

Original Article

Silencing of TIPE3 suppresses proliferation of human glioma cells via AKT signaling pathway

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Abstract: TIPE3, a member of TNFAIP8 (tumor necrosis factor- α -induced protein 8, or TIPE) family, was reported to promote tumorigenesis by acting as a transfer protein of lipid second messenger. Glioma is the most common and lethal brain tumor in central nervous system. However, the role of TIPE3 on glioma remains unknown. In the present study, siRNA interference was performed to silence TIPE3 expression. CCK-8, colony formation assay, EdU incorporation assay and flow cytometry analysis were employed to examine glioma cell proliferation and cell cycle after TIPE3 knockdown. Western blot was used to investigate the expression of AKT, p-AKT, cyclinD1, p21 and p-HistoneH3. The results showed that silencing of TIPE3 inhibited glioma cell proliferation and caused cell cycle arrest. TIPE3 depletion suppressed activity of phosphorylation AKT pathway consistent with down-regulating cyclinD1 expression and elevating p21 levels. Taken together, our study indicates down-regulation of TIPE3 inhibits glioma cell proliferation via PI3K/AKT pathway. Moreover, our study suggests that TIPE3 may act as a therapeutic target for the treatment of glioma.

Keywords: TIPE3, glioma, proliferation, cell cycle, AKT

Introduction

Gliomas are the most common tumors within central nervous system (CNS). The World Health Organization (WHO) classifies gliomas into four categories [1] according to histological and clinical features: pilocytic astrocytoma (WHO Grade I), diffuse astrocytoma (WHO Grade II), anaplastic astrocytoma (WHO Grade III), glioblastoma (WHO Grade IV). Glioblastomas, the most malignant type among gliomas, are characterized by high morbidity, high recurrence rate and poor prognosis. Despite improvement in comprehensive treatment including surgical resection [2], radiotherapy [3] and chemotherapy, the prognosis is poorly a median survival of less than 15 months [4]. The progression of initiation, proliferation and invasion in glioma are complicated biological events [5-7]. Researchers are urged to elucidate the molecular mechanism on glioma and find potential therapeutic targets.

TIPE3 (TNFAIP8L3) is a member of TNFAIP8 (tumor necrosis factor- α -induced protein 8, or TIPE) family. Recent studies reportedly showed

that TIPE3 acts as a transfer protein of phosphoinositide second messengers that promote tumorigenesis [8]. It was reported that TIPE3 is a cytoplasmic protein and expressed at high level in most human carcinoma cells [9]. However, the roles of TIPE3 on glioma have not been reported yet.

In the present study, TIPE3 expression was examined in glioma patients' samples. We investigated the effect of silencing TIPE3 on glioma cell proliferation. Moreover, the possible mechanism by which TIPE3 depletion affects glioma cell proliferation was elucidated.

Materials and methods

Cell culture

Human U251, U118 and U87 glioma cell lines were purchased from Shanghai Cell Bank, Chinese Academy of Science (Shanghai, China). Cells were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin and 0.1 g/mL streptomycin (Biosharp, China). Cells were incubated in 5% CO₂ at 37°C.

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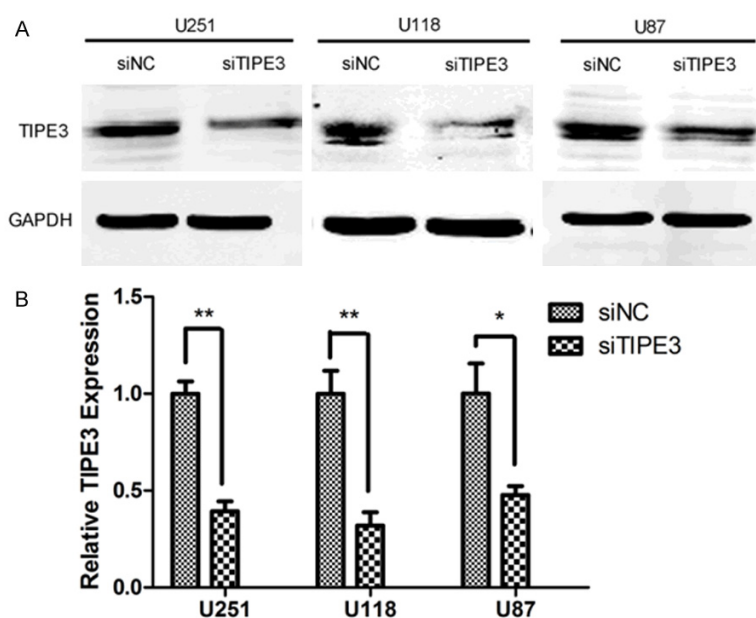


Figure 1. TIPE3 knockdown efficiency in glioma cells by RNAi. A. Western blot was used to measure TIPE3 expression levels of U251, U118 and U87 cells after siRNA transfection. GAPDH served as loading control. siNC represents siRNA-scrambled (negative control group). siTIPE3 represents siRNA targeting-TIPE3 (knockdown group). B. Graphical analysis of protein expression was presented. Relative TIPE3 expression/GAPDH of siNC group was set as 1. * $P < 0.05$ and ** $P < 0.01$. Data represents mean \pm SD. The experiment was repeated three times with similar results.

siRNA Transfection

siRNA-TIPE3 (siTIPE3, or TIPE3 knockdown group) and siRNA-Scramble (siNC, or negative control group) were synthesized by RiboBio Incorporation (Guangzhou, China). Cells were plated in six-well plates at 3×10^5 cells/well. When cells fusion reached 30-50%, culture medium was replaced by antibiotics-free and serum-free medium. siRNA and X-tremeGENE siRNA Transfection Reagent (Roche, Mannheim, Germany) complexes were incubated in Opti-MEM (Gibco, USA) for 20 min and were added to cells. After 4-6 hours, the medium was replaced by complete medium. Knockdown efficiency was assessed by western blot.

Cell counting kit-8 assay

Cell viability was examined by CCK-8 (Dojindo, Japan). About 3000 cells were plated in 96-well plates per well. After siRNA transfection, 5 μ l CCK-8 reagents were added to cells at 24 h, 48 h and 72 h. Then cells were incubated in 37°C for one hour. Absorbance was measured by

PerkinElmer victor 1420 at a wavelength of 450 nm.

Colony formation assay

Clone forming abilities of cells were assessed by colony formation assay. After transfection, 400 cells were seeded into 6-well plates per well and incubated in 5% CO₂ at 37°C for 2 weeks. The culture medium was replaced every 4 days. Cells were fixed with 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet. Plates were washed with PBS for 3 times. When the plates dried, visible colonies were counted.

5-ethynyl-2'-deoxyuridine (EdU) incorporation assay

Cell proliferation was assessed by Cell-Light™ EdU Imaging Kit (RiboBio, Guangzhou, China). The experiment was performed according to

the manufacturer's protocols. Cells were cultured in 10 μ M EdU for 24 hours. Cells were fixed with 4% paraformaldehyde for 20 min, then permeabilized in 0.5% Triton X-100. After being washed with PBS for 5 min, cells were incubated in 1 \times Apollo reaction cocktail for 30 min. Then DNA was stained with 1 \times Hoechst 33342 for 30 min. Cells were visualized under a fluorescence microscope (Olympus, BX51). Images were snapped by Image Pro Plus.

Flow cytometry

siRNA transfection was done as previously described, and then cells were incubated for 48 hours. Subsequently, cells were harvested and centrifuged at 1000 rpm for 5 min. Cells were washed twice with PBS and fixed in 75% ethanol at -20°C overnight. After fixation, cells were washed twice with cold PBS and incubated with 100 μ g/mL RNase A at 37°C for 30 min. Then cells were incubated with 50 μ g/mL propidium iodide (PI) at 4°C for 30 min. Samples were examined using BD FACSCalibur and the cell cycle distribution was calculated by Modfit software.

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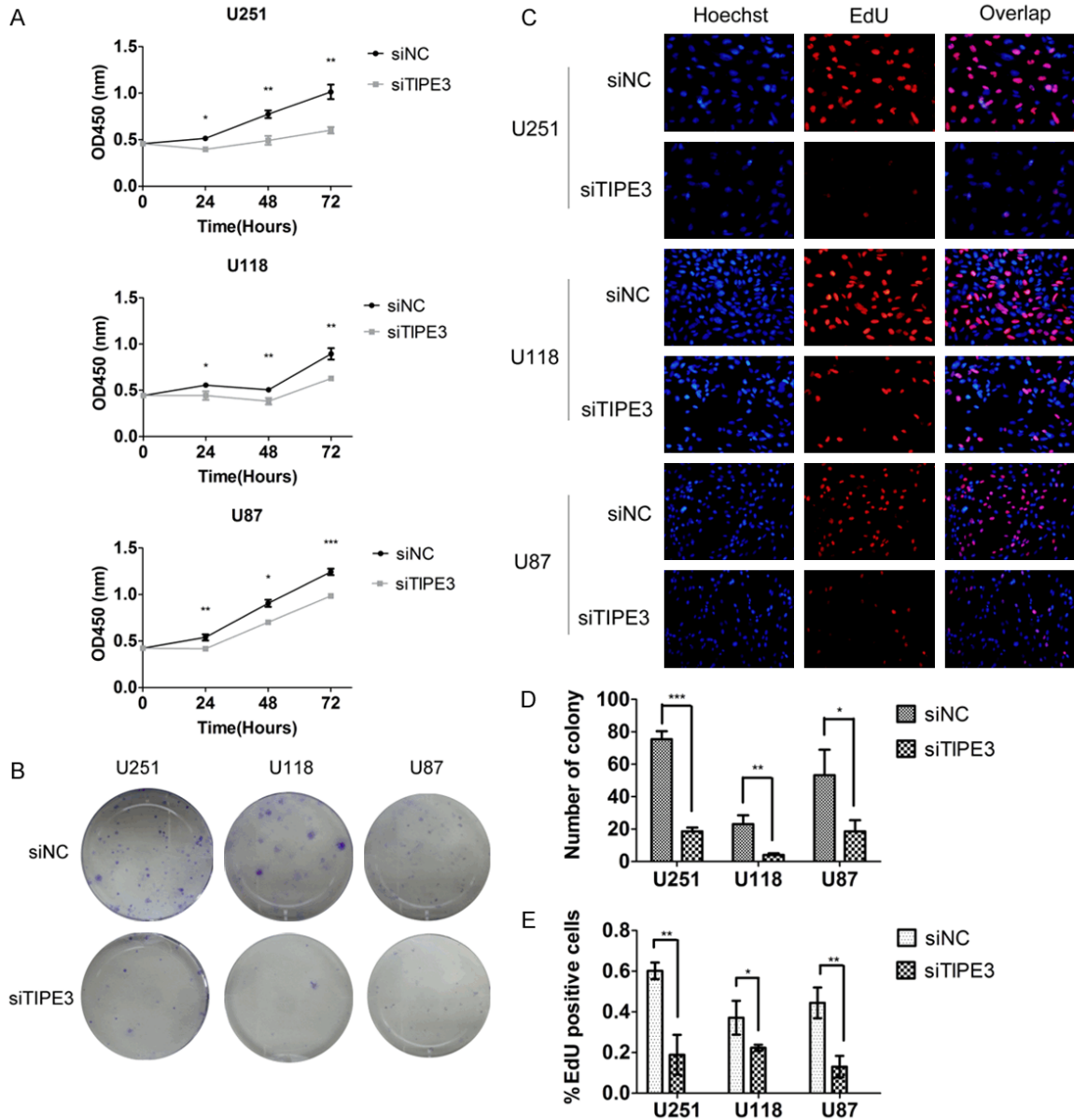


Figure 2. Silencing of TIPE3 inhibits proliferation of glioma cells. A. CCK-8 assay was performed to measure cell viability of U251, U118 and U87 after transfection at indicated times. B and D. Colony formation assay was performed to measure clone formation of U251, U118 and U87 cells after transfection. C and E. EdU incorporation assay was used to measure DNA replication of U251, U118 and U87 cells after transfection. Representative images were shown (magnification, 400×). Proliferative cell was labeled with EdU (red) and cell nuclei were stained with Hoechst (blue). Percentage of EdU positive cell was shown in graphical analysis. *P<0.05 **P<0.01 and ***P<0.0001. Data represents mean ± SD. Experiment was repeated three times with similar results.

Western blot

In 48 hours after transfection, cells were lysed in RIPA lysis buffer containing protease and phosphatase inhibitors for 20 min at 4°C, and then centrifuged at 12000 rpm for 10 min at 4°C. Protein concentration was measured by

BCA kit (Biosharp, China). Supernatant was collected and heated for 5 min at 100°C. Equal amounts of protein was separated by 12% SDS-PAGE and transferred into a PVDF membrane (Millipore, USA). The PVDF membrane was blocked in TBST containing 1% bovin serum albumin (Biosharp, China) for 1 hour. Sub-

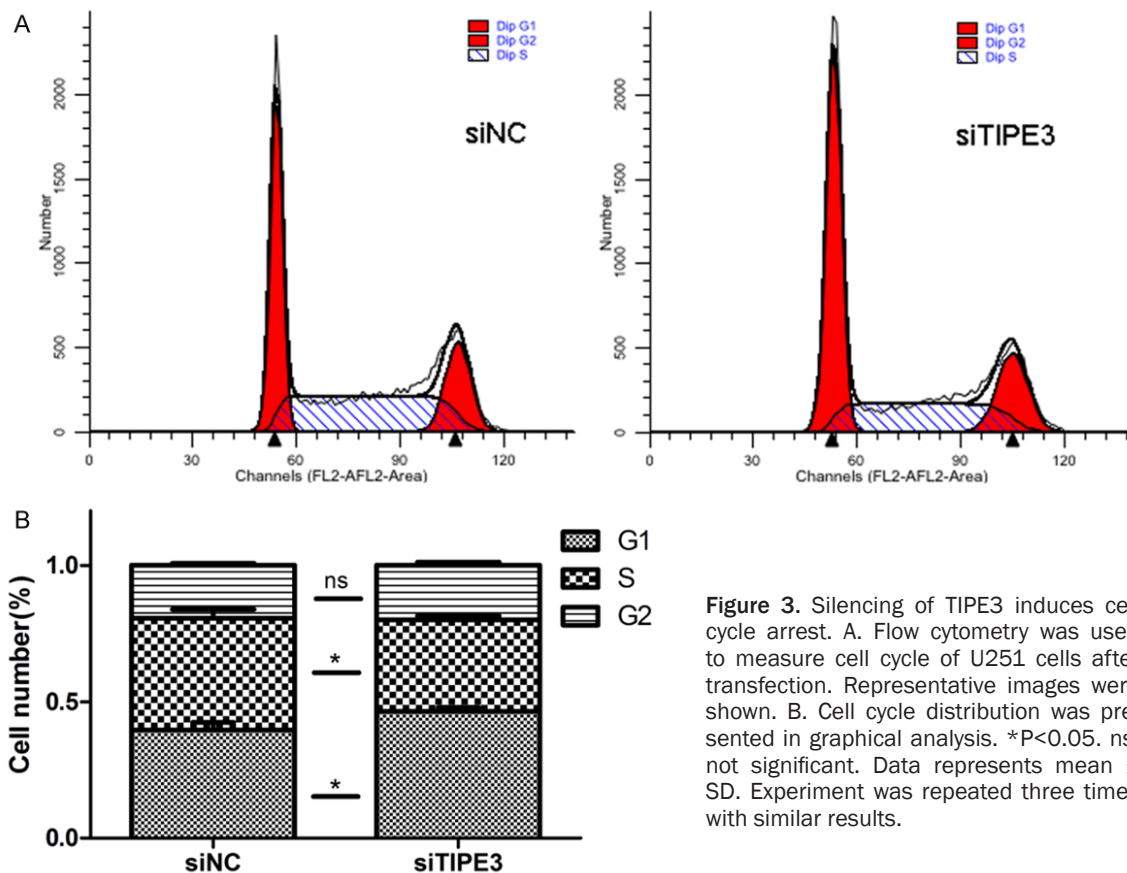


Figure 3. Silencing of TIPE3 induces cell cycle arrest. **A.** Flow cytometry was used to measure cell cycle of U251 cells after transfection. Representative images were shown. **B.** Cell cycle distribution was presented in graphical analysis. * $P < 0.05$. ns, not significant. Data represents mean \pm SD. Experiment was repeated three times with similar results.

sequently, membrane was probed with indicated primary antibodies overnight and followed by Alex Fluor 680/790-labeled goat anti-rabbit IgG (LI-COR Bioscience, USA). Bands were visualized using LI-COR Odyssey Infrared Imaging System. Images were analyzed by Quantity One (Bio-Rad, USA).

Primary antibodies include: anti-TIPE3 (1:100, Wuhan Boster Biological Incorporation, China), anti-cyclinD1 (1:1000, Cell Signaling Technology, USA), anti-AKT (1:1000, Cell Signaling Technology, USA), anti-p-AKT1/2/3 (Thr 308) (1:200, Santa Cruz, USA), anti-p21 (1:1000, Cell Signaling Technology, USA), anti-p-HistoneH3 (1:200, Santa Cruz, USA), anti-GAPDH (1:1000, Cell Signaling Technology, USA).

Statistical analysis

The results were representative of the three independent experiments and presented as mean \pm SD. P values < 0.05 were considered statistically significant. SPSS 19.0 (IBM, USA) was used for all statistical analyses.

Results

Assessment of TIPE3 expression after siRNA transfection

To explore the function of TIPE3 in glioma cells, a siRNA transfection experiment was performed. The knockdown efficiency was assessed by western blot. As depicted in **Figure 1A** and **1B**, TIPE3 protein expression significantly reduced after siRNA-TIPE3 transfection in human glioma cell lines (U251, U118 and U87).

Knockdown of TIPE3 inhibits cell proliferation in human glioma cells

The cell viability was determined by Cell Counting Kit-8 (CCK-8). As shown in **Figure 2A**, the growth curves for cells transfected with siRNA-TIPE3 were lower than those for cells transfected with siRNA-Scramble (the negative control). The colony formation assay indicated that the colony-forming capabilities of U251, U118 and U87 cells were significantly impaired due to TIPE3 depletion (**Figure 2B** and **2D**).

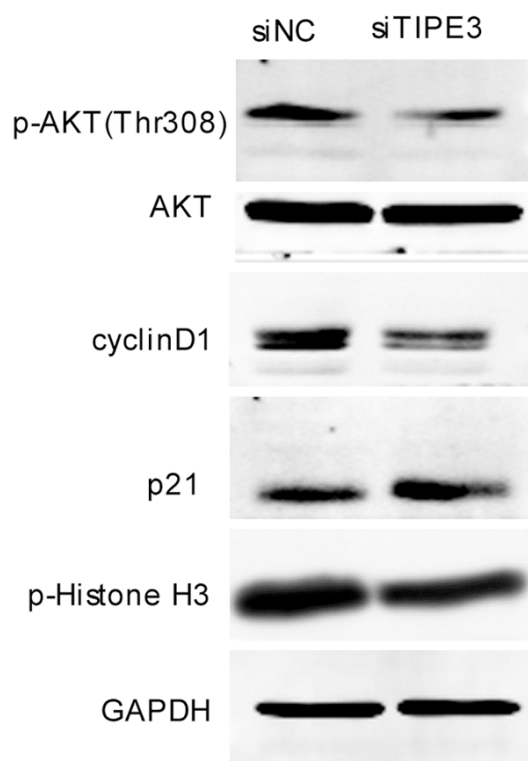


Figure 4. Silencing of TIPE3 inhibits AKT signaling pathway. Western blot was performed to assess AKT, p-AKT, cyclinD1, p21 and p-Histone H3 expression levels in U251 cells with siRNA transfection. GAPDH served as loading control.

To investigate the effect of TIPE3 depletion on DNA replication, the EdU incorporation assays were performed in U251, U118 and U87 cell lines. As shown in **Figure 2C** and **2E**, EDU-positive cells, when transfected with siTIPE3, experienced a significant drop in its percentage in comparison with the negative control group.

All the results above indicate that down-regulation of TIPE3 suppresses the proliferation of glioma cells in vitro.

Knockdown of TIPE3 arrests cell cycle in human glioma cells

To explore whether the effects of TIPE3 depletion on cell proliferation was related to cell cycle, we performed flow cytometry analysis. The percentage of S phase cells reduced after silencing of TIPE3, accompanied by an increased level of G0/G1 phase cell (**Figure 3A** and **3B**). These results indicated a cell cycle arrest of G1-S transition delay due to TIPE3 knockdown.

The mechanism TIPE3 regulates the proliferation of glioma cells

To illustrate the molecular mechanism by which TIPE3 regulates the proliferation of glioma cells, western blots were performed to detect the related protein expression. As depicted in **Figure 4**, Silencing of TIPE3 inhibits AKT phosphorylation. Considering the inhibitory role of TIPE3 knockdown in cell cycle progression, we examined the expression levels of CyclinD1, and its downstream regulator, p21. As shown in **Figure 4**, CyclinD1 was at a low level in knockdown group than that in negative control group. And its downstream regulator, p21, was at a high level due to TIPE3 knockdown (**Figure 4**). phosph-HistoneH3, a marker of proliferation, reduced after TIPE3 knockdown (**Figure 4**).

Discussion

Recent studies reported that TIPE3, as a newly found lipid transfer protein, promotes tumorigenesis by elevating PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ levels in cells [8]. Unlike other members of the TNFAIP8 family playing an inhibitory role in cancer [10, 11], TIPE3 contributes to tumorigenesis in a mouse model injected with NIH 3T3-Ras^{V12} cells while TIPE3 depletion inhibits proliferation of lung, bladder and colorectal cancer cell lines [8]. However, no studies about the biological functions of TIPE3 in human glioma have been reported.

We, for the first time, reported that silencing of TIPE3 inhibits proliferation of glioma cells (**Figure 2**). By CCK-8 assay, colony formation assay and EdU incorporation assay, we find that there were reduced viability and proliferation abilities in U251, U118 and U87 cells after TIPE3 knockdown.

Proliferation is a complicated process and in part regulated by cell cycle control [12]. To investigate whether silencing of TIPE3 deregulate cell growth through cell cycle blockage, we conducted FACS flow cytometry analysis. The results indicate G1-S transition arrest due to TIPE3 depletion (**Figure 3**). Cell cycle is regulated by a series of activators and inhibitors. CyclinD1 promotes G1-S transition progression [13]. P21 causes cell cycle arrest [14] by inhibiting CDKs and DNA replication [15, 16]. Our results show decreased cyclinD1 expression and increased p21 expression after silencing

TIPE3 (**Figure 4**). Moreover, our study shows decreased expression of p-HistoneH3 (**Figure 4**), a marker of proliferation. With reduced EDU positive cells after TIPE3 depletion (**Figure 2C** and **2E**), DNA replication showed attenuation. In that case, we assume that silencing of TIPE3 inhibits glioma cell proliferation by arresting G1-S transition of cell cycle, thereby attenuating DNA replication.

Phosphatidylinositol 3-kinase (PI3K) and its downstream target AKT, are in the center of cell signaling pathways [17, 18], controls different cellular processes [19] and hallmarks of cancer [20]. It was reported that AKT pathway activation promoted the conversion of anaplastic astrocytoma to glioblastoma multiform in model [21]. Inhibition of AKT has an anti-proliferative effect on gliomas [22]. More importantly, AKT phosphorylation is reported to be enhanced by TIPE3 overexpression [8]. Taken together, we examined the expression of total AKT and p-AKT by western blot, finding that p-AKT expression was reduced but the total AKT level stayed unchanged (**Figure 4**).

AKT activation phosphorylates glycogen synthase kinase-3 β (GSK-3 β), thereby suppressing the activity of GSK-3 β [23]. CyclinD1 can be phosphorylated by GSK-3 β , and such phosphorylation promotes cyclinD1 translocation from nucleus to cytoplasm and its subsequent degradation therein [13]. Therefore, AKT-mediated phosphorylation stabilizes cyclinD1 through inactivating GSK-3 β [24]. P21 inhibits cell proliferation and cell cycle progression in two ways [25]. One is to inhibit CDKs activity, and the other to bind PCNA as a competitor thus inhibiting DNA replication [15, 16]. AKT-mediated phosphorylation of p21 prevents it from binding PCNA and inhibits the function of p21 as CDKIs [26]. Therefore, AKT activation relieves p21's inhibitory effects on cell growth. Taken together, we assume that silencing of TIPE3 inhibits AKT activity, and subsequently changes the expression of downstream effector cyclinD1 and p21, and finally suppresses glioma cell proliferation.

In conclusion, we for the first time prove the inhibitory role of TIPE3 depletion on glioma cell proliferation via AKT signaling pathway. Our study suggests that TIPE3 is a potential therapeutic target for glioma. More efforts are required to investigate the function of TIPE3 on glioma in vivo.

Acknowledgements

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Disclosure of conflict of interest

None.

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