

Original Article

Downregulation of TMEM176A suppresses cell growth and proliferation in non-small cell lung cancer

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Abstract: As a member of membrane-spanning 4A family, transmembrane protein 176A (TMEM176A) has been recently reported to be overexpressed in various cancers. However, the biological functions of TMEM176A in non-small cell lung cancer (NSCLC) remain unclear. In this study, we investigated the expression of TMEM176A in publicly available Oncomine database and found it was upregulated in lung cancer tissues compared with normal lung tissues. Similar results were also found in fresh primary NSCLC tumor tissues and corresponding normal tissues using Western blot analysis. Then the expression of TMEM176A was efficiently silenced in human NSCLC cell lines, A549 and 95D by RNA interference. Knockdown of TMEM176A obviously inhibited cell growth and colony formation, induced cell arrest at G0/G1 phase and promoted cell apoptosis in NSCLC cells, as determined by MTT, clonogenic assay and flow cytometry assays. Mechanistically, inhibition of TMEM176A significantly reduced the expression of CDK4 and Cyclin D1, but increased the expression of caspase-3, PARP cleavage and bad. In conclusion, these results firstly demonstrated that TMEM176A might serve as a potential oncogene that involved in the cell proliferation of NSCLC.

Keywords: Non-small cell lung cancer, TMEM176A, cell growth, apoptosis, caspase-3

Introduction

Non-small cell lung cancer (NSCLC), account for approximately 85% of all lung cancer, is the leading cause of cancer related death in the world [1]. Recently, despite lower toxicity and higher efficiency in epidermal growth factor receptor tyrosine kinase inhibitors compared with traditional postoperative chemotherapy [2], patients with NSCLC exhibited worse 5-year survival rate of only 15% [3]. The main factors associated with NSCLC are tumor uncontrolled proliferation. Therefore, identification of new molecular targets regarding the cellular and molecular mechanisms involved in the pathogenesis of NSCLC might provide a novel therapeutic strategy for this malignancy.

Transmembrane proteins (TMEMs) family is a group of ca. 310 different proteins and predicted as components of cellular membranes, including lysosomes and mitochondrial mem-

branes [4]. At present, members of TMEMs have been reported to be abnormally expressed in various tumors. TMEM7, acts as a tumor suppressor gene, was downregulated in renal cell carcinoma (RCC) tissues in comparison to normal human kidney [5]. Additionally, TMEM22 is overexpressed and likely play a crucial role in growth of renal cell carcinoma (RCC) [6]. TMEM16A is highly expressed and contributes to tumor invasion in human gastric cancer [7]. TMEM45A and TMEM158 have been demonstrated to play a positive role in cell proliferation and invasion in human ovarian cancer cells [8, 9]. Similarly, TMEM176A, belongs to membrane-spanning 4A family of proteins [10], was first identified as tumor-associated protein in hepatocellular carcinoma (HCC) [11]. Previously, the expression of TMEM176A was shown to be significantly increased in lung cancer [12]. However, the biological functions of TMEM176A in NSCLC have remained largely uncovered.

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To investigate the role of TMEM176A in NSCLC, we firstly compared its expression between NSCLC tissues and normal tissues. The effects of TMEM176A silencing on cell growth of NSCLC cells, as well as the underlying mechanisms, were then determined. Our findings suggest that TMEM176A might be a promising molecular target for NSCLC therapy via inhibition of TMEM176A.

Materials and methods

Clinical samples and cell lines

Total 6pairs of fresh primary NSCLC tumor tissues and adjacent normal tissues were collected from patients undergoing surgery at Department of Thoracic Surgery, Union Hospital, Tongji Medical College between 2013 and 2015, which were subjected to compare the expression of TMEM176A between NSCLC tissues and paired normal lung tissues. All the participants were required to sign informed consent and approved by the ethics committee of Union Hospital, Tongji Medical College. All the patients were pathological diagnosed and confirmed received none of preoperative chemotherapy or radiotherapy.

Cell culture and transfection

NSCLC cell lines (A549 and 95D) were purchased from Cell Bank of the Chinese Academy of Sciences, Shanghai, China and maintained in RPMI-1640 (Hyclone, Biowest) supplemented with 10% fetal bovine serum (FBS). All cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C. To further investigate the function of TMEM176A in NSCLC in cellular level, cells were cultured in six-well plates and transfected with 100 nmol/l of TMEM176A siRNA or control siRNA (Invitrogen, Carlsbad, CA, USA) using Lipofectamine 2000 transfection reagent kit (Invitrogen, Carlsbad, California, USA). After 48 h transfection, cells were underwent knockdown efficiency determination by western blotting.

Western blot analysis

Total protein was obtained from tissue samples and cell lines using 2 mL ice-cold RIPA lysis buffer (Beyotime, Shanghai, China). Equal amount of proteins (30 µg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel

(SDS-PAGE) and transferred to a PVDF membrane (Millipore, Billerica, MA, USA) using Bio-Rad semidry transfer system. Then the membrane was blocked with TBST (Tris-buffered saline, 0.1% Tween-20) containing 5% non-fat dry milk for 1h at room temperature, and probed with the corresponding primary antibodies, including anti-TMEM176A, CDK4, Cyclin D1, Bad, caspase-3 and PARP overnight. After washed by TBST, the membrane was then incubated with appropriated horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature. The membranes were washed and immunoreactive bands were visualized using super ECL detection reagent (Pierce Biotechnology, USA).

MTT assay

The effect of TMEM176A silencing on cell viability was determined in A549 and 95D cells using MTT assay. After 48 h of infection, cells (3,000 cells per well) were seeded into 96-well plates in triplicates and then added into 20 µL MTT solution (5 mg/mL) at 1, 2, 3, 4, and 5 days after transfection. After another 4 h incubation, 200 µL dimethyl sulfoxide (DMSO) was added into each well. The OD value was measured using an ELISA reader (Bio-Rad, USA) at wavelength of 595 nm. Each experiment was performed in triplicate.

Clonogenic assay

For the clonogenic assays, A549 and 95D cells (500 cells/well) were reseeded in 6-well plates after infection for 48 h. Then cells were allowed to grow for an additional 7 days to form natural colonies. The adherent cells were washed twice with PBS and then stained with 0.5% crystal violet solution to visualize the colonies. Colonies that contain > 50 cells were counted under fluorescence microscope.

Flow cytometry analysis

The effects of TMEM176A silencing on cell cycle distribution and apoptosis were measured using flow cytometry analysis. In brief, cells were washed with cold PBS twice after 48 h transfection and re-seeded into 6-cm dishes at a density of 60,000 cells per dish. Then cell were collected and stained with PI for cell cycle assay or Annexin V/7-AAD solution for apopto-

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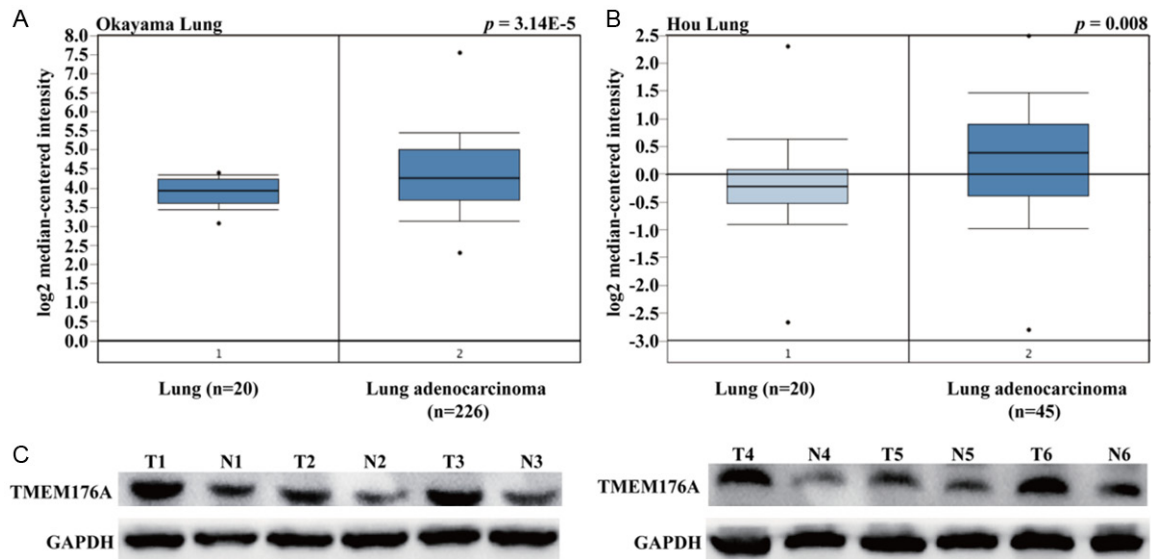


Figure 1. TMEM176A is commonly upregulated in NSCLC tissues compared with the adjacent normal tissues. TMEM176A expression in Okayama Lung (A) and Hou Lung (B) grouped by normal lung (1) and lung adenocarcinoma (2); (C) Western blot was employed to detect the expression levels of TMEM176A protein in NSCLC and corresponding normal tissues. NSCLC, non-small cell lung cancer; GAPDH was used as internal control.

sis assay according to the manufacturer's instructions. The stained cells were analyzed using flow cytometer (FACS Calibur, BD Biosciences).

Statistical analysis

Statistical analyses on cellular level were performed by SPSS software version 10.0 (SPSS Inc, United States) and expressed as mean \pm standard deviation (SD) of three independent experiments. Paired Student's t test was used to compare differences between siCon and siTMEM176A groups and $P < 0.05$ was considered as significant.

Results

TMEM176A was overexpressed in NSCLC tissues

To investigate the association of TMEM176A with NSCLC progression, we firstly determined the TMEM176A mRNA levels using the datasets from the publicly available Oncomine database (www.oncomine.org) and found it was significantly upregulated in human lung cancer tissues compared with that in normal tissues using Okayama Lung (**Figure 1A**, $P = 3.14E-5$) and Hou Lung (**Figure 1B**, $P = 0.008$) datasets. To further confirm this result, Western blot analysis were used to determine the expression of TMEM176A in fresh primary NSCLC tumor tissues and corresponding normal tis-

ues. As shown in **Figure 1C**, the protein level of TMEM176A was significantly elevated in 6 pairs of NSCLC tissues compared with the adjacent normal tissues ($P < 0.001$). These data suggested that TMEM176A might play an important role in the development of NSCLC.

Downregulation of TMEM176A remarkably suppressed cell growth

Considering the expression TMEM176A was significantly upregulated in NSCLC, we further investigated the cellular functions of TMEM176A in NSCLC cell lines by loss-of-function assays. NSCLC cell lines, A549 and 95D were selected to knock down the expression of TMEM176A by siRNA transfection. As shown in **Figure 2A**, the expression levels of endogenous TMEM176A proteins were obviously inhibited in these two cell lines, as confirmed by Western blot analysis. Subsequently, we investigated whether knockdown of TMEM176A inhibited uncontrolled cell proliferation in NSCLC cells. The results from the MTT assay demonstrated that the cell growth rate was significantly reduced in both A549 and 95D cells transfected with siTMEM176A compared with cells transfected with siCon (**Figure 2B**, $P < 0.001$). In addition, clonogenic assay showed that the numbers of colonies were significantly reduced in TMEM176A-downregulated A549 and 95D cells (**Figure 2C**, $P < 0.001$). From this point, we

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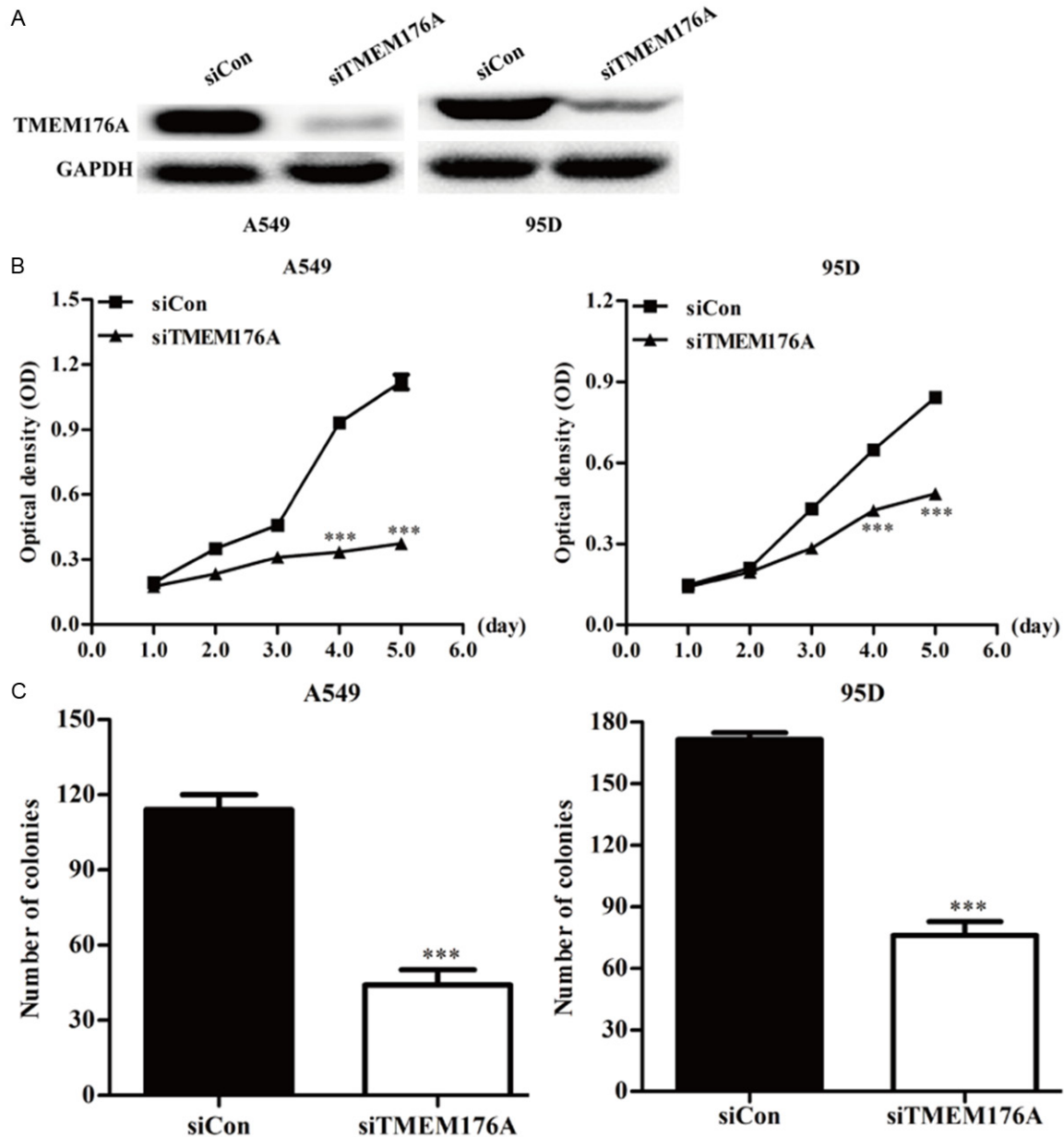


Figure 2. TMEM176A silencing induced by siRNA significantly inhibited NSCLC cell growth and proliferation. A. The protein level of TMEM176A was remarkably down regulated by specific siRNA targeting TEM176A (siTMEM176A) compared with control siRNA (siCon) in A549 and 95 D cells. B. MTT assay indicated that the growth rate of A549 and 95 D cells was significantly decreased after TMEM176A silencing. C. Clonogenic assay showed knockdown of TMEM176A significantly impaired cell colony formation ability in A549 and 95 D cells. NSCLC, non-small cell lung cancer; SiTMEM176A: Cells were treated with siRNA targeting TMEM176A; SiCon: Cells were treated with control siRNA. ***P<0.001 as compared with control cells; GAPDH was used as an internal control.

speculated TMEM176A could promote cell growth in NSCLC.

TMEM176A silencing induces cell cycle arrest and apoptosis

To further investigate the mechanism underlying the inhibition of cell proliferation, cell cycle

distribution and apoptosis were detected on A549 and 95D cells after lentivirus infection using flow cytometric assay. As shown in **Figure 3A** and **3B**, the percentage of cells in G0/G1 phase was significantly increased (P<0.001), whereas the number of cells in S phase was remarkably decreased in A549 cells following TMEM176A knockdown (P<0.001). Similarly,

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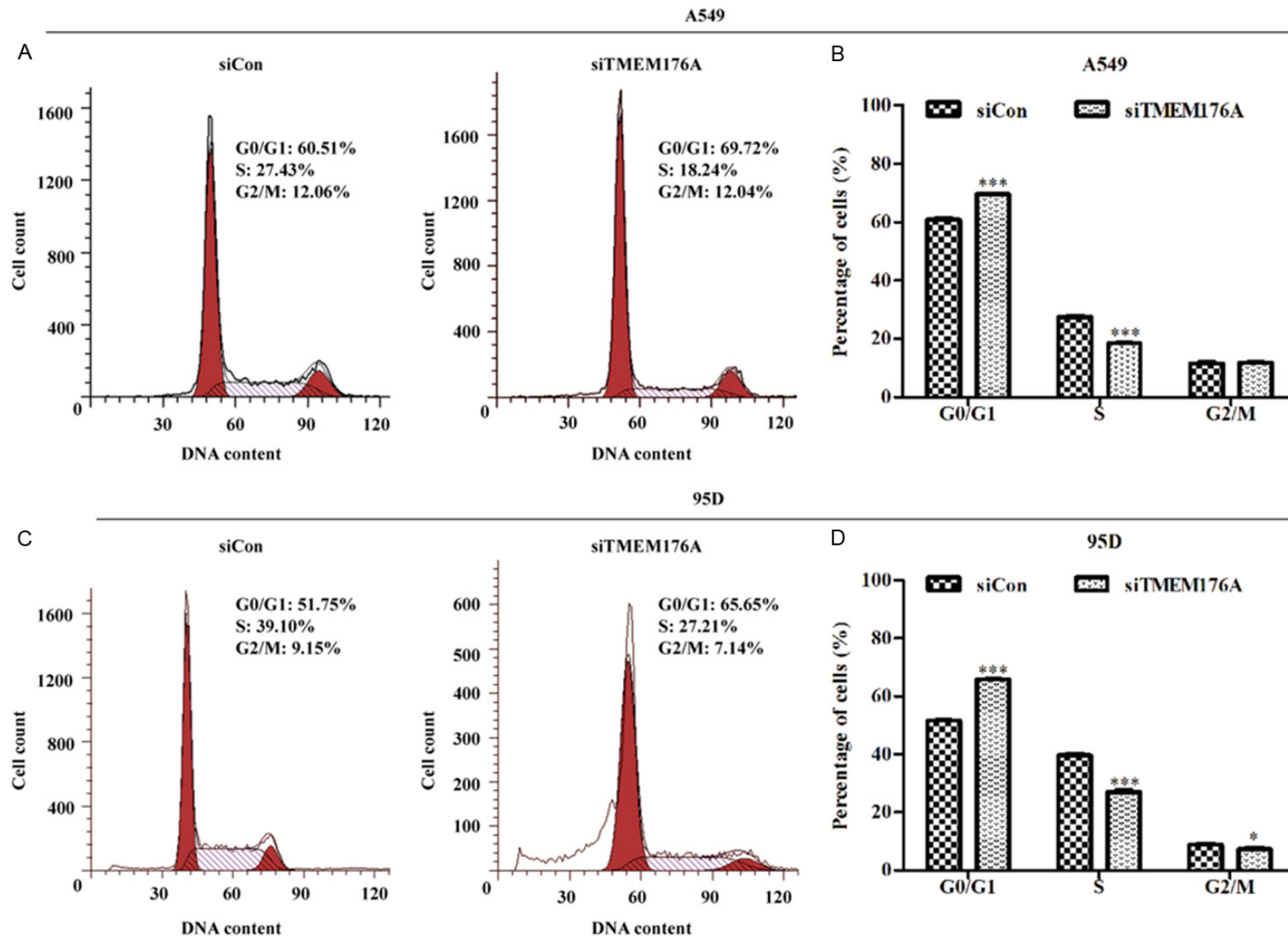


Figure 3. TMEM176A silencing induced cell cycle arrest at G0/G1 phase. The profile of cell percentages in G0/G1, S and G2/M phases in A549 (A) and 95D (C) cells following TMEM176A knockdown. Statistical analysis of G0/G1, S and G2/M phase population in siCon- and siTMEM176A-treated A549 (B) and 95D (D) cells. siTMEM176A: Cells were treated with siRNA targeting TMEM176A; siCon: Cells were treated with control siRNA. * $P < 0.05$, *** $P < 0.001$ as compared with control cells.

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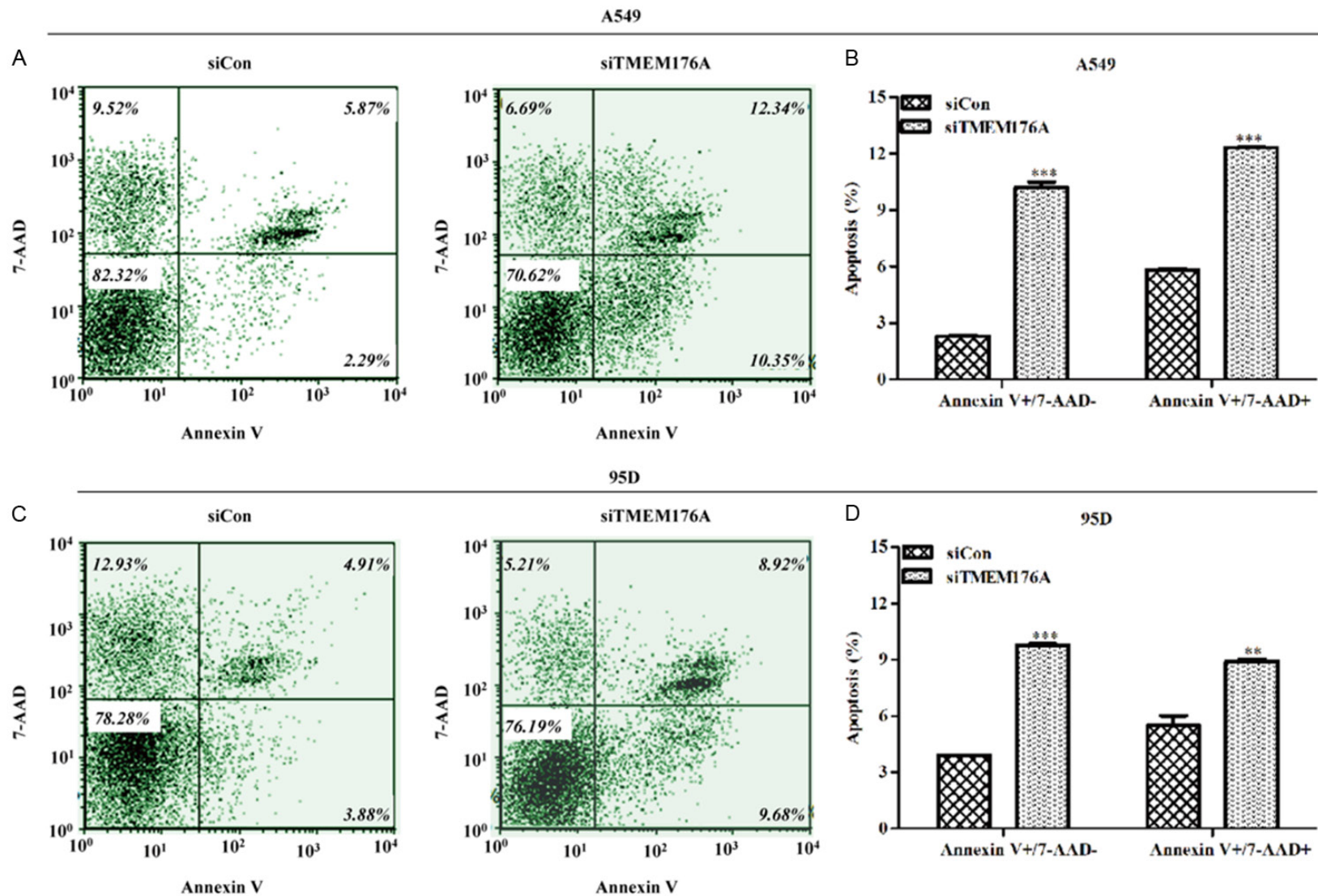


Figure 4. TMEM176A silencing promoted NSCLC cell apoptosis. Apoptosis of cells infected with siTMEM176A or siCon after Annexin V/7-AAD staining, including early-stage (Annexin V⁺/7-AAD⁻) and late-stage (Annexin V⁺/7-AAD⁺) apoptosis in A549 (A) and 95D cells (C). Quantitation of apoptotic cells in A549 (B) and 95D cells (D) following TMEM176A silencing. siTMEM176A: Cells were treated with siRNA targeting TMEM176A; SiCon: Cells were treated with control siRNA. ** $P < 0.01$, *** $P < 0.001$ as compared with control cells.

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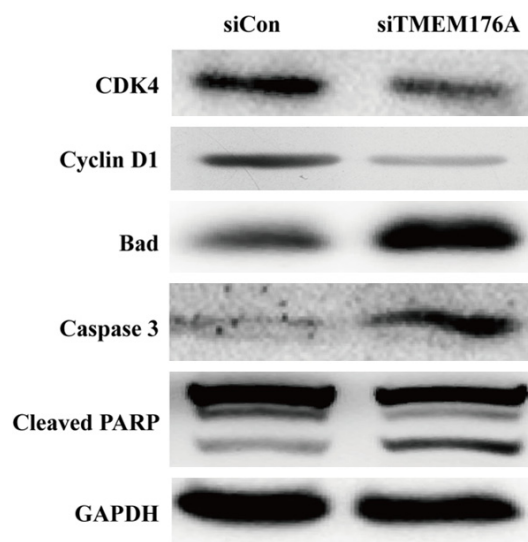


Figure 5. Western blot analysis of proteins associated with cell cycle progression and apoptosis. SiTMEM176A: Cells were treated with siRNA targeting TMEM176A; SiCon: Cells were treated with control siRNA. $**P<0.01$ as compared with control cells. GAPDH was used as an internal control.

knockdown of TMEM176A significantly elevated the percentage of cells in G0/G1 phase ($P<0.001$), while reduced the cell counts in S ($P<0.001$) and G2/M ($P<0.05$) phases in 95D cells (**Figure 3C** and **3D**).

What's more, siTMEM176A did cause significant change in the profile of Annexin V/7-AAD-staining cell populations corresponding to viable and non-apoptotic (Annexin V-/7-AAD+), early (Annexin V+/7-AAD-) and late apoptotic (Annexin V+/7-AAD+) cells in A549 and 95D cells. Moreover, more cells presented Annexin V+/7-AAD- and Annexin V+/7-AAD+ signals in siTMEM176A than that in siCon groups in A549 and 95D cells (**Figure 4A** and **4C**). Statistical analysis further demonstrated the overall apoptotic rate of siTMEM176A group cells was about 22.69% and 18.60% in A549 and 95D cells, respectively, which was significantly higher than that in corresponding siCon groups (**Figure 4B** and **4D**). Collectively, TMEM176A might play an important role in regulating cell cycle progression and apoptosis.

Downregulation of TMEM176A regulated cell cycle and apoptotic markers

Furthermore, we detected the expression alterations of some cell cycle regulators and apop-

totic markers. As shown in **Figure 5**, knockdown of TMEM176A significantly downregulated the expression levels of CDK4 and Cyclin D1, but upregulated the expression levels of bad, caspase-3 and cleaved PARP in xenografts. Therefore, these findings suggest that TMEM176A might be essential for cell proliferation of NSCLC.

Discussion

Recently, TMEMs have been identified to be involved in tumor progression and development. TMEM176A, as a member of TMEM family, has been reported to be significantly upregulated in lung carcinoma [12], but limited functional data describing their detailed involvement in NSCLC is lack. In the present study, re-analysis of the data obtained from Oncomine database showed TMEM176A was commonly upregulated in lung cancer tissues. In line with this observation, we found TMEM176A was upregulated in NSCLC and adjacent normal tissues using Western blot analysis, which suggest TMEM176A might be a putative oncogene in NSCLC.

To further investigate the functions of TMEM176A in NSCLC, the expression of TMEM176A was suppressed in two NSCLC cell lines, A549 and 95D, by means of siRNA treatment. Loss-of-function assay indicated that knockdown of TMEM176A drastically suppressed cell growth and colony formation ability of NSCLC. It has been reported that cell cycle arrest and promoting cell apoptosis is an important mechanism in inhibiting tumor cell growth [13, 14]. Thus, we determined the effects of TMEM silencing on NSCLC cell cycle distribution and apoptosis. Based on our data, knockdown of TMEM176A could arrest cell cycle at G0/G1 phase and promoted cell early and late apoptosis, which suggested that TMEM176A plays an essential role in NSCLC cell growth. In line with this finding, TMEM14A, also belongs to a member of TMEMs, has a pro-tumorigenic effect in ovarian cancer cells through increasing cell proliferation [15]. Overexpression of TMEM14A could inhibit N-(4-hydroxyphenyl) retinamide-induced cell apoptosis in glioma [16].

To further uncover the mechanisms underlying the inhibitory effects induced by TMEM176A knockdown, we detected the expression of found that TMEM176A knockdown downregu-

lated the expression of several cell cycle regulators and apoptotic markers. CDK4 and Cyclin D1 have been demonstrated to be responsible for initiation and completion of DNA replication [17, 18], which were both downregulated by knockdown of TMEM176A. Further data revealed that the TMEM176A silencing upregulated the expression of caspase-3, and cleaved PARP. Generally, cell apoptosis consists of two distinct signaling pathways, including the extrinsic and intrinsic pathway, in which caspase family proteases can be activated [19, 20]. Caspase-3 is the key enzyme required in the caspase activation and execution [21]. PARP is reported to promote programmed cell death, in which it was cut by the caspase-3, leading to cell apoptosis [22, 23]. In addition, the activation of caspase is also regulated by Bcl-2 family proteins [24]. Bad, as a member of Bcl-2 family, has been considered as a pro-apoptotic factor in promoting cell death [25]. Therefore, our results provide evidence implicate TMEM176A silencing as an essential inhibitor during NSCLC progression.

In summary, our study is the first to report that TMEM176A is closely correlated with NSCLC tumorigenesis by regulating cell proliferation, as well as provide a mechanistic basis for the further exploration of TMEM176A as a diagnostic and therapeutic target for NSCLC. Furthermore, it is still necessary to further investigate the potential application of TMEM176A-targeted therapy using lentivirus-mediated shRNA approach in more preclinical and clinical studies.

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Disclosure of conflict of interest

None.

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