made in 30 pairs of NSCLC tissues and adjacent non-tumor tissues. As shown in **Figure 1A**, the levels of miR-106a significantly increased in NSCLC tissues in comparison to adjacent non-tumor tissues ($P < 0.05$). Moreover, expression of miR-106a in three lung cancer cell lines A549, H1299, and H460 was significantly increased compared with that in human normal bronchial epithelial cell line 16HBE cells (**Figure 1B**, $P < 0.05$). These data indicated that miR-106a may play critical roles in the development and progression of NSCLC.

Decreased expression of miR-106a inhibited NSCLC cell growth and metastasis

We then investigated the role of miR-106a in the regulation of cell growth and metastasis of NSCLC cells, A549 cells were transfected with miR-106a inhibitor (anti-miR-106) or control inhibitor (anti-ctrl). From qRT-PCR results, we found that the expression of miR-106a transfected with anti-miR-106 significantly decreased compared to control group in A549 cells (**Figure 2A**, $P < 0.05$). Moreover, the proliferation rate of cells that transfected anti-miR-106a also remarkably decreased (**Figure 2B**, $P < 0.05$). Besides, transfection of anti-miR-106a also significantly inhibited colony formation of A549 (**Figure 2C**, $P < 0.05$). To test the effect of miR-106a on the motility of NSCLC cells, in vitro migration and invasion assays were performed. Likewise, our studies revealed that transfection of anti-miR-106a significantly inhibited the migration and invasion abilities of A549 cells

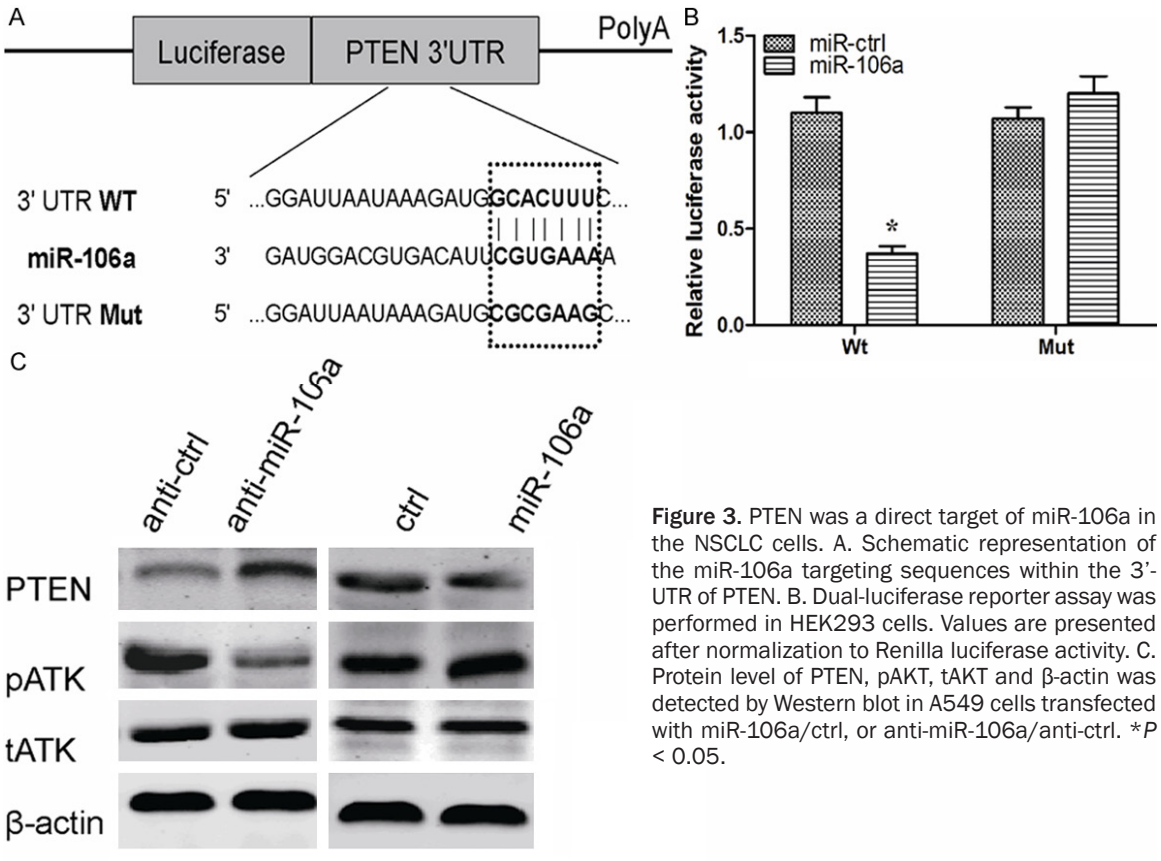

Figure 3. PTEN was a direct target of miR-106a in the NSCLC cells. A. Schematic representation of the miR-106a targeting sequences within the 3'-UTR of PTEN. B. Dual-luciferase reporter assay was performed in HEK293 cells. Values are presented after normalization to Renilla luciferase activity. C. Protein level of PTEN, pAKT, tAKT and β-actin was detected by Western blot in A549 cells transfected with miR-106a/ctrl, or anti-miR-106a/anti-ctrl. *$P < 0.05$.

(**Figure 2D** and **2E**, $P<0.05$). These data demonstrated that down-regulated miR-106a expression could suppress the development and progression of NSCLC.

PTEN was a target of miR-106a in NSCLC cells

To detect the molecular mechanism by which miR-106a suppress the growth and metastasis of lung cancer cells, we predicted the putative target genes of miR-106a in human cells using the tool miRanda, PicTar and TargetScans. Among the predicted candidates, PTEN was predicted to be a target of miR-106a (**Figure 3A**). And then the dual-luciferase activity assay showed that miR-106a significantly suppressed the luciferase activity of the wild-type (WT) 3'-UTR of PTEN, without effect on its mutant (Mut) (**Figure 3B**, $P < 0.05$). In addition, increased expression of miR-106a significantly inhibited PTEN protein level, on the contrary, pAKT protein expression significantly increased, and total AKT protein stayed the same, while inhibition of miR-106a showed opposite effects (**Figure 3C**).

miR-106a expression was inversely correlated with PTEN in NSCLC tissues

To further explore the relationship between miR-106a and PTEN expression in vivo, we examined the expression of PTEN mRNA in 30 pairs of NSCLC tissues and their matched non-tumor tissues using qRT-PCR. Results showed that PTEN mRNA was significantly decreased in NSCLC tissues compared with the matched non-tumor tissues (**Figure 4A**, $P<0.05$). Moreover, PTEN was negatively correlated with miR-106a expression in the same NSCLC tissues (**Figure 4B**, $P < 0.05$). These data further indicated that PTEN was a target of miR-106a in NSCLC. In addition, we investigated 58 NSCLC cases and found those people that expressed high miR-106a level had a shorter overall survival time than that expressed low level (**Figure 4C**, $P < 0.05$).

Discussion

The miRNAs have been reported to play essential roles in progression of various cancers including NSCLC via regulation of expression of

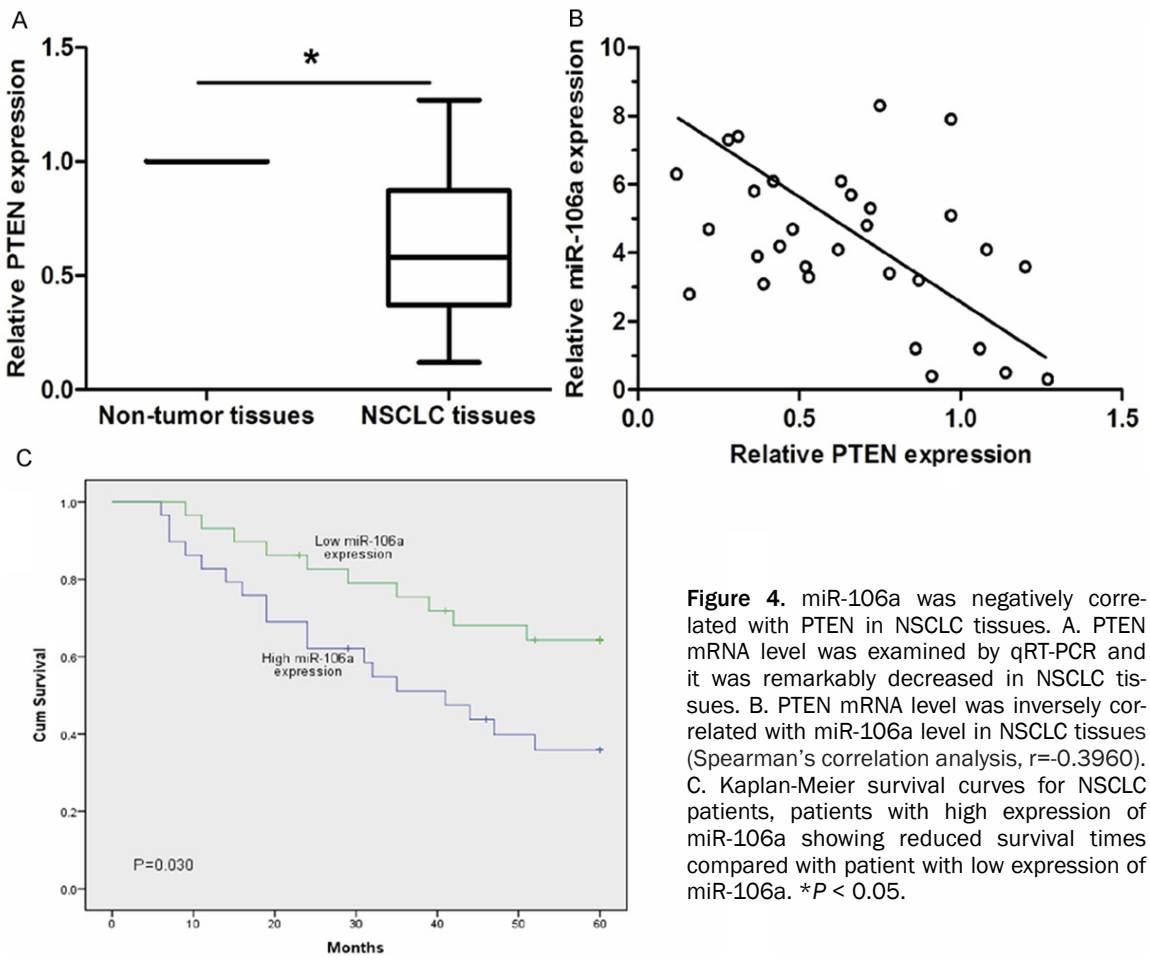

Figure 4. miR-106a was negatively correlated with PTEN in NSCLC tissues. A. PTEN mRNA level was examined by qRT-PCR and it was remarkably decreased in NSCLC tissues. B. PTEN mRNA level was inversely correlated with miR-106a level in NSCLC tissues (Spearman's correlation analysis, r=-0.3960). C. Kaplan-Meier survival curves for NSCLC patients, patients with high expression of miR-106a showing reduced survival times compared with patient with low expression of miR-106a. *$P < 0.05$.

multiple target genes involved in the progression and metastasis [18, 19]. So, identification of specific miRNAs and their targets involved in carcinogenesis would provide valuable insight for the diagnosis and therapy of patients with human malignancies. In the present study, our studies showed that miR-106a expression was increased in NSCLC tissues. Kncok-down expression of miR-106a was able to inhibit cell growth and metastasis in A549 cells. Therefore, our study, for the first time, identified that miR-106a might be an oncogenic MicroRNA in the progression of NSCLC. However, the role of miR-106a in NSCLC carcinogenesis remains unclear.

In addition, at the molecular level, our study indicated that PTEN is a direct target of miR-106a in non-small cell lung cancer cells. PTEN was originally discovered as the tumor suppressor gene frequently lost on chromosome 10q23 [20]. Heterozygous loss of PTEN in the mouse resulted in the development of cancer of multiple origins, as well as in a lethal lymphoproliferative disease [21]. In humans, germline loss and mutation of PTEN is observed in a group of autosomal dominant syndromes (PTEN hamartoma tumor syndromes (PHTS)), which are characterized by neurologic disorders, multiple hamartomas, and cancer susceptibility [22]. Importantly, loss of PTEN results in prolonged activation of Akt and subsequently in increased cell proliferation, migration, and invasion, which forms molecular mechanisms of PTEN contribution to tumorigenesis and progression of malignant tumor [23]. What's more a previous study showed that the PTEN/PI3K/pAkt pathway may play an important role in lung cancer carcinogenesis [24].

In conclusion, our study demonstrated that miR-106a was significantly increased in NSCLC tissues and cell lines. Down-regulated expression of miR-106a could inhibit tumor growth

and metastasis of NSCLC cells by increasing PTEN expression. Together, the present study suggested that miR-106a could act as an oncogene in NSCLC and represent a potential molecular target for NSCLC therapy.

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Disclosure of conflict of interest

None.

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