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MiR-26a enhances metastasis potential of lung cancer cells via AKT pathway by targeting PTEN

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ABSTRACT

Lung cancer is the leading cause of cancer related death, 90% of lung cancer patients die of metastasis. Many microRNAs (miRNAs) are deregulated in cancer. They are involved in tumorigenesis and function as oncogenes or tumor suppressor genes. Recent studies show that miRNAs may be responsible for tumor metastasis. Several functional studies show that miR-26a plays an important role in carcinogenesis; however, none of these studies is related to tumor metastasis. In the present study, we investigated the effect of miR-26a on metastasis potential of lung cancer cells. Our data showed that miR-26a expression level was higher in lymph node metastasis tumor tissues than in primary tumor tissues. Ectopic expression of miR-26a dramatically enhanced lung cancer cell migration and invasion abilities. Metastasis-related genes matrix metallopeptidase 2 (MMP-2), vascular endothelial growth factor (VEGF), Twist and β-catenin were upregulated. Phosphatase and tensin homolog (PTEN) was a direct target of miR-26a. Further mechanistic study revealed that miR-26a increased AKT phosphorylation and nuclear factor kappa B (NFκB) transcriptional activation. Our study demonstrated that miR-26a enhanced lung cancer cell metastasis potential via modulation of metastasis-related gene expression, and activation of AKT pathway by PTEN suppression, suggesting that miR-26a might be a potential therapeutic candidate in patients with metastatic lung cancer.

1. Introduction

Lung cancer is the most common cause of death in men and second only to breast cancer in women. It accounts for 12.7% (1.6 million) of the total cancer cases and 18.2% (1.4 million) of the total cancer deaths [1]. The most common form of lung cancer is non-small-cell lung cancer (NSCLC), which comprises approximately 80% of all lung cancers. The overall 5-year survival rate associated with NSCLC is 15%.

About 40% of patients with NSCLC present at an advanced stage, with metastatic or locally advanced disease, and nearly 90% of lung cancer patients die of metastasis [2]. The metastatic cascade is a series of biological processes that enable the movement of tumor cells from the primary site to a distant location and the establishment of a new cancer growth. It includes the following steps: invading the surrounding tissue, entering the microvasculature of the lymph and blood systems (intravasation), surviving and translocating to microvessels of distant tissues, exiting from the bloodstream (extravasation), surviving in the microenvironment of distant tissues, and finally adapting to the foreign microenvironment of these tissues in ways that facilitate cell proliferation and the formation of a secondary tumor (colonization) [3]. However, the mechanism of cancer cell metastasis is still poorly understood.

MicroRNAs (miRNAs) are small non-coding RNAs of 20–22 nucleotides. It represses gene expression through interaction with 3′ untranslated regions (3′-UTRs) of mRNAs. miRNAs are predicted to target over 50% of all human protein-coding genes, enabling them to have numerous regulatory roles in many physiological and developmental processes, including development, differentiation, apoptosis and proliferation, through imperfect pairing with target mRNAs of protein-coding genes and the transcriptional or post-transcriptional regulation of their expression. Many miRNAs are deregulated in cancer. They are involved in tumorigenesis and function as oncogenes or tumor suppressor genes [4,5]. Recent studies show that some miRNAs control tumor cell invasion and metastasis. For instance, miR-10b is highly expressed in metastatic breast cancer cells and positively regulates cell migration and invasion [6]; miR-373 and miR-520c promote breast cancer cell invasion and metastasis by suppression of CD44 [7]; miR-126 inhibit NSCLC cell invasion by targeting Crk [8]. The role of miR-26a in cancer cells seems controversial as it served as an oncogene in glioma and T-cell acute lymphoblastic leukemia but as a tumor suppressor in nasopharyngeal carcinoma, breast cancer and liver cancer cells [9–14]. However, there is no evidence of miR-26a in cancer metastasis been documented so far.

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In this study, we investigated the potential role of miR-26a in lung cancer cell metastasis. We assessed the expression level of miR-26a in human lung cancer specimens and lung cancer cell lines, and examined its effects on cell growth, migration, invasion and expression of metastasis-related genes. Furthermore, we explored the target of miR-26a in lung cancer cells, and the underlining mechanism of its function. This will provide better understanding of lung cancer metastasis.

2. Materials and methods

2.1. Materials

miR-26a mimics, inhibitor and control were purchased from Genepharma (Shanghai, China). Antibodies against PTEN, NFκB p65, AKT, p-AKT, MMP-2, Twist and PCNA were purchased from Cell Signaling (Beverly, MA), antibodies against VEGF, β-catenin, histone H3 and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), secondary antibodies coupled to HRP were purchased from ZSGB-BIO (Beijing, China). Reverse transcription kit and real-time PCR kit were purchased from TaKaRa Biotechnology (Dalian, China). pNFκB-luc and pAP-1-luc were purchased from Clontech (Mountain View, CA). Nuclear extraction kit was purchased from Beyotime Institute of Biotech (Jiangsu, China).

2.2. Cell lines and clinical specimens

Human lung cancer cell lines A549, H661, SK-MES-1 and BEAS-2B human normal lung bronchial epithelium cell line were purchased from American Type Culture Collection (Manassas, VA). H661 cells were grown and maintained in RPMI-1640 medium, A549 and SK-MES-1 cells were grown and maintained in DMEM medium at 37 °C, 5% CO_2. Medium was supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin. BEAS-2B cells were grown and maintained in Keratinocyte Supplementary Free Medium with Supplement (GIBCO BRL, Grand Island, NY).

Lung cancer specimens were obtained from patients at the Tianjin Cancer Hospital. Informed consent was obtained from each patient. The study has been approved by the Hospital's Ethical Review Committee.

2.3. Cell proliferation assay

Cells were seeded at an initial density of 2×10^5 cells/mL in a 6-well plate and incubated with 0–200 nM of miR-26a mimics for 48 h at 37 °C. The cell viability was determined by Vi-CELL Cell Viability Analyzer (BECKMAN COULTER, Brea, CA), following the manufacturer's instruction.

2.4. Wound healing assay

Cell migration was examined by wound healing assay as previously described [15]. Briefly, cells were cultured in six-well plates to 100% confluence. A plastic pipette tip was used to generate a clean wound area across the center of the well. Cell debris was removed by washing with PBS, and cells were allowed to migrate in the medium. The wound was assessed by a microscope (Nikon, Tokyo, Japan) at ×40 magnification at indicated time points. Cells in each field of view were counted by photographing through the microscope, and the average number of cells present in each scrape with each treatment was determined. At least five wound areas were investigated on each plate to quantify the migration.

2.5. Cell invasion assay

The tumor cell invasion activity was assessed by Cell Invasion Kit (CHEMICON INTERNATIONAL Inc., Billerica, MA) as previously described [15]. It was performed in an Invasion Chamber, a 24-well tissue culture plate with cell culture inserts. The inserts contain an 8 μm pore size polycarbonate membrane, over which a thin layer of ECMatrixTM is dried. Cells were suspended to a final concentration of 2×10^5 cells/mL in serum free medium with 0.1% BSA. Cell suspensions (300 μL) were added to the upper compartment, medium collected from NIH3T3 cell culture was added with 0.1% BSA, then added to the lower compartment, and incubated for 48 h at 37 °C in 5% CO_2 atmosphere. Invasive cells on the lower surface of the membrane were stained following the manufacturer's instruction, and counted by photographing the membrane through the microscope (×100 magnification).

2.6. Western blotting analysis

Western blottings were performed as previously described [16]. Briefly, cells were incubated with miR-26a mimics for 48 h, washed with PBS, and the cell pellets were prepared in lysis buffer (20 mM Tris (pH7.5), 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na3VO4, leupeptin). Lysates were electrophoresed on SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with 5% milk protein, 0.1% Tween 20 in PBS, then were probed with rabbit anti-PTEN, Akt, p-Akt antibodies at 1:1000 dilution in 0.1% Tween 20 in PBS with 5% BSA overnight at 4 °C. After washing, the membranes were probed with HRP-conjugated goat anti-rabbit antibody at 1:5000 dilution in 0.1% Tween 20 in PBS with 3% milk protein for 1 h. The blots were developed with the Phototope HRP Western Blot Detection system (Cell Signaling).

2.7. Real-time PCR

Total RNA was extracted from cells using Trizol (Invitrogen, Carlsbad, CA). Reverse transcription was performed as preciously described [17] using TaKaRa kit following manufacturer's instruction.

For gene expression, primers were synthesized by SBS Genetech (Beijing, China). SYBR Green was used to quantify mRNA levels. All real-time PCR reagents were purchased from TaKaRa Biotechnology Co. PCR reactions were performed as preciously described [17] in the ABI Prism 7500 Sequence Detector System (ABI, Foster City, CA). The primers for MMP-2, VEGF, Twist, β-catenin and GAPDH were listed in Table 1. The expression of miR-26a was detected using Hairpin-it miRNAs qPCR Quantitation kit (GenePharma), following manufacturer's instruction. U6 was used for normalization.

2.8. Construction of expression plasmid

The following primers were used to amplify and clone the 3′-UTR of human *PTEN* into the pGL3 luciferase vector (Promega) according to manufacturer's instruction. For predicted binding site one: forward 5′-GCTCTAGACAATCATAATACCTGCTGTGGA-3′, reverse 5′-CTAGCTAGC TAAAGCACATGTAGGACAATTTC-3′. For predicted binding site two:

Table 1
PCR primer sequences.

Primers		Sequence (5′–3′)	Length of amplicons (bp)
MMP-2	Forward	GCGGCGGTCACAGCTACTT	71
	Reverse	CACGCTCTTCAGACTTTGGTTCT	
VEGF	Forward	AGGAGGAGGGCAGAATCATCA	76
	Reverse	CTCGATTGGATGGCAGTAGCT	
Twist	Forward	GCCAATCAGCCACTGAAAGG	83
	Reverse	TGTTCTTATAGTTCCTCTGATTGTTACCA	
β-Catenin	Forward	GCTGGGACCTTGCATAACCTT	86
	Reverse	ATTTTCACCAGGGCAGGAATG	
GAPDH	Forward	TGCACCACCAACTGCTTAGC	87
	Reverse	GGCATGGACTGTGGTCATGAG	

MMP-2, matrix metallopeptidase 2; *VEGF*, vascular endothelial growth factor; *GAPDH*, Glyceraldehyde-3-phoshate dehydrogenase.

forward 5′-GCTCTAGACCATCCCCATAGAATTTGAC-3′, reverse 5′-CTAGCTAGCTGATGCTGCCGGTAAACTC-3′. The forward primer includes an Xba I restriction site, and the reverse primer has a Nhe I restriction site (underlined) to facilitate ligation into the vector. Predicted miR-26a binding sites were mutated using the TaKaRa MutanBEST Kit.

The following primers were used to amplify and clone the human PTEN into the pCMV-tag2B vector (Agilent Technologies, Santa Clara CA) according to manufacturer's instruction. Forward 5′-CGGGATCCGACATGACAGCCATCATCAAAG-3′, reverse 5′-CCGCTCGAGTCAGACTTTTGTAATTTGTGTATG-3′. The forward primer includes a Bam HI restriction site, and the reverse primer has an Xho I restriction site (underlined) to facilitate ligation into the vector.

2.9. Transfection and luciferase assay

Cell transfection was carried out using lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the manufacturer's instruction. Briefly, cells were plated in a 24-well plate at 1×10^5 cells/well. Cells were transfected with 100–200 nM of miR-26a mimics, or 100 ng of pNFκB-luc, or 200 ng of 3′UTR of PTEN, and 1 ng of pRL-SV40 as an internal control. Both miR-26a mimics and miR control were composed of RNA duplexes with the following sequence, miR-26a mimics: sense 5′-UUCAAGUAAUCCAGGAUAGGCU-3′, anti-sense: 5′-CCUAUCCUGGAUUACUUGAAUU-3′. Control: sense 5′-UUCUCCGAACGUGUCACGUTT-3′, anti-sense 5′-ACGUGACACGUUCGGAGAATT-3′. The sequence of miR-26a inhibitor was 5′-AGCCUAUCCUGGAUUACUUGAA-3′. The sequence of miR-26a inhibitor control was 5′-CAGUACUUUUGUGUAGUACAA-3′. Luciferase assay was performed 24 h later using the Dual-luciferase Reporter Assay System (Promega) following the manufacturer's instruction, on BERTHOLD TriStar LB 941 (BERTHOLD TECHNOLOGIES, Bad Wildbad, Germany). The sequences of siRNA duplex for PTEN: 5′-GGCGCUAUGUGUAUUAUUATT-3′, anti-sense: 5′-UAAUAACACAUAGCGCCTT-3′.

2.10. Statistical analysis

The data were presented as mean ± standard deviation (SD). Variance analysis between groups was performed by one-way ANOVA and significance of difference between control and treatment groups was analyzed using Dunnett multiple comparison test. The differences with $p<0.05$ were considered statistically significant.

3. Results

3.1. MiR-26a was downregulated in human lung cancer cell lines and clinical specimens

To define the role of miR-26a in human lung cancer tumorigenesis, we compared the expression levels of miR-26a in three human lung cancer cell lines, A549 (adenocarcinoma), SK-MES-1 (squamous cell carcinoma), H661 (large cell carcinoma), with BEAS-2B (human

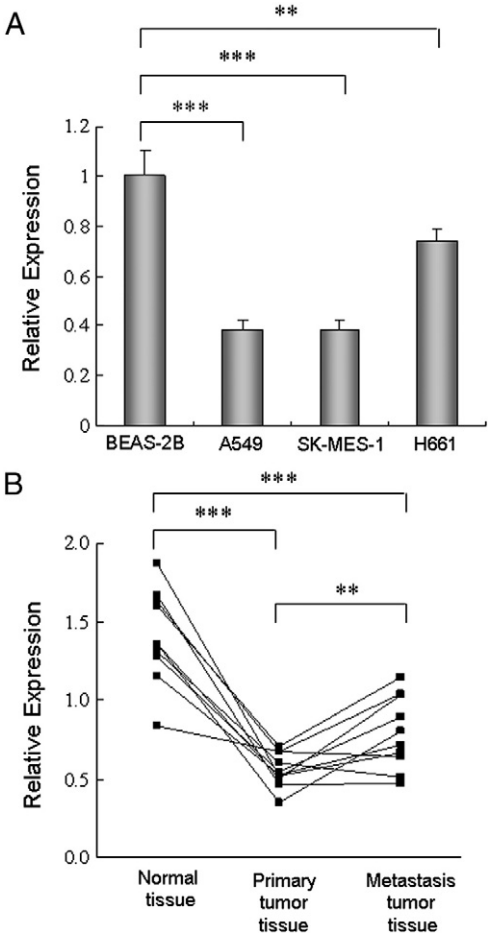


Fig. 1. Expression of miR-26a in human lung cancer cell lines and clinical specimens. A) Relative expression of miR-26a in BEAS-2B cells and lung cancer cell lines A549, SK-MES-1 and H661. B) Relative expression of miR-26a in human lung normal tissues, primary tumor tissues and lymph node metastasis tumor tissues ($n=10$). Each set of paired specimens was connected by lines. miRNA abundance was normalized to U6 RNA. Values represent the mean ± SD from three independent measurements. Columns, mean; bars, SD. ** $P<0.01$, *** $P<0.001$ versus control.

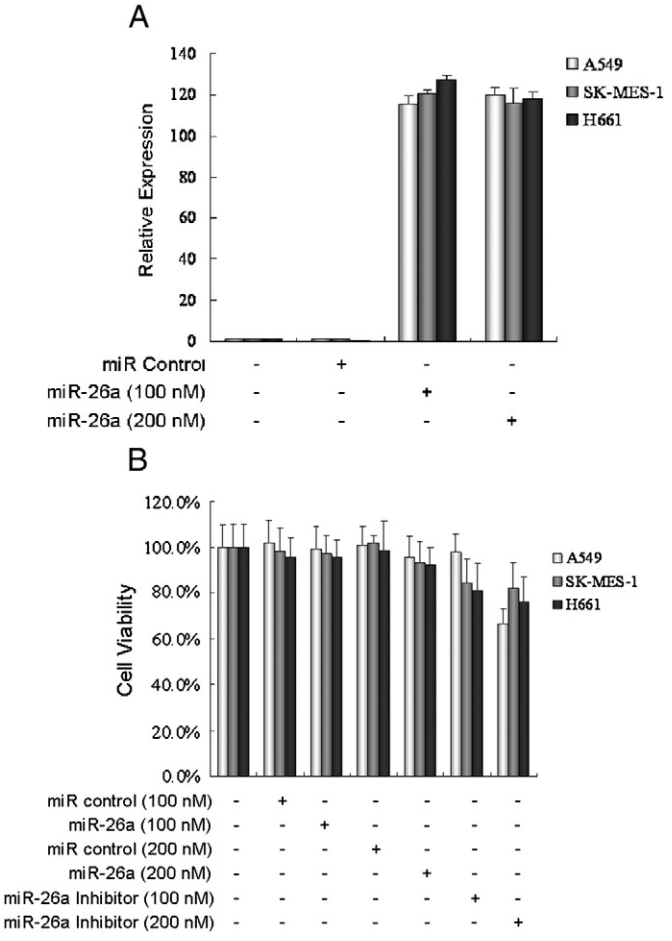


Fig. 2. The effect of miR-26a on growth of human lung cancer cells. A) A549, SK-MES-1 and H661 cells were transfected with 100–200 nM of miR-26a. The miR-26a expression levels were detected by real-time-PCR. B) A549, SK-MES-1 and H661 cells were transfected with 100–200 nM miR-26a. Cells were collected 48 h after and counted by a Vi-CELL Cell Viability Analyzer. Values represent the mean ± SD from three independent measurements.

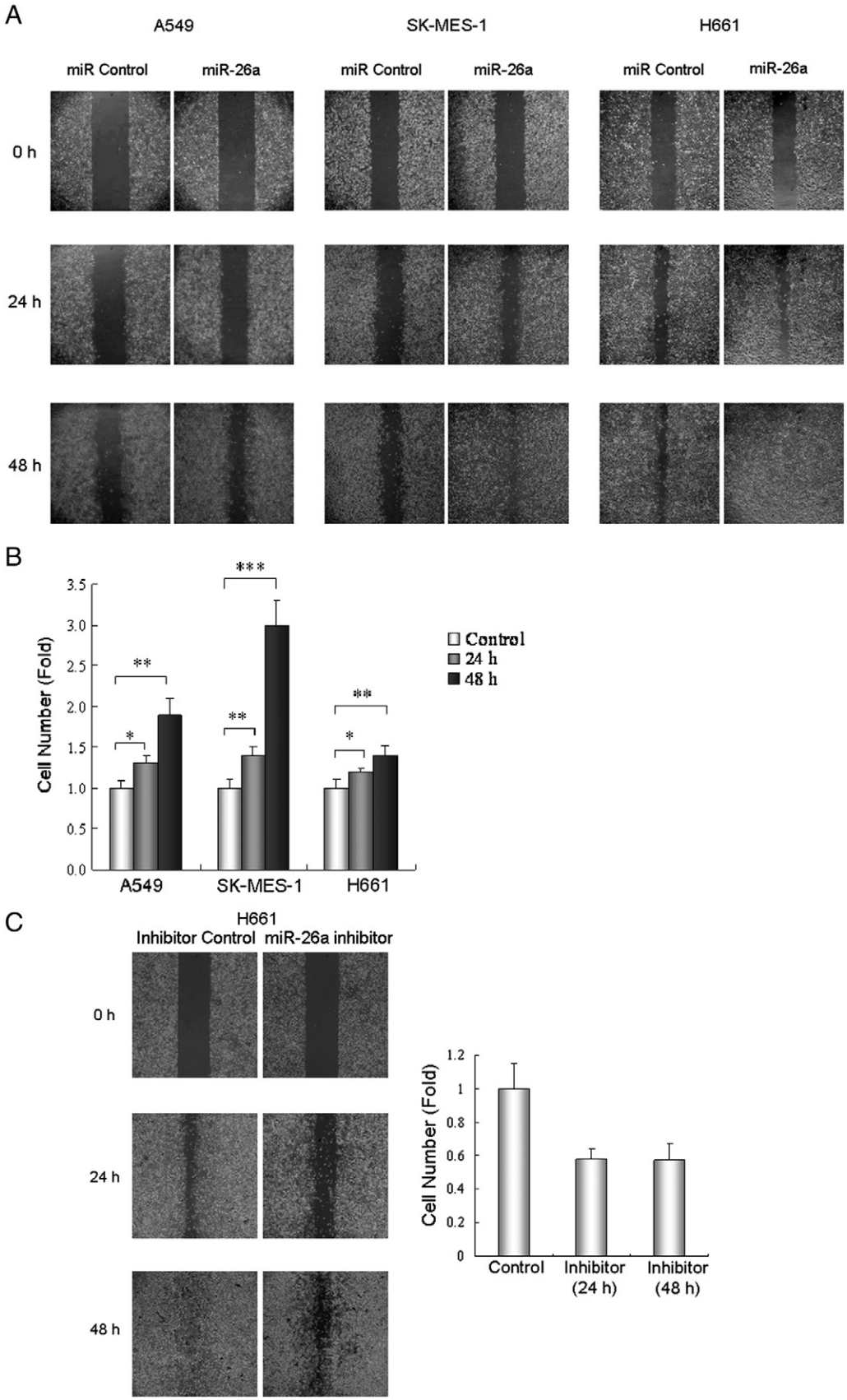


Fig. 3. MiR-26a enhanced the migration of lung cancer cells. A549, SK-MES-1 and H661 cells were transfected with 100 nM of miR-26a. Wound healing assays were performed to assess cell migration after 24 and 48 h. A) Representative photographs of cells are presented (×40 magnification). B) Number of cells migrated at 24 and 48 h time point is presented. C) H661 cells were transfected with 100 nM of miR-26a inhibitor. Wound healing assays were performed to assess cell migration after 24 and 48 h. Values represent the mean ± SD from three independent measurements. * $P<0.05$,** $P<0.01$, *** $P<0.001$ versus control.

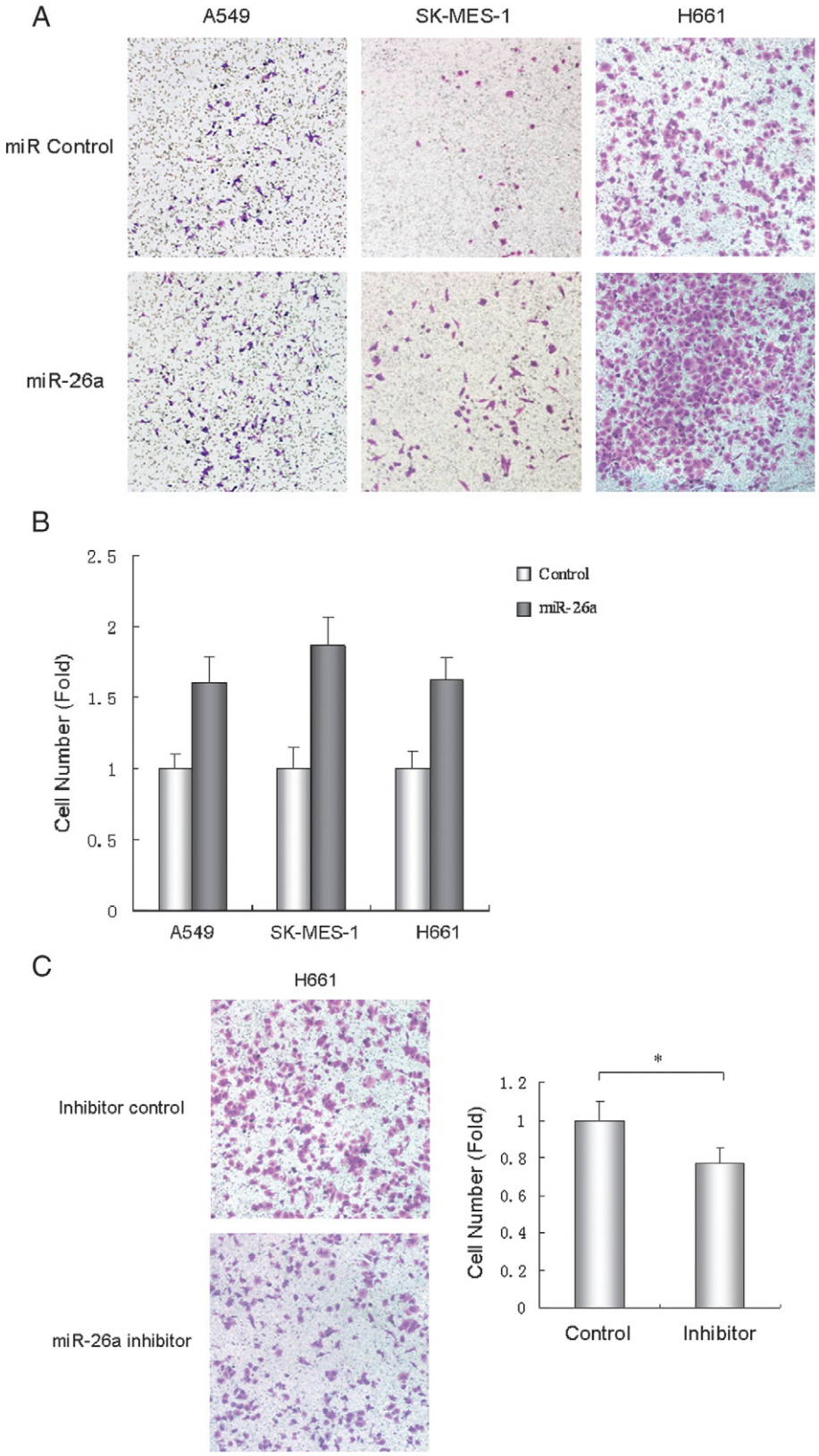


Fig. 4. MiR-26a enhanced the invasion of lung cancer cells. A549, SK-MES-1 and H661 cells were transfected with 100 nM of miR-26a. Invasion Chamber assays were performed to assess cell migration after 48 h. A) Representative photographs of cells are presented (×100 magnification). B) Number of cells invaded after 24 h is presented. C) H661 cells were transfected with 100 nM of miR-26a inhibitor. Invasion Chamber assays were performed to assess cell migration after 48 h. Values represent the mean ± SD from three independent measurements. * $P<0.05$ versus control.

normal lung bronchial epithelium) cell line. In H661 cells, the expression level of miR-26a was reduced to 74.1% compared with that of BEAS-2B cells. In A549 and SK-MES-1 cells, the expression levels of miR-26a were further reduced to 38.5% and 38.4%, respectively (Fig. 1A). These results indicated that miR-26a is significantly downregulated in human lung cancer cells.

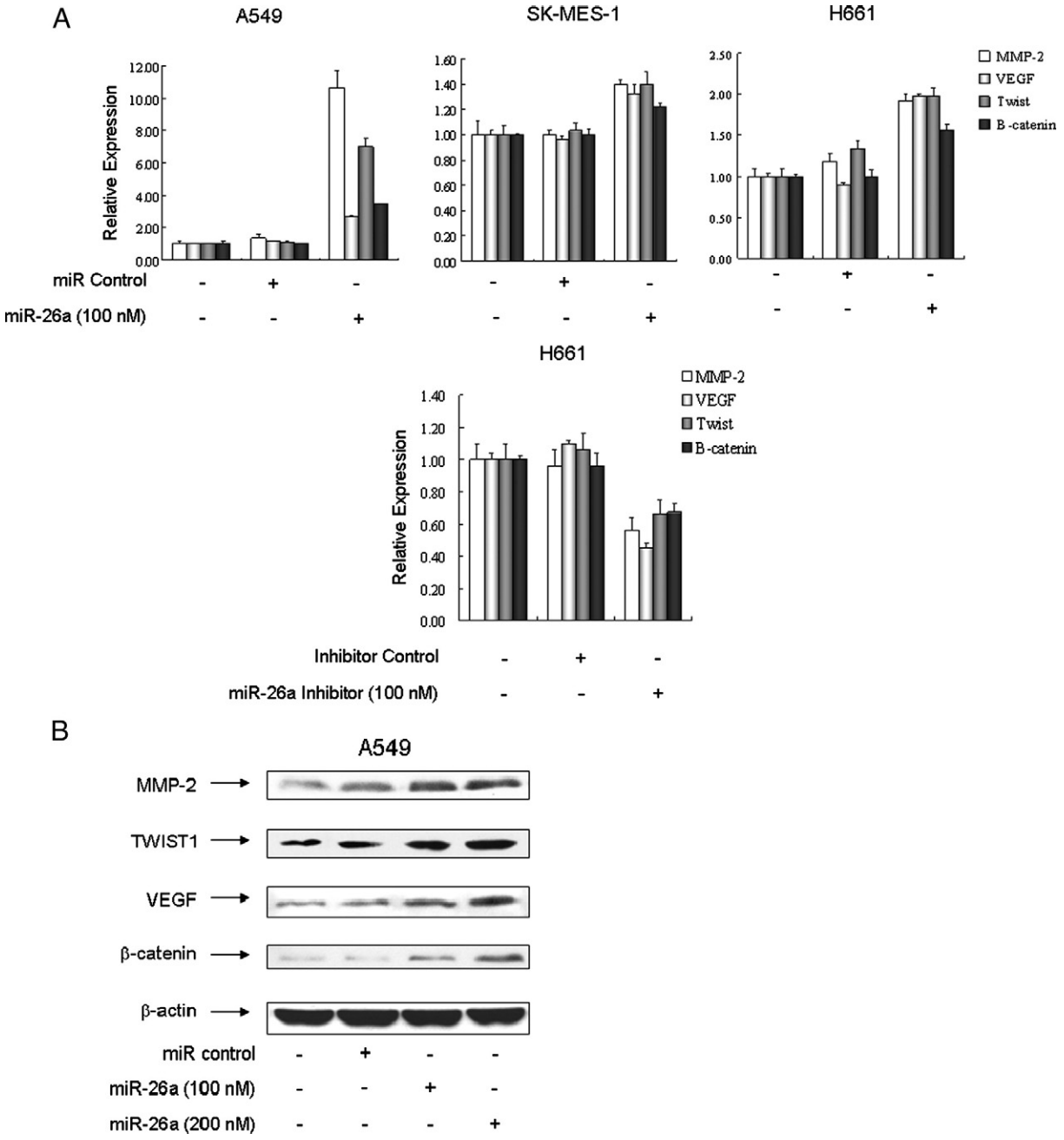


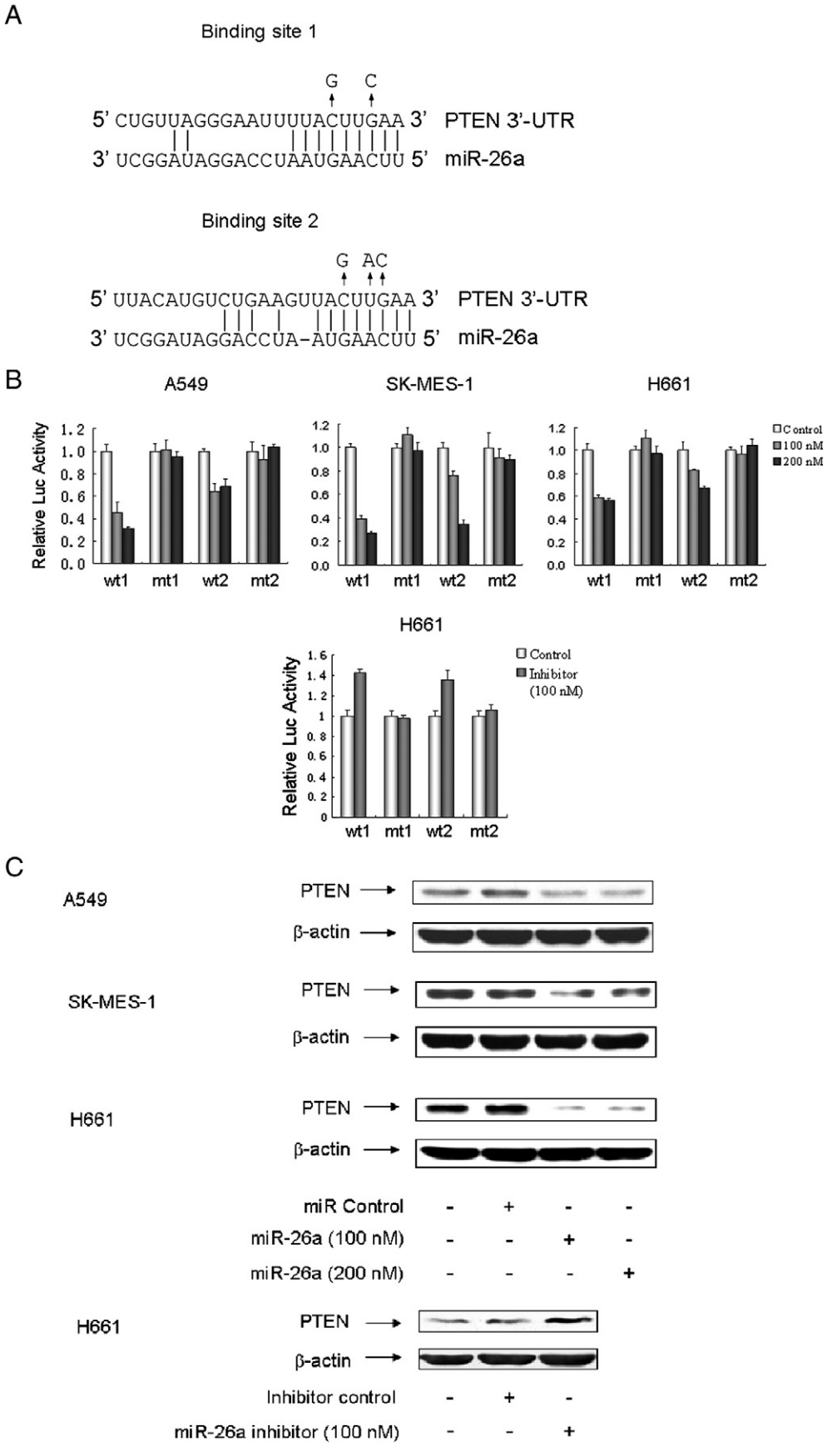
Fig. 5. MiR-26a modulated metastasis-related gene expression. A549, SK-MES-1 and H661 cells were transfected with 100 nM of miR-26a or miR-26a inhibitor. A) mRNA expression of MMP-2, VEGF, Twist and β-catenin was detected 24 h after by real-time PCR. B) Protein expression level of MMP-2, VEGF, Twist and β-catenin in A549 cells was detected by Western blotting analysis. Similar results were obtained in three independent experiments.

We further detected the expression level of miR-26a in lung cancer specimens. 10 sets of lung cancer specimens were collected, including normal tissues, primary tumor tissues and lymph node metastasis tumor tissues. Our data indicated that miR-26a is downregulated in lung cancer specimens (*** $P<0.001$). Interestingly, its expression level was higher in lymph node metastasis tumor tissues than in primary tumor tissues (** $P<0.01$) (Fig. 1B).

3.2. MiR-26a had no effect on growth of human lung cancer cells

To investigate the function of miR-26a in the development of lung cancer, we evaluated the effect of miR-26a on the growth of lung cancer cells by transient transfection. We first assessed the transfection efficiency of miR-26a. A549, SK-MES-1 and H661 cells were transfected with 100 or 200 nM of miR-26a, the miR-26a levels were detected by stem-loop RT-PCR. The miR-26a levels were increased significantly by an average of 120-fold. Interestingly, the transfection of 200 nM of miR-26a did not reach higher efficiency compared with transfection of 100 nM of miR-26a (Fig. 2A).

Next we investigated the effect of miR-26a on lung cancer cell growth. Cell proliferation was detected by Vi-CELL Cell Viability Analyzer. Fig. 2B showed that both 100 and 200 nM of miR-26a had no effect on cell proliferation, and 100 nM of miR-26a inhibitor had no effect on cell proliferation. However, 200 nM of miR-26a inhibitor slightly inhibited A549 cell growth. This may be due to the toxicity of high concentration of miR-26a inhibitor in A549 cells.


A
Binding site 1
G C
5' CUGUUAGGGAAUUUUACUUGAA 3' PTEN 3'-UTR
3' UCGGAUAGGACCUAAUGAACUU 5' miR-26a
Binding site 2
G AC
5' UUACAUGUCUGAAGUUACUUGAA 3' PTEN 3'-UTR
3' UCGGAUAGGACCUA-AUGAACUU 5' miR-26a
B
A549
SK-MES-1
H661
Relative Luc Activity
wt1 mt1 wt2 mt2
Control
100 nM
200 nM
H661
Control
Inhibitor (100 nM)
C
A549
SK-MES-1
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PTEN
β-actin
miR Control
miR-26a (100 nM)
miR-26a (200 nM)
Inhibitor control
miR-26a inhibitor (100 nM)

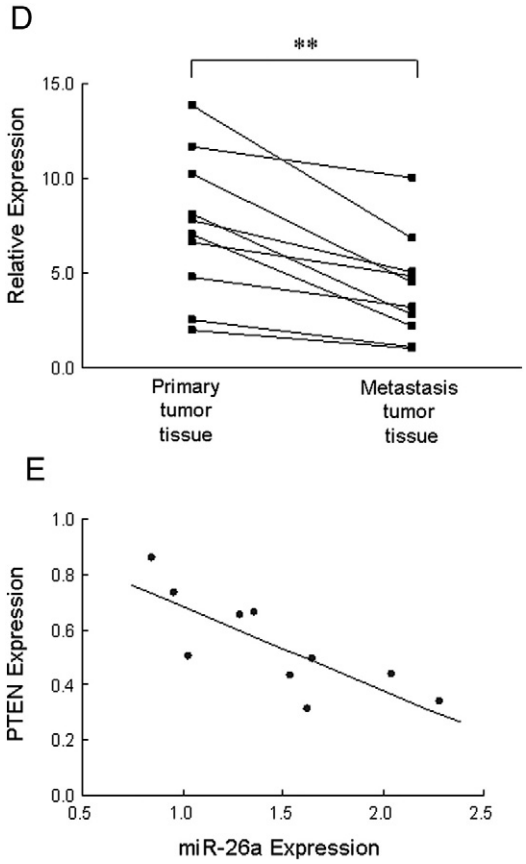

Fig. 6. PTEN is a direct target of miR-26a. A) Putative miR-26a binding sites in 3′-UTR of PTEN. Nucleotide changes for binding site mutants are indicated. B) Relative activity of the luciferase gene fused with the wild type (wt) or mutant (mt) 3′-UTR of PTEN. A549, SK-MES-1 and H661 cells were transfected with 100 and 200 nM of miR-26a, or 100 nM of miR-26a inhibitor. The luciferase activities were measured after 24 h. C) MiR-26a reduced the expression of PTEN. A549, SK-MES-1 and H661 cells were transfected with 100 and 200 nM of miR-26a, or 100 nM of miR-26a inhibitor. The expression levels of PTEN were detected by Western blot after 24 h. Similar results were obtained in three independent experiments. D) Relative expression of PTEN in human primary tumor tissues and lymph node metastasis tumor tissues ($n=10$). Each set of paired specimens was connected by lines (** $P<0.01$). E) Inverse correlation between miR-26a and PTEN level in lung cancer specimens (Spearman's correlation analysis, $r=-0.792$; ** $P<0.01$).

3.3. MiR-26a enhanced the migration of lung cancer cells

The effect of miR-26a on migration of lung cancer cells was examined by wound healing assay. A549, SK-MES-1 and H661 cells were transfected with 100 nM of miR-26a, and cell migration was detected after 24 and 48 h. As shown in Fig. 3A, B, the cell motility was enhanced in a time-dependent manner. The migration levels were increased to 1.3-, 1.4- and 1.2-fold of control after 24 h for A549, SK-MES-1 and H661 cells, respectively; increased to 1.9-, 3.0- and 1.4-fold of control after 48 h, respectively. The migration of SK-MES-1 cells was affected most dramatically. To confirm the effect of miR-26a on cell migration, miR-26a level was further knocked down by transfection with 100 nM of miR-26a inhibitor. Since H661 cells express the highest basal level of miR-26a among these cancer cell lines, we did this test in H661 cells. The migration levels were significantly decreased (Fig. 3C).

3.4. MiR-26a enhanced the invasion of lung cancer cells

Another important step for cancer cell metastasis is cell invasion. The effect of miR-26a on cell invasion was assessed by transwell assay. Cells were transfected with 100 nM of miR-26a and grown on reconstituted extracellular matrix (ECM). Invasive cells were stained and counted under microscope after 24 h. A significant increase in the number of invasive cells was observed. The levels of invasion were increased to 1.6-, 1.9- and 1.6-fold of control for A549, SK-MES-1 and H661 cells, respectively (Fig. 4A, B). SK-MES-1 cells were affected most dramatically. H661 cells were further transfected with 100 nM of miR-26a inhibitor; the level of invasion was decreased (Fig. 4C). This was consistent with the wound healing assay.

3.5. MiR-26a modulated metastasis-related genes expression

As our results showed that miR-26a enhanced both cell migration and invasion ability, this drove us to further investigate its effect on metastasis-related genes. MMP-2, VEGF, Twist and β-catenin play important roles in cancer metastasis, and they promote lung cancer metastasis. By using real-time PCR technique, we detected mRNA expression levels of these four genes 24 h after transfection with 100 nM of miR-26a. All these four metastasis-related genes were up-regulated in A549, SK-MES-1 and H661 cells (Fig. 5A). In particular, they were regulated most significantly in A549 cells, in which MMP-2 level was increased by 7.7-fold of control, VEGF level was increased by 2.3-fold of control, Twist level was increased by 6.5-fold of control, and β-catenin level was increased by 3.5-fold of control, respectively. Meanwhile, they were modulated moderately in SK-MES-1 cells, in which these four genes were upregulated by 1.2–1.4-fold. To confirm the effect of miR-26a on these genes, miR-26a was knocked down in H661 cells by transfection with miR-26a inhibitor. Our data showed that these four genes were downregulated by 0.45–0.67-fold. Fig 5B showed that protein levels of these genes in A549 cells were increased by miR-26a transfection; this was constant with mRNA results.

3.6. PTEN was a direct target of miR-26a

To elucidate the underlying mechanisms by which miR-26a executes its function, we explored miR-26a targets using the TargetScan bioinformatics algorithm. In particular, we focused on tumor suppressor genes. We found that there were two potential binding sites in the 3′-UTR of *PTEN* (Fig. 6A). This makes *PTEN* a particularly attractive target for further study.

We performed luciferase reporter assay to determine whether miR-26a could directly target the 3′-UTR of *PTEN* in lung cancer cells. As 1247–1268 nt and 2604–2626 nt, oriented from 5′ end of 3′-UTR, were putative binding sites for miR-26a in 3′-UTR of *PTEN*, we cloned these target sequences (wt) or the mutant sequence (mt) into pGL3 luciferase vector immediately downstream of the firefly luciferase gene. Nucleotide changes for binding site mutants were indicated in Fig. 6A. A549, SK-MES-1 and H661 cells were then transfected with wt or mt *PTEN* 3′-UTR vectors, and miR-26a mimic or miR control. The results showed that the miR-26a mimic, at both 100 and 200 nM, significantly decreased the luciferase activities of wt 3′-UTR vector when compared with miR control. Whereas, luciferase activities of mt 3′-UTR vector were unaffected by transfection with miR-26a (Fig. 6B). MiR-26a was then knocked down in H661 cells by transfection with miR-26a inhibitor. Our data showed that miR-26a inhibitor increased the luciferase activities of wt 3′-UTR vector (Fig. 6B).

We further investigated whether miR-26a expression regulated the expression level of PTEN. A549, SK-MES-1 and H661 cells were transiently transfected with miR-26a mimic or miR control, and PTEN expression levels were detected by Western blot. As shown in Fig. 6C, both 100 and 200 nM of miR-26a reduced the expression level of PTEN in three cell lines. MiR-26a was then knockdown in H661 cells by transfection with miR-26a inhibitor, and the expression level of PTEN increased. Taken together, all these results strongly indicated that PTEN was a direct target of miR-26a.

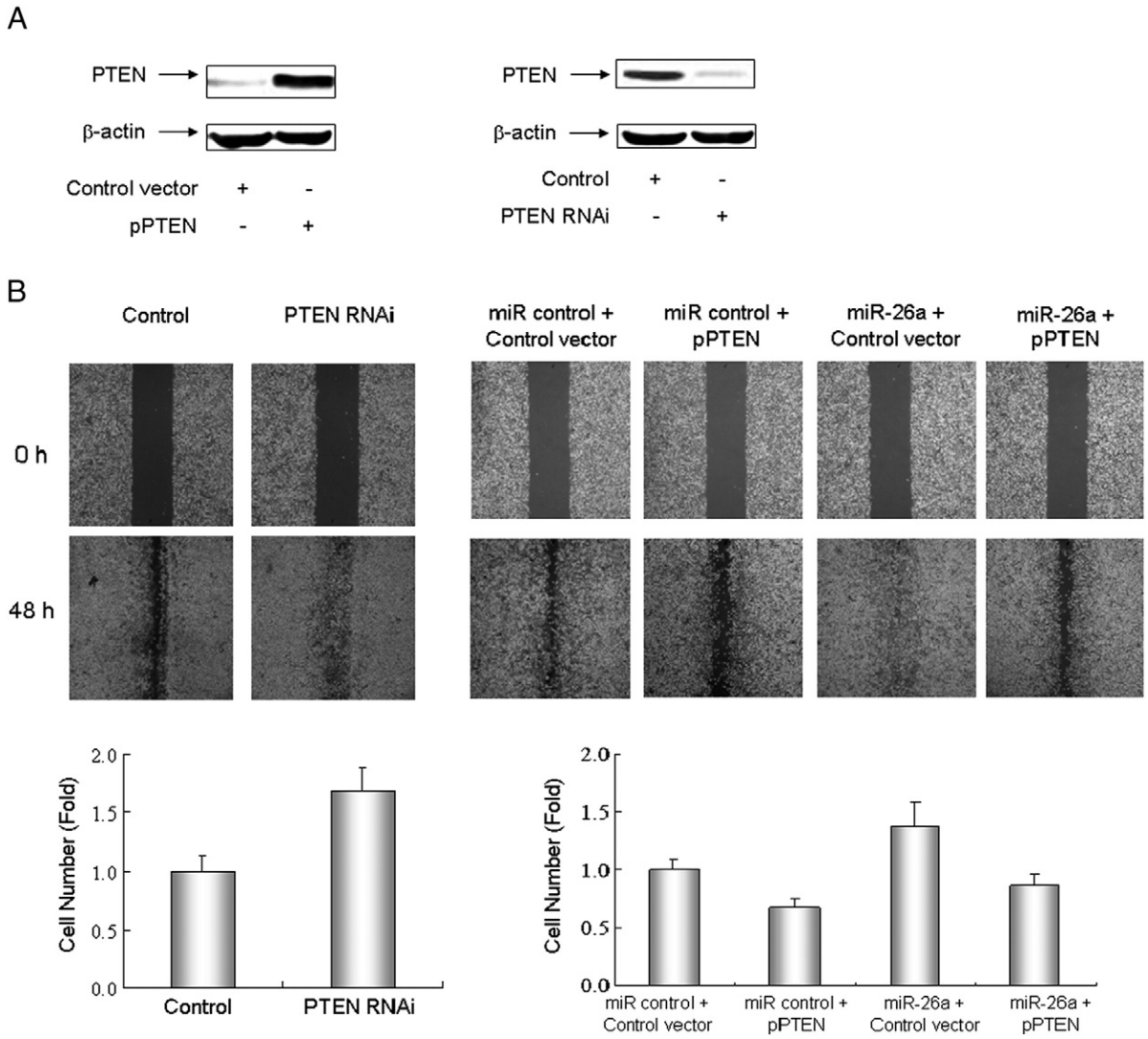

Fig. 7. PTEN mediated the effect of miR-26a on migration and invasion of lung cancer cells. A) H661 cells were transfected with 2 μg of pPTEN vector or 100 nM of siRNA duplex. The expression levels of PTEN were detected by Western blot. B) Wound healing assays were performed to assess cell migration after 48 h. C) Invasion Chamber assays were performed to assess cell migration after 48 h.

Next we detected the expression level of PTEN in lung cancer specimens. We found that PTEN expression level was lower in lymph node metastasis tumor tissues than in primary tumor tissues (** $P<0.01$) (Fig. 6D), and inversely correlated with miR-26a level ($r=-0.792$; ** $P<0.01$) (Fig. 6E).

3.7. PTEN mediated the effect of miR-26a on migration and invasion of lung cancer cells

To investigate the effect of PTEN on migration and invasion of lung cancer cells, PTEN was overexpressed by pPTEN vector or knockdown by RNA interfering in H661 cells (Fig. 7A). Wound healing assay showed that PTEN knockdown enhanced cell motility, and PTEN overexpression abolished the enhancement of cell migration by miR-26a (Fig. 7B). Similarly, transwell assay also demonstrated that PTEN knockdown enhanced cell invasion, and the enhancement of cell invasion by miR-26a was attenuated by PTEN overexpression (Fig. 7C).

3.8. AKT pathway contributed to the enhanced metastasis potential induced by miR-26a

There are several signaling pathways involved in cancer cell metastasis, one of them is the AKT pathway. PTEN functions as a tumor suppressor, and it negatively regulates AKT signaling pathway. This promoted us to investigate the effect of miR-26a on AKT pathway. AKT activation was detected by Western blot. As shown in Fig. 8A, after transfection with 100 and 200 nM of miR-26a, the phosphorylation levels of AKT in A549, SK-MES-1 and H661 cells were increased dramatically, whereas the total AKT levels remained unchanged. When H661 cells were transfected with 100 nM of miR-26a inhibitor, the phosphorylation levels of AKT decreased. We next examined the downstream molecule of AKT, the nuclear factor NFκB. The transcriptional activation of NFκB was assessed by luciferase reporter assay. After transfection with miR-26a, the luciferase activities were increased in all three cell lines (Fig. 8B). NFκB activation was also assessed by nuclear translocation assay in H661 cells. Western blot results showed that after transfection with miR-26a, the nuclear NFκB level increased, whereas cytoplasmic NFκB level decreased. When cells were transfected with miR-26a inhibitor, the nuclear NFκB level decreased, whereas cytoplasmic NFκB level increased (Fig. 8C).

The transcription factor activator protein 1 (AP-1) is one of the genes regulated by NFκB; it controls a number of cellular processes including cell proliferation, differentiation, apoptosis and metastasis. We examined the AP-1 transcriptional activation by luciferase reporter assay. Our data showed that miR-26a increased AP-1 transcriptional

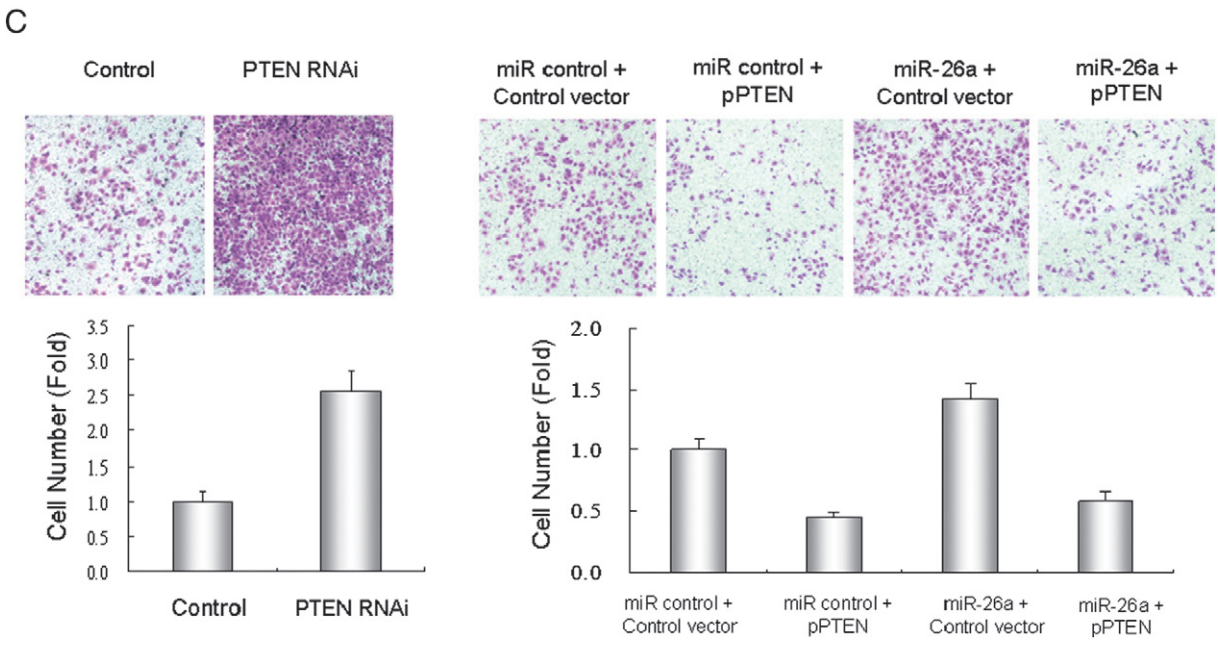

Fig.7 (*continued*).

activation in A549, SK-MES-1 and H661 cells (Fig. 8D). The effect of miR-26a was also examined by miR-26a inhibitor in H661 cells by luciferase reporter assay. MiR-26a inhibitor decreased transcriptional activation of NFκB and AP-1 (Fig. 8E). Taken together, these results demonstrated that miR-26a stimulated AKT pathways.

4. Discussion

Metastasis is the most significant process affecting the clinical management of cancer patients. However, the molecular pathways underlying each step still remain unclear. Although the oncogenic or tumor-suppressing functions of miRNAs have been characterized, the mechanistic roles played by miRNAs specifically in mediating metastasis have been addressed only recently [18,19].

There is a study showing that miR-26a was downregulated in lung cancer tissues, but no reports are available about its functions in lung cancer [20]. Our results showed that the expression levels of miR-26a were lower in tumor tissues than in adjacent normal lung tissues, and lower in lung cancer cell lines than in normal lung bronchial epithelium. Interestingly, we found that the expression levels of miR-26a in lymph node metastasis tissues were higher than in primary tumor tissues. Jiang et al. reported that expression level of miR-125a-5p was lower in NSCLC tissues than in adjacent normal lung tissues, but it enhanced cell migration and invasion [21]. This drove us to investigate the role of miR-26a in tumor cell metastasis. We selected A549, SK-MES-1, H661 cells, which exhibit similar clinical phenotypes of lung adenocarcinoma, squamous cell carcinoma and large cell carcinoma, respectively, as models for further study.

We first examined the effect of miR-26a on cell growth. Our results showed that miR-26a had no effect on lung cancer cell growth. Numerous studies showed that miR-26a suppressed cell proliferation in nasopharyngeal carcinoma, breast cancer and liver cancer cells [11–13], but promoted cell proliferation in glioma [9,10]. Interestingly, in leukemia, miR-26a inhibited acute myeloid leukemia cell growth, whereas stimulated T-cell acute lymphoblastic leukemia cell proliferation [14,22]. These controversial results suggested that miR-26a might play different roles depending on tumor and tissue types.

Metastasis is the most common cause of death in cancer patients. To elucidate the effect of miR-26a on lung cancer cell metastasis potential, we performed wound healing and transwell chamber assays. Our results demonstrated that miR-26a enhanced lung cancer cell migration and invasion *in vitro*. We then detected the effect of miR-26a on expression of metastasis-related genes. Matrix metalloproteinases (MMPs) are essential proteases involved in adhesion, invasion and migration of cancer cells. MMP-2 belongs to MMPs family. It highly expresses in tumor tissue, activates several key molecules leading to rapid cellular proliferation, increased motility, invasion and angiogenesis. Downregulation of MMP-2 inhibited tumor cell invasion [23,24]. Angiogenesis is the process of new blood vessel formation, and it is critical for the growth and metastasis of tumors. VEGF is a pro-angiogenic growth factor, and plays important role in tumor metastasis. Recently, antiangiogenesis agents that target the VEGF/VEGF receptor pathway have become an important part of standard therapy in multiple cancer indications [25,26]. Twist is a master regulator of epithelial–mesenchymal transition (EMT) and metastasis, and it contributes to metastasis by promoting EMT. Twist over-expression correlates with tumor metastasis, and depletion of Twist efficiently suppresses cell migration and invasion [27,28]. Cancer metastasis is often associated with activation of the Wnt/β-catenin signaling pathway. Upregulation of β-catenin promotes metastasis whereas downregulation of β-catenin inhibits metastasis [29,30]. We found that miR-26a upregulated MMP-2, VEGF, Twist and β-catenin. These data demonstrated that miR-26a promoted lung cancer cell metastasis by modulating metastasis-related genes.

To further explore the underlying mechanism, we investigated the targets of miR-26a. There have been several miR-26a targets reported, such as EZH2 [11], MTDH [13], GSK3β [31], interleukin-6 [32], interleukin-2 [33], SMAD1 [34], CCND2 [12], CCNE2 [12], RB1 [10] and MAP3K2 [10]. Also, two studies showed that PTEN is an miR-26a target in glioma [9,10]. However, none of these studies was related to tumor metastasis. In this study, we found a novel function of miR-26a on cancer metastasis. MiR-26a enhanced lung cancer cell metastasis potential by directly targeting PTEN. PTEN is a tumor suppressor, and it negatively regulates AKT/PKB signaling pathway. PTEN is one of the most mutated and deleted tumor suppressors in human cancer, and it is also found partially downregulated in cancer in the

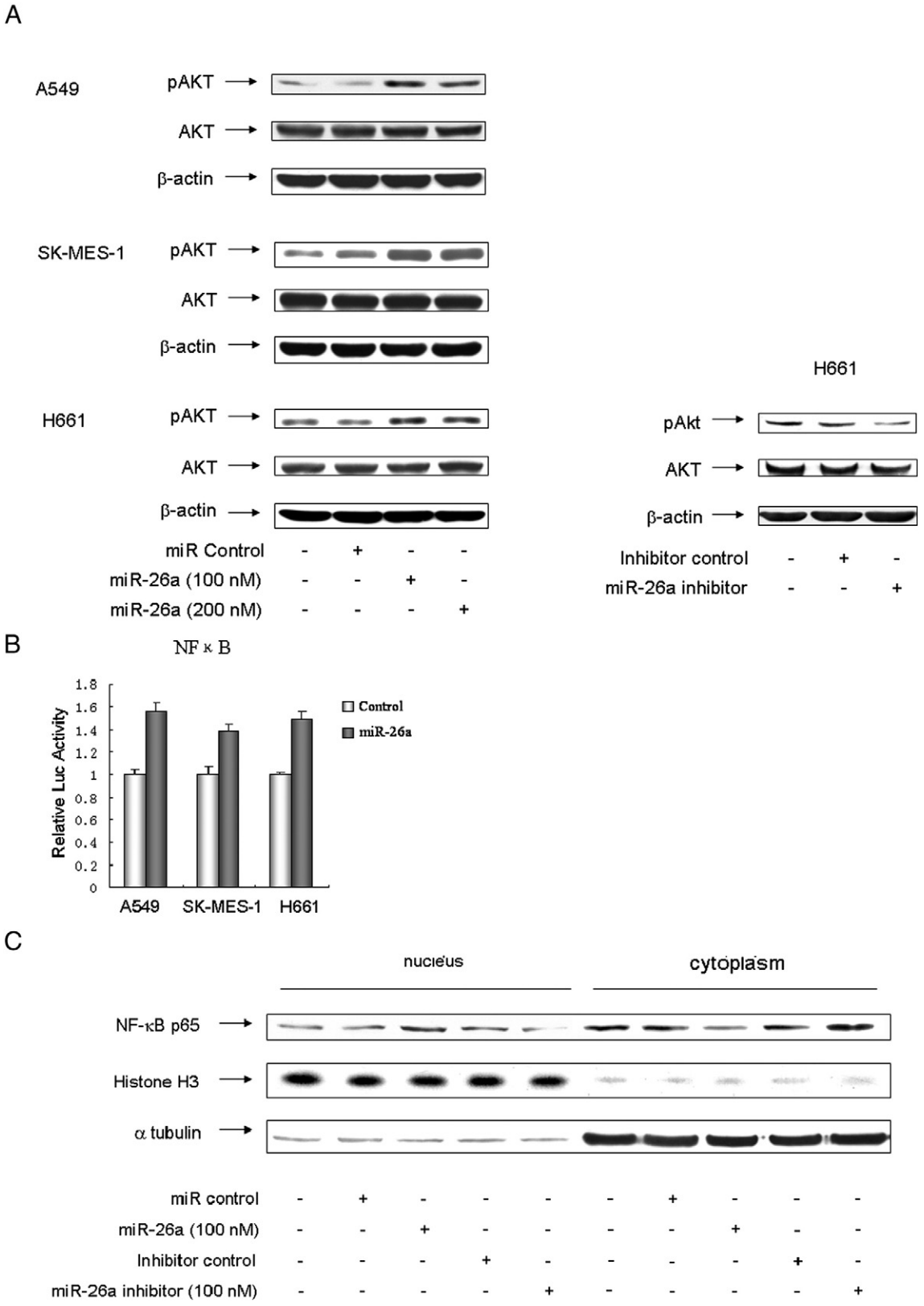

Fig. 8. MiR-26a stimulated AKT signaling pathway. A) A549, SK-MES-1 and H661 cells were transfected with 100 nM and 200 nM of miR-26a, or 100 nM of miR-26a inhibitor. AKT and pAKT levels were detected by Western blot. B) Cells were transfected with 100 nM of miR-26a and pNFκB-luc. NFκB transcriptional activation was detected by luciferase reporter assay. C) H661 cells were transfected with 100 nM of miR-26a or miR-26a inhibitor, and NFκB nuclear translocation was detected by Western blot. D) Cells were transfected with 100 nM of miR-26a and pAP-1-luc. AP-1 transcriptional activation was detected by luciferase reporter assay. E) H661 cells were transfected with 100 nM of miR-26a inhibitor and pNFκB-luc or pAP-1-luc. NFκB and AP-1 transcriptional activation was detected by luciferase reporter assay. Values represent the mean ± SD from three independent experiments.

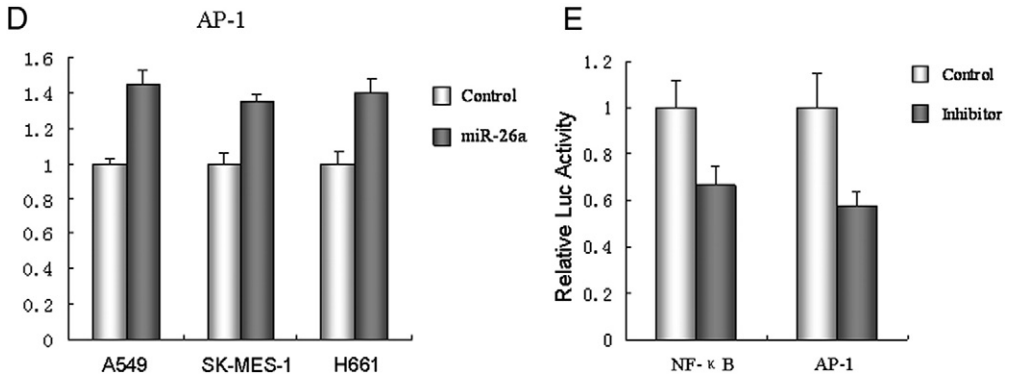

Fig. 8 (*continued*).

absence of genetic loss or mutation [35,36]. PTEN plays an important role in tumor metastasis. Loss of PTEN is associated with gallbladder adenocarcinoma metastasis [37], melanoma metastasis [38] and mammary metastasis [39,40]. Our results showed that miR-26a repressed PTEN expression, increased AKT phosphorylation and NFκB transcriptional activation. Our findings demonstrated that miR-26a enhanced lung cancer cell metastasis potential via activation of the AKT/NFκB pathway by PTEN suppression.

5. Conclusion

This study elucidates a novel function of miR-26a. miR-26a enhanced lung cancer cell metastasis potential by modulating metastasis-related genes, and targeting PTEN/AKT/NFκB pathway. Further *in vivo* study is needed for a deeper analysis of its functional roles, before miR-26a becomes a potential target for treatment of lung cancer and even other cancers.

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