

MicroRNA-28 promotes the proliferation of non-small-cell lung cancer cells by targeting PTEN

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Abstract. Non-small-cell lung cancer (NSCLC) is the fundamental form of lung cancer and the leading cause of cancer-related mortality in humans. Numerous studies have identified a role for microRNAs (miRs) in cell proliferation, invasion and metastasis in numerous types of cancer, including lung cancer. In the present study, the functional roles and molecular mechanisms of miR-28 in NSCLC tumorigenesis were investigated. Reverse transcription-quantitative PCR (RT-qPCR) was used to measure miR-28 expression levels in NSCLC tumor tissues and cell lines. A dual-luciferase assay was performed to observe the direct interaction between miR-28 and PTEN in A549 cells. Furthermore, the effect of miR-28 on the mRNA and protein expression levels of PTEN was examined by RT-qPCR and western blotting, respectively. A Cell Counting kit-8 assay was performed to identify the relationship between the miR-28/PTEN axis and tumor cell proliferation using cells infected with lentivirus (LV)-anti-miR-28 or LV-anti-miR-28 + short hairpin RNA-PTEN. miR-28 expression was upregulated in NSCLC tumor tissues and cell lines compared with the control groups. PTEN was identified as the downstream gene of miR-28 in NSCLC and was negatively regulated by miR-28. In addition, miR-28 knockdown suppressed the proliferation of A549 and H292 cells. Cells infected with LV-anti-miR-28 + short hairpin RNA-PTEN promoted tumor cell proliferation in A549 and H292 cells compared with cells infected with LV-anti-miR-28. Taken together, the present study suggested that miR-28 might serve as the promoter in the development of NSCLC by

targeting PTEN. Therefore, the miR-28/PTEN axis may serve as a potential diagnostic and therapeutic target for NSCLC.

Introduction

Lung cancer is a malignant carcinoma, and a leading cause of morbidity and mortality worldwide (1,2). The disease can be divided into two main types, according to clinicopathological features, namely, small-cell lung cancer and non-small-cell lung cancer (NSCLC) (3). NSCLC accounts for 80-90% of all cases of lung cancer (4,5). Although the diagnostic and clinical therapeutic targets of NSCLC have significantly improved in the last decade, the high recurrence rate and poor 5-year survival rate remain (6). Thus, the molecular mechanism underlying NSCLC tumorigenesis requires further investigation.

Previous studies have confirmed the involvement of microRNAs (miRNAs/miRs) in the development of NSCLC (4,7). miRNAs are a series of short (~22 nucleotides), single-stranded, non-coding RNAs, which are mainly transcribed from introns or exons of protein-coding genes (8). miRNAs are involved in the post-transcriptional regulation of gene expression in multicellular organisms; they regulate genes by binding to the 3'-untranslated regions (3'-UTRs) of target mRNAs, thus decreasing the level of oncoprotein translation (5,9). Previous studies have reported that miRNA dysregulation occurs in numerous types of human carcinoma, and that miRNAs are involved in tumor cell proliferation, apoptosis and metastasis (10,11). According to the functional roles of downstream genes, miRNAs can act as oncogenes or tumor suppressors in different types of human cancer, including NSCLC (12,13). For example, aberrant miR-28 expression has been detected in gastric cancer (14), ovarian cancer (15), B-cell lymphoma (16), colorectal cancer (17), hepatocellular carcinoma (18) and renal cell carcinoma (19). However, the detailed molecular mechanism of miR-28 in NSCLC has not been fully elucidated.

The present study aimed to identify the expression levels, functional roles and molecular mechanisms of miR-28, and to investigate the target genes associated with tumor cell proliferation, in NSCLC progression. The present study suggested that miR-28 acted as a promoter in the process of NSCLC cell proliferation by targeting PTEN. Furthermore, it was proposed that the miR-28/PTEN axis may serve as a novel clinical therapeutic target for NSCLC.

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Materials and methods

NSCLC tissues and cell lines. Tumor tissues and matched adjacent non-tumor tissues (<3 cm from the tumor margin) were harvested from 33 patients with NSCLC (21 men and 12 women; mean age, 48.9 years; age range, 35–72 years) at Jingzhou Central Hospital between October 2015 and December 2018. Regarding TNM staging (20), 15 (45.45%) patients were classified as TNM stage I or II, whereas 18 (54.55%) patients were classified as TNM stage III or IV. Total tissues were stored at -80°C for tissue repository establishment. The present study was approved by the Ethical Committee of the Jingzhou Central Hospital. Written informed consent was obtained from all patients.

The cell lines, including BEAS-2b, A549, H1650, H292, H1944 and H1299, were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences. All cell lines were cultured using RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin at 37°C with 5% CO₂.

Prediction of target genes of miR-28. TargetScan (version 3.1; www.targetscan.org/mamm_31) software was used to determine the candidate downstream target genes of miR-28.

Reverse transcription-quantitative PCR (RT-qPCR). The NSCLC tumor and matched adjacent normal tissues (100 mg) were ground in liquid nitrogen and the resulting cells underwent RNA extraction. The aforementioned cell lines were also used for RT-qPCR. Total RNA was extracted from the cells using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (cat. no. 1708890; Bio-Rad Laboratories, Inc.), according to the manufacturer's protocol. Subsequently, TaqMan[®] MicroRNA assays (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used to detect the expression of miR-28 (miR-28-5p, forward, 5'-CGGATCCAGGCCCTTCAAGGACTTTCT-3' and reverse, 5'-CGAATTCACAGAGCTCCTGCTGTGTCA-3'), according to the manufacturer's protocol. miR-28 expression levels were normalized to the internal reference gene U6 (forward, 5'-GCTTCGGCAGCATATACTAAAT-3' and reverse, 5'-CGCTTCACGAATTGCGTGTCTAT-3'). SYBR Premix Ex Taq II (Takara Bio, Inc.) was used to determine PTEN mRNA expression levels. The following primer pairs were used for qPCR: PTEN forward, 5'-TGGATTGACTTAGACTTGACCT-3' and reverse, 5'-GGTGGGTTATGGTCTTCAAAGG-3'; and GAPDH forward, 5'-GACTCATGACCACAGTCCATGC-3' and reverse, 5'-AGAGGCAGGGATGATGTTCTG-3'. PTEN mRNA expression levels were normalized to the internal reference gene GAPDH. PCR was performed using the 7500 Realtime PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following thermocycling conditions: 12 min at 96°C; followed by 35 cycles of 15 sec at 96°C and 1 min at 58°C; and a final extension at 72°C for 5 min. Relative expression levels were quantified using the 2^{-ΔΔC_q} method (21).

Cell transfection and infection. The miR-28 mimic (mimic-miR-28; GAGUUAUCUGACACUCGAGGAA)

and its negative control (mimic-NC; UUCUCCGAACGU GUCACGU) were purchased from Shanghai GenePharma Co., Ltd. A549 cells (2x10⁵ cells/well) were transfected with mimic-miR-28 or mimic-NC (50 nmol/l) to investigate the interaction between miR-28 and PTEN. Lentivirus (LV)-short hairpin RNA (sh)-PTEN (CCACAGCUAGAACUUAUCA AA), LV-anti-miR-28 (CUCAAUAGACUGUGAGCUCCU U), LV-anti-miR-28 + sh-PTEN or the LV-NC (UUCUCC GAACGUGUCACGU) were infected (MOI=3) into A549 and H292 cells (2x10⁵ cells/well). The lentiviruses used in the present study were purchased from Shanghai GenePharma Co., Ltd. Mimic transfection was performed using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following incubation for 6 h at 37°C, the cell culture medium was replaced with fresh medium. At 24 h post-transfection, cells were used for subsequent experiments.

Luciferase activity assay. The luciferase reporter plasmid pGL3 (Shanghai GenePharma Co., Ltd.) was used to perform the luciferase activity assay. *Renilla* luciferase was used as the normalization control. A549 cells (5x10⁵ cells/well) were seeded into a 6-well plate. The sequences of the wild-type (WT) PTEN 3'-UTR (UCCCAAGUCCUUUGUAGCUCCUC) and the mutant (MUT) PTEN 3'-UTR (UCCCAAGUCCUUUGU UCGAGGAC) were obtained from Shanghai GenePharma Co., Ltd. and were cloned into the pGL3 vector. Subsequently, A549 cells were transfected with pGL3-WT-PTEN-3'-UTR or pGL3-WUT-PTEN-3'-UTR (1 μg/ml) using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated for 6 h at 37°C. A549 cells were also transfected with mimic-miR-28 or mimic-NC (50 nmol/l) using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated for 6 h at 37°C. Subsequently, the cell culture medium was replaced with fresh medium. After 48 h, the Dual-Luciferase Reporter assay system (Promega Corporation) was used to measure the luciferase activity, according to the manufacturer's instructions.

Western blotting. Total protein was extracted from A549 and H292 cells using ~1 ml RIPA buffer (Beyotime Institute of Biotechnology). Total protein was quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Protein (25 μg) was separated by SDS-PAGE using 10% gels and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk at room temperature for 2 h. Subsequently, the membranes were incubated with primary antibodies against PTEN (cat. no. 60300-1-Ig; 1:1,000; Proteintech Group, Inc.) and GAPDH (cat. no. 60004-1-Ig; 1:20,000; Proteintech Group, Inc.) overnight at 4°C. The membranes were then washed with TBS-0.5% Tween 20 and incubated with a horseradish peroxidase-conjugated secondary antibody (cat. no. ab205719; 1:20,000; Abcam) for 2 h at room temperature. Protein bands were visualized using an ECL kit (EMD Millipore) and were imaged using a Bioshine ChemiQ 4600 Mini Chemiluminescence Imaging system (Ouxiang). Protein expression was quantified using Image J software (version 1.49; National Institutes of Health).

Cell Counting Kit-8 (CCK-8) assay. The CCK-8 assay was used to determine the proliferation of A549 and H292 cells

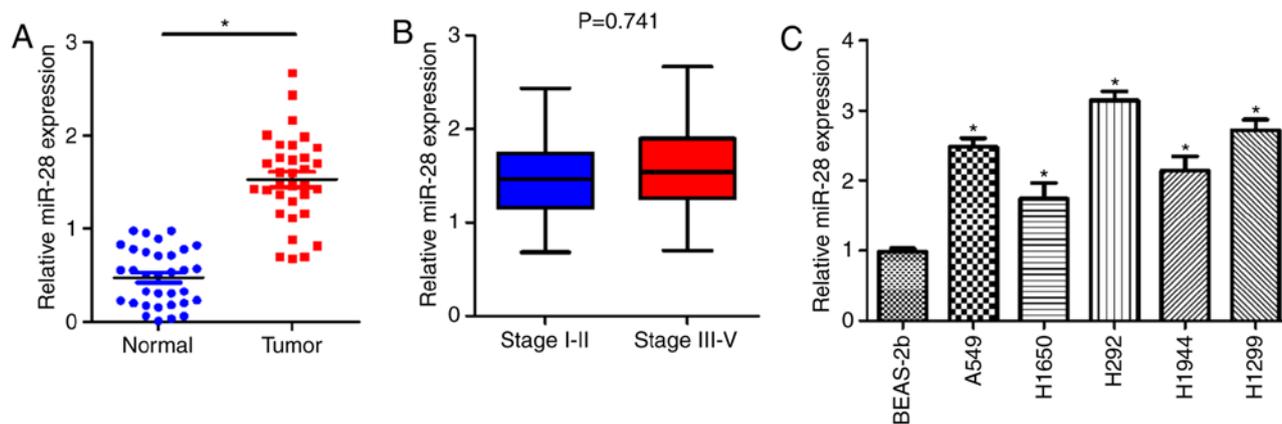


Figure 1. miR-28 expression is upregulated in NSCLC tumor tissues and cell lines. (A) Expression levels of miR-28 were measured in tumor tissues and paired adjacent non-tumor tissues of 33 patients with NSCLC. * $P < 0.05$. (B) Expression levels of miR-28 in NSCLC tumor tissues at different TNM stages. (C) Expression levels of miR-28 in a series of NSCLC cell lines, including A549, H1650, H292, H1944 and H1299. The BEAS-2b cell line represents normal human pulmonary epithelial cells and acted as the control group. * $P < 0.05$ vs. the BEAS-2b group. All experiments were performed in triplicate. miR, microRNA; NSCLC, non-small-cell lung cancer.

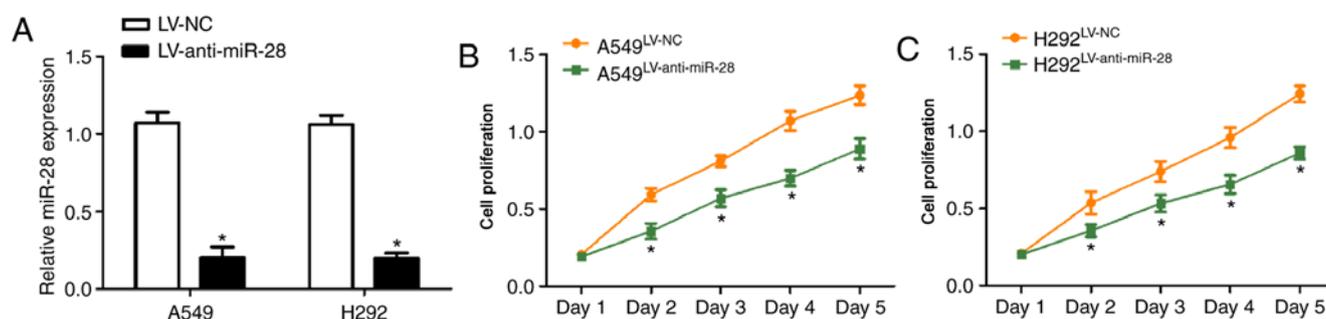


Figure 2. miR-28 knockdown reduces proliferation of A549 and H292 cells. (A) Expression levels of miR-28 in A549 and H292 cells infected with LV-anti-miR-28 or LV-NC. miR-28 expression levels were decreased following infection with LV-anti-miR-28. Cell proliferation was measured by a Cell Counting kit-8 assay in (B) A549 and (C) H292 cells infected with LV-anti-miR-28 or LV-NC, every 24 h for 5 days. All experiments were performed in triplicate. * $P < 0.05$. LV, lentivirus; miR, microRNA; NC, negative control.

(5×10^3 cells/well). The CCK-8 reagent (10 μ l; APEX BIO Technology) was added to each well and subsequently, the cells were counted every 24 h for 5 days using a Fluoroskan Ascent microplate fluorometer (Thermo Fisher Scientific, Inc.). The optical density at a wavelength of 450 nm was measured. After 5 days, the growth curve was generated.

Statistical analysis. All experiments were performed in triplicate and repeated three times. The data are presented as the mean \pm SD. A paired Student's t-test was used for the comparison of miR-28 expression between NSCLC tumor and matched adjacent normal tissues. One-way ANOVA followed by Tukey's post hoc test was used to measure the differences between quantitative variables. $P < 0.05$ was considered to indicate a statistically significant difference. GraphPad Prism (version 7.0; GraphPad Software, Inc.) and R studio (version 3.5.1; www.r-project.org) software were used to perform the statistical analyses.

Results

Upregulation of miR-28 expression in NSCLC tissues and cell lines. To investigate whether miR-28 was associated with

NSCLC development, RT-qPCR was performed to measure the expression levels of miR-28 in NSCLC tumor tissues and cell lines. miR-28 expression was significantly upregulated in NSCLC tissues compared with matched adjacent non-tumor tissues (Fig. 1A). There was no significant difference between the expression levels of miR-28 in early (1.497 in stage I/II) and late TNM stage tumors (1.553 in stage III/IV; $P > 0.05$; Fig. 1B). The expression levels of miR-28 in A549, H1650, H292, H1944 and H1299 cell lines were significantly higher than in the BEAS-2b cell line (Fig. 1C), which is a normal lung bronchus epithelial cell line and acted as the control group. The results suggested that miR-28 was not only involved in the progression of NSCLC, but was also upregulated in NSCLC tumor tissues and cell lines.

miR-28 knockdown reduces proliferation of A549 and H292 cells. To determine the biological functions of miR-28 in the development of NSCLC, A549 and H292 cells were infected with LV-anti-miR-28. miR-28 expression levels were significantly decreased in both cell lines following infection with LV-anti-miR-28 compared with those infected with LV-NC (Fig. 2A), indicating that the infection was successful. The cell proliferation curves generated by performing the

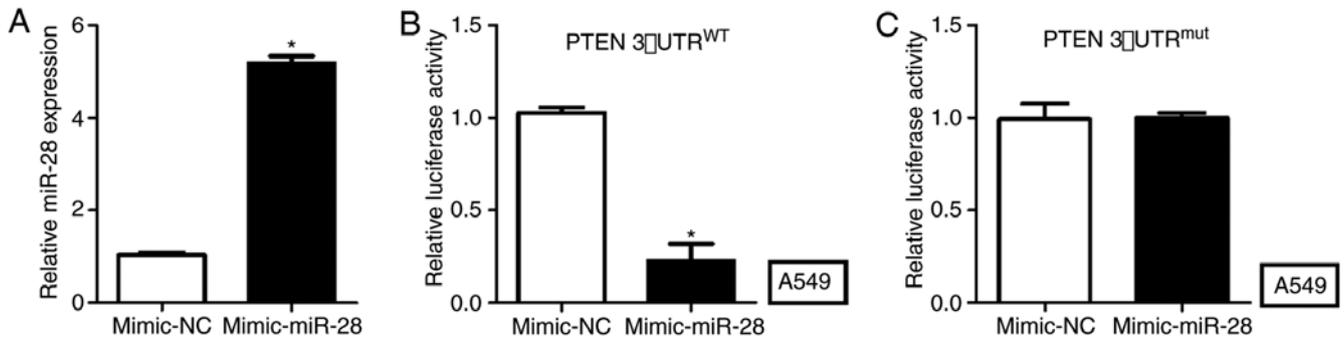


Figure 3. PTEN is the direct target gene of miR-28. (A) Expression levels of miR-28 in A549 cells transfected with mimic-miR-28 or mimic-NC. A dual-luciferase assay was performed to detect the relative luciferase activity in A549 cells co-transfected with mimic-miR-28 or mimic-NC and (B) WT-PTEN 3'-UTR or (C) MUT-PTEN 3'-UTR. *P<0.05 vs. mimic-NC. 3'-UTR, 3'-untranslated region; miR, microRNA; MUT, mutant; NC, negative control; WT, wild-type.

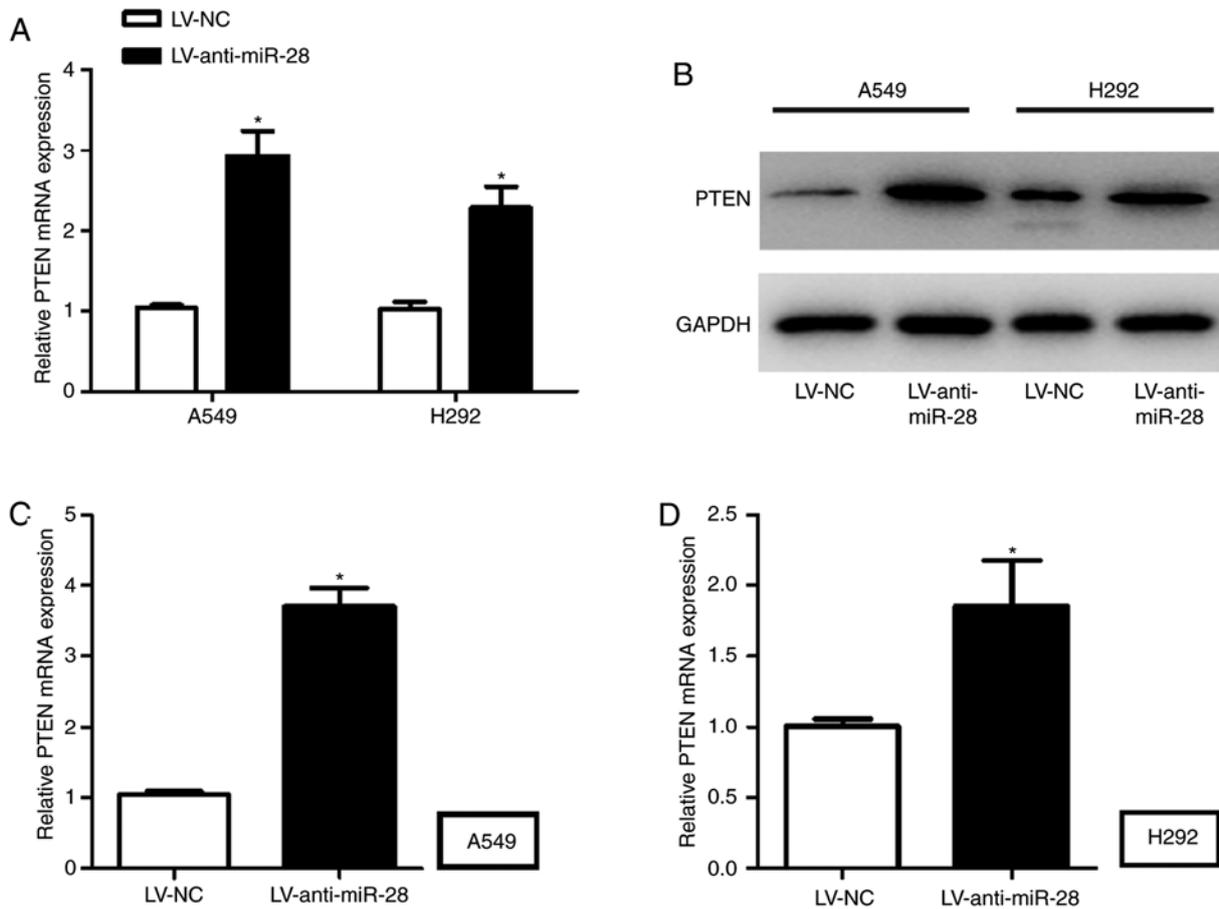


Figure 4. PTEN is negatively regulated by miR-28. (A) mRNA expression levels of PTEN in A549 and H292 cells infected with LV-anti-miR-28 or LV-NC. (B) Protein expression levels of PTEN in A549 and H292 cells infected with LV-anti-miR-28 determined by western blot analysis. Semi-quantification of PTEN protein expression in (C) A459 and (D) H292 cells infected with LV-anti-miR-28 or LV-NC. All experiments were performed in triplicate. *P<0.05. LV, lentivirus; miR, microRNA; NC, negative control.

CCK-8 assay suggested that miR-28 knockdown inhibited proliferation of A549 and H292 cells compared with the LV-NC group (Fig. 2B and C). The results indicated that miR-28 might act as a promoter in NSCLC progression.

PTEN is the direct target gene of miR-28 and is negatively regulated by miR-28. PTEN was predicted as the downstream gene of miR-28 using TargetScan (www.targetscan.org). To verify whether miR-28 directly targeted PTEN in NSCLC, a

dual-luciferase activity assay was performed in A549 cells. miR-28 expression levels were significantly increased in A549 cells transfected with mimic-miR-28 compared with those transfected with mimic-NC (Fig. 3A). The luciferase activity in A549 cells co-transfected with WT-PTEN-3'-UTR and mimic-miR-28 was significantly decreased compared with the mimic-NC group (Fig. 3B). The luciferase activity of A549 cells transfected with MUT-PTEN-3'-UTR was not significantly altered between cells co-transfected with mimic-miR-28 or mimic-NC (Fig. 3C).

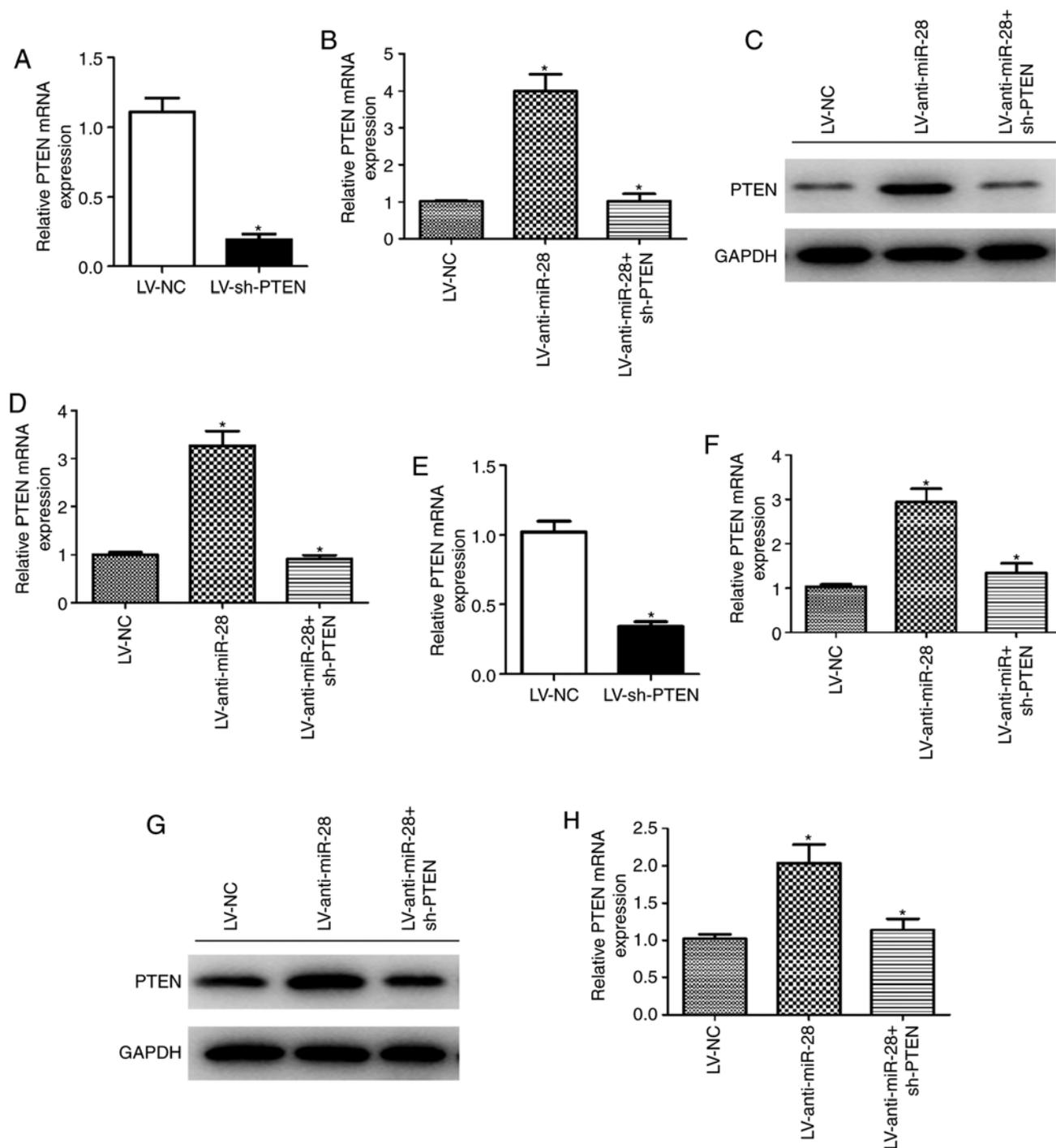


Figure 5. PTEN knockdown reverses the effect of miR-28 on PTEN expression in A549 and H292 cells. mRNA expression levels of PTEN in A549 cells infected with (A) LV-NC or LV-sh-PTEN, and (B) LV-anti-miR-28, LV-anti-miR-28 + sh-PTEN or LV-NC. Protein expression levels of PTEN in A549 cells infected with LV-anti-miR-28, LV-anti-miR-28 + sh-PTEN or LV-NC were (C) determined by western blot analysis and (D) semi-quantified. (E) mRNA expression levels of PTEN in H292 cells infected with (E) LV-NC or LV-sh-PTEN, and (F) LV-anti-miR-28, LV-anti-miR-28 + sh-PTEN or LV-NC. Protein expression levels of PTEN in H292 cells infected with LV-anti-miR-28, LV-anti-miR-28 + sh-PTEN or LV-NC (G) determined by western blot analysis and (H) semi-quantified. All experiments were performed in triplicate. * $P < 0.05$ vs. LV-NC. LV, lentivirus; miR, microRNA; NC, negative control; sh, short hairpin RNA.

To explore the direct effect of miR-28 on PTEN, RT-qPCR and western blotting were performed to measure the mRNA and protein expression levels of PTEN in A549 and H292 cells infected with LV-anti-miR-28. A549 and H292 cells infected with LV-anti-miR-28 displayed significantly higher PTEN mRNA and protein expression levels compared with A549 and H292 cells infected with LV-NC (Fig. 4A-D).

Collectively, these results suggested that PTEN may be the downstream gene of miR-28 in NSCLC; therefore, miR-28 knockdown promoted the expression of PTEN.

PTEN knockdown reverses the effect of anti-miR-28 on PTEN expression and cell proliferation in NSCLC. sh-PTEN was used to investigate whether PTEN affected the progression of

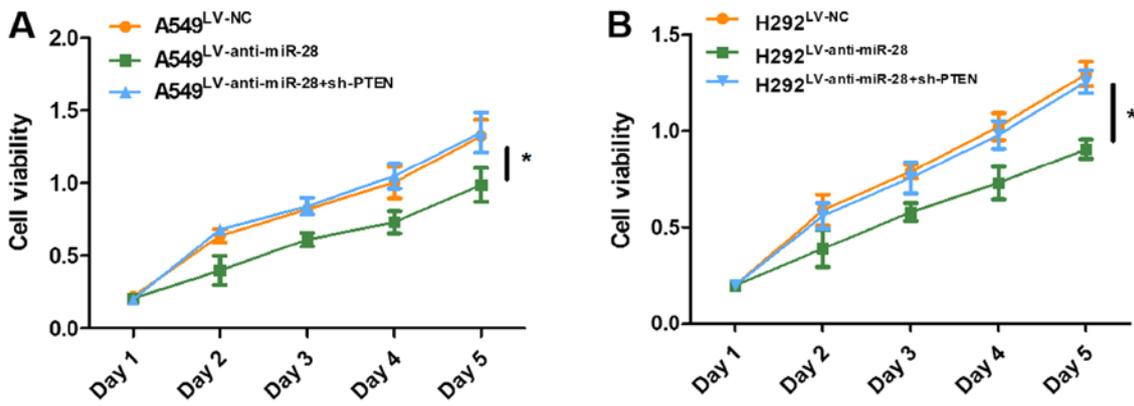


Figure 6. PTEN knockdown reverses the effect of anti-miR-28 on proliferation of A549 and H292 cells. Proliferation of (A) A549 and (B) H292 cells infected with LV-anti-miR-28, LV-anti-miR-28 + sh-PTEN or LV-NC; proliferation was detected every 24 h for 5 days. All experiments were performed in triplicate. * $P < 0.05$. LV, lentivirus; miR, microRNA; NC, negative control; sh, short hairpin RNA.

NSCLC. PTEN expression levels were significantly decreased in A549 and H292 cells infected with LV-sh-PTEN compared with those infected with LV-NC (Fig. 5A and E). The mRNA and protein expression levels of PTEN in A549 and H292 cells co-infected with LV-anti-miR-28 + sh-PTEN were significantly lower compared with cells infected with LV-anti-miR-28 (Fig. 5B-D and F-H). The CCK-8 assay suggested that A549 and H292 cells co-infected with LV-anti-miR-28 + sh-PTEN displayed higher levels of cell proliferation compared with A549 and H292 cells infected with LV-anti-miR-28 (Fig. 6). The results indicated that PTEN knockdown reversed the inhibitory effect of LV-anti-miR-28 on cell proliferation. Furthermore, the results suggested that miR-28 might play a role as an oncogene in NSCLC by targeting PTEN.

Discussion

In the present study, the underlying functional roles of miR-28 in NSCLC tumorigenesis were investigated. miR-28 expression levels were significantly upregulated in NSCLC tumor tissues and cell lines compared with the matched adjacent non-tumor tissues and the control cell line, respectively. Additionally, PTEN was identified as the downstream gene of miR-28 during NSCLC development. miR-28 knockdown increased PTEN expression levels and reduced tumor cell proliferation *in vitro*. PTEN knockdown reduced the effect of miR-28 on NSCLC tumor cell proliferation. Overall, it could be suggested that miR-28 acted as a promoter in NSCLC by targeting PTEN. Therefore, the miR-28/PTEN axis may serve as a potential clinical target for NSCLC diagnosis, treatment and prognosis.

miR-28, which is located at chromosome 3q28, has two main subtypes, miR-28-3p and miR-28-5p (22). In previous studies, miR-28 and its two subtypes have been reported to be aberrantly expressed by certain pathological mechanisms. Zhou *et al* (23) reported that miR-28-3p expression levels were increased in the plasma of patients with pulmonary embolism. With regards to lymphocytic leukemia, miR-28-5p expression was significantly increased in patients compared with healthy controls (24). Platelet miR-28 expression was also identified as being upregulated in patients with myeloproliferative neoplasm (25). The aforementioned results indicated that

miR-28 upregulation commonly occurs in diseases, which is consistent with the findings of the present study.

miR-28 can act as an oncogene or a tumor suppressor in various types of malignant carcinoma. Schneider *et al* (16) reported that overexpression of miR-28 inhibited cell proliferation in B-cell lymphoma. Xu *et al* (15) demonstrated that miR-28-5p induced the proliferation of ovarian cancer cells, as well as their migration and invasion, by targeting NEDD4-binding protein 1. Wu *et al* (17) reported that miR-28-5p had a suppressive effect on colorectal cancer progression by interacting with the downstream gene structure specific recognition protein 1. However, another similar study suggested that miR-28-3p acted as a tumor promoter in colorectal cancer cell migration and invasion (22). Therefore, the aforementioned studies illustrate that the effects of miR-28 are not always identical in different types of cancer and that the different subtypes of miR-28 may have opposite functions in the same cancer. The present study suggested that miR-28 served as an oncogene in NSCLC cell proliferation, which is supported by Wang *et al* (26) who reported that miR-28 is one of the potential oncogenes in lung cancer.

PTEN, located at chromosome 10q23.31, has been identified as a tumor suppressor gene via the PI3K/AKT pathway in a number of different forms of cancer (27), including renal cancer (28), gastric cancer (29), endometrial cancer (30), breast cancer (31) and malignant melanoma (32). Previously, emerging miRNAs have been identified as promoters of tumor cell growth, metastasis or apoptosis by targeting PTEN, including miR-1297 (33), miR-200 (34), miR-130a (35), miR-26a (36) and miR-17 (37). In the present study, PTEN was also identified as the target gene of miR-28. Additionally, the oncogenic role of miR-28 in NSCLC proposed in the present study was the same as that of miRNAs reported previously (33-37). These results suggested that miR-28 directly targeted PTEN and may promote tumorigenesis in NSCLC.

A previous study investigated the interaction of the miR-28/PTEN axis in carcinoma. Li *et al* (14) reported that miR-28 acts as an oncogene in gastric cancer growth and invasion by targeting PTEN, via the PI3K/AKT signaling pathway, which strengthened the present findings. Moreover, the function of the miR-28/PTEN axis in other types of human cancer requires further investigation and could identify additional biomarkers for cancer research.

In conclusion, the present study suggested that miR-28 was upregulated in NSCLC tumor tissues and cell lines. Moreover, miR-28 promoted NSCLC cell proliferation by targeting PTEN, which serves as a suppressor of pathogenesis in numerous diseases (27). The present study identified a potential biomarker for the clinical diagnosis, treatment and prognosis of NSCLC. Further studies investigating whether there are other target genes of miR-28 in NSCLC and whether the miR-28/PTEN axis functions in other types of cancer are required.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HQ conceived and designed the present study. FC and QZ performed the experiments and analyzed the data. FC, QZ and KX helped to design the study and interpreted the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of the Jingzhou Central Hospital. Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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