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Growth inhibition of subcutaneous tumor with glioma cells in nude mice by silencing TLR4

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Abstract

This study investigated the regulatory impact of Toll-like receptor 4 (TLR4) gene on glioma cell proliferation and apoptosis, elucidating the molecular mechanisms underlying TLR4-induced growth inhibition in vivo. U-87MG-Sh and U-87MG-NC cells, with silenced TLR4 and negative control plasmid respectively, were established. Eighteen nude mice, divided into transfection, negative control, and blank control groups, were inoculated with corresponding cells. Over four weeks, the transfection group exhibited significantly reduced tumor growth rates, smaller mass and volume, and lower growth activity compared to controls. Histological analysis revealed sparse tumor cells, increased fibrous connective tissue, and slower angiogenesis in the transfection group. Flow cytometry demonstrated a lower proliferation index and increased G0/1 cell count in the transfection group. mRNA levels of TLR4, NF-κB, and CyclinD1 were significantly lower in the transfection group. TLR4 silencing correlated with U-87MG cell proliferation regulation, growth inhibition, NF-κB and CyclinD1 modulation, and induction of cell cycle arrest and apoptosis. These findings suggest TLR4 as a potential gene therapy target for glioma.

Keywords: Toll like receptor 4; Glioma; Nude mice; Transplanted tumor; Cell proliferation; Apoptosis

1. Introduction

Glioma is a common malignant tumor of nervous system [1] and is associated with a high morbidity and high mortality, which seriously threatens people's life and health. It is of great significance to explore the mechanism of occurrence, development, invasion and metastasis of glioma for improving the early diagnosis and treatment of glioma.

Toll like receptors (TLRs) are an important part of pattern recognition receptors. They mainly recognize exogenous and endogenous ligands, trigger signal transduction and promote the release of inflammatory mediators, which play a key role in the innate and adaptive immune response [2]. Besides, they can also regulate cell apoptosis, proliferation and angiogenesis [3-5]. The expression of TLRs are associated with the occurrence and development of many different types of tumors [6, 7]. For instance, TLR4 has a significant role in tumor cell proliferation, tumor formation, apoptosis, invasion, and immune escape [8]. Nevertheless, the exact mechanisms of TLR4 in glioma remain unclear.

Previously, we have demonstrated that silencing TLR4 expression by RNA interference inhibited the proliferation and invasion of glioma cells in vitro [9]. The following study further investigated the effects of TLR4 in vivo using a nude mice animal model, to explore the relationship between the high expression of TLR4 and the development of glioma. Nuclear factor-kappa B (NF-κB) is the downstream target gene of TLR4 which regulates cell proliferation through downstream target gene NF-κB. This study aimed to provide new targets for early diagnosis and treatment of glioma by further verifying the regulatory roles of TLR4 in glioma cell proliferation and tumor growth.

2. Materials and methods

2.1. Reagents and equipment

Major reagents and equipment were obtained as follows: AnnexinV-PE/7AAD kit (BD, USA); All-in-OneTM qPCR Primer, Catalog number: HQP054754 (GeneCopoeia, USA); H-TLR4-shRNA (1-3) Plasmid (Catalog No.: HSH054754-CU6-a,b,c; CSHCTR001-CU6) (GeneCopoeia, USA); RNA isolated total RNA extraction reagent, HiScript II 1st Strand cDNA synthesis Kit, qPCR SYBR Green Master Mix reagent (Vazyme, China); MEM medium (Gibco, USA); FC-500 type flow cytometer (Beckman Coulter, USA); PCR amplifier (Eppendorf, Germany); and MX3000p PCR cycler (Agilent, USA).

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2.2. Cell lines

The U-87MG cell line (cat. no. CL-0238) was obtained from Procell Life Science & Technology Co., Ltd. STR profiling, Y-chromosome paint and Q-band assay confirmed that the cell line is male in origin (performed by Procell Life Science & Technology Co., Ltd). Based on current literature, the cell line is likely a glioblastoma of unknown origin [10]. The U-87MG cell line had a stable passage in the laboratory and was cultured in MEM containing 10% FBS, 100 U/ml penicillin and streptomycin. Cells were sustained in an incubator at 37˚C with 5% CO_2. The U-87MG-Sh and U-87MG-NC cells previously constructed were selected using complete medium supplemented with 300 ng/mL puromycin (Sigma-Aldrich; Merck KgaA) [9].

2.3. Establishment of tumor-bearing nude mouse model and experimental grouping

U-87MG-Sh, U-87MG-NC and U-87MG cells in logarithmic growth phase were collected and suspended in saline. The cell concentration was adjusted to 3×10^7/ml. The above cell suspension was subcutaneously inoculated to the right forelimb of nude mice at a volume of 200 μl/mouse.

A total of 18 nude mice were randomly divided into three groups (n=6): blank control group, negative control group and transfection group, half male and half female. The blank control group was inoculated with U-87MG cells; the negative control group with U-87MG-NC cells; and the transfection group with U-87MG-Sh cells. The experiment lasted for 4 weeks. Mice conditions, diet and defecation were monitored on a daily basis.

Body weight and tumor diameters were measured once a week; tumor size was measured using standard caliper; tumor volume calculation method: V tumor= $ab^2/2$ (a and b represent the longest and shortest diameter, respectively).

2.4. Detecting the growth activity of subcutaneously transplanted tumor in nude mice

The tumor burden (mm^2) was calculated by quantification of tumor-bioluminescence surface area detected with the imaging device. Briefly, nude mice were anesthetized using 0.04 ml of 2% pentobarbital sodium and were laid in a dark box with the tumor facing upward. Consequently, mice were exposed to a bioluminescent flash (475 nm) for 60 s. High-resolution images (512×512 pixels) were then taken using a sensitive CCD camera.

2.5. Flow cytometry detection of tumor cell apoptosis and cell cycle

The single cell suspension of tumor tissue was prepared by mesh rubbing method with 150-mesh sieve, washed with PBS three times, and adjusted to 1×10^7/ml.

The cell suspension (0.1 ml) was washed in PBS once and re-suspended by 0.1ml 1 × binding buffer. Next, 5 μl Annexin V-PE reagent was added and placed away from light for 5 min, and then 10 μl 7AAD staining solution was added and placed away from light for 15 min. Before the apoptosis was detected by flow cytometry, 385 μl 1 × binding buffer was added to the cell suspension.

The cell suspension (0.1 ml) was incubated with 1ml of DNA dye at 4°C for 30min. Then, cell proliferation cycle was detected by flow cytometry and analyzed by the Multicycle AV software (Beckman coulter, USA). The proliferation index (PI) was calculated according to the following formula: $PI=(S+G_2/M) /(G_{0/1}+S+G_2/M) \times 100\%$.

2.6. HE staining to observe the histomorphology of subcutaneously transplanted tumor in nude mice

Tumor tissue was washed in PBS once, fixed in 4% polyoxymethylene, embedded in paraffin, sectioned to a thickness of 4 μm, and stained by H&E.

2.7. Fluorescent RT-PCR detection of the mRNA expression of TLR4, NF-κB and CyclinD1

A certain amount of subcutaneous transplanted tumor tissue was sampled, washed with cold PBS once, mixed with 1ml RNA isolation reagent, and extracted with the total RNA according to routine one-step method. Then, reverse transcription was performed according to instructions; cDNA products were used as templates for the following PCR amplification. Human GAPDH was used as internal reference for standardization. According to the standard real-time PCR process, SYBR Green I was used as fluorescent dye, and each sample was repeated three times.TLR4 sequence was synthesized by GeneCopoeia, USA, Catalog number:HQP054754; upstream primer of NF-κB 5'–CACCCTGACCTTGCCTAT-3'; downstream primer of NF-κB 5 '- CCCAGACTCCACCATTTT-3'; upstream primer of CyclinD1 5 '- CTGTCCCACTCCTACGATA-3'; downstream primer of CyclinD1 5 '- CTTGCCTCAAAGTCCTGCTTG-3'. The internal reference GAPDH upstream primer 3 '- CACTACCGTACCTGACACCA-5'; GAPDH downstream primer 3 '- ATGTCGTTGTCCCACCACCT-5'. The relative expression of TLR4 (NF-κB or CyclinD1) mRNA was calculated by $2^{-\Delta Ct}$ method, ΔCt = TLR4 (NF - κ B or CyclinD1) CT value - GAPDH CT value. The experiment was repeated three times.

2.8. Statistical analysis

Statistical analysis was performed using the SPSS 21 software (IBM Corp.). Data were presented as mean ± SD. Multi-group comparison of means was done by one-way ANOVA followed by Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Establishment of glioma tumor model in nude mice and TLR4 silencing inhibited U-87MG tumor growth in vivo.

After inoculating U-87MG, U-87MG-NC and U-87MG-Sh cells, the transplanted tumor began to form on the 7th day, and gradually increased, and the tumor formation rate reached 100%. The transplanted tumors were round or oval-shaped, and the surface was smooth (Figure 1A). The nude mice bearing tumor showed good growth status, with normal diet and activities; in addition, no significant adverse reactions were observed.

The growth curve showed that the growth of subcutaneously transplanted tumor in transfection group was slower than that in blank control group and negative control group, (Figure 1B, $P < 0.01$).

The weight and volume of subcutaneously transplanted tumor in transfection group were significantly smaller than those in blank control group and negative control group ($P < 0.01$, Figure 1C).

Furthermore, the growth of tumor expressing green protein was further observed by in vivo imaging sys-

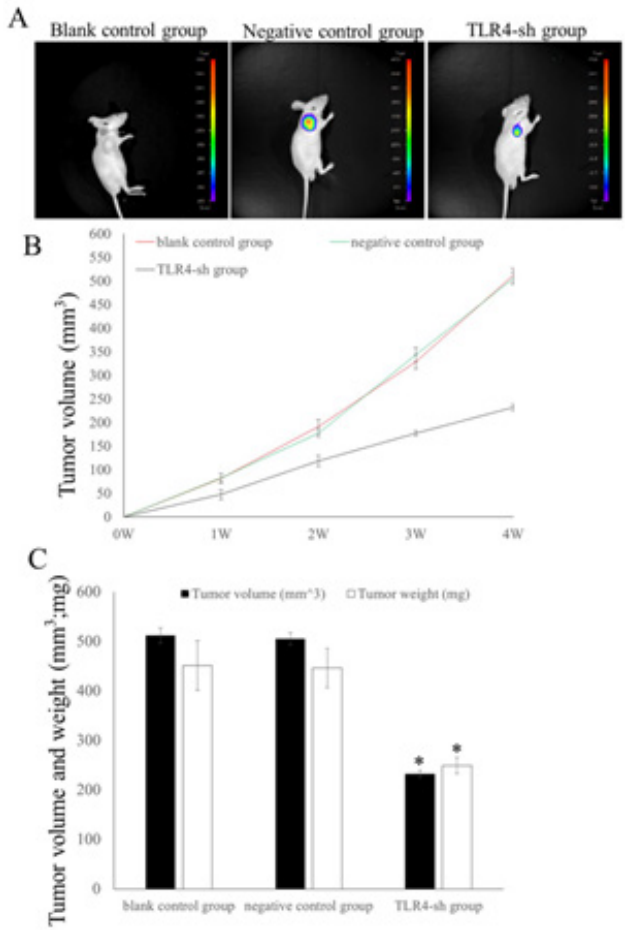

Fig. 1. Establishment of glioma tumor model in nude mice and TLR4 silencing inhibited U-87MG tumor growth in vivo. (A) After inoculating U-87MG, U-87MG-NC and U-87MG-Sh cells, the transplanted tumor began to form on the 7th day, and gradually increased, and the tumor formation rate reached 100%. The transplanted tumors were round or oval-shaped, and the surface was smooth. The growth of tumor-expressing green protein was further observed by in vivo imaging system. When compared with the blank and negative control groups, tumors in the transfection group showed slower growth, lower activity, and smaller size. (B) The growth curve showed that the growth of subcutaneously transplanted tumor in transfection group was slower than that in blank control group and negative control group. (C) The weight and volume of subcutaneously transplanted tumor in transfection group were significantly lower than those in blank control group and negative control group ($P < 0.01$). * $P < 0.01$ Vs. blank control group and negative control group.

tem. When compared with the blank and negative control groups, tumors in the transfection group showed slower growth, lower activity, and smaller size (Figure 1A). This indicated that TLR4 silencing might decrease the growth of transplanted U-87MG cells in nude mice.

3.2. TLR4 gene silencing inhibited glioma cell growth and induced massive fibrous connective tissue proliferation around the tumor.

There was no difference in the morphology of tumor tissue between the blank control group and the negative control group. Both the subcutaneous transplanted tumor cells showed obvious atypia and different cell sizes. The necrosis of tumor tissue cells was mainly confined to the central area, with less necrosis in the peripheral area. The tumor cells in the subcutaneous transplanted tumor tissue were abundant, with small intercellular spaces and tightly arranged. In the transfection group, there was extensive necrosis in tumor tissue without obvious regionality, tumor cells scattered in the distribution and the intercellular space was enlarged. There was a large number of lymphocyte infiltration in the interstitial space, and fibrous tissue increased (Figure 2).

3.3. Cell cycle, proliferation and cell apoptosis of subcutaneously transplanted tumor in nude mice detected by flow cytometry.

The $G_{0/1}$ phase of subcutaneous transplanted tumor cells in the transfection group was significantly higher than that in the negative control group and the blank control group ($P < 0.01$), while the proliferation index (PI) was significantly decreased ($P < 0.01$), Figure 3A.

The apoptosis rate of subcutaneous transplanted tumor cells in transfection group was significantly higher than that in blank control group and negative control group, P

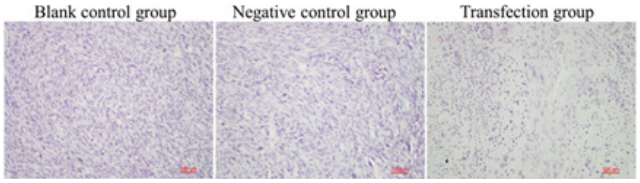

Fig. 2. The histomorphology of subcutaneous transplanted tumor in mice was detected by H&E staining (×200). There was no difference in the morphology of tumor tissue between the blank control group and the negative control group, which showed that the necrosis of tumor tissue cells was mainly confined to the central area, with less necrosis in the peripheral area and the tumor cells in the subcutaneous transplanted tumor tissue were abundant, with small intercellular spaces and tightly arranged. In the transfection group, extensive necrosis in tumor tissue without obvious regionality was detected, tumor cells were scattered in the distribution, the intercellular space was enlarged, a large number of lymphocytes were infiltrated in the interstitial space, and fibrous tissue was increased.

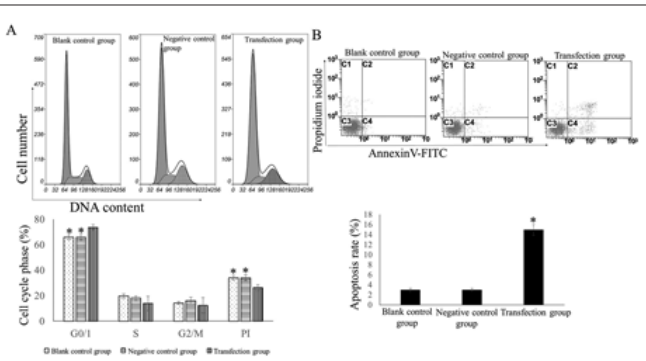

Fig. 3. Flow cytometry detection of cell apoptosis, cell cycle and cell proliferation in transplanted tumor tissue, (n=6, mean±SD). (A) flow cytometry detection of cell cycle. The $G_{0/1}$ phase of subcutaneous transplanted tumor cells in the transfection group was significantly higher than that in the negative control group and the blank control group ($P < 0.01$), while the proliferation index (PI) was significantly decreased ($P < 0.01$). *$P < 0.01$, vs. transfection group. (B) flow cytometry detection of cell apoptosis. The apoptosis rate of subcutaneous transplanted tumor cells in transfection group was significantly higher than that in blank control group and negative control group, $P < 0.01$. *$P < 0.01$, vs. negative and blank control groups. PI, proliferation index.

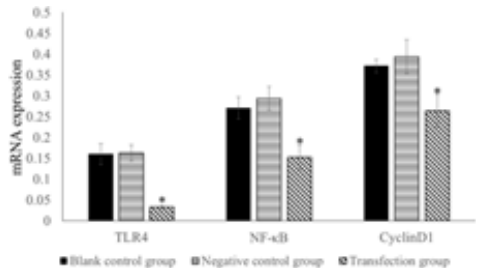

Fig. 4. The expression of mRNA of TLR4, NF-κB and CyclinD1 in subcutaneous transplanted tumor cells of nude mice was detected by fluorescence quantitative RT-PCR. The expression levels of mRNA of TLR4, NF-κB and CyclinD1 in the transfection group were significantly lower than those in the negative and blank control groups (P<0.01). * P<0.01, vs. blank and negative control groups.

< 0.01, Figure 3B.

3.4. Expression of mRNA of TLR4, NF-κB and CyclinD1 in subcutaneous transplanted tumor cells of nude mice detected by qRT-RCR.

The expression levels of mRNA of TLR4, NF-κB and CyclinD1 in the transfection group were significantly lower than those in the negative control group and blank control group ($P < 0.01$). Compared with the blank control group, the expression of mRNA of TLR4, NF - κ B and CyclinD1 in the negative control group were not significantly different, $P > 0.05$, Figure 4.

4. Discussion

Glioma is a common malignant tumor in the central nervous system with the characteristics of high invasiveness and poor prognosis. It has become one of the malignant tumors with high mortality [11]. The conventional treatments for glioma include surgery, chemotherapy and radiotherapy. However, even if surgery combined with radiotherapy and chemotherapy are used, the therapeutic effect of glioma is still unsatisfactory, and the 5-year survival rate is still low. Exploring the molecular mechanism of the occurrence and development of glioma is of great significance for early diagnosis and targeted treatment.

Toll like receptor (TLR) is a kind of membrane recognition receptor, which can recognize antigen-related molecular patterns and injury-related molecular patterns. TLR can regulate immune function, cell apoptosis and cell proliferation and so on [12]. It is of great significance to maintain the balance of the body. Besides, It has been reported that TLR4 is closely related to the occurrence and development of tumors [13, 14]. For instance, Sun *et al*. [14] studied that paclitaxel activated TLR4/NF - κ B pathway, which induced the expression of ABCB1 gene and made ovarian cancer cells to develop drug resistance; Dianne H Dapito *et al*. [15] showed that TLR4 promoted the occurrence and progression of HCC via regulating the proliferation and inhibiting apoptosis of HCC cells; Rong-Rong Yao *et al*. [16] showed that TLR4/STAT3 pathway promoted the migration and epithelial-mesenchymal transition of hepatocellular carcinoma cells through M2 polarized tumor-associated macrophages; Nilesh Pandey *et al*. [17] reviewed that TLR4 expression in solid tumor cells could promote tumor cell proliferation and metastasis; Caixia Jiang *et al*. [18] studied that high expression of TLR4 in ovarian cancer promoted cell proliferation, invasion and metastasis; Peter J Olbert *et al*. [19] showed that TLR4 promoted the development of bladder cancer. However, there are few reports on the relationship between TLR4 and gliomas. In this paper, the relationship between TLR4 and the occurrence and development of gliomas in nude mice was studied.

The glioma cells named U-87MG-Sh with low expression of TLR4 gene were established by gene silencing method [9], and inoculated subcutaneously to the nude mice to form subcutaneous transplantation tumor, which was called the transfection group. During the whole experiment period, the nude mice did not experience side effects such as weight loss, loss of appetite and hypokinesia. The growth speed and activity of subcutaneous transplanted tumor in transfection group significantly decreased than that in blank control group (subcutaneously inoculated with U-87MG cells) and negative control group (subcutaneously inoculated with U-87MG-NC cells), and the expression of TLR4 gene in subcutaneous transplanted tumor cells of transfection group was significantly lower than that of blank control group and negative control group, suggesting that the growth of glioma U-87MG cells can be inhibited by reducing TLR4 gene expression. Flow cytometry was used to detect the cell apoptosis and cell proliferation cycle of subcutaneously transplanted tumor in nude mice. The results showed that the apoptosis rate of transplanted tumor cells in transfection group was significantly higher than that in blank control group and negative control group, the cell with the period of $G_{0/1}$ was significantly increased, and the cell proliferation index was significantly lower than that of the negative control group and the blank control group. Therefore, it is suggested that the decrease of TLR4 gene expression in glioma cells might be related to the induction of apoptosis, cell cycle arrest and inhibition of cell proliferation.

Nuclear factor-κB (NF-κB) is an important nuclear transcription factor in cells and it is involved in inflammatory response, immune response, regulation of cell apoptosis and cell proliferation, which is an important factor for balancing the normal faction of cells. Besides, it is related to a variety of human diseases. In recent years, it has been found that the expression of NF-κB is related to the occurrence and development of tumors. For instance, Olga Sokolova *et al.* [20] reviewed that NF-κB promoted the occurrence, development and drug resistance of gastric cancer; Kartick C Pramanik *et al.* [21] reviewed the high expression of NF-κB in pancreatic cancer and its role in the occurrence, development and drug resistance of pancreatic cancer; Meera Patel *et al.* [22] reviewed that NF-κB could promote the occurrence and development of colorectal cancer by regulating cell apoptosis and cell proliferation cycle; Jan P Nicolay *et al.* [23] have shown that inhibition of the NF-κB expression could promote the apoptosis of cutaneous T-cell lymphoma cells; Wenjun Li *et al.* [24] have shown that cystic fibrosis transmembrane conduction regulators inhibited the growth and metastasis of esophageal cancer cells by down-regulating the NF-κB expression. Also, studies have shown that NF-κB is the downstream target gene of TLR4, and TLR4 can regulate the cell proliferation cycle by regulating the expression of NF-κB. For instance, M Rathore *et al.* [25] have shown that knockdown of TLR4 expression could inhibit the NF-κB expression, thus inhibiting the invasion and metastasis of melanoma cells. In this study, it was detected that the

expression of NF-κB in the transfection group was significantly lower than that in the blank control group and the negative control group, suggesting that knockdown of TLR4 expression in glioma cells can down-regulate the expression of NF-κB.

Cyclin D1 is the main factor in cell cycle regulation and is associated with regulating the cell cycle G_1/S checkpoint, which can promote cell cycle from G_1 to S phase, thus promoting cell proliferation. Therefore, the abnormal expression of Cyclin D1 in tissue cells is closely related to tumorigenesis. For instance, it has been reported that high expression of Cyclin D1 has been detected in a variety of tumor cells [26]; Soni Kumari [27] and other studies have shown that Cyclin D1 and Cyclin E2 were highly expressed in gastric cancer and involved in the early occurrence of gastric cancer; P Ramos-García [28] reviewed that the high expression of Cyclin D1 in oral cancer promoted the occurrence of oral cancer; Pradeep Kumar Kopparapu [29] and other studies have showed that Cyclin D1 was highly expressed in bladder cancer, and Cyclin D1 is involved in the invasion and metastasis of bladder cancer. In this study, it was detected that the expression level of Cyclin D1 in the transfection group was significantly lower than that in the negative control group and the blank control group, suggesting that silencing TLR4 gene could down-regulate the NF-κB expression, leading to the down-regulation of Cyclin D1 expression, which could result in cell cycle arrest and inhibition of cell proliferation. At the same time, it was also detected that the proliferation index of subcutaneous transplanted tumor cells in the transfection group was significantly lower than that in the blank control group and the negative control group, while the $G_{0/1}$ phase was significantly increased, and the cells were arrested in the $G_{0/1}$ phase. Flow cytometry was used to detect the apoptosis rate of subcutaneously transplanted tumor cells and it was found that the apoptosis rate of subcutaneous transplantation tumor cells in transfection group was significantly higher than that in blank control group and negative control group, which suggested that silencing TLR4 gene in glioma cells could induce cell apoptosis. There are several similar reports of these results. For instance, Huasong Chang *et al*. [30] have shown that ethanol extraction of propolis and its main component caffeic acid phenethyl ester can induce apoptosis and autophagy of breast cancer cells by inhibiting TLR4 pathway; Biqiong Ren *et al.* [31] showed that curcumin induced cell apoptosis and inhibited cell proliferation by down-regulating the TLR4 expression. The results of the study are consistent with those reports, which indicate that inhibiting the TLR4 gene expression can induce apoptosis of U-87MG cells.

In conclusion, TLR4 is involved in the occurrence and development of glioma. Inhibiting the expression of TLR4 gene can inhibit cell proliferation, block cell cycle to $G_{0/1}$ phase and induce cell apoptosis. It is expected that TLR4 will be used as a biomarker for the diagnosis of glioma and a specific target for targeting therapy of glioma.

Conflict of Interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
No human or animals were used in the present research.

Informed Consent
The authors declare that no patients were used in this study.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
Zhansen An and Yingzi Liu designed the study and performed the experiments, Cuihong Zheng, Jing Liu and Zefeng He collected the data, Xuan Yang, Liang Liu and Jing Tong analyzed the data, Zhansen An and Yingzi Liu prepared the manuscript. All authors read and approved the final manuscript.

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