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ORIGINAL ARTICLE

Down-regulation of STIP1 regulate apoptosis and invasion of glioma cells via TRAP1/AKT signaling pathway

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Abstract

Background: In recent years, many studies have confirmed that *STIP1* (phosphorylation-induced protein 1) is involved in the development and progression of various tumors. However, its potential role in glioma progression and the underlying mechanisms of glioma development remain unclear.

Methods: We analyzed the expression of *STIP1* in 35 human glioma tissue specimens of different grades, using 6 normal brain tissues for comparison. We transfected U87 and U251 cell lines with small interfering RNA (siRNA) to downregulate STIP1, and set up a negative control group and a blank group for comparison. The MTT assay was used to detect cell proliferation, and cell cycle progression and apoptosis were analyzed through flow cytometry. Transwell experiments were employed to detect the invasion and migration of STIP1-depleted and control U87 and U251 cells and western blotting was used to detect the expression of TRAP1/Akt pathway proteins. In addition, immunohistochemical analysis was used to reveal differences in expression and localization between transplanted tumor specimens of each group.

Results: We observed a high expression of STIP1 in glioblastoma, MTT assay revealed a decreased cell proliferation rate in the STIP1-downregulated cells. Cell cycle analysis revealed an increased proportion of cells in G1 phase, as well as an increase in apoptosis, upon STIP1 downregulation. Western blotting showed that TRAP1, pAkt, and MMP2 expression was decreased upon *STIP1* downregulation. In addition, TRAP1, ki-67, and MMP2 displayed a decreased expression in vivo.

Conclusions: STIP1 is highly expressed in glioblastoma compared to normal brain tissues. Downregulation of *STIP1* in glioma cells reduces cell proliferation rate and invasion and increases cell apoptosis.

Keywords Glioblastoma, STIP1, TRAP1, Apoptosis, Invasion.

Introduction

Glioma is the most common primary malignant tumor in the brain; it has the characteristics of "three highs and one low" and is characterized by and its high incidence, high recurrence rate, high mortality rate, and low cure rate [1,2]. Glioblastoma is the most common type of glioma. The median overall survival time for glioblastoma is approximately 14.6 months, and with standard treatment and diagnosis, the 5-year survival rate is less than 9.8% [3,4]. It is therefore imperative to study the molecular mechanisms of glioma and identify new treatment strategies. Recently, there have been multiple molecular studies of glioma in attempts to provide a deeper theoretical basis for targeted therapy.

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The main biological function of phosphorylation stress-inducing proteins is as linker molecules for the heat shock proteins Hsp70 and Hsp90 [5]. In cells, phosphorylation stress induces the binding of phosphorylation stress-inducing proteins to Hsp70 and Hsp90 to form a complex. This complex regulates the functional complementation of Hsp70 and Hsp90 in the folding of various transcriptional factors and kinases, including oncogenic proteins and prions [6–9]. Recent studies have shown that phosphorylation stress-inducing proteins are also involved in tumor cell development, stress response, bovine hormone production, and cell differentiation and proliferation [10].

We have previously confirmed a positive correlation between *STIP1* and *TRAP1* in-silico using the cBioportal for Cancer Genomics, and also that STIP1 mRNA and protein levels are significantly higher in glioblastoma than normal brain tissues. However, the molecular mechanism of action of STIP1 in glioma is still unclear. Concurrently, other studies have shown that the inhibition of TRAP1 expression leads to an increased expression and phosphorylation of p70S6K and RSK1, which affects tumor cell invasion [11,12]. Based on the important role of the TRAP1/Akt pathway in tumorigenesis, we hypothesized that STIP1 may promote glioma development via the TRAP1/Akt pathway. We tested our hypothesis in patient samples, glioma cell lines, and an intracranial xenograft model and confirmed that STIP1 regulates glioma cell growth and invasion through regulation of the TRAP1/AKT pathway.

Materials and methods

Glioma tissue specimen

35 cases of glioma specimens (liquid nitrogen cryopreservation) were selected, including 13 low-grade glioma tissues (WHO class II, oligodendroglioma in 2 cases, astrocytoma in 11 cases). Twenty-two high-grade glioma tissues (11 cases of WHOIII grade included 1 case of anaplastic oligodendroglioma, 7 cases of astrocytoma, 3 cases of interstitial ependymoma; 11 cases of WHO IV grade). Six cases of non-neoplastic brain tissue (both from the Department of Neurosurgery, the First Affiliated Hospital of Soochow University from 2000 to 2016), the study was approved by the local ethics committee, and all patients in the First Affiliated Hospital of Suzhou University used their samples get permission.

Cell culture

Glioma cell line U87 U251 (purchased from Institute of Biological cells, Shanghai Academy of Life Sciences, China) was cultured in DMEM medium supplemented with 10% fetal bovine serum. The incubator was set at 37 °C and 5% CO_2. Change liquid 5 ml daily, make sure the cell is in good condition, take it in logarithmic phase for experiment.

siRNA transfection

siRNA-NC synthesized by Gima Gene Co., Ltd. and three STIP1-siRNA sequences(labeled1,2,3,respectively)(siRNA-1:GCTACTCCGAAGCTATTAAGC;siRNA-2:GCCAGAGCCAATGGAAGAAGA; siRNA-3: GGAGACTACCAGAAGGCTTAT). Adjust the cell density to 15 $\sim$ 20 $\times$ 104 /ml, and pour in the 6-well plates. the cell confluence rate is required to be 80 to 90% for transfection. First, using lipofectamine 2000 as a transfection reagent, mix 20 μmol/L siRNAolgio 5 μl with 250 μl DMEM and incubate for 5 min at room temperature. At the same time, mix 5 μl of lipofectamine 2000 with 250 μl of DMEM medium to form a suspension. Finally, mix the two and incubate for 20 min at room temperature to form a transfection complex of siRNA and lipofectamine 2000 dilution. They were added to U87 and U251 cell culture media, and the final concentration of 3 siRNA interference chains and siRNA Negative Control chains was 100 μmol / L. U87 and U251 cells were treated with 3 interference chains and negative control strands, respectively, including blanks without any treatment. RNA was extracted 24 h later, and the negative control was used as a reference.

Quantitative PCR

TRIzol reagent (Invitrogen, USA) was used to extract tissue or cellular RNA, and the extracted RNA was measured with DEPC water as a control. The concentration and purity were determined by spectrophotometer. RNA with 260/280 ratio between 1.9 and 2.0 was selected. The RNA was reverse transcribed into cDNA, and GAPDH was used as the internal reference. Three replicate wells were set for each sample. We used 20 ul system, and each replicate was added with the following reagents 20 μl, 2 $\times$ All-in-One qPCMix 10 μl.miRNAqPCR Primer 2 μl, Uni, each set of three duplicate wells, placed at 37 °C, 5% CO_2 incubator incubation, 12 h, 24 h, 48 h, versal Adaptor PCR Primer 2 μl, cDNA 2 μl, 50 $\times$ ROX Referenc Dye 0.4 ul, ddH2O3.6 μl. Finally, the method of $2^{-\triangle\triangle CT}$ was used to compare the content of STIP1 in normal brain tissue and glioma specimens.

Mtt experiment

After transfection for 24 h, the blank control group, siRNA-NC, STIP1-siRNA were prepared into single cell suspensions, 2 $\times$ 104 fine/ml, and 100 ul of cell suspension (2000 cells per well) was added to the 96-well plate. We detected cell growth index every four hours.

Matrigel invasion experiment

40 ul of diluted matrigel gel was added to the transwell upper chamber at 37 °C for 30 min. The glioma cells were discarded and the medium was rinsed three times with PBS. Then, it was digested with 0.25% trypsin, centrifuged, and a cell suspension (5 $\times$ 10 5 /ml) was prepared using a serum-free medium. 100 ul of cell suspension and 200 ul of serum-free medium were added to the transwell plate, and 500 ul of complete medium containing 0.05% FBS was added to the lower chamber of the transwell plate. 5% CO_2, cultured in a 37 °C incubator for 48 h. Wipe the cells on the matrigel with a cotton swab. Remove the upper chamber and add 4% paraformaldehyde for 30 min, 0.1% Crystal violet stained for 2 min and was transparent with xylene. Observe the surface cells attached

to matrigel under high magnification (400×) and observe the average of 6 fields of view.

Cell cycle

The cell suspension was adjusted to a concentration of 1×10^6 /ml, and a negative control group was set. The supernatant was centrifuged, and 70% of cold ethanol was added to the cells to fix it overnight. Add 100 ul of RNaseA solution to the cell pellet for 30 min at 37 °C, then add 400 ul of PI staining solution to mix at 4 °Cfor 30 min in a dark water bath. On-machine detection, the red fluorescence at the excitation wavelength 488 was recorded.

Protein extraction and western blot

The cell culture medium was removed, and the cell lysate RIPA 400 and 4 ul of PMSF were added to each 107 cells and lysed at 4 °C for 30 min, and centrifuged at 12,000 rpm for 15 min to take the supernatant. The Biyuntian protein concentration assay kit was used to determine the protein concentration and balance at the wavelength of 560 nm. An equal amount of protein sample was separated and transferred to a nitrocellulose membrane by 6–10% SDS-PAGE, and membrane albumin (BSA) (Amresco, USA) was blocked with 5% bovine serum at constant voltage for 2 h at room temperature, then Incubation with a primary antibody of 1:1000 at 4 °C and 1:3000 HRP coupled secondary overnight antibody (Beyotime Institute of Biotechnology) followed by washing with PBST three times for 5 min each. Use enhanced chemiluminescence detection signal (ECL) (Thermo)

Immunofluorescence

Transfect U87 and U251 cells with STIP1-siRNA,Negative control and blank group, Fix with 4% paraformaldehyde for 30 min and block Work with BSA (Amresco) for 30 min. Incubate cells Anti-STIP1, anti- TRAP1(CST, USA) primary antibody Overnight at 4 °C, then incubated with tetramethylrhodamine Isothiocyanate-labeled secondary antibody(diluted at 1:500) for 30 min at 37 °C. Cell stainingImaging with a fluorescence microscope using DAPI (Olympus BX50 / BXFLA / DP70; Japan Olympus).Three independent experiments were repeated.

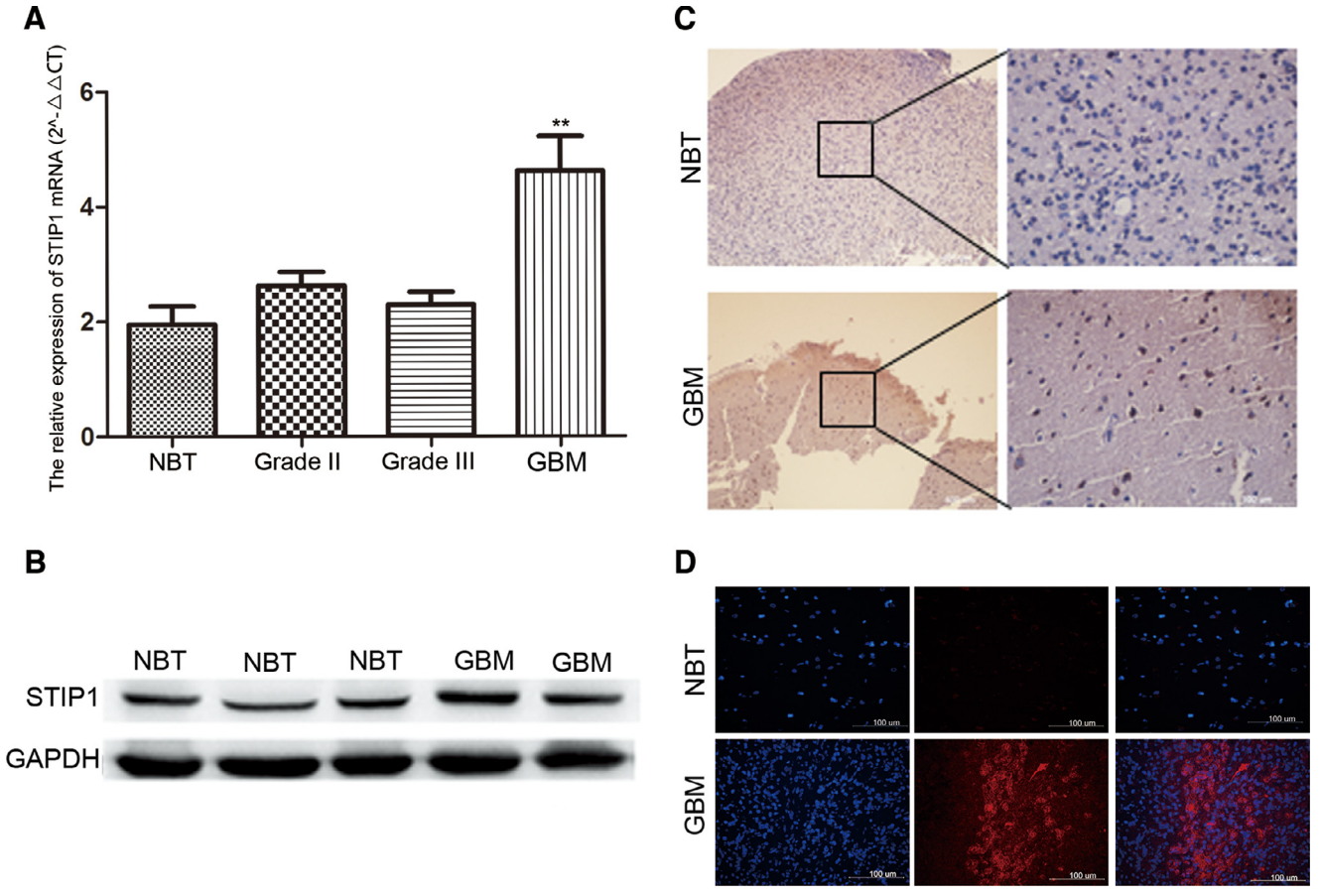

Fig. 1 The expression of STIP1 in non-tumor brain tissues, low-grade glioma and GBM.
A shows qRT-PCR analysis (data were reported as 2−△△CT) of the expression of STIP1in five non-tumor brain tissues, ten gradeIItissue, nine gradeIIItissue and ten GBM tissue.
B Western blotting analysis of STIP1 expression in NBT and GBM.
C Immunohistochemistry staining of STIP1 of NBT tissue and GBM tissue.
D the image of immunofluorescence of NBT and GBM.

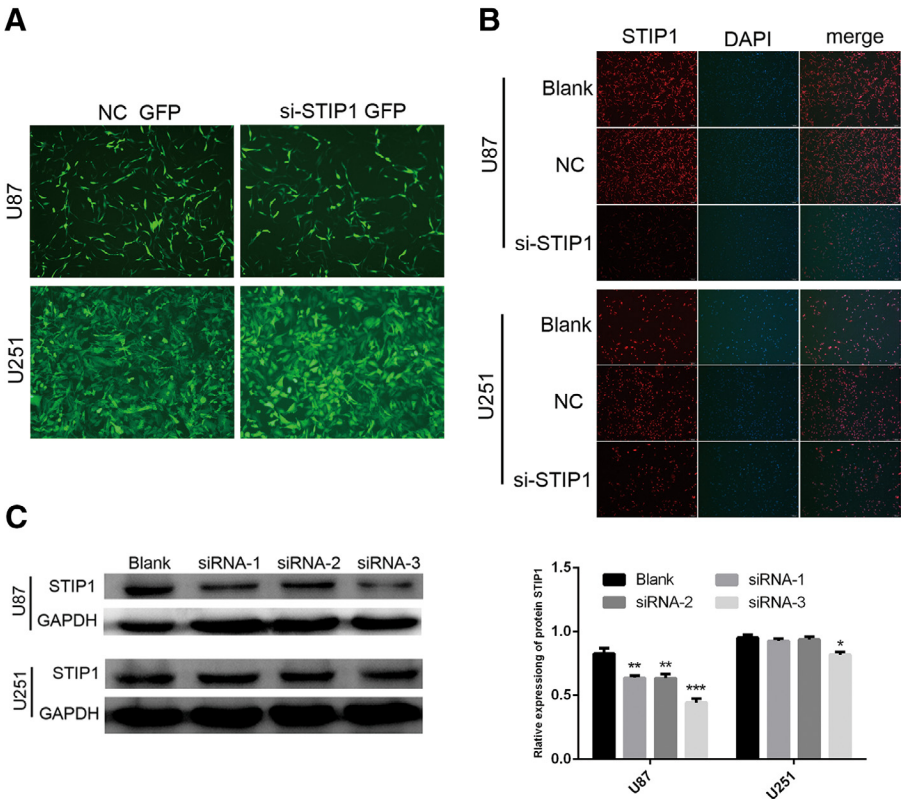

Fig. 2 The Transfection efficiency of STIP1 in U87 and U251.
A Transfection efficiency of u251 and u87 detected under fluorescence microscope.
B Fluorescence images of U87 and U251 transfected with STIP1si-RNA and negative controlled group
C the result of western blot shows the protein expression of STIP1 in Three groups transfected with STIP1-siRNA
D Column chart of protein expression of STIP1 in Three groups transfected with STIP1-siRNA.data were reported as means ± SD (*P < 0.05,**P<0.01,***P<0.001).

Immunohistochemistry

U87 transplanted tumor is embedded in paraffin and precision sliced into 6 μm sections with microtome. Sections were subsequently incubated with the Tissue Staining Kit HRP-DAB system (R&D Systems, Minneapolis,MN), according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were conducted with Swith GraphPad Prism 5 program. Student's *t*-test or ANOVA were used to test the difference between the groups. Data were considered statistically significant at an alpha value of $p < 0.05$.

Results

Expression of STIP1 in glioma and normal brain tissues

Quantitative PCR was used to assess mRNA levels of STIP1 and TRAP1 in 35 glioma tissue samples of different grades and 6 non-cancerous brain tissues. The results showed that the expression levels of STIP1 in glioblastoma tissues (grade II, grade III) were significantly higher than those in non-cancerous brain tissues (p<0.05; Fig. 1A). However, no significant difference was found in STIP1 expression levels between low-grade gliomas and non-tumor brain tissues (p>0.05; Fig. 1A). Similar results were obtained for STIP1 protein expression through western blotting (Fig. 1B), immunohistochemistry (Fig. 1C), and immunofluorescence (Fig. 1D).

Efficiency of STIP1 downregulation by siRNA in U87 and U251 cells

We used three siRNAs to transfect U87 and U251 cells in order to select the most efficient siRNA for subsequent experiments. The transfection efficiency in U87 and U251 cells was over 90% for all three siRNAs (Fig. 2A). Western blot analysis showed a significant decrease in the expression of STIP1 in cells transfected with each siRNA compared to the blank group. However, the third transfection sequence was the most efficient (p<0.01; Fig. 2C). Immunofluorescence also confirmed significant down-regulation of STIP1 in the U87 and U251 cells transfected with siRNA (Fig. 2B).

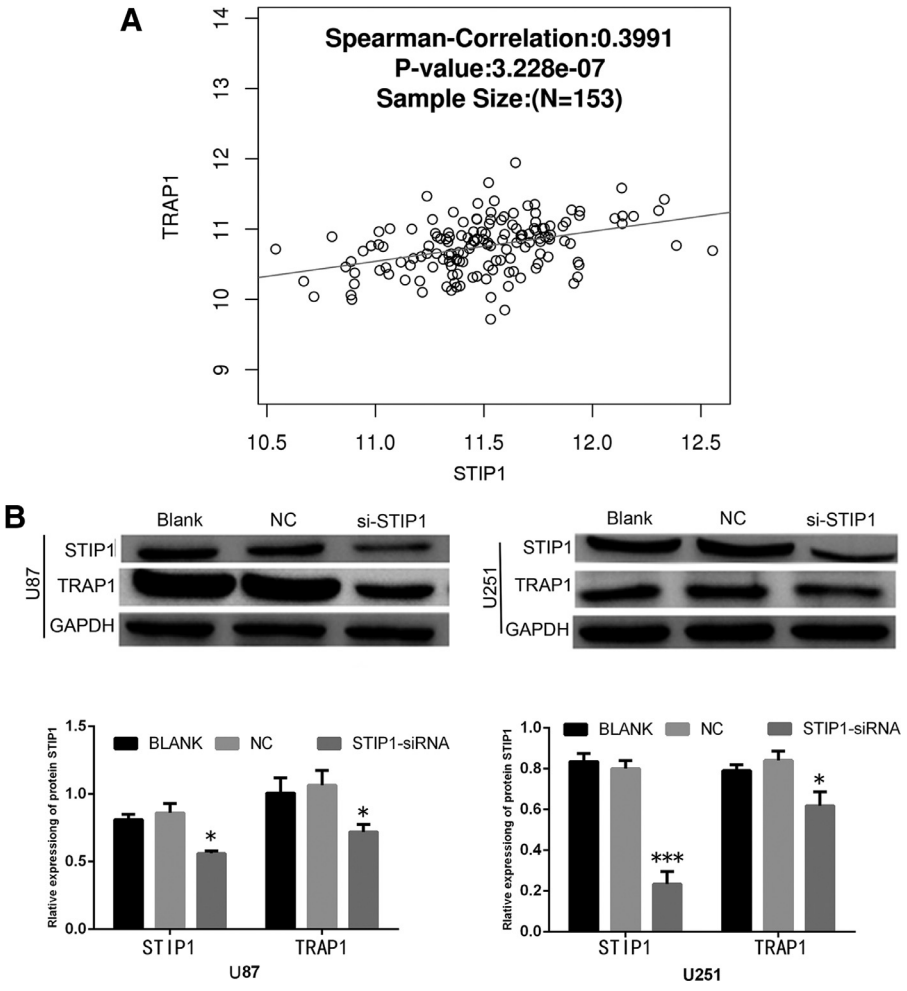

Fig. 3 The relationship between STIP1 with TRAP1.
A the image of bioinformatics analysis between STIP1 and TRAP1.
B the expression of TRAP1 after down-regulation of stip1 in u87n and u251
C Column chart of the relative expression of protein TRAP1 after down-regulation of STIP1 in u87n and u251.data were reported as means ± SD (*$P < 0.05$,***$P<0.001$).

Relationship between STIP1 and TRAP1 in glioma

We performed bioinformatic analyses for 153 glioblastoma patient samples and found that STIP1 and TRAP1 expression levels were positively correlated (correlation coefficient=0.3991, Fig. 3A). Western blot analysis showed that the expression of TRAP1 in U251 and U87 cells transfected with STIP1-siRNA was significantly lower than that in the negative control group ($p<0.05$, Fig. 3B).

Effect of downregulation of STIP1 on proliferation and apoptosis of U87 and U251 cells

The MTT assay showed that the U251 and U87 cell lines both showed a significant decrease in proliferative capacity on downregulation of STIP1 compared with the control and blank groups ($p<0.05$, Fig. 4A). Flow cytometry was used to analyze cell cycle progression and apoptosis in STIP1 siRNA-transfected cells as well as the blank and negative treatment groups (Fig. 4B). The results of three independent replicates showed an increase in the G1 phase of the cell cycle in STIP1 downregulated cells (U87: 64.14±8.11%, U251: 83.34±6.03%) compared with the blank and the negative control groups (Fig. 4C, D). Furthermore, there were obvious apoptotic peaks in the profiles of STIP1 downregulated cells, and the proportion of apoptotic cells was significantly increased (U87: 16.38±8.33%, U251: 30.47±10.79%) compared with the blank and negative control groups (Fig. 4D).

Down-regulation of STIP1 reduces the expression of MMP2 to inhibit invasion via the TRAP1/Akt signaling pathway

The results of our transwell invasion assay showed that the invasive ability of STIP1 downregulated cells was significantly

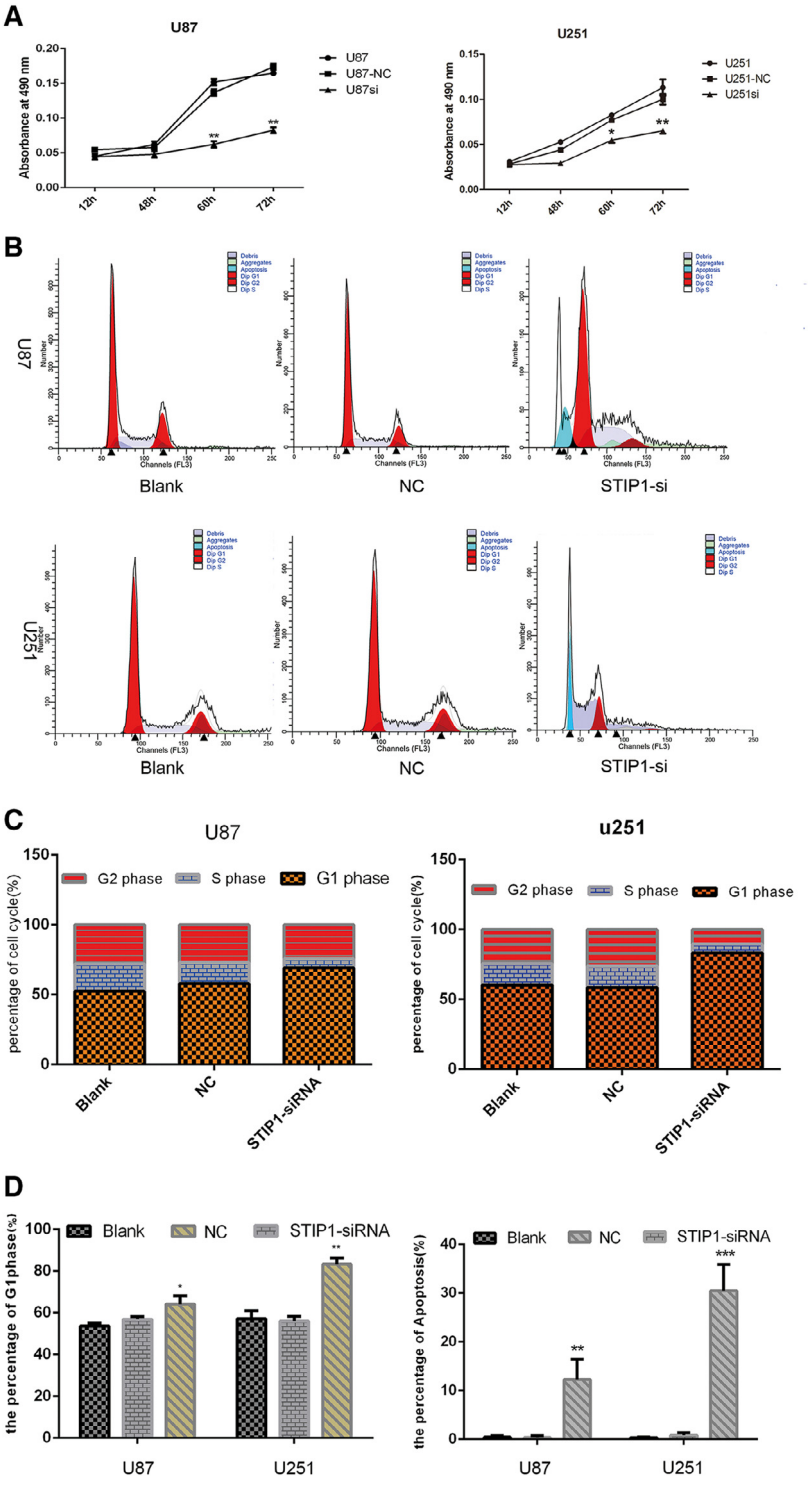

Fig. 4 Mtt assay and Flow cytometry to detect the rate of proliferation, cell cycle and apoptosis of STIP1-si group compared with NC group and Blank group.

A Mtt was used to detect The cell proliferation rate in U87 and U251 tansfected with STIP1-siRNA,negative controlled, and the blank group($^{*}P<0.05$,$^{**}P<0.01$)

B Flow cytometry results of each phase cell rate in U87 and U251 tansfected with STIP1-siRNA,negative control group and blank group.

C Column chart of each phase cell rate of U87 and U251 tansfected with STIP1-siRNA, negative control group and blank group.

D Column chart of the apoptosis cells and each phase proportion of U87 and U251 tansfected with STIP1-siRNA, negative control group and blank group.data were reported as means ± SD ($^{*}P < 0.05$,$^{**}P<0.01$,$^{***}P<0.001$).

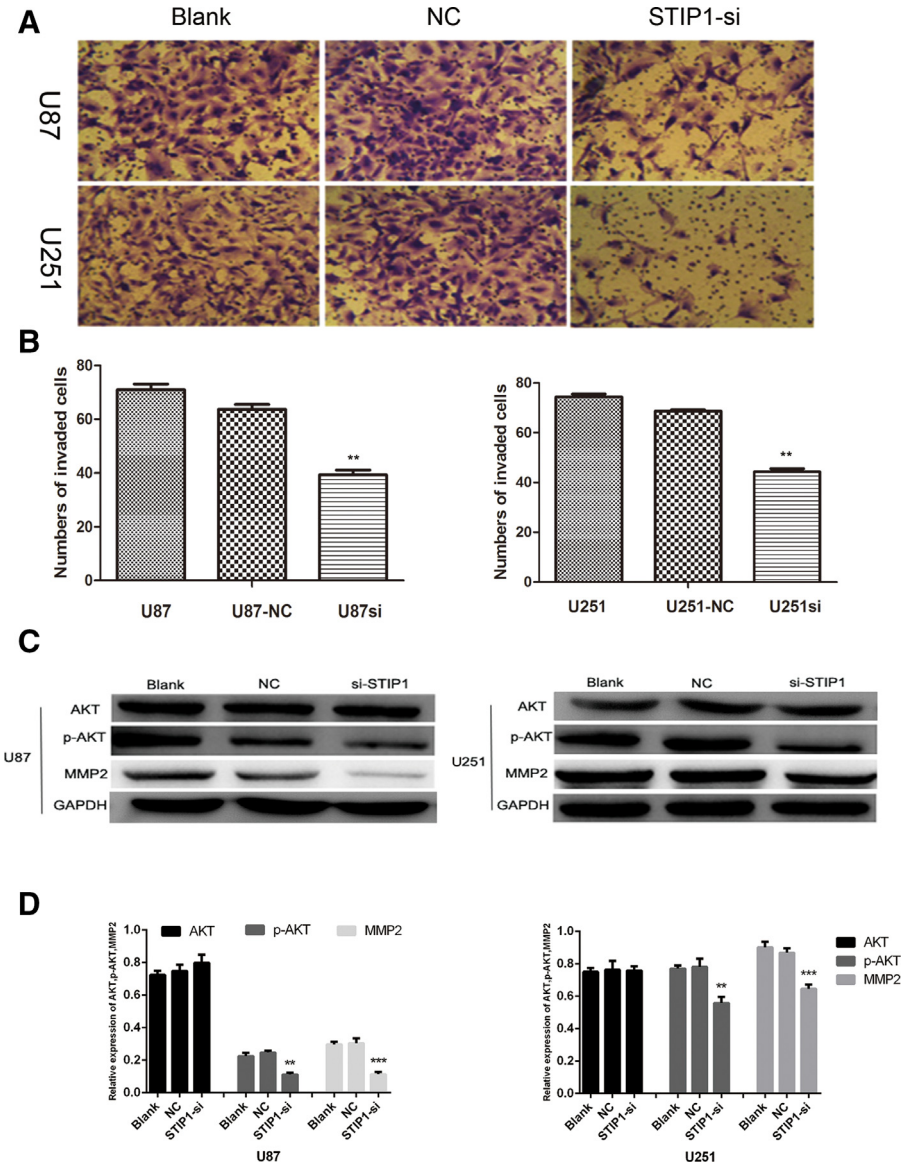

Fig. 5 Investigating STIP1-si group's invasive ability and molecular mechanism by Transwell assay and Western blot compared with NC group and Blank group.
A Transwell assay exhibits the invasion of U87 and U251 cells transfected with STIP1-siRNA,negative control group and blank group
B Column chart of the invasion of U87 and U251 cells tansfected with STIP1-siRNA,negative control group and blank group.data were reported as means ± SD (**$P < 0.01$,***$P<0.001$).
C Western blotting analysis of AKT,P-AKT,MMP2 expression in U87 and U251 cells tansfected with STIP1-siRNA, negative control group and blank group. Data were reported as means ± SD (**$P < 0.01$,***$P<0.001$).

reduced ($p<0.05$; Fig. 5A, B) compared to empty vector and untreated cells. Western blot analysis revealed that, although Akt expression was not significantly changed ($p>0.05$, Fig. 5C, D), TRAP1, p-Akt and MMP2 protein expression were all significantly decreased ($P<0.05$, Fig. 5C, D) after downregulation of STIP1. Therefore, downregulation of STIP1 expression suppressed activation of the TRAP1/Akt pathway, which explains the impeded cell invasion ability observed in these cells.

Expression of molecular indicators of proliferation and invasion was decreased upon STIP1-siRNA transfection in vivo

U87 cells transfected with STIP1 siRNA were injected into the brains of nude mice in order to form intracranial xenografts. Hematoxylin and eosin staining revealed reduced invasive ability compared to the blank group (Fig. 6A). Immunohistochemistry showed that the expression of STIP1, TRAP1,

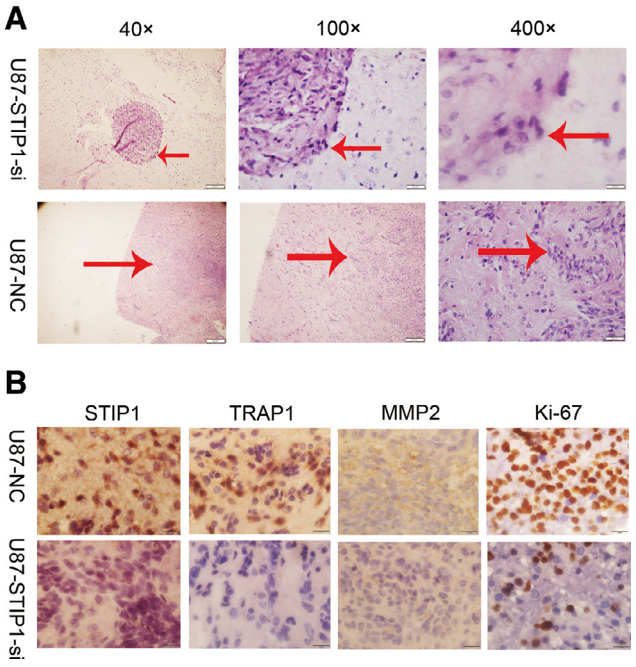

Fig. 6 Down-regulation STIP1 inhibited tumor proliferation and invasion in vivo.
A Hematoxylin–eosin staining of the intracranial tumors of the mice in STIP1-si and blank groups.
B Immunohistochemistry staining of STIP1, TRAP1,KI-67and MMP2 of the intracranial tumors of the mice in both groups (400 ×).

ki-67 and MMP2 were all lower in STIP1 siRNA xenografts compared with the blank group (Fig. 6B).

Conclusion

Our study showed that STIP1 is highly expressed in glioblastoma tissu Down-regulation of STIP1 in U25 and U87 cells resulted in decreased cell proliferation, an increase in the proportion of cells in G1 phase, increased apoptosis, and decreased cell invasiveness. TRAP1 mRNA and protein expression levels were significantly reduced, and p-AKT and MMP2 protein expression decreased significantly with down-regulation of STIP1. Therefore, down-regulation of STIP1 limits its glioma cell proliferation, induces apoptosis and inhibits glioma cell invasion through the TRAP1/Akt pathway.

Discussion

We found that the expression of STIP1 in glioblastoma was higher than in normal brain tissue, but there was no significant increase in STIP1 expression in low grade glioma (grade II, grade III). The decreased expression of TRAP1 resulting from silencing of STIP1 results in inhibition of the Akt pathway, leading to decreased proliferation, increased apoptosis and reduced invasiveness.

Studies have shown that STIP1 is involved in many biological processes in various tumor cell types. In 2007, Erlich et al. found that STIP1 can be secreted by glioblastoma cell lines and induces tumor cell proliferation, but does not induce normal glia cell proliferation [13]. Recent studies have found that increased expression of STIP1 in glioma-associated glial cells/macrophages and infiltrating lymphocytes is associated with tumor progression. Furthermore, the expression of STIP1 in lymphocytes appears to be regulated by the microenvironment of the brain tumor [14]. By comparing the protein profiles between ovarian tumors and normal interstitial fluid, Wang et al. found that STIP1 can be secreted by ovarian cancer tissue into the peripheral blood of patients, resulting in a significant increase in serum STIP1 levels. This increase in serum STIP1 in ovarian cancer patients may suggest that the combined use of CA125 and STIP1 as screening markers may improve early detection of ovarian cancer [15]. Later studies also found that silencing of STIP1 in epithelial ovarian cancer cells inhibited their proliferation and invasion [16], while the detection of CA125 and STIP1 did indeed provide a valuable reference for early screening of ovarian cancer. Multiple studies have also found that STIP1 expression is associated with cancer cell proliferation and invasion in other cancer cells, such as breast cancer [17], and papillary thyroid carcinoma [23]. It has therefore been suggested that STIP1 in serum could be used as a candidate biomarker for early cancer cell detection for several human cancer types. In most cases, STIP1 levels are increased in cancer cells compared to normal cells, and higher STIP1 levels are also found in metastatic, drug-resistant or aggressive tumors [17]. As its expression levels increase, STIP1 appears to promote the proliferation and differentiation of malignant cells, and these cancer cell characteristics are reversed when STIP1 expression levels are reduced. These results are consistent with the conclusions of our study.

TRAP1 is one of the major members of the heat shock protein family [24]. It has important roles in resisting apoptosis induced by oxidative stress, maintaining mitochondrial integrity, and sustaining the steady state of the intracellular environment [25,26]. Agliarulo et al. found that silencing TRAP1 leads to enhanced cell motility in vitro, and this effect is mediated by the akt/p70s6k pathway [27]. Matrix metalloproteins (MMPs) degrade the extracellular matrix and basement membrane, promoting tumor migration. It has been shown that MMP2, an important member of the MMP family, can be targeted by Akt to affect glioma invasion [28].

In our study, we found that STIP1 is highly expressed in glioblastoma, but not in low-grade gliomas. This may be due to genetic differences in individuals, and further studies with higher sample numbers will confirm whether this is true of larger populations. In conclusion, *STIP1* is low expressed in normal cells and plays an important biological role, but is significantly higher in breast cancer [17], pancreatic cancer [18], esophageal squamous cell carcinoma [19], colon cancer [20], hepatocellular carcinoma [21], renal cell carcinoma [22] and glioblastoma,. It is suggested that STIP1 may play an important role as a proto oncogene in the carcinogenesis and development of tumors.

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Supplementary materials

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