Saikosaponin D improves chemosensitivity of glioblastoma by reducing the its stemness maintenance

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\textbf{ABSTRACT}

\textbf{Objective:} Chemotherapy is one of the important adjuvant methods for the treatment of glioblastoma (GBM), and chemotherapy resistance is a clinical problem that neurooncologists need to solve urgently. It is reported that Saikosaponin D (SSD), an active component of \textit{Bupleurum chinense}, had various of antitumor activities and could also enhance the chemosensitivity of liver cancer and other tumors. However, it is not clear whether it has an effect on the chemosensitivity of glioma and its specific mechanism.

\textbf{Methods:} The CCK8 assay, Wound healing assay and Matrigel invasion assay were used to detect the effect of SSD on the phenotype of GBM cells. We detected the effect of SSD on the chemosensitivity of GBM by Flow cytometry, LDH content and MTT assay. Then, we used cell plate cloning, semi-quantitative PCR and western blotting experiments to detect the effect of SSD on the stem potential of GBM cells. Finally, the effect of SSD on the chemosensitivity of GBM and its potential mechanism were verified by nude mouse experiments in vivo.

\textbf{Results:} firstly, we found that SSD could partially inhibit the malignant phenotype of LN-229 cells, including inhibiting migration, invasion and apoptosis, and increasing the apoptosis rate and lactate dehydrogenase (LDH) release of LN-229 cells under the treatment of temozolomide (TMZ), that is to say, increasing the chemotherapy effect of TMZ on the cells. In addition, we unexpectedly found that SSD could partially inhibit the colony forming ability of LN-229 cells, which directly related to the stemness maintenance potential of cancer stem cells. Subsequently, our results showed that SSD could inhibit the gene and protein expression of stemness factors (OCT4, SOX2, c-Myc and Klf4) in LN-229 cells. Finally, we verified that SSD could improve the chemotherapeutic effect of TMZ by inhibiting the stem potential of glioblastoma in vivo nude mice.

\textbf{Conclusion:} this research can provide a certain theoretical basis for the application of SSD in the chemotherapy resistance of GBM and its mechanism of action, and provide a new hope for the clinical treatment of glioblastoma.

\section{1. Introduction}

Glioblastoma (GBM) may be originated from glial stem or progenitor cells [1]. It is the most common and most malignant primary intracranial tumor in adults, with great morphological and genetic heterogeneity [2, 3]. The five-year survival rate is only 5%, which is considered to be one of the most difficult tumors in the field of neurosurgery [4]. The treatment of glioblastoma is based on surgery, radiotherapy, chemotherapy and other comprehensive treatments. Among them, temozolomide (TMZ) based chemoradiotherapy has become the standard therapy for...
glioablastoma. Despite ongoing research, there has not been improvement in survival in glioblastoma, especially the chemoresistance of TMZ is the main challenge in the treatment of GBM [5]. In recent years, many scholars have discovered that natural products play a significant role in the sensitization of tumor radiotherapy and chemotherapy, but their applications in GBM are very few [6-8].

Saikosaponin D (SSD) is a triterpenoid compound extracted from the traditional Chinese medicine *Radix Bupleuri* [9]. Recent studies have shown that SSD has a variety of biological activities and pharmacological effects, including anti-tumor [10], liver protection [11], anti-inflammatory [12], sedative [13], anti-epileptic [14], immune enhancement [15] and estrogen-like effects [16]. The anti-tumor effect is its most important pharmacological effect [17]. And SSD has been widely applied to a variety of cancers up to now [17], including liver cancer, thyroid cancer, prostate cancer, lung cancer, cervical cancer, breast cancer, and so on. In addition, recent studies have shown that SSD can also enhance the sensitivity of liver cancer to radiotherapy and chemotherapy [18,19].

For GBM, it is reported that SSD can induce apoptosis of U87 cells [20], but whether it can enhance the chemotherapeutic sensitivity of glioma is still unclear. Here, we found that SSD can inhibit the malignant phenotype of LN229 cells, including CCK-8 cell proliferation experiment, Wound Healing assay, and Transwell invasion test. And also increase the content of lactate dehydrogenase (LDH) and the apoptotic ability of LN229 cells under TMZ stimulation. Fortunately, we unexpectedly discovered that SSD can promote the colony sphere formation of LN-229 cells. This discovery prompted us to test the effect of SSD on the expression of stemness factors in the cells. The results show that SSD can indeed reduce the expression ability of the stemness factor of glioblastoma cells, which provides certain research ideas for its mechanism of enhancing chemosensitivity on GBM.

2. Materials and methods

2.1. Drugs

SSD (SS8010, purity ≥98%) and TMZ (IT1330, purity ≥98%) were both purchased from Solarbio Science & Technology Co. Ltd (Beijing, China).

2.2. Cell lines and cell culture

LN-229 cells were obtained from the American Type Culture Collection (ATCC R CRL-2611, Maryland, USA) and mycoplasma contamination were consistently negative. The cells were seeded in Dulbecco’s modified Eagle’s medium (DMEM, High glucose) with 10% fetal bovine serum (FBS) and 1 × Pen Strep (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, US) at 37 °C and 5%CO₂, in a humidified atmosphere.

2.3. Cell proliferation experiment (CCK-8)

100 μl (2 × 10³ cells) of the cell suspension in the logarithmic growth phase were seeded into a 96-well plate and cultured overnight until adhered. The cells were treated with various concentrations of SSD and incubated for 24 h. The fresh medium was replaced, and 10 μl CCK8 solution was added to each well, and the cells were continued to culture for 2 h. The absorbance was measured at 450 nm on the microplate reader. Meanwhile, the blank control group was also set up.

2.4. Wound healing assay

The cells in the logarithmic growth phase, culture medium (DMEM) and culture-Insert were all prepared in advance. The cells were digested and resuspended in serum-free medium. Then the cell suspension was seeded into the culture-Insert in the middle of Petri dish. When the insert area is full of cells, the culture-Insert could be removed by tweezers to produce a scratch with a width of 500 μm. The cells continued to be cultured routinely for 24 h, and pictures recorded the width of scratch were taken every 6 h. The experimental results were analyzed according to the collected image data.

2.5. Matrigel invasion assay (Transwell)

The Matrigel frozen at −80 °C was thawed overnight, diluted with a serum-free medium containing 10 g/L BSA, and then coated the bottom membrane of the Transwell upper chamber with 50 μl per well, and placed in a 37 °C incubator for 4 h 200 μl of the LN-229 cell suspension (1 × 10⁵ cells/ml) in the logarithmic growth phase was added to the upper chamber, and SSD was added into the corresponding wells according to the experimental group. 500 μl of DMEM containing 10% FBS was added to the lower chamber and incubated at 37 °C for 24 h. Took out the upper chamber of Tanswell, discarded the medium, washed the cells twice with PBS, wiped off the cells on the upper surface with a cotton swab, and fixed for 30 min with absolute ethanol. Next, the cells were washed twice with PBS, stained with 0.1% crystal violet for 15 min; then continued to wash with PBS three times, and dried. Finally, observed and counted cells under microscope.

2.6. Lactate dehydrogenase (LDH) release detection

LN-229 cells in logarithmic growth phase were inoculated into 96 well cell culture plate to make the cell density reach 80~90%. The cells were treated with TMZ (10 μg/mL) or SSD (10 μM) or TMZ (10 μg/mL) combined with SSD (10 μM) for 24 h after they adhered to the bottom of plate. Then added the LDH release reagent provided by the kit, mix well, and continued to incubate for 1 h. The cell culture plate was centrifuged at 400g for 5 min. Next, 120 μl of supernatant was added to the corresponding well of a new 96 well plate, and 60 μl of LDH detection working solution was added, respectively. Mix well and incubate at room temperature for 30min. Then the absorbance was measured at 490 nm.

2.7. Cell apoptosis detection (Flow cytometry)

The cells were digested with trypsin without EDTA and collected into a centrifuge tube after being dispersed. The cells were centrifuged at 1000 rpm for 5 min. The cell precipitate was resuspended with PBS precooled at 4 °C and centrifuged again. The supernatant was carefully removed; the cell precipitate was resuspended with 1 × Binding buffer and adjusted the concentration to 3 × 10⁶ cells/ml. 100 μl of cell suspension was put into a 5 ml flow cytometer; 5 μl annexin V/Alexa fluor 488 was added, and then incubated at room temperature for 5 min in the dark. Finally, PI and PBS were added for flow cytometry immediately.

2.8. The median lethality rate (IC50) assay

The cells (1 × 10⁵ cells/well) were seeded in 96-well plates, 24 h before treatment. Cells were treated with TMZ (1, 5, 10, 15 and 20 μg/mL), or SSD (1, 5, 10, 15 and 20 μg/mL), or TMZ (0.5, 2.5, 5.5, 7.5 and 10 μg/mL) combined with SSD (0.5, 2.5, 5, 7.5 and 10 μg/mL), with the vehicle control (DMSO), and further incubated for 48 h. Cell viability was assessed through a modified Alamar blue assay. Briefly, a solution was prepared of DMEM medium with 10% of a resazurin salt dye stock solution (Sigma. St. Louis, MO, USA) at a concentration of 0.1 mg/mL, which was further added to each well for 48 h treatment. After 4 h of incubation at 37 °C and 5% CO₂, the absorbance of the plate was read at 570 and 600 nm in a BioTek (BioTek Instruments, Inc., Winooski, VT, USA). Cell viability was then calculated in accordance with the following equation: Cell viability (%) = (A570-A600) of treated cells/ (A570-A600) of control cells × 100%. Half-maximal inhibitory concentration (IC₅₀) values were further calculated using GraphPad Prism.
Software v.7.04 (GraphPad Software, Inc.).

2.9. Colony sphere formation assay

The cells in logarithmic growth phase were digested with trypsin and pipetted into single cells, and then resuspended in DMEM medium containing 10% FBS. The cell suspension was diluted in gradient multiples, seeded into 96 well plates and cultured in DMEM/F12 medium containing B27 (both from Gibco; Thermo Fisher Scientific, Inc.), 20 ng/ml basic fibroblast growth factor (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and 20 ng/ml epidermal growth factor (Provitro Biosciences, LLC, Mount Vernon, WA, USA), and were incubated at 37 °C, 5% CO₂ and saturated humidity incubator for 3 weeks. The volume of cell clones was measured under an inverted microscope (Olympus CX41; Olympus Corporation, Tokyo, Japan). The number of clones with more than 10 cells was counted under the microscope. Finally, the clone formation rate was calculated.

2.10. Semi-quantitative PCR

We perform extract for PCR and Wester blotting at 24 h. The total RNA was extracted according to the instructions of Trizol reagent. After determining the mass concentration, the cDNA was synthesized by reverse transcription and analyzed by semi-quantitative PCR. The primers were synthesized as follows: For OCT4, 5′-CGGGTACCGGTCGCCACCATG-TATTCTTCCGGTATGGAGAAACCCAGCAGCTCCCGCGACGA-3′ and 5′-CCGGATCTCAGTCATCTCATCTCCTTGATGTCGCCACAA-GATTCGCTAG-3′; For Klf4, 5′-CGGGATCTCAGTCATCTCATCTCCTTGATGTCGCCACAA-GATTCGCTAG-3′; For SOX2, 5′-CGGGATCTCAGTCATCTCATCTCCTTGATGTCGCCACAA-GATTCGCTAG-3′; For c-Myc, 5′-CGGGATCTCAGTCATCTCATCTCCTTGATGTCGCCACAA-GATTCGCTAG-3′; For α-tubulin, 5′-CGGGATCTCAGTCATCTCATCTCCTTGATGTCGCCACAA-GATTCGCTAG-3′. The expression of target genes was calculated by the 2^(-ΔΔCt) method and normalized to the expression of β-actin. All experiments were repeated at least three times independently. A value of P < 0.05 was considered statistically significant.

2.11. Western blotting

The cells of each group were lysed by RIPA Lysis Solution (P0013C, Beyotime) and protease inhibitor PMSF. The cell lysates were loaded and separated by 5% and 10% SDS-PAGE gels. After determining the concentration of the cell lysates by the BCA method, the samples were loaded and separated by 5% and 10% SDS-PAGE gels. Then the proteins were transferred to PVDF membrane (0.2 μm, Milipore, USA), and blocked with 5% non-fat dry milk in TBST for 1 h at room temperature. Then, specific antibodies against SOX2 (ab137385, 1:1000, Abcam), OCT4 (ab18976, 1:1000, Abcam), c-Myc (ab18227, 1:2000, Abcam) used as endogenous control were detected by Odyssey detection system (Licor) and the expression of proteins were quantified by ImageJ version 1.48.

2.12. Nude mice tumorigenicity experiment

The nude mice (BALB/c, male, 4 weeks old, weighing 14–16 g, provided by Beijing Wei tong Li hua Biotechnology Co., Ltd.) used in this experiment were approved by the Animal Ethics Committee of Tianjin Fifth Central Hospital (Tianjin, China). Before the experiment, they were bred adaptively for 1 week, drank water and ate independently. Then, 100 μl of LN-229 cell suspension (1 × 10^6 cells/ml) was inoculated subcutaneously into nude mice. When the tumor volumes all reached 100 mm³, mice were randomly divided into four groups with 8 mice in each group. And mice were intraperitoneally injected with vehicle (0.9%NaCl), SSD (10 mg/kg) [20], TMZ (15 mg/kg) [21], SSD and TMZ every two days for 33 consecutive days. At the same time, the subcutaneous tumor volume and the weight of mice were monitored. Finally, the mice were anesthetized with isoflurane, and the tumor tissues were dissected, collected, weighed, and the tumor masses were subjected to in-situ apoptosis experiments.

2.13. Statistical analysis

All experiments were repeated at least three times independently. All experimental values were reported as mean ± standard deviation (SD), and analyzed by GraphPad Prism 8.0 software (San Diego, CA). The statistical significance of differences between groups were compared with Student’s t-test, while one-way analysis of variance (ANOVA) was used for the comparison of multiple groups. A value of P < 0.05 was considered statistically significant.

3. Results

3.1. SSD inhibits the malignant phenotype of GBM cells

First of all, we tested the effects of SSD with different concentrations and times on the proliferation of LN-229 cells by the CCK8 cell viability assay. The results showed that the cell viability of LN-229 decreased significantly with the increased concentration of SSD in a dose-dependent manner. And with the extension of the administration time, SSD could significantly reduce the cell viability of LN-229 (Fig. 1A).

Subsequently, in order to further confirm the influence of SSD on the malignant phenotype of GBM, we conducted wound healing assay and Transwell invasion experiments respectively. The results showed that the relative migration distance of LN-229 cells treated with SSD (10 μM) for 24 h was significantly shorter than that of the control group (Fig. 1B). Consistently, as shown in Fig. 1C, the number of invasion cells was measured using Matrigel invasion assays. And compared with control, SSD significantly reduced the number of cells that penetrated Matrigel (Fig. 1C).

The above results revealed that SSD had an obvious inhibitory effect on the malignant phenotype of GBM (including cell viability, migration and invasion ability), which was consistent with the results reported in the literature that SSD could markedly suppress cell proliferation in a dose-dependent manner and enhance apoptosis, excluding migration (not mentioned) in human U87 GBM cells [22]. We speculate that the more poorer effect of SSD on the LN229 cells compared to U87 cells possibly based on the different genetic phenotypes and biological characteristics between different GBM lines.

3.2. SSD increases the chemosensitivity of TMZ on GBM

GBM is the most malignant glioma [23]. Due to the aggressive growth characteristics of malignant glioma and the special anatomical location, recurrence is inevitable despite surgery and radiotherapy [24]. Chemotherapy plays an important role in further killing the remaining glioma cells [25]. TMZ is currently the only oral chemotherapy drug approved by the FDA for the treatment of malignant glioma [26]. But chemoresistance to TMZ is a major challenge in the treatment of glioblastoma (GBM).

Here, we firstly tested whether SSD affected the chemotherapy effect of TMZ on GBM. The LN-229 cells were randomly divided into 4 groups and then treated with blank solution, SSD, TMZ and the combination of both. After 24 h, used to the content of the release of LDH was detected according to the instructions of the LDH ELISA kit. As shown in Fig. 2A, both of SSD and TMZ increased the release of LDH in LN-229 cells, and their combination significantly increased this effect (Fig. 2A). It is inferred that SSD could enhance the chemotherapeutic effect of TMZ on GBM. In order to further confirm this inference, we treated LN-229 in the same way. After 24 h, the cell apoptosis was detected by flow cytometry Annexin V/PI staining. The results showed that the treatment with SSD or TMZ significantly increased the apoptosis rate compared with the
controls, and the percentage of apoptotic cells was even higher under the stimulated by the combination of two (Fig. 2B-C). A similar trend was shown by the IC$_{50}$ assay results (Fig. 2D).

3.3. SSD reduces the stem cell maintenance potential of GBM

Tumor recurrence and metastasis are the main reasons leading to the failure of malignant tumor treatment [27]. The existence of cancer stem cells (CSCs) is considered to be the root of tumor occurrence and recurrence [28]. Essentially, CSCs play an important role in tumor survival, proliferation, metastasis and recurrence through their self-renewal and immortal proliferation [29]. Traditional radiotherapy and chemotherapy can kill tumor cells in the state of proliferating, but CSCs in a resting state may develop drug resistance through mutation and escape [30]. So, is the chemo-sensitization effect of SSD on GBM related to the stemness potential of tumor cells? Recently, we unexpectedly found that SSD could inhibit the colony formation rate of LN-229 cells (Fig. 3A). Subsequently, we tested the expression changes of stem factors (OCT4, SOX2, c-Myc and Klf4) in LN-229 cells treated with SSD through PCR (Fig. 3B) and western blotting experiments (Fig. 3C), and the results showed that SSD significantly inhibit the expression of these four factors. It can be concluded that the chemo-sensitization effect of SSD on TMZ-induced GBM may be related to the inhibition of stem cell stem potential. To this end, we also carried out the following nude mice tumorigenicity experiment to verify the mechanism in vivo.

3.4. SSD increases the chemosensitivity of GBM to TMZ by reducing the stemness of tumor tissue in vivo

In order to verify the above effects and mechanisms, we established a tumor xenograft model of GBM by subcutaneous inoculation of LN-229 cells, and observed the volume changes of tumors after intraperitoneal administration. The results showed that the combination of SSD and TMZ made the tumor volume growth rate significantly lower than that of the other groups (Fig. 4A). Then the weight of tumor in each group was measured at the end of administration, and the results were consistent with the changes of volume growth (Fig. 4B). These results suggested that SSD enhanced the chemosensitivity of TMZ to GBM.

Finally, we detected the expression differences of stem factors in the...
tumors of each group by western blotting, and found that the expression of OCT4, SOX2, c-Myc and Klf4 proteins in SSD combined with TMZ group was significantly lower than that of other groups (Fig. 4C).

Therefore, it is inferred that the mechanism of SSD increasing the sensitization of TMZ on GBM is closely related to the lower stemness potential of GBM stem cells.

4. Discussion

In recent years, despite the rapid development of global medical science and technology [31], GBM is still a major problem to be solved in the neuro-tumor field, and the treatment concept of surgery combined with chemoradiotherapy has reached the bottleneck stage [32]; we still need to adopt the comprehensive treatment mode of surgery combined with chemotherapy before the emergence of more lethal tumors of each group by western blotting, and found that the expression of OCT4, SOX2, c-Myc and Klf4 proteins in SSD combined with TMZ group was significantly lower than that of other groups (Fig. 4C).

Therefore, it is inferred that the mechanism of SSD increasing the sensitization of TMZ on GBM is closely related to the lower stemness potential of GBM stem cells.

1) SSD is expected to be a sensitizing regulator for chemotherapy of various cancers

Anti-tumor effect is the most important pharmacological activity of SSD [34]. Up to now, a large number of literatures have reported the inhibitory effects of SSD on a variety of tumors, including pancreatic cancer cells [10], hepatocellular carcinoma [35], renal cell carcinoma [36], human osteosarcoma [37], undifferentiated thyroid cancer cells [38], prostate cancer cells [39], lung cancer cells [40], breast cancer cells [41] and so on. This shows that it has broad prospects in cancer treatment.

In addition, SSD can also enhance the sensitivity of tumor cells to radiotherapy and chemotherapy. For example, SSD can enhance the radiosensitivity of liver cancer cells by inhibiting hypoxia-inducible factor-1α (HIF-1α) [42] or adjusting cell cycle G0/G1 and G2/M [43]. SSD also made various of cancer cells (including cervical cancer, ovarian cancer and non-small cell lung cancer) sensitive to cisplatin-induced cell death through the increase of reactive oxygen species (ROS) and caspase activation [44]. SSD can even enhance radiation-induced DNA damage, and more importantly, increase antioxidant levels after radiotherapy [45].

In terms of chemo-sensitization, SSD inhibited liver cancer cells and enhance chemosensitivity through SENP5-dependent inhibition of Gli1 SUMOylation under hypoxia [20], and also enhanced the sensitivity of human non-small cell lung cancer cells to Gefitinib [18]. It also reversed P-glycoprotein-mediated multidrug resistance in breast cancer MCF-7/adriamycin cells [19]. Here, we also preliminarily confirmed that SSD can reduce the stemness potential of GBM and enhance its chemosensitivity to TMZ. It can be inferred that SSD will become a chemosensitizer for various drug-resistant tumors.

However, it was elusive that we tested the effect of SSD on glioblastoma LN-229 and found that 10 μM SSD can inhibit 22% cell proliferation than the control group (CCK-8 test results), but induce LDH release about 6 times (LDH ELISA results), and induce cell apoptosis about 2 times that of the control group (Annexin/PI flow cytometry results). We speculate that it may be due to the different initial states of cells when exposed to drugs in different experiments, and different experimental principles may lead to greater variability in experimental results.

2) The inhibitory effect of SSD on the stemness potential of GBM stem cells is its key mechanism as a sensitizer

Chemotherapy is an indispensable treatment for GBM. But the high drug resistance of chemotherapy is a thorny problem. At present, there are many opinions about the causes of chemotherapy resistance [45]. And most of the viewers believe that the poor therapeutic effects of tumors, including radiotherapy and chemotherapy, are closely related to the existence of tumor stem cells [46]. Therefore, it is of great
significance to find out the factors influencing the stemness of cancer stem cells and their mechanism of action, so as to understand the internal mechanism of tumor resistance and recurrence and metastasis. Klf4, Sox2, Oct4 and c-Myc are considered to be key genes for maintaining the stemness of cancer stem cells. In recent years, many studies have shown that OCT4, SOX2, c-Myc and Klf4 are not only involved in the normal development of the body, but also closely related to the occurrence and development of tumor, especially the chemosensitivity of tumor cells [47]. Da et al. found that there was obvious expression of OCT4 in human gliomas, especially in gliomas with high degree of malignancy, it was as high as 100%, and all of them were strongly positive [48]. And the expression of OCT4 was not related to the gender of glioma patients, but was significantly related to tumor recurrence and chemoradiotherapy, which was consistent with the function of OCT4 in maintaining stem cell characteristics [49]. At the same time, the important role of SOX2 in the mechanism of TMZ resistance has been gradually revealed. The overexpression of SOX2 is a molecular marker of the subtype of precellar neurons in glioma cells, which is the most resistant subtype to chemoradiotherapy [50].

The above research results indicate that OCT4 and SOX2 are important factors leading to GBM resistance. Fortunately, this article found that SSD can significantly inhibit OCT4, SOX2, c-Myc and Klf4, and then inhibit the stemness potential of GBM, thereby providing a new opportunity for SSD to enhance the chemosensitivity mechanism of GBM. However, since this study did not validate these results in other glioblastoma cell lines (eg SNB19, U87, U251), the above conclusions appear thin and more experiments need to be done in more cell lines, even GBM patient-derived cells. Indeed, commercially purchased cell lines lose some of the original tumor characterization due to long-term in vitro culture. Conversely, patient-derived cells more accurately reflect the true characteristics of the tumor. However, patient-derived tumors are highly individual and often require a larger number of accumulations to explore a mechanism. In this study, we first identified LN229 cells express markers of stemness (SOX2 and OCT4), which are just suitable for the study of the mechanism of glioma stemness maintenance, so we chose LN229 cells as the experimental object. Therefore, as long as conditions permit, the conclusions of this study should be demonstrated from more patient-derived tumor cells. In addition,
whether SSD causes cytotoxicity should also be examined in further studies.

However, the chemoresistance mechanism of glioblastoma is intricate and often involves abnormal changes in more than a few signaling pathways, and is likely to be regulated by endocrine, immune, and nervous systems, it is ultimately difficult to find more effective therapeutic targets for glioblastoma. In fact, this study only provides an immature reference for improving the chemosensitivity of glioblastoma, and more in-depth and comprehensive mechanisms need to be verified by more experiments and more rigorous design.

**Ethics statement**

Here, the animal study was reviewed and approved by Ethics Committee of the Fifth Central Hospital of Tianjin (NO. TJWZX20200216).

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**Declaration of competing interest**

The authors declare that they have no commercial or other relationships conflicts of interests.

**Data availability**

Data will be made available on request.

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Fig. 4. SSD increases the chemosensitivity of GBM to TMZ by reducing the stemness of tumor tissue in vivo. LN-229 cells were inoculated subcutaneously in nude mice. After tumorigenesis, they were treated with different drugs, and the volume changes of the nude mice’s subcutaneous tumors were observed at any time (A). After the administration, the subcutaneous tumor mass of nude mice in each group was stripped, weighed (B), and tissue proteins were extracted to detect the changes in the expression of stemness factors in each group (C). Data are shown as means ± SD (n = 3); *P < 0.05 when compared with the control (Student’s t-test).
