European Review for Medical and Pharmacological Sciences 2020; 24: 4368-4381

Study on the role and mechanism of β4GalT1 both *in vivo* and *in vitro* glioma

X. YANG[1], Z.-Y. LI[2], C.-L. YUAN[1], Y.-F. TAN[1], N.-D. ZHANG[1], L.-H. LIU[1], J.-X. SUN[1]

[1]Department of Laboratory Medicine, Deyang People's Hospital, Deyang, P.R. China
[2]Institute of Neuroscience, Chongqing Medical University, Chongqing, P.R. China

Abstract. – OBJECTIVE: To discuss the role and mechanism of β4GalT1 both *in vivo* and *in vitro* glioma, observe whether pathophysiological processes of glioma can be improved after β4GalT1 is knocked down, and study whether β4GalT1 plays a role in malignant biological processes of glioma by regulating the apoptosis and immune processes.

PATIENTS AND METHODS: Firstly, the distribution difference of β4GalT1 in tumor tissues and normal tissues was analyzed by Gene Expression Profiling Interactive Analysis (GEPIA) tumor analysis system to deduce the possible role of β4GalT1 in glioma. Secondly, whether the malignant degree of glioma was related to the expression of β4GalT1 and its immunity using human tumor tissues and blood lymphocyte subsets was analyzed. Thirdly, interfere lentivirus vector with β4GalT1 and knockdown β4GalT1 was analyzed to observe whether the malignant degree of glioma has changed. Fourthly, interfere lentivirus vector with recombinant β4GalT protein and β4GalT1 was analyzed to verify the effect of β4GalT *in vitro* test. Fifth, interfere lentivirus vector with recombinant β4GalT protein and β4GalT1 was analyzed to verify effect of β4GalT *in vivo* test. Finally, we discuss whether β4GalT is involved in the biological process of glioma through inflammatory reaction.

RESULTS: In the GEPIA tumor analysis system, the expression in tumor was significantly higher than that in normal tissues. The expression of β4GalT1 in glioma tissues was higher than that in normal tissues, and the higher the malignancy of the tumor, the higher the expression of β4GalT1 in the glioma tissues, and the lower the immune level was. The expression of IDH1, MGMT, and ki-67 was reduced, and the survival rate of the mice with glioma was improved after β4GalT1 was knocked down. *In vitro* tests, the activity of tumor cells and their reproductive ability can be reduced after β4GalT1 was knocked down, the immune level of the body can be improved, and the level of tissue apoptosis can be reduced. After recombinant β4GalT1 was given alone, the result was opposite to that of β4GalT1 knocked down group. *In vivo* tests, gross tumor volume can be reduced after β4GalT1 was knocked down, the immune level of the body can be improved, and the level of tissue apoptosis can be reduced. After recombinant β4GalT1 was given alone, the result was opposite to that of β4GalT1 knocked down group. After knocking down β4GalT1, the expression of inflammatory factors can be reduced both *in vivo* and *in vitro*, and the inflammatory microenvironment of tumors can be improved. After recombinant β4GalT1 was given alone, the result was opposite to that of β4GalT1 knocked down group.

CONCLUSIONS: The level of β4GalT1 expression in tumor tissues was increased. The malignant degree of glioma is related to the expression of β4GalT1 and its immunity. The level of tumor marker can be decreased, and the survival rate of glioma model mice can be increased after β4GalT1 is knocked down. Apoptosis and immune injury caused by tumor can be improved and gross tumor volume can be deduced after β4GalT1 is knocked down. During the development of glioma, β4GalT1 may play a malignant biological role through inflammatory response.

Key Words:
β4GalT1, Glioma, Apoptosis, Inflammation.

Introduction

Glioma is the most common malignant tumor in the central nervous system of human beings, which seriously threatens human health[1]. The role of tumor inflammatory microenvironment is increasingly prominent in the development of glioma[2]. Due to the existence of blood-brain barrier, it is difficult for biological macromolecules and peripheral immune cells to enter brain tissues and ventricle[3]. The local immune response in the brain is mainly performed by microglia of innate immune cells in the brain[4]. It was found that the number of microglia in gliomas was

Corresponding Author: Xi Yang, MD; e-mail: 340343543@qq.com

significantly higher than that in normal parts[5]. Malignant astroglioma cells expose tumor antigens, which are recognized by microglia cells in the tumor area and promote the activation of microglia cells[6,7]. Activated microglia can secrete TNF-α, IL-1β, and other cytokines, which can further act on itself to form a positive feedback cycle of inflammatory cytokines secretion, promotes the inflammatory microenvironment of glioma[8-10], and also acts on glioma cells to accelerate the occurrence and development of glioma. Meanwhile, glioma cells can also secrete a variety of cytokines, which can act on themselves and promote the occurrence and development of glioma[11,12]. It can also act on local microglia cells of the tumor, thereby affecting the immune function of microglia cells and causing immune escape of glioma[13]. The immune level is directly related to the local apoptosis of the body, and the apoptosis of nerve cells caused by the occurrence of glioma is one of the important mechanisms that cause the deterioration of glioma[14]. Therefore, targeted intervention on the occurrence and development of glioma based on the above two mechanisms will provide a theoretical basis for the treatment of glioma.

The protein glycosylation regulated by β1 4-galactosyltransferase 1 (β4GalT1) is closely related to microglial cell activation and its mediated inflammatory response[15]. All the members of the 4GalT family where 4GalT1 is located have a short amino terminal cytoplasmic region, a transmembrane stem region and a carboxy-terminal catalytic domain that plays a catalytic role in the coelomic surface of Golgi, mainly located in Golgi[16-18]. β4GalT1 plays an important role in egg fertilization, neural crest cell migration, endothelial cell migration, autoimmune diseases, and other processes[19-22]. β4GalT1 can catalyze TNF-α and other inflammatory factors to induce immune response, thus aggravating the local inflammatory response[23]. Besides, it plays an important role in the inflammatory activation of glial cells in the nervous system, and the expression of β4GalT1 in the process of cerebral inflammation and microglial inflammation is significantly increased[24,25]. In normal astrocytes, overexpression of β4GalT1 can promote malignant transformation of cell biological behavior[26]. It was also found that the expression of β4GalT1 is closely related to the inflammatory microenvironment of glioma, and intervention with β4GalT1 can inhibit the secretion of multiple cytokines in glioma cells and the proliferation, migration, and invasion of glioma cells induced by inflammatory factors[27]. Therefore, it is of great significance to further explore the molecular mechanism of the development of inflammatory response mediated by activation of small glial cells during the occurrence and development of glioma, to control the occurrence of glioma, to find therapeutic targets for glioma, and to conduct comprehensive treatment.

The purpose of this study is to investigate the role and mechanism of β4GalT1 both *in vivo* and *in vitro* gliomas, to observe whether β4GalT1 knocked down can improve the pathophysiological process of gliomas, and to investigate whether β4GalT1 plays a malignant biological process of gliomas by regulating apoptosis and immune processes.

Materials and Methods

Human Glioma Specimen

30 patients who underwent craniotomy tumor resection and biopsy and were diagnosed as glioma by pathology in the Neurosurgery Department of Chongqing Medical University from 2015 to December 2018 were selected, and the normal tissues around the tumor were used as control group. There were 19 males and 11 females, with an average age of (46.41 ±10.22) years old. According to the Central Nervous System Histology Classification Standard (WHO, 2016), the patients were divided into Grade II (n = 11), Grade III (n = 8), and Grade IV (n = 11). Inclusion criteria: patients with no history of radiotherapy and chemotherapy, craniocerebral trauma, stroke, and intracranial infection, patients diagnosed by pathological diagnosis, patients with complete data, and regular follow-up. Exclusion criteria: patients with multiple intracranial space occupying, patients with other malignant tumors, patients with poor pathological quality or lack of results. All experimental procedures were approved by Ethics Committee of Deyang People's Hospital.

Experimental Grouping Design

Patients were divided into four groups, including Con group (administrated with β4GalT1 control lentivirus), Con+β4GalT1 group (β4GalT1 control lentivirus and recombinant β4GalT1), LEN group (β4GalT1 lentivirus), and LEN+β4GalT1 group (β4GalT1 lentivirus and recombinant β4GalT1).

Culture, Passage, Cryopreservation and Seed Plate of Cells

C6, U251, and SVG P12 were taken from the liquid nitrogen tank and melted in the water solution tank. Each cell line was inhaled into each cryotherapy tube, and culture solution of 5 times the volume was added and centrifuged at 1000 r/min for 5 min. The extract was discarded and centrifuged with 10 times the volume culture medium 1000 r/min for 5 min. The extract was discarded, and 3 ml medium was added to the culture medium under 5% CO_2.

The cells with density of more than 80% were selected for passage. Then, the culture medium was sucked out and washed with PBS. 1 ml of 0.25% trypsin was added, and turned upside down, when the cells start to become round, the trypsin was absorbed. 6 ml of culture medium was added, and the cells at the bottom of the bottle were blown off and separated two culture bottles to continue to culture.

After digestion with trypsin, the cells were centrifuged at 1000 rpm for 5 min, and the supernatant cells were collected. 10% dimethyl sulfoxide (DMSO) and 90% fetal bovine serum (FBS) were added into the suspension, and 3.0×106 cells /1 ml/ tube were labeled and frozen at -80°C.

1 ml trypsin was added for digestion for 30 s, and DMSO was added to terminate digestion. Individual cells were blown off and the suspension was transferred into a 15 ml centrifuge tube, centrifuged at 1000 r for 5 min. The supernatant solution was added into 1 ml of culture solution to suspend the cells and count. The suspension concentration of C6 and SVG P12 was 8.0×103/ well and U251 was 1.0×104/well, respectively, which were inoculated on 96-well plates for MTT and cloning experiments.

In cell intervention, β4GalT1 control lentivirus (GenePharma, Shanghai, China) was added to Con and Con+ β4GalT1 groups; β4GalT1 lentivirus (GenePharma, Shanghai, China) was added to LEN and LEN+ β4GalT1 groups; 1nM of β4GalT1 (3609-GT-010, R&D systems, Oakville, ON, Canada) was added to Con+ β4GalT1 or LEN+ β4GalT1 groups.

Establishment of Glioma Mouse Model[28]

5-7 weeks old Balb/c male nude mice were fed in SPF environment. U251 cells were digested and subcutaneously injected into the right armpit of nude mice with 2 × 106 phosphate-buffered saline (PBS) dissolved in 0.1 ml. They were divided into 4 groups. 10 μl of recombinant β4GalT1 was injected into the lateral ventricles of Con+ β4GalT1 and LEN+ β4GalT1 groups. 2 μM of β4GalT1 lentivirus or control lentivirus was injected to the lateral ventricles. All animals used in this study were cared for in strict accordance with Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). All experimental procedures were approved by Animal Ethics Committee of Deyang People's Hospital. Measures were taken to minimize animal suffering.

Determination of Lymphocyte Subsets

100 μl of anticoagulant blood was added to CD3-FITC, CD4-APC, and CD8-PE with 20 μl for each and incubated in the dark for 20 min. 2 ml of red blood cell lysate was added and kept for 10 min avoiding light. The cells were washed twice with PBS, and 5000 lymphocytes were collected by flow cytometry and the percentage of positive cells was analyzed by CellQuest software.

Western Blot

In vitro test, the cells were precooled and washed twice. Then, the radioimmunoprecipitation assay (RIPA) lysate containing protease inhibitor was added to the cell lytic solution for cell lysis, and the culture fluid was collected after centrifugation. In the *in vivo* test, the tumor tissue was cut off after cardiac perfusion. The total protein concentration was determined by bicinchoninic acid (BCA) assay. 10% separation adhesive and 5% concentrated adhesive were prepared. The gel and electrophoresis cell were installed, the comb was pulled out, the target protein was connected with marker in turn. After connecting the power supply, the samples were separated to the bottom of the glue at 80 V and 120 V respectively, transferred to the electric-to-liquid working tank, and placed with polyvinylidene difluoride (PVDF) film at 250 mA electric-to-liquid for 1 h. It was sealed with 5% skim milk for 1 h and incubated overnight with the primary antibody of the target protein, rinsed with Tris-Buffered Saline and Tween-20 (TBST) solution for 3 times, and incubated with secondary antibody for 1 h after removing the membrane. TBST solution was rinsed 3 times to develop with developer. All the data were detected with the ChemiDoc^{TM} Touch Imaging System and analyzed with the Image lab 3.0 software (Bio-Rad, Irvine, CA, USA). The antibodies used in this test are as follows: β4GalT1 (ABIN6259493, Biotech

Co., Ltd, Luzern, Switzerland); β-actin (M01263-2, Boster, Wuhan, China); IDH1 (#66969s, Cell Signaling Technology, Danvers, MA, USA); MGMT (#58121s, Cell Signaling, Danvers, USA); ki-67 (#9449s, Cell Signaling, Danvers, USA); BAX (ab32503, Abcam, Cambridge, MA, USA); BCL-2 (ab182858, Abcam, Cambridge, MA, USA); cleaved Caspase-3 (ab2302, Abcam, Cambridge, MA, USA); MMP4 (ab39973, Abcam, Cambridge, MA, USA), TLR4 (ab13556, Abcam, Cambridge, MA, USA), NF-κB (BM3940, Boster, Wuhan, China), TNF-α (ab1793, Abcam, Cambridge, MA, USA) and IL-1β (ab9722, Abcam, Cambridge, MA, USA), followed by secondary antibodies conjugated to horseradish peroxidase (HRP) anti-rabbit IgG (H+L) (AS014, ABclonal, Wuhan, China) and anti-mouse IgG (H+L) (AS003, ABclonal, Wuhan, China).

ELISA

All the caspase-3, caspase-9, TNF-α, and IL-1 β enzyme activities were operated by enzyme-linked immunosorbent assay (ELISA) kit instructions provided by Wuhan Ph.D. Company. Glioma tissues were used *in vivo* and U251 cell line was used *in vitro*.

TUNEL Immunofluorescence

TUNEL was performed as previously described[29]. TUNEL reaction solution (ab66108, Abcam, Cambridge, MA, USA).

Flow Cytometry[30]

The cell culture solution was placed in a centrifuge tube, washed with PBS, digested with trypsin, and blew off individual cells. The culture solution was collected into a 15 ml centrifuge tube, centrifuged at 1000 rpm for 6 min, and the supernatant was discarded. 1 ml of precooled PBS was added and the cells were resuspended. It was counted. Fifty to one hundred thousand resuspended cells were centrifuged, and supernatant was discarded. 195 µl of Annexin V-FITC binding solution was added and mixed with 5 µl of Annexin V-FITC thoroughly. 10 µl of propidium iodide (PI) dyeing solution was added to blow off and absorb and mix well. Flow cytometry was performed after 20 min under dark exposure.

Calculation of Volume of Tumor and Spleen Index

After modeling for 24 d, tumor tissues were taken, and tumor volume = 0.5× length × width^2 (mm^3). Spleen index = (spleen weight/body weight) ×10.

MTT

The target cell culture medium was placed in 96-well plate with 3 compound wells for each sample of 100 µl per well. 0 µmol/L was taken as control group. The 96-well plate was incubated at 37°C and 5% CO^2 for 48 h, and 5 mg/ml of MTT solution was added to each well for 20 mg /ml solution for 4 h. After the supernatant was discarded, 150 µl of DMSO was added to each well to shock away light for 10 min. The absorbance at 490 nm was determined by microplate reader.

Cloning Experiment

The target cell culture solution was placed in 96-well plates with 3 multiple wells for each sample. It was washed with PBS twice and added with new culture solution for 10 d. When cloning was visible to the naked eye, culture was terminated, and PBS was washed. It was fixed with 4% paraformaldehyde for 20 min and washed with PBS twice. It was dyed with 0.4% trypan blue for 20 min, and the fixative was washed with flowing water and calculated. Clone formation rate = (clone number) number of inoculated cells × 100%.

Statistical Analysis

Data were expressed by mean ± annotation difference and analyzed by GraphPad Prism 6.0 (San diego, CA, USA). SPSS 19.0 (IBM, Armonk, NY, USA) was used for survival analysis. One-way ANOVA was used to analyze the overall difference, and Sidak test was used for inter-group comparison. *p*-value less than 0.05 was considered statistically significant.

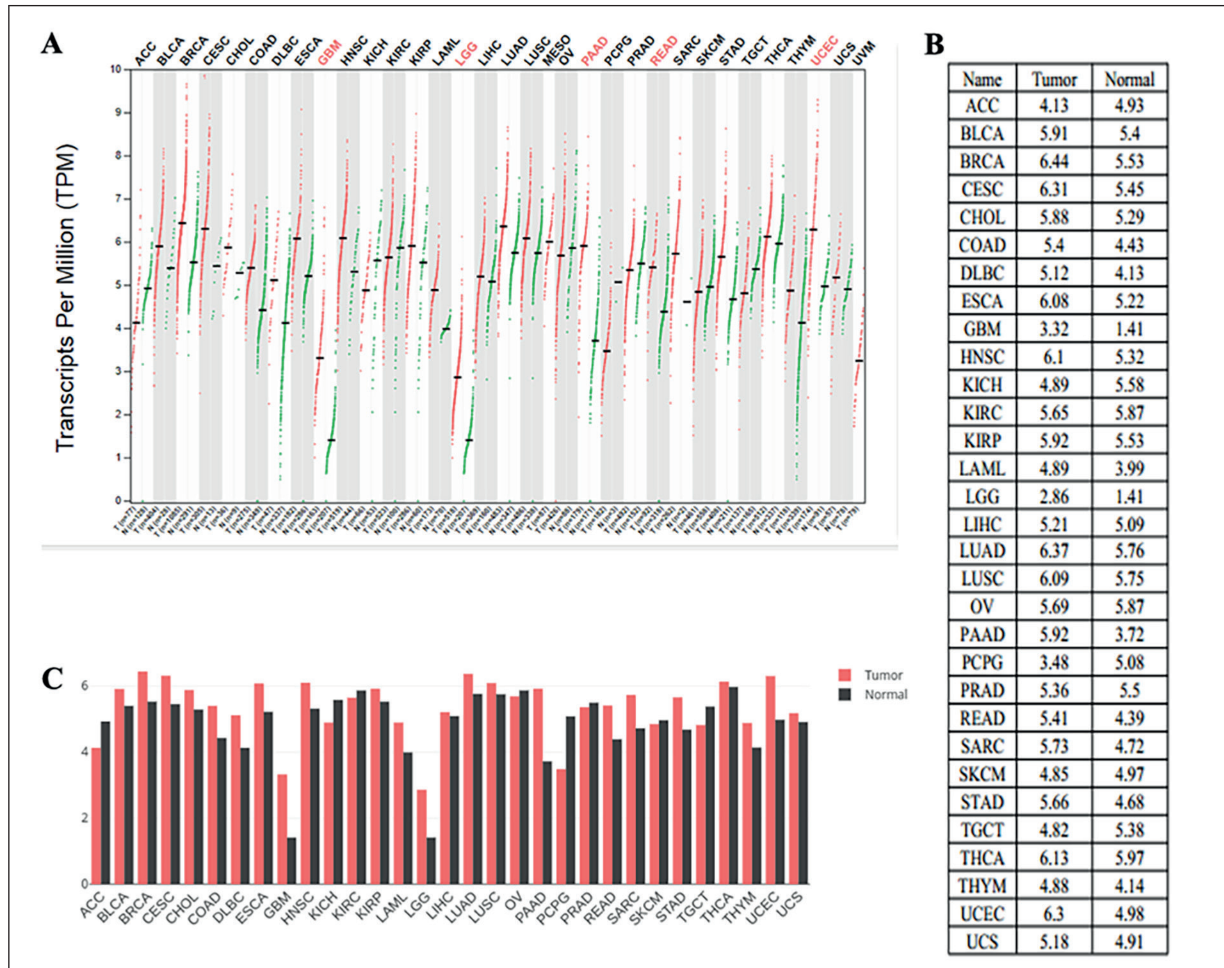
Results

Distribution and Expression of β4GalT1 in Tumor Tissues and Normal Tissues

As shown in Figure1A, 1B, and 1C, GBM was a glioma in the human GEPIA tumor analysis system, and its expression in the tumor was significantly higher than that in normal tissues (3.32 *vs.* 1.41).

Degree of Malignancy of the Glioma is Related to the Expression of β4GalT1 and Its Immune Status

Figure 2A and 2B showed that the expression of β4GalT1 in G1, G2, and G3 was significantly increased. The expression level of β4GalT1 in the two levels of patients was compared in Figure



Name	Tumor	Normal
ACC	4.13	4.93
BLCA	5.91	5.4
BRCA	6.44	5.53
CESC	6.31	5.45
CHOL	5.88	5.29
COAD	5.4	4.43
DLBC	5.12	4.13
ESCA	6.08	5.22
GBM	3.32	1.41
HNSC	6.1	5.32
KICH	4.89	5.58
KIRC	5.65	5.87
KIRP	5.92	5.53
LAML	4.89	3.99
LGG	2.86	1.41
LIHC	5.21	5.09
LUAD	6.37	5.76
LUSC	6.09	5.75
OV	5.69	5.87
PAAD	5.92	3.72
PCPG	3.48	5.08
PRAD	5.36	5.5
READ	5.41	4.39
SARC	5.73	4.72
SKCM	4.85	4.97
STAD	5.66	4.68
TGCT	4.82	5.38
THCA	6.13	5.97
THYM	4.88	4.14
UCEC	6.3	4.98
UCS	5.18	4.91

Figure 1. A, TPM of β4GalT1 in GEPIA tumor analysis system. All of (**B**) and (**C**) were the relative expression of β4GalT1 in each tumor tissue.

2C, and it can be seen that the expression level was higher in the high group, and the malignant degree of glioma was directly related to β4GalT1. Indexes of lymphocyte subsets in selected patients were observed to further research the relationship between inflammatory β4GalT1 and inflammatory reaction. In Figure 2E and 2F, it can be seen that CD3+ and CD4+ in high group were lower than that in low group, while CD8+ was lower than that in low group. At the same time, the trend of CD4+/CD8+ was the same as that of CD3+ and CD4+. The subcellular subsets of each patient were correlated with the level of β4GalT1 in the tumor tissues that were removed. As shown in Figure 2G and 2H, the higher the level of β4GalT1, the poorer the patient's cellular immunity and the higher the malignancy of the tumor.

The Level of Tumor Markers Can Be Decreased and the Survival Rate of Glioma Model Mice Can Be Improved After β4GalT1 Was Knocked down

In Figure 3A, 3B, and 3C, it was shown in the results of Western blot that the expressions of IDH1, MGMT, and ki-67 in LEN group were significantly decreased after the interference of β4GalT1 lentivirus, which indicated that the malignant degree of glioma decreased after β4GalT1 was knocked down. Figure 3D shows the survival analysis of glioma model mice. Con and LEN selected 30 mice for each. It can be seen that the survival rate of LEN group is significantly higher than that of Con group, which indicates that the survival rate of mice can be improved after β4GalT1 was knocked down.

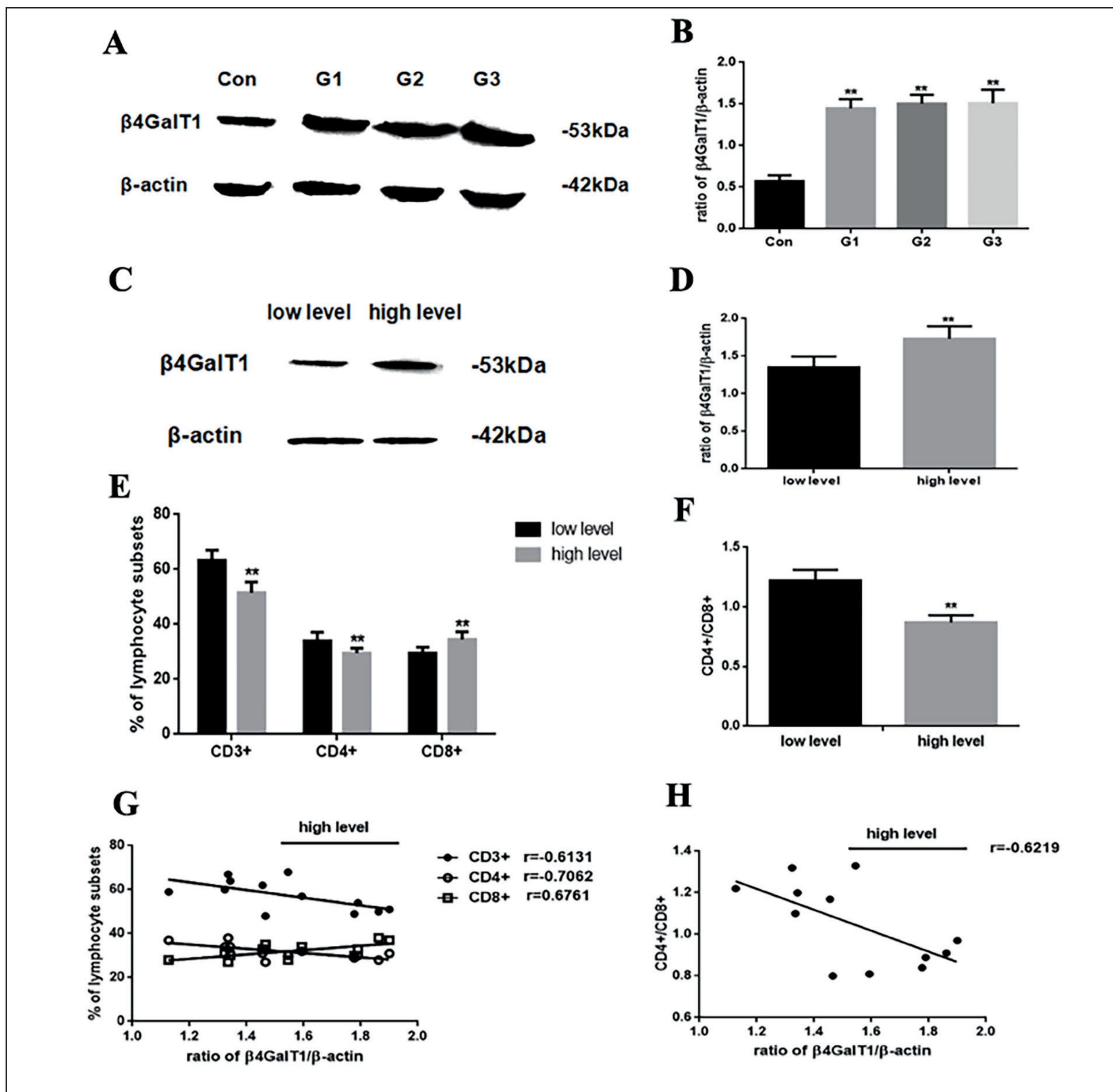


Figure 2. A, Western blot assay of β4GalT1 expression in Con and glioma human sample. **B**, Quantification of β4GalT1 expression, the protein levels were normalized to β-actin (G1, G2, G3 *vs.* Con group $^{**}p<0.05$, n=6 per group). **C**, Western blot assay of β4GalT1 expression in low level and high-level human body tissues. **D,** Quantification of β4GalT1 expression, protein levels were normalized to β-actin (low level *vs.* high level group, $^{**}p<0.05$, n=6 per group). **E,** % of lymphocyte subsets in low level and high-level patient's blood sample. **F,** CD4+/CD8+in low level and high-level patient's blood sample (low level *vs.* high level group, $^{**}p<0.05$, n=6 per group). **G, H,** Correlation analysis of β4GalT1 expression and lymphocyte subsets or CD4+/CD8+(n=6 per group).

Verification of the Role of β4GalT1 in Glioma in Vitro

Figure 4A shows MTT. After giving β4GalT1, MTT level of SVG P12 cell line decreased, which fully indicates the harm of β4GalT1. The cell activity of Con β4GalT1 group was the strongest, and that of LEN group was the lowest, and the results of C6 and U251 were consistent. Figure 4B was the cloning experiment. The cloning ability of Con β4GalT1 group was the strongest, and that of LEN group was the lowest. The results of C6 and U251 were consistent. Figure 4C, 4D, 4E, 4F mainly describe the apoptosis-promoting effect of β4GalT1. Figures 4C and 4D show that BAX and cleaved caspase-3 were apoptotic genes, BCL-2 was anti-apoptotic gene, the levels of BAX and cleaved caspase-3 were the high-

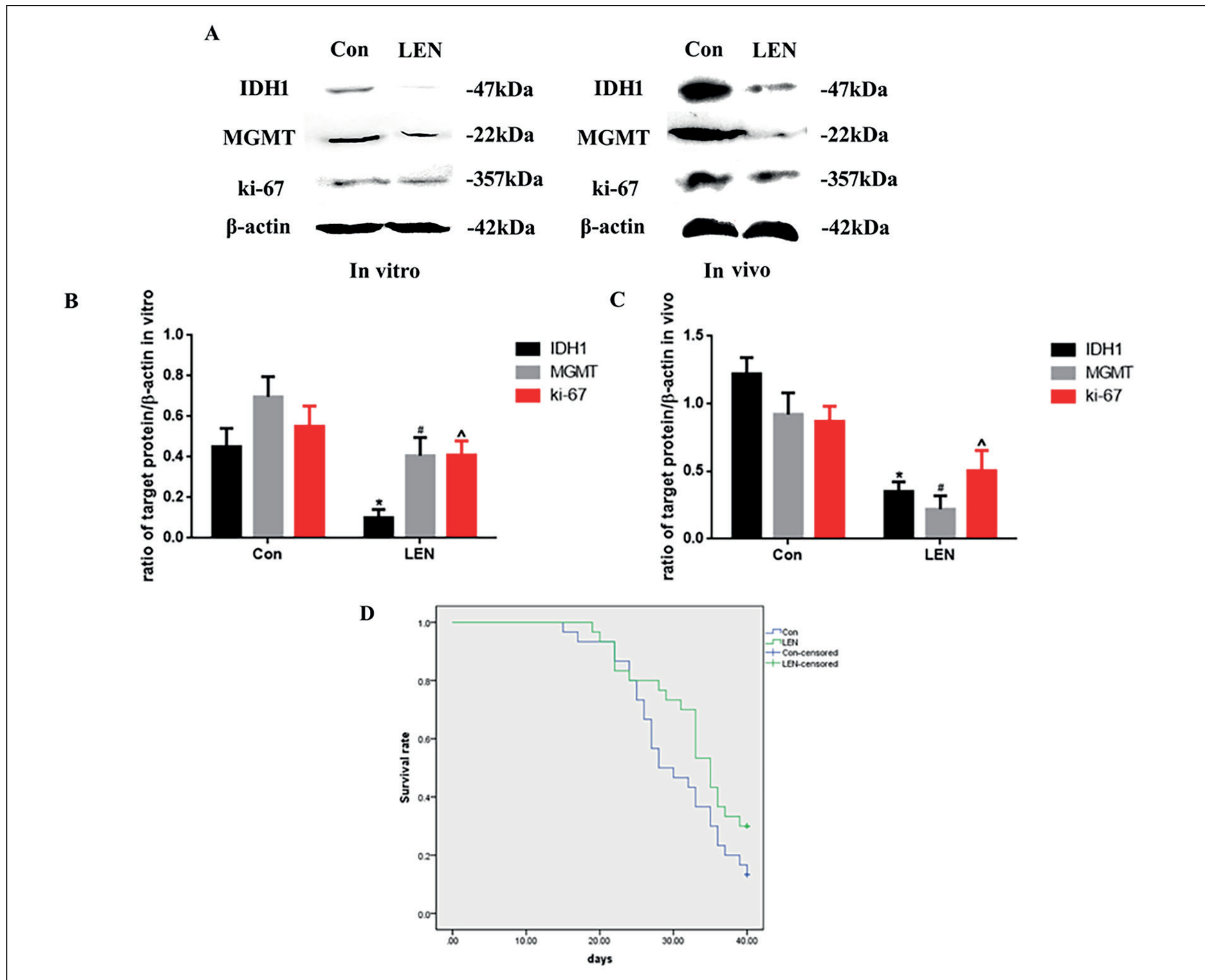


Figure 3. A, Western blot assay of IDH1, MGMT, ki-67 expression in Con and LEN group *in vitro* and *in vivo*. **B**, **C**, Quantification of IDH1, MGMT, ki-67 expression *in vitro* and *in vivo*. Protein levels were normalized to β-actin (LEN *vs.* Con group $^{**}p<0.05$, n=6 per group). **D,** The survival analysis of Con and LEN mice.

est in Con+β4GalT1 group, the levels of BAX and cleaved caspase-3 ("it" refers to what? BAX and cleaved caspase-3 or Con+β4GalT1 group?) b) were the lowest in LEN group, the lowest in Con+β4GalT1 group, and the highest in Con+β4GalT1 group. Figures 4E and F show flow cytometry. It could be seen that the level of apoptotic cells in Con+β4GalT1 group was the highest, and that in LEN group was the lowest. Figure 4G shows the activity of caspase-3 and caspase-9. It can be seen that the activity of caspase-3 and caspase-9 is the highest in Con+β4GalT1 group and the lowest in LEN group.

Verification of the Role of β4GalT1 in Glioma in Vivo

Figure 5A shows that the tumor volume was the highest in Con+β4GalT1 group and the lowest in LEN group. Figure 5B shows that the spleen index was the lowest in Con+β4GalT1 group and the highest in LEN group. Figure 5C shows WB data, which was consistent with Figure 4. Figure 5E and F show the data of TUNEL immunofluorescence staining, and it can be seen in the figure that the apoptosis of Con+β4GalT1 group was the strongest and LEN group was the lowest. Figure 5G shows the ELISA data, which was consistent with the 4 parts.

β4GalT1 Can Play A Role in Glioma by Stimulating Inflammatory Response

Figure 6A, 6B, and 6C show that MMP4, TLR4, NF- κ B, TNF- α, and IL-1 β were the highest in Con+β4GalT1 group and the lowest in LEN group. Figures 6D and E show the ac-

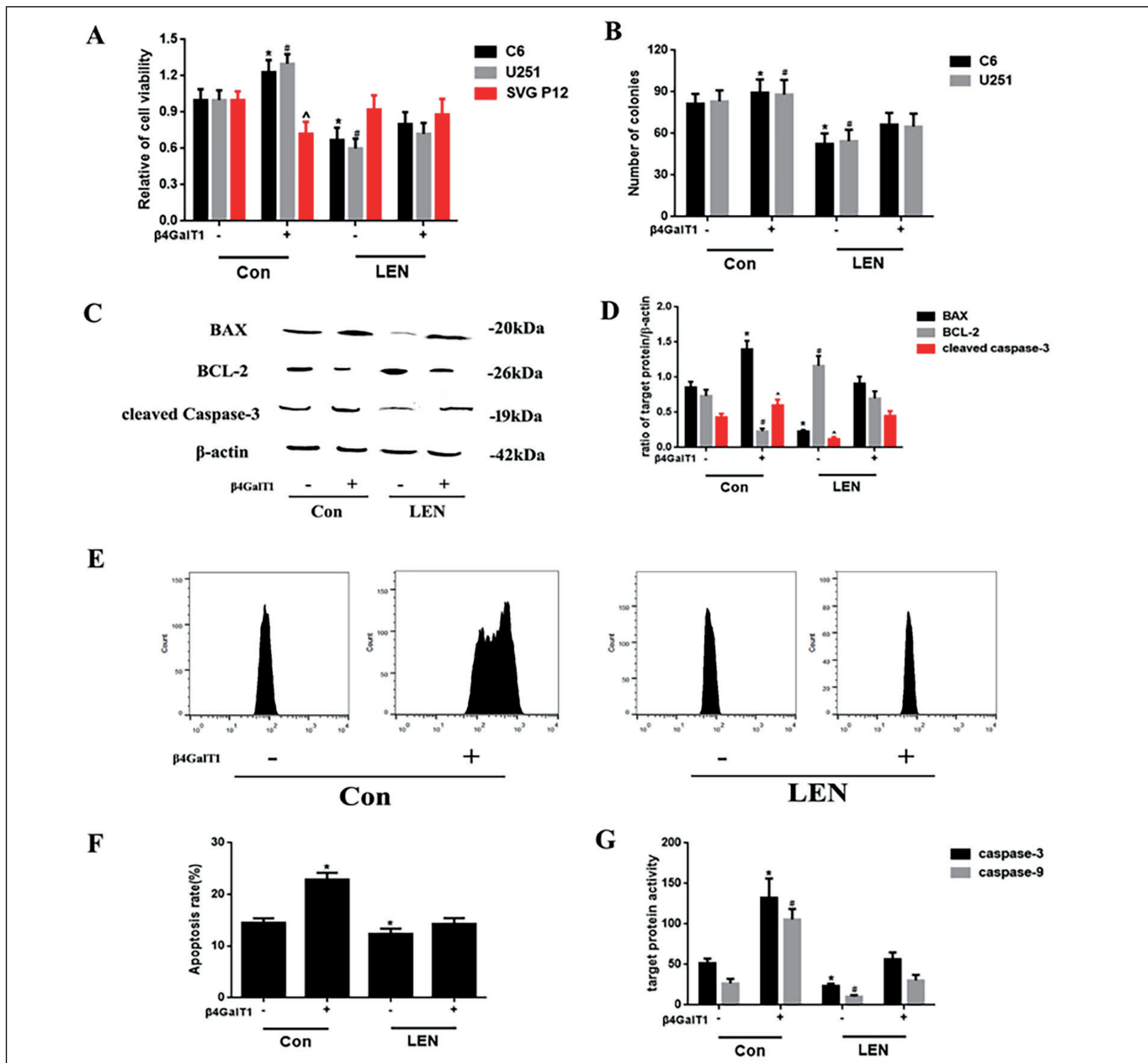


Figure 4. A, MTT assay in Con, Con+β4GalT1, LEN, LEN+β4GalT1 group of C6, U251, and SVG P12 cell line. **B,** Colonies assay in Con, Con+β4GalT1, LEN, LEN+β4GalT1 group of C6, U251 cell line. **C,** Western blot assay of BAX, BCL-2, cleaved caspase3 expression in Con, Con+β4GalT1, LEN, LEN+β4GalT1 group *in vitro*. **D,** Quantification of BAX, BCL-2, cleaved caspase3 expression, protein levels were normalized to β-actin (LEN *vs.* Con group, Con+β4GalT1 *vs.* Con group, $^{**}p<0.05$, n=6 per group). **E,** Apoptosis assay in Con, Con+β4GalT1, LEN, LEN+β4GalT1 group. **F,** Apoptosis rate in Con, Con+β4GalT1, LEN, LEN+β4GalT1 group. **G,** ELISA assay of caspase3 and caspase9 in Con, Con+β4GalT1, LEN, LEN+β4GalT1 group.

tivity data of TNF-α and IL-1β *in vitro*. It can be seen that the activities of TNF-α and IL-1β in Con+β4GalT1 group were the highest, and those in LEN group were the lowest. Figures 6F and G show the data of TNF-α and IL-1β activities *in vivo*. It can be seen that the activities of TNF-α and IL-1β in Con+β4GalT1 group were the highest, and those in LEN group were the lowest.

Discussion

Glioma is the most common and malignant primary malignant brain tumor in human beings. Most gliomas, especially high-grade gliomas, are characterized by extensive invasive growth without clear boundaries. This feature not only makes the operation more difficult, but also leads to the

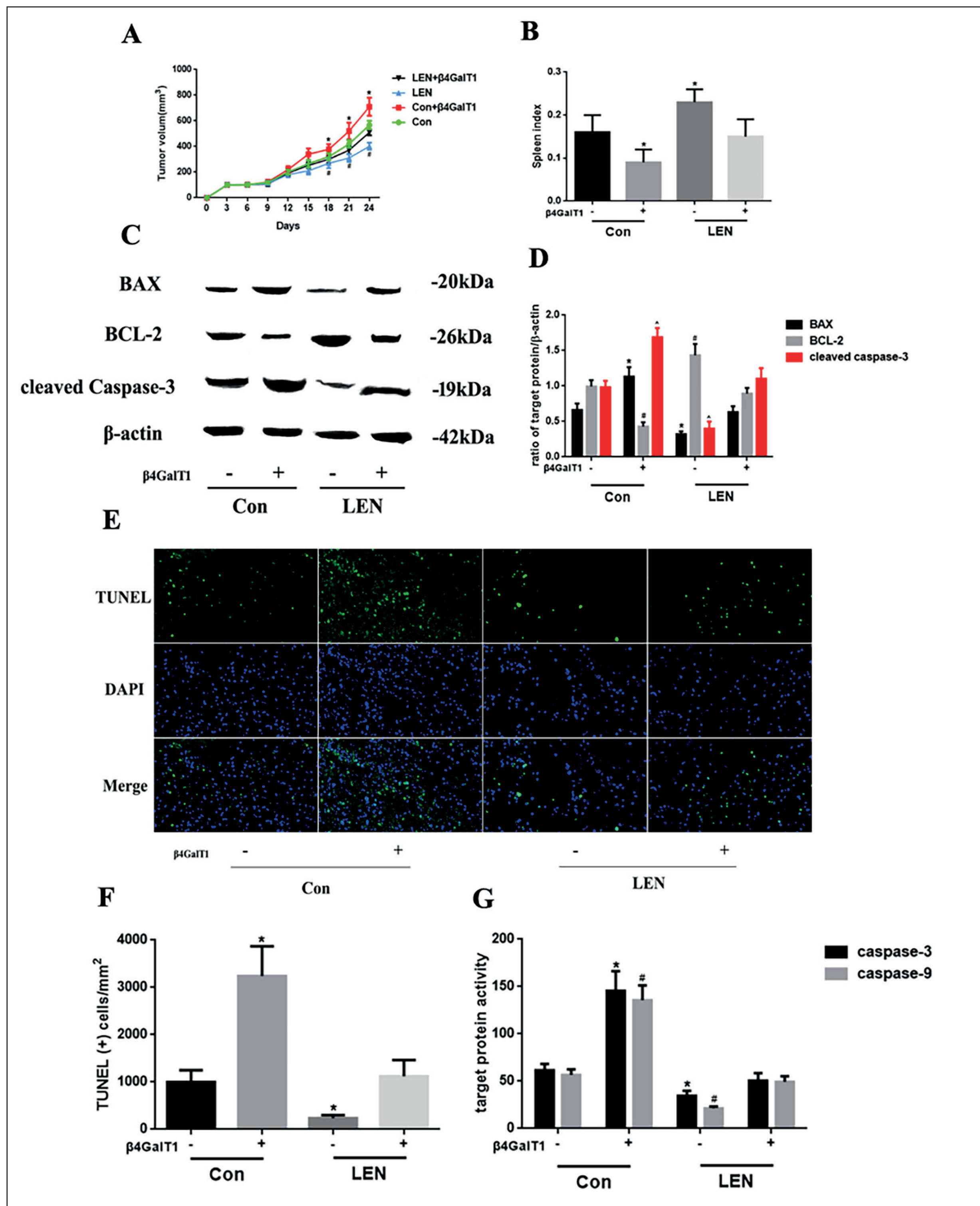


Figure 5. A, Tumor volume analysis in Con, Con+β4GalT1, LEN, LEN+β4GalT1 group. **B,** Spleen index in Con, Con+β4GalT1, LEN, LEN+β4GalT1 group. **C,** Western blot assay of BAX, BCL-2, cleaved caspase3 expression in Con, Con+β4GalT1, LEN, LEN+β4GalT1 group *in vivo*. **D,** Quantification of BAX, BCL-2, cleaved caspase3 expression, protein levels were normalized to β-actin (LEN *vs.* Con group, Con+β4GalT1 *vs.* Con group, **$p<0.05$, n=6 per group). **E,** TUNEL staining of Con, Con+β4GalT1, LEN, LEN+β4GalT1 group (magnification ×400). **F,** TUNEL(+) cells number in Con, Con+β4GalT1, LEN, LEN+β4GalT1 group. **G,** ELISA assay in Con, Con+β4GalT1, LEN, LEN+β4GalT1 group.

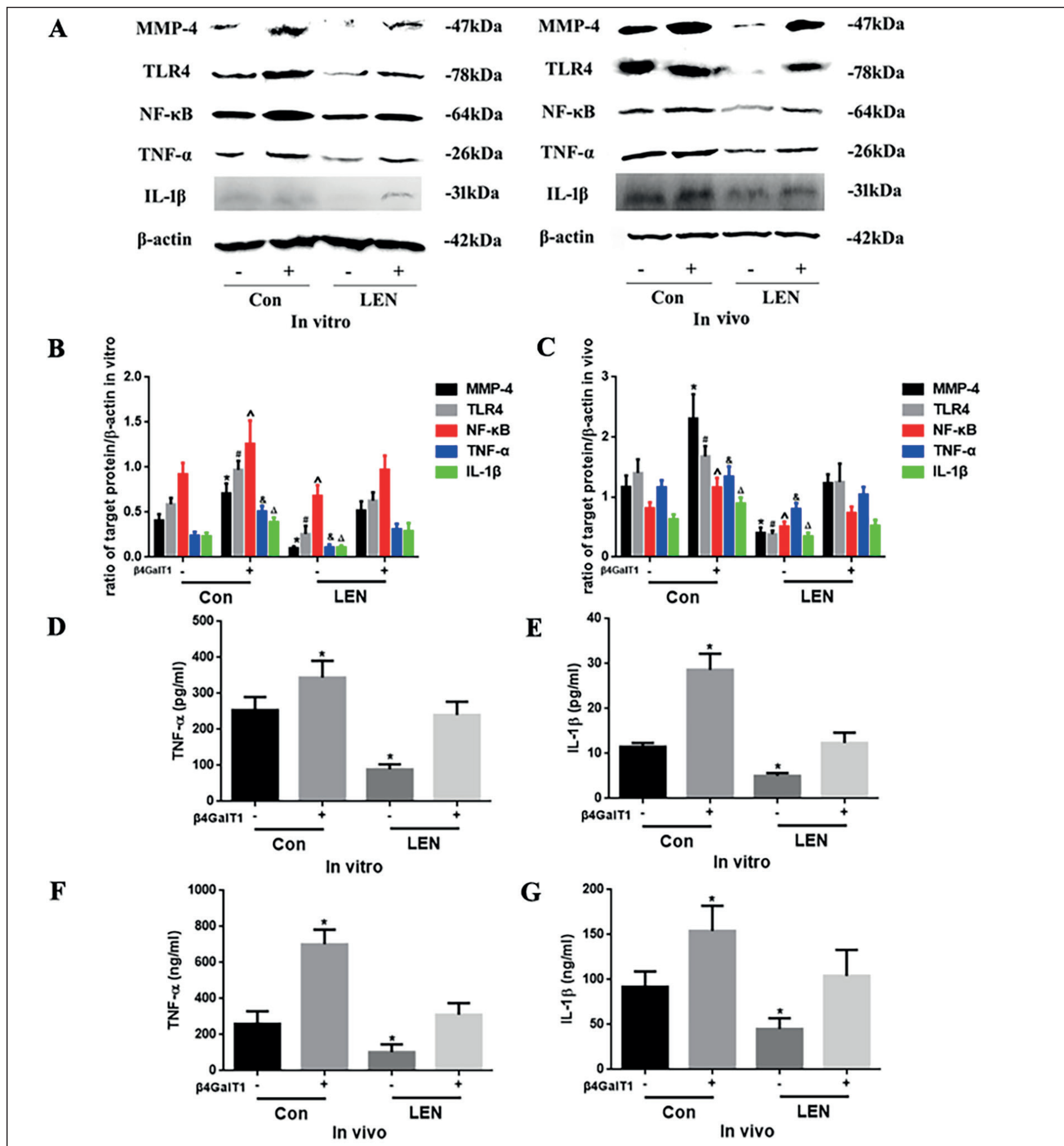


Figure 6. A, Western blot assay of MMP4, TLR4, NF-κB, TNF-α, and IL-1β expression in Con Con+β4GalT1, LEN, LEN+β4GalT1 group *in vitro* and in *vivo*. (**B**) and (**C**), Quantification of MMP4, TLR4, NF-κB, TNF-α, and IL-1β expression *in vitro* and in *vivo*, protein levels were normalized to β-actin (LEN *vs.* Con group, Con+β4GalT1 *vs.* Con group, $^{**}p<0.05$, n=6 per group). **D, E,** ELISA assay of TNF-α and IL-1β of Con, Con+β4GalT1, LEN, LEN+β4GalT1 group *in vitro.* **F, G,** ELISA assay of TNF-α and IL-1β of Con, Con+β4GalT1, LEN, LEN+β4GalT1 group *in vivo.* (LEN *vs.* Con group, Con+β4GalT1 *vs.* Con group, $^{**}p<0.05$, n=6 per group).

fact that the operation cannot be completely resected. Radiotherapy combined with chemotherapeutic drugs is often needed after operation[1,4,5,31].

Inflammatory response of microglia activation mediated plays an important role in glioma. Activated microglia can secrete many kinds of cytokines, such as TNF-α, IL-1 β and so on, which can not only further act on themselves, form positive feedback circulation of inflammatory factor secretion and promote the inflammatory micro-

environment of glioma[32], but also act on glioma cells, accelerate the occurrence and development of gliomas, induce apoptosis of nerve cells, and deteriorate the pathophysiological process of gliomas[33].

Glycosylation modification of protein regulated by β4GalT1 is closely related to the activation of microglia and its mediated inflammatory response, and plays an important role in oocyte fertilization, neural crest cell migration, endothelial cell migration, autoimmune diseases and so on[34]. β4GalT1 can catalyze inflammatory factors such as TNF-α to induce immune response, thus exacerbating local inflammatory response in the injured area. It plays an important role in the inflammatory activation of glial cells in the nervous system, and the expression of β4GalT1 is significantly increased in the process of brain inflammatory response and inflammatory activation of microglial cells. Overexpression of β4GalT1 promotes malignant transformation of its cellular biological behavior[35]. It was also found that the expression of β4GalT1 is closely related to the inflammatory microenvironment of glioma. Intervention with β4GalT1 can inhibit the secretion of multiple cytokines in glioma cells and the proliferation, migration, and invasion of glioma cells induced by inflammatory factors[36]. Therefore, β4GalT1 can be used as a new therapeutic target for glioma.

In this study, we first applied the GEPIA tumor analysis system to analyze the expression of β4GalT1 in various tumor tissues. In the field of glioma, the expression of β4GalT1 in tumor tissues was significantly increased, which indicated that β4GalT1 was associated with the occurrence and development of glioma, so β4GalT1 may have some influence on the degree of malignancy of glioma. So, whether there is a correlation between the degree of malignancy of glioma and β4GalT1. We selected human tissues with higher tumor grade and tissues with lower grade for protein analysis. The data showed that the higher the degree of malignancy of glioma, the higher the expression of β4GalT1. This suggests that 4GalT1 may be associated with the degree of malignancy of glioma, further indicating that β4GalT1 plays an important role in the occurrence and development of glioma. However, how β4GalT1 affects the occurrence and development of glioma needs further discussion. Previous studies by have pointed out that β4GalT1 can promote the secretion of cytokines and the proliferation and migration of cells induced by inflammatory factors. Therefore, we explored whether β4GalT1 can further aggravate glioma by promoting inflammatory response. At the same time, we also found that the higher the degree of malignancy of glioma, the lower the level of cellular immunity of the body, which further proved that the degree of malignancy of glioma was directly related to the tumor inflammatory microenvironment. Then, whether the cellular immune level of the body is related to β4GalT1. We did a correlation analysis of the two and found that cellular immunity levels were positively correlated with β4GalT1, suggesting that the malignancy of the glioma was correlated with β4GalT1 and the inflammatory level induced by β4GalT1. Therefore, we can see that the increased expression of β4GalT1 and the inflammatory cascade induced by it may be one of the causes of glioma deterioration in the immune dysfunction microenvironment of glioma.

To further verify the relationship between malignancy of glioma and β4GalT1, we applied *in vivo* and external experiments. In cells and animal tissues, the expression of IDH1, MGMT, and ki-67, tumor markers related to gliomas, was down-regulated after the intervention of β4GalT1. These three markers are all associated with the occurrence and development of glioma and are related to the malignant degree of the tumor. Their down-regulated expression fully indicates that there is a positive relationship between β4GalT1 and the malignant degree of glioma. In addition, we studied the survival rate of glioma mice. After intervention with β4GalT1, the survival rate of glioma mice was significantly improved, indicating that β4GalT1 was associated with the malignant degree of glioma again.

Apoptosis plays an important role in the development of glioma, and normal nerve cell apoptosis can aggravate the malignant degree of glioma[7,15,30], which can also be caused by the injury of the inflammatory response. In the previous results, we concluded that β4GalT1 can promote the inflammatory cascade amplification reaction and lead to the deterioration of glioma. However, whether β4GalT1 can promote inflammatory cascade amplification and induce glial cell apoptosis remains to be further verified. Therefore, we observed the relationship between β4GalT1 and nerve cell apoptosis. *In vitro*, with the exogenous intervention of β4GalT1, the expressions of pro-apoptotic BAX and cleaved caspase-3 were significantly increased, while the expression of apoptotic inhibitor Bcl-2 was significantly decreased. However, after β4GalT1 knocked

down, the expression levels of BAX and cleaved caspase-3 were significantly down-regulated, while Bcl-2 levels were significantly increased, indicating that β4GalT1 can induce nerve cell apoptosis in glioma and promote the deterioration of glioma. In addition, the protease activity of caspase-3 and caspase-9 was observed, and the data showed that β4GalT1 promoted the inflammatory microenvironment of glioma, accelerated the occurrence and development of glioma, induced the apoptosis of nerve cells, and worsened the pathophysiological process of glioma. *In vivo*, the expression trend of BAX, cleaved caspase-3, and bcl-2, as well as the protease activity of caspase-3 and caspase9 after exogenous intervention with β4GalT1 or interference with lentivirus intervention were completely consistent with the cell test. In addition, tumor volume and immune level were monitored, and the data showed that spleen index decreased significantly after exogenous intervention of β4GalT1, while increased after interference of β4GalT1, indirectly indicating the pro-inflammatory effect of β4GalT1, and directly indicating the biological role of β4GalT1 in glioma. Based on the above results, we concluded that β4GalT1 aggravated the tumor microenvironment of glioma and induced the apoptosis of normal glioma cells by promoting the inflammatory response, further aggravating the symptoms, thus causing the deterioration of glioma and increasing the mortality rate of mice.

To further test the relationship between β4GalT1 and inflammation in gliomas, our colleagues monitored inflammatory factor levels in *in vitro* and *in vivo*. The data showed that inflammatory factors MMP4, TLR4, NF-κB, TNF-α, and IL-1β were significantly increased in the Con+β4GalT1 group, while decreased in the LEN group, which fully indicated that the local inflammatory microenvironment of the body could be up-regulated after the administration of β4GalT1 alone in glioma, the above cytokines could be released, and the inflammatory microenvironment of the tumor could be aggravated, again demonstrating the importance of β4GalT1 in the inflammatory response of glioma, while the experimental results showed the opposite after the intervention of β4GalT1, fully demonstrating that the release of inflammatory cytokines was improved after blocking β4GalT1. In addition, the enzyme activity of TNF-α and IL-1β was measured, and the trend was consistent with the protein analysis.

Conclusions

The results of the present study indicated that β4GalT1 may be involved in the occurrence and development of glioma by regulating the inflammatory microenvironment of tumors. β4GalT1 can promote microglial cell activation and its mediated inflammatory response, while regulating neuronal apoptosis, thereby promoting malignant biological behavior of gliomas. We concluded that the expression of β4GalT1 was increased in tumor tissues. The malignancy of glioma is related to the expression of β4GalT1 and its immune status. Knocking down β4GalT1 can reduce the level of tumor markers and improve the survival rate of glioma mice. Knocking down of β4GalT1 can improve apoptosis and immune damage caused by tumors and reduce tumor volume. During the development of glioma, β4GalT1 may play a malignant biological role through inflammatory response.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

This study was supported by the issue of the role of β4GalT1 in the Development and Progression of Glioma and Its Inflammatory Mechanism of Deyang Key Science and Technology Research (No. 2018SZS060).

References

1) Chen D, Li D, Xu XB, Qiu S, Luo S, Qiu E, Rong Z, Zhang J, Zheng D. Galangin inhibits epithelial-mesenchymal transition and angiogenesis by downregulating CD44 in glioma. J Cancer 2019; 10: 4499-4508.
2) Riva M, Wouters R, Weerasekera A, Belderbos S, Nittner D, Thal DR, Baert T, Giovannoni R, Gsell W, Himmelreich U, Van Ranst M, Coosemans A. CT-2A neurospheres-derived high-grade glioma in mice: a new model to address tumor stem cells and immunosuppression. Biol Open 2019; 8. pii: bio044552.
3) Liu CJ, Shamsan GA, Akkin T, Odde DJ. Glioma cell migration dynamics in brain tissues assessed by multimodal optical imaging. Biophys J 2019; 117: 1179-1188.
4) Galstyan A, Markman JL, Shatalova ES, Chiechi A, Korman AJ, Patil R, Klymyshyn D, Tourtellotte WG, Israel LL, Braubach O, Ljubimov VA, Mashouf LA, Ramesh A, Grodzinski ZB, Penichet ml, Black KL, Holler E, Sun T, Ding H, Ljubimov AV, Ljubimova JY. Blood-brain barrier permeable nano immunocon-

jugates induce local immune responses for glioma therapy. Nat Commun 2019; 10: 3850.

5) Bian E, Chen X, Xu Y, Ji X, Cheng M, Wang H, Fang Z, Zhao B. A central role for MeCP2 in the epigenetic repression of miR-200c during epithelial-to-mesenchymal transition of glioma. J Exp Clin Cancer Res 2019; 38: 366.

6) Yi S, Yang F, Jie C, Zhang G. A novel strategy to the formulation of carmustine and bioactive nanoparticles co-loaded PLGA biocomposite spheres for targeting drug delivery to glioma treatment and nursing care. Artif Cells Nanomed Biotechnol 2019; 47: 3438-3447.

7) Meng Q, Li S, Liu Y, Zhang S, Jin J, Zhang Y, Guo C, Liu B, Sun Y. Circular RNA circSCAF11 accelerates the glioma tumorigenesis through the miR-421/SP1/VEGFA Axis. Mol Ther Nucleic Acids 2019; 17: 669-677.

8) Scobie MR, Houke HR, Rice CD. Modulation of glioma-inflammation crosstalk profiles in human glioblastoma cells by indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804) and 7-bromoindirubin-3'-oxime (7BIO). Chem Biol Interact 2019; 312: 108816.

9) Elmaci İ, Bilir A, Ozpinar A, Altinoz MA. Gemcitabine, vinorelbine and cyclooxygenase inhibitors in the treatment of glioblastoma. Ultrastructural analyses in C6 glioma *in vitro*. Tissue Cell 2019; 59: 18-32.

10) Liang R, Li J, Tang X, Liu Y. The prognostic role of preoperative systemic immune-inflammation index and albumin/globulin ratio in patients with newly diagnosed high-grade glioma. Clin Neurol Neurosurg 2019; 184: 105397.

11) Wu L, Wang F, Xu J, Chen Z. PTPN2 induced by inflammatory response and oxidative stress contributed to glioma progression. J Cell Biochem 2019; 120: 19044-19051.

12) Guo Y, Hong W, Wang X, Zhang P, Körner H, Tu J, Wei W. MicroRNAs in microglia: how do microRNAs affect activation, inflammation, polarization of microglia and mediate the interaction between microglia and glioma? Front Mol Neurosci 2019; 12: 125.

13) Ramaswamy P, Goswami K, Dalavaikodihalli Nanjaiah N, Srinivas D, Prasad C. TNF-α mediated MEK-ERK signaling in invasion with putative network involving NF-κB and STAT-6: a new perspective in glioma. Cell Biol Int 2019; 43: 1257-1266.

14) Wu M, Zhang H, Tie C, Yan C, Deng Z, Wan Q, Liu X, Yan F, Zheng H. MR imaging tracking of inflammation-activatable engineered neutrophils for targeted therapy of surgically treated glioma. Nat Commun 2018; 9: 4777.

15) Khoder-Agha F, Harrus D, Brysbaert G, Lensink MF, Harduin-Lepers A, Glumoff T, Kellokumpu S. Assembly of B4GALT1/ST6GAL1 heteromers in the Golgi membranes involves lateral interactions via highly charged surface domains. J Biol Chem 2019; 294: 14383-14393.

16) Asadollahpour Nanaei H, Dehghani Qanatqestani M, Esmailizadeh A. Well-genome resequencing reveals selection signatures associated with milk production traits in African Kenana dairy zebu cattle. Genomics 2019 Jun 3. pii: S0888-7543(19)30136-3. doi: 10.1016/j.ygeno.2019.06.002. [Epub ahead of print].

17) Zhang Y, Fang Z, Guo X, Dong H, Zhou K, Huang Z, Xiao Z. lncRNA B4GALT1-AS1 promotes colon cancer cell stemness and migration by recruiting YAP to the nucleus and enhancing YAP transcriptional activity. J Cell Physiol 2019; 234: 18524-18534.

18) Harrus D, Khoder-Agha F, Peltoniemi M, Hassinen A, Ruddock L, Kellokumpu S, Glumoff T. The dimeric structure of wild-type human glycosyltransferase B4GalT1. PLoS One 2018; 13: e0205571.

19) Li Z, Wang Y, Hu R, Xu R, Xu W. LncRNA B4GALT1-AS1 recruits HuR to promote osteosarcoma cells stemness and migration via enhancing YAP transcriptional activity. Cell Prolif 2018; 51: e12504.

20) Xie H, Zhu Y, Zhang J, Liu Z, Fu H, Cao Y, Li G, Shen Y, Dai B, Xu J, Ye D. B4GALT1 expression predicts prognosis and adjuvant chemotherapy benefits in muscle-invasive bladder cancer patients. BMC Cancer 2018; 18: 590.

21) Nilius V, Killer MC, Timmesfeld N, Schmitt M, Moll R, Lorch A, Beyer J, Mack E, Lohoff M, Burchert A, Neubauer A, Brendel C. High β-1,4-Galactosyltransferase-I expression in peripheral T-lymphocytes is associated with a low risk of relapse in germ-cell cancer patients receiving high-dose chemotherapy with autologous stem cell reinfusion. Oncoimmunology 2018; 7: e1423169.

22) Wu J, Xiao L, Zhou H, Liu H, Ge Y, Yang J, Li Y, Wu D, Zhao Y, Zhang X. ZFX modulates the growth of human leukemic cells via B4GALT1. Acta Biochim Biophys Sin (Shanghai) 2018; 50: 522.

23) Liu YX, Wang L, Liu WJ, Zhang HT, Xue JH, Zhang ZW, Gao CJ. MiR-124-3p/B4GALT1 axis plays an important role in SOCS3-regulated growth and chemo-sensitivity of Cml. J Hematol Oncol 2016; 9: 69.

24) Xie H, Zhu Y, An H, Wang H, Zhu Y, Fu H, Wang Z, Fu Q, Xu J, Ye D. Increased B4GALT1 expression associates with adverse outcome in patients with non-metastatic clear cell renal cell carcinoma. Oncotarget 2016; 7: 32723-32730.

25) Al-Obaide MA, Alobydi H, Abdelsalam AG, Zhang R, Srivenugopal KS. Multifaceted roles of 5'-regulatory region of the cancer associated gene B4GALT1 and its comparison with the gene family. Int J Oncol 2015; 47:1393-1404.

26) Geisler C, Mabashi-Asazuma H, Kuo CW, Khoo KH, Jarvis DL. Engineering β1,4-galactosyltransferase I to reduce secretion and enhance N-glycan elongation in insect cells. J Biotechnol 2015; 193: 52-65.

27) Zhou H, Ma H, Wei W, Ji D, Song X, Sun J, Zhang J, Jia L. B4GALT family mediates the multidrug resistance of human leukemia cells by regulating the hedgehog pathway and the expression of

p-glycoprotein and multidrug resistance-associated protein 1. Cell Death Dis 2013; 4: e654.

28) Wu L, Xu D, Wang Y, Liao B, Jiang Z, Zhao L, Sun Z, Wu N, Chen T, Feng H, Yao J. Study of in vivo brain glioma in a mouse model using continuous-wave terahertz reflection imaging. Biomed Opt Express 2019; 10: 3953-3962.
29) Lai Y, Deng J, Wang M, Wang M, Zhou L, Meng G, Zhou Z, Wang Y, Guo F, Yin M, Zhou X, Jiang H. Vagus nerve stimulation protects against acute liver injury induced by renal ischemia reperfusionvia antioxidant stress and anti-inflammation. Biomed Pharmacother 2019; 117: 109062.
30) Zhou H, Zhang Z, Liu C, Jin C, Zhang J, Miao X, Jia L. B4GALT1 gene knockdown inhibits the hedgehog pathway and reverses multidrug resistance in the human leukemia K562/adriamycin-resistant cell line. IUBMB Life 2012; 64: 889-900.
31) Yang H, Chen Y, Yan H, Wu H. Effects of dexmedetomidine on glioma cells in the presence or absence of cisplatin. J Cell Biochem 2020; 121: 723-734.
32) Choi HJ, Chung TW, Kim CH, Jeong HS, Joo M, Youn B, Ha KT. Estrogen inducedβ-1,4-galactosyltransferase 1 expression regulates proliferation of human breast cancer MCF-7 cells. Biochem Biophys Res Commun 2012; 426: 620-625.
33) Shinzaki S, Iijima H, Fujii H, Kuroki E, Tatsunaka N, Inoue T, Nakajima S, Egawa S, Kanto T, Tsujii M, Morii E, Takeishi S, Asano M, Takehara T, Hayashi N, Miyoshi E. Altered oligosaccharide structures reduce colitis induction in mice defective in β-1,4-galactosyltransferase. Gastroenterology 2012; 142: 1172-1182.
34) Hou X, Tashima Y, Stanley P. Galactose differentially modulates lunatic and manic fringe effects on Delta1-induced NOTCH signaling. J Biol Chem 2012; 287: 474-483.
35) Guillard M, Morava E, de Ruijter J, Roscioli T, Penzien J, van den Heuvel L, Willemsen MA, de Brouwer A, Bodamer OA, Wevers RA, Lefeber DJ. B4GALT1-congenital disorders of glycosylation presents as a non-neurologic glycosylation disorder with hepatointestinal involvement. J Pediatr 2011; 159: 1041-3.e2.
36) Chen J, Lu L, Shi S, Stanley P. Expression of Notch signaling pathway genes in mouse embryos lacking beta4galactosyltransferase-1. Gene Expr Patterns 2006; 6: 376-382.