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LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

Identification of the role of Smad interacting protein 1 (SIP1) in glioma

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Abstract Glioma is an extremely aggressive and lethal form of brain cancer. Despite recent advances in diagnostics and treatments, prognosis for advanced patients suffering from these diseases remains poor. Therefore, identification of new therapeutic targets for glioma is of significant importance. In this study, we identified the important role of Smad interacting protein 1 (SIP1; also known as ZEB2) in glioma. We firstly found that SIP1 expression was high in four tumorigenic glioma cell lines but low in two nontumorigenic glioma cell lines. By knockdown or overexpression assay, we discovered that knockdown of SIP1 expression statistically significantly inhibited cell migration and invasion of tumorigenic glioma cells, while overexpression of SIP1 promoted cell migration and invasion of nontumorigenic glioma cells. SIP1 knockdown inhibits and overexpression promotes glioma cell clonogenicity in vitro. Further studies identified that SIP1 overexpression inhibits expression of E-cadherin and enhances expression of mesenchymal proteins such as fibronectin and vimentin. This study supports the rationale for developing SIP1 as a potential therapeutic and diagnostic target for gliomas.

Keywords Smad interacting protein 1 (SIP1) · Glioma · E-cadherin · Migration · Invasion

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Introduction

Brain tumors can be classified into four general categories: glioma, meningioma, schwannoma, and medulloblastoma. Glioma occurs in the glial cells and is the most common type of brain tumor in adults, accounting for about 42% of all adult brain tumors. Glioblastoma multiforme (GBM), which is classified as a World Health Organization (WHO) grade IV tumor that originates from poorly differentiated astrocytes, is an extremely aggressive and lethal form of brain cancer [1]. Unfortunately, it is the most common form of brain tumor in adults, accounting for 67% of all astrocytomas. Because the glia cells are diffusely distributed in the brain, glioma is highly resistant to chemotherapy and radiotherapy. It is an aggressive, highly invasive, and neurologically destructive tumor considered to be among the deadliest of human cancers. Despite recent advances in diagnostics and treatments, prognosis for advanced patients suffering from these diseases remains poor [2, 3]. One of the important reasons is that active cell migration and invasion of glioma cells ultimately lead to ubiquitous tumor recurrence and patient death. Although our understanding of glioma carcinogenesis has been

steadily improving, the factors that mediate glioma invasion are still poorly understood [4]. Therefore, discovery of critical carcinogenic pathways and identification of new therapeutic targets for glioma are of significant importance for global and local public health.

Smad interacting protein 1 (SIP1; also known as ZEB2) belongs to the zinc finger E-box binding protein (ZEB) family. ZEB was first isolated from a *Drosophila* complementary DNA (cDNA) expression library [5]. Previous studies reported expression of SIP1 in various tumors, including ovarian carcinoma, gastric tumors, pancreatic tumors, and squamous cell carcinoma, which associated with various clinicopathological features such as effusions, histological type, differentiation grade, and overall survival [6–9]. SIP1 was also identified in a large-scale screen for cancer-related genes, which demonstrates its putative role in oncogenic transformation [10]. However, expression and function of SIP1 in gliomas has not yet been assessed. In this study, we examined the expression of SIP1 in different glioma cell lines and investigated the role of SIP1 in malignant glioma. These findings will be useful in identifying potential candidates for targeted therapeutic intervention of glioma.

Materials and methods

Cell culture and reagents

Human glioma cell lines (U87, U373, U138, T98G, U118, SW1088) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in minimum Eagle's medium (MEM) (U87, U373, U138, and T98G), Dulbecco's modified Eagle's medium (DMEM) (U118) or Leibovitz's L-15 (SW1088) medium supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin/streptomycin. All antibodies were bought from Santa Cruz Biotechnology (Santa Cruz, CA). SuperScript First-Strand synthesis system for reverse transcription polymerase chain reaction (RT-PCR) (cat #11904-018) was a product of Invitrogen. All primers were synthesized from Sigma-Proligo (The Woodlands, TX, USA). BD BioCoat Matrigel Invasion Chambers were products of BD company (BD Biosciences, Bedford, mA).

RNA preparation and semiquantitative RT-PCR

Cells were grown to reach 70–80% confluence. Total RNA was harvested using TriZol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. cDNA was synthesized using the SuperScript First-Strand cDNA synthesis kit (Invitrogen). cDNAs were used for PCR analysis using the following oligonucleotide primers: SIP1: forward, 5′-cgcttgacatcactgaagga-3′, and reverse, 5′- cttgccacactctgtgcatt-3′; E-cadherin: 5′- tgcccagaaaatg aaaaagg-3′ and reverse, 5′-gtgtatgtggcaatgcgttc-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, 5′-tgcctcctgcaccaccaact-3′, and reverse, 5′-cccgttcagctcag ggatga-3′; Platinum® Taq DNA polymerase (Invitrogen) PCR conditions were as follows: 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 2 min, and final extension at 72°C for 10 min for ZEB2 (28 cycles), E-cadherin (30 cycles), and GAPDH (28 cycles). The PCR products were resolved by electrophoresis on 1% ethidium-bromide–stained agarose gels, and band intensity was quantitated with the use of ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Transfection of glioma cells with siSIP or pcDNA3-SIP1

Small-interfering RNA (siRNA) duplex oligonucleotides targeting human SIP1 messenger RNA (mRNA) were synthesized by Sigma-Proligo (The Woodlands, TX) according to a previously published sequence (siSIP1; 5′-CCUCUUGUCAUCUGUACUUTT-3′) [11]. cDNAs for wild-type SIP1 were cloned in the pcDNA3 vector (Invitrogen). The construct was sequenced and named pcDNA3-SIP1. One day before transfection, U373 or U138 cells at 80% confluence were detached by treatment with 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA; Invitrogen) and plated onto a 24-well plate at 5×10^4 cells per well. U373 cells were then transfected by incubation with siSIP1 (siLuc as a control) at final concentrations of 100 nM and Oligofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. U138 cells were transfected with pcDNA3-SIP1, or empty expression vector pcDNA3 (Invitrogen) in the presence of Fugene HD (Roche). Cells were collected for the following assay after 48 h or for RNA and protein extraction after 72 h.

Wound healing assay

U373 or U138 cells were transfected with siSIP1 or pcDNA3-SIP1 as mentioned above. When cell confluence reached about 90% at 48 h post transfection, an artificial homogenous wound was created on the monolayer with a sterile plastic 200-μl micropipette tip. After wounding, the debris was removed by washing the cells with serum-free medium. Migration of cells into the wound was observed at different time points. Cells that migrated into the wounded area or cells with extended protrusion from the border of the wound were visualized and photographed under an inverted microscope (40× objective) (Leica, Solms, Germany). A total of three areas were selected randomly from each well and the cells in three wells of each group were quantified in each experiment.

In vitro Matrigel invasion assay

Cell invasiveness in vitro was reflected by the ability of cell to transmigrate a layer of extracellular matrix in Biocoat Matrigel Invasion Chambers (Becton–Dickinson Labware, Bedford, MA). U373 or U138 cells were transfected with siSIP1 or pcDNA3-SIP1 as mentioned above. At 48 h post transfection, cells were trypsinized and plated at a density of 5.0×10^4 per insert. Medium with 10% FBS was added to the lower chamber as chemoattractant. After incubation for 24 h, noninvading cells were removed from the top of the Matrigel with a cotton-tipped swab. Invasive cells, which had the ability to push themselves through the 8-μm pores and grow on the lower surface, were fixed with 100% methanol and stained with 1% toluidine blue (Sigma) before counting under an inverted microscope. In all experiments, data were collected from three chambers.

Soft agar colony-formation assay

Soft agar colony-formation assay was used to determine the effect of SIP1 knockdown or overexpression on the transformation capability of glioma cells. In brief, a bottom layer (0.6% low-melt agarose) was prepared with MEM containing 10% FBS and 100 units/ml penicillin/streptomycin. A top layer (0.3% agar) was prepared with MEM and the same media as described above but containing 1,000 U373 or U138 cells which were transfected with siSIP1 or pcDNA3-SIP1 as mentioned above. Plates were incubated at 37°C in 5% CO_2 in a humidified incubator for 2 weeks. The plates were then scanned and photographed, and the number of colonies was quantified.

Western blotting

U138 cells were transfected with pcDNA3-SIP1 as mentioned above. At 72 h post transfection, cells were washed twice with phosphate-buffered saline (PBS) and solubilized in radioimmunoprecipitation assay lysis buffer. The supernatants containing the whole-cell protein extracts were obtained after centrifugation of the cell lysates at $12{,}000 \times g$ for 10 min at 4°C. The protein concentrations were determined by bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Heat-denatured protein samples (20 μg per lane) were resolved by sodium dodecyl sulfate (SDS)—polyacrylamide gel electrophoresis (PAGE) and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was incubated with primary and secondary antibodies, respectively, for 1.5 h at room temperature. The primary antibodies against SIP1, E-cadherin, fibronectin, vimentin, and β-actin were purchased from Santa Cruz Biotechnology. Signals were developed with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Inc.).

Statistical analysis

Data are presented as mean ± standard deviation. Data were analyzed using the SPSS software version 12.0 for Windows. Statistical analyses were done by analysis of variance (ANOVA) or Student's t test. P value <0.05 was considered statistically significant.

Result

Expression of SIP1 and E-cadherin in different glioma cell lines

Previous studies showed that an inverse correlation between SIP1 and E-cadherin expression levels was observed in some breast cancer cell lines and the intestinal type of gastric carcinomas [6, 12]. We first examined the expression of SIP1 and E-cadherin in three human glioma cell lines that were established from high-grade tumors (U87 MG, U373 MG, and U138 MG). SIP1 expression was high in U87 and U373 cells and low in U138 cells, while E-cadherin expression was low in U87 and U373 cells and high in U138 cells (Fig. 1). U87 and U373 cells are tumorigenic in nude mice, while U138 was the only cell line studied that was not tumorigenic in nude mice according to data from the American Type Culture Collection (http://www.atcc.org/).

SIP1 knockdown inhibits and overexpression promotes glioma cell migration and invasion in vitro

To investigate the functional role of SIP1 on migration and invasion of glioma cells in vitro, we used siRNA to specifically knockdown SIP1 expression or constructed wild-type SIP1 expression vector pcDNA3-SIP1 to overexpress SIP1 expression (Fig. 2). Then U373 cells were transfected by incubation with siSIP1, and U138 cells were transfected with pcDNA3-SIP1. Post transfection (48 h), wound healing and Matrigel invasion assays were performed to examine the effect of SIP knockdown or overexpression on glioma cell migration and invasion. Our results showed that SIP1 knockdown inhibits U373 cell migration and invasion, while SIP1 overexpression promotes U138 cell migration (Fig. 3) and invasion (Fig. 4).

SIP1 knockdown inhibits and overexpression promotes glioma cell clonogenicity in vitro

Colony-formation assay is considered the most stringent assay in vitro for measuring malignant transformation and

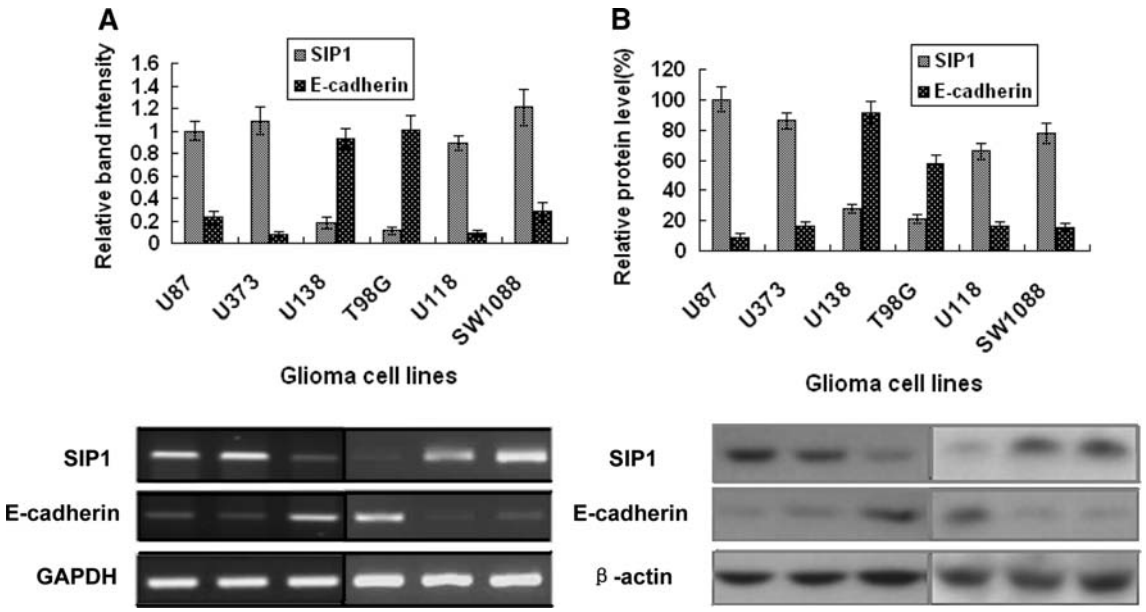

Fig. 1 SIP1 and E-cadherin expression in human glioma cell lines. **a** SIP1 and E-cadherin mRNA expression. Representative agarose gel images showing mRNA expression for SIP1, E-cadherin, and the loading control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in glioma cell lines as measured by RT-PCR. The intensities of products for SIP1, E-cadherin, and GAPDH were quantified with the use of ImageQuant software. The relative mRNA levels of SIP1 and E-cadherin represent the GAPDH-normalized mRNA levels. **b** SIP and E-cadherin protein expression. Western blotting analysis of SIP1 and E-cadherin levels in glioma cell lines; β-actin loading control is shown in the below panel. The relative protein levels of SIP1 and E-cadherin represent the β-actin-normalized protein levels; $n = 3$ experiments

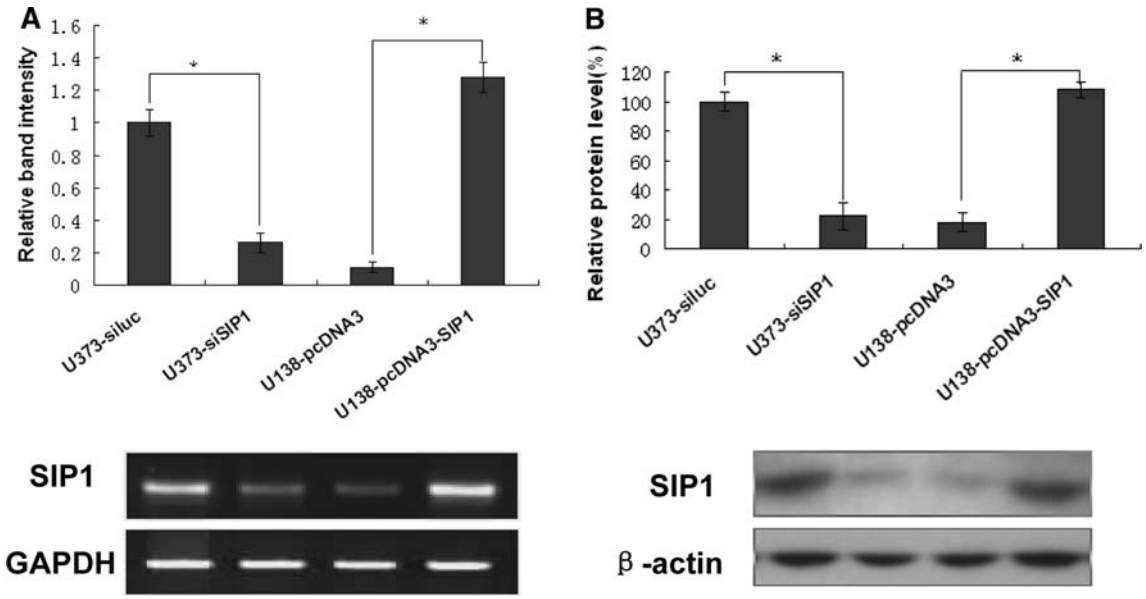

Fig. 2 Effect of siSIP1 and pcDNA3-SIP1 on SIP1 expression in U373 or U138 cells. U373 or U138 cells were transfected with siSIP1 or pcDNA3-SIP1 as mentioned in the "Materials and methods" section. Total RNA and protein were extracted from the transfected cells 72 h after transfection. **a** RT-PCR analysis of SIP1 expression in each group. The bar graphs show the GAPDH mRNA-normalized levels of SIP1. **b** Immunoblot analysis of SIP1 and β-actin protein expression in each group at 72 h after transfection. Mean values and upper 95% confidence intervals of three independent transfection experiments are shown. * $P < 0.05$, statistically significant difference

tumorigenesis ability. To determine the effect of SIP1 on the clonogenicity of glioma cells, we performed colony-formation assay in vitro. Our results demonstrated that colony numbers of U373 cells treated with siSIP1 were markedly lower than the numbers of control group. In contrast, treatment of U138 cells with pcDNA3-SIP1

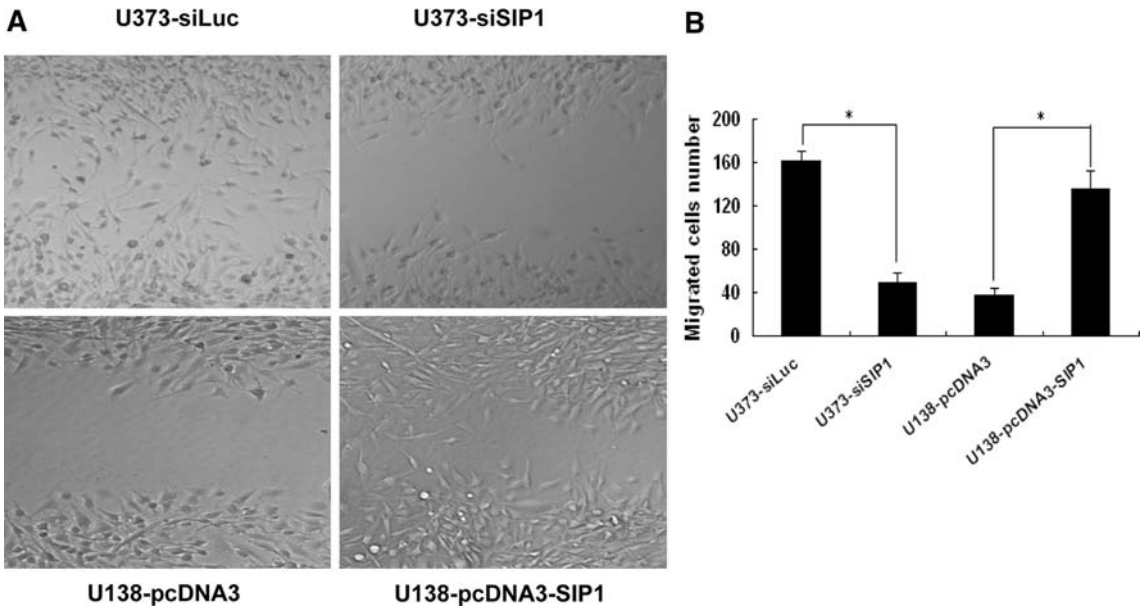

Fig. 3 Effect of SIP1 knockdown or overexpression on glioma cell migration in vitro. U373 or U138 cells were transfected with siSIP1 or pcDNA3-SIP1 as mentioned in the "Materials and methods" section. **a** Twenty hours after wounding, cells with extended membrane protrusion moved into the wounded areas. **b** The number of migrated cells in each treatment groups ($n = 3$). * $P < 0.05$, statistically significant difference

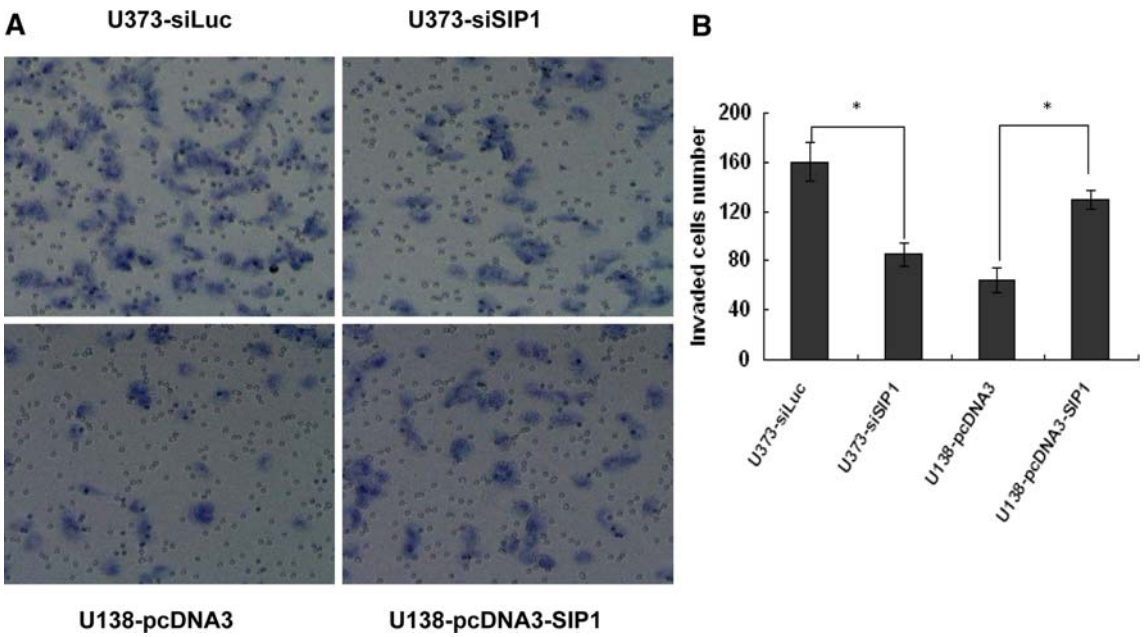

Fig. 4 Effect of SIP1 knockdown or overexpression on glioma cell invasion in vitro. U373 or U138 cells were transfected with siSIP1 or pcDNA3-SIP1 as mentioned in the "Materials and methods" section. **a** The invasive cells were stained and counted under microscope. **b** Quantitative results for the transmembrane ability of each group cells ($n = 3$). * $P < 0.05$, statistically significant difference

increased colony-formation numbers (*$P < 0.05$, Fig. 5). Thus, knockdown of SIP1 significantly inhibited in vitro transformation capability of U373 cells, and overexpression of SIP1 promoted in vitro transformation of U138 cells.

SIP1 overexpression inhibits expression of E-cadherin and enhances expression of mesenchymal proteins

Loss of E-cadherin triggers invasion, metastasis, and dedifferentiation in various epithelial carcinomas. Recently, it has

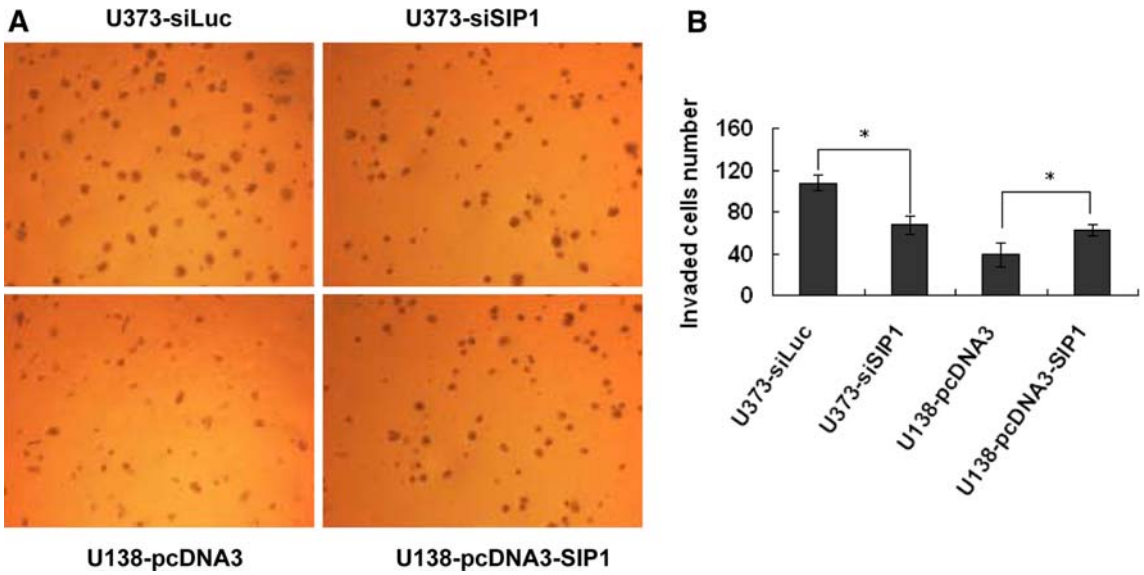

Fig. 5 Effect of SIP1 knockdown or overexpression on glioma cell colony formation. U373 or U138 cells were transfected with siSIP1 or pcDNA3-SIP1 as mentioned in the "Materials and methods" section. Then cells were collected and seeded at 1,000/well density in 6-cm plates. After 14 days, cell colonies were photographed. **a** Representative field photograph by colony-forming assay (×100). **b** The number of colonies quantified. Each value is the mean ± standard deviation (SD) of five different fields (* $P < 0.05$)

been reported that several transcription factors strongly repress transcription of the E-cadherin gene (*CDH1*) by binding E-box on E-cadherin promoter, including members of the Snail, ZEB, and basic helix-loop-helix (bHLH) families. We therefore examined whether SIP1 overexpression influences the expression of E-cadherin and whether such change in E-cadherin contributed to the subsequent induction of cell migration and invasion on glioma cells. Examination of the protein level indicated that expression of E-cadherin was significantly inhibited after pcDNA3-SIP1 transfection compared with the control plasmid transfection (Fig. 6). This result suggests that SIP1 overexpression inhibits E-cadherin expression.

Previously studies have showed that expression of SIP1 and de novo expression of vimentin are frequently involved in epithelial-to-mesenchymal transitions (EMTs) under both normal and pathological conditions [13]. EMT is thought to promote cancer cell migration and invasion. EMT has been observed downregulation of epithelial-specific proteins such as E-cadherin while simultaneously expressing mesenchymal proteins such as N-cadherin, fibronectin, and vimentin [14]. Therefore, we examined the expression level of mesenchymal proteins, fibronectin, and vimentin. The results show that the expression of these mesenchymal cell markers was increased after pcDNA3-SIP1 transfection compared with the control plasmid transfection (Fig. 6). These data indicate that SIP1 overexpression enhances expression of mesenchymal proteins.

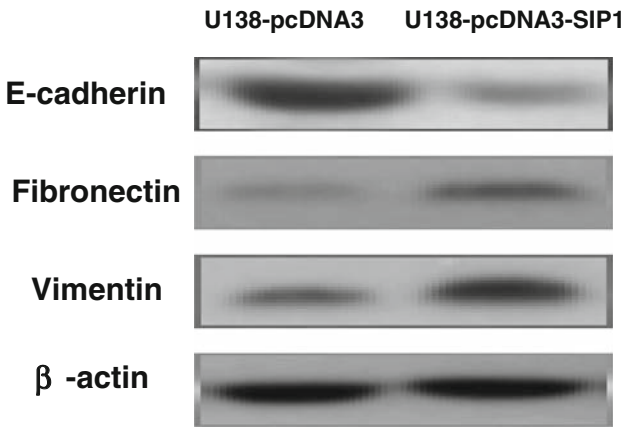

Fig. 6 SIP1 overexpression inhibits expression of E-cadherin and enhances expression of mesenchymal proteins. Expression level changes of E-cadherin, fibronectin, and vimentin in U138 cells at 72 h after pcDNA3-SIP1 transfection. β-actin is used as a loading control

Discussion

The ZEB family of transcription factors contains two members (ZEB1, also known as δEF1, TCF8, AREB6; and ZEB2, also known as SIP1) encoded by independent genes (*ZFHX1A* and *ZFHX1B*, respectively) [15]. The expression of ZEB has been reported in different tumor series. SIP1 (ZEB2) expression has been analyzed in ovarian, squamous cell carcinomas, gastric, and pancreatic tumors [6–9], while ZEB1 expression has mainly been studied in colorectal

tumors [16, 17] and uterine cancers, where it has been associated with aggressive behavior [15, 18]. In this study, we found SIP1 expression to be high in two tumorigenic glioma cell lines but low in two nontumorigenic glioma cell line. We further showed that knockdown of SIP1 expression statistically significantly inhibited cell colony formation in soft agar, and migration and invasion of tumorigenic glioma cells, while overexpression of SIP1 promotes cell colony formation in soft agar, and migration and invasion of nontumorigenic glioma cells. These results firstly suggest that SIP1 is associated with glioma cell transformation, migration, and invasion capability in vitro.

Tumor invasion and metastasis are complex processes and appear to be controlled by a coordinated series of cellular and molecular processes that enable tumor cells to dissociate and migrate from the primary tumor. The acquisition of the invasive phenotype of glioma probably involves alterations in cell–cell adhesion and increases in cell motility [19, 20]. In this process, E-cadherin loss ostensibly promotes metastasis by enabling the first step of the metastatic cascade: disaggregation of cancer cells from one another [21]. Utsuki et al. investigated the relationship between E-cadherin expression and tumor grade in astrocytomas. They found that E-cadherin was not expressed [22]. Reports have shown that several transcription factors [such as Snail (SNAI1 and SNAI2), ZEB (ZEB1 and ZEB2), and basic helix-loop-helix (bHLH: E47 and TWIST) families] strongly repress E-cadherin and they are now thought to be involved in tumor progression. Nevertheless, the specific role of these different repressors in tumorigenesis is not fully understood [15]. Here, in further studies we found that SIP1 overexpression inhibits expression of E-cadherin.

The changes in cell adhesion and migration during tumor invasion are reminiscent of an important developmental process termed epithelial–mesenchymal transition (EMT) [23]. EMT is the cellular and molecular processes by which epithelial cells lose cell–cell interactions and apico-basal polarity at the same time as acquiring mesenchymal and migratory properties [15]. The loss of functional E-cadherin and overexpression of mesenchymal cell markers such as N-cadherin, fibronectin, and vimentin are hallmarks of EMT and carcinoma cell invasiveness [15, 23]. Our data indicate that SIP1 overexpression inhibits expression of E-cadherin and enhances expression of mesenchymal proteins, fibronectin and vimentin. In conclusion, our results support the rationale for developing SIP1 as a potential therapeutic and diagnostic target for glioma. Further studies aimed at investigating the role of SIP1 and E-cadherin in gliomagenesis in different grades of glioma patient samples and identifying possible relationships between SIP1 and E-cadherin mRNA or protein levels and various clinicopathological and prognostic parameters of gliomas.

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