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The Expression Level of CD47 in Glioma and Its Impact on Invasiveness

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Abstract

Objective: To investigate the expression level of CD47 in glioma and assess its influence on the invasiveness of tumor cells. **Methods:** A total of 30 glioma brain tissue specimens and 10 normal brain tissue control specimens were selected. Real-time fluorescence quantitative PCR (RT-PCR), Western Blot analysis and invasion assay were used to analyze the effects of low and high expression levels of CD47 on the aggressiveness of glioma cells. **Results:** The expression of CD47 in glioma cells was found to be significantly high. Conversely, a notable decrease in CD47 expression resulted in a significant inhibition of tumor cell aggressiveness. On the other hand, an increased expression of CD47 was observed to significantly promote tumor cell aggressiveness ($P<0.05$). **Conclusion:** CD47 is highly expressed in glioma cells, and a decrease in CD47 expression of CD47 significantly inhibits the aggressiveness of tumor cells. CD47 serves as a significant prognostic factor and potential therapeutic target in glioma.

Keywords

CD47 expression, Glioma, Tumor cells, Invasiveness

1. Introduction

Glioma is the most common intracranial tumor, with more than 350,000 cases diagnosed each year. Glioma is characterized by high incidence, high recurrence rate and high mortality [1]. Currently, surgical resection, radiation therapy, and chemotherapy have been widely prescribed to treat gliomas, but the overall survival rate of patients' remains low. Therefore, it is very important to develop new therapeutic drugs to improve the survival rate of glioma patients [2, 5]. As a cell membrane protein, CD47 is a member of the immunoglobulin superfamily. It is not only expressed on the surface of a variety of normal cells, but also highly expressed in most human tumor cells, which can be used as a molecular marker for tumor diagnosis and prognosis [6]. As a member of the immunoglobulin superfamily, CD47 can be used as a molecular marker for tumor diagnosis and prognosis. Play a variety of normal cell physiological functions such as regulating cell movement, nerve axon development and immune regulation, while anti-CD47 treatment can significantly inhibit the tumor growth level of solid tumors in vivo and in vitro. CD47 is highly expressed in a variety of solid tumor cells (such as ovary, lung, liver, pancreas, breast, colon, and glioma) and malignant hematoma cells (such as acute myeloid leukemia, lymphocytic leukemia, and non-Hodgkin lymphoma), and its expression level is positively correlated with disease progression [7, 9]. As a membrane protein, CD47 plays an important role in tumor immune escape. However, the molecular mechanism of CD47 expression regulation in glioma remains unclear. In this study, glioma brain tissue samples were obtained from 30 patients with glioma who were surgically resected and confirmed by pathology to investigate the expression level of CD47 in glioma and its

influence on the aggressiveness of tumor cells, as reported below.

2. The materials and methods

2.1 Specimen Collection

Glioma brain tissue samples were collected from 30 patients with glioma who were surgically resected and confirmed by pathology, and 10 normal brain tissue samples were collected as control group. The tissue type of glioma was determined according to the classification standard of the World Health Organization (WHO), and each sample was determined by two pathologists with the title of associate chief physician or above. The sample was stored in a refrigerator at -80℃. Inclusion criteria : (1) Patients who did not receive radiotherapy, chemotherapy or adjuvant therapy before surgery; (2) no other tumors; (3) Hemoglobin ≥110g/L; (4) no serious cardiopulmonary insufficiency, liver disease, kidney disease or dysfunction.

2.2 Methods

(1) Main reagents and consumables: Glioma cells U251, T98G and U87 and normal astrocytes HEB were purchased from Institute of Biophysics, Chinese Academy of Sciences (Beijing, China); DMEM culture-medium and fetal bovine serum (FBS) from Gibco (USA); FBS (10%), streptomycin and penicillin (100 U/ mL) (Gibco, Shanghai, China) were added and cultured in 10% petri dishes at 37℃ and 5% CO_2.

(2) CD47 expression vector construction and transfection: Using pOTB7-CD47 plasmid as template, CD47 fragment was amplified by PCR, and the PCR product CD47 and the target vector pcDNA3.1-3xFlag were double-digested with XhoI and HindIll, respectively. The CD47 gene fragment recovered by enzyme digestion was linked to pcDNA3.1-3xFlag vector fragment. DH5α receptor cells were directly transformed from the overnight connected mixture, and the recombinant plasmid was amplified and extracted. The CD47-overexpressed plasmid pcDNA3.1-3xFlag-CD47 and empty Vector were transfected into glioma cells with a cell density of 60% to 70%. Transfection was performed according to the instructions using transfection reagents. The cells were washed with phosphate buffered saline before transfection, and the protein was extracted 72h later to verify the transfection efficiency and subsequent experiments were conducted.

(3) Real-time fluorescent quantitative PCR (RT-PCR): TRIzol reagent was used to extract total ribonucleic acid. To quantify CD47 transcription, genomic DNA (gDNA) removal and complementary DNA (cDNA) synthesis were performed with the help of transgenic biotechnology to generate cDNA by hyperhelix. Oligonucleotide primers for GAPDH and CD47 are as follows: GAPDH: 5'-GCA CCG TCAAGG CTG AGA AC-3' (sense) and 5' -TGG TGA AGACGC CAG TGGA-3' (antisense); CD47:5' -AGA TCCGGT GGT ATG GAT GAGA-3' (sense) and 5' -GTCACA ATT AAA CCA AGG CCA GTAG-3' (antisense). Each procedure is conducted in triplicate. The qRT-PCR products were identified by 2% agarose gel electrophoresis and melting curve, and quantitatively analyzed by formula (ΔCt). ΔCt= target gene Ct- internal reference gene Ct. CD47 cDNA cycle threshold (Ct) normalized GAPDH according to ΔΔCt method.

(4) Western Blot analysis: 6-well plates were applied to cell culture until confluent to 80% in serum-free DMEM medium of 100 U/ mL double antibody (penicillin/streptomycin). The medium was removed and the cells were washed with PBS twice. M-PER mammalian protein extraction reagent was added to the cells, then the cells were scraped off a plate and centrifuged at 4 ℃ at 14 000 g for 10 min, and then the supernatant was heated for 5 min with the help of Laemmli specimen buffer supplemented with MTT (50 mmol/L). An equal amount of cracked and heated protein (30 μg/well) was electrophoretic with the help of a polyacrylamide gel (10%; BioRad, Shanghai, China). The isolated proteins were transferred to PVDF membranes and then treated at room temperature in skim milk (5%; BioRad) with the help of closed 1h. At 4 ℃, the membrane was incubated overnight with the help of the first antibody. Concentrations were determined as directed, then the second antibody was incubated with a horseradish peroxide-bound membrane at room temperature for 1 h, and finally the protein was detected using an enhanced chemiluminescence's kit (GE Healthcare).

(5) Invasion experiment: A Transwell chamber (24-well insert, 8-μm aperture) of the basement membrane (200 μg /mL) was pre-added for the invasion experiment. 24 h after transfection, the cells were treated with mitomycin C (10μg /mL) for 2 h. The cells were treated with trypsin and planted in the upper chamber in a serum-free medium with a density of 1×10^5 cells per chamber. The chamber below is filled with DMEM-F12 medium containing FBS (15%), which acts as a chemical inducer. Incubation lasted 48h, followed by the elimination of cells located on the upper chamber surface, and the invasion cells located in the lower chamber were stained and immobilized with methanol and crystal violet. The number of cells in 5 random fields was evaluated by light microscopy.

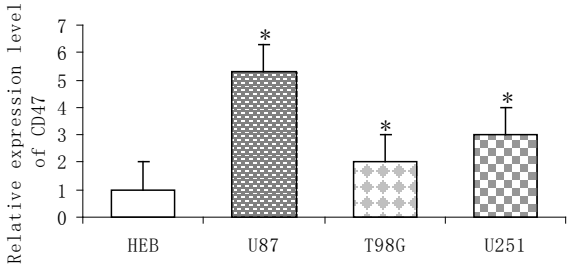
2.3 Statistical Analysis

SPSS26.0 was used for data processing, measurement data were expressed as mean ± standard deviation, and T-test was used for comparison. χ^2 test was used for statistical data, and P<0.05 was considered statistically significant.

3. Results

3.1 Expression level of CD47 in glioma

The expression of CD47 in 30 glioma specimens and 10 normal control specimens was evaluated by qRT-PCR. The results showed that CD47 existed in most glioma tissues. Compared with normal astrocytes HEB, U251, T98G and U87 cells not only promoted transcription, but also promoted CD47 translation. Compared with HEB, CD47 expression in U87 was the most significant (χ^2=12.285, P=0.000), as shown in Figure 1.

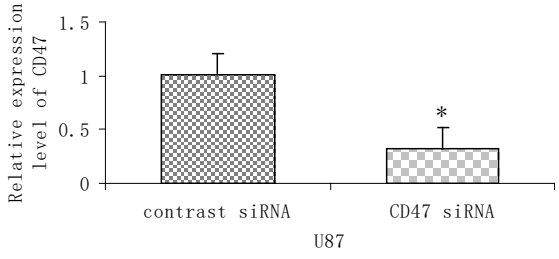


Note: Compared with HEB, *P<0.05.

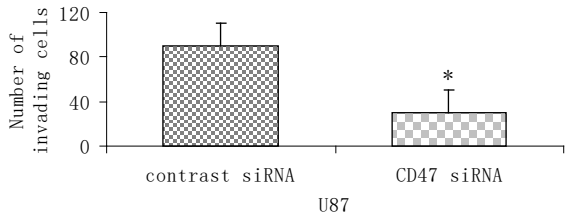
Fig.1. Expression level of CD47 in glioma.

3.2 Effects of low and overexpression of CD47 on invasiveness of glioma cells

(1) Effects of low expression of CD47 on glioma invasivity: CD47 and contrast siRNA were transfected into U87 cells respectively, and CD47 cells were blocked from transcription and translation by interfering with the siRNA of CD47 (CD47-siRNA), and CD47 of U87 cells was knocked out. Compared with contrast siRNA, CD47 knockout significantly inhibited the invasion of U87 (χ^2=8.362, P=0.003; χ^2=8.957, P=0.002), as shown in Figure 2.



A



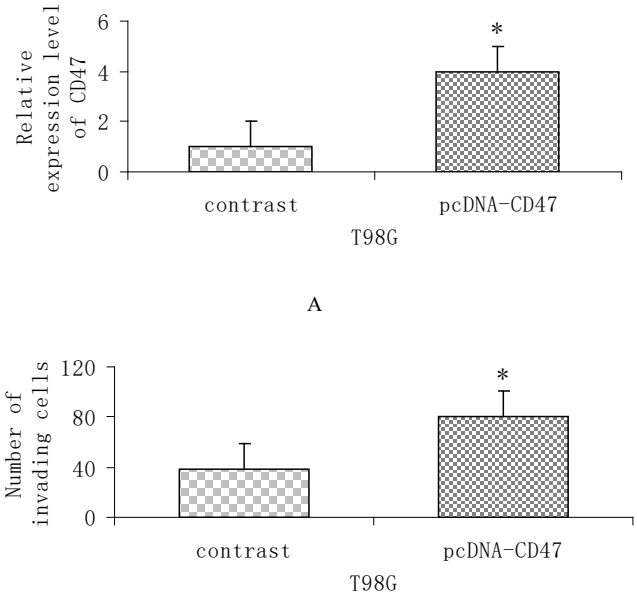
Note: Compared with contrast siRNA, *P<0.05.

B

A: CD47 transcription was evaluated by RT-PCR 24 h after transfection. B: The aggressiveness of siRNA was compared with that of CD47 siRNA

Fig. 2. Effect of low expression of CD47 on the aggressiveness of glioma cells.

(2) Effects of overexpression of CD47 on invasiveness of glioma cells: pcDNA3.1-3xFlag-CD47 plasmid was transfected into T98G cells, and then Western Blott and qRT-PCR were used to confirm the excessive expression of CD47 in T98G cells. Compared with the control group, overexpression of CD47 significantly enhanced the aggressiveness of T98G cells (χ^2=9.382, P=0.000; χ^2=7.159, P=0.008), as shown in Figure 3.



A: CD47 transcription was evaluated by RT-PCR 24 h after transfection. B: Aggressiveness of control group versus pcDNA-CD47

Fig. 3. Effect of overexpression of CD47 on invasiveness of glioma cells.

4. Discussion

At the beginning of glioma, there are usually no typical symptoms. As the tumor continues to grow, it will show the following symptoms: first, increased intracranial pressure and other general symptoms, such as headache, vomiting, vision loss, double vision, seizures and mental symptoms. The other is the local symptoms caused by the compression, infiltration and destruction of the brain tissue by the tumor, and the local symptoms vary according to the location of the tumor growth [10, 12]. Glioma is an invasive growth, which has no obvious boundary with normal brain tissue, is difficult to completely resect, is not very sensitive to radiotherapy and chemotherapy, and is very easy to relapse. Benign and malignant tumors growing in important parts of the brain are difficult to resect or cannot be operated on at all [13, 14]. Due to the influence of blood-brain barrier and other factors, the efficacy of chemical drugs and general anti-tumor Chinese medicine is not ideal, so glioma is still one of the worst prognosis tumors in the whole body.

4.1 Structure and regulation of CD47

(1) Structure of CD47: In the immune system, CD47 is the only 5-transmembrane (5-TM) receptor. It consists of three parts: the heavy glycosylated N-terminal extracellular domain (ECD), the 5-TM transdomain, and the short C-terminal domain (CTD). ECD includes V-set immunoglobulin superfamily domains that bind to SIRPα. CTD can be divided into four subtypes, from type I to type IV, which are expressed differently in different cells. All of these structures and splicing isomers are critical to the function of CD47 [15].

(2) CD47 binding protein: CD47 interacts with other extracellular proteins located on the membrane and inside the cell. It owes much of its functionality to its bonding partner. The most well-studied CD47 binding proteins are thromboreactive protein 1 (TSP-1) and SIRPα. In addition to SIRP-α, because its extracellular domain is similar to SIRP-α, SIRP-γ also binds to CD47, but its affinity is 10 times lower. TSP-1 was the first ligand identified as CD47. It interacts with CD47 through the C-terminal RFYVVMWK sequence (4N1K) of the CBD domain. CD47-TSP-1

interaction inactivates vascular endothelial growth factor receptor 2 (VEGFR2) and inhibits angiogenesis to inhibit tumor growth. Therefore, TSP-1 is also considered to be a potent inhibitor of tumor growth and metastasis. The CD47-TSP-1 interaction also inhibits inflammatory responses, such as cytokine secretion. The lack of TSP-1 in macrophages limits their phagocytosis. In addition, the interaction of CD47 with TSP-1 promotes stem cell regeneration by upregulating stem cell transcription factors KLF4, Sox2, c-Myc, and Oct4. SIRPα was identified as an endogenous ligand of CD47. It is also a transmembrane glycoprotein, which is mainly expressed on macrophages, monocytes and DC. It contains one transmembrane domain, three IG-like domains, and four tyrosine phosphorylation sites. In the tail of the cytoplasm, there are two tyrosine-based immunoreceptor inhibitor motifs (ITIMs). The interaction between SIRP-α and CD47 is mediated by the N-terminal of SIRP-α and a single Ig-V domain of CD47.

(3) Regulation of CD47: CD47 expression is regulated at different levels. First, transcription factors such as Myc, hypoxia-inducing factor-1 (HIF-1), and nuclear respiratory factor-1 (NRF-1) enhance the expression of nuclear factor κB (NF-κB) CD47. In addition, cytokines such as tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), and interleukin can enhance CD47 expression. In contrast, various microRNAs and long non-coding Rnas (lncRNAs) have negative regulatory effects on CD47 [16, 17]. At the post-translational level, pyroglutamylation and glycosylation of CD47 have been studied intensively. Lautenberg *et al*., Shana *et al*., and our group reported that CD47 is a substrate of QPCTL, and the N-terminal region of CD47 is pyroglutamated. QPCTL catalyzes the pyroglutamylation of CD47. This modification is essential for the recognition of CD47 by SIRP-α and contributes to their interaction.

4.2 Expression level of CD47 in glioma and its influence on invasiveness

The innate immune system plays an important role in resisting infection and inhibiting malignant transformation of cells. Antigen-presenting cells such as monocytes, dendritic cells and macrophages play an important role in the innate immune system by capturing and eliminating malignant transformed cells through phagocytosis. More importantly, antigen-presenting cells can act as a "bridge" to present tumor-derived antigens to T cells, activate T cells, and thus activate the downstream acquired immune system [18]. However, tumor cells often promote autoimmune escape by overexpressing immune checkpoint proteins. Integrin-associated proteins (CD47), a class of highly glycosylated five-time transmembrane proteins, are often overexpressed in hematologic or solid tumors, enabling tumor cells to evade surveillance by the innate immune system. CD47 binds to and activates signal regulatory protein a (SIRPa), an inhibitory molecule expressed in myeloid cells such as macrophages. Activated SIRPa initiates a series of signals that inhibit phagocytosis of macrophages. Therefore, CD47 acts as an anti-phagocytic or "don't eat me" signal to protect the corresponding cell from its elimination by phagocytic cells. At present, the use of CD47 blocking antibodies for tumor treatment has entered the stage of clinical trials.

In solid tumors, CD47 monoclonal antibody also has a good therapeutic effect, such as in breast cancer, ovarian cancer, bladder cancer, liver cancer and other tumors in vitro experimental studies, CD47 monoclonal antibody can significantly inhibit tumor growth and metastasis, and prolong survival. Glioma is the most common intracranial tumor, the incidence of glioma has been increasing year by year in recent years, especially in the elderly population. The prognosis of glioma patients is poor, easy to relapse, and the fatality rate is high. The main reason is that glioma invasiveness makes it easy to relapse and stubborn. In this study, the expression of CD47 in glioma tissue cells was compared with that in normal brain specimens and astrocytes, and it was found that CD47 was highly expressed in glioma cells and the expression of CD47 was most obvious in U87. By interfering with the siRNA of CD47 (CD47-siRNA), CD47 cells were prevented from transcription and translation, and CD47 of U87 cells was knocked out. It was found that the knockdown of CD47 significantly inhibited the invasion of U87. The silencing of CD47 reduced the apoptosis, proliferation and migration of glioma cells, and inhibited the growth of tumor cells in xenografted mice by inducing apoptosis of glioma U87 cells, which became a very important indicator to judge the degree of tumor cell activity. In this study, pcDNA3.1-3xFlag-CD47 plasmid was transfected into T98G cells, and then Western Blott and qRT-PCR were used to confirm the excessive expression of CD47 in T98G cells. By comparison, it was found that excessive CD47 expression significantly promoted the aggressiveness of T98G cells.

The data suggest that CD47 expression levels enhance the aggressiveness of glioblastoma by activating the PI3K/AKT signaling pathway. PI3K/AKT is one of the most important signaling pathways in intracellular signal transduction pathways, playing an important role in regulating cell growth, motility, survival, proliferation, protein synthesis, autophagy, transcription and angiogenesis. The pathway consists of three main driving molecules: P13 kinase (P13K), serine/threonine kinase (AKT), and mammalian target of rapamycin (mTOR). For astrocytoma, the PI3K/AKT pathway induces glycolysis by regulating the location of glucose transporter Glut1 on the cell surface and activates hexokinase, which phosphorylates the IP3 receptor of AKT as an enzyme in glycolysis and inhibits the apoptosis of U87 cells. AKT also regulates various cellular responses to mTOR in gliomas. mTOR is an atypical

serine, which is mainly involved in the regulation of cell growth, survival, and movement.

5. Conclusion

In short, the results of this study indicate that CD47 is highly expressed in glioma cells, and CD47 enhances the aggressiveness of glioma by activating the PI3K/AKT signaling pathway, and CD47 is an important predictor and therapeutic target for glioma.

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