

Ste20-like kinase is upregulated in glioma and induces glioma invasion

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The malignant development and poor prognosis of gliomas are associated with a high degree of invasion and a high recurrence rate. However, the molecular mechanism underlying the invasiveness of glioma remains to be elucidated. Ste20-like kinase (SLK) is one of the members of the Ste20 family, which has been implicated in cellular migration and invasion. In this study, we intended to explore the expression of SLK significantly related to clinicopathologic stages of gliomas. Immunohistochemical staining and western blot analysis demonstrated that SLK was highly expressed in human glioma tissues and cell lines. Kaplan-Meier analysis revealed that poor survival was associated with high SLK expression. The inhibition of SLK by RNA interference significantly suppressed the invasion ability of glioma, and on protein level, knock-down of SLK led to an up-regulation of E-cadherin and a down-regulation of Vimentin in glioma cells. Collectively, this research shed light on mechanisms of invasion and progression of malignant gliomas and suggested that SLK may be a potential therapeutic strategy for glioma.

Key words: glioma, SLK, E-cadherin, invasion, knock-down

Gliomas are the most common malignant tumors in the central nervous system and are characterized by highly aggressive growth [1]. The highly invasive nature of glioma contributes to the diffuse infiltration and migration of glioma cells. Thus, the glioma regularly results in restrictive and incomplete surgical resection in the surrounding glioma tissues. Although enormous improvements have been made in neurosurgery, chemotherapy, and radiotherapy, the prognosis of patients with glioma is still poor. [1–3]. Previously, more and more evidence demonstrated that epithelial-mesenchymal transition (EMT) was tightly involved in enhancing the invasive capability of gliomas and was correlated with histologic malignancy. During EMT, the cancer cells obtained plasticity with mesenchymal-type movement and became highly invasive. Hence, EMT may be one of the most infusive fields in oncobiology, especially in invasion and metastasis of glioma. [4–7]. Therefore, a better understanding of the EMT in gliomas is a key to research effective therapeutic strategy that abolishes the metastasis and invasion of glioma cells.

Numerous studies indicated that massive cellular and molecular processes may play an essential role in the signaling networks of cell migration [8, 9]. Ste20-like kinase (SLK) is

one of the members of the Ste20 family which is located in the nucleus, cytosol, microtubules and centrosomes. It has been implicated in a variety of biological processes, such as growth, proliferation, survival and cell migration [10–13]. Previous studies have shown that the activation of SLK was required to regulate cell migration [14–16]. Recently, our study also confirmed the function of SLK in the signaling networks of cell migration. Additionally, we have shown that SLK was recruited to the leading edge of migrating cells and co-localized with adhesion complex components such as the microtubules, Rac1 and Paxillin [12]. In human breast cancers, the expression of dominant-negative SLK could result in impaired motility and invasion [15]. Taken together, all available evidence suggests that SLK might be a new biological marker for the development and progression of human cancers. Nevertheless, whether SLK is involved in the modulation of invasiveness of glioma cells still remains to be investigated.

Currently, we revealed that the expression of SLK was markedly higher in glioma tissues and cell lines. Meanwhile, SLK expression level was upregulated in clinical glioma samples and had a negative correlation with E-cadherin. Knock-down of SLK decreased the invasive ability of glioma cells.

Patients and methods

Patients and tissue samples. Fresh-frozen human glioma specimens were gained and in accordance with an institutional review board (IRB) protocol approved by the Partners Human Research Committee at Affiliated Hospital of Nantong University from 2005 to 2012. Normal brain specimens were donated by individuals who died in traffic accidents and had no pathologically detectable lesions. All tumor tissues came from newly diagnosed glioma patients without chemotherapy or radiation therapy before sample collection. All tissues were immediately frozen in liquid nitrogen after surgery and stored at -80°C until use.

Immunohistochemistry. The tissue sections were dehydrated with gradient ethanol and xylene dewaxing and soaked in 0.3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. We treated the sections with 0.1 M citrate buffer (pH6.0) and heated them at 105°C in autoclave for 10 minutes to retrieve the antigen. After rinsing in PBS (pH7.2), the sections were incubated with rabbit anti-human SLK antibody and anti-E-cadherin antibody for 2 h at room temperature. Then, the peroxidase reaction was visualized by incubating the sections with DAB (0.02% diaminobenzidine tetrahydrochloride, 0.1% phosphate buffer solution, and 3% H_2O_2) after washing in PBS. At last, sections were counterstained with hematoxylin, dehydrated, and mounted in resin mount. Immunohistochemical staining was done by two independent scientists and the staining was observed under a microscope. The percentage of glioma cells was scored as follows: 0, <1%; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, >75%. The staining intensities were as follows: 1 (weak staining), 2 (moderate staining), and 3 (strong staining). From each of the two scale scores, each section was classified as: low/no SLK/E-cadherin expression or high SLK/E-cadherin expression.

Western blot analysis. Glioma tissues and cell proteins were homogenized instantly in a homogenization buffer containing 1 M Tris HCl pH7.5, 1% Nonidet p-40 (NP-40), 1% Triton X-100, 10% sodium dodecyl sulfate (SDS), 0.5 M EDTA, 0.5% sodium deoxycholate, leupeptin 10 $\mu\text{g}/\text{mL}$, aprotinin 10 $\mu\text{g}/\text{mL}$, and 1 mM PMSE, and then centrifuged for 30 minutes to gather the supernatant liquid. The supernatant was deliquated in $2\times$ SDS loading buffer and denatured at 100°C for 15 minutes. An equivalent number of proteins from each specimen was electrophoresed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to the PVDF membrane (Millipore, Bedford, Tumor Biol.MA). Being blocked in 5% non-fat milk after Tris Physiological Buffered Saline and Tween 20 (TBST) (20 mM Tris, 150 mM NaCl, 0.05% Tween-20) for 2 h at room temperature, the membranes were incubated overnight at 4°C with the primary antibodies. After washing with TBST for three times, each time for 5 minutes, the membranes were incubated with horseradish peroxidase-linked IgG as the secondary antibody for 2 h at

room temperature. The membrane was developed using the ECL detection systems. The experiments were conducted on three different occasions.

Antibodies. The antibodies used in this study included the following: anti-SLK antibody (1:300; Santa Cruz Biotechnology), E-cadherin (1:500; Santa Cruz Biotechnology), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000; Santa Cruz Biotechnology), Vimentin (1:1000; Santa Cruz Biotechnology), Snail (1:1000; Santa Cruz Biotechnology).

Cell culture. Glioma cell lines, including U87, U251, H4, and U118 were purchased from Cell Library, China Academy of Science, and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY) with 10% fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin-streptomycin mixture (GibCo BRL) at 37°C with 5% CO_2 . The medium was replaced every 2–3 days, and cultures were split using 0.25% trypsin.

Expression plasmid and transient transfection. All oligonucleotides containing the short hairpin RNA (shRNA) target sequences were designed and synthesized by Shanghai Genechem (China). The sequences are siSLK-1: 5'-GCCAU-AACCAGAACCUGAA-3'; siSLK-2: 5'-GGUUGAGAUUGA CAUUAUA-3'. Transient transfections were performed using Lipofectamine 2000, following the manufacturer's recommendation (Life Technologies).

Transwell migration assay. Glioma cells were transfected with SLK shRNAs and starved overnight in DMEM media with 0.1% FBS and then trypsinized and resuspended into DMEM containing 0.1% bovine serum albumin. Cells (1×10^5) were added to the apical chamber of 24-well transwell plates (Corning, 8- μm pore size), and DMEM with 10% FBS was added to the bottom chambers. After cultured overnight, top (non-migrated) cells were removed and bottom (migrated) cells were fixed. Then the cells were stained with crystal violet to visualize nuclei. The number of migrating cells of five fields was counted under $\times 200$ magnification, and the means for each chamber were identified. All experiments were carried out in triplicate and repeated twice.

Monolayer wound-healing assays. Glioma cells were seeded to almost complete confluence in a monolayer in 6-well plates and grown to confluence overnight. After transfected forty-eight hours, cells were serum starved for 12 h and then scratched within the confluent cell layer by using a 10- μL micropipette tip. Then the cells were cultured in 5% fetal bovine serum-Dulbecco's modified eagle medium (FBS-DMEM) at 5% CO_2 and 37°C . Collected pictures at 0, 24, and 48 h time points by an inverted Leica phase-contrast microscope (Leica DFC 300 FX).

Statistical analysis. All statistical analyses were performed with SPSS 13.0 software (SPSS, Inc, Chicago, IL). The Chi square (χ^2) test was used to analyze the relationship between SLK expression and E-cadherin expression and the clinical features expression. The expression of SLK and E-cadherin was studied by using the Spearman rank correlation test

because the data were not in widespread distribution. The Kaplan-Meier survival plots were used to analyze survival data, and curves were compared using the log-rank test. Data were calculated as (mean \pm SD). P-values <0.05 in all cases were considered to be statistically significant.

Results

SLK overexpression in primary glioma. To confirm whether SLK was expressed in gliomas, our study explored the expression of SLK in normal brain tissue specimens and fresh glioma tissues by western blot. The results revealed that the expression level of SLK, at both protein and mRNA levels, was markedly higher in glioma tissues compared with normal brain tissues and increased with the malignant degree (Figure 1A, B). To further analyze the association of SLK with clinicopathologic grades of gliomas, we examined 104 paraffin-embedded glioma tissues, including 38 cases of grade II gliomas, 36 cases of grade III gliomas,

and 30 cases of grade IV gliomas by immunohistochemistry. As shown in Figure 1C, SLK staining intensity from grade II to grade IV tumors was significantly higher than in normal brain tissues and its expression increased as the malignant degree increased. These data show that the expression of SLK is significantly associated with glioma grades.

Correlation between the expression of SLK and glioma patients' survival. We further analyzed the connection between clinical relevance of SLK and glioma patients' survival by summarizing the clinicopathological data of patients (Table 1). The percentage of the SLK positive cells in each specimen was calculated in the 104 samples using immunohistochemical evaluation. As presented in Figure 2A, we found that the expression level of SLK was strongly related to the clinicopathologic grades ($p=0.031$). Further analysis showed that no significant correlation was observed between SLK expression and patient's age, gender, extent of resection or tumor size in 104 glioma cases. However, its expression was negatively correlated with E-cadherin expression, which

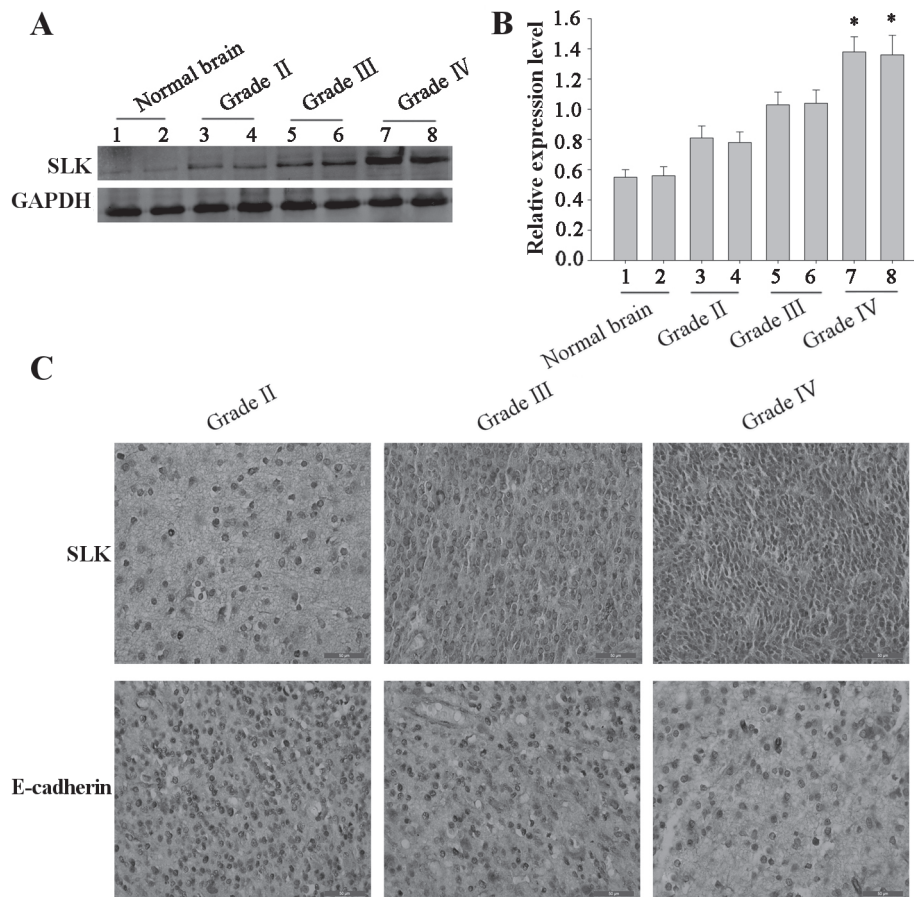


Figure 1. Expression of SLK in human glioma tissues. (A) Expression of SLK protein in two normal brain tissues and glioma tissues (grades II–IV). (B) The bar chart shows the ratio of SLK protein to GAPDH by densitometry. The data are mean \pm SD, $p<0.05$. (C) Paraffin-embedded glioma tissue sections (grades II–IV) were stained with SLK antibodies and anti-E-cadherin antibodies followed by counterstaining with hematoxylin (SP $\times 400$).

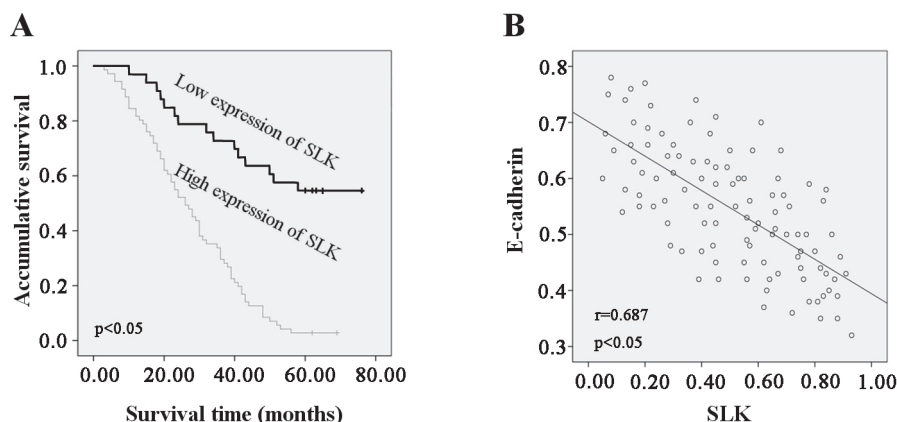


Figure 2. SLK correlated with the glioma patients' survival. (A) Kaplan-Meier postoperative survival curve for high SLK expression and low SLK expression in 104 patients of glioma. Patients in the high-expression of SLK group had poor survival. ($p < 0.05$). (B) The relationship between SLK and E-cadherin.

Table 1. SLK expression and clinicopathologic characteristics of 104 glioma specimens.

Variables	Total	SLK expression		p-value*
		Low	High	
Age				
<45	41	14	27	0.669
>45	63	19	44	
Gender				
Female	45	16	29	0.464
Male	59	17	42	
Tumor location				
Frontal	27	9	18	0.902
Parietal	15	5	10	
Occipital	10	4	6	
Temporal	35	9	26	
Unknown	17	6	11	
Surgery				
Partial resection	41	12	23	0.031
Gross total resection	63	15	48	
Tumor diameter				
<4 cm	36	19	17	0.001
>4 cm	68	14	54	
Necrosis				
Absence	84	15	69	0.000
Presence	20	18	2	
WHO grade				
II	38	18	20	0.031
III	36	9	27	
IV	30	6	24	

Statistical analyses were performed by the Pearson χ^2 -test
* $p < 0.05$ was considered significant

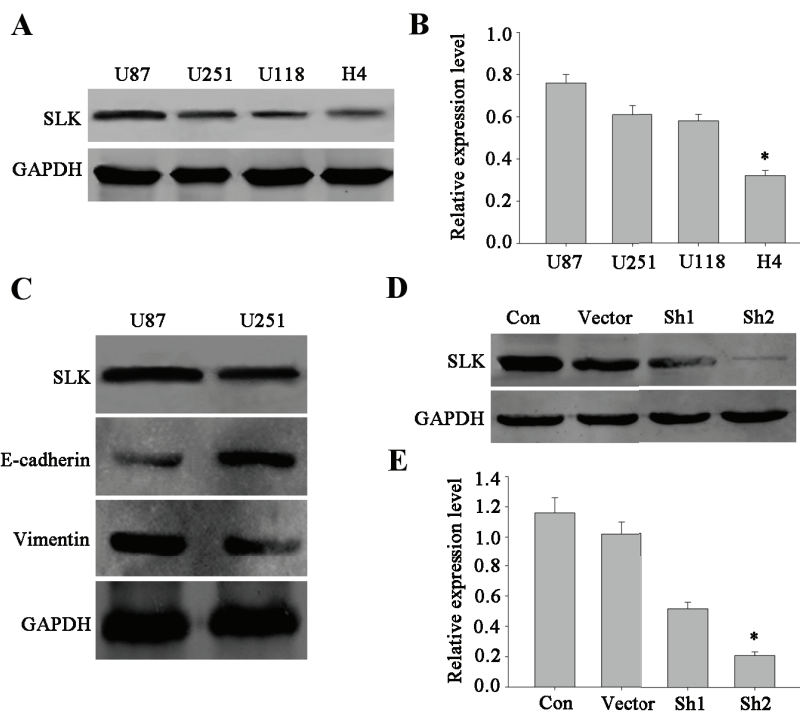


Figure 3. SLK regulated the EMT process of glioma cells. (A) Expression of SLK protein in cultured glioma cell lines (U87MG, U251MG, U118 and H4). (B) The bar chart demonstrates the ratio of SLK protein to GAPDH by densitometry. The data are mean \pm SD. (C) SLK, E-cadherin, Vimentin, and GAPDH protein in U87 and U251 cell lines analyzed by western blot analysis. (D) and (E) Silencing SLK expression by transfection with SLK-siRNA in U87 cells.

is the marker of epithelial cells (Figure 2B). The Kaplan-Meier survival curves revealed that high SLK level significantly correlated with poor survival.

Silencing SLK expression inhibited glioma cell migration. In order to explore the biological significance of endog-

enous SLK in the development and progression of glioma cells, we examined the expression of SLK in human glioma cell lines by western blot analysis, including U87, U251, H4, and U118. As shown in Figure 3A and 3B, SLK was highly expressed in U87 and U251 cells. EMT is an important

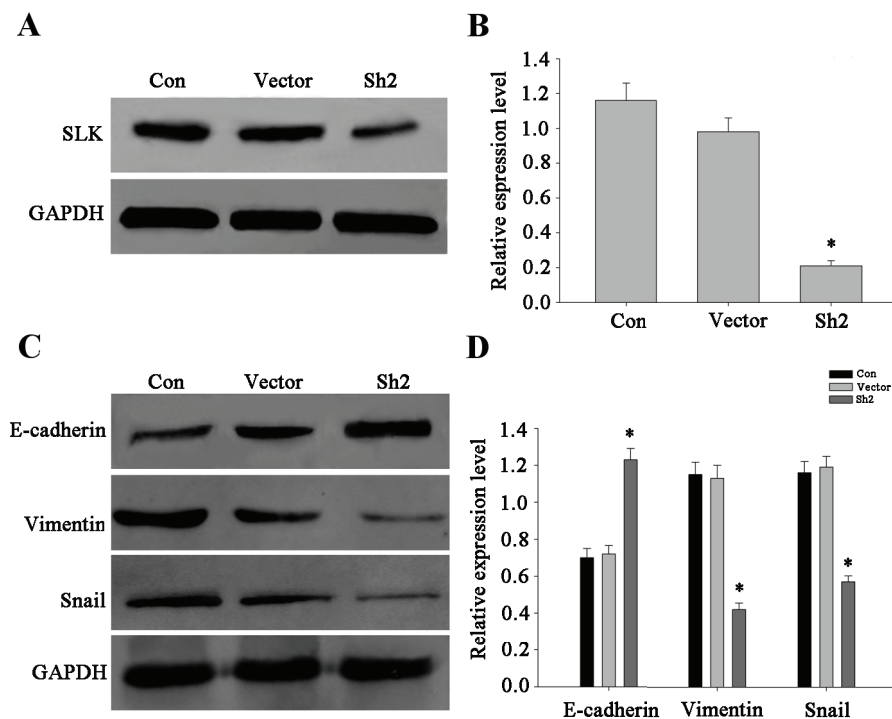


Figure 4. Silencing SLK expression downregulated E-cadherin expression in U87 cells. (A) and (B) Analysis of SLK in control, vector, sh2. (C) Western blot analysis of E-cadherin, Vimentin, and Snail in control, vector, and sh2 cells. (D) The bar chart shows the ratio of E-cadherin, Vimentin, and Snail to GAPDH by densitometry. The data are mean \pm SD. $p < 0.05$.

process in glioma migration and invasion. Hence, we were prompted to examine the effects of SLK on the EMT process in human glioma cells. We studied the gene expression of two EMT marker proteins (E-cadherin, Vimentin) in glioma cells, and as presented in Figure 3C, the expression of E-cadherin was decreased in U87 cells. In contrast, the expression level of mesenchymal markers such as Vimentin was opposite to E-cadherin. Then, the dysfunction of SLK in human glioma cell models with RNA interference (RNAi)-mediated SLK knockdown was tested. U87 cells were transiently transfected with SLK-siRNA or control siRNA. 48 h later, siRNA targeting SLK markedly inhibited the expression of SLK protein in siSLK-2-transfected U87MG cells compared to the cells treated with siSLK-1 and control- siRNA (Figure 3D and 3E).

Therefore, we decided to use siSLK-2 to carry out the following experiments. As shown in Figure 4A and 4B, SLK expression was decreased in U87 cells transfected with siSLK-2. Then we found that knockdown of SLK increased E-cadherin expression and reduced Vimentin expression. Previous studies demonstrated that E-cadherin could be inhibited by Snail. Hence, we sought to explore the expression level of Snail. As expected, its expression was decreased in shSLK/U87MG cells (Figure 4C and 4D). These findings suggest that SLK regulates the EMT process that might contribute to glioma metastasis and invasion.

Modulation of the invasive ability of glioma cells by SLK *in vitro*. To determine whether the invasiveness of glioma

cells was biologically and clinically related to the expression of SLK, representative photo-micrographs were taken at 0, 24, and 48 hours after the glioma cell surfaces were scratched. We found that silencing the endogenous SLK expression dramatically inhibited cell migration in glioma cell lines compared with the control and vector cells, as revealed by wound healing assay (Figure 5A and 5B). Furthermore, the SLK RNAi-transduced U87MG glioma cells markedly reduced the ability of glioma invasion compared with the vector control-transduced cells, as examined by transwell assays (Figure 5C and 5D). Taken together, these results provide evidence that SLK expression modulates the aggressiveness of glioma cells.

Discussion

The malignant glioma is usually associated with high morbidity and mortality and is a serious public health problem all over the world. The outstanding feature of aggressive growth of human gliomas has imposed some obstacles to thorough surgical removal of the tumor and might be the key factor leading to poor prognosis [2, 17–18]. Currently, the study findings provided new insights into the potential role of SLK in promoting the invasiveness of gliomas.

Previous findings, including those made by ourselves, suggested that SLK was required for focal adhesion turnover and cell migration. SLK was required for invasion of breast

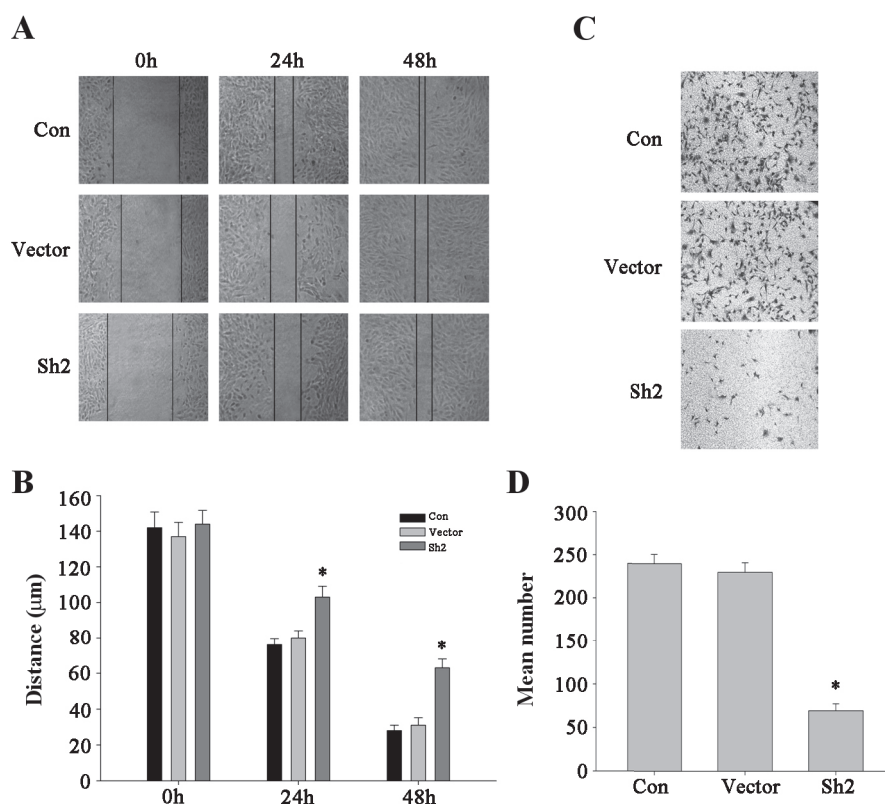


Figure 5. SLK modulated the invasive ability of glioma cells *in vitro*. (A) and (B) Wound healing assays with control, vector and transfected with the SLK siRNA cells. Migration of glioma cells to the wound was visualized at 0, 24, and 48 h with an inverted Leica phase contrast microscope ($\times 200$ magnification). $p < 0.05$. (C) and (D) Knockdown of SLK inhibited the migration ability in transwell assays. The quantification of penetrated cells was represented as the mean of three different experiments.

cancer cells, contributing to promotion of breast cancer progression and activation of relevant signaling pathways [14, 15]. It promoted us to explore whether SLK plays a role in glioma invasion and has a prognostic effect on glioma patients. For this purpose, experiments were done to examine the effect of SLK expression on the invasion of glioma, as western blot analysis showed that SLK was markedly overexpressed in glioma tissue specimens. Together with the immunohistochemical results based on 104 glioma samples, the SLK expression significantly correlated with the WHO histologic grading ($p = 0.031$). This result strongly indicates that SLK is involved in the progression of glioma and might be a new prognostic indicator of glioma. E-cadherin is a transmembrane glycoprotein that mediates homotypic intercellular adhesion and establishes interaction with intracellular proteins and coordination of epithelial cell morphology, polarity and function [19–21]. Knockdown of E-cadherin was related to metastasis and invasion of tumors including breast cancer and HCC [22]. Furthermore, previous study has reported that E-cadherin was down-regulated in the majority of glioma tissues. To access the relationship between SLK and EMT of glioma, the expression of E-cadherin in human glioma tissues was also evaluated. As shown in Figure 3 and 4, clearly positive expression of Vimentin and relatively

weak expression of E-cadherin were detected in glioma cells. Especially, the SLK expression was found to be positively associated with the expression of Vimentin and negatively associated with E-cadherin expression, which suggested that SLK promoted the EMT of glioma.

To further validate the histological results, we altered the SLK expression in glioma cells by siRNA techniques to access its functional levels at the cell level. Interestingly, we found that silencing SLK drastically repressed E-cadherin expression accompanied by a down-regulation of Vimentin on mRNA and protein level in glioma cells. These results suggest that SLK could enhance the expression of Vimentin and repress the expression of E-cadherin in glioma cells. Epithelial-mesenchymal transition leading to malignant cancer progression has been confirmed to be correlated with loss of the epithelial traits and acquisition of the mesenchymal traits, such as increased expression of Vimentin and decreased expression of E-cadherin. Cancer cells with mesenchymal transition transformed into active cancer cells which increased invasive ability [23, 24]. Both, transwell migration assay and monolayer wound-healing assays, showed that depletion of endogenous SLK drastically inhibited the glioma invasion. Hence, these results suggest that SLK-induced EMT in glioma cells promotes the invasiveness of glioma cells.

In summary, our current study provides the first demonstration that SLK, a potential oncogene, is evidently overexpressed in gliomas and is associated with glioma grades as well as poor prognosis. Moreover, we found that silencing SLK expression reduced the ability of glioma invasion with a possible involvement of E-cadherin and Vimentin. Our findings indicate that SLK may be a novel therapeutic target for glioma. However, further study is required to explore the precise mechanism of SLK in the development of gliomas.

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