Original Article

ODF3B affects the proliferation and apoptosis of glioma via the JAK/STAT pathway

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Abstract: The pathogenesis of glioma has remained unclear. In this study, it was found that high expression of the outer dense fibers of sperm tail 3B (ODF3B) in gliomas was positively correlated with the grade of glioma. The higher the grade, the worse the prognosis. ODF3B is closely related to the growth and apoptosis of glioma. In terms of mechanism, ODF3B was found to affect the proliferation and apoptosis of glioma through the JAK1 and JAK2/STAT3 pathways. ODF3B was also found to affect the growth and apoptosis of glioma in vivo. We conclude that ODF3B affects glioma proliferation and apoptosis via the JAK/STAT pathway and is a potential therapeutic target.

Keywords: Glioma, ODF3B, proliferation, apoptosis, JAK/STAT

Introduction

Glioma is the most common and aggressive intracranial malignant tumor [1, 2]. Although progress has been made in the multimodal treatment of glioma in recent years, including surgery, radiotherapy, chemotherapy, targeted therapy and supportive therapy, the overall prognosis is still poor and the survival is short, especially for glioma multiforme (GBM), which has the highest degree of malignancy [3, 4]. Therefore, it is necessary to find new targets and treatments.

“The trophoblast theory of cancer” states that cancer originates from primordial germ cells that fail to complete their migration to germ cells during the embryonic period [5]. The recent “cancer stem cell” theory can be seen in some ways as a reformulation of this theory [6]. During germ cell development, some transient proteins are proto-oncogenes or tumor suppressor genes, but they play an important role in spermatogenesis and reproduction [7]. These proteins are highly expressed in cancer cells, while they are less expressed or even not expressed in normal tissues. These proteins are called oncotesticular antigens, which are tumor-related proteins [8].

Outer dense fibers of sperm tail 3B (ODF3B) is a protein-coding gene located in the nucleus, cytoskeleton, cytosolute, mitochondria and other parts, and is highly expressed in brain tissue, esophagus, etc. [9, 10]. Some studies have found that ODF3B is also expressed in the brain of rats, so we speculate that ODF3B may be a cancerous testicular antigen, which is related to the occurrence and development of central nervous system tumors such as glioma. ODF3B has been scarcely reported in tumors, and is worthy of further study.

The JAK-STAT pathway is a signal transduction pathway stimulated by cytokines, which is involved in many important biological processes such as cell growth, apoptosis, and immune regulation [11-16]. Previous studies have shown
that the JAK/STAT pathway is involved in the occurrence and development of a variety of tumors, especially JAK2/STAT3, which is involved in the malignant biological behavior of liver cancer, colon cancer, lung cancer and glioma [17-20]. Therefore, we conclude that ODF3B-mediated JAK/STAT pathway may be involved in the malignant biological behavior of glioma.

This study explored the role of ODF3B in the occurrence and development of glioma and the related mechanism. Currently, there are no relevant reports on ODF3B in glioma. We used biological databases (The Cancer Genome Atlas, Chinese Glioma Genome Atlas), clinical samples, glioma cells, and the in situ tumor formation model in nude mice, and studied the relevant mechanisms by the protein immnobilotyping methods.

Materials and methods

Patients and samples

In this study, samples from patients diagnosed with glioma in the Department of Neurosurgery, Affiliated Hospital of Southwest Medical University from June 2018 to October 2022 were used. The use and relevant importance of all samples were communicated to the patients and their families before surgery, and consent was obtained. This study was approved by the Ethics Committee of the Affiliated Hospital of Southwest Medical University and carried out in accordance with the relevant provisions of the Declaration of Helsinki. All specimens were stored in liquid nitrogen within 20 min after collection, and the whole process followed the principle of aseptic operation.

We used The Cancer Genome (The Cancer Genome Atlas, TCGA, https://portal.gdc.cancer.gov/) and gene mapping of China Glioma (Chinese Glioma Genome Atlas, CGGA, http://www.cgga.org.cn/) databases. The correlation between ODF3B gene expression and glioma was analyzed. The TCGA and CGGA data were processed by special R software.

Cell culture

The cells NHA, U87-MG, U373, U251, A172, LN229 and T98 used in this study were purchased from the cell bank of the Chinese Academy of Sciences, and all cells were identified by STR. The cells were cultured in DMEM high-glucose medium with 10% fetal bovine serum, 50 units/mL penicillin and 50 μg/mL streptomycin at 37°C and 5% CO₂.

Quantitative real-time polymerase chain reaction

TRIZol reagent was used to extract the total RNA in the cells to be tested, a cDNA chain was synthesized using a reverse transcription reagent (Takara Company), and quantitative gene detection was performed using SYBR Premix Ex Taq. Real-time quantitative PCR was conducted using an ABI7500 quantitative fluorescence PCR instrument. All experiments were repeated 3 times, and Folds = 2^-ΔΔCT was used to assess the expression of the target gene between the experimental group and the control group.

Immunohistochemistry

All the samples were placed in liquid paraffin wax, frozen and sliced, dewaxed, and antigen repair was then performed. Goat serum was added for sealing, primary antibody was added and incubated at room temperature for 1 h, secondary antibody was added and incubated at room temperature for 30 min, streptomycin protein-alkaline phosphatase was added, incubated at room temperature for 30 min and then rinsed. A color development agent was added, images were collected after color development was terminated, and Image J software was used to calculate the percentage of positive cells.

Western blotting

RIPA lysis solution containing phosphatase inhibitors and protease inhibitors was used to extract total protein for 20 min on ice, and the protein concentration was determined by the BCA method. The membranes were isolated on 10% SDS-PAGE gel and transferred to PVDF membranes, which were sealed at room temperature for 20 min with a rapid blocking solution and then combined with primary antibodies. The membranes were then incubated with the second antibody and the bands corresponding to specific proteins were observed by the electrochemiluminescence detection system.
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*Cell transfection*

Lentivirus construction, ODF3B knockdown and control lentivirus construction were undertaken by Beijing Hopson Gene Biotechnology Co., Ltd.

Cells in good condition were inoculated into 6-well plates, and the plates were prepared using the standard cell culture technology, with each well containing approximately $10^4$ cells. Appropriate infection conditions and multiplicity of infection (MOI) values were selected, and samples were added according to the following formula: Virus addition amount ($\mu$L) = (cell number × MOI value/virogen titer) × 10^{-3}. Fluid change was performed 12-24 h after virus infection according to cell status. Seventy-two hours after infection, the culture medium was added with purinomycin for screening, and the transfection efficiency was observed under a fluorescence microscope.

*CCK8 proliferation experiment*

The cells were digested and collected, and cell counts were performed after resuspension. The experiment was carried out using 96-well plates, with 2000 cells added to each well and at least three wells in each group. After 24 h, the culture solution was gently removed and 100 $\mu$L DMEM and 10 $\mu$L CCK8 solution were added to each well. Optical density (OD) values at 450 nm were measured by enzyme-labeling after incubation for 2 h in the dark. OD values were measured at 48 h, 72 h, 96 h and 120 h, and the data were mapped using Prism software.

*Cell colony formation experiment*

Pancreatic enzymes were digested and the cells collected for counting. The cells were diluted and transferred to 6-well plates for culture, with 200 glioma cells/well. Cell growth was observed after 2 weeks of culture. The culture solution was removed, the cells were washed twice with PBS, and methanol 500 $\mu$L was added to each well for 30 min. 0.1% crystal violet was added for 30 min, and then removed. The cells were washed twice with PBS, dried and photographed. The photographs were analyzed using Imagine J software.

*Apoptosis was detected by flow cytometry*

The Annexin V FITC Apoptosis Detection Kit was used to digest tumor cells using pancreatic enzymes and the cells counted. Each sample contained about 20 $\times$ 10^4 cells which were washed once with PBS. 1 $\times$ binding buffer was prepared, and 10 $\times$ buffer was diluted with PBS to 1:9. To each sample, 100 $\mu$L buffer, 5 $\mu$L Annexin V and 5 $\mu$L 7-AAD liquid were added and incubated for 15 min away from light. 100 $\mu$L buffer was added to each sample and apoptosis was then determined.

*An in situ tumor formation model was established in nude mice*

Twelve healthy female BALA/C mice aged 4-5 weeks and weighing approximately 11-18 g were selected and acclimatized in an SPF animal house for 1 week. Then 6 mice were included in the U373-NC group and the remaining 6 mice were included in the U373-SH group. Nude mice were anesthetized intraperitoneally with 1% pentobarbital (7 $\mu$L/mg), and were fixed on the stereotactic apparatus after anesthesia. After disinfection, appropriate needles were selected to drill holes in the right lateral ventricle (the fontanel was 2 mm forward and the center line was 2 mm to the right side) to avoid brain parenchyma damage. 5 $\mu$L of cell suspension was collected with a microsyringe, containing approximately 100 $\times$ 10^4 cells, and injected slowly. After injection, the needle was slowly withdrawn. The wounds were disinfected, and the mice were kept in an SPF feeding environment. The state of each nude mouse was observed every day, and the survival time of each mouse was recorded. A survival curve of the data was created using Prism software, and brain tissue was fixed using paraformaldehyde. Following brain tissue sectioning, H-E staining was performed to observe tumor size and immunohistochemistry was performed to determine malignant biological behavior. All experiments were repeated three times.

*Statistical analysis*

All data in this study were analyzed using SPSS 22.0 software, R software (version 4.1.0) and GraphPad Prism 7.0. Comparisons were conducted between the two groups using the Student’s test, and results were analyzed by ANOVA and Bonferroni tests between multiple
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Results

ODF3B is highly expressed in glioma and positively correlated with grade

In order to explore the potential role of ODF3B in gliomas, we used the TCGA database to explore the expression of ODF3B in common tumors, and found that ODF3B was highly expressed in most tumors, and was highly expressed in both GBM and low-grade (LGG) gliomas (Figure 1A). We then used the TCGA dataset, CGGA dataset and 54 samples collected from the Department of Neurosurgery of the Affiliated Hospital of Southwest Medical University to verify ODF3B expression in normal tissues and glioma tissues, and found that ODF3B was highly expressed in tumor tissues (Figure 1B-D), and a positive correlation was found in both the database and clinical samples (Figure 1E-G). We verified the expression of ODF3B in the normal brain cell line NHA and human glioma cell lines U87-MG, U373, U251, LN229, A172 and T98, and found that ODF3B was highly expressed in both mRNA and protein levels in glioma cell lines (Figure 1H, 1I). Therefore, ODF3B was highly expressed in gliomas and positively correlated with grade.

Relationship between ODF3B expression and clinicopathological features

To elucidate the potential role of ODF3B in the malignant progression of glioma, we analyzed the expression of ODF3B at different ages in the CGGA and TCGA databases. According to the median age of the patients, they were divided into the elderly group (> 40 years old) and the young group (≤ 40 years old). The results showed that the expression of ODF3B in the elderly group was significantly higher than that in the young group (Figure 2A, 2B). IDH mutation is considered to be the main driver of glioma, with an incidence of more than 70%; thus, we investigated the relationship between ODF3B expression and IDH status. In the TCGA and CGGA databases, the expression of ODF3B was positively correlated with IDH wild-type (Figure 2C, 2D). At the same time, 1p19q co-deletion is an important pathological feature in the occurrence and development of glioma, and the prognosis of patients with co-deletion is generally better than that of patients without co-deletion. We therefore evaluated the relationship between ODF3B expression and 1p19q state in the database. We found that MD2 expression was significantly up-regulated in the 1p19q group without co-deletion (Figure 2E). Moreover, ODF3B was found to be correlated with the histology of glioma, and the expression of ODF3B in GBM was significantly higher than that in LGG (Figure 2F). These results suggest that the high expression of ODF3B is related to the occurrence and development of glioma.

High expression of ODF3B predicts poor prognosis

We divided patients based on the expression of ODF3B into the high expression group and low expression group according to the median. The CGGA database indicated that the higher the expression of ODF3B, the lower the overall survival (OS) (Figure 3A, 3B). OS, disease-specific survival (DSS) and progression-free interval (PFI) in the TCGA database were all negatively correlated with ODF3B expression (Figure 3C, 3D). These results suggest that ODF3B is associated with the prognosis of glioma, and the higher the expression, the worse the prognosis.

Decreased ODF3B affects the proliferation and apoptosis of glioma

Lentivirus was used to perform transient knockdown in U87-MG and U373 cell lines with the highest expression, and their knockdown efficiency was verified. We found that sh2-ODF3B had the highest knockdown efficiency (Figure 4A, 4B). Therefore, sh2-ODF3B was selected for the functional test. During the process of cell culture, it was found that the number of glioma cells in the knockdown group decreased significantly 24 h after ODF3B knockdown. Therefore, CCK8 was used to verify the effect of ODF3B knockdown on the proliferation of glioma cell lines, and it was found that the proliferation ability of U87-MG and U373 cells decreased after ODF3B knockdown (Figure 4C, 4D). The effect of ODF3B knock-
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B

C

D

E

F

G

H

I

J

ODF3B

GAPDH

NHA  U87  U373  U251  A172  LN229  T98

Normal  Grade II  Grade III  Grade IV
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In order to verify the effect of ODF3B knockdown on glioma, the Annexin V-FITC/PI apoptosis detection kit was used to detect the changes in apoptosis capacity of glioma cells. It was found that after ODF3B knockdown, apoptosis of U87-MG and U373 cells increased (Figure 4G). These results suggest that ODF3B affects the apoptosis of glioma cells. Changes in apoptosis-related proteins were then examined, and it was found that after ODF3B knockdown, expression of apoptosis-related protein Bcl2 decreased, and expression of cleaved-caspase 3 increased (Figure 4H, 4I). Therefore, the knockdown of ODF3B weakens the proliferation capacity of glioma and increases the apoptosis capacity, delaying the malignant progression of glioma.

**ODF3B affects the proliferation and apoptosis of glioma through the JAK/STAT pathway**

In order to explore the related functions of ODF3B in glioma, KEGG analysis was performed according to the expression of ODF3B, and enrichment of the KEGG pathway showed that ODF3B was correlated with herpes simplex virus type 1 infection, Epstein-Barr virus infection, human immunodeficiency virus type 1 infection, the JAK-STAT signaling pathway, toll-like receptor signaling pathway, etc. (Figure 5A, 5B). The JAK-STAT pathway is a signal transduction pathway stimulated by cytokines, which is involved in many important biological processes such as cell growth, apoptosis and immune regulation. Therefore, it was speculated that ODF3B may affect the proliferation and apoptosis of glioma through JAK-STAT, and thus affect its progression. In order to verify this hypothesis, western blotting (WB) verification was performed, and it was found that p-JAK1, p-JAK2 and p-STAT3 expression decreased after ODF3B knockdown (Figure 5C). Therefore, ODF3B affected the proliferation and apoptosis of glioma through the JAK1 and JAK2/STAT3 pathways.

To further determine whether ODF3B affects the characteristics of glioma proliferation and apoptosis through the JAK-STAT pathway, we used Colivelin TFA, a neuroprotective peptide, to interact with glioma U87-MG and U373 cells. Colivelin TFA, which has brain permeability, is a potent activator of STAT3. When Colivelin TFA was added, p-STAT3 expression levels in shODF3B+Colivelin TFA group were restored, similarly, apoptosis-related protein Bcl2 expression was increased, and cleaved-caspase 3 expression was decreased (Figure 5D). Moreover, Colivelin TFA restored the proliferative ability of shODF3B and reversed its apoptosis (Figure 5E-H). Therefore, these results suggest that ODF3B regulates glioma proliferation and apoptosis through the JAK-STAT pathway.

**Down-regulation of ODF3B can affect the development of glioma in vivo**

We also examined the effect of ODF3B on tumorigenesis in an in situ implantation model. The results showed that ODF3B deletion significantly inhibited tumor formation in vivo. In fact, tumors were significantly smaller in the ODF3B knockdown group than in the control group (Figure 6A). Moreover, immunohistochemical results in the ODF3B knockdown group indicated decreased expression of apoptosis-associated protein Bcl2, and increased expression of cleaved-caspase 3 (Figure 6B). In addition, knockdown of ODF3B significantly extended the survival time of mice (Figure 6C). The results also indicated that ODF3B knockdown affected the proliferation and apoptosis of glioma, and affected the progression of glioma.

**Discussion**

Glioma is the most common primary malignant tumor in the brain. Due to cell heterogeneity...
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and heredity, to date, there is no cure and the prognosis is poor, especially for GBM [21-23].

Current research on ODF3B is insufficient, and it has been found that it is correlated with the

Figure 2. Relationship between ODF3B expression and clinicopathological features. A, B. The expression of ODF3B in the TCGA database and CGGA database was correlated with age. C, D. ODF3B expression was correlated with IDH mutation status in the TCGA and CGGA databases. E. The relationship between 1p19q deletion and ODF3B expression in the TCGA database. F. The relationship between histology and ODF3B expression in the TCGA database (*P < 0.05, **P < 0.01, ***P < 0.001).

Figure 3. High expression of ODF3B predicts poor prognosis. A. ODF3B in the CGGA was correlated with overall survival (OS). B. ODF3B in the TCGA database was correlated with OS. C. ODF3B was associated with disease-specific survival (DSS) in the TCGA database. D. ODF3B was associated with progression-free interval (PFI) in the TCGA database.
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occurrence and development of multiple sclerosis, while the research on tumors is still at an early stage [24]. It has been shown in the database that glioma is highly expressed in the central nervous system, and originates from glial cells in the central nervous system. Therefore, we speculated that ODF3B may be related to the occurrence and development of glioma. Firstly, through the TCGA database, we found that ODF3B was highly expressed in a variety of tumors compared with normal tissues, and there were significant differences in both LGG and GBM. Therefore, we collected glioma specimens to verify the difference in the expression of ODF3B in glioma tissues of different grades and in normal tissues, and found that the higher the degree of malignancy, the higher the expression of ODF3B, and this was related to prognosis. ODF3B was found to be associated with biomarkers closely related to glioma, such as IDH, MGMT, 1p19q, etc. Therefore, we speculated that ODF3B affected the development of glioma. Next, we continued to verify our hypothesis at the cellular level, and found that the expression of ODF3B in glioma cell lines was significantly higher than that in normal human brain cell lines. We used lentivirus to knock down ODF3B, and found that the knockdown of ODF3B affected the proliferation of glioma cells and promoted apoptosis. We also performed in situ tumor formation, and found that ODF3B knockdown affected tumor growth and was related to survival time, and immunohistochemistry suggested that it was related to proliferation and apoptosis.

In studies on glioma apoptosis, we found that apoptosis-related protein Bcl2 decreased with ODF3B knockdown through WB, and cleaved-caspase3 expression increased. Members of the Bcl-2 protein family are important regulators of the programmed cell death pathway and can inhibit apoptosis [25, 26]. As a protein that directly causes the disintegration of apoptotic cells, the caspase family is closely related to apoptosis. When caspase 3 is increased, the apoptotic ability is inhibited [27, 28]. According to the WB results, ODF3B affected apoptosis during the development of glioma, and when ODF3B was highly expressed, it inhibited apoptosis in glioma.

In order to explore the related mechanism of ODF3B's influence on the proliferation and apoptosis of glioma, KEGG analysis was performed according to the expression of ODF3B. Enrichment of the KEGG pathway showed that ODF3B was associated with herpes simplex virus type 1 infection, Epstein-Barr virus infection, human immunodeficiency virus type 1 infection, the YAK-STAT signaling pathway, toll-like receptor signaling pathway, etc. The JAK/STAT pathway is involved in biological processes such as growth, apoptosis, differentiation, and immune regulation of glioma cells [29-31]. Therefore, we speculated that ODF3B can affect the proliferation and apoptosis of gliomas through the JAK/STAT pathway. We conducted WB and found that p-JAK1, p-JAK2 and p-STAT3 expression decreased after ODF3B knockdown. Therefore, ODF3B affects the pro-
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Figure 5. ODF3B affects the proliferation and apoptosis of glioma through the JAK/STAT pathway. A. In TCGA database, KEGG analysis of relevant pathways. B. In the TCGA database, gene set enrichment analysis (GSEA) was used, and JAK/STAT was a significantly enriched tumor-related pathway in patients with high ODF3B expression. C,
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In order to fully verify that ODF3B affects the progression of glioma through the JAK/STAT pathway, we activated the pathway using Colivelin TFA, an agonist of the JAK/STAT pathway, and found that the proliferation and apoptosis of glioma cells were reversed after activation of the JAK/STAT pathway.

**Conclusion**

Our study has demonstrated that ODF3B is highly expressed in glioma, and is closely related to glioma related factors such as IDH, MGMT, and 1p19q, and to pathological grade and survival prognosis. After knocking down ODF3B, *in vivo* and *in vitro* experiments showed that the proliferation capacity of glioma was weakened, the apoptosis capacity was

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**Figure 6.** Down-regulation of ODF3B can affect the development of glioma *in vivo*. A. Comparison of tumor size between the two groups after *in situ* tumor formation in nude mice. B. Changes in tumor Ki-67 and cleaved-caspase 3 in nude mice after knocking down ODF3B. C. Comparison of survival time between the two groups of nude mice after knocking down ODF3B (*P* < 0.05, **P** < 0.01, ***P** < 0.001).
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increased, and the survival time was affected. ODF3B was found to affect the progression of glioma via the JAK/STAT pathway, and the apoptosis of glioma cells was reversed after activation of the JAK/STAT pathway. This suggests that ODF3B is a potential therapeutic target for glioma.

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Written informed consent was obtained from all individual participants.

Disclosure of conflict of interest

None.

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