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TM4SF1 facilitates non-small cell lung cancer progression through regulating YAP-TEAD pathway

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Abstract. – OBJECTIVE: Transmembrane-4-L-Six-Family-1 (TM4SF1) has been found involved in the development and progression of tumor. This study aims to investigate the effect of TM4SF1 on the proliferation, migration, and invasion of non-small cell lung cancer (NSCLC) and reveal its underlying mechanisms.

MATERIALS AND METHODS: qRT-PCR, immunohistochemical analysis, and Western blot were used to evaluate the expression of TM4SF1 in human NSCLC tissues and cells. Cell proliferation was measured by CCK-8 and colony formation assay. Cell apoptosis was evaluated by flow cytometry assay. Cell migration and invasion were detected by wound healing and transwell assays. Co-immunoprecipitation (Co-IP) assay was used to examine the interactions between proteins. Expression levels of related proteins were determined by Western blot. For *in vivo* experiment, xenograft tumor models were used.

RESULTS: TM4SF1 was upregulated in NSCLC tissues and cell lines and closely correlated to survival time, tumor size, lymph node metastasis, distant metastasis, and clinical stage. Gain-of function and loss-of function experiments demonstrated the oncogenic effect of TM4SF1 on NSCLC cell proliferation, apoptosis, migration, and invasion. Notably, mechanism studies showed that TM4SF1 regulated the interaction between YAP and TEAD and the level of downstream target genes. Besides, sh-YAP or Peptide 17 treatment (YAP-TEAD protein-protein interaction inhibitor) reversed the effect of TM4SF1 on NSCLC cells. The *in vivo* research suggested that the knockdown of TM4SF1 inhibited the growth of xenograft tumor of NSCLC.

CONCLUSIONS: This is the first evidence demonstrating that TM4SF1 could promote proliferation, migration, and invasion in NSCLC, at least partially through a potential YAP-TEAD signaling pathway-dependent mechanism. This study might provide a potential therapeutic target for the treatment of NSCLC.

Key Words:
Non-small cell lung cancer, TM4SF1, Proliferation, Migration, Invasion, YAP-TEAD signaling pathway.

Introduction

Lung cancer is one of the most common tumors worldwide and it has the highest incidence[1,2]. As the most frequent type of lung cancer, non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancer cases[3]. Despite the rapid development in early diagnosis and treatment, the 5-year overall survival rate of NSCLC patients is still unsatisfactory[4]. Therefore, it is necessary to investigate the potential molecular mechanisms underlying the progression of NSCLC for developing effective therapy.

Transmembrane-4 L6 family member 1 (TM4SF1) is located at chromosomal region 3q21-3q25 and belongs to the transmembrane-4 protein L6 superfamily (also known as the tetraspanin family). TM4SF1 is known as a complete membrane glycoprotein that transmits extracellular signals to the cytoplasm[5]. TM4SF1 was demonstrated to exert a significant effect on tumor cell proliferation, invasion, ROS metabolism, angiogenesis, resistance, and self-renewal in multiple cancers including pancreatic cancer[6,7], prostate cancer[8], breast cancer[9], bladder cancer[10], esophageal cancer[11], etc. Previous studies[12,13] have illuminated that TM4SF1 was highly expressed in NSCLC patients and A549 cells and it was associated with shorter median overall survival (OS) and progression-free survival (PFS). Nevertheless, the role and regulatory mechanisms of TM4SF1 in the progression of NSCLC are still elusive and need to be clarified.

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The present work demonstrated that TM4SF1 was abnormally highly expressed in NSCLC tissues and cell lines. TM4SF1 promoted cell proliferation, migration, and invasion and inhibited cell apoptosis. Of note, the YAP/TEAD signaling pathway was involved in this process. In addition, TM4SF1 silencing inhibited the growth of xenograft tumors of NSCLC *in vivo*. These data suggest an oncogenic role of TM4SF1 and provides new insights for the treatment or diagnosis in NSCLC.

Materials and Methods

Clinical Samples and Ethics Statement

NSCLC and adjacent non-tumor tissues were collected from 36 patients who underwent surgical resection at Taizhou People's Hospital (Taizhou, Jiangsu, China). All enrolled patients did not receive chemotherapy, radiation therapy or immunotherapy before surgery. This investigation was approved by the Medical Research Ethics Committee of Taizhou People's Hospital. Informed consent was obtained from each patient.

Immunohistochemistry

All tumor tissues were fixed in formalin, embedded in paraffin, sectioned (4-μm- thick), and incubated with anti-TM4SF1 rabbit polyclonal antibodies, anti-Ki-67, anti-caspase-3, and anti-E-cadherin (all were purchased from Abcam, Cambridge, UK) at 4°C overnight. Immunostaining was performed based on the streptavidin-peroxidase technique. The sections were washed with phosphate-buffered saline (PBS) and incubated with an horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody for 1 h at room temperature. Peroxidase was visualized using 3, 3′-diaminobenzidine, and haematoxylin was used for lightly counterstain.

Cell Culture and Transfection

Five NSCLC cell lines (A549, H1975, SPC-A1, H1299, H460) and human bronchial epithelioid cell line 16HBE were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Roswell Memorial Park Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum, 100 IU/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator with 5% CO_2 at 37°C. For overexpression of TM4SF1 in H1975 and H460 cells, the plasmids pcDNA3.1-TM4SF1 and empty vector plasmids were purchased from Genechem (Shanghai, China). For knockdown of TM4SF1 in A549 and SPC-A1 cells, the sequences of shRNA targeting for TM4SF1 and shCTRL were purchased from Gene Pharma. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for transfection. The YAP-TEAD interaction Inhibitor Peptide 17 was purchased from Selleckchem (Houston, TX, USA).

Reverse Transcription and Quantitative Real-Time PCR (qRT-PCR)

The total RNAs of cells and tissues were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into cDNA using Super M-MLV reverse transcriptase (Beyotime Biotechnology, Shanghai, China) followed manufacturers' instructions. Quantitative PCR was performed using SYBR Green (Sigma-Aldrich, St. Louis, MO, USA) and data were analyzed using the $2^{-\Delta\Delta CT}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal reference. Real-time primers were summarized in Table I.

Western Blot Assay

The cells and tissues were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China). After quantification with bicinchoninic acid assay (BCA) kit, the total proteins were fractionated by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, the proteins were transferred onto polyethylene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), sealed with 5% skim milk, and incubated with the following primary rabbit antibodies at 4°C overnight: anti-TM4SF1 (1:2000, #ab113504), anti-p21 (1:1000, #ab188224), anti-p27 (1:1000, #ab32034), anti-PCNA(1:1000, #ab92552), anti-cleaved caspase-3 (1:1000, #ab49822), anti-Bcl-2 (1:800, #ab59348), anti-Bax (1:2000, #ab32503), anti-E-cadherin (1:500, #ab15148), anti-N-cadherin (1:1000, #ab76057), and anti-GAPDH (1:2000, #ab181602). Subsequently, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:2000, #ab6721) for 1 h at room temperature. All antibodies were obtained from Abcam (Cambridge, UK). The expressions of the protein were analyzed by an imaging system (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed by Image J software (NIH, Bethesda, Maryland, USA).

Table I. List of primers used for qRT-PCR.

DNAs	Primers
TM4SF1	Forward: 5′-TCG CGGCTAATATTTTGCTT-3′ Reverse: 5′-TGCAATT CCAATGAGAGCAG-3′
YAP	Forward: 5′-AGCAGGATGGTGGGACTCAA-3′- Reverse: 5′-AGGTGCCACTGTTAAGGAAAGGAT-3′
CTGF	Forward: 5′-AGGAGTGGGTGTGTGACGA-3′ Reverse: 5′-CCAGGCAGTTGGCTCTAATC-3′
Cyr61	Forward: 5′-CCTTGTGGACAGCCAGTGTA-3′ Reverse: 5′-ACTTGGGCCGGTATTTCTTC-3′
Birc5	Forward: 5′-GCCACGCATCCCAGCTT-3′ Reverse: 5′-TTTCAAAATACCACTGTCTCCTTCTC-3′
GAPDH	Forward: 5′-GGGTGGAGCCAAACGGGTC-3′ Reverse: 5′-GGAGTTGCTGTTGAAGTCGCA-3′

Cell Counting Kit-8 (CCK-8) Assay

The cells were seeded into 96-well plate (4×10^3 cells/well) and transfected with the plasmids, then added with 10 μl of CCK-8 solution (GLP-BIO, Montclair, CA, USA) and incubated for two hours at day 1, 2, 3, and 4 after transfection. Optical density value at 450 nm was detected by a microplate reader (Bio-Rad, Hercules, CA, USA).

Clone Formation Assay

The cells (1×10^3) were seeded into 6-well plates and transfected the plasmids, then maintained with RPMI-1640 medium for two weeks until the cell clone was visible. Cells were then fixed in paraformaldehyde (PFA, Sigma-Alrdich, St. Louis, MO, USA) for 15 min and stained with 0.2% crystal violet (Solarbio, Beijing, China) for 20 min. Staining cells were washed in PBS and photographed using a camera.

Wound Healing Assay

Wound healing assay was performed to evaluate the cell migration capacity. 2×10^5 cells with or without transfection were seeded in 6-well plates and maintained overnight. When cells reached 85% confluence, cells were treated with 10 μg/ml mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) for 2 h. The monolayer cells were scratched with a 200-μl pipette tip on the bottom of each plate. Then, washed out the cell debris with PBS and the rest of the cells continued to be cultured in serum-free medium at 37°C with 5% CO_2. At 0 h and 24 h after scratch, images were captured using a microscope (Olympus, Tokyo, Japan).

Transwell Assay

Transwell assay was performed to measure the cell invasion capacity. The cells (5×10^4) were seeded in the top chamber (Millipore, Billerica, MA, USA) pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Serum-free culture medium was added into the top chambers while the bottom chambers were filled with 500 μl cultured medium containing 10% FBS. After incubation for 24 h, cells in the bottom chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet and counted under a microscope.

Flow Cytometry

Cell apoptosis was evaluated using Annexin V-FITC Apoptosis Detection Kit (Invitrogen, Carlsbad, CA, USA). In brief, the treated cells digested by 0.025% trypsin and washed with PBS. Then, the cells were collected and resuspended with binding buffer and then stained with 5 μl Annexin V-FITC and 10 μl propidium iodide (PI) in the dark for 15 min at room temperature. The samples were detected with a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Protein Co-Immunoprecipitation (Co-IP)

Cells were washed with PBS twice and then resuspended in non-denaturing Co-IP lysis buffer (50 mM Tris, pH 7.4, 1% Nonidet P-40, 0.25% deoxycholate Na, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT and 1× protease inhibitors). After centrifugation, the cell supernatant was incubated with the anti-Myc agarose beads overnight. The protein-antibody-bead complexes were rinsed with PBS and denatured with Laemmli buffer and the protein fractions were used for Western blot.

Tumor Xenograft

All animal experiments were performed according to the guideline for the care and use of laboratory animals with the approval of the Taizhou People's Hospital. Female athymic BALB/c mice (5-6 weeks old) were obtained from the animal

experimental center of the Taizhou People's Hospital (Taizhou, Jiangsu, China). A549 cells (5×10^6) stably transfected with shCtrl or shTM4SF1 were subcutaneously injected into either side of flanks of nude mice (n = 6 mice/group). Tumor volumes were measured every 3 days and the calculation formula was: volume= (length × width^2 × 0.5). Mice were euthanized 33 days after inoculation, and tumors were excised and weighed. The tumor tissues were fixed for subsequent IHC assay.

Statistical Analysis

Statistical Product and Service Solutions (SPSS, version 20.0, IBM Corp., Armonk, NY, USA) and GraphPad Prism 7 (San Diego, CA, USA) were used for data analysis. All results were shown as means ± SD, and mean values were compared using Student's *t*-test or one-way ANOVA. All experiments were repeated more than three times and *p*-value<0.05 was considered as statistically significant.

Results

TM4SF1 is Upregulated in NSCLC Tissues and Correlated with Poor Prognosis

QRT-PCR assay showed that TM4SF1 mRNA level was significantly higher expressed in NSCLC tissues as compared to that in non-tumor tissues (36 pairs; Figure 1A). The elevated TM4SF1 expression was also confirmed by Western blot and IHC assays (Figure1B-C). In addition, the relationship between TM4SF1 expression and clinicopathologic characteristics in 36 NSCLC patients was also analyzed. It was noteworthy that tumor size (T1-T2 *vs.* T3-T4) (p=0.019), lymph node metastasis (N0-N1 *vs.* N2-N3) (p=0.019), distant metastasis (M0 *vs.* M1) (p=0.040), and clinical stage (I-II *vs.* III-

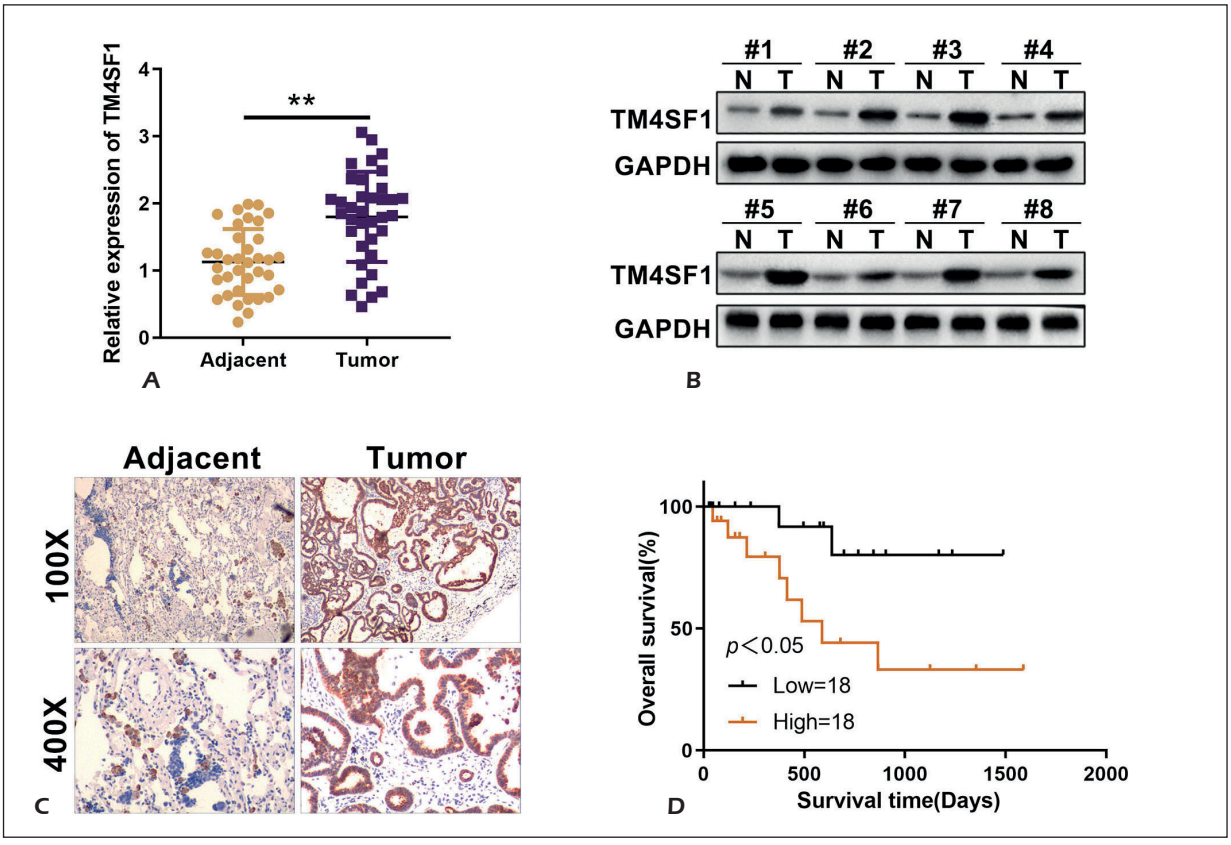

Figure 1. TM4SF1 is up-regulated in NSCLC tissues and correlated with poor prognosis. **A**, mRNA level of TM4SF1 in NSCLC tissues and normal adjacent tissues was measured by qRT-PCR (n=36). **B**, Protein level of TM4SF1 in NSCLC tissues and normal adjacent tissues was measured by Western blot (n=8). **C**, IHC staining on TM4SF1 in NSCLC and normal adjacent tissues. Representative images were shown. Magnification × 100, and magnification × 400. **D**, Kaplan-Meier curves for the overall survival rate in NSCLC patients according to the relative expression levels of TM4SF1 (dichotomized at the median). *p<0.05; **p<0.01.

IV) (p=0.003) were correlated with TM4SF1 expression (Table II). In contrast, there was no significant relationship between TM4SF1 expression and age (p=0.738), gender (p=0.729), smoking history (p=0.494) pathology (p=0.513), and differentiation (p=0.502) of patients. Kaplan-Meier analysis demonstrated that higher TM4SF1 expression was significantly correlated with shorter overall survival (p<0 .05; Figure 1D).

TM4SF1 Enhances NSCLC Cell Proliferation and Inhibits Cell Apoptosis

To further explore the potential role of TM4SF1 in the progression of NSCLC, we detected the mRNA and protein levels of TM4SF1 in five NSCLC cell lines (A549, H1975, SPC-A1, H1299, and H460) and human bronchial epithelioid cell line (16HBE) using qRT-PCR and Western blot assays. The results indicated that TM4SF1 was highly expressed in NSCLC cell lines. Particularly, A549 and H1299 displayed the highest TM4SF1 level, and H1975 and H460 displayed the lowest TM4SF1 level (Figure 2A-B). Thus, H1975 and H460 cell lines were transfected with pcDNA3.1-TM4SF1 for loss-of-function experiments, whereas A549 and H1299 cell lines were transfected with shTM4SF1 for gain-of-function experiments. The transfection efficiency was verified by Western blot assay, as illustrated in figure 2C, TM4SF1 was successfully overexpressed in both H1975 and H460 cells, and both shRNAs showed significant inhibition of TM4SF1. Indeed, shTM4SF1#2 was more efficient than shTM4SF1#1 in both A549 and SPC-A1 cells and thus chosen for following investigations.

CCK-8 assay revealed that overexpression of TM4SF1 dramatically promoted the proliferative ability of H1975 and H460 cells. Depletion of TM4SF1 suppressed the viability of A549 and H1299 cells (Figure 2D). The effect of TM4SF1 on promoting proliferation was also confirmed by clone formation assay (Figure 2E). By flow

Table II. Analgesic effect of midazolam combined with sufentanil in rats with pancreatitis.

Characteristics	No. of patients	TM4SF1 Low expression (≤ medin)	TM4SF1 High expression (≤ medin)	*p*-value
No.	36	18	18	
Ages (years)				0.738
<65	17	8	9	
≥65	19	10	9	
Gender				0.729
Female	13	7	6	
Male	23	11	12	
Smoking				0.494
Yes	22	12	10	
No	14	6	8	
Pathology				0.513
Squamous cell carcinoma	11	7	4	
Adenocarcinoma	22	10	12	
Others	3	1	2	
Differentiation				0.502
Well	8	5	3	
Moderately	17	9	8	
Lowly	11	4	7	
T classification				0.019
T1+T2	17	12	5	
T3+T4	19	6	13	
N classification				0.019
N0+N1	19	13	6	
N2+N3	17	5	12	
Distant metastasis				0.040
M0	22	14	8	
M1	14	4	10	
Clinical stage				0.003
I-II	17	13	4	
III-IV	19	5	14	

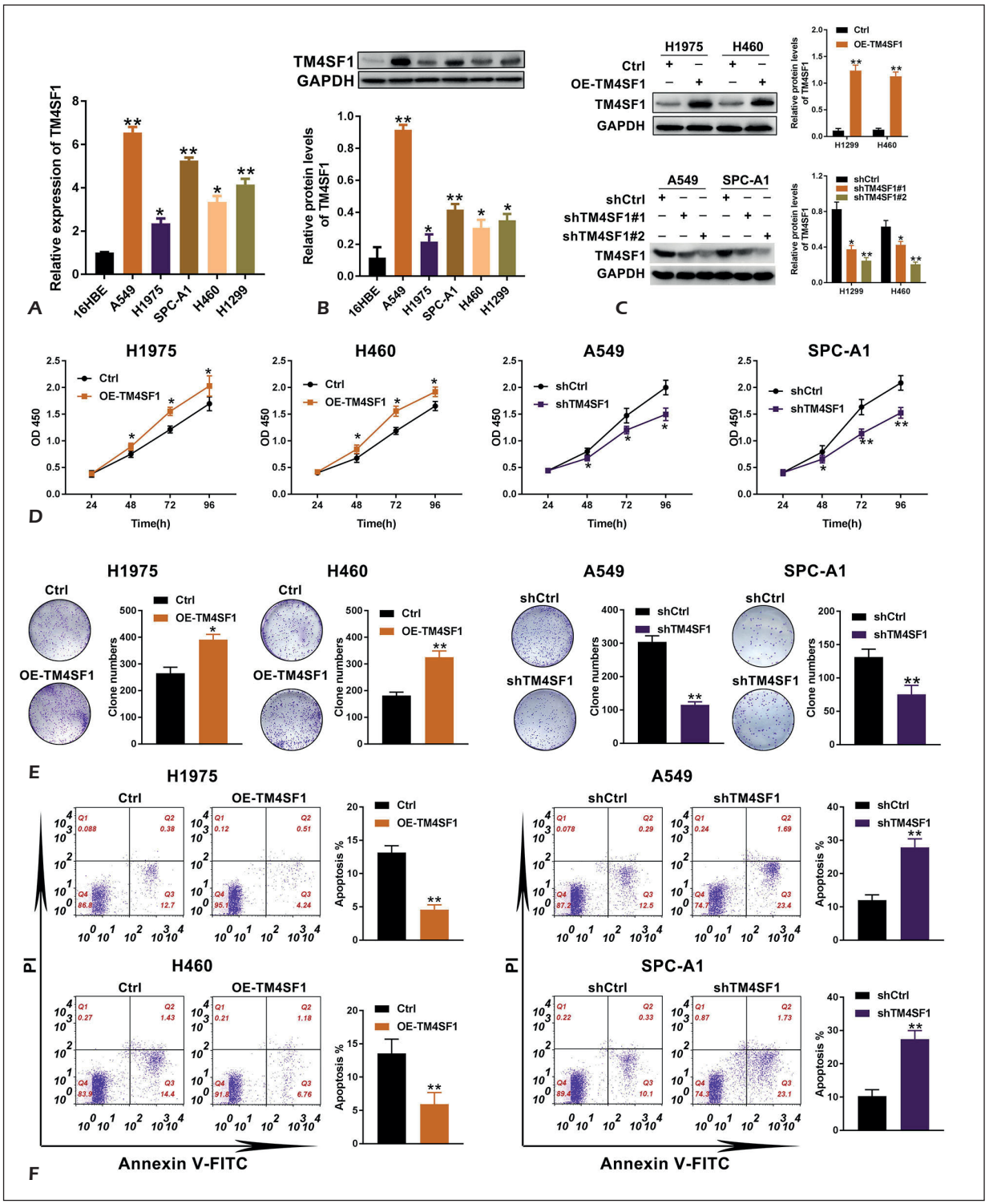

Figure 2. TM4SF1 enhances NSCLC cell proliferation and inhibits cell apoptosis. **A-B**, Expression of TM4SF1 in NSCLC cell lines and normal 16HBE cells was measured by qRT-PCR and Western blot. **C**, Transfection efficiency of TM4SF1 was evaluated by Western blot. **D**, Proliferation of NSCLC cells was assessed by CCK-8 assay. **E**, Clone forming assay was also performed to evaluate cell proliferation. **F**, Flow cytometry was used to assess cell apoptosis. *$p<0.05$; **$p<0.01$.

cytometry assay, overexpression of TM4SF1 reduced while knockdown of TM4SF1 enhanced the ratios of apoptotic cells (Figure 2F). These data indicated that TM4SF1 could promote proliferation and reduce apoptosis of NSCLC cells *in vitro*.

TM4SF1 Promotes NSCLC Cell Migration and Invasion

The effect of TM4SF1 on the migratory ability of NSCLC cells was measured by wound healing assay. By detecting the changes in the spacing between cells after scratching, we found that OE-TM4SF1 could accelerate migratory capacity whereas sh-TM4SF1 caused the opposite effect (Figure 3A). Meanwhile, transwell invasion assay revealed that OE-TM4SF1 significantly increased the number of invasive cells whereas sh-TM4SF1 clearly decreased the number of invasion cells (Figure 3B). Of note, these results indicated that TM4SF1 could increase migration and invasion of NSCLC cells.

TM4SF1 Enhances the Transcriptional Activity of YAP-TEAD Interaction Through Promoting YAP Expression

Western blot was performed to assess the effect of TM4SF1 on YAP expression. The results illustrated that the protein level of YAP was increased while p-YAP protein level was reduced by OE-TM4SF1 in H1975 cells. However, knockdown of YAP had the opposite effect in A549 cells (Figure 4A). Furthermore, Co-IP assay suggested that overexpression of TM4SF1 promoted the interaction between YAP and TEAD while TM4SF1 knockdown limited it (Figure 4B). The

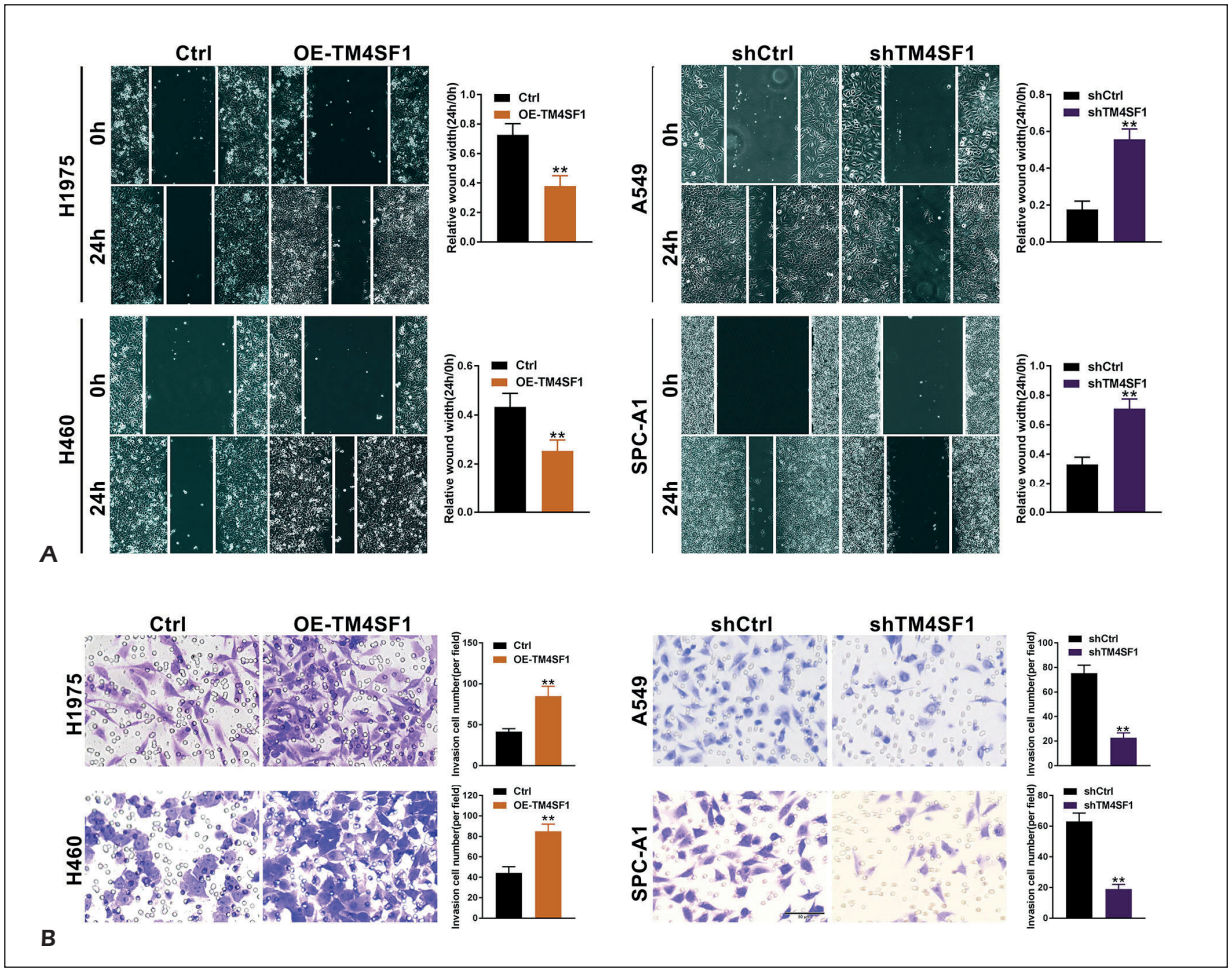

Figure 3. TM4SF1 promotes NSCLC cell migration and invasion. **A**, Migration of NSCLC cells was measured by wound healing assay. Magnification × 200. **B**, Invasion of NSCLC cells was assessed by transwell assay. Magnification × 400. **p*<0.05; ***p*<0.01.

results of qRT-PCR assay pointed out that the expression of transcriptional targets of YAP/TEAD pathway, CTGF, Cyr61, and Birc5, were elevated significantly with OE-TM4SF1 transfection in H1975 cells but decreased observably with shTM4SF1 transfection in A549 cells (Figure 4C). In general, the molecular mechanism underlying the role of TM4SF1 in NSCLC is dependent on the regulation in YAP-TEAD transcriptional activation.

TM4SF1 Regulates the Expression of Proteins Involved in Cell Proliferation, Apoptosis and Epithelial–Mesenchymal Transition (EMT) via YAP-TEAD Pathway

For further analysis, rescue experiments were performed in H1975 cells and divided into four groups: Ctrl, OE-TM4SF1, OE-TM4SF1 + shYAP, and OE-TM4SF1 + Peptide 17(YAP-TEAD protein-protein interaction inhibitor). As Figure 5A exhibited, the protein levels of p21, p27, cleaved

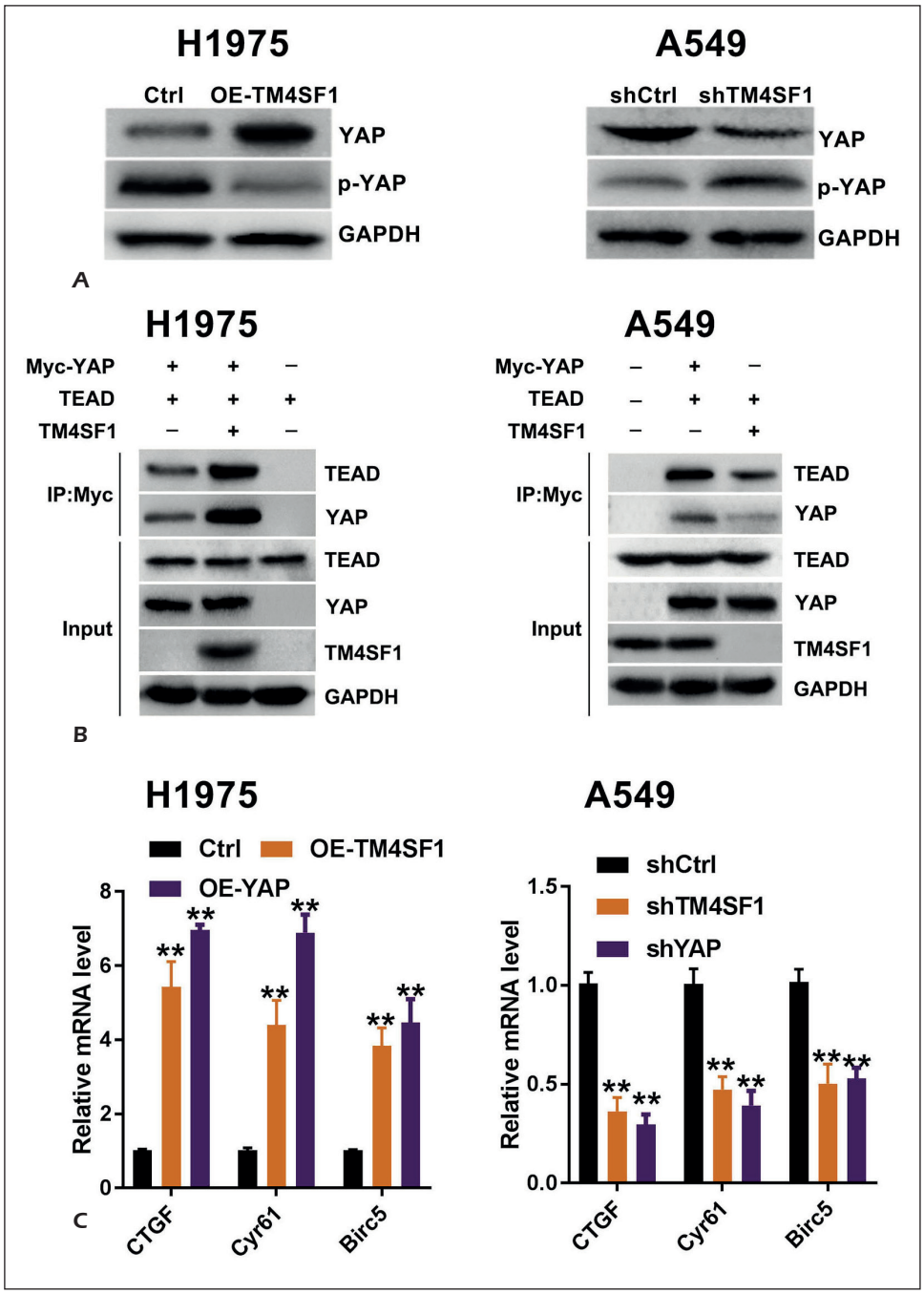

Figure 4. TM4SF1 enhances the transcriptional activity of YAP-TEAD interaction through promoting YAP expression. **A**, Protein levels of YAP and p-YAP in H1975 and A549 cells were measured by Western blot. **B**, Interaction between YAP and TEAD was assessed by Co-IP assay. **C**, Expression of CTGF, Cyr61, and Birc5 was measured by qRT-PCR. *p<0.05; **p<0.01.

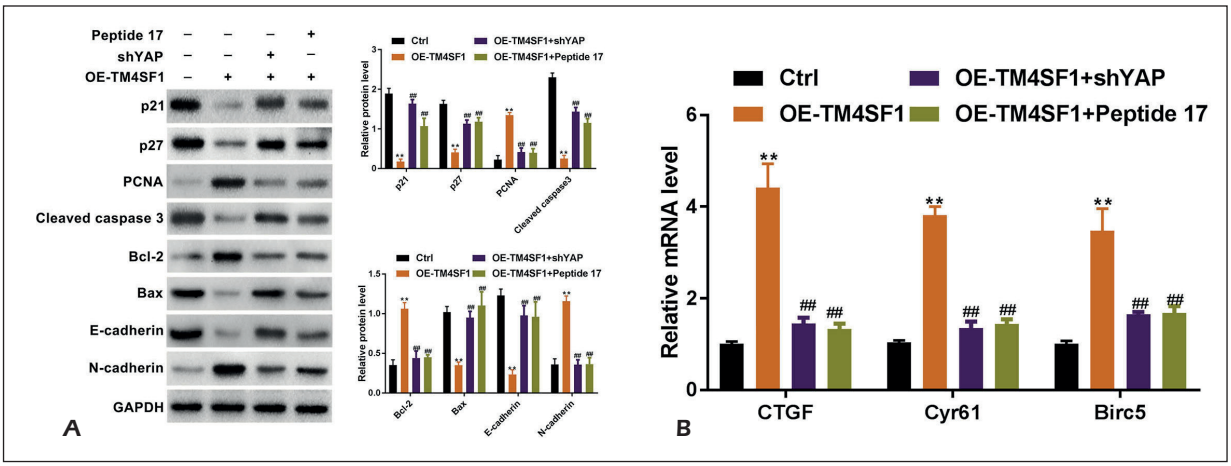

Figure 5. TM4SF1 regulates the expression of proteins involved in cell proliferation, apoptosis, and epithelial-mesenchymal transition (EMT) via YAP-TEAD pathway. **A**, Protein levels of p21, p27, PCNA, Cleaved caspase-3, Bcl-2, Bax, E-cadherin, and N-cadherin in H1975 cells were measured by Western blot. **B**, Expression of CTGF, Cyr61, and Birc5 was measured by qRT-PCR. *p<0.05; **p<0.01.

caspase 3, Bax, and N-cadherin were significantly decreased, while the protein levels of PCNA, Bcl-2, and E-cadherin were dramatically increased in the OE-TM4SF1 group in comparison to the Ctrl. However, above effects of TM4SF1 were reversed by simultaneous knockdown of YAP or using Peptide 17. QRT-PCR assay demonstrated that Peptide 17 and OE-TM4SF1 transfection partially abolished the promotive effects of TM4SF1 on the mRNA expression of CTGF, Cyr61, and Birc5 (Figure 5B). Together, these results suggested that TM4SF1 could regulate cell proliferation, apoptosis, and EMT through mediating YAP-TEAD pathway.

TM4SF1 Knockdown Inhibits Tumor Growth in NSCLC Xenograft Model

The effects of TM4SF1 on the NSCLC xenograft model were explored. A549 cells transfected with shCtrl or shTM4SF1 were subcutaneously injected into mice, and significant reductions in tumor volume and weight were observed in shTM4SF1 group (Figure 6A-B). IHC analysis of tumor sections indicated that shTM4SF1 decreased the expression of TM4SF1 and Ki-67 and increased the expression of E-cadherin and caspase 3 (Figure 6C). Taken together, these results suggested that TM4SF1 promoted tumor growth, and regulated apoptosis- and metastasis-related proteins *in vivo*, which was consistent with the *in vitro* results.

Discussion

Some studies have verified that TM4SF1 was over-expressed in various cancers and involved in tumor progression. Similarly, this research demonstrated that TM4SF1 expression was evaluated in NSCLC patients and cells. High TM4SF1 expression predicted poor prognosis, and was correlated with tumor size, lymph node metastasis, distant metastasis, and clinical stage. These investigations were consistent with the previous studies[12,13]. However, the underlying mechanism is still unclear. Here, this study confirmed for the first time that TM4SF1 could promote NSCLC cell proliferation, migration, and invasion *in vitro* and accelerate tumor growth *in vivo*. Hippo pathway (the main downstream effectors are YAP and TAZ) is a newly discovered pathway and has become a hotspot of research due to its role in regulating cancer growth and development[14,15]. In this research, mechanism experiments showed that TM4SF1 regulated tumor progression via raising the expression of YAP and enhancing YAP-TEAD interaction, then resulting in up-regulated expression of downstream target genes, CTGF, Cyr61, and Birc5.

TM4SF1 was first identified as a protein of the L6 family in epithelial cancer cells[16,17]. Several previous studies indicated that TM4SF1 exhibits carcinogenic function and is considered to be associated with malignant progression via different regulatory mechanisms. Allioli et al[8] demonstrated that TM4SF1 was a direct target gene for androgen

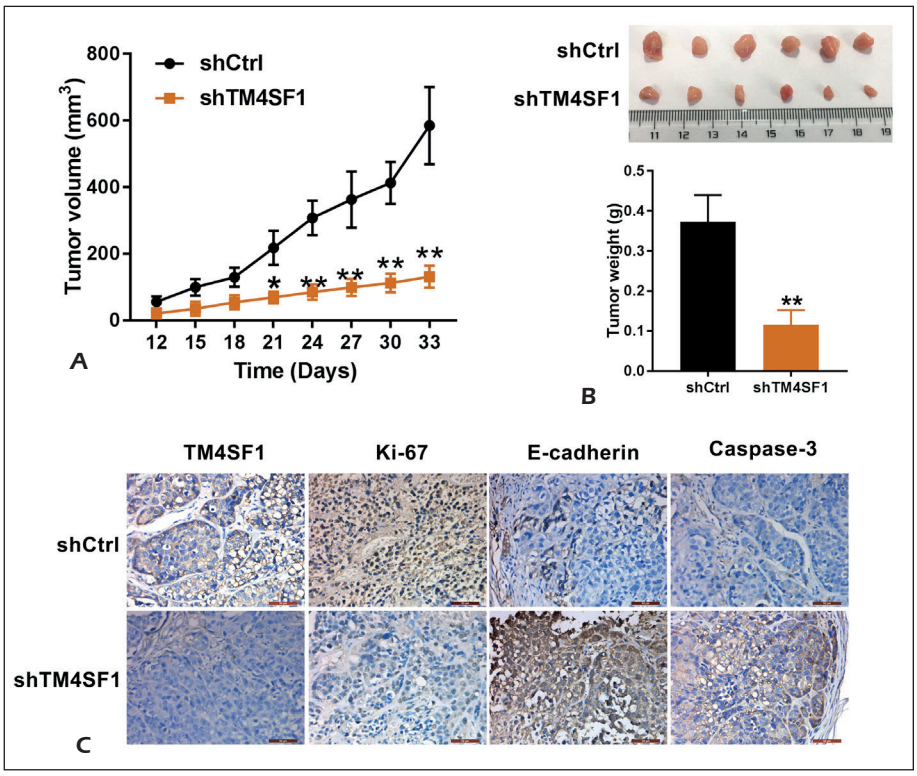

Figure 6. TM4SF1 knockdown inhibited tumor growth tumor growth in NSCLC xenograft model. **A-B**, Tumor weight and tumor volume were measured after TM4SF1 silencing. **C**, Expression of YAP, Ki-67, E-cadherin, and Cleaved caspase-3 in tumors was determined by IHC. Magnification × 200. *p<0.05; **p<0.01.

receptor and highly expressed in prostate cancer tissues, the inhibition of TM4SF1 inhibits cell migration. The role of TM4SF1 in liver cancer occurs through inhibiting cell apoptosis but increasing migration *in vitro*, and promoting tumor growth and metastasis *in vivo*[18,19]. Cao et al[20] indicated that the downregulation of TM4SF1 inhibited the migration and invasion of pancreatic cancer cells by regulating the expression and activity of MMP-2 and MMP-9. TM4SF1 also promoted breast cancer cell migration by regulating PI3K-AKT-mTOR pathway[9] and the expression of DDR1[21]. Cao et al[10] clarified the new function of TM4SF1 in regulating reactive oxygen metabolism through the feedback loop of PPARγ-SIRT1 in bladder cancer. In addition, TM4SF1 has been reported to promote drug resistance and self-renewal of stem cells in cancers[7,11]. This study revealed that TM4SF1 could promote NSCLC proliferation, migration, and invasion *in vitro*. In agreement with our *in vitro* findings, knockdown of TM4SF1 inhibited tumor growth and promoted the expression of apoptosis (caspase-3) and epithelial markers (E-cadherin).

The Hippo signaling pathway, also known as the Salvador/Warts/Hippo (SWH) pathway, is primarily derived from the protein kinase Hippo (Hpo) in Drosophila and is a key regulator in the pathway[22]. YAP, as an oncogene, is a transcriptional activator of the Hippo pathway, and abnormal Hippo-YAP pathway can lead to the imbalance of cell proliferation, apoptosis, and EMT[23-26]. YAP is firstly phosphorylated by activating MST1/2 -LATS1/2, which interacts with cytoskeletal proteins and is retained in the cytoplasm to perform its transcriptional activation function, thereby regulating the size and volume of organs[27-29]. Non-phosphorylated YAP is translocated to the nucleus and interacts with TEAD family transcription factors to stimulate the expression of apoptosis- and proliferation- genes, such as CTGF, Cyr61, and Birc5[30]. In addition, increasing evidence has confirmed that the Hippo signaling pathway plays an important role in cancer development, tissue regeneration, and functional regulation of stem cells that involved in the progression of lung cancer[27,31-34]. Our findings suggested that the potential mechanisms of TM4SF1 in regulating lung cancer progression occur through upregulating the expression of YAP and inhibiting p-YAP and enhancing the YAP-TEAD interaction.

Conclusions

We uncovered the biological and clinical significance of TM4SF1 in NSCLC. TM4SF1 increases NSCLC proliferation, migration, and in-

vasion, relying on the direct promotion of YAP and YAP-TEAD interaction. These results indicate that the targeting of the TM4SF1-YAP-TEAD axis may be a novel approach for inhibiting the growth and metastasis of NSCLC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

References

1) Takayuki N, Keiko T, Junji U, Yoshiko K, Nobuyo T, Tadaaki Y, Koichi T. Advanced non-small-cell lung cancer in elderly patients: patient features and therapeutic management. Biomed Res Int 2018; 2018.
2) Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394-424.
3) Goldstraw P, Ball D, Jett JR, Le Chevalier T, Lim E, Nicholson AG, Shepherd FA. Non-small-cell lung cancer. Lancet 2011; 378: 1727-1740.
4) Martin P, Leighl NB. Review of the use of pretest probability for molecular testing in non-small cell lung cancer and overview of new mutations that may affect clinical practice. Ther Adv Med Oncol 2017; 9: 405-414.
5) Wright MD, Ni J, Rudy GB. The L6 membrane proteins—a new four-transmembrane superfamily. Protein Sci 2000; 9: 1594-1600.
6) Xu L, Li Q, Xu D, Wang Q, An Y, Du Q, Zhang J, Zhu Y, Miao Y. hsa-miR-141 downregulates TM4SF1 to inhibit pancreatic cancer cell invasion and migration. Int J Oncol 2014; 44: 459-466.
7) Cao J, Yang J, Ramachandran V, Arumugam T, Deng D, Li Z, Xu L, Logsdon CD. TM4SF1 promotes gemcitabine resistance of pancreatic cancer in vitro and in vivo. PLoS One 2015; 10: e0144969.
8) Alllioli N, Vincent S, Vlaeminck GV, Decaussin PM, Ragage F, Ruffion A, Samarut J. TM4SF1, a novel primary androgen receptor target gene over-expressed in human prostate cancer and involved in cell migration. Prostate 2011; 71: 1239-1250.
9) Sun Y, Xu Y, Xu J, Lu D, Wang J. Role of TM4SF1 in regulating breast cancer cell migration and apoptosis through PI3K/AKT/mTOR pathway. Int J Clin Exp Pathol 2015; 8: 9081.
10) Cao R, Wang G, Qian K, Chen L, Ju L, Qian G, Wu CL, Dan HC, Jiang W, Wu M. TM4SF1 regulates apoptosis, cell cycle and ROS metabolism via the PPARγ-SIRT1 feedback loop in human bladder cancer cells. Cancer Lett 2018; 414: 278-293.
11) Xue L, Yu X, Jiang X, Deng X, Mao L, Guo L, Fan J, Fan Q, Wang L, Lu SH. TM4SF1 promotes the self-renewal of esophageal cancer stem-like cells and is regulated by miR-141. Oncotarget 2017; 8: 19274.
12) Ma YS, Yu F, Zhong XM, Lu GX, Cong XL, Xue SB, Xie WT, Hou LK, Pang LJ, Wu W. miR-30 Family Reduction Maintains Self-Renewal and Promotes Tumorigenesis in NSCLC-Initiating Cells by Targeting Oncogene TM4SF1. Mol Ther 2018; 26: 2751-2765.
13) Seo DC, Sung JM, Cho HJ, Yi H, Seo KH, Choi IS, Kim DK, Kim JS, Am EA, Shin HC. Gene expression profiling of cancer stem cell in human lung adenocarcinoma A549 cells. Mol Cancer 2007; 6: 75.
14) Harvey KF, Zhang X, Thomas DM. The Hippo pathway and human cancer. Nat Rev Cancer 2013; 13: 246.
15) Yu FX, Guan KL. The Hippo pathway: regulators and regulations. Genes Dev 2013; 27: 355-371.
16) Hellström I, Horn D, Linsley P, Brown J P, Brankovan V, Hellström KE. Monoclonal mouse antibodies raised against human lung carcinoma. Cancer Res 1986; 46: 3917-3923.
17) O'Donnell RT, DeNardo SJ, Shi XB, Mirick GR, DeNardo GL, Kroger LA, Meyers FJ. L6 monoclonal antibody binds prostate cancer. Prostate 1998; 37: 91-97.
18) Huang YK, Fan XG, Qiu F. TM4SF1 promotes proliferation, invasion, and metastasis in human liver cancer cells. Int J Mol Sci 2016; 17: 661.
19) Du X, Fan W, Chen Y. microRNA-520f inhibits hepatocellular carcinoma cell proliferation and invasion by targeting TM4SF1. Gene 2018; 657: 30-38.
20) Cao J, Yang JC, Ramachandran V, Arumugam T, Deng DF, Li ZS, Xu LM, Logsdon C D. TM4SF1 regulates pancreatic cancer migration and invasion in vitro and in vivo. Cell Physiol Biochem 2016; 39: 740-750.
21) Yang JC, Zhang Y, He SJ, Li MM, Cai XL, Wang H, Xu LM, Cao J. TM4SF1 promotes metastasis of pancreatic cancer via regulating the expression of DDR1. Sci Rep 2017; 7: 45895.
22) Harvey K, Tapon N. The Salvador–Warts–Hippo pathway—an emerging tumour-suppressor network. Nat Rev Cancer 2007; 7: 182.
23) Ling HH, Kuo CC, Lin BX, Huang YH, Lin CW. Elevation of YAP promotes the epithelial-mesenchymal transition and tumor aggressiveness in colorectal cancer. Exp Cell Res 2017; 350: 218-225.
24) Liu Y, He K, Hu Y, Guo X, Wang D, Shi W, Li J, Song J. YAP modulates TGF-β1-induced simultaneous apoptosis and EMT through upregulation of the EGF receptor. Sci Rep 2017; 7: 45523.
25) Marti P, Stein C, Blumer T, Abraham Y, Dill MT, Pikiolek M, Orsini V, Jurisic G, Megel P, Makowska Z. YAP promotes proliferation, chemoresistance, and angiogenesis in human cholangiocarcinoma through TEAD transcription factors. Hepatology 2015; 62: 1497-1510.
26) Basu S, Totty NF, Irwin MS, Sudol M, Downward J. Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis. Mol Cell 2003; 11: 11-23.

27) Zhao B, Tumaneng K, Guan KL. The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. Nat Cell Biol 2011; 13: 877.
28) Halder G, Dupont S, Piccolo S. Transduction of mechanical and cytoskeletal cues by YAP and TAZ. Nat Rev Mol Cell Biol 2012; 13: 591.
29) Hao Y, Chun A, Cheung K, Rashidi B, Yang X. Tumor suppressor LATS1 is a negative regulator of oncogene YAP. J Biol Chem 2008; 283: 5496-5509.
30) Zhao B, Ye X, Yu J, Li L, Li W, Li S, Yu J, Lin JD, Wang CY, Chinnaiyan AM. TEAD mediates YAP-dependent gene induction and growth control. Genes Dev 2008; 22: 1962-1971.
31) Pan D. The hippo signaling pathway in development and cancer. Dev Cell 2010; 19: 491-505.
32) Yang W, Han W, Qin A, Wang Z, Xu J, Qian Y. The emerging role of Hippo signaling pathway in regulating osteoclast formation. J Cell Physiol 2018; 233: 4606-4617.
33) Andl T, Zhang Y. Reaping Wnt after calming Hippo: Wnt and Hippo signaling cross paths in lung cancer. J Thorac Dis 2017; 9: 4174.
34) Lu T, Li Z, Yang Y, Ji W, Yu Y, Niu X, Zeng Q, Xia W, Lu S. The Hippo/YAP1 pathway interacts with FGFR1 signaling to maintain stemness in lung cancer. Cancer Lett 2018; 423: 36-46