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TM4SF1 promotes glioma malignancy through multiple mechanisms

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Transmembrane-4 L Six Family member 1 (TM4SF1) belongs to a family of integral membrane proteins implicated in cell growth and tumor progression. Glioma is the most common and aggressive malignant brain tumor in adults. In this study, we showed that TM4SF1 was highly expressed in glioma tumor tissues and cell lines. The expression levels of TM4SF1 were negatively correlated with patients' survival rates. Silencing TM4SF1 by RNA interference inhibited the proliferation, migration, and invasion of glioma cells. Moreover, TM4SF1 silencing induced glioma cell cycle arrest and early apoptosis. In contrast, overexpression of TM4SF1 in glioma cells exhibited the opposite effects. Mechanistically, we found that loss of TM4SF1 reduced phospho-ATK, Cyclin D1, Bcl-2, and MMP-9 levels in glioma cells. Taken together, these findings provide novel insights into glioma pathogenesis and suggest that TM4SF1 may represent a novel target for glioma intervention.

Key words: glioma; TM4SF1; cell proliferation; migration; invasion

Glioma is the most common and aggressive malignant brain tumor in adults. At present, the treatment for glioma is surgical removal followed by chemotherapy and radiotherapy [1–3]. Despite much progress in the diagnosis and treatment, glioma remains one of the deadliest malignant tumors due to its high proliferation, invasiveness, and therapeutic resistance [4, 5]. Therefore, it is urgent to study its pathogenesis and explore new targets for better treatment.

Transmembrane-4 L Six Family member 1 (TM4SF1), also known as tumor-associated antigen L6, is abundantly expressed in the lung, breast, colon, and ovarian carcinomas [6]. The L6 antigen is a 24 kDa protein and belongs to a family of integral membrane proteins implicated in cell growth [7]. Previous studies have shown that overexpression of TM4SF1 enhances tumor growth, promotes cell migration and invasion, and shows the opposite effect by suppressing TM4SF1 in various malignant tumors, such as liver, lung, pancreatic, and esophageal cancer [8–11]. Knockdown of TM4SF1 by siRNA inhibits cell proliferation but promotes apoptosis by reducing the expression of Bcl-2 and increasing the expression of caspase 3 and Bax in gastric cancer cells [12]. TM4SF1 may interact with DDR1, and both of them participate in the formation and function of invadopodia that support cell migration and invasion in pancreatic cancer [13]. Furthermore, TM4SF1 is upregulated in 209 invasive breast cancer patients and may serve as a poor prognostic indicator in invasive breast cancer [14]. Wang's study showed that TM4SF1 is overexpressed in human glioma tissues relative to control brain tissues and high expression of TM4SF1 increases with ascending tumor grade and leads to poor survival [15]. These studies suggest TM4SF1 may be a potential target for anti-invasion/metastasis in glioma.

So far, the underlying mechanisms of TM4SF1-promoted glioma progression are not clear. In this study, we further studied the role of TM4SF1 in proliferation, migration, invasion, cell cycle, and apoptosis in glioma, and explored the molecular mechanism, in order to provide experimental evidence for clinical diagnosis and treatment of glioma.

Materials and methods

Cell lines and culture conditions. The glioma cell lines (U87, U251, U373, A172, SNB19, SF295, LN428, DBTRG, T98G, SW1783, and LN229) and immortalized normal human astrocytes (NHA) were purchased from the Chinese Academy of Sciences cell bank and were maintained in Dulbecco's modified Eagle's medium (DMEM, HyClone)

supplemented with 10% fetal bovine serum (FBS, BI) at 37 °C in a humidified incubator with 5% CO_2.

Gene transduction. Two pairs of siRNAs that targeted TM4SF1 were designed and synthesized: siTM4SF1#1: 5'-GGCUCUUGGUGGAAUUGAATT-3' (forward), 5'-UUCAAUUCCACCAAGAGCCTT-3' (reverse); siTM4SF1#2: 5'-GCUCUCACCAACAGCAAUATT-3' (forward), 5'-UAUUGCUGUUGGUGAGAGCTT-3' (reverse). The negative control siRNA (siNC) was scrambled and the sequence was: 5'-UUCUCCGAACGUGUCACGUTT-3' (forward), 5'-ACGUGACACGUUCGGAGAATT-3' (reverse). For transient transfections, the cells were cultured to 60–80% confluency and transfected with 20 nM siRNA with Lipofectamine ®RNAiMAX (Thermo Fisher) according to the manufacturer's instructions. The transfected cells continued to grow for 48 h before further experiments.

The full-length TM4SF1 coding sequence was cloned into the pLV [Exp]-EGFP: T2A: Puro plasmid (VectorBuilder China) to produce a TM4SF1 expression vector of pLV-EGFP-TM4SF1. The plasmid pLV-EGFP-TM4SF1 and the control plasmid pLV-EGFP-Null were transfected into cells as mentioned above.

Real-Time PCR. Total RNA was isolated from glioma cell lines and reverse transcribed using cDNA Reverse Transcription Kit HiScript® III All-in-one RT SuperMix Perfect (Vazyme) for qPCR. The mRNA expression of GAPDH and TM4SF1 was measured by using a ChamQ SYBR qPCR Master Mix (Vazyme) according to the manufacturer's instructions. The primer for GAPDH was 5'-AGAAGGCTGGGGCTCATTTG-3' (forward), 5'-AGGGGCCATCCACAGTCTTC-3' (reverse), and the primer for TM4SF1 was 5'-TCCTGCCAGCATTTGTCTTC-3' (forward), 5'-CTGCCACAATGACACAGTAG-3' (reverse).

Cell proliferation assay. Cell proliferation was analyzed using the Cell Counting Kit-8 (CCK8, Beyotime) according to the manufacturer's protocols. Cells were plated on 96-well plates and grown in a 10% serum-containing medium. Cell numbers were estimated at day 0, 1, 2, 3, and 4. 10 μl CCK-8 solution was added to each well and incubated for 2 h in an atmosphere containing 5% CO_2 at 37 °C. Then the absorbance was measured at 450 nm using a multifunctional microplate reader (Infinite 200 PRO, Tecan).

Wound-healing assay. Cells were seeded in 6-well plates and incubated at 37 °C until 90% confluent. The wounds were created by scratching using a sterile 200 μl pipette tip and the cells were cultured in serum-free DMEM for 24 h. The images of 0 h and 24 h were taken at the same location. The width of the wound at 0 h (Width 1) and 24 h (Width 2) was measured and the cell migration efficiency was calculated. Cell migration efficiency = (Width 1–Width 2)/Width 1×100%.

Cell invasion assay. Before the cell invasion study, the Transwell chambers (8 μm pore size) were coated with Matrigel (Corning). Cells were suspended in a 200 μl serum-free DMEM and added into the upper chamber with a density of 3×10^4 cells/insert, while the lower chamber was filled with 500 μl DMEM containing 10% FBS. Cells were allowed to invade the lower chamber for 24 h. The invaded cells on the bottom surface were fixed with 4% paraformaldehyde and stained with 1% crystal violet. The pictures were taken, and the invaded cells were counted in at least eight randomly selected fields in each membrane.

Flow cytometry analysis (FACS) of the cell cycle and apoptosis. For the cell cycle analysis, the cells were collected and fixed in 75% ice-cold ethanol at 4 °C overnight. Then the cells were incubated with 100 μg/ml RNase A at 37 °C for 30 min, and stained with 50 μg/ml PI at 4 °C in the dark for 30 min and detected using a flow cytometer (Cytoflex, Beckman).

The Annexin-V FITC Apoptosis Detection Kit (BD Biosciences) was used to measure cell apoptosis. The cells were collected and suspended in 1× binding buffer, then stained with Annexin V-FITC in dark for 15 min and PI for 5 min at room temperature. After that, apoptosis was detected by FACS.

Western blot. Cells were lysed in RIPA buffer (Beyotime) mixed with PMSF (100×), protease inhibitor cocktail (100×, CST), and PhosSTOP (Roche). 30 μg protein of each sample was separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with rabbit anti-TM4SF1 (Abcam, #ab113504, 1:1000 dilution), rabbit anti-p-AKT (Selleck, #A5030, 1:1000 dilution), rabbit anti-AKT (CST, #4691, 1:1000 dilution), rabbit anti-Cyclin D1 (Selleck, #A5035, 1:1000 dilution), rabbit anti-Bcl-2 (Selleck, #A5010, 1:1000 dilution), rabbit anti-MMP-9 (Beyotime, #AF5234, 1:500 dilution). and rabbit anti-β-actin (CST, #4967, 1:8000 dilution) overnight at 4 °C, followed by an incubation with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Proteintech, #SA00001-2, 1:5000 dilution) for 1 h at room temperature. The signals were detected using an ECL kit (Millipore) and a chemiluminescent image system (GeneGnome XRQ, Syngene).

Statistical analysis. All the data were representative of three independent experiments and the results are shown as mean±standard deviation (SD). Differences among groups were analyzed by Student's t-test. Statistical analysis was performed with SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA). The graph was made by GraphPad Prism version 6.0. A value of $^*p<0.05$, $^{**}p<0.01$, or $^{***}p<0.001$ was considered statistically significant.

Results

TM4SF1 is highly expressed in glioma tissues and in cell lines and its expression is inversely correlated with patients' survival rate. We first detected the level of TM4SF1 expression in glioma cell lines by western blot. TM4SF1 level was higher in 10 of the 11 (90.91%) glioma cell lines than that in NHA (Figure 1A). By using the Cancer Genome Atlas (TCGA) dataset, we analyzed the mRNA expression levels

and found that TM4SF1 was upregulated in all of the four glioblastoma (GBM) subtypes when compared to the normal brain tissues (Figure 1B), with higher levels in mesenchymal and proneural subtypes (*p<0.05, **p<0.01, compared with normal). In addition, the Chinese Glioma Genome Atlas (CGGA) dataset showed that high expression levels of TM4SF1 are associated with poor prognosis of glioma patients (Figure 1C).

TM4SF1 enhances glioma cell proliferation, migration, and invasion. To investigate the functional role of TM4SF1 in glioma cancer cells, we first did loss-of-function studies using U87 and A172 cell lines, which express the higher level of endogenous TM4SF1 (Figure 1A). The two cell lines were transiently transfected with siRNA (including siNC, siTM4SF1#1, or siTM4SF1#2) and confirmed silencing of TM4SF1 in the cells by qPCR. As shown in Figure 2A, TM4SF1 mRNA expression in U87 and A172 cells was downregulated effectively in both siTM4SF1#1 and siTM4SF1#2 groups (***p<0.001) compared with the WT group, while not reduced in the siNC group. We also examined the expression of TM4SF1 in the two cell lines by western blot. The siRNA siTM4SF1#1 and siTM4SF1#2 significantly downregulated the TM4SF1 expression in these two cell lines (Figure 2B). CCK-8 assay showed that TM4SF1 knockdown markedly inhibited cell proliferation in siTM4SF1#1 and siTM4SF1#2 groups in both of the two cell lines, and the cell viability decreased significantly from day 2 (***p<0.001, Figure 2C). To assess the role of TM4SF1 on cell migration, we plated the U87 and A172 cells, which were transfected with siRNA, on 6-well plates. The wound was created when cells grew to 90% confluency. After 24 h, we observed that the width of the scratch in the siTM4SF1#1 and siTM4SF1#2 group was much wider than that of the WT group (Figure 3A). The cell migration efficiency was calculated as the ratio of migrated distance to scratch width. As is shown in Figure 3A, the U87 cell migration efficiency was 89%, 90%, 54% (**p<0.01), and 75% (*p<0.05) in WT, siNC, siTM4SF1#1, and siTM4SF1#2 group, respectively. While in A172 cells, the data was 59%,

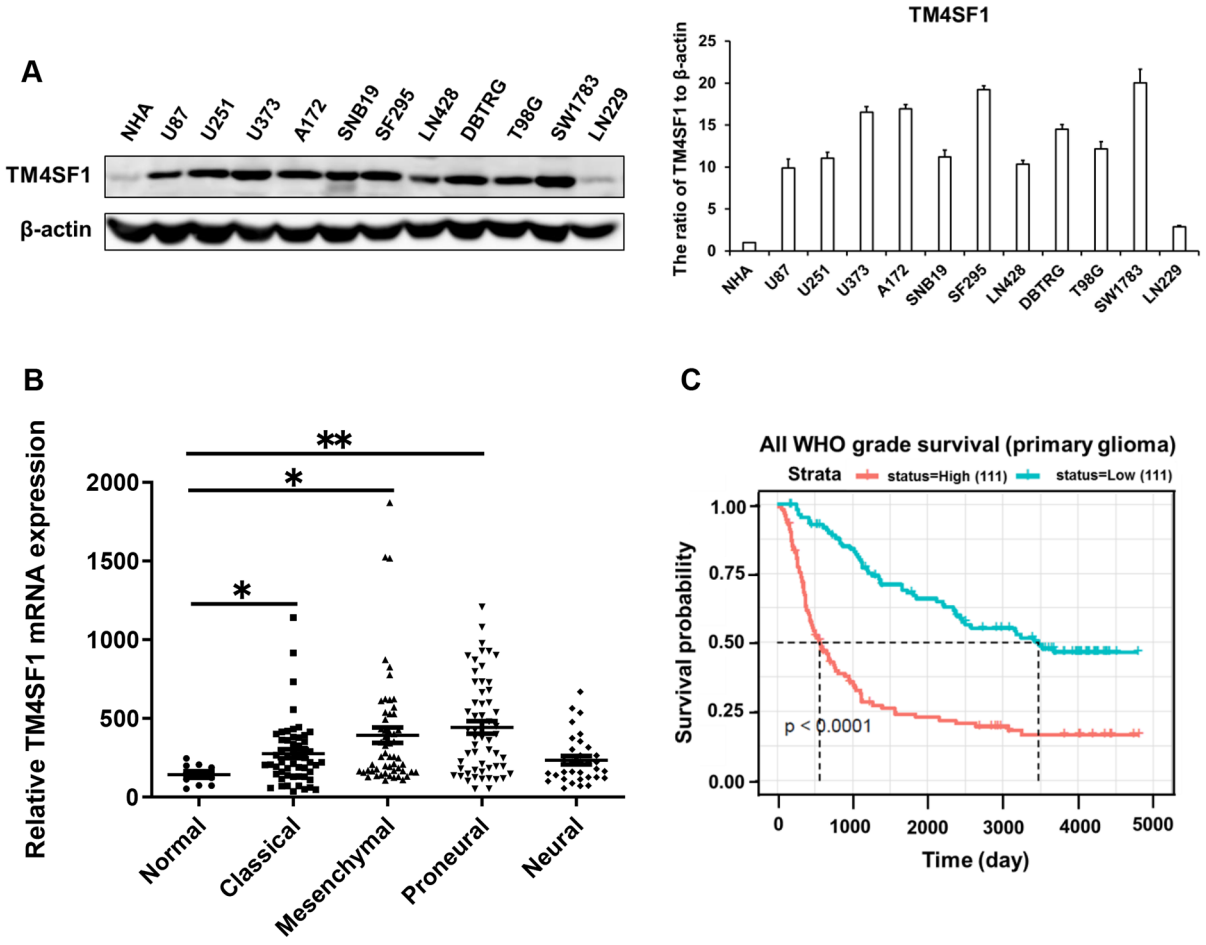

Figure 1. TM4SF1 is upregulated in glioma cell lines and predicts a poor prognosis. A) Western blot analysis of TM4SF1 expression in human glioma cell lines and immortalized normal astrocytes NHA. β-actin was used as a loading control. B) TCGA dataset analysis of the TM4SF1 mRNA expression levels in GBM subtypes and normal brain tissues. *p<0.05, **p<0.01, compared with normal. C) CGGA dataset analysis of the relationship between the expression levels of TM4SF1 and the prognosis of glioma patients.

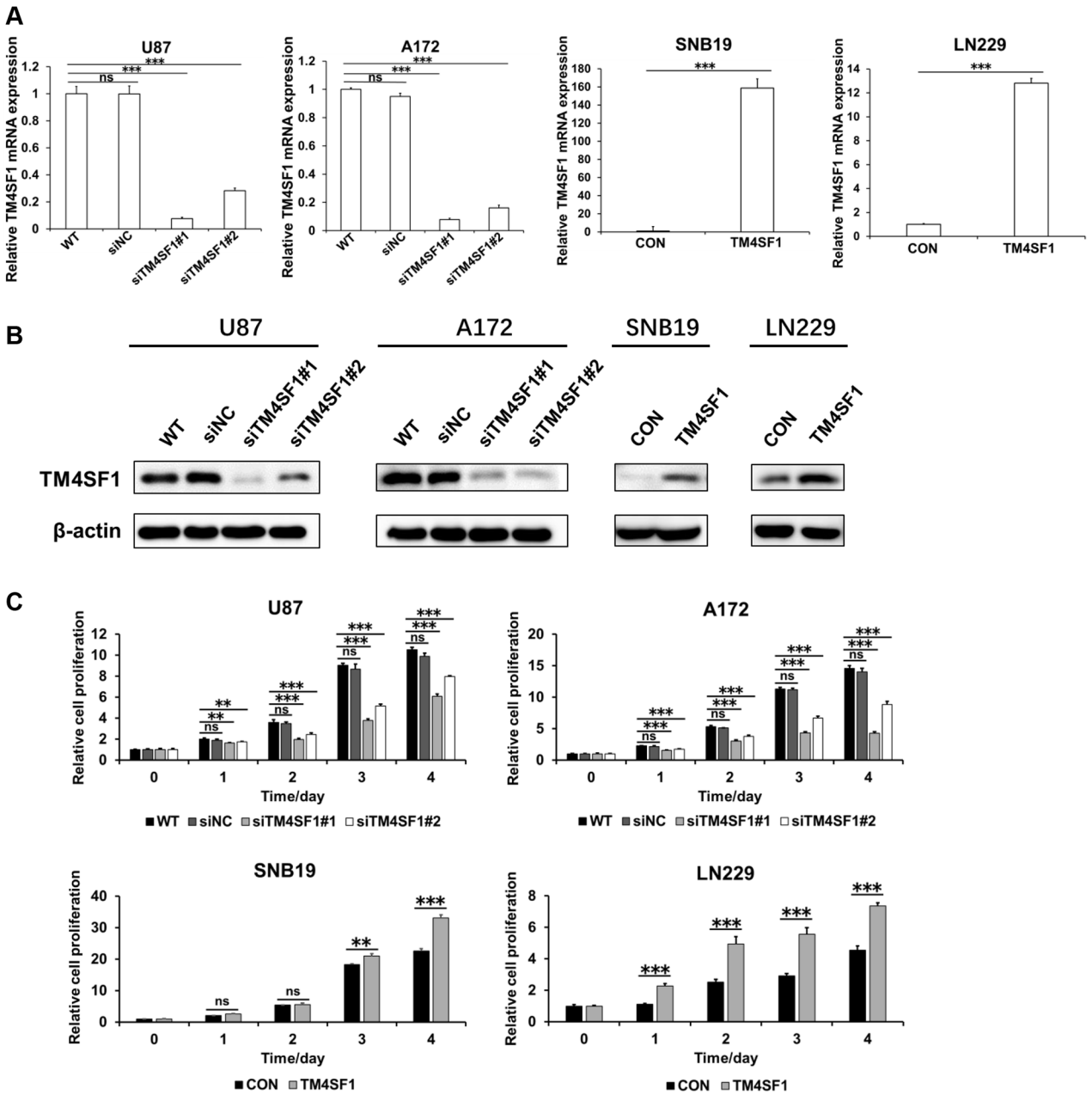

Figure 2. TM4SF1 enhances glioma cell proliferation. qPCR (A) and western blot (B) analysis of TM4SF1 expression in U87 and A172 cells transfected with siRNA (including siNC, siTM4SF1#1, or siTM4SF1#2), as well as SNB19 and LN229 cells transfected with pLV-EGFP-Null or pLV-EGFP-TM4SF1. GAPDH was used as a loading control in qPCR and β-actin in western blot. C) Effect of the TM4SF1 knockdown or overexpression on glioma cell proliferation. **p<0.01, ***p<0.001

60%, 38% (**p<0.01), and 41% (*p<0.05), respectively. Thus, the wound-healing assay showed that the cell migration efficiency was significantly reduced by the knockdown of TM4SF1 in siTM4SF1#1 and siTM4SF1#2 groups. Moreover, the invasion assay was performed. As shown in Figure 3C, the cells invaded the lower chamber in siTM4SF1#1 and siTM4SF1#2 group much less than that in group WT and group siNC. The invaded cell number was 366±23 per field for the WT group and 362±34 for the siNC group in U87 cells, while 113±5 (**p<0.01) and 170±6 (**p<0.01) for the siTM4SF1#1 and siTM4SF1#2 group, respectively. As for the A172 cells, the invaded cell number was 81±4, 80±3, 13±2 (***p<0.001), and 31±7 (***p<0.001) for WT, siNC, siTM4SF1#1, and siTM4SF1#2, respectively (Figure 3C).

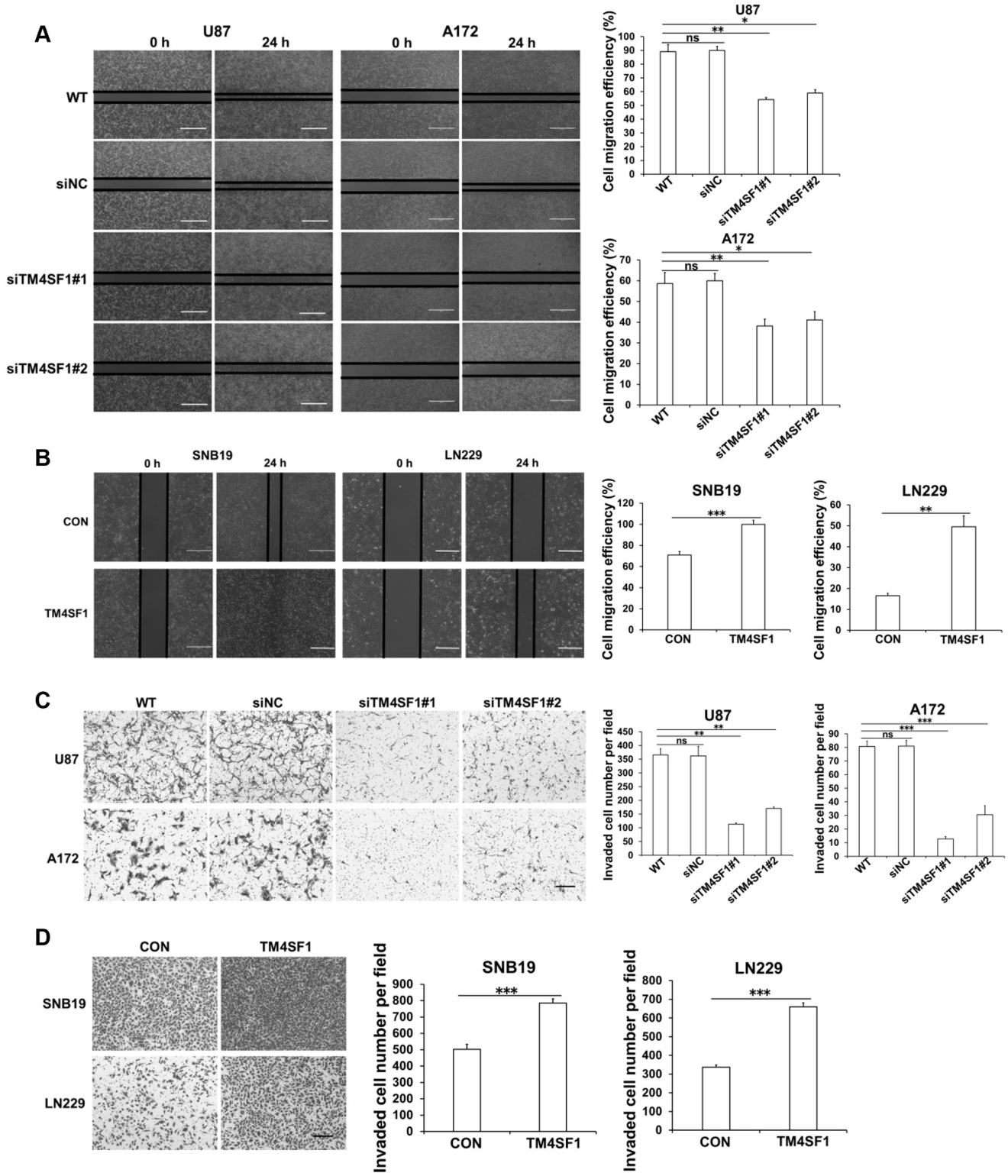

Figure 3. TM4SF1 promotes glioma cell migration and invasion. A, B) Effect of TM4SF1 knockdown or overexpression on glioma cell migration assessed by wound-healing assay after 24 h. Cell migration efficiency = (Width 1–Width 2)/Width 1×100%. C, D) Effect of TM4SF1 on the cell invasion by the Matrigel Transwell assay, and the invaded cell number was counted and shown as a bar graph. Scale bar: 1000 μm (A, B), 100 μm (C, D). *p<0.05, **p<0.01, ***p<0.001

To further validate the role of TM4SF1 in glioma cells, we also performed gain-of-function studies by using SNB19 and LN229 cell lines, which expressed relatively lower levels of TM4SF1 (Figure 1A). The cells were transfected with pLV-EGFP-TM4SF1 and named SNB19-TM4SF1 or LN229-TM4SF1, while the cells transfected with the control plasmid pLV-EGFP-Null were named SNB19-CON or LN229-CON. And the TM4SF1 expression level was significantly higher in SNB19-TM4SF1 and LN229-TM4SF1 than that in CON confirmed by qPCR and western blot (Figures 2A, 2B). Importantly, we found that the overexpression of TM4SF1 significantly promoted the proliferation, migration, and invasion of SNB19 and LN229 cells (Figures 2C, 3B, 3D). The results suggested that the overexpression of TM4SF1 enhances glioma cells proliferation, migration, and invasion.

Knockdown of TM4SF1 induces cell cycle arrest and early apoptosis. In order to clarify whether TM4SF1 expression could affect the cell cycle, we performed the flow cytometry analysis (Figure 4A). The flow cytometry results showed the percentage of the G0/G1 phase was 72.2±1.25% and 69.18±5.01% in the WT and siNC group of U87 cells, respectively, while that of siTM4SF1#1 and siTM4SF1#2 group increased to 79.73±3.06% (*p<0.05) and 80.63±4.52% (*p<0.05), respectively. In A172 cells, the percentage of G0/

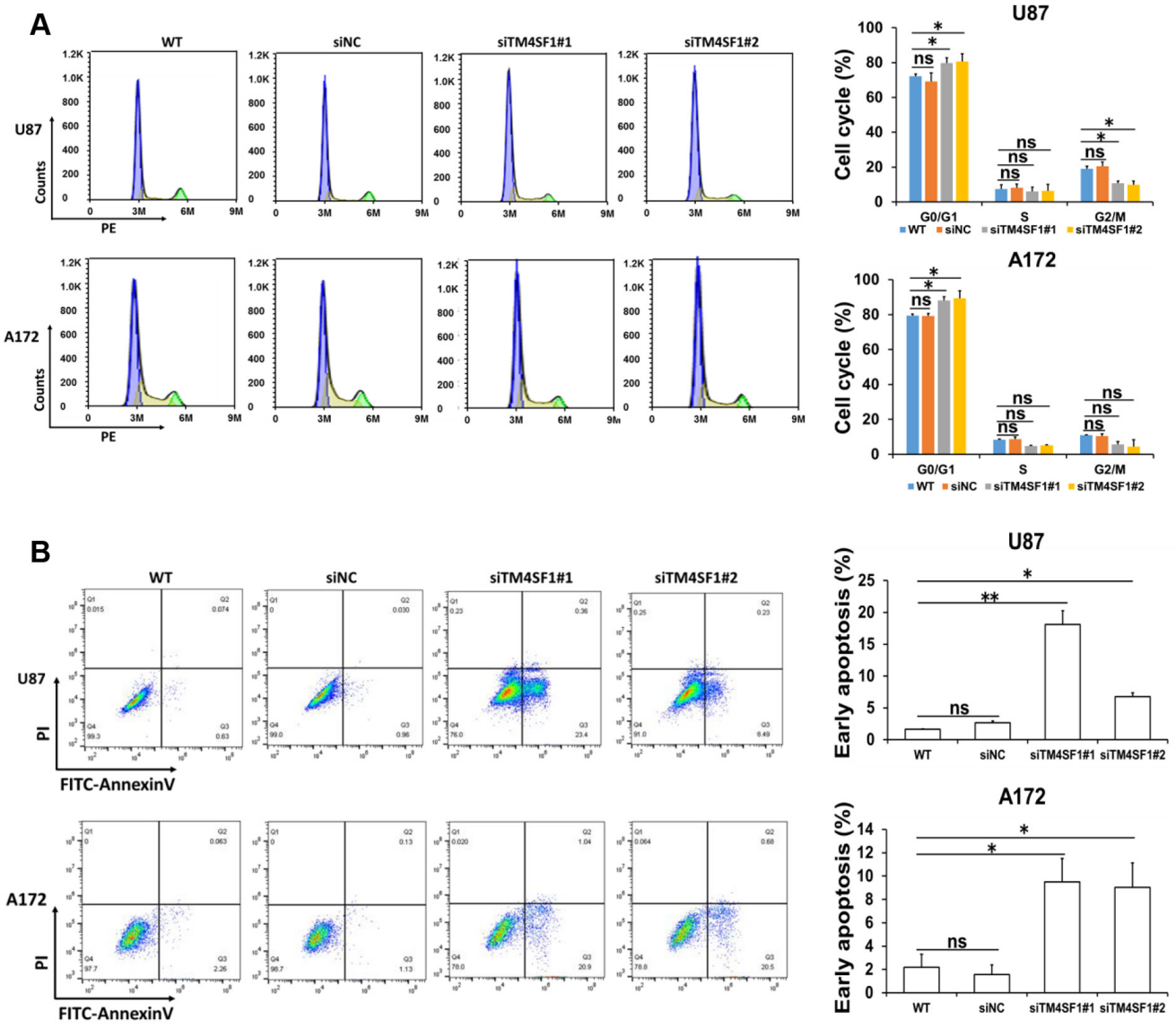

Figure 4. Knockdown of TM4SF1 induces cell cycle arrest and early apoptosis. A) The flow cytometric analysis histograms of the cell cycle are shown and the percentage of cells at the G0/G1, S, and G2/M phase is shown as a bar graph. B) The scatter diagrams of cell apoptosis are shown and the percentage of early apoptosis is shown as a bar graph. *p<0.05, **p<0.01

G1 was 79.39±0.93% in the WT group and 79.21±1.51% in the siNC group, and that increased to 88.06±2.15% (*p<0.05) in the siTM4SF1#1 group and 89.35±4.26% (*p<0.05) in the siTM4SF1#2 group (Figure 4A). The apoptosis detected by FACS showed that in the WT group of U87 cells, the early apoptotic rate was 1.63±0.08%, and in siTM4SF1#1 and siTM4SF1#2 group was 18.12±2.17% (**p<0.01) and 6.75±0.59% (*p<0.05), respectively. In A172 cells, the percentage of early apoptosis was 9.51±2.01% (*p<0.05) and 9.03±2.09% (*p<0.05) in siTM4SF1#1 and siTM4SF1#2 group, respectively, which was significantly higher than in the WT group (2.19±1.12%) (Figure 4B). Taken together, the data suggested that the silencing of TM4SF1 induces cell cycle arrest at the G0/G1 phase and early apoptosis.

TM4SF1 regulates glioma cells proliferation, migration, and invasion through multiple mechanisms. Finally, we sought to identify the TM4SF1-regulated signaling pathways involved in glioma pathogenesis. AKT has been reported to participate in the glioma cells invasion [16–17]. We examined the related molecules of the major signaling pathways involved in glioma pathogenesis by western blot. As shown in Figure 5, the siRNA siTM4SF1#1 and siTM4SF1#2 significantly suppressed the protein level of TM4SF1 and p-AKT both in U87 and A172 cells. In addition, we observed downregulation of the expression of the G1/S-specific Cyclin D1, anti-apoptotic protein Bcl-2, and MMP-9, which participates in cell migration and invasion when TM4SF1 was silenced in both U87 and A172 cells. Consistent with this, the overexpression of TM4SF1 induced higher expression of p-AKT, Cyclin D1, Bcl-2, and MMP-9 in SNB19 and LN229 cells. Thus, these results suggested that TM4SF1 promotes glioma cells proliferation, migration, and invasion through multiple mechanisms.

Discussion

In recent years, more and more reports show that the TM4SF1 gene is involved in the migration and invasion of various cancers. It was found that the expression of TM4SF1 in non-small-cell lung cancer (NSCLC) tissue is significantly higher than that in normal tissue, and the prognosis of patients was worse. TM4SF1 promotes proliferation, migration, invasion, and anti-apoptotic ability of NSCLC cells through the YAP-TEAD signaling pathway [18]. It was found that miR-141 could downregulate the expression of TM4SF1 and inhibit the invasion and migration of pancreatic cancer cells but has no significant effect on proliferation and cycle progression [19]. Morphological studies showed that the epithelial cells or fibroblasts with high expression of the TM4SF1 gene could extend more nano-pseudopods and promote cell polarization and migration [20]. However, there are few studies on the role of TM4SF1 in glioma. In this current study, we examined the expression of TM4SF1 in glioma cell lines by western blot and found that the expression levels of TM4SF1 were increased significantly relative to NHA (Figure 1A). Using the TCGA and CGGA datasets, we also found that the expression levels of TM4SF1 were significantly higher in all of the four subtypes of glioblastoma and were negatively correlated with the prognosis of the patients (Figure 1C).

Although the association between TM4SF1 and glioma has been reported, the biological function of TM4SF1 on glioma remains unclear. In this study, the siRNAs siTM4SF1#1 and siTM4SF1#2 significantly downregulated the TM4SF1 expression in the two cell lines U87 and A172. CCK-8 assay showed that TM4SF1 knockdown markedly inhibited cell proliferation of U87 and A172 cells (Figure 2C). And the

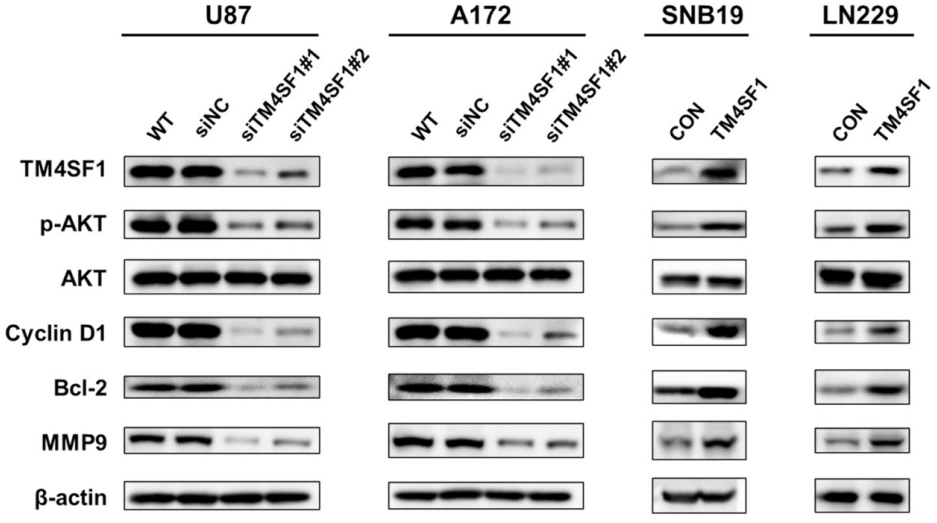

Figure 5. Effect of TM4SF1 on p-AKT, Cyclin D1, Bcl-2, and MMP-9 expression in glioma cells. The protein levels of TM4SF1, p-AKT, AKT, Cyclin D1, Bcl-2, and MMP-9 in U87, A172, SNB19, and LN229 cells were determined by western blot. β-actin was used as an endogenous control.

cell migration efficiency and invasion were significantly reduced by the knockdown of TM4SF1 in siTM4SF1#1 and siTM4SF1#2 groups (Figure 3). We also found that the overexpression of TM4SF1 in SNB19 and LN229 cells showed the opposite effect (Figures 2 and 3). Similar biological functions have been reported in other tumors. It was found that downregulation of TM4SF1 in NSCLC A549 and H1299 cells by siRNA suppresses cell growth, migration, and invasion [21]. Overexpressing TM4SF1 in the liver cancer cell HepG2 reduces apoptosis and increases cell migration *in vitro* and enhances tumor growth and metastasis *in vivo*, whereas the silencing of TM4SF1 has the opposite effect [8]. Similarly, TM4SF1 expression is markedly higher in colorectal cancer (CRC) tissues than in non-tumor tissues and is positively correlated with poor prognosis. Downregulation of TM4SF1 by shRNA inhibits the migration, invasion, and tumor-sphere formation of SW480 and LoVo cells [22].

It was reported that silencing of TM4SF1 induces cell apoptosis in NSCLC cells A549 and H1299 cells, while the percentage of cells in the G2/M phase increases significantly compared with the control [21]. Three bladder cancer cell lines (T24, EJ, and UM-UC-3) were transfected with siRNA and the percentage of apoptotic cells was higher than the control group. And knockdown of TM4SF1 in bladder cancer cells resulted in an increase in the proportion of cells at the G0/G1 phase [23]. Similarly, the FACS results in our study showed that the silencing of TM4SF1 induced cell cycle arrest at the G0/G1 phase and early apoptosis.

AKT is activated frequently in cancer cells and involved in their growth, migration, and invasion. The meta-analysis indicated that p-AKT overexpression was significantly associated with worse survival in NSCLC patients [24], and p-AKT may be an indicator of poor prognosis in patients with gastric cancer [25]. In addition, it was reported that the antitumor gene CAMTA1 regulates glioma cells' proliferation and cell cycle by inhibiting AKT phosphorylation [26]. In our study, the p-AKT expression levels were significantly inhibited by siRNA siTM4SF1#1 and siTM4SF1#2 in U87 and A172 cells and increased by the TM4SF1 overexpression in SNB19 and LN229 cells. The G1/S-specific Cyclin D1 regulates cell proliferation and participates in tumorigenesis, cellular senescence, and apoptosis [27]. It was reported that knockdown ARID4B in glioma cells LN229 reduces the mRNA level of Cyclin D1 [28]. It is well known that Bcl-2 inhibits cell apoptosis through kinds of mechanisms [29]. Aclidinium bromide effectively induces apoptosis in U251 and U87 cells, and the antiapoptotic proteins Bcl-2, Cyclin D1, and p-AKT are decreased significantly [30]. Similarly, we found that siRNA siTM4SF1#1 and siTM4SF1#2 reduced the expression of CyclinD1 and Bcl-2, thus inducing cell cycle arrest and early apoptosis. The migration and invasion ability of glioma cells are closely related to the expression level of matrix metalloproteinase genes. It was found that the knockdown of Foxo3a reduces the ability of invasion in U251 cells and Foxo3a promotes the expression of matrix metalloproteinase MMP-9, suggesting that Foxo3a participates in the invasion of glioma cells by MMP-9 [31]. The NF-κB-specific inhibitor BAY 11-7082 suppressed the increased migration and invasion induced by IL-33 in U251 cells, and decreased the secretion of MMP-2 and MMP-9, indicating that IL-33 may be involved in the cell migration and invasion of glioma by promoting MMP-2 and MMP-9 expression via the NF-κB signaling pathway [32]. In our study, silencing TM4SF1 inhibited the migration and invasion of glioma cells by downregulating the expression of MMP-9, while promoting TM4SF1 expression enhanced the invasion by upregulating MMP-9 expression.

In conclusion, TM4SF1-promoted proliferation, migration, and invasion of glioma cells are involved in the PI3K-AKT pathway activation and regulating the expression of Cyclin D1, Bcl-2, and MMP-9. Targeting TM4SF1 may represent a new strategy for glioma intervention.

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