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Original Article

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Down-regulation of microRNA-26b modulates non-small cell lung cancer cells chemoresistance and migration through the association of PTEN

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Abstract

To explore the effect of microRNA-26b (miR-26b) in non-small cell lung cancer (NSCLC) cells, we investigated the mRNA levels of miR-26b in 4 NSCLC cell lines and 10 clinical samples from human patients with NSCLC by quantitative reverse transcriptase polymerase chain reaction. It was found that miR-26b was significantly down-regulated in both NSCLC cells and human carcinoma tissues. Synthetic oligonucleotides were used to up-regulate or down-regulate miR-26b in NSCLC cell lines H1299 and A549 cells. Results showed that both down-regulating and up-regulating miR-26b had no effect on cancer cell proliferation in H1299 or A549 cells, whereas miR-26b over-expression increased cancer cell migration and reduced cisplatin chemosensitivity. Phosphatase and tensin homolog (PTEN) was confirmed to be directly bound by miR-26b by dual-luciferase reporter assay, and was down-regulated in miR-26b over-expressing NSCLC cells. Finally, when PTEN was up-regulated in NSCLC cells, it reversed the effects of miR-2b over-expression on NSCLC migration and cisplatin chemosensitivity. In conclusion, our data showed a functional mechanism of miR-26b in regulating NSCLC. It indicates that miR-26b may regulate NSCLC migration and chemosensitivity through the regulation of PTEN.

Key words: NSCLC, miR-26b, PTEN, migration, cisplatin

Introduction

Lung cancer is one of the most common cancers in both men and women worldwide. As estimated in 2013, lung cancer may account for 28% of all male cancer deaths and 26% of all female cancer deaths [1]. The most common form of lung cancer is non-small cell lung cancer (NSCLC), which accounts for ~80% of all lung cancers in the world. Unfortunately, although great progress has been made in terms of providing best possible treatment for patients with NSCLC, including surgery and targeted chemotherapy [2,3], the overall 5-year survival rate associated with NSCLC is still <20% [4,5]. Therefore, it is very important to understand the molecular mechanisms underlying NSCLC growth, migration, or chemosensitivity, and to develop optimal treatments for patients with NSCLC.

MicroRNAs (miRNAs) are a class of non-coding RNAs of 20–24 nucleotides. It can regulate gene expression through binding with the 3′-untranslated region (3′-UTR) of targeted mRNAs, thus having profound modulatory functions in various physiological and developmental processes, such as embryogenesis, cell and tissue development,

cell apoptosis, and pathology [6–8]. In human cancers, many of the miRNA families are deregulated, therefore playing critical roles in tumorigenesis [9,10], tumor development or proliferation [11,12], and tumor apoptosis or metastasis [13–16]. Among many cancer-associated miRNAs, miR-26b has been reported to be a cancer regulator in breast cancer [17] and pituitary cancer [18]. miR-26b was found to be under-expressed in lung carcinoma [19], yet its functional role remains elusive.

The tumor suppressor gene, phosphatase and tensin homolog (*PTEN*) is located on human chromosome 10q23 [20]. Down-regulation of PTEN was reported in many human primary tumors, including NSCLC [21]. In addition, PTEN has been reported to be regulated by miR-21 and miR-26a in NSCLC [22–24]. However, it is not clear whether PTEN is directly associated with miR-26b in regulating NSCLC development.

In the present study, we first measured the expression levels of miR-26b in both NSCLC cancer cell lines and clinical specimens from patients with NSCLC. The endogenous expression level of miR-26b was regulated by either down-regulating or up-regulating its gene expression, in NSCLC cell lines H1299 and A549. The effects of miR-26b down-regulation or over-expression on cell proliferation, migration, and cisplatin chemosensitivity were assessed. Moreover, we examined whether PTEN is the direct target of miR-26b in NSCLC cells. Our data would help to understand the underlying mechanisms of miRNA regulation in lung cancer.

Materials and Methods

Clinical specimens

The clinical specimens were obtained from patients with NSCLC in the Department of Pathology and the Department of Rheumatology at Peking Union Medical College Hospital. All patients signed consent forms. The procedures were also approved by the Clinical Research and Ethic Committee at Peking Union Medical College Hospital. For each individual patient, both NSCLC carcinoma tissues (T) and adjacent non-carcinoma tissues (ANT) were included in the study.

Cell culture

Four NSCLC cell lines including H1299, A549, H661, and H1703 were used in the present study. For non-carcinoma control cell line, a fetal lung fibroblast cell line MRC5 was used. All cell lines were commercially obtained from Shanghai Institute of Cell Biology, China Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, USA), and supplemented with 10% fetal bovine serum (FBS; Sigma), 100 μg/ml streptomycin, and 100 U/ml penicillin (Pen/Strep, Sigma), at 37°C with 95% O_2 and 5% CO_2.

Down-regulation and up-regulation of miR-26b

The synthetic oligonucleotides of miR-26b mimic (miR-26b-mimic), miR-26b inhibitor (miR-26b-inhibitor), and negative control oligonucleotides (miR-C) were purchased from RiboBio (RiboBio, Guangzhou, China). NSCLC cell lines, H1299 and A549 cells, were seeded in 6-well plates (2×10^5 cells/well). miR-26b-mimic and miR-26b-inhibitor were transfected into the cells at the final concentration of 200 or 500 nM with Lipofectamine 2000 (Sigma) according to the manufacturer's instruction. Twenty-four hours later, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed to examine the efficiency of miRNA regulation.

qRT-PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, USA). Reverse transcription was conducted using TaKaRa kit (TaKaRa, Dalian, China) according to the manufacturer's instruction. To quantify gene expression, the synthesized primers were purchased from SBS Genetech (SBS Genetech, Beijing, China). For *PTEN*, the forward primer was 5′-CAATGTTCAGTGGCGGAACTTG-3′, and the reverse primer was 5′-GAACTTGTCTTCCCGTCGTGTG-3′. For miR-26b, the forward primer was 5′-CGCCGCTTCAAGTAATTCAG GAT-3′, and the reverse primer was 5′-GTGCAGGGTCCGAGGT-3′. The qRT-PCR was conducted with SYBR Premix Ex Taq (TaKaRa) by an ABI Prism 7500 Sequence Detector System (Applied Biosystems, Foster City, USA), according to the manufacturer's instruction. The expression of miR-26a was detected using a Hairpin-it miRNAs qPCR Quantitation kit (GenePharma, Guangzhou, China), according to the manufacturer's instruction. U6 was used as the internal control.

Western blot analysis

Total proteins were extracted from H1299 and A549 cells with a RIPA buffer with 0.5% sodium dodecyl sulfate (SDS) and 3% proteinase inhibitor cocktail (Sigma) according to the manufacturer's instruction. Cell lysates were then separated by 10% SDS–PAGE and transferred onto PVDF membranes (Invitrogen). After blocking with 5% bovine serum albumin at room temperature for 1 h, membranes were incubated with primary antibody against PTEN (1:200, Abcam, Hong Kong, China) at 4°C overnight. Membranes were washed three times and incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Blots were visualized by an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, USA).

Cell viability and proliferation assay

H1299 and A549 cells were initially transfected with miR-C, miR-26b-mimic, or miR-26b-inhibitor for 48 h. The cell viability was determined by Vi-CELL Cell Viability Analyzer (Beckman Coulter, Pasadena, USA) according to the manufacturer's instruction. For proliferation assay, the viable cells were transferred into 24-well plates (2×10^5 cells/well) and continuously cultured for another 5 days. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to measure the relative optical intensity by a CytoFluor II multiwell plate reader (PerSeptive Biosystems, Framingham, USA) for each experimental condition.

Cell migration assay

Cell migration assay was performed using a quantitative cell migration kit (ECM500, Chemicon, Billerica, USA), according to the manufacturer's protocol. In brief, H1299 and A549 cells were cultured in ECM chambers (2×10^4 cells/well). Serum-free DMEM (300 μl) was added to the ECM layer and allowed to hydrate for 1 h. Cells were then dissociated, re-suspended in L-DMEM and allowed to adhere for another 1 h. Migration medium with miR-C, miR-26b-mimic, or miR-26b-inhibitor was added to the bottom chamber. Two days later, cells in the lower chamber were immunostained. The relative optical density at 560 nm was measured by a standard microplate reader (Sunrise, Tecan, Switzerland) according to the manufacturer's instruction to determine cell migration.

Cisplatin chemosensitivity assay

H1299 and A549 cells were seeded in 96-well plates (1×10^5 cells/well), and transfected with miR-C, miR-26b-mimic, or miR-26b-inhibitor. Twenty-four hours after transfection, culture medium was changed to

freshly prepared DMEM plus 10% FBS with the addition of cisplatin (0, 10, 20, 50, and 100 μg/ml). Cells were cultured for another 72 h, followed by the examination of cell viability.

Dual-luciferase reporter assay

The predicted binding site of miR-26b on 3′-UTR of PTEN was amplified through conventional RT-PCR from the cDNA library of H1299 cells. A recombinant mutant 3′-UTR of PTEN was also generated by a Site-Directed Mutagenesis Kit (SBS Genetech). The amplified wild-type and mutant PTEN 3′-UTRs were then inserted into a pmiR-REPORT luciferase reporter vector (Ambion, Austin, USA) to generate Luc-PTEN and Luc-mPTEN (mutant) vectors according to the manufacturer's instruction. A negative control luciferase vector, Luc-C was also generated. Then, all three vectors were co-transfected with β-galactosidase and miR-26b-mimic (RiboBio) into HEK293T cells by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. HEK293T cells were then cultured at 37°C with 5% CO_2 for another 24 h. The dual-luciferase activities were measured by a luciferase reporter assay system (Promega, Madison, USA) according to the manufacturer's instruction, and normalized to β-galactosidase activity transfected with Luc-C.

Over-expression of PTEN

In order to endogenously over-express PTEN in NSCLC cell lines, we obtained the plasmid pcDNA3.1 from Clontech (Clontech, Palo Alto, USA). PTEN sequence was amplified from human cDNA library and inserted into pcDNA3.1 to generate PTEN over-expression plasmid (pcDNA-PTEN). A control plasmid with empty insertion was also generated (pcDNA-C). H1299 and A549 cells were seeded in 6-well plates (1×10^5 cells/well), and transfected with expression vector pcDNA-PTEN or pcDNA-C with a Lipofectamine 2000 reagent according to the manufacturer's instruction. Two days after transfection, the PTEN over-expression efficiency was confirmed by western blot analysis.

Statistical analysis

All data were presented as the mean ± standard error of the mean. Statistical analysis was performed with SPSS software (version 13.0). One-way analysis of variance (ANOVA) was used to analyze cell assay results, and two-tail unpaired Student's *t*-test was used for other assays. A *P*-value of <0.05 was considered significant difference. All experiments were repeated at least three times.

Results

miR-26b is down-regulated in NSCLC cell lines and clinical specimens

In the present study, qRT-PCR was used to examine the mRNA expression levels of miR-26b in four NSCLC cell lines, H1299, A549, H661, and H1703 cells. It was found that miR-26b was significantly down-regulated in all four NSCLC cell lines compared with that in a control cell line MRC5 (**Fig. 1A**, $*P < 0.05$). The mRNA levels of miR-26b were also compared between cancer specimens (T) and adjacent non-cancer specimens (ANT) in patients with NSCLC. The results showed that, similar to the expression pattern in NSCLC cell lines, miR-26b was markedly down-regulated in carcinoma tissues compared with in non-carcinoma tissues of the 10 examined clinical specimens from patients with NSCLC (**Fig. 1B**, $*P < 0.05$).

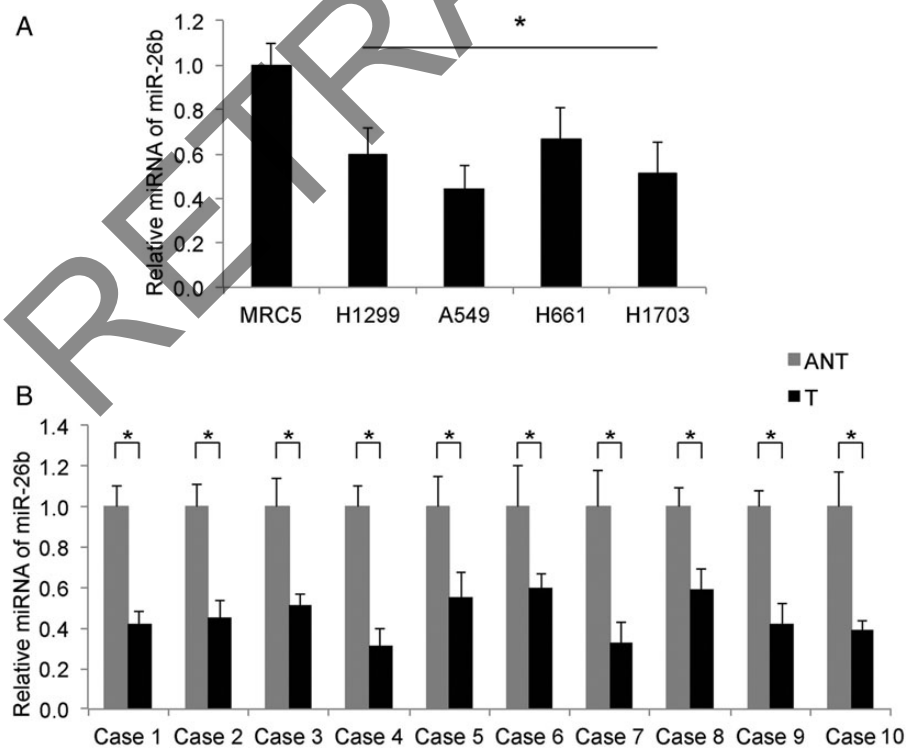

Figure 1. Down-regulation of miR-26b in NSCLC cell lines and clinical specimens (A) The expression levels of miR-26b were assessed by qRT-PCR in NSCLC cell lines H1299, A549, H661, H1703, and a fetal lung fibroblast cell line MRC5 ($*P < 0.05$). (B) The expression levels of miR-26b were also compared between carcinoma tissues (T) and adjacent non-carcinoma tissues (ANT) in 10 paired clinical specimens extracted from patients with NSCLC ($*P < 0.05$).

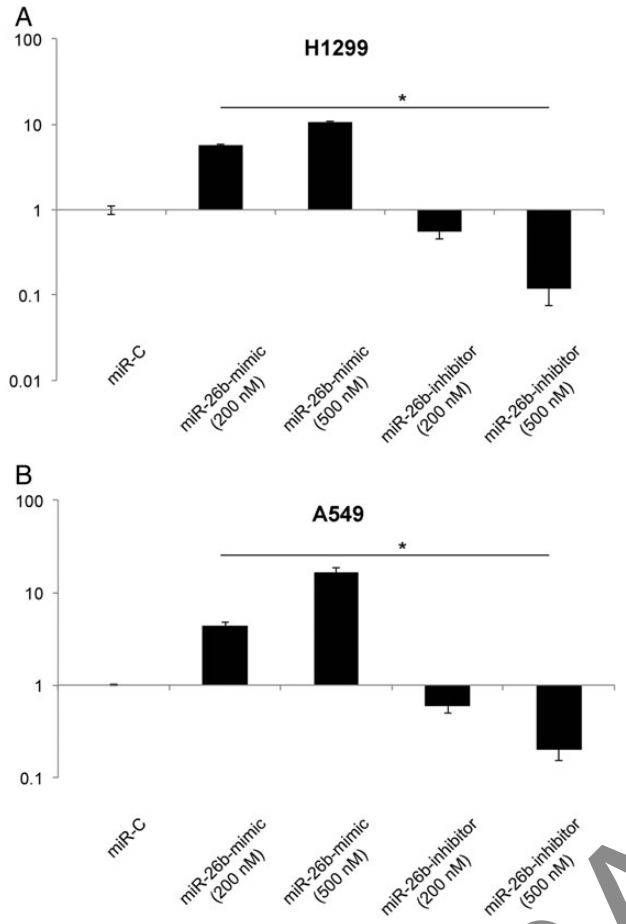

Figure 2. Up-regulation and down-regulation of miR-26b in NSCLC cell lines
H1299 cells (A) and A549 cells (B) were transfected with miR-26b-mimic (200 or 500 nM), miR-26b-inhibitor (200 or 500 nM), or a control miRNA (miR-C, 200 nM). Twenty-four hours later, the efficiencies of miR-26b up-regulation or down-regulation were assessed by qPCR (*$P < 0.05$ compared with miR-C).

Change in miR-26b has no effect on cancer proliferation of NSCLC cells

Since the endogenous mRNA expression levels of miR-26b were found to be different in four NSCLC cell lines and the normal lung cell line, we wonder whether miR-26b plays any functional role in regulating NSCLC growth.

The synthesized miRNA oligonucleotides miR-26b-mimics and miR-26b-inhibitors were transfected into NSCLC cell lines H1299 and A549 cells to up-regulate or down-regulate endogenous miR-26b. miR-C was also transfected into H1299 and A549 cells as a control. Twenty-four hours after transfection, qRT-PCR assay was used to examine the transfection efficiencies. It was found that the endogenous miR-26b mRNAs were significantly up-regulated by 200 and 500 nM miR-26b-mimics, or down-regulated by 200 and 500 nM miR-26b-inhibitors in both H1299 cells (**Fig. 2A**, *$P < 0.05$) and A549 cells (**Fig. 2B**, *$P < 0.05$).

Then, we assessed whether the regulation of the expression of miR-216b would have any cytotoxic effects on NSCLC cells. Forty-eight hours after transfection, higher concentration (500 nM) of synthetic oligonucleotides caused significant decrease in viabilities in both cell lines, whereas low concentration (200 nM) of miR-26b-mimic and miR-26b-inhibitor did not (**Fig. 3A,B**, *$P < 0.05$). Thus, considering the regulation on miR-216b and the cytotoxic effects, we used 200 nM of miR-26b-mimic or miR-26b-inhibitor in the subsequent experiments.

Furthermore, we examined the effects of miR-26b-mimic or miR-26b-inhibitor on the growth of NSCLC cells. H1299 and A549 cells were transfected with miR-26b-mimic (200 nM), miR-26b-inhibitor (200 nM), or miR-C (200 nM), and the cell growth was examined for 5 days by cell proliferation assay. The results showed that neither up-regulation nor down-regulation of miR-26b had significant impact on the growth of NSCLC cells (**Fig. 3C,D**, $P > 0.05$, one-way ANOVA).

miR-26b up-regulation enhances the migration of NSCLC cells

An important characteristic for cancer cell metastasis is cell migration. Thus, we examined the effects of up-regulation or down-regulation of miR-26b on cell migration through a migration assay. H1299 and A549 cells were transfected with 200 nM of miR-26b-mimic, miR-26b-inhibitor, or miR-C. Two days later, migrated cells were stained and counted under microscope. It was found that in H1299 cells, migrated cells were significantly increased with miR-26b up-regulation, and no significant change was observed in miR-26b down-regulated cells (**Fig. 4A**). The numbers of invasive H1299 cells increased to 2.3 folds of control in the miR-26b up-regulation group (**Fig. 4B**, *$P < 0.05$), and there was no significant change in the miR-26b down-regulation group and control group (**Fig. 4B**, $^{\Delta}P > 0.05$). The enhanced effect of miR-26b up-regulation on NSCLC migration was more dramatic in A549 cells (**Fig. 4C**), which showed a 3.5-fold increase compared with the control (**Fig. 4D**, *$P < 0.05$). Similar to the effect in H1299 cells, down-regulation of miR-26 had no significant effect on the migration of A549 cells (**Fig. 4C,D**, $^{\Delta}P > 0.05$).

miR-26b up-regulation reduces the chemosensitivity of NSCLC cells

Chemotherapy is an efficient treatment for cancer patients. Thus, it is very important to assess the effect of miR-26b regulation on NSCLC chemosensitivity. To test this hypothesis, H1299 and A549 cells were transfected with miR-26b-mimic, miR-26b-inhibitor, or miR-C (200 nM) for 24 h. Then, H1299 cells were treated with different concentrations of cisplatin (0, 10, 20, 50, and 100 μg/ml) for 72 h, followed by measuring the cell proliferation. The results showed that up-regulation of miR-26b significantly decreased the chemosensitivity of H1299 cells against cisplatin at concentrations of 20, 50, and 100 μg/ml, whereas down-regulation of miR-26b had no effect on cisplatin chemosensitivity (**Fig. 5A**, *$P < 0.05$). The effect of miR-26b over-expression on decreased cisplatin chemosensitivity was more obvious in A549 cells, resulting in significant resistance against cisplatin from 10 to 100 μg/ml (**Fig. 5B**, *$P < 0.05$).

PTEN is the target of miR-26b in NSCLC cells

As we demonstrated the functional roles of miR-26b in NSCLC cells, we further explored the underlying signaling pathways associated with miR-26b in NSCLC cells. Through the online bioinformatics tools including miRana and Target Scan, we predicted tumor suppressor gene *PTEN* might be the direct target of miR-26b (**Fig. 6A**). To test this hypothesis, we conducted a dual-luciferase reporter assay. The predicted PTEN 3′-UTR binding site was cloned into a luciferase vector to form the recombinant plasmid Luc-PTEN, and co-transfected it with miR-26b-mimic and β-galactosidase in HEK293 cells. The luciferase vector including a mutant sequence of PTEN 3′-UTR binding site

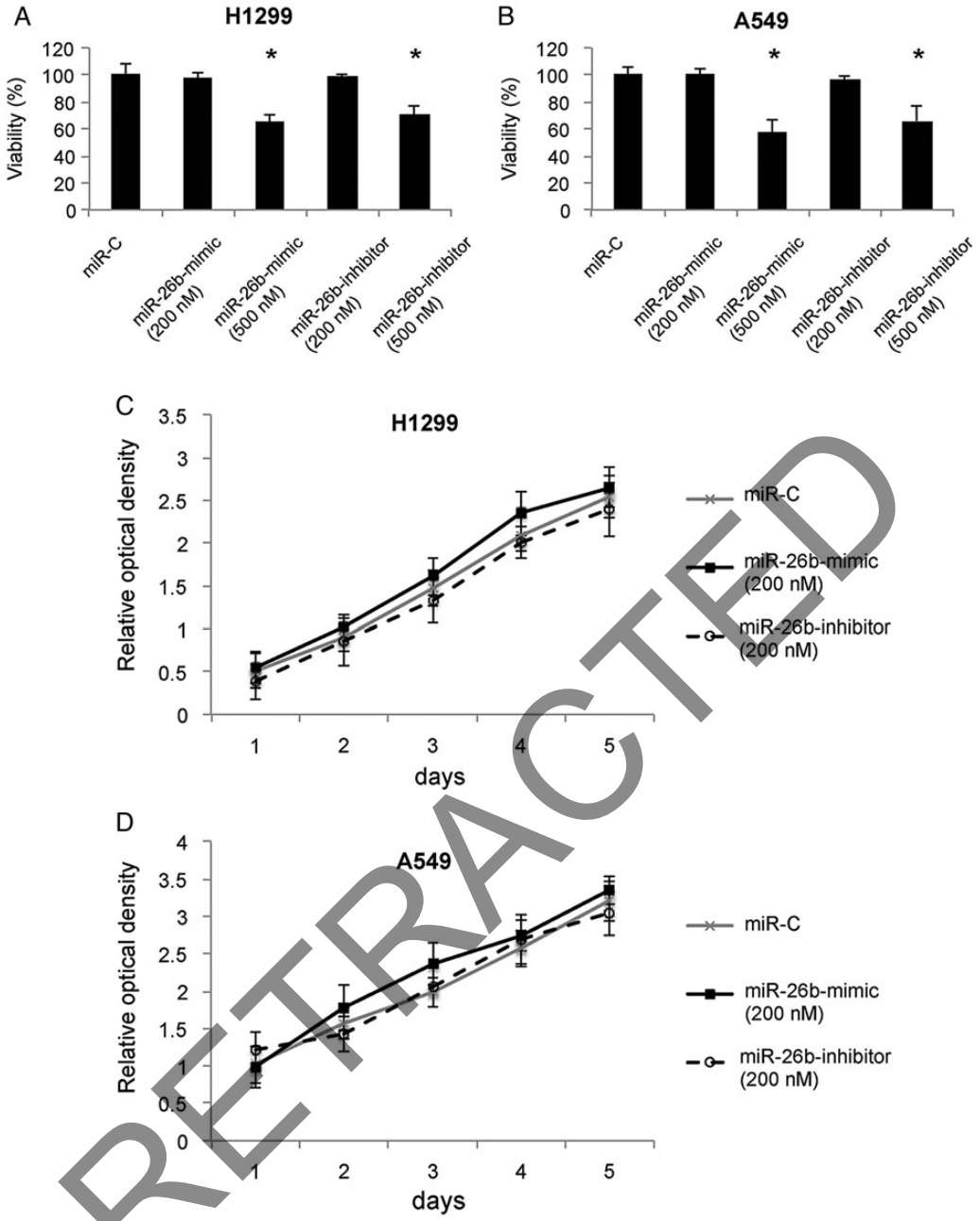

Figure 3. MiR-26b has no effect on the growth of NSCLC cells H1299 cells (A) and A549 cells (B) were transfected with miR-26b-mimic (200 or 500 nM), miR-26b-inhibitor (200 or 500 nM), or a control miRNA (miR-C, 200 nM). Forty-eight hours later, the cell viabilities were assessed (*$P < 0.05$ compared with miR-C). H1299 cells (C) or A549 cells (D) were transfected with miR-26b-mimic (200 nM), miR-26b-inhibitor (200 nM), or miR-C (200 nM). The growth of NSCLC cells was assessed by cell proliferation assay from the first to the fifth day after transfections.

(Luc-mPTEN) or a control luciferase vector (Luc-C) was also included in the dual-luciferase reporter assay. The results showed that, compared with Luc-C transfected cell, the relative luciferase activity was significantly decreased by Luc-PTEN, but not by Luc-mPTEN (**Fig. 6B**, *$P < 0.05$, $^{\Delta}P > 0.05$), suggesting that PTEN is the direct target of miR-26b. To further investigate whether PTEN was modulated by miR-26b in NSCLC cells, we transfected H1299 cells and A549 cells with 200 nM miR-C or miR-26b-mimic. Forty-eight hours after transfection, western blot analysis showed that PTEN protein levels were significantly down-regulated by miR-26b up-regulation in both H1299 cells (**Fig. 6C**) and A549 cells (**Fig. 6D**).

PTEN over-expression reverses the effects of miR-26b up-regulation in NSCLC cells

Finally, we wondered whether PTEN was directly involved in the miR-26b regulation in NSCLC cells. To test this hypothesis, PTEN sequence was cloned into a eukaryotic expression vector pcDNA3.1 (pcDNA-PTEN) to endogenously up-regulate PTEN proteins in NSCLC cells. The western blot analysis showed that, after miR-26b over-expression, the endogenous PTEN proteins were significantly up-regulated in both H1299 cells and A549 cells transfected with pcDNA-PTEN, when compared with the cells transfected with a non-specific control vector of pcDNA-C (**Fig. 7A,B**). At 24 h after

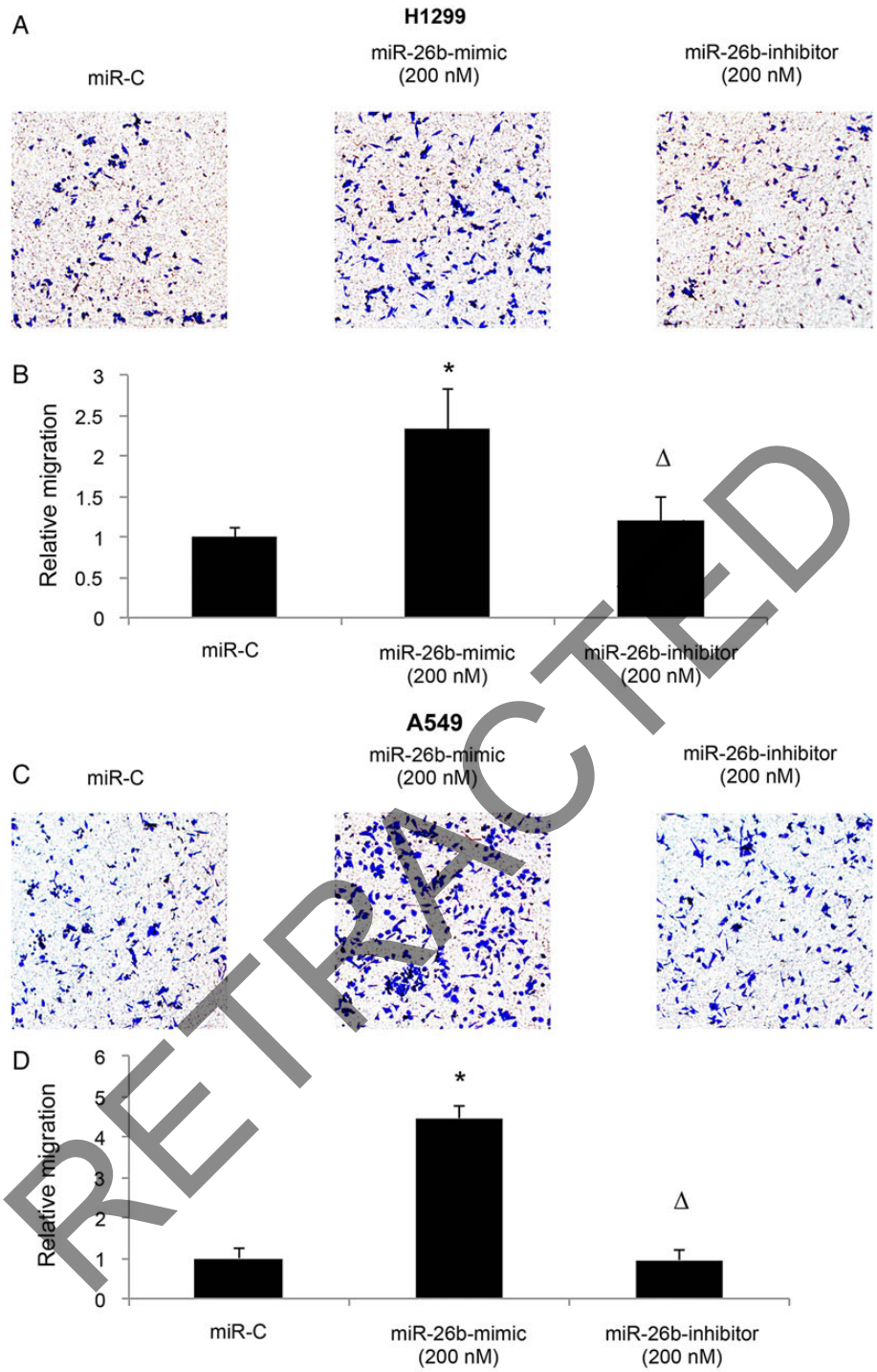

Figure 4. Over-expression of miR-26b increases migration of NSCLC cells (A) H1299 cells were transfected with 200 nM of miR-26b-mimic, miR-26b-inhibitor, or miR-C. Forty-eight hours later, migration assay was performed and representative photographs were shown for stained migrated cells. (B) The numbers of migrated H1299 cells were counted for each transfection conditions and normalized to the number under control (miR-C) condition (*$P < 0.05$; $^{\Delta}P > 0.05$). (C) A549 cells were transfected with 200 nM of miR-26b-mimic, miR-26b-inhibitor, or miR-C. Forty-eight hours later, migration assay was performed and representative photographs were shown for stained migrated cells. (D) The numbers of migrated A549 cells were counted for each transfection conditions and normalized to the number under control (miR-C) condition (*$P < 0.05$; $^{\Delta}P > 0.05$).

transfecting H1299 and A549 cells with 200 nM miR-26b-mimic, either pcDNA-PTEN or pcDNA-C was further transfected into the cells. Forty-eight hours later, a migration assay was performed. The results demonstrated that ectopic PTEN over-expression reversed the enhanced migration induced by miR-26b up-regulation in both H1299 cells (**Fig. 7**C, *$P < 0.05$) and A549 cells (**Fig. 7**D, *$P < 0.05$). Seventy-two hours after PTEN up-regulation, cisplatin chemosensitivity assays were performed. The results showed that PTEN

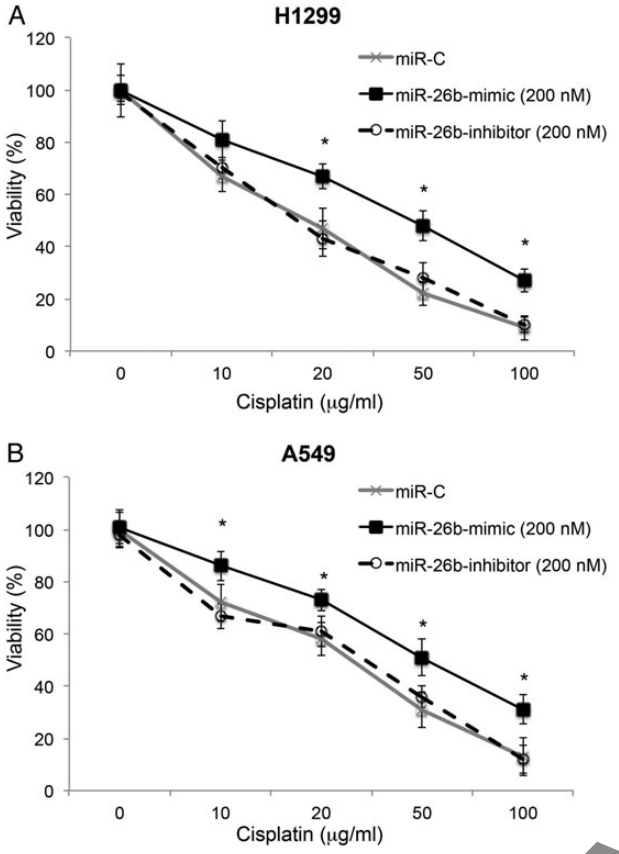

Figure 5. Over-expression of miR-26b decreases cisplatin chemosensitivity of NSCLC cells (A) H1299 cells and (B) A549 cells were transfected with 200 nM of miR-26b-mimic, miR-26b-inhibitor, or miR-C. Twenty-four hours after transfection, different concentrations of cisplatin (0, 10, 20, 50, and 100 μg/ml) were added into the culture and a proliferation assay was conducted at 72 h to compare cisplatin chemosensitivity among different transfection conditions (*$P < 0.05$ compared with miR-C).

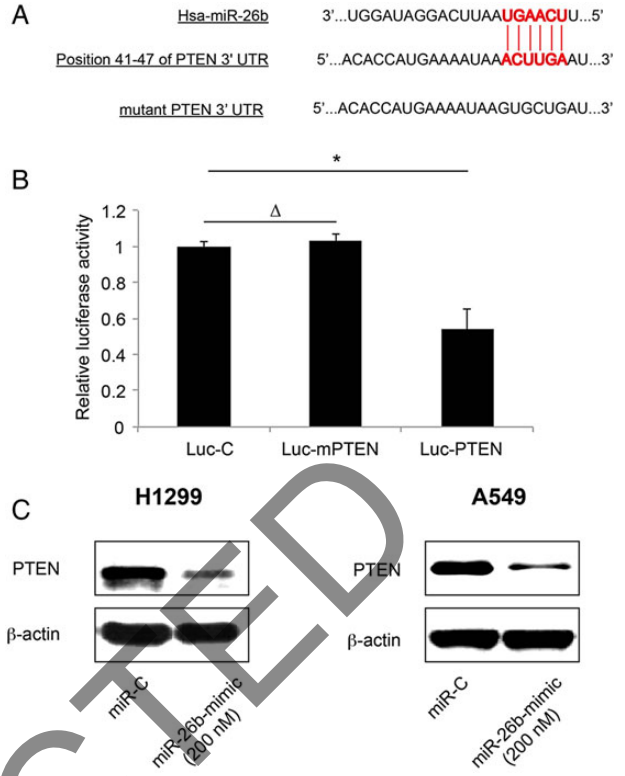

Figure 6. PTEN is the target of miR-26b in NSCLC cells (A) The diagram is shown for the predicted binding of miR-26b with PTEN 3′-UTR. A mutant PTEN 3′-UTR sequence at binding site was also shown. (B) Wild-type PTEN 3′-UTR and mutant PTEN 3′-UTR were inserted into luciferase reporter vector pmiR-REPORT to make the wild-type luciferase vector Luc-PTEN and mutant luciferase vector Luc-mPTEN. A blank control luciferase vector (Luc-C) was also made. In dual-luciferase reporter assays, HEK293T cells were then co-transfected with β-galactosidase, miR-26b-mimics, and one of the three luciferase vectors, Luc-C, Luc-mPTEN, or luc-PTEN, for 24 h, followed by measuring the relative luciferase activities (*$P < 0.05$; $^{\Delta}P > 0.05$). (C) H1299 cells and (D) A549 cells were transfected with either 200 nM miR-C or miR-26b-mimic for 48 h, followed by western blot analysis of the expression of PTEN.

over-expression reversed chemoresistance at cisplatin concentrations of 20 and 50 μg/ml in H1299 cells (**Fig. 7E**, *$P < 0.05$), as well as at cisplatin concentrations of 10, 20 and 50 μg/ml in A549 cells (**Fig. 7F**, *$P < 0.05$).

Discussions

In the present study, we examined the mRNA expression levels of miR-26b in four NSCLC cell lines, and paired tumor/non-tumor clinical specimens from 10 patients with NSCLC. It was demonstrated that miR-26b was significantly down-regulated in NSCLC cell lines compared with that in normal lung fibroblast cell line MRC5, and markedly under-expressed in lung carcinomas, when compared with that in adjacent non-carcinomas in patients with NSCLC. Our results confirmed the data from an original report demonstrating that miR-26b was under-expressed in NSCLC [19]. However, in that study, the functional role of miR-26b was not disclosed [19]. In another report, it was shown that miR-26b was up-regulated, and over-expressing miR-26b inhibited ATF2 expression in gamma-irradiated H1299 cells [25]. But it was not clear whether miR-26b could directly regulate NSCLC development.

Therefore, in the present study, we presented the functional mechanisms of miR-26b in regulating NSCLC migration and cisplatin chemosensitivity for the first time. It was demonstrated that miR-26b up-regulation in H1299 and A549 cells increased cancer cell migration and reduced cisplatin chemosensitivity. On the other hand, we showed that miR-26b down-regulation played no functional roles in NSCLC. These results are in agreement with a previous study showing that down-regulating miR-26b in pituitary tumor cells inhibited cancer growth [18], suggesting that miR-26b acts as an oncogene in both NSCLC and pituitary cancer. Interestingly, while miR-26b is down-regulated in breast cancer as it is in NSCLC, it decreases viability and induces apoptosis in breast cancer [17], thus acting as a tumor suppressor gene, contradictory to the oncogenic role of miR-26b in NSCLC. The possible explanation for its controversial effects may be that the complex underlying signaling pathways determine whether miR-26b acts as an oncogene or tumor suppressor gene in different forms of cancers.

Also in the present study, we demonstrated that PTEN was the direct target of miR-26b, and the expression of PTEN was down-regulated by miR-26b up-regulation in NSCLC cell lines H1299 and A549 cells. As a tumor suppressor gene, PTEN is known to be down-regulated in NSCLC [21], and its down-regulation in NSCLC is associated with increased lung cancer growth and invasion [24]. Thus, the results of our study showing that ectopic up-regulation of PTEN in

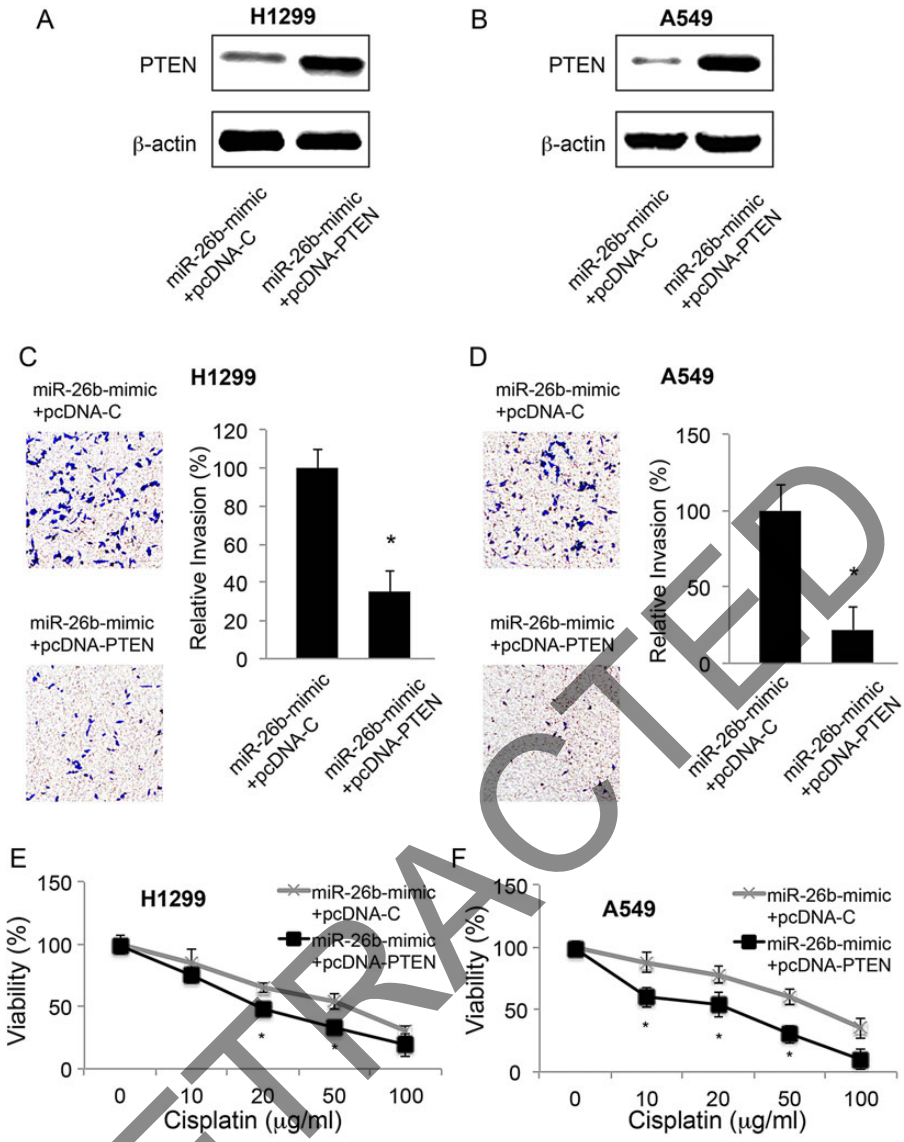

Figure 7. Over-expression of PTEN reverses the effects of miR-26b up-regulation on NSCLC cells H1299 and A549 cells were transfected with miR-26-mimic for 24 h, followed by second transfection with eukaryotic expression vector pcDNA-PTEN to endogenously up-regulate PTEN in NSCLC cells. A non-specific expression vector (pcDNA-C) was also included in the experiment as the parallel control. Forty-eight hours after second transfection, western blot assay was conducted to assess PTEN protein in H1299 cells (A) and A549 cells (B). Migration assay was conducted at 48 h after PTEN over-expression in H1299 cells (C) and A549 cells (D) (*$P < 0.05$). Cisplatin chemosensitivity assay was also performed at 72 h after PTEN over-expression in H1299 cells (E) and A549 cells (F) (*$P < 0.05$).

NSCLC cells reverses the proliferative effects of miR-26b up-regulation on cancer migration and cisplatin chemosensitivity further confirms that miR-26b is an oncogenic factor in NSCLC.

In conclusion, we demonstrated a novel mechanism of miR-26b in regulating NSCLC *in vitro*. Over-expression of miR-26b promotes cancer cell migration and reduces cisplatin chemosensitivity in NSCLCs, potentially through suppressing the PTEN signaling pathway. Our findings on functional roles of miR-26b as well as its downstream signaling pathway might help to develop clinical therapies to treat patients with NSCLC.

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