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SENP1-Mediated deSUMOylation Regulates the Tumor Remodeling of Glioma Stem Cells Under Hypoxic Stress

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Abstract

Objectives: This study aimed to investigate the effect of specific small ubiquitin-like modifier (SUMO) proteases 1 (SENP1)-mediated deSUMOylation on the malignant behavior of glioma stem cells (GSCs) under hypoxia conditions and evaluate the clinical value of prevention in glioma patients. **Introductions:** Under hypoxic conditions, upregulated hypoxia-inducible factor 1α (HIF1α) expression in GSCs activates Wnt/β-catenin signaling pathways, which provide rich nutritional support for glioblastoma (GBM). SENP1-mediated deSUMOylation stabilizes the expression of HIF1α and β-catenin, leading to the occurrence of GSCs-initiated tumorigenesis. Targeting SENP1-mediated deSUMOylation may suppress the malignancy of GSCs and disrupt GBM progression. **Methods:** The expression of SENP1 in different World Health Organization grades was observed by immunohistochemistry and western blot. Lentivirus-packaged *SENP1* shRNA downregulated the expression of SENP1 in GSCs, and the downregulated results were verified by western blotting and polymerase chain reaction. The effects of LV-SENP1shRNA on the migration and proliferation of GSCs were detected by scratch and cloning experiments. The effect of LV-*SENP1* shRNA on the tumor formation ability of GSCs was observed in nude mice. Immunoprecipitation clarified the mechanism of SENP1 regulating the malignant behavior of GSCs under hypoxia. The correlation between the expression level of SENP1 and the survival of glioma patients was determined by statistical analysis. **Results:** SENP1 expression in GSCs derived from clinical samples was upregulated in GBM. SUMOylation was observed in GSCs *in vitro*, and deSUMOylation, accompanied by an increase in SENP1 expression, was induced by hypoxia. SENP1 expression was downregulated in GSCs with lentivirus-mediated stable transfection, which attenuated the proliferation and differentiation of GSCs, thus diminishing tumorigenesis. Mechanistically, HIF1α induced activation of Wnt/β-catenin, which depended on SENP1-mediated deSUMOylation, promoting GSC-driven GBM growth under the hypoxia microenvironment. **Conclusion:** Our findings indicate that SENP1-mediated deSUMOylation as a feature of GSCs is essential for GBM maintenance, suggesting that targeting SENP1 against GSCs may effectively improve GBM therapeutic efficacy.

Keywords

glioblastoma, glioma stem cells, HIF1α, SENP1, SUMOylation

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Introduction

Glioblastoma (GBM) is the most aggressive malignant tumor with an unfavorable prognosis, characterized by diffuse infiltration and high vascularization.[1] Despite the currently available treatments against GBM, the prognosis of the disease remains poor, with a very low 5-year survival rate.[2]

Glioma stem cells (GSCs) are central to GBM growth, angiogenesis, and therapeutic resistance, as well as the root cause of postoperative recurrence.[3] GSCs can survive even in the hypoxic microenvironment, possessing similar characteristics to noncentral nervous system tumor stem cells.[4] As malignancy

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increases, GSCs possess higher self-renewal, differentiation, and proliferation capabilities.[5] What is worse, excessive proliferation and metabolic remodeling lead to hypoxic microenvironment formation, thus increasing hypoxia-inducible factor 1α (HIF1α) expression.[6] HIF1α is a transcription factor that is induced in hypoxic or inflammatory environments.[7] After binding with HIF-1β into a heterodimer, it can recognize and bind the hypoxic response elements into the nucleus, thus initiating the transcription of related tumor genes.[7] HIF1α plays a pivotal role in promoting tumor cell proliferation, metabolism, and apoptosis, and participates in various transduction pathways that have an impact on the occurrence and development of different diseases.[7,8] Under hypoxic conditions, upregulated HIF1α expression in GSCs activates Wnt/β-catenin signaling pathways, which induces GSCs differentiation into perivascular cells, causing internal vascularization of the tumor and antagonizing the effectiveness of anti-angiogenic drugs, and which can also provide rich nutritional support for GBM, resulting in more aggressive and prone to distant invasiveness.[9,10]

The expression of HIF1α and β-catenin in activation of the relevant signaling pathways can be modified by small ubiquitin-like modifier (SUMO)ylation.[11,12] SUMOylation is a ubiquitination-like posttranslational modification, which binds to lysine residues to promote its ubiquitination and protein degradation process.[13] Protein SUMOylation is in a dynamic equilibrium state, and deSUMOylation mediated by SUMO-specific protease 1 (SENP1) can cleave the SUMO–SUMO chain linked to lysine residues, resulting in protein overexpression.[14] In the current study, SENP1-mediated deSUMOylation stabilizes the expression of HIF1α and β-catenin, leading to the occurrence of GSCs-initiated tumorigenesis. Targeting SENP1-mediated deSUMOylation may suppress the malignancy of GSCs and disrupt GBM progression.

In this study, we used surgical glioma clinical samples to find that the expression of SENP1 gradually increased according to malignancy grades. To verify the characteristics of malignant behavior of GSCs by SENP1-mediated deSUMO modification, we investigated extensive SUMO modification of GSCs and disclosed the reduced level of SUMO modification under hypoxia conditions. After SENP1 expression was downregulated, the migration and proliferation ability of GSCs significantly decreased, and the tumorigenic ability of GSCs *in vivo* declined as well.

Materials and Methods

Ethics Statement

All the animal experimental methods and protocols were performed in strict accordance with the guidelines that were approved by the Research Ethics Committee and Animal Care Committee of Soochow University (Approval No. SUDA20210708A03). We followed the "Guide for the Care and Use of Laboratory Animals" and the reporting of this study conforms to ARRIVE 2.0 guidelines.[15]

Clinical Specimens

Clinical glioma tissue specimens ($n = 40$) and adjacent NBT were collected from patients who underwent surgical removal and were diagnosed with glioma according to World Health Organization (WHO) pathological criteria at the Department of Neurosurgery, the Second Affiliated Hospital of Soochow University. The pathological diagnosis of glioma was independently achieved by 2 senior and experienced pathologists. Ethical approval was obtained from the Ethics Committee of Soochow University (JD-LK2022-148-01). Informed consent was obtained from all subjects.

Cell Line, Cell Culture, and Hypoxic Conditions

The cell line of human GSCs-SU3 (from a surgical specimen of a glioma patient) was established and maintained according to the methods as previously reported.[16] Briefly, for tumorsphere culture of GSCs, SU3 cells were cultured using DMEM/F12 medium (Gibco, Grand Island, NY, USA) containing bFGF (Gibco, 20 ng/mL), EGF (Gibco, 20 ng/mL), and 1 × B27 in an incubator (Sanyo, Japan) with humidified atmosphere at 37 °C with 5% CO_2, as described previously. For an adhesive culture of GSCs, we planted the suspended SU3 cells in a polylysine-treated culture dish with DMEM/F12/N2 (Gibco, Grand Island, NY, USA) containing 0.01 poly-L-ornithine (Sigma).

Throughout the study, hypoxia was achieved by maintaining SU3 cells in a hypoxic incubator (Hera Cell 150, Thermo Electron Corporation). For long-term cultures, cells were maintained at 1% oxygen levels.

Lentivirus Transfection

We constructed shRNA-*SENP1* sequences (sh-1: AACTACAT CTTCGTGTACCTC and sh-2: CTAAACCATCTGAAT TGGCTC) using the lentiviral plasmid. The procedure of lentiviral transfection was carried out according to the manufacturer's manual (Shanghai GenePharma Co, Ltd).

Cell Counting Kit-8 Assay

SU3 cell viability was measured using cell counting kit-8 (CCK-8, DOJINDO, China) according to the manufacturer's protocol. SUV3 cells were seeded into 96-well plates at 2.0×10^3 cells per well and incubated in 5% CO_2 at 37 °C for 1, 2, 3, and 4 days. Then, the cells were incubated with CCK-8 solution for 1 hour, and the absorbance was measured at 450 nm using a microplate luminometer (BioTek, USA).

Transwell Assay

SU3 cells (1.0×10^4 cells) were diluted in serum-free DMEM, 200 μL SU3 cells suspension was added into the upper part of the Cell Culture Insert (24-well format; 353097, Corning, USA), and the lower chamber was filled with medium supplemented with containing 20% fetal bovine serum. For the

Transwell invasion assay, first, Matrigel was added to the lower chamber. The upper chamber was carefully immersed in the lower chamber with sterile forceps. After incubation for 24 hours in a 37°C hypoxic incubator, cells in the upper chamber. Transwell chambers containing the invading cells were fixed with a 2.5% crystal violet stain solution. The number of invading cells was observed by optical microscope and photographed.

RNA Extraction and Quantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated by Trizol kit (Invitrogen, USA). Complementary DNA was synthesized using the cDNA synthesis kit (Takara, People's Republic of China) according to the manufacturer's instructions. Real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed with the double-stranded DNA dye SYBR Green PCR Core Reagents (PE Biosystems, UK) using the ABI PRISM7300 system (PerkinElmer, USA). To detect the expression of SENP1 in SU3 cells, PCR was done in triplicate in one assay, and SDs representing experimental errors were calculated. All data were analyzed using ABI PRISM SDS 2.0 software. Gene-specific primers used in the study were: *SENP1* forward, 5′ATCAGGCAGTGAAACGTTGGAC3′ and reverse, 5′GCAGGCTTCATTGTTTATCCCA3′; *HIF1α* forward, 5′GTCTGAGGGGACAGGAGGAT3′ and reverse, 5′CTCCTCAGGTGGCTTGTCAG3′ *β-catenin* forward, 5′GAAGGTGTGGCGACATATGCAGCT3′ and reverse, 5′ATCCAAGGGGTTCTCCCTGGGC3′; *β-actin* forward, 5′CTTTTCCAGCCTTCCTTCTTGG3′ and reverse, 5′CAGCACTGTGTTGGCATAGAGG3′.

Western Blotting and SUMOylation Analysis

Protein extracts were equally loaded onto 10% SDS-PAGE gel, electrophoresed, and transferred to nitrocellulose membranes (Amersham Bioscience, UK). After blocking with 5% nonfat milk in phosphate-buffered saline, the membranes were incubated with primary antibodies diluted to the recommended concentration overnight at 4 °C. The samples were incubated with an HRP-conjugated secondary antibody at room temperature for 2 hours, and the protein bands were detected using a chemiluminescence device. The SUMOylation protocol was similar to that used for the western blot analysis with the exception that SUMOylation was used instead of the primary antibodies. Immunoblotting was performed using antibodies as indicated: SENP1 (Abcam, UK, ab236094, 1:500), HIF1α (Abcam, UK, ab308433, 1:1000), β-catenin (Abcam, UK, ab32572, 1:5000), and β-Actin (Abcam, UK, ab8226, 1:5000), SUMOylation (Cell Signaling Technology, USA, #4930).

Tumor Sphere Formation Assay

SU3 cells were seeded in 6-well plates (3×10^3 cells per well) and incubated in complete DMEM for 24 h. The medium was then replaced by CM with 10% FBS, and the cells were cultured in an incubator at 37 °C with 5% CO_2 for 2 weeks until they grew into colonies. The medium was subsequently removed, and the SU3 colonies were stained with 0.1% crystal violet and counted.

Immunofluorescence Staining

SU3 cells (1×10^4) were seeded in 24-well plates for growth for 6 days and then were fixed with 4% paraformaldehyde (Sangon Biotech, Shanghai, China). After blocking with 5% normal goat serum (Sangon Biotech, Shanghai, China), cells were stained using the primary antibodies (anti-SENP1, anti-SOX2, anti-HIF1α, respectively, dilution 1:200), and then the second antibody (Thermo Fisher, dilution 1:1000). Nuclei were counterstained with DAPI. Images were captured under the Zeiss LSM800 confocal imaging system.

Immunohistochemistry

Immunohistochemistry was performed as previously described. Briefly, the tissue sections of 5 μm thickness were baked in a 58°C incubator for 2 hours, and soaked in gradient ethanol for 5 min each. The primary antibody was added and stored at 4 °C overnight, then the biotinylated secondary antibody at room temperature for 2 hours, followed by ABC-peroxidase for 1 hour. Next, diaminobenzidine and hematoxylin were applied to stain and counterstain the tissue section. Finally, images were captured under a microscope (Olympus) and evaluated in a blinded manner by 2 senior pathologists independently. From the total scores, 0, 1–3, 4–6, and 7–9 were recorded as −, +, ++, and +++, respectively. These scores were defined as no or low expression when the score was < 4; and positive or high expression when the score was ≥ 4. The scores were accepted if 2 senior pathologists agreed with the values. Otherwise, the values were reestimated until a consensus was reached.

Xenograft Model in Nude Mice

Eight-week-old male BALB/c nu/nu mice were obtained from Soochow University. All animal experiments were approved by the Ethical Committee of Soochow University. Our team make efforts to minimize the number of animals utilized and to decrease their suffering. A xenograft mouse model was established as previously described.[15] Control cells, SU3 cells under hypoxia conditions, and SU3 cells stably transfected with SENP1shRNA lentivirus were injected into the right flank of BALB/C nude mice. Tumor burden was monitored every week starting on day 7 after implantation. Tumor volume and body weight in both groups were measured every day. At 4 weeks after transplantation, the mice were sacrificed by cervical dislocation under deep 3% isoflurane anesthesia. All procedures in the experiments were in compliance with the Guide for the Care and Use of Laboratory Animals. Tumor size was

calculated according to the formula: length × width^2 × 0.5, and the average tumor size for individual groups was determined.

Immunoprecipitation

Cell pellets were washed 3 times with cold 0.01 M PBS and lysed in Radioimmune Precipitation Assay buffer for 30 min on ice. After centrifugation at 12,000 r/min for 15 min at 4 °C, the lysates were collected and precleared by Protein G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then incubated with an anti-SUMO1 antibody (1:50; Cell Signaling Technology, Boston, MA, USA) with continual shaking for 2 hours. The protein–antibody complexes were collected with 20 μL of Protein G PLUS Agarose at 4 °C with continual shaking overnight. Subsequently, the immunoprecipitates were washed three times with lysis buffer and analyzed by 12% SDS-PAGE and immunoblotting.

Statistical Analysis

Statistical significance was analyzed by the GraphPad Prism software (Version 8.0.2, CA, USA). The results are presented as $M \pm SD$. One-way analysis of variance was used to evaluate the differences among at least 3 groups. Student's *t*-test was used to determine the differences between groups. A *P*-value <.05 was considered statistically significant. All experiments were repeated 3 times independently.

Results

The SENP1 Expression in Glioma Specimens

Immunohistochemical staining and Western Blotting were performed to detect the SENP1 expression in glioma specimens with different WHO malignancy grades. Forty glioma specimens were divided into 4 grades according to WHO pathological criteria.[17] Six specimens were investigated for each WHO grade. The results showed that the expression of SENP1 increased gradually following a rise of malignancy degrees, indicating that malignant progression of gliomas was associated with SENP1 (Figure 1A-B).

GSCs Have Extensive SUMOylation Modification and the SUMOylation Modification Level Decreases Under Hypoxia Condition

Hypoxia is an important factor responsible for the malignant biological properties of GSCs.[18] To clarify whether GSCs are mainly modified by SUMOylation or deSUMOylation under hypoxic conditions, immunoblotting techniques were applied to detect the expression of SUMOylation in SU3 cells under hypoxic conditions, which showed that SUMOylation existed from 12 kDa to 120 kDa in SU3 cells (Figure 2A), indicating that a large number of proteins were SUMOylated in SU3 cells. Immunoblotting results also showed that the expression of SENP1 and HIF1α were significantly upregulated in SU3 cells after 72 hours of hypoxia compared to SU3 cells cultured under normoxic conditions (Figure 2B).

Furthermore, immunofluorescence (IF) staining was performed to detect the expressions of SENP1 and HIF1α under hypoxic conditions, which disclosed that the expressions of SENP1 and HIF1α increased significantly in SU3 cells after hypoxia for 72 hours compared with the control group (Figure 2C).

Downregulating the Expression of SENP1 can Attenuate the Proliferation and Clonogenicity of GSCs Under Hypoxic Condition

SU3 cells were cultured in normoxia or hypoxia states, respectively. Microscopic observations showed that the tumor spheres of SU3 cells floating in the medium significantly enlarged after hypoxic treatment for 12 hours, and the number of adherent cells also obviously increased after 72 hours (Figure 3A). Also, higher expression of SOX2 in SU3 cells after hypoxic treatment for 72 hours was disclosed via IF staining (Figure 3B), indicating the enhanced viability of SU3 cells under hypoxic conditions.

LV-*SENP1*shRNA packaged with lentivirus was synthesized, and stable transfection into SU3 cells was performed. Western Blotting and real-time quantitative PCR were applied to verify the *SENP1* knocking-down efficiency (Figure 4A-B). CCK8 assay was conducted to detect the effect of SENP1 downregulation on the proliferation of SU3 cells under hypoxia (Figure 4C). As shown in Figure 4C, the proliferation activity of SU3 cells was significantly enhanced after hypoxia treatment for 72 hours, compared with GSCs cultured under a normoxia state. Interestingly, stable downregulation of SENP1 in SU3 cells reduced the proliferation ability of GSCs under hypoxia.

SU3 cells were inoculated into a 12-well plate with an adhesive growth pattern to evaluate clone formation ability. The results showed that SU3 cells cultured under hypoxia for 72 hours exhibited stronger clone formation ability than SU3 cells cultured under normoxia. SENP1 downregulation significantly reduced clone formation ability of SU3 cells under hypoxia (Figure 4D). Cell scratching assay showed that the relative migration area of SU3 cells increased after hypoxia treatment for 72 hours compared with SU3 cells cultured under normoxia, and SENP1 downregulation reduced the proliferation ability of SU3 cells under hypoxia (Figure 4E).

SENP1-Mediated deSUMOylation Affects the Tumorigenicity of GSCs In Vivo

In vitro experiments, we confirmed that downregulation of SENP1 can reduce the migration and proliferation ability of GSCs. To further investigate the effect of SENP1 on the downregulation tumorigenic ability of GSCs *in vivo*, SU3 cells (1×10^6/mouse) in different treatment groups under hypoxia were injected into the subcutaneous right flank of BALB/C nude mice. The results showed that the tumor size and growth rate increased significantly after hypoxia compared with the

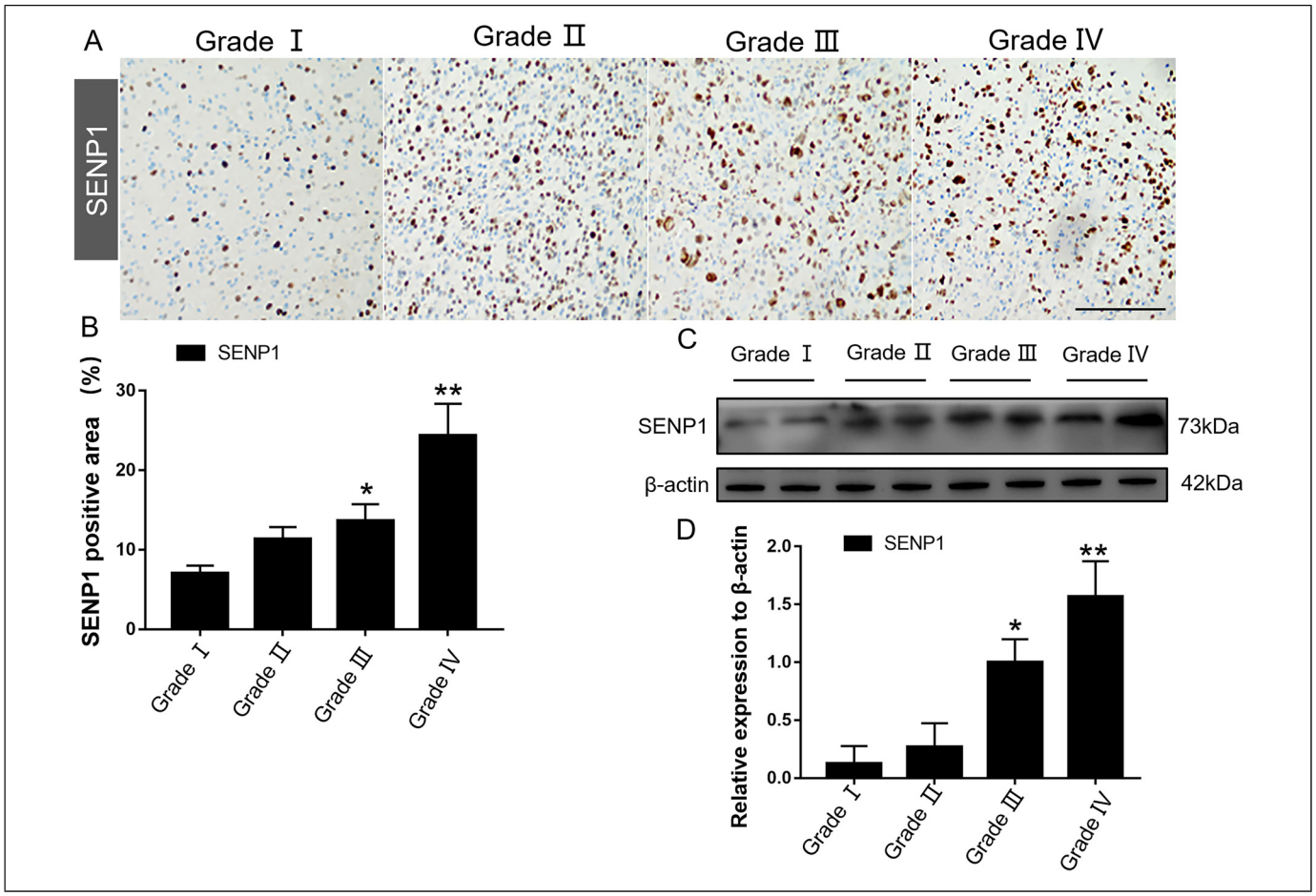

Figure 1. The expression of SENP1 in different WHO tumor grades was detected by immunohistochemical analysis and western blotting. (A-B): The expression of SENP1 in surgical specimens of glioma with different malignant degrees was detected by immunohistochemical staining and quantitative analysis. (C-D): The expression of SENP1 in tumor tissues was detected by Western blotting and quantitative analysis. *$P < .05$, Grade III *versus* Grade I. **$P < .01$, Grade IV *versus* Grade I. The results are shown as the $M \pm SD$. The results shown are representative of the 3 independent experiments. Scale bar: 100 μm.
Abbreviations: SENP1, small ubiquitin-like modifier proteases 1; WHO, world health organization.

control group. However, compared with the hypoxic group, the tumor size and growth rate of GSCs with downregulated SENP1 expression decreased obviously (Figure 5A-C). After tumor-bearing mice were sacrificed, immunohistochemical staining was performed to observe the expression of SENP1 in the xenografts, which showed SENP1 expression increased in hypoxic GSCs tumorigenic tissues, while decreased in SENP1 downregulated GSCs tumorigenic tissues (Figure 5D).

Activation of HIF1α and Wnt/β-Catenin Signaling Pathways Depends on SENP1-Mediated deSUMOylation

HIF1α is an important protein for tumor adaptation to hypoxia and is also an important mechanism for activating Wnt/β-catenin signaling pathways to promote GSC growth.[8] The expressions of HIF1α and β-catenin are regulated by SUMOylation. To verify the effect of deSUMOylation mediated by SENP1 on HIF1α and Wnt/β-catenin signaling pathways, qRT-PCR was performed. After the downregulation of SENP1 in SU3 cells, the mRNA levels of HIF1α and β-catenin were not descending (Figure 6A). However, the protein levels of HIF1α and β-catenin decreased in SU3 cells with downregulation of SENP1, due to enhanced SUMOylation via SENP1 downregulation (Figure 6B).

SENP1 Is Associated With the Prognostic Survival Period of Glioma Patients

To evaluate the association between SENP1 protein expression and the prognosis of glioma patients, 40 glioma patients were allocated to 2 groups, the low and high SENP1 expression groups. A log-rank test and Kaplan–Meier analysis were performed to assess the effect of SENP1 expression on patient survival. As shown in Table 1 and Figure 7, the result showed that the median survival time of all glioma patients was 29.3 months. The median survival time of patients with high SENP1 expression was 21.15 months. The median survival time of patients with low SENP1 expression was 37.45 months. The median survival time of patients with high

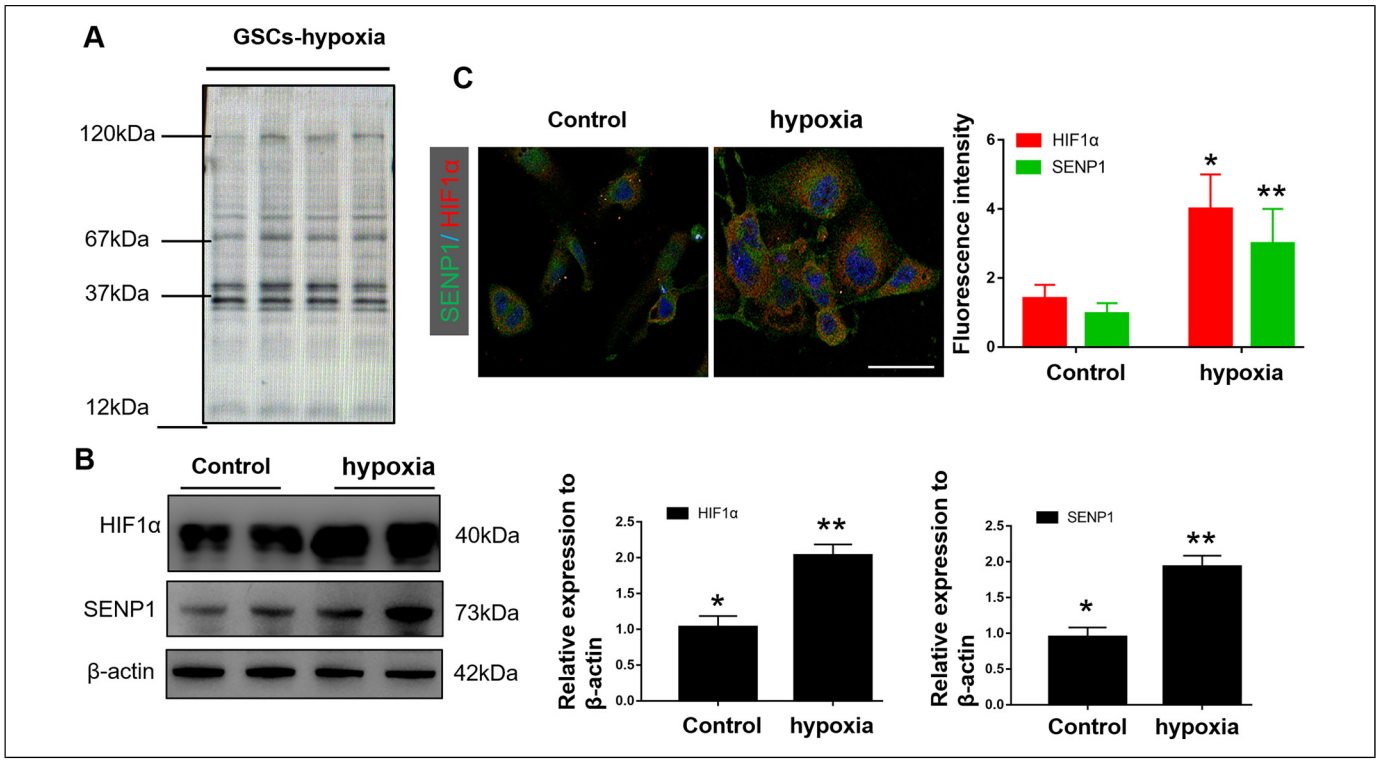

Figure 2. The level of SUMOylation in GSCs in each group was detected by western blotting, and the expressions of SENP1 and HIF1α were detected by immunofluorescence staining and immunoblotting. (A). The level of SUMOylation in SU3 cells in each group was detected by succinylation antibody in western blotting. (B-C). The expressions of SENP1 and HIF1α were detected by immunofluorescence staining, immunoblotting, and quantitative analysis. *$P < .01$, control group *versus* hypoxia. **$P < .01$, control group *versus* hypoxia. The results are shown as the $M \pm SD$. The results shown are representative of the 3 independent experiments. Scale bar: 100 μm.
Abbreviations: SENP1, small ubiquitin-like modifier proteases 1; SUMO, small ubiquitin-like modifier; GSC, glioma stem cell; HIF1α, hypoxia-inducible factor 1α.

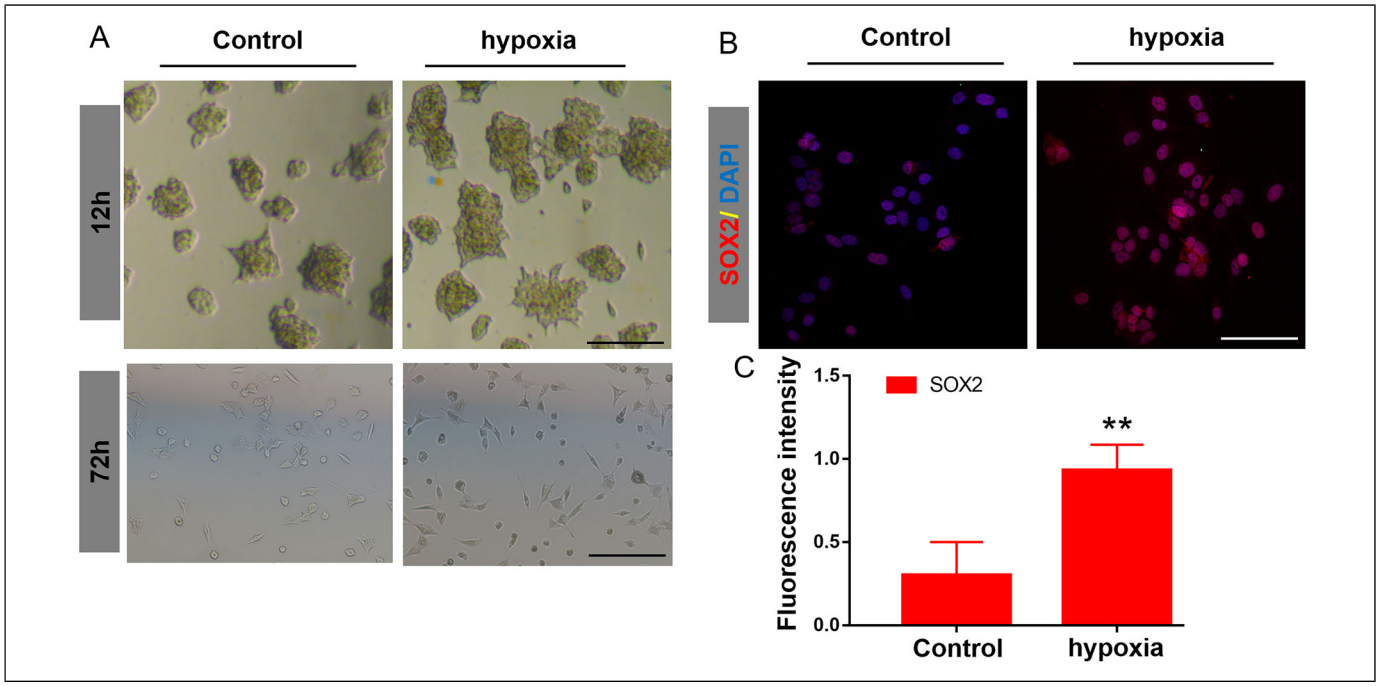

Figure 3. The growth of GSCs under hypoxia was observed under microscopic view and the expression of SOX2 in GSCs under hypoxia was analyzed by immunofluorescence staining. (A). Microscopic view of SU3 cells *in vitro* under different culture conditions. (B-C). The expression of SOX2 in SU3 cells under different culture conditions was analyzed by immunofluorescence staining and quantitative analysis. **$P < .01$, control group *versus* hypoxia. The results are shown as the $M \pm SD$. The results shown are representative of the 3 independent experiments. Scale bar: 100 μm.
Abbreviations: GSC, glioma stem cell.

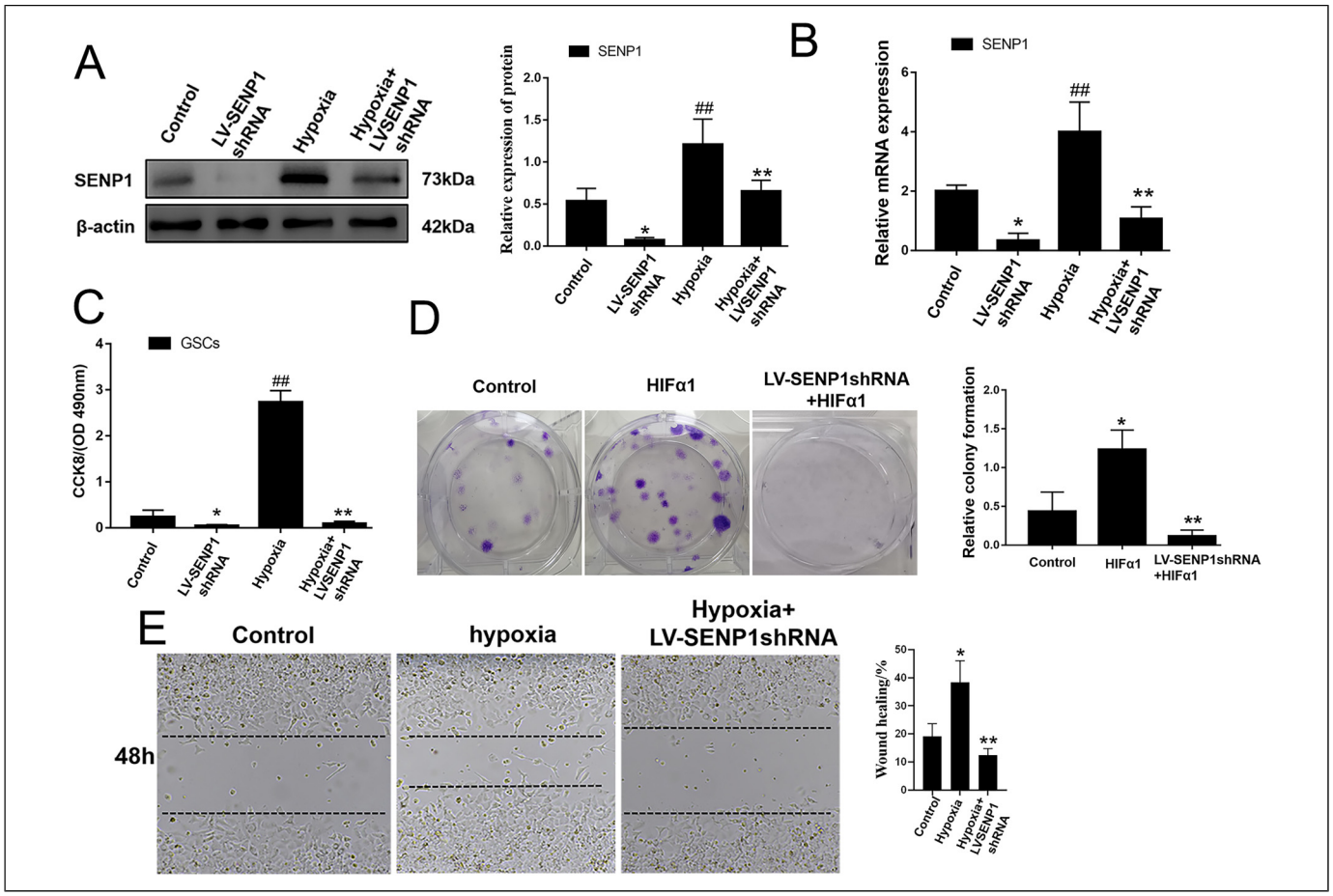

Figure 4. The effects of SENP1 downregulation on the proliferation and migration of GSCs were detected by CCK8, cloning, and cell scratch assay. (A). SENP1 expression assessed by western blotting in vitro. (B). SENP1 expression assessed by qRT-PCR analysis in vitro. $*P<.01$, control group *versus SENP1* knocking-down group. $^{\#\#}P<.01$, control group *versus* hypoxia group. ** $P<.01$, the *SENP1* knocking-down group versus the hypoxia group. (C). The growth of SU3 cells in each group was analyzed by CCK8. (D). The proliferation of SU3 cells in each group was observed by Clone formation assay. (E). The migration of SU3 cells in each group was observed and compared by scratch assay. $*P<.01$, control group *versus* hypoxia. ** $P<.01$, the *SENP1* knocking-down group versus the hypoxia group. The results are shown as the $M \pm SD$. The results shown are representative of the 3 independent experiments.

Abbreviations: SENP1, small ubiquitin-like modifier proteases 1; GSC, glioma stem cell; CCK8, cell counting kit-8; qRT-PCR, quantitative reverse transcription–polymerase chain reaction.

SENP1 expression was significantly lower than that of patients with low SENP1 expression ($P<.01$).

Discussion

In this study, SENP1 was found to be upregulated mostly in GBM based on analysis of clinical samples. DeSUMOylation was observed in GSCs under hypoxic conditions. SENP1 downregulation reduced proliferation, and migration induced by hypoxia in GSCs, inhibiting tumorigenicity as well. Under hypoxic conditions, the expression of HIF1α and β-catenin in GSCs depends on SENP1-mediated deSUMOylation. This study fully demonstrates the effect of SENP1-mediated deSUMOylation on the malignant biological properties of GSCs under hypoxic conditions, laying a clinical translation foundation for the development of target therapy against GBM.

GBM contains a small heterogeneous population of tumor cells known as GSCs, which play an important role in the occurrence, development, therapeutic resistance, and recurrence of tumors.[19] The tumor microenvironment-hypoxia is an unavoidable local niche for GSC growth, which can promote GSC proliferation, drug resistance, suppressive immunity, migration, invasion, and angiogenesis.[20,21] At present, there is still a lack of relevant research related to the regulation of GSC biological behavior and participation in glioma progression. The current study disclosed that deSUMOylation mediated by SENP1 can affect the malignant characteristics of GSCs under hypoxia, and SENP1 is closely related to the prognosis of glioma patients.

It has been found that in the malignant evolution of GBM, tumor microenvironment can induce the expression of abnormal cellular proteins, which in turn affects the changes of downstream signaling pathways, accelerating or delaying the

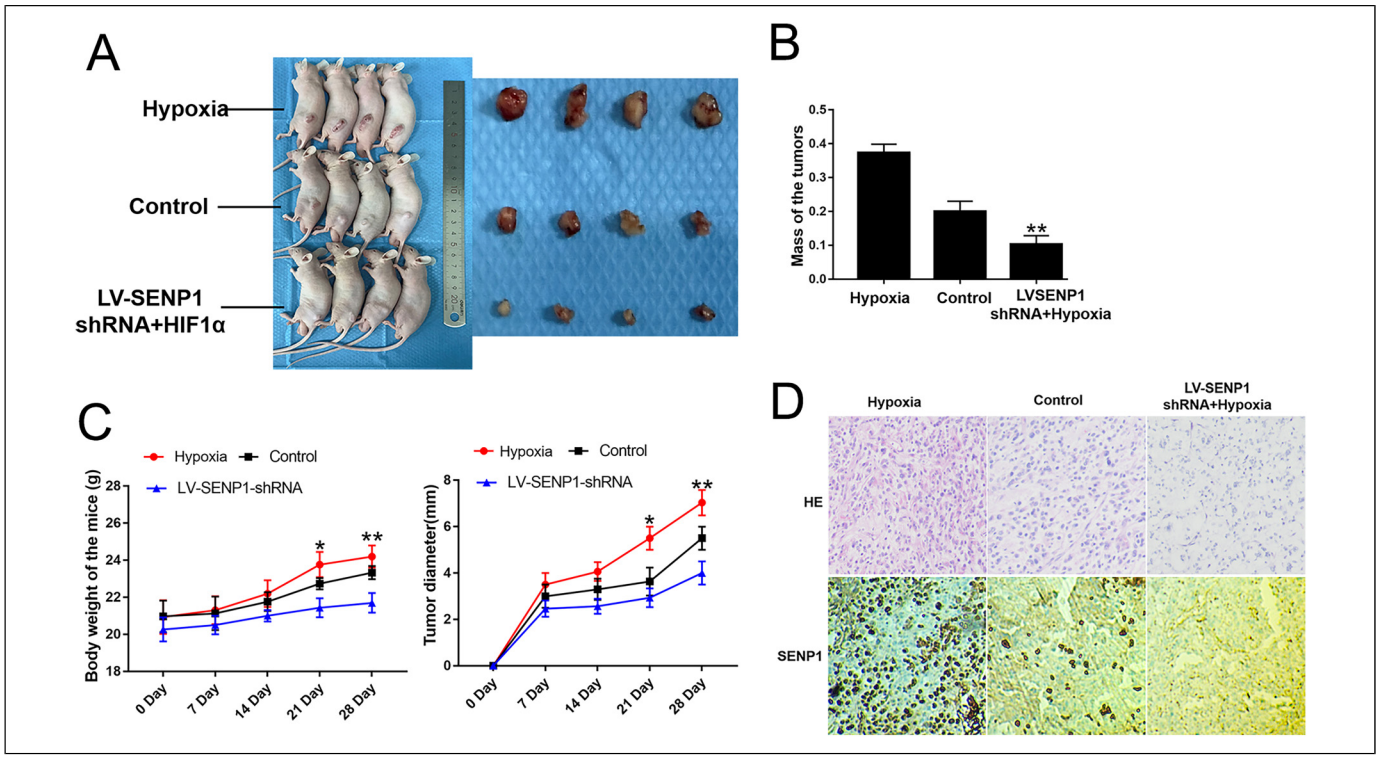

Figure 5. Downregulation of SENP1 expression results in a decrease in the tumorigenic ability of GSCs. (A). Tumor-bearing mice in each group. (B). The weight of the xenografts was shown in the dot chart. (C). The changes in the body weights of the mice are shown in the line chart. (D). The tumor was marked by HE staining and the expression of SENP1 in different tumorigenic tissues was observed by immunohistochemistry. Scale bar: 100 μm. The results are shown as the $M \pm SD$. The results shown are representative of the 3 independent experiments.
Abbreviations: SENP1, small ubiquitin-like modifier proteases 1; GSC, glioma stem cell.

progression of GBM.[22,23] SUMOylation can promote cells sensing extracellular stimuli, mobilize specific signal transduction pathways in cells, change the biological behaviors of cells, and adapt to changes in the extracellular environment.[24,25] Among them, SENP1 is one of the hot spots in the progression of tumor.[26] SENP1 is a key factor in the SUMOylation cycle that plays a decisive role in the degree of SUMOylation of substrate proteins.[27] We found that SENP1 expression gradually increased following malignancy degrees of glioma. However, this only indicates that SENP1 expression is associated with glioma progression, but whether it participates in the malignant phenotypes of GSCs has not been fully elucidated.

Recent research has already confirmed that the acidity (hypoxic microenvironment) of solid tumors is higher than that of normal tissues, and can increase with the expansion of the tumor.[28] In the study of xenografts of GBM *in vivo*, it was found that there was indeed an acidic hypoxic microenvironment in GBM, which promotes the self-renewal ability, mitochondrial activity, and ATP production of GSCs, thus promoting and maintaining the stemness of GSCs.[29] In our studies, it was confirmed that the expression of SENP1 in clinical samples of glioma patients is positively correlated with the malignancy degrees of glioma. To observe the correlation between SENP1 and the malignant characteristics of GSCs under hypoxia, and whether GSCs under hypoxia were mainly modified by SUMOylation and/or deSUMOylation, our investigations showed that GSCs were widely modified by SUMOylation, but the SUMOylation level of GSCs under hypoxia decreased, which indicated that the malignant behaviors of GSCs under hypoxia depend on deSUMOylation.

The effect of a hypoxic microenvironment remodeled by GSCs is characterized by high expression of HIF1α.[30] The elevated expression of HIF1α can activate multiple intracellular signaling pathways, such as the Wnt/β-catenin axis, to maintain the stemness of GSCs, promote the expression and clonal proliferation of GSCs, and finally induce GSCs to differentiation into surrounding progeny tumor cells, further promoting tumor angiogenesis and enhancing invasion ability of tumor.[31,32] SENP1 can maintain HIF1 α stability, and activation of multiple signaling pathways under hypoxic conditions, promoting tumor growth and invasiveness.[33] Based on the phenomenon that the malignant behaviors of GSCs under hypoxia depended on deSUMOylation revealed by our experimental results, it is reasonable to deduce that SENP1-mediated deSUMOylation can serve as an important target for regulating the malignant behaviors of GSCs, which implied that downregulation of SENP1 not only inhibited the proliferation, migration, and differentiation abilities of GSC but also prevented tumor tissue remodeling.

Consistent with the previous studies, SENP1 affects the malignant characteristics of GSCs under hypoxia and is related to the regulation of HIF1α stability. At present, it has

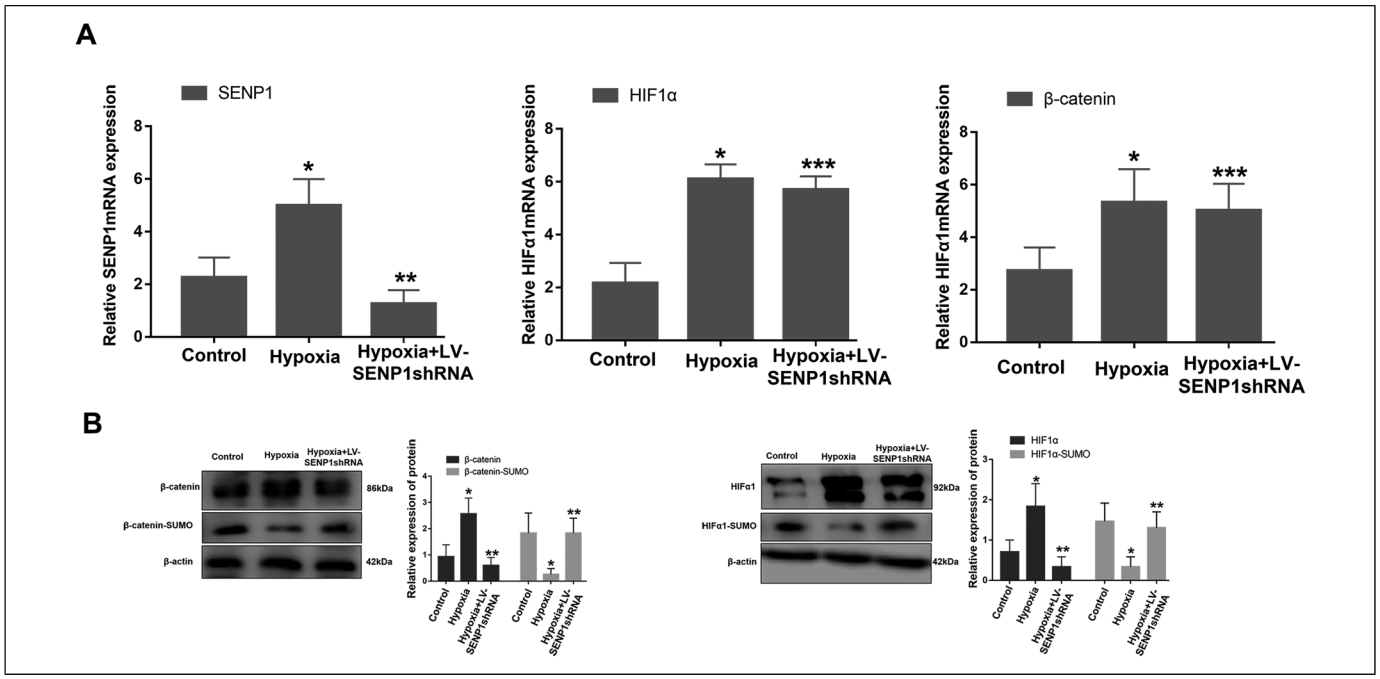

Figure 6. SENP1 regulates the activation of HIF1α and WNT/β-Catenin in GSCs *in vitro*. (A) mRNA levels of endogenous *SENP1, HIF1α,* and *β-Catenin* were assessed with qRT-PCR in different groups. QRT-PCR analysis indicated that *HIF1α* and *β-Catenin* mRNA levels did not change in the presence of LV-*SENP1*-shRNA following induction with TGF-β1. *$P < .01$, **$P < .01$, ***$P > .05$. *, the control group *versus* the hypoxia group; **, the *SENP1* knockdown group versus the hypoxia group; ***, the *SENP1* downregulation group versus the hypoxia group. (B) HIF1α and β-Catenin levels in total cell lysates were assessed with Western blotting. SUMOylation analysis of the immunoprecipitated HIF1α and β-Catenin proteins. HIF1α and β-Catenin were immunoprecipitated from whole cell lysates using anti-HIF1α and anti-β-Catenin antibodies, respectively. The immunoprecipitated proteins were subjected to Western Blotting, and the blots were probed with SUMOylation. *$P < .01$, **$P < .01$. *, the *SENP1* knockdown group versus the hypoxia group (HIF1α and β-Catenin); **, the *SENP1* knockdown group versus the hypoxia group (HIF1α-SUMO and β-Catenin-SUMO). All data are presented as the mean ± SEM of three independent experiments. The results shown are representative of the 3 independent experiments.

Abbreviations: SENP1, small ubiquitin-like modifier proteases 1; GSC, glioma stem cell; CCK8, cell counting kit-8; HIF1α, hypoxia-inducible factor 1α; qRT-PCR, quantitative reverse transcription–polymerase chain reaction.

Table 1. Mean survival time of patients with high and low expression of SENP1.

SENP1 expression	Patients, *n*	Mean survival time, months	[95% CI]	$\chi 2$	*P*-value
Low[a]	20	21.15	[13.391–28.909]	8.315	.004
High	20	37.45	[28.068–46.832]		
Overall	40	29.3	[22.767–35.833]		

aPatients were divided into high and low expression groups based on the median SENP1 expression value in glioma tissues.

been confirmed that HIF1α activates a variety of tumor-related signaling pathways, among which the activation of HIF1α and β-catenin has certain significance for the prediction and evaluation of the malignancy and invasion ability of GBM.[30,34] HIF1α and β-catenin are both SUMOylation target proteins.[11,12] SENP1 activation under hypoxia causes deSUMOylation of HIF1α and β-catenin, which can be stably expressed and activate downstream target genes, resulting in tumor malignant events.[35,36] Therefore, it is of great significance to inhibit the SENP1/HIF1α pathway to control GSCs-initiated tumor remodeling. Although downregulation of SENP1 did not affect the transcription level of HIF1α and β-catenin, downregulation of SENP1 inhibited the expression of the abovementioned proteins by increasing the SUMOylation level of HIF1α and β-catenin, which indicates that downregulation of SENP1 blocks the activity of the corresponding signaling pathway by increasing the SUMOylation level of the target protein.

Some articles reported the correlation between SENP1 and the prognosis of oral tumors, lung cancer, and other organ malignancies, thus proving that SENP1 can be used as a biomarker to predict the survival of patients.[36] Studies have confirmed that the expression level of SENP1 in tumor tissues is an important indicator of tumor progression and the degree of lymph node metastasis, and we also found that SENP1 is positively correlated with the clinicopathological grades of gliomas. Therefore, we statistically analyzed the correlation between the expression level of SENP1 and the survival time of patients, and the results showed that the higher the expression

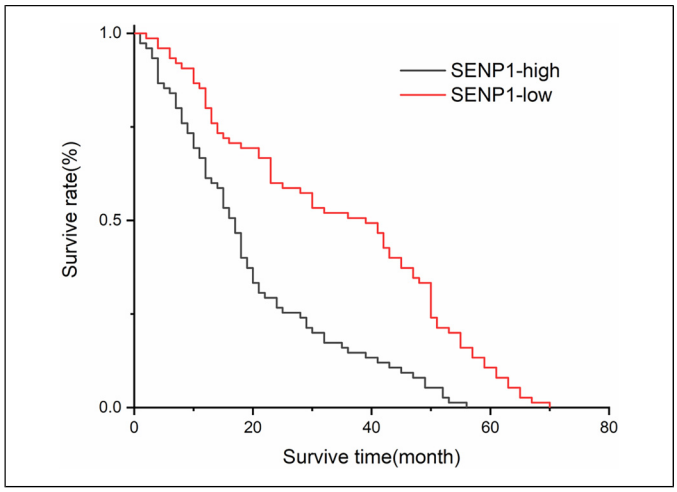

Figure 7. Correlation between the expression of SENP1 and the prognosis of glioma patients.
Abbreviation: SENP1, small ubiquitin-like modifier proteases 1.

level of SENP1, the shorter the survival time of glioma patients. This also indirectly reflects that the expression level of SENP1 protein in glioma tissue has certain application value for clinical diagnosis, treatment, and prognosis prediction of glioma patients.

Conclusions

In this study, we explored the role of SENP1-mediated deSUMOylation on GSCs-associated tumor microenvironment remodeling. Our findings indicate that SENP1-mediated deSUMOylation as a feature of GSCs is essential for GBM maintenance, suggesting that targeting SENP1 against GSCs may effectively improve GBM therapeutic efficacy.

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Author Contributions

Ping Wen performed the research; Jun Dong designed the study; Liang Liu, Xinglei Liu and Zhipeng Xu analyzed the data; Haoran Li Provided clinical samples; Ping Wen and Jun Dong wrote the paper. All authors read and approved the final manuscript.

Data Availability

Original data can be requested from the corresponding author.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics Approval and Consent to Participate

Investigations were conducted following the ethical standards of the Declaration of Helsinki and approved by the Research Ethics Committee and Animal Care Committee of Soochow University (Approval No. SUDA20210708A03).

The pathological diagnosis of glioma was independently achieved by 2 senior and experienced pathologists. Ethical approval was obtained from Soochow University (SUDA20210708A02). Informed consent was obtained from all subjects.

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