Effects of syndecan-1 on the expression of syntenin and the migration of U251 glioma cells

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Abstract. Glioma is the most frequently occuring primary brain tumor. Syndecan-1 (SDC1) expression is related to poor prognosis of numerous human malignancies including glioma. Syndecan binding protein (SDCBP) is an important partner for SDC1. The present study investigated whether SDC1 and SDCBP are expressed in glioma and their functions on glioma cell migration. An immunohistochemical assay revealed that SDC1 and SDCBP were expressed and were positively related to malignant level of glioma (SDC1, r_s=0.576, P=0.001; SDCBP, r_s=0.661, P<0.001). Moreover, the protein levels of SDC1 were positively correlated with those of SDCBP in glioma tissues (r_s=0.628, P=0.001). In U251 glioma cells, protein levels of SDC1 and SDCBP were both upregulated in U251 cells with SDC1 overexpression, while downregulated with SDC1 knockdown. Transwell assay and scratch-wound healing assay showed that SDC1 overexpression significantly increased U251 cell migration, while SDC1 knockdown had the opposite effects. Rac1 activity, signal transducer and activator of transcription 3 (STAT3) phosphorylation, as well as expression of matrix metalloproteinase 2 (MMP2) and MMP9 was significantly increased by SDC1 overexpression, while was decreased by SDC1 knockdown. In conclusion, SDC1 overexpression upregulated SDCBP expression, and promoted glioma cell migration via Rac1 activation.

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Introduction

Glioma refers to malignant tumors that are frequently found in the central nervous system (1), accounting for 45% of primary intracranial tumors with an incidence rate of ~5/100,000 per year (2). Despite the significant advance in the surgical and medical treatment of this disease, the prognosis for glioma patients is still poor (3). Therefore, the study of the pathophysiology of glioma will help to develop new therapeutic strategies.

Syndecan-1 (SDC1, also known as CD138) is a major member of syndecans, which belong to the transmembrane heparan sulphate proteoglycan (HSPG) family. HSPGs consist of an extracellular domain carrying glycosaminoglycan (GAG) chains, a transmembrane domain and a highly conserved cytoplasmic domain (4). Studies have shown that SDC1 participates in diverse cellular processes, including cell proliferation (5,6), migration (7,8), invasion and angiogenesis (9,10). SDC1 expression is dysregulated in many human cancers such as glioma and its expression is related to poor prognosis and high metastatic potential of these cancers (11-20). Syndecan binding protein (SDCBP, also known as syntenin), a scaffold protein containing two PDZ domains, has been identified as a molecule binding to the cytoplasmic domain of SDC (21). Recent studies on glioma have also demonstrated that SDCBP is an important mediator of invasion (22,23). SDC1 may form a functional complex with SDCBP to play key roles in regulating tumor cell adhesion and migration, as well as the biogenesis of exosomes (24,25). The association of SDC1 and SDCBP in glioma has not be investigated. Rac1, as a member of Rho GTPase family proteins, it regulates cell migration, reorganization of cytoskeletal as well as cell proliferation (26). The migration of glioma cells was suppressed by the inactivation of Rac1 (27). The regulatory function of SDC1 and SDCBP on Rac1 activity has been reported. Heparanase stimulated the clustering of SDC1 and enhanced cell spreading, thus promoting cell adhesion and spreading, by PKC, Src and Rac1 (28). SDCBP knockdown suppressed Rac1 activity and the invasion of breast cancer cells (29). It is not known whether SDC1/SDCBP expression affects Rac1 activity in glioma.

In the present study, we compared SDC1 and SDCBP expression among high grade gliomas, low grade gliomas and non-tumorous brain tissues, and explored the correlation between SDC1 and SDCBP expression in glioma tissues. We

also manipulated the expression of SDC1 in U251 glioma cells and studied the functions of SDC1 and SDCBP expression, cell migration as well as Rac1 activity. This study may provide an experimental basis for the identification of novel therapeutic target in glioma.

Materials and methods

Tissue specimens. Archival formalin-fixed and paraffin-embedded tissue specimens were obtained at the First People's Hospital of Zunyi and the First Affiliated Hospital of Zunyi Medical College (Zunyi, Guizhou, China). Thirty-two patients with glioma admitted from 2014 to 2016 were enrolled in this study. The glioma was confirmed by pathological examination after operation. These patients included 7 cases of World Health Organization (WHO) level I glioma, 7 specimens of grade II glioma, 9 specimens of level III glioma as well as 9 specimens of level IV glioma [glioblastoma multiforme (GBM)]. The glioma patients did not receive any surgical treatment, preoperative radiotherapy and/or chemotherapy before surgery. Non-neoplastic brain tissues were available at the inside decompression of five patients who received temporal lobe resection for epilepsy. The study was approved by the Clinical Research Ethics Committee of the First People's Hospital of Zunyi and the First Affiliated Hospital of Zunyi Medical College (Zunyi). All participants gave their written concent agreement.

Immunohistochemical analysis. Tissue specimens were sectioned, deparaffinized and hydrated. After antigen retrieval, slices were incubated with rabbit anti-human SDC1 (dilution, 1:100; cat. no. ab128936) or SDCBP monoclonal antibody (dilution, 1:50; cat. no. ab133267) (both from Abcam, Cambridge, MA, USA) instantly at 4˚C. Then washed with phosphate-buffered saline (PBS), the Donkey anti-Rabbit IgG H&L secondary polyclonal antibody (dilution, 1:1,000; cat. no. ab98488; Abcam) was applied for half an hour at 23˚C. The slices were stained with diaminobenzidine (DAB; Zhongshan, Beijing, China) and counterstained with hematoxylin. Images were captured with Leica Application Suite 4.6.0 program (Leica, Deerfield, IL, USA) at magnification, x200. Five fields were chosen at random for each slice. Intensity of immunoreactivity (IRS) was scored as: IRS = percentage of positive cells (PP) + staining intensity (SI). The SI was 0, 1, 2 and 3, respectively, when the staining was negative, weak, intermediate and powerful. The score of PP was 0, 1, 2, 3 and 4, respectively, when the percentage was <5, 5-25, 26-50, 51-75 and >75%. The IRS was set as 0-1, negative (-); 2 weakly positive (+); 3-4 moderately positive (++); ≥5 strongly positive (+++). The evaluation of staining results was carried out by two investigators independently.

Cell culture and transfection. U251 cells were from China Center for Type Culture Collection of Wuhan University (CCTCC; Wuhan, China) and cultured in Eagle's Minimum Essential Medium (MEM; HyClone, Logan, UT, USA) coupled with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA). U251 cells were infected with SDC1 expression plasmid (SDC1-OE), control plasmid (vector) (Shanghai Genechem Co., Ltd., Shanghai, China), SDC1 siRNA (5'-AGGAGGAAUUCUAUGCCUGA-3') or control siRNA (GenePharma Co., Ltd., Shanghai, China) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Two days after infection, transfected cells were harvested and SDC1 expression was evaluated by western blot analysis.

Western blot analysis. The cells were lysed with radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China). Besides, concentrations of protein were quantified with BCA assay kit (Thermo Fisher Scientific, Rockford, IL, USA). The extracted protein was influenced by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred onto the membrane of a polyvinylidene difluoride (PVDF). Immune-detection of specific proteins was performed with enhanced chemiluminescence (ECL; Millipore, Bradford, PA, USA) following the incubation of primary antibodies (rabbit anti-human SDC1; dilution, 1:200; cat. no. ab128936) or SDCBP monoclonal antibody (dilution, 1:500; cat. no. ab133267) and donkey anti-rabbit IgG H&L (HRP) (dilution, 1:1,000; cat. no. ab6802). All the important antibodies and secondary antibodies were purchased from Abcam. Rabbit anti-human β-actin (dilution, 1:1,000; cat. no. ab8227) or GAPDH monoclonal antibody (dilution, 1:1,000; cat. no. ab9485) served as a loading control. The Image Pro Plus 6.0 was used to quantify immunoblot intensities (Media Cybernetics, Bethesda, MD, USA). The experiments were performed three times.

Transwell assays. The migration ability of cells was assessed by Transwell assay. U251 cells were transfected with plasmids and siRNAs as described above. Forty-eight hours after transfection, U251 cells ($3x10^4$/well) were seeded onto the upper chamber (8-μm pore size; Corning, Lowell, MA, USA) containing MEM. The lower chamber contained MEM with 10% FBS. After being incubated for 24 h, non-migrated cells were completely removed, then stain and fix the remaining cells with crystal violet. The cell numbers were calculated by using a microscope (Nikon, Tokyo, Japan) in five sections for each group. Carry out the experiment in triplicate and repeat it for three times independently.

Scratch-wound healing assay. Cells migration capacity was assessed with a scratch-wound healing assay. Cells were transfected with plasmids and siRNAs and grown to >90% confluence. A linear wound was created by scraping the cell monolayer with a sterile 200 μl pipette tip. Then, cells were then cleaned up with MEM three times and cultured for 0 and 12 h before being photographed with a microscope (Nikon). The formula is the percentage of wound closure: (original wound area actual wound area)/area of the original wound x100. The experiment was repeated three times.

Pull-down assay of GST-PAK1-binding domain (GST-PBD). U251 cells in 10 cm culture dishes were transfected with plasmids or siRNAs as described above. At 48 h post-transfection, GST-PBD pull-down assay was carried out using active Rac1 pull-down and detection kit (Thermo Fisher Scientific) following the manufacturer's protocol. The precipitated samples were analyzed via western blotting.

Table I. Results of immunohistochemical staining.

Protein	Expression	Control (n=5)	I-II (n=14)	III-IV (n=18)	Kruskal-Wallis H test		Mann-Whitney U test		
					H	P-value		Z	P-value
SDC1	-	4	7	2	12.784	0.002[a]	III-IV vs. control	-2.852	0.003[c]
	+	1	4	4			III-IV vs. I-II	-2.806	0.004[c]
	++	0	2	5			I-II vs. control	-1.251	0.305
	+++	0	1	7					
SDCBP	-	5	8	2	16.27	<0.001[b]	III-IV vs. control	-3.193	0.001[c]
	+	0	3	2			III-IV vs. I-II	-3.120	0.001[c]
	++	0	2	6			I-II vs. control	-1.689	0.175
	+++	0	1	8					

-, negative staining; +, weak positive; ++, moderate positive; +++, strong positive. [a]P<0.01, [b]P<0.001 and [c]P<0.0167. SDC1, syndecan-1; SDCBP, syndecan binding protein.

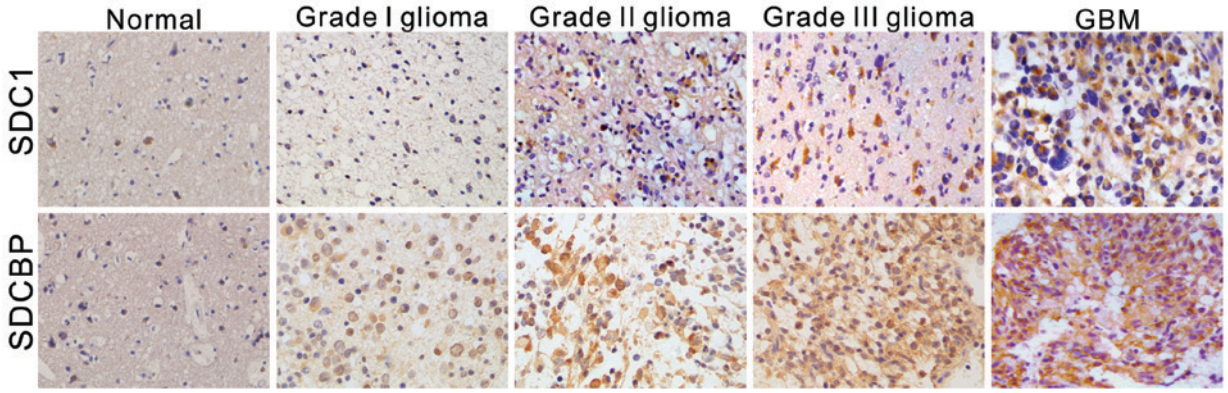

Figure 1. Immunohistochemical staining of syndecan-1 (SDC1) and syndecan binding protein (SDCBP) in normal brain tissues and glioma (x200).

Statistical analysis. SPSS software was used to carry out statistical analysis (version 17.0; SPSS Inc., Chicago, IL, USA). Ordered variables in different groups were analyzed with Kruskal-Wallis H test. Mann-Whitney U test as well as Bonferroni modification (α'=0.0167) was used to compare the difference between two groups. Associations between two factors were assessed by Spearman's correlation analysis. One-way ANOVA followed by LSD-t experiment (for the same variance) or Tamhane's T2 experiment (for different variance) was used to analyze the mean values for different groups (α=0.05). P-values <0.05 were regarded as statistically significant.

Results

SDC1 and SDCBP are upregulated in human glioma tissues. Immunohistochemical analysis was carried out in 32 glioma and 5 non-tumorous brain tissues with antibodies against SDC1 or SDCBP (Fig. 1). SDC1 mainly located in the cell membrane and cytoplasm, while SDCBP localized in the cytoplasm. In non-tumorous brain tissues, the signals were weak and the positive-staining cells were scattered. In low grade glioma tissues (grade I-II), the signals were weak to moderate. High grade gliomas (grade III-IV) had the strongest staining intensity and the highest positive-staining cell number. The positive rate of SDC1 in non-tumorous brain tissues, low level gliomas and high level gliomas were 20.0% (1/5), 50.0% (7/14) and 88.9% (16/18), respectively. The positive rate of SDCBP in non-tumorous brain tissues, low level gliomas as well as high level gliomas were 0% (0/5), 42.9% (6/14) and 88.9% (16/18), respectively. By Kruskal-Wallis H test (Table I), we found that the expression of SDC1 and SDCBP showed significant difference among the three groups. According to the findings of Mann-Whitney U test experiment, there was an obvious difference in the expression of SDC1 as well as SDCBP between high grade gliomas and control brain tissues, as well as between high grade gliomas and low grade gliomas. There was no significant difference of SDC1 and SDCBP expression between low level gliomas and control brain tissues. Moreover, SDC1 and SDCBP expression was increased with the malignant grade of glioma, by Spearman's correlation analysis (SDC1, r_s=0.576, P=0.001; SDCBP, r_s=0.661, P<0.001).

SDC1 expression was positively related to SDCBP expression in glioma tissues. Spearman experiment was then employed to assess the relationship between SDC1 expression as well as SDCBP expression in glioma tissues (Table II). The results showed a positive relation between protein levels of SDC1 and SDCBP among the glioma tissues (r_s=0.628, P=0.001).

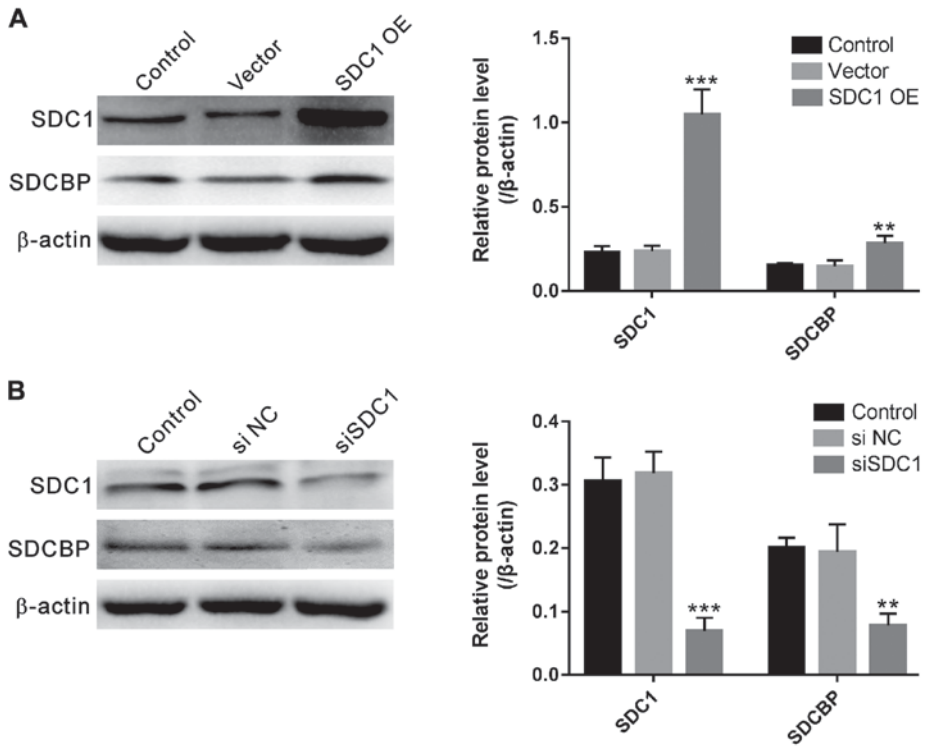

Figure 2. Protein levels of syndecan-1 (SDC1) and syndecan binding protein (SDCBP) in U251 cells were evaluated by western blotting. β-actin was employed as an internal control. Image Pro Plus 6.0 was used to quantify immunoblot intensities. The experiment was repeated three times independently. (A) U251 cells were transfected with SDC1 expression plasmid (SDC1-OE) or control plasmid (vector). At 48 h post-transfection, the protein levels of SDC1 and SDCBP were detected by western blotting. **P<0.01 and ***P<0.001 vs. control and vector-transfected cells. (B) U251 cells were transfected with SDC1 siRNA (siSDC1) or control siRNA (siNC). The protein levels of SDC1 and SDCBP were detected at 48 h after transfection. **P<0.01 and ***P<0.001 vs. control and siNC-transfected cells.

SDC1 affects SDCBP expression in U251 cells. U251 cells were infected with SDC1 expression plasmid (SDC1-OE), control plasmid (vector), SDC1 siRNA (siSDC1) or control siRNA (siNC). Forty-eight hours after the infection, SDC1 as well as SDCBP's protein levels were determined by western blotting. As shown in Fig. 2A, SDC1 was significantly increased in U251 cells transfected with SDC1-OE (1.049±0.146) as compared to control cells (0.228±0.038) or cells transfected with vector (0.237±0.031) (P<0.001). The levels of protein in SDCBP were also increased in cells transfected with SDC1-OE (SDC1-OE, 0.284±0.044; control, 0.153±0.013; vector, 0.146±0.036; P=0.004). The protein levels of SDC1 and SDCBP in cells transfected with vector had no significant difference compared to control cells. On the contrary, SDC1 siRNA transfection significantly decreased the expression of SDC1 (SDC1-OE, 0.069±0.021; control, 0.306±0.037; vector, 0.318±0.033; P<0.001) and SDCBP (siSDC1, 0.077±0.019; control, 0.201±0.015; siNC, 0.193±0.044; P=0.004) (Fig. 2B). siNC transfection had no effects on the expression of the proteins as compared to control cells. These data suggested the association between the protein expression of SDC1 and SDCBP in glioma cells.

Effects of SDC1 expression on the migration ability of U251 cells. To determine whether SDC1 influences the migration of U251 cells, Transwell assay (Fig. 3) and Scratch-wound healing assay (Fig. 4) were performed. U251 cells transfected with SDC1-OE showed a significant increase in cell migration compared to vector-transfected cells or control cells (SDC1-OE, 257±15; control, 144±11; vector, 144±8; P<0.001). Complementary to the results from SDC1 overexpression, SDC1 knockdown remarkably reduced the migration of U251 cells (siSDC1, 68±6; control, 143±7; siNC, 146±9; P<0.001).

Scratch-wound healing assay showed that SDC1 overexpression significantly enhanced the migration of U251 cells (SDC1-OE, 89.74±6.94%; control, 51.32±1.56%; vector, 50.36±3.78; P<0.001) (Fig. 4A). On the contrary, U251 cells transfected with siSDC1 displayed a notable decrease in the migration (siSDC1, 34.89±2.19%; control, 66.06±4.29%; siNC, 61.16±5.16%; P<0.001) (Fig. 4C).

Effects of SDC1 expression on the expression of matrix metalloproteinase 2 (MMP2) and MMP9. The expression of MMP2 and MMP9, two major MMP associated with cell migration (30), was detected. As illustrated in Fig. 5, MMP2 as well as MMP9 expression was significantly increased in SDC1 overexpressed cells, but remarkably decreased in SDC1 knockdown cells.

Effects of SDC1 expression on Rac1 activity and STAT3 phosphorylation. GST-PBD pull-down assay was conducted to test Rac1 activity, which is critical for glioma cell migration (27). According to Fig. 6A, ectopic expression of SDC1 notably induced Rac1 activity (SDC1-OE, 1.02±0.19; control, 0.49±0.12; vector, 0.50±0.12; P=0.007), while Rac1 expression was not affected by SDC1. Complementary data were obtained in SDC1 knockdown cells (siSDC1, 0.197±0.048; control, 0.422±0.074; siNC, 0.442±0.070; P=0.006). Judging

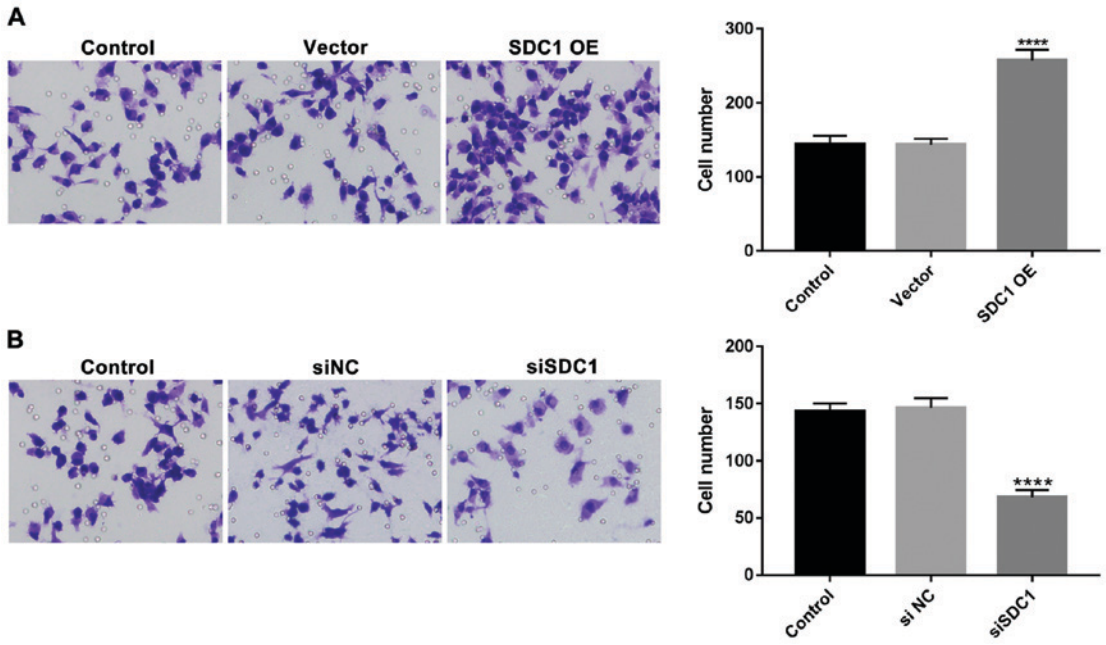

Figure 3. Effects of syndecan-1 (SDC1) expression on the migration ability of U251 cells. U251 cells were transfected with (A) plasmids or (B) siRNAs as described above. Transwell assay was performed as described in Materials and methods. Crystal violet staining images are shown in the left panel (x200). Quantification of the number of migrated cells are shown as mean ± SD on the right panel. (A) ****P<0.0001 vs. control and vector-transfected cells. (B) ****P<0.0001 vs. control and control siRNA (siNC)-transfected cells.

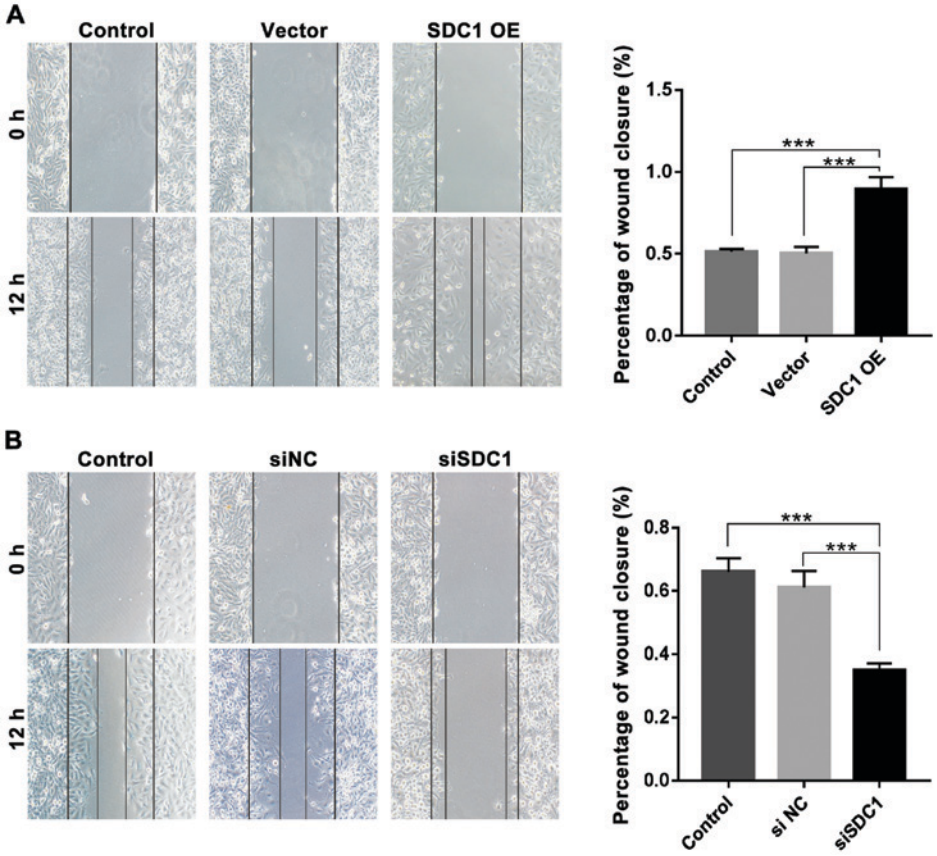

Figure 4. Effects of syndecan-1 (SDC1) expression on the migration of U251 cells. Scratch wound-healing assay was carried out to assess cell migration ability of U251 cells. Images were taken at 0 and 12 h after the creation of the scratch wound (x100). The proportion of wound closure was counted. (A) ****P<0.0001 vs. control and vector-transfected cells. (B) ***P<0.001 vs. control and control siRNA (siNC)-transfected cells.

from these data, SDC1 may influence glioma by inducing Rac1 activity.

Signal transducer and activator of transcription 3 (STAT3) has been identified to be essential for activation of Rac1 for

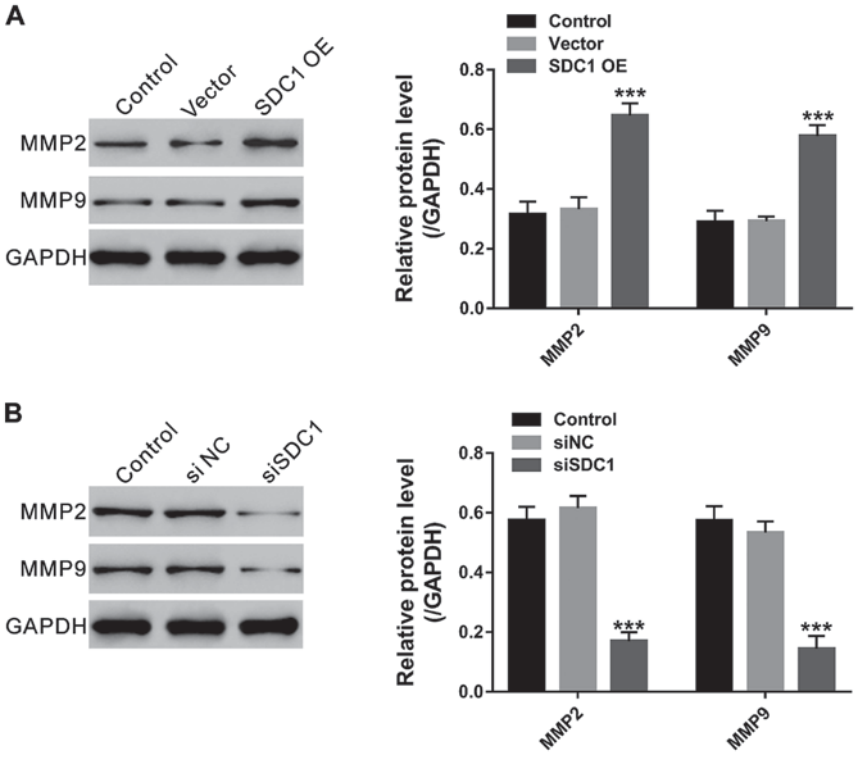

Figure 5. Effects of syndecan-1 (SDC1) expression on matrix metalloproteinase 2 (MMP2) and MMP9 expression. U251 cells were transfected with plasmids or siRNAs as described. The protein expression of MMP2 and MMP9 was evaluated by western blotting. (A) **P<0.01 and ***P<0.001 vs. control and vector-transfected cells. (B) **P<0.01 and ***P<0.001 vs. control and control siRNA (siNC)-transfected cells.

Table II. Correlation analysis between the protein levels of SDC1 and SDCBP in glioma tissues.

	SDCBP			
SDC1	Positive	Negative	r_s	P-value
Positive	20	3	0.628	0.001[a]
Negative	2	7		

[a]P<0.01. SDC1, syndecan-1; SDCBP, syndecan binding protein.

the regulation of cell proliferation and migration (31). As shown in Fig. 6B, SDC1 overexpression notably increased the levels of phosphorylated STAT3 (SDC1-OE, 0.74±0.04; control, 0.24±0.04; vector, 0.23±0.03; P<0.0001), while complementary data were obtained in SDC1 knockdown cells (siSDC1, 0.35±0.04; control, 0.74±0.05; siNC, 0.75±0.03; P<0.0001).

Discussion

SDC1 is the most studied member of the HPSGs. Deregulated SDC1 expression has been used as a prognostic factor in human malignancies. Decreased SDC1 expression is a negative prognostic factor in colorectal (13), head and neck (14) and hepatocellular carcinoma (18). In contrast, strong stromal SDC1 expression has been shown to correlate with a poor prognosis in patients with glioma (12), prostate (11), breast (17), ovarian (19) and gastric cancer (20). Studies have shown that SDC1 binds to its partners through the cytoplasmic domain, thus involving in cell proliferation (5,6), migration (7,8), invasion and angiogenesis (9,10). The EFYA motif is crucial for the interaction of SDC and PDZ-domain proteins, including SDCBP (21). SDCBP was overexpressed in various tumor samples (32-35). In the present study, by immunohistochemcal staining, the expression levels of SDC1 and SDCBP in glioma tissues were higher than in normal brain tissues, and the expression levels of both proteins were increased with the malignant grade of glioma, consistently with previous findings (12,35). Importantly, the protein levels of SDC1 and SDCBP were positively correlated in glioma tissues. We then overexpressed or knocked down SDC1 expression in U251 glioma cells, and found that SDCBP expression was substantially affected by the manipulation of SDC1 expression. These data suggested an association between SDC1 and SDCBP during glioma tumorigenesis.

Migration is the critical process for metastasis of cancer. SDC1 is actively involved in cell migration (7,8) and angiogenesis (9,10). SDCBP can promote the invasion of glioma cells (22,23). MMP2 and MMP9 are major MMPs associated with cell migration (30). In the present study, ectopic expression of SDC1 in glioma cells significantly enhanced their migration ability and the expression of MMP2/9 as indicated by Transwell assay and western blotting, respectively, which provided evidence that SDC1 expression was associated with metastatic potential of glioma.

Rac1 and its downstream effector STAT3 have been suggested to be a part of regulating cell migration (27,31). Previous studies have shown that SDCBP and SDC1 could regulate Rac1 activity (28,29). Here, we found that ectopic expression of SDC1 in U251 cells significantly increased

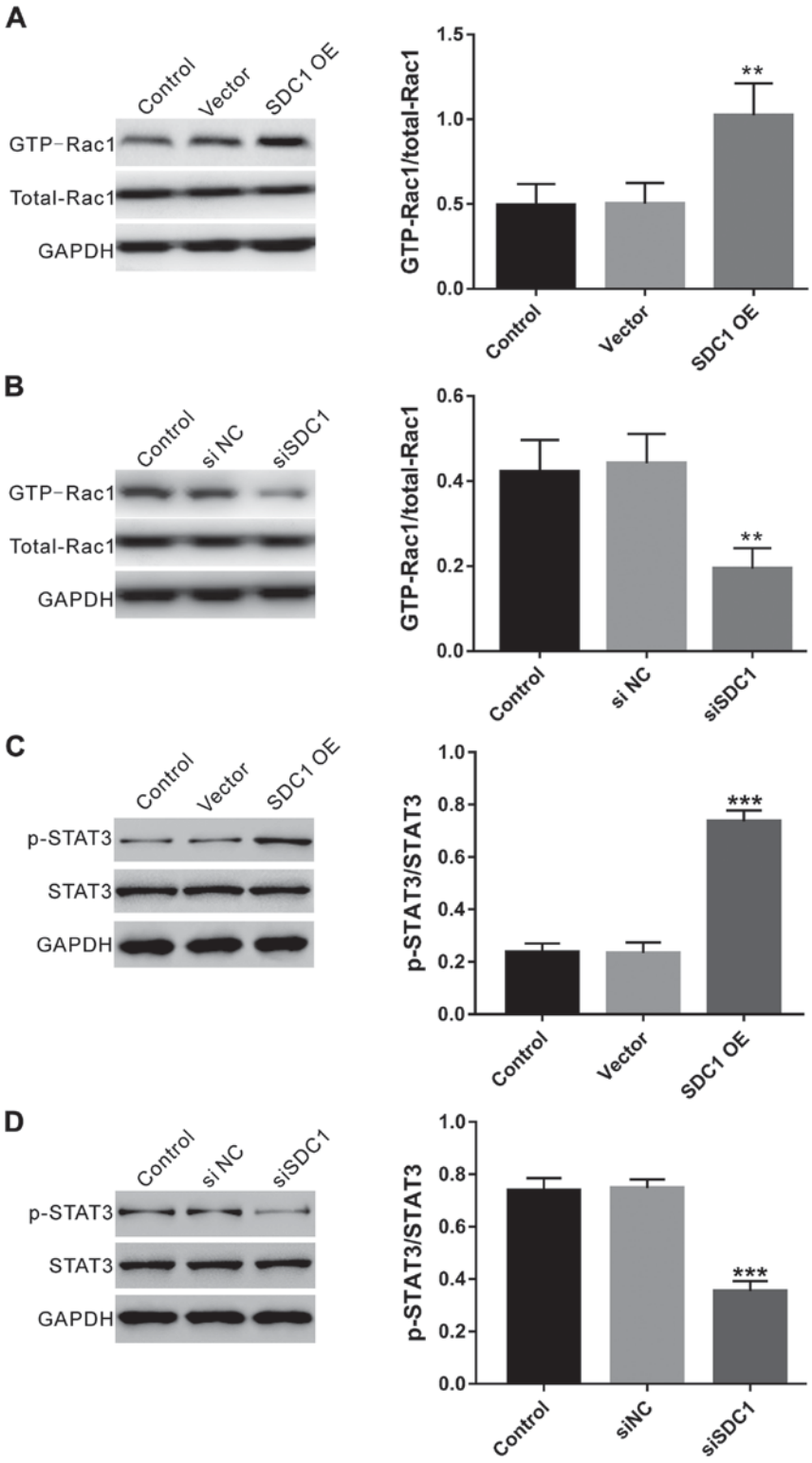

Figure 6. Effects of syndecan-1 (SDC1) expression on Rac1 activity and signal transducer and activator of transcription 3 (STAT3) phosphorylation. U251 cells were transfected with plasmids or siRNAs as described. At 48 h after transfection, (A and B) activation of Rac1 and (C and D) phosphorylation of STAT3 was evaluated by GST-PAK1-binding domain (GST-PBD) pull-down assay and western blotting, respectively. (A and C) **P<0.01 and ***P<0.001 vs. control and vector-transfected cells. (B and D) **P<0.01 and ***P<0.001 vs. control and control siRNA (siNC)-transfected cells.

SDCBP expression, Rac1 activity and STAT3 phosphorylation. Complementary data were obtained in SDC1 knockdown cells. These findings indicated that SDC1 induced SDCBP expression and Rac1/STAT3 pathway, and that the association between SDC1 and SDCBP may promote the development of glioma.

In conclusion, SDC1 may promote the migration of glioma cells by forming a complex with SDCBP and activating the Rac1 signaling pathway. SDC1 and SDCBP may be clinical markers for glioma progression, prognosis, and treatment outcome evaluation. However, tumor metastasis is a complicated process, which require a series of orderly steps (36).

Further detailed investigation is needed to explore how SDC1 and SDCBP interact with tumor microenvironment and participate in glioma carcinogenesis.

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