Knockdown of Serine Threonine Tyrosine Kinase 1 (STYK1) Inhibits the Migration and Tumorigenesis in Glioma Cells

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Pediatric glioma is a devastating brain tumor. Serine threonine tyrosine kinase 1 (STYK1) is a member of the protein tyrosine kinase family and plays a significant role in the formation of several malignant tumors. However, the expression pattern and role of STYK1 in glioma are not yet clear. The aim of this study was to investigate the role and molecular mechanism of STYK1 in glioma. The results showed that STYK1 was highly expressed in glioma cell lines. We also found that knockdown of STYK1 inhibited cell proliferation, migration, and invasion in vitro as well as tumorigenesis in vivo. Furthermore, knockdown of STYK1 significantly decreased the expression levels of phosphorylation of PI3K and Akt in glioma cells. Taken together, our data suggest that STYK1 plays an important role in the development and progression of glioma. Therefore, STYK1 may represent a novel therapeutic target for the treatment of glioma.

Key words: Serine threonine tyrosine kinase 1 (STYK1); Glioma; Invasion; PI3K/Akt pathway

INTRODUCTION

Pediatric glioma is a devastating brain tumor and is highly aggressive, resistant to treatment, and prone to recurrence[1]. Despite recent advances in the diagnosis and therapy of pediatric glioma, the general survival rate of patients with pediatric glioma is less than 2 years[2–4]. The molecular mechanisms underlying pediatric glioma metastasis remain unclear. Therefore, there is an urgent need to explore the molecular mechanisms responsible in order to find novel treatments for pediatric glioma.

Serine threonine tyrosine kinase 1 (STYK1), a member of the protein tyrosine kinase family, contains a single putative transmembrane domain and an intracellular domain possessing tyrosine kinase activity. However, it lacks an extracellular domain for binding specific ligands[5]. Increasing evidence has reported that STYK1 was implicated in the development and progression of several types of cancers including ovarian cancer, intrahepatic cholangiocarcinoma, lung cancer, and breast cancer[6–9]. For example, Wang et al. confirmed that the expression of STYK1 was significantly upregulated in human hepatocellular carcinoma (HCC) tissues and cell lines, and overexpression of STYK1 promoted cell proliferation, migration, and invasion in vitro as well as tumorigenesis

and metastasis in vivo[10]. However, the expression pattern and role of STYK1 in glioma are not yet clear. The aim of this study was to investigate the role and molecular mechanism of STYK1 in glioma. Our data demonstrated that STYK1 plays an important role in glioma development and progression, and knockdown of STYK1 inhibited the migration and tumorigenesis in glioma cells.

MATERIALS AND METHODS

Cell Culture

Human glioma cell lines (U87MG, U251, and LN229) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Bio-Rad, Hercules, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were incubated in an atmosphere of 37°C with 5% CO_2.

Short Hairpin RNA and Cell Transfection

Short hairpin RNA targeting STYK1 (sh-STYK1) and empty vector (vector) were purchased from Invitrogen (Carlsbad, CA, USA). For STYK1 silencing, U87MG cells were transfected with sh-STYK1 or a vector using

Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocols.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was prepared from glioma cells using the TRIzol reagent (Invitrogen). RNA (1 μg) was utilized to synthesize cDNA using the SuperScript First-Stand Synthesis system (Invitrogen). qRT-PCR was performed using a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with FastStart Universal SYBR Green Master (Roche, USA). The following primer sequences were designed according to the corresponding human genes: STYK1, 5′-AAA TCT AGA ATG GGC ATG ACA CGG ATG-3′ and 5′-AAA GCG GCC GCT CAA AGC ATG CTA TAG TTG TAG AAG-3′; β-actin, 5′-TGG CAC CCA GCA CAA TGA A-3′, and 5′-CTA AGT CAT AGT CCG CCT AGA AGC A-3′. Reaction specificity was confirmed by analyzing melting curves and by electrophoresis on 2.0% agarose gel analysis of products. Subsequently, the relative expression of target genes was analyzed by the ΔCt method and expressed as the fold changes.

Western Blot

Total protein was extracted from glioma cells using RIPA lysis buffer (Beyotime, Nantong, P.R. China). Equal amounts of protein were loaded, separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). After the nitrocellulose membranes were blocked by 5% nonfat dry milk in TBST, they were incubated overnight with STYK1, E-cadherin, N-cadherin, p-PI3K, PI3K, p-Akt, Akt, and GAPDH antibodies (1:20,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were then rinsed and incubated with goat anti-mouse horseradish peroxidase-conjugated IgG (Santa Cruz) for 1 h at room temperature. Protein bands were detected using enhanced chemiluminescence (ECL) reagents (Pierce, Rockford, IL, USA).

Cell Proliferation Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to detect cell proliferation. Briefly, U87MG cells at a density of 1×10^4 cells/well were plated in 96-well plates and transfected with sh-STYK1 or vector. Then 100 μl of MTT (0.5 mg/ml) was added to each well at different time points, and cells were incubated at 37°C for 4 h. The medium was then carefully removed, and dimethyl sulfoxide (DMSO; 150 μl) was added into each well. The absorbance of solubilized blue formazan was read at a wavelength of 450 nm using a microplate reader.

Cell Migration and Invasion Assays

Cell migration assay was performed using Transwell™ chambers (Costar, Washington, DC, USA). The transfected cells were seeded into the upper chamber of the Transwell plates; the lower wells of the plates were filled with 600 μl of DMEM supplemented with 10% FBS as an attractant. After 24 h, cells that transferred to the lower surface of the filter were fixed, stained with Giemsa, and counted under a microscope (magnification: ×100). The invasion assay was done by the same procedure, except that the membrane was precoated with Matrigel (BD Biosciences, Bedford, MA, USA) to form a matrix barrier.

In Vivo Experiment

The animal study was approved by the Institutional Animal Care and Use Committee of the Second Affiliated Hospital, Medical College of Xi'an Jiaotong University (P.R. China). Female nude mice (4–6 weeks, 18–22 g) were purchased from the Laboratory Animal Center of the Medical College of Xi'an Jiaotong University. U87MG cells (1×10^6 cells/0.1 ml) transfected with sh-STYK1 or vector were injected subcutaneously into the flank of nude mice ($n = 5$ per group). Tumor size was measured every 5 days using a caliper and calculated using the formula: volume = length × width^2 × π/6. After 20 days, the mice were sacrificed, and tumors were excised and weighed.

Statistical Analysis

All values were expressed as mean ± SD. Statistical significance was analyzed with one-way factorial ANOVA or Student's two-tailed t-test. A value of $p < 0.05$ was considered to be statistically significant.
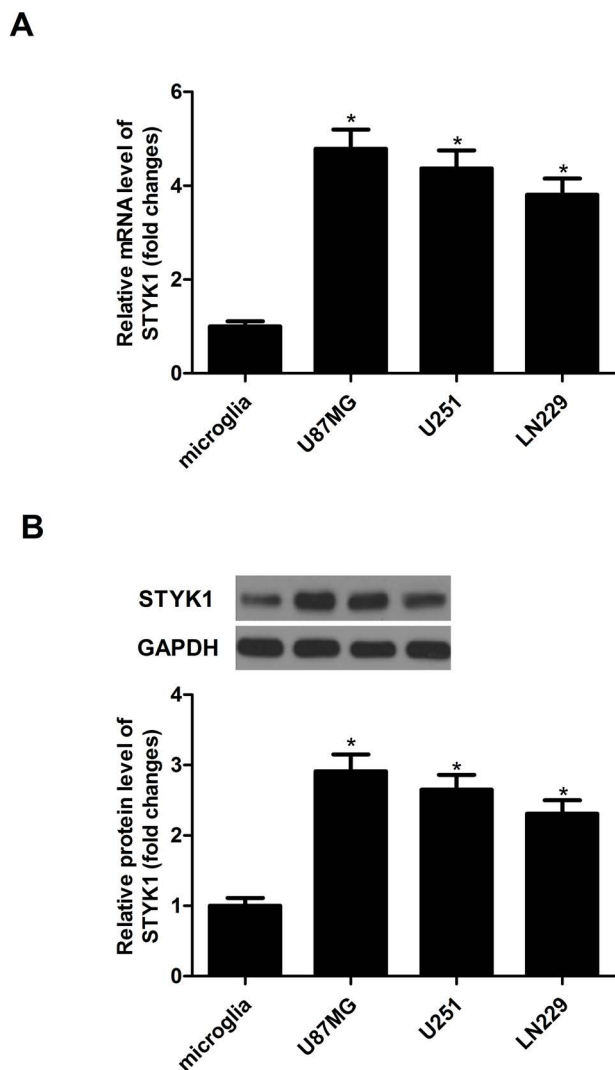
RESULTS

STYK1 Is Highly Expressed in Glioma Cell Lines

We first determined the mRNA expression of STYK1 in human glioma cell lines by qRT-PCR. The results demonstrated that the mRNA expression levels of STYK1 were remarkably increased in all cell lines compared with that in the microglia cells (Fig. 1A). We then analyzed the protein expression of STYK1 in human glioma cell lines. The results of the Western blot indicated that, compared to the microglia cells, the protein expression levels of STYK1 were significantly elevated in human glioma cell lines (Fig. 1B).

Knockdown of STYK1 Inhibits the Proliferation of Glioma Cells

To investigate the effect of STYK1 on cell proliferation in vitro, we stably transfected U87MG cells with sh-STYK1 for establishing STYK1-silencing glioma cells. Transfection efficiency was confirmed by qRT-PCR and Western blot. After transfection, the expression levels

A

B

Figure 1. STYK1 is highly expressed in glioma cell lines. (A) The mRNA expression levels of STYK1 in human glioma cell lines were detected by qRT-PCR. (B) The protein expression levels of STYK1 in human glioma cell lines were evaluated by Western blot, and GAPDH served as the loading control. The values shown represent the mean±SD. *$p < 0.05$.

of STYK1 of both mRNA (Fig. 2A) and protein (Fig. 2B) were obviously downregulated in U87MG cells. The effect of STYK1 on glioma cell proliferation was then detected using the MTT assay. Compared with the vector group, knockdown of STYK1 markedly suppressed the proliferation of U87MG cells (Fig. 2C).

Knockdown of STYK1 Inhibits the Migration and Invasion of Glioma Cells

We next evaluated the effect of STYK1 on glioma cell migration and invasion. The number of migrated U87MG cells was greatly decreased after transfection with sh-STYK1 when compared with the vector group

(Fig. 3A). Similarly, the results of the Matrigel invasion assay demonstrated that knockdown of STYK1 significantly reduced the invasion of U87MG cells (Fig. 3B). Furthermore, we detected the effect of STYK1 on EMT-related marker expression in U87MG cells. The results of the Western blot assay indicated that knockdown of STYK1 significantly upregulated the protein expression of E-cadherin and downregulated the protein expression of N-cadherin in U87MG cells, compared with the vector group (Fig. 3C).

Knockdown of STYK1 Inhibits the Activation of PI3K/Akt Pathway in Glioma Cells

To explore the molecular mechanisms by which STYK1 affected these malignant features, we examined the effect of STYK1 on the phosphorylation levels of PI3K and Akt in U87MG cells. The Western blot revealed that knockdown of STYK1 markedly inhibited phosphorylation levels of PI3K and Akt in U87MG cells when compared with those transfected with vector alone (Fig. 4A and B). An Akt inhibitor (wortmannin) was used to observe the effects on U87MG cell proliferation and invasion. Because administration of 100 nM wortmannin had no significant effect on cell viability, 100 nM wortmannin was chosen for additional experiments (Fig. 4C). Furthermore, we observed that wortmannin significantly enhanced the inhibitory effects of sh-STYK1 on U87MG cell proliferation (Fig. 4D) and invasion (Fig. 4E).

Knockdown of STYK1 Attenuates Tumor Growth in a Xenograft Model

To further explore the role of STYK1 on glioma growth in vivo, the xenografted tumor in nude mice was employed. The experiment in vivo showed that, compared with the vector group, knockdown of STYK1 significantly suppressed tumor volume (Fig. 5A) and weight (Fig. 5B).
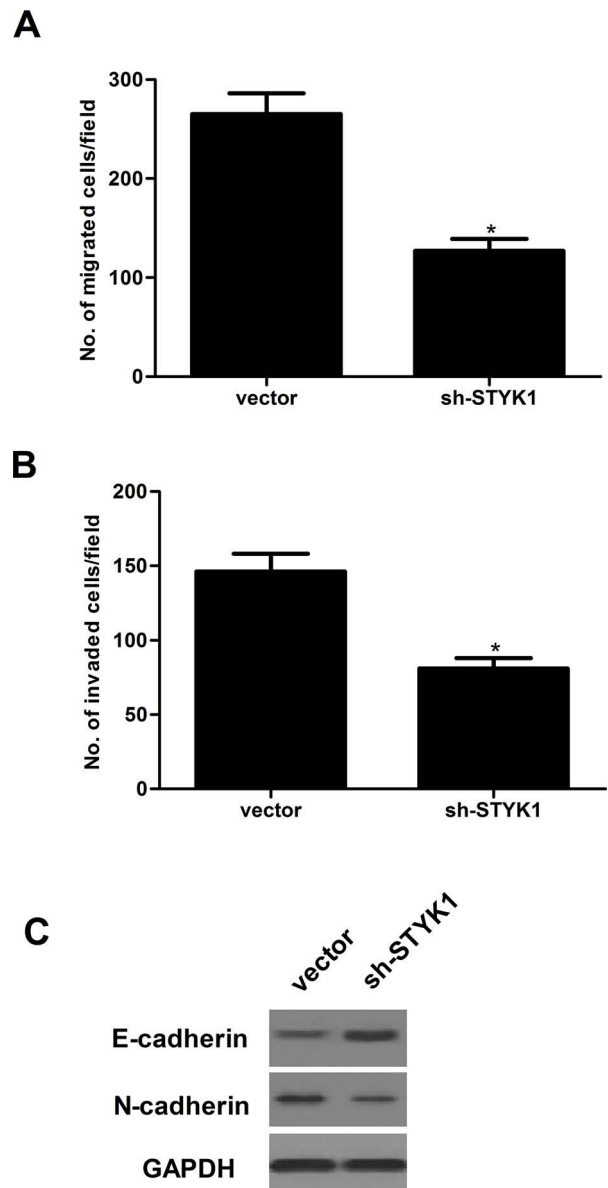
DISCUSSION

In this study, we showed that STYK1 expression is upregulated in human glioma cell lines. Knockdown of STYK1 inhibited cell proliferation, migration, and invasion in vitro and tumor growth in vivo. Furthermore, knockdown of STYK1 markedly inhibited phosphorylation levels of PI3K and Akt in U87MG cells.

Previous studies have shown that STYK1 has been identified as an oncogene and promotes proliferation in vitro and tumor growth in vivo in many types of cancers. Jackson et al. confirmed that the expression of STYK1 is higher in malignant ovarian tissues than that of normal ovarian tissues[6]. Kimbro et al. reported high levels of STYK1 expression even in the early stages of breast cancer[11]. Furthermore, a recent study demonstrated that downregulation of STYK1 was closely related with decreased

A

B

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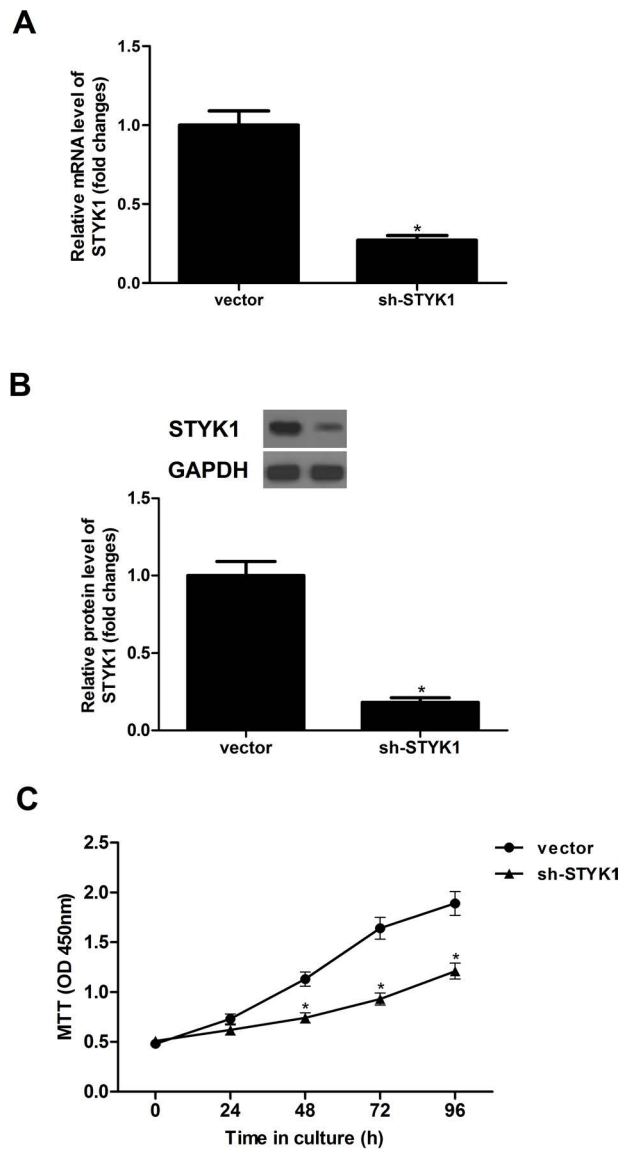
Figure 2. Knockdown of STYK1 inhibits the proliferation of glioma cells. U87MG cells were transfected with sh-STYK1 or vector for 24 h. (A) The mRNA expression level of STYK1 in U87MG cells was detected by qRT-PCR. (B) The protein expression level of STYK1 in U87MG cells was evaluated by Western blot. (C) Cell proliferation was detected using the MTT assay. The values shown represent the mean±SD. *$p<0.05$.

A

B

C

Figure 3. Knockdown of STYK1 inhibits the migration and invasion of glioma cells. U87MG cells were transfected with sh-STYK1 or vector for 24 h. (A) Cell migration was evaluated by the Transwell migration assay. (B) Cell invasion was evaluated by the Matrigel invasion assay. (C) The protein expression levels of E-cadherin and N-cadherin were measured using Western blot analysis. The values shown represent the mean±SD. *$p<0.05$.

proliferation of intrahepatic cholangiocarcinoma cells, as well as decreased tumor volume and weight in vivo[12]. In accordance with previous observations, the current study demonstrated that the expression levels of STYK1 in both mRNA and protein were obviously upregulated in human glioma cell lines. In addition, we observed that knockdown of STYK1 inhibited glioma cell proliferation in vitro and tumor growth in vivo. These data from in vitro and in vivo

models suggest that STYK1 may behave as an oncogene in the development and progression of glioma.

Metastasis and invasion are thought to be responsible for ~90% of glioma-associated mortality[13]. EMT is a complex process, which involves cytoskeletal remodeling and cell–cell and cell–matrix adhesion, leading to the
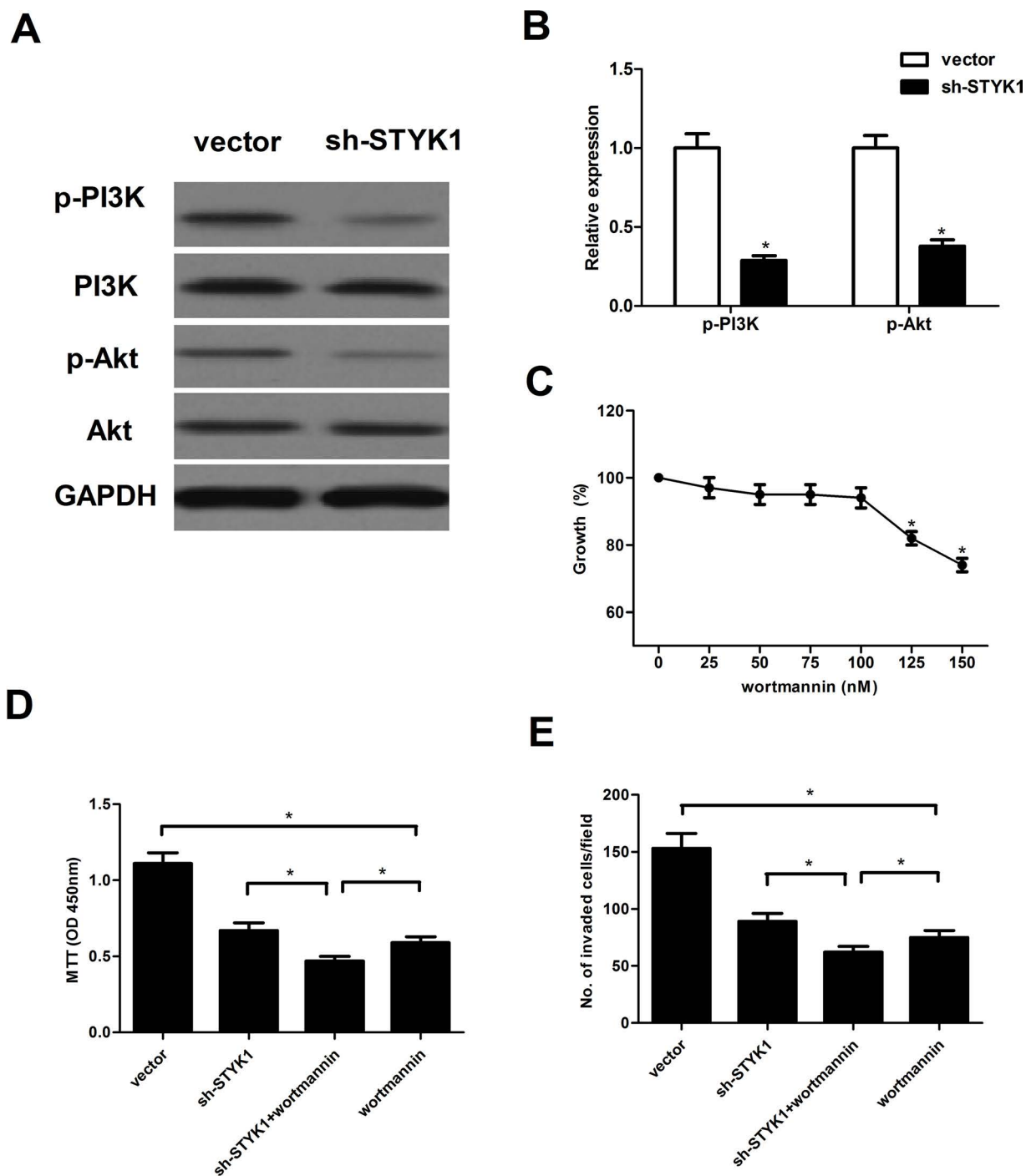
Figure 4. Knockdown of STYK1 inhibits the activation of the PI3K/Akt pathway in glioma cells. U87MG cells were transfected with sh-STYK1 or vector for 24 h. (A) The protein expression levels of PI3K, p-PI3K, Akt, and p-Akt were determined by Western blot. (B) Quantification analysis was performed using Gel-Pro Analyzer version 4.0 software. (C) U87MG cells were treated with different concentrations of wortmannin for 24 h, and cell viability was detected. U87MG cells were transfected with sh-STYK1 or vector in the presence or absence of the wortmannin (100 nM) for 24 h. (D) Cell proliferation was detected by the MTT assay. (E) Cell invasion was evaluated by the Matrigel invasion assay. The values shown represent the mean±SD. *$p < 0.05$.

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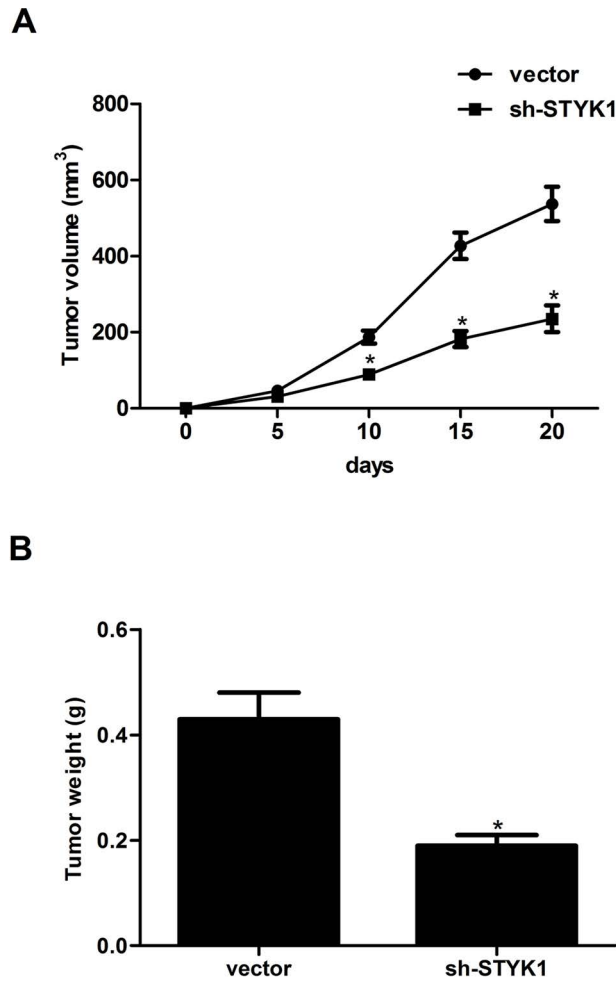
Figure 5. Knockdown of STYK1 attenuates tumor growth in a xenograft model. U87MG cells (1×10^6 cells/0.1 ml) transfected with sh-STYK1 or vector were injected subcutaneously into the flank of nude mice. (A) The tumor volumes were calculated every 5 days. (B) After 20 days, the mice were sacrificed, and tumors were excised and weighed. The values shown represent the mean±SD. *$p < 0.05$.

transition from a polarized, epithelial phenotype to a highly motile mesenchymal phenotype[14]. E-cadherin is often lost in cancer progression, and loss of E-cadherin can promote invasive and metastatic behavior in many epithelial tumors[15,16]. The current study demonstrated that knockdown of STYK1 significantly suppressed glioma cell migration and invasion, upregulated the expression of E-cadherin, and downregulated the expression of N-cadherin in U87MG cells. These data suggest that STYK1 positively regulates the EMT phenotype, consequently affecting glioma cell migration and invasion in vitro.

The PI3K/Akt signaling pathway plays an important role in human cancer initiation and progression[17,18]. Previous studies reported that the PI3K/Akt pathway is

constitutively upregulated in the majority of gliomas[19–21]. Moreover, the phosphorylation of Akt activates downstream target genes involved in proliferation, cell cycle progression, and migration of tumor cells, as well as the EMT process[22–24]. Thus, inhibition of the PI3K/Akt signaling pathway may represent a promising approach to the treatment of glioma. It was reported that buparlisib, a PI3K inhibitor, effectively inhibits glioma cell proliferation in vitro and growth of glioma xenografts in nude rats[25]. The current study demonstrated that knockdown of STYK1 markedly inhibited phosphorylation levels of PI3K and Akt in U87MG cells. These results suggest that knockdown of STYK1 inhibited the proliferation, invasion, and tumorigenesis partly via the inactivation of PI3K/Akt signaling pathway in glioma cells.

In conclusion, we demonstrated for the first time that STYK1 plays an important role in the development and progression of glioma. Knockdown of STYK1 inhibited the proliferation, invasion, and tumorigenesis in glioma cells. Thus, our findings suggest that STYK1 may serve as a potential therapeutic target for the treatment of glioma.

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