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ORIGINAL ARTICLE

SPZ1 promotes glioma aggravation via targeting CXXC4

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Summary

Purpose: *The purpose of this study was to detect the expression pattern of SPZ1 in glioma samples and to clarify its biological functions in the malignant progression of glioma. Our results provide a novel molecular target for glioma.*

Methods: *SPZ1 levels in 40 pairs of glioma and non-tumoral ones were detected by quantitative real-time polymerase chain reaction (qRT-PCR). The differences in clinical indicators and prognosis between glioma patients expressing high and low levels of SPZ1 were compared. After knockdown of SPZ1 by transfection of sh-SPZ1, migratory and invasive abilities of A172 and U251 cells were examined by transwell migration and invasion assays. The interaction between SPZ1 and its target gene CXXC4 was finally explored by Western blot and dual-luciferase reporter assay.*

Results: *SPZ1 was upregulated in glioma tissues than non-tumoral ones, and the difference was statistically significant. Cell function experiments showed that knockdown of SPZ1 weakened the migratory and invasive abilities of A172 and U251 cells. CXXC4 was identified as the target gene binding to SPZ1. Knockdown of CXXC4 abolished the role of SPZ1 knockdown in inhibiting glioma progression.*

Conclusions: *SPZ1 stimulates glioma's malignant progression via targeting CXXC4*

Key words: *SPZ1, CXXC4, Glioma, malignant progression*

Introduction

Glioma is a common primary central nervous system (CNS) tumor, which is responsible for 44% of CNS cases. Its recurrence is very high and the prognosis is extremely poor [1-3]. In particular, glioblastoma is the most lethal subtype of glioma. Its overall survival ranges only about 16-18 months, and the 5-year survival (5%) ranks third in cancers following pancreatic cancer and lung cancer [1,2]. So far, the exact mechanism of glioma remains unclear [4]. Surgical resection, chemotherapy and radiotherapy are currently preferred methods to glioma patients [5,6]. In recent years, novel therapeutic strategies for glioma, including anti-neovascularization, immunotherapy and viral therapy, are also being actively explored. However, optional drugs for glioma are very limited [3,7]. Owing to the complexity of glioma-associated signaling transductions, glioma heterogeneity and the presence of blood brain barrier, great challenges are faced in developing targeted drugs [8,9]. More importantly, a large number of glioma patients develop tumor relapse in a short period [10,11]. Therefore, prevention and alleviation of glioma malignant progression should be well concerned.

SPZ1 (spermatogenic leucine zipper 1) is a transcription factor containing the helix-loop-helix basic region/leucine zipper. It is initially discovered in mouse sperm library and functionally related to spermatogenesis. SPZ1 is mainly expressed

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in the embryonic development stage [12,13]. In transgenic mouse and nude mouse models, activated SPZ1 triggers the expressions of downstream genes [14,15]. Differentially expressed SPZ1 has been identified in colorectal cancer, breast cancer and hepatocellular carcinoma [13,15,16]. Through bioinformatics analysis, CXXC4 could bind to SPZ1 3'UTR. CXXC4 is located on human chromosome 4q24, and its encoded protein inhibits the formation of DVL1 and Axin complex through binding the PDZ region of DVL1. As a negative regulator of the Wnt pathway, CXXC4 functions as an anticancer gene [17,18]. In this paper, we mainly explored the potential regulations of SPZ1 on glioma malignant progression via targeting CXXC4, which may provide a novel biomarker for glioma.

Methods

Glioma samples

Forty pairs of glioma tissues and the adjacent normal tissues were collected from glioma patients during the surgical resection. None of them had received preoperative anti-cancer treatment. This study was approved by the Ethics Committee of the First Medical Centre, Chinese PLA General Hospital and informed consent was obtained from each subject.

Cell lines and reagents

Glioma cell lines U251, U87, T98-G, A172 and the normal human glia (HEB) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) in a humidified incubator with 5% CO_2 at 37°C.

Transfection

SPZ1 sh-RNA (sh-SPZ1), the negative control (sh-NC), CXXC4 siRNA (si-CXXC4) and si-NC were purchased from GenePharma (Shanghai, China). Cells were resuspended in antibiotic-free DMEM and re-seeded into 50% density and transfected using Lipofectamine™2000 (Sigma-Aldrich; Merck KGaA).

Transwell migration and invasion assay

Transwell chambers were pre-coated with 100 μL of diluted Matrigel in serum-free medium (1:100) overnight (Corning, Corning, NY, USA). Cells were suspended in serum free medium, and 10^5 cells were added into the upper chamber. Then, the lower chamber was filled with 1 mL DMEM containing 10% fetal bovine serum (FBS). After 48-h incubation, transwell chambers were taken out. The cells that migrated to the bottom surface of the membrane were fixed with 100% methanol and stained with 0.5% crystal violet for 2 min. Invasive cells were counted in 5 randomly selected fields per sample. Thereafter, Transwell migration assay was similarly performed using transwell chambers without pre-coating Matrigel.

Wound healing assay

Cells were prepared into suspension with 5×10^5 cells/mL, and implanted in 6-well plates. Until 90% of cell attachment, an artificial wound was made using a sterilized pipette tip. Cells were washed in phosphate buffered saline (PBS) for 2-3 times and cultured in the medium containing 1% FBS (Hyclone, South Logan, UT, USA). Twenty-four h later, wound closure was taken and the wound healing percentage was calculated.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the tissues and cells using the TRIzol kit (Invitrogen, Carlsbad, CA, USA), respectively, followed by measurement of RNA concentration using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The complementary DNA (cDNA) was synthesized according to the instructions of the PrimeScriptTM RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). QRT-PCR was conducted using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Japan) on the StepOne Plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The relative level was calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to that of glyceraldheyde 3-phosphate dehydrogenase (GAPDH). SPZ1: forward: 5'-ACAGGAAGCAGAACACTGGAG-3', reverse: 5'-CAGCTCATGCTTAGCCGACA-3'; CXXC4: forward: 5'-CTCATCAACTGTGGCGTCTG-3', reverse: 5'-TTAGTTTGCCCTTCATTTCC-3'; GAPDH: forward: 5'-GGAGTCAACGGATTTGGT-3', reverse: 5'-GTGATGGGATTTCCATTGAT-3'.

Western blot

Total protein was extracted from treated cells by RIPA (radioimmunoprecipitation assay) solution (Yeasen, Shanghai, China). Protein sample was separated by electrophoresis on 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). Samples were then transferred to polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland). After membranes were blocked with skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membranes were then washed with Tris-buffered Saline with Tween 20 (TBST) and incubated with secondary antibody at room temperature for 1 h. Finally, the protein blot on the membrane was exposed by chemiluminescence.

Dual-luciferase reporter assay

A172 and U251 cells were inoculated in 24-well plates. Binding sequences in the 3'UTR of SPZ1 pairing to CXXC4 were used for generating wild-type and mutant-type pmirGLO-SPZ1 vectors. They were co-transfected into glioma cells with either NC or pcDNA-CXXC4, respectively. At 48 h, luciferase activity (Promega, Madison, WI, USA) was measured.

Statistics

SPSS 22.0 (IBM, Armonk, NY, USA) was used for statistical analyses. Measurement data were compared using the Student's t-test, whereas categorical variables

were analyzed by x^2 test or Fisher's exact test. Data were presented as mean ± standard deviation (SD). P<0.05 was considered as statistically significant.

Results

SPZ1 was highly expressed in glioma

SPZ1 was highly expressed in glioma tissues compared with non-tumoral ones (Figure 1A, 1B). Forty recruited glioma patients were classified into two groups based on the cut-off value of SPZ1 level in the corresponding glioma tissues. The differences in age, gender, tumor grade, and malignant progression between groups were analyzed. It was shown that SPZ1 level was positively related to the incidence of malignant progression in glioma (Table 1). Consistently, higher abundance of SPZ1 was detected in glioma tissues (Figure 1C). In addition, SPZ1 was upregulated in glioma cell lines. A172 and U251 cells expressed the highest level of SPZ1 among the four tested glioma cell lines, so they were selected for the following cell function experiments (Figure 1D).

Knockdown of SPZ1 inhibited glioma metastasis

SPZ1 knockdown model was generated in A172 and U251 cells by transfection of sh-SPZ1 (Figure 2A). Migratory and invasive abilities of glioma cells were examined by Transwell migration and invasion assays. Transfection with sh-SPZ1 in A172 and U251 cells largely decreased the numbers of migratory and invasive cells (Figure 2B). Meanwhile, wound healing percentage was decreased in glioma cells with SPZ1 knockdown, indicating the inhibited migratory ability (Figure 2C).

A negative interaction between SPZ1 and CXXC4

As bioinformatics analysis revealed, there were binding sequences in the 3'UTR of SPZ1 pairing to CXXC4. Based on the predicted binding sequences, wild-type and mutant pmirGLO-SPZ1 vectors were constructed. Besides, overexpression of CXXC4 decreased luciferase activity in pmirGLO-SPZ1-WT, whereas it had no influence on luciferase activity in the mutant-type one, confirming the binding between SPZ1 and CXXC4 (Figure 3A). Protein level of CXXC4 was upregulated in A172 and U251 cells

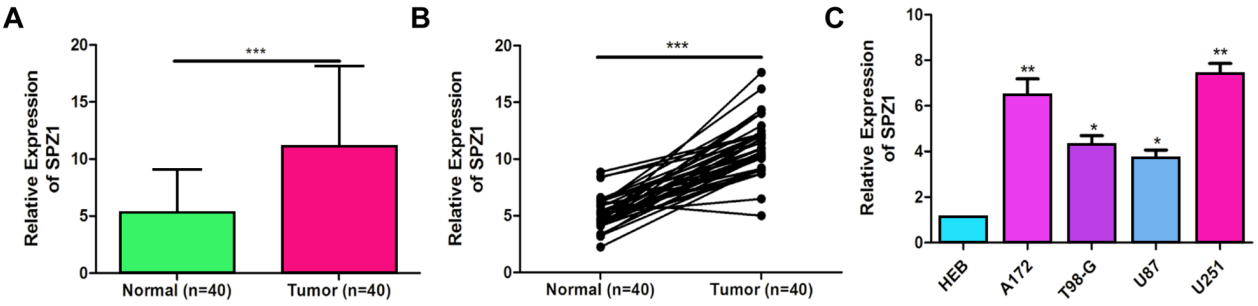

Figure 1. SPZ1 was highly expressed in glioma. SPZ1 levels in (A-B) glioma tissues (n=40) and non-tumoral tissues (n=40); **(C)** SPZ1 levels in glioma cell lines; *p<0.05, **p<0.01, ***p<0.001.

Table 1. Association of SPZ1 expression with clinicopathologic characteristics of glioma

Parameters	*Number of cases*	*Groups*		*p*
		Low SPZ1 (n=20)	*High SPZ1 (n=20)*	
Age (years)				0.525
<55	22	12	10	
≥55	18	8	10	
Gender				0.527
Male	20	11	9	
Female	20	9	11	
T stage				0.327
T1-T2	25	14	11	
T3-T4	15	6	9	

transfected with sh-SPZ1 than those transfected with sh-NC (Figure 3B). Compared with non-tumoral tissues, CXXC4 was markedly downregulated in glioma ones (Figure 3C).

SPZ1 promoted glioma malignant progression by targeting CXXC4

CXXC4 level was much lower in glioma cells with co-silenced CXXC4 and SPZ1 than those with SPZ1 knockdown, verifying the transfection efficacy of si-CXXC4 (Figure 4A). Compared with cells with solely knockdown of SPZ1, both migratory and invasive abilities were found to be stronger in A172 and U251 cells co-transfected with sh-SPZ1 and si-CXXC4 (Figure 4B). As expected, wound closure percentage was higher in glioma cells with co-knockdown of SPZ1 and CXXC4 than those with SPZ1 knockdown (Figure 4C). It could be concluded that CXXC4 was able to abolish the regulatory effect of SPZ1 on glioma malignant progression.

Discussion

Glioma is the most common and aggressive primary tumor in the CNS. It is featured with high recurrence, high mortality and low curative rate [1-3]. It is reported that malignant glioma is the second most common cause from death in people younger than 34 years old, and the third one in people aged 35-54 years [2-4]. The strong invasiveness of glioma cells results in the blurred boundary between glioma lesions and normal brain regions, thus causing difficulties in complete surgical resection. The remaining glioma lesions lead to a high rate of tumor relapse [5-7]. Moreover, chemotherapy and/or radiotherapy resistance can easily develop in glioma patients [7,8]. The existence of the blood-brain barrier makes the drug penetration rate low, which is also a challenge in developing targeted drugs for glioma [8,9]. Therefore, therapeutic strategies for glioma require to be largely improved [10,11].

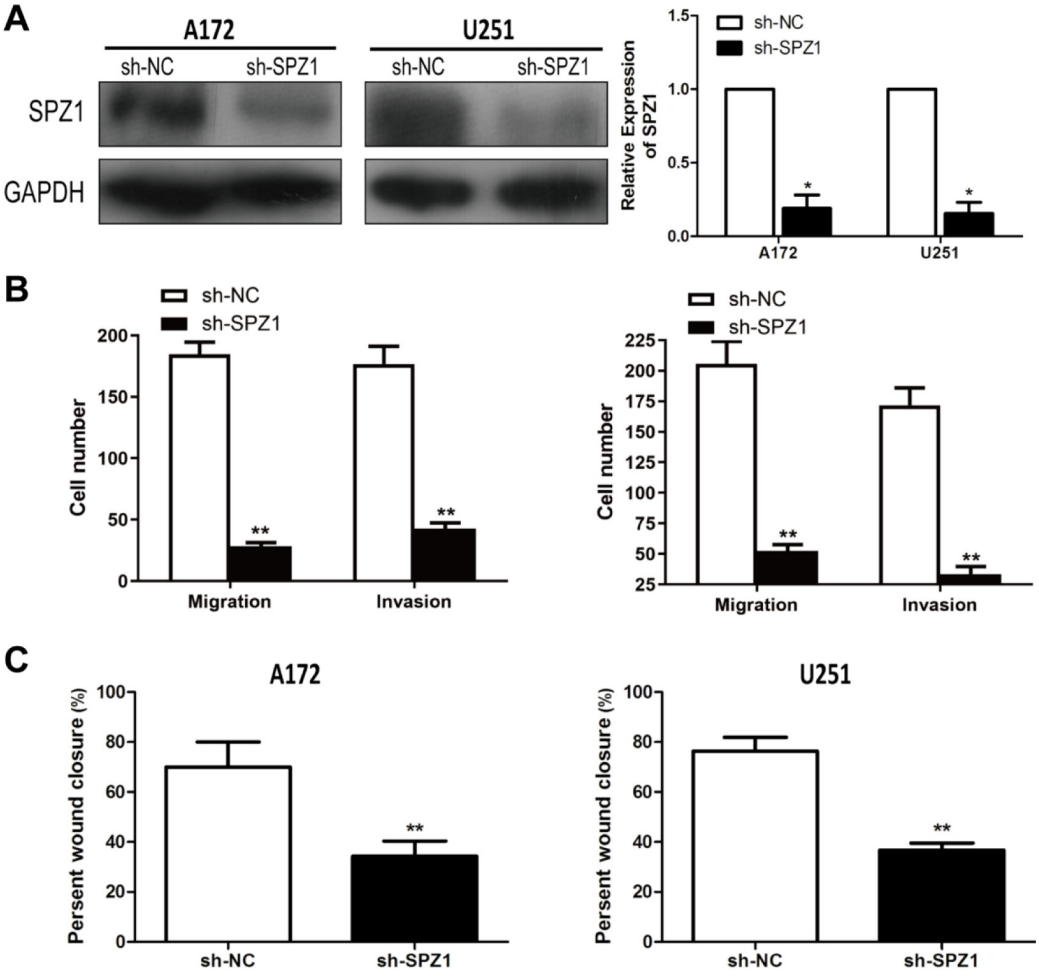

Figure 2. Knockdown of SPZ1 inhibited glioma malignant progression. **A:** Protein and mRNA levels of SPZ1 in A172 and U251 cells transfected with sh-NC or sh-SPZ1. **B:** Migration and invasion in A172 and U251 cells transfected with sh-NC or sh-SPZ1. **C:** Wound closure in A172 and U251 cells transfected with sh-NC or sh-SPZ1. *p<0.05, **p<0.01.

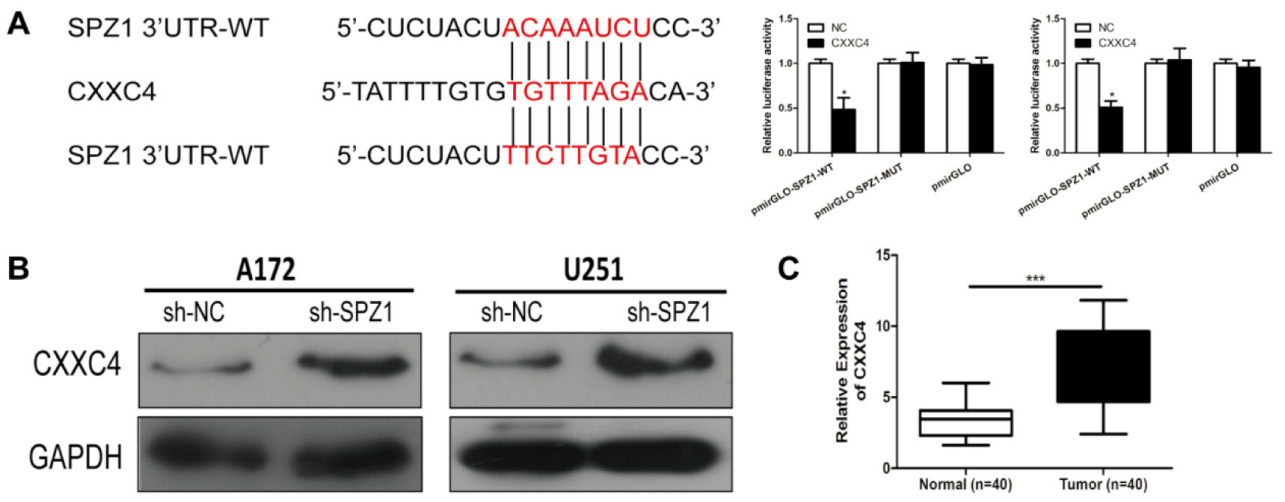

Figure 3. A negative interaction between SPZ1 and CXXC4. **A:** Luciferase activity in A172 and U251 cells; **B:** Protein level of CXXC4 in A172 and U251 cells transfected with sh-NC or sh-SPZ1; **C:** CXXC4 levels in glioma tissues (n=40) and non-tumoral tissues (n=40). *p<0.05, ***p<0.001.

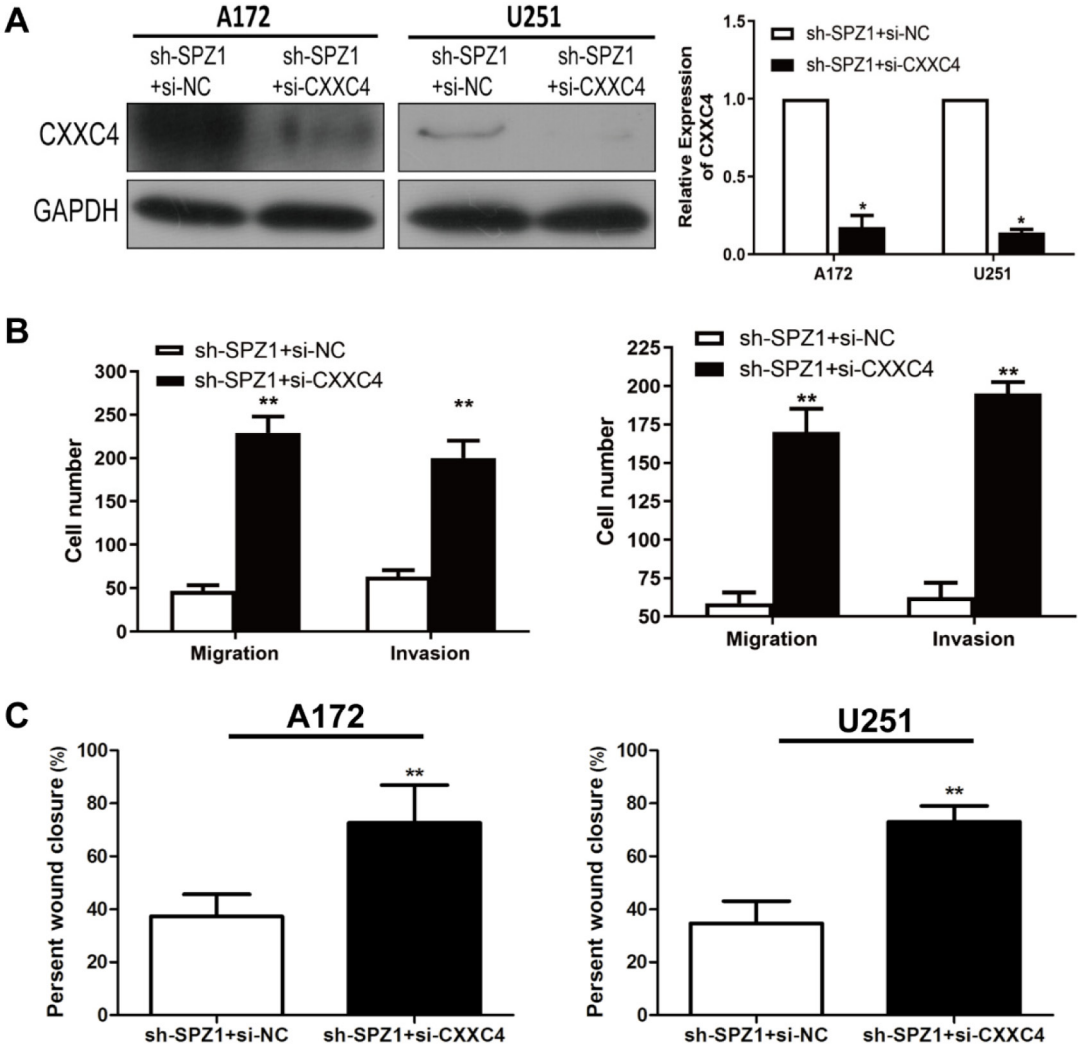

Figure 4. SPZ1 promoted glioma malignant progression by targeting CXXC4. **A:** Protein and mRNA levels of SPZ1 in A172 and U251 cells co-transfected with sh-SPZ1+si-NC or sh-SPZ1+si-CXXC4; **B:** Migration and invasion in A172 and U251 cells co-transfected with sh-SPZ1+si-NC or sh-SPZ1+si-CXXC4. **C:** Wound closure in A172 and U251 cells co-transfected with sh-SPZ1+si-NC or sh-SPZ1+si-CXXC4. *p<0.05, **p<0.01.

Gene mutations accumulated during tumor progression create a favorable microenvironment for tumor cell growth, further aggravating the progression [19,20]. It is already known that mutations of EGFR, IDH1, TP53 and PTEN are involved in the progression of glioma [21,22]. However, the exact mechanism of glioma remains unclear [23]. SPZ1 is the downstream gene of the Ras/Raf/MAPK signaling, and it is involved in tumorigenesis [12-16]. This study mainly explored the role of SPZ1 in regulating biological functions of glioma. SPZ1 was found to be upregulated in glioma tissues than in non-tumoral ones, and it was identically upregulated in glioma cell lines. Highly expressed SPZ1 was positively related to the incidence of progression in glioma patients. Subsequently, SPZ1 knockdown was achieved by transfection of sh-SPZ1 in A172 and U251 cells. Transwell and wound healing assay showed that knockdown of SPZ1 markedly inhibited migratory and invasive abilities in glioma. Hence, it was proved that SPZ1 might be an oncogene in aggravating glioma progression. The specific molecular mechanism, however, is unknown.

Using the bioinformatics tool, CXXC4 was identified to be the potential candidate binding to SPZ1, and this prediction was further confirmed by dual-luciferase reporter assay. Western blot analysis showed the upregulated CXXC4 in glioma cells with SPZ1 knockdown. In addition, a negative expression correlation was identified between CXXC4 and SPZ1 in glioma tissues. The regulatory effects of SPZ1 on glioma cell metastasis were partially abolished by CXXC4. To sum up, CXXC4 is involved in SPZ1-induced aggravation of glioma.

Conclusions

SPZ1 is closely related to the malignant progression in glioma due to targeting CXXC4.

Conflict of interests

The authors declare no conflict of interests.

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