CD47 is highly expressed in gliomas and targeting CD47 is a promising therapeutic strategy

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Abstract
Gliomas are very malignant brain tumors that are difficult to treat. CD47 is an antiphagocytic molecule that binds to SIRPα on phagocytes. It is overexpressed on the plasma membranes of multiple human tumor cell types and is an important diagnostic and prognostic biomarker in many types of cancer. However, the association between CD47 protein expression in glioma tissue and clinicopathological stage has not been investigated in detail. A total of 80 surgical glioma specimens were stained with anti-CD47 antibody to assess the relationship between CD47 protein expression and clinicopathological stage of the glioma. Wound healing assays were performed to analyze the influence of CD47 on the migration and invasion of glioma cells, and near-infrared fluorescence localization assays in a U-87 MG-bearing xenograft model were used to determine the distribution of anti-CD47 antibody in vivo. MTT assays and administration of anti-CD47 to a U251-bearing xenograft model were used to analyze the inhibitory effects of the antibody on gliomas. CD47 expression was higher in high-grade gliomas than in low-grade gliomas, and high CD47 expression was positively correlated with histology and tumor clinicopathological stage. CD47 over-expression promoted the growth and motility of two glioma cell lines (U-87 MG and U251) and a laboratory-developed anti-CD47 antibody accumulated at the glioma site. Proliferation of U251 and U-87 MG cells was not significantly inhibited by the anti-CD47 antibody in vitro, but the antibody significantly inhibited U251 growth in vivo. It also enhanced inhibition capacity by Taxol. Our results suggest that CD47 plays a critical role in the progression of gliomas from stage I to IV and may be a potential target for the treatment of gliomas. CD47 appears to play a critical role in the progression of gliomas from stage I to IV and an anti-CD47 antibody prepared in the laboratory may inhibit the growth of gliomas.

Keywords
CD47, combination, gliomas, immunohistochemical staining, Taxol

Introduction
Gliomas are the most common primary malignant solid brain tumors, with an annual incidence of 5.26 per 100,000 population.1,2 Their incidence is significantly higher in males than females and
increases with age. Both genetic and environmental factors increase the risk of developing brain tumors. Gliomas are difficult to treat and their prognosis is generally poor.3,4

CD47 is a 50 kDa transmembrane glycoprotein that binds to SIRPα, which is expressed on various phagocytic cells such as dendritic cells and macrophages, and generates the signal “don’t eat me.”5,6 CD47 inhibits macrophage phagocytosis and its expression helps cells evade innate immunity. It is strongly expressed in human and murine malignancies such as melanoma, leukemia, gastric cancer, bladder cancer, astrocytoma, and prostate cancer. CD47 expression is correlated with the progression, metastasis and outcomes of tumors, and increased CD47 expression in some solid tumors is associated with poor survival.7–10 Willingham et al.10 reported the correlation of CD47 expression and survival of glioma patients. Blockade of CD47 significantly improves antitumor responses and enhances T-cell mediated antitumor immunity. However, the association between CD47 protein expression and glioma clinicopathological stage is unclear.11,12

Chemotherapy remains an important strategy for treating solid cancers.13,14 Paclitaxel (PTX), a compound extracted from the bark of Taxus chinensis, is the best microtubule-stabilizing drug; it has significant anti-cancer activity and has been approved by the FDA for the treatment of various cancers.15,16 However, it can cause a variety of severe side effects because of a lack of specificity and its efficacy is restricted. Therefore, new drug combination that improve the anti-cancer activity of PTX and reduce adverse reactions are needed.17,18

In the present study, we investigated the expression of CD47 in gliomas and its association with clinicopathological features. We also explored the impact of CD47 overexpression in glioma cells, and carried out a preliminary examination of the effects on glioma cells of an anti-CD47 antibody, developed in our laboratory, alone or in combination with PTX.

Materials and methods

Materials

Patient specimens. The study material are paraffin-embedded tumor tissue samples from the patients diagnosed with gliomas. We collected all gliomas specimens from our hospital between 2013 and 2018 and totally 80 cases. A total of 80 patients consist of 44 males and 36 females, and their mean age are 58 ± 20 years. The study was approved by the Ethics Committee of the People’s Hospital of Lan Ling, Shandong. All patients signed informed consent forms and agreed to publication of this study. The gliomas had all been staged and pathological diagnoses had been made, before collection of the patient specimens.

Anti-CD47 antibody. The anti-CD47 antibody used in these experiments was prepared, screened, identified, and purified by the National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing. The antibody is a mouse monoclonal antibody from the hybridoma 6B9-1E12, IgG1 isotype, with known binding and blocking activities (unpublished data).

Cell lines and other materials. The cell lines U251 and U-87 MG were obtained from the ATCC. Taxol was purchased from MedChemExpress. Anti-his antibody and anti-β-actin antibody, IgG1 isotype and immunohistochemical reagents were purchased from Beijing Zhongshan Jinqiao Biological Technology Co.

Methods

Immunohistochemistry (IHC) and assessment of IHC staining. Tissue sections (5 μm) were de-paraffinized in xylene and hydrated in decreasing concentrations of ethanol. Antigen was retrieved by heating for 30 min in citric acid buffer solution. Peroxide 3% was used to block endogenous peroxidase and normal goat serum was used to block non-specific binding in tissue sections. Primary anti-CD47 antibody (10 μg/mL) was added and sections were incubated at 4°C overnight. They were then incubated with HRP-conjugated secondary antibody (Jackson ImmunoResearch) for 1 h at room temperature, stained with DAB and visualized by light microscope.

Levels of immunostaining were evaluated blind by two researchers according to the proportion and intensity of CD47 positive stained cells. Weak staining was light yellow and there were 10% or less positive cells, moderate staining was yellow and involved 11%–50% positive cells, strong staining was yellow brown and involved 50%–70% positive cells, and very strong staining was brown and involved 70% or more positive cells.
**Statistical analysis.** Images of five randomly-selected fields of immunohistochemically-stained sections were captured. Levels of staining with CD47 antibody were assessed and scored based on the percentages of positive cells and intensities of staining with Image J. The specimens were divided into four groups according to clinical diagnoses based on WHO pathological grading criteria. The average values of CD47 staining intensity of specimens of each pathological grade were compared by ANOVA. Statistical significance was accepted at $p < 0.05$.

**Cell proliferation assays.** U251 and U-87 MG cells were seeded at 4000 cells/well in 96-well plates and incubated with anti-CD47 antibody diluted in two-fold steps from 10 to 0.625 μg/mL. After 48 or 72 h, relative cell numbers were determined with a modified MTT assay. MTT solution was added to each well followed by incubation for 4–6 h at 37°C; the reaction was then stopped with lysis buffer and absorbance was read the following day at 570 nm in an MD5 microreader (MD Corp.).

**Cell growth and migration assays.** 30 μg CD47 expression plasmid or control plasmid was introduced transiently into U-87 MG or U251 cells cultured in 10 cm dishes with Lipofectamine® 3000 according to the manufacturer’s instructions (Thermo Fisher Scientific), and at 18 h after transfection the CD47-transfected cells and control plasmid-transfected cells were seeded into 96-well plates at a density of $1 \times 10^3$ cells. After 24 h, cell proliferation was determined with a modified MTT assay. A wound healing assay was used for evaluating the migration of human glioma cancer cell lines. Briefly, $5 \times 10^6$ cells were inoculated into 6-well plates. After the cells reached 90% confluence, they were scraped off an area with a 200 μL tip, washed and incubated further for 48 h. Wound widths were observed and photographed by microscope. The expression of transfected CD47 plasmid was detected by western blotting. For this purpose, $1 \times 10^6$ CD47 transfected cells or control plasmid-transfected cells were lysed with RIPA (Radioimmunoprecipitation assay buffer) protein lysis buffer containing 150 mmol/L NaCl, 1 mmol/L EGTA, 50 mmol/L Tris-HCl (pH 7.4), 1% NP-40, 1× protease inhibitor cocktail (Thermo Fisher Scientific, A32963). Clarified lysates were fractionated by SDS-PAGE, transferred to nitrocellulose membranes, incubated with anti-his tag antibody overnight at 4°C and detected with HRP-conjugated secondary antibodies. The blots were also probed with β-actin antibody.

**In vivo imaging studies of anti-CD47 antibody.** Six-week-old nude mice were purchased from Beijing Vital River Laboratory Animal Technology. The mice received a total of $5 \times 10^6$ U-87 MG cells by inoculation subcutaneously in the right flank. After the tumors reached about 100 mm³, 100 μg Cy7-labeled anti-CD47 antibody was injected into the tail veins of nude mice and total three mice were injected for observing the distribution of fluorescence in mice at different times. The mice were anesthetized with isoflurane and images were captured by X-ray and fluorescence imaging after 6, 12, and 24 h, and overlaid according to the instructions of the manufacturer. The mice were handled during daylight hours. All experiments and protocols involving mice were approved by the Institutional Animal Care and Use Committee of Beijing Normal University and in accord with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

**FACS analysis.** U251 and U87-MG cells were suspended at $1 \times 10^6$/mL; anti-CD47 antibody or Ig G isotype control was added and the cells were incubated for 1 h at 4°C. After 3 washes with PBS, PE-labeled secondary antibody was added for 30 min at room temperature. The stained cells were washed and analyzed with a FACSCalibur™ flow cytometer (Becton-Dickinson, USA). Data were analyzed with CellQuest Pro software (BD Biosciences, USA).

**The U251-bearing BALB/c nude mouse model and anti-CD47 administration.** 6–8 week-old female BALB/c nude mice were used for anti-CD47 antibody administration. A U251 cell suspension ($5 \times 10^6$ cells) was transplanted into the right armpit of each mouse. On the eighth day after inoculation the mice were randomly divided into two groups of six mice each, receiving daily intraperitoneal injections (0.2 mL) of IgG1 isotype control and anti-CD47 antibody, respectively, at 40 mg/kg each. Tumor volumes were measured and calculated using the formula $(length \times width^2)/2$ every 2 days starting 1 week after tumor challenge. After 10 days, the tumors were removed and weighed.
Results

**CD47 expression in the tumor tissues of glioma patients.** We collected 80 glioma tissue specimens to evaluate CD47 protein expression and these specimens had been classified into different clinicopathological stages according to the size and the scope of glioma tumor by a clinical pathologist. About 75% of the specimens were from patients with grade II or III clinical diagnoses, and 47 specimens were from male patients and 33 from female patients. The average patient age was 58 years. CD47 expression was evaluated via IHC with anti-CD47 antibody. As shown in Figure 1, A-D, CD47 expression in stage I patients was rare and weak (Figure 1(a)), and the frequency and intensity of CD47-positive cells gradually increased with clinicopathological stage. CD47 expression in stage IV patients was very strong and the fraction of CD47-positive cells increased further (Figure 1(b)–(d)). CD47 was mainly localized at the cell membrane, with some expression in the cytoplasm (Figure 1(e)).

**Association between CD47 expression and clinicopathological stage.** CD47 expression at successive clinicopathological stages was analyzed by image J. The results indicated that expression increased with stage. Stage I patients had lower CD47 levels, averaging 1.48 ± 0.65. Stage II patients had higher CD47 levels, averaging 1.66 ± 0.65. The average CD47 level of patients at stage III was 1.79 ± 0.41 and the CD47 level of the patients at stage IV was the greatest, averaging 1.94 ± 0.31. There was a statistically significant difference between Stage I and II with a p value of 0.013 by ANOVA, in terms of average value of CD47 staining extent. Stage IV tumors were stained with some extent difference trend compared to stage III tumors with a p value of 0.053 by ANOVA. The data show that CD47 expression is positively correlated with glioma TNM stage (Table 1).

**CD47 promotes the growth and migration of glioma cells.** To see whether CD47 was expressed by the cells of glioma cell lines, we examined U251 and U-87 MG cells by living cell FACS using anti-CD47 antibody. The results showed that CD47 was highly expressed on the plasma membranes of these two cancer cell lines (Supplemental Figure 1A and B). To assess the effect of CD47 on growth of the glioma cells, we determined the impact of CD47 overexpression on their growth. The results showed that CD47 overexpression increased cell growth approximately two-fold (Figure 2(a) and (b)). We also analyzed the impact of CD47 over-expression on glioma cell migration. Migration assays showed that CD47 over-expression significantly enhanced the migration of the U-87 MG and U251 cancer cells (Figure 2(c) and (d)). Expression of the transfected CD47 plasmid in these cells was confirmed with anti-his tag antibody (Figure 2(e)).

**Anti-CD47 antibody accumulates at glioma tumor sites.** CD47 is highly expressed on the plasma membrane of glioma cells. To examine the accumulation of anti-CD47 antibody at tumor sites, the
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Fluorescent dye-Cy7-labeled anti-CD47 antibody was injected intravenously into U-87 MG tumor-bearing nude mice and fluorescence intensities were captured in the mice with time. Fluorescence clearly accumulated in the tumors (Figure 3(a)) and ex vivo-fluorescence images of excised tumors confirmed this accumulation (Figure 3(b)). These results indicated the anti-CD47 antibody accumulates in glioma cells.

**Table 1.** Association between clinicopathological stage and CD47 expression in glioma tissue.

| Groups   | Cases | Age  | Male/female | Mean CD47 value | p Value |
|----------|-------|------|-------------|-----------------|---------|
| I Grade  | 12    | 28–48| 7 cases/5 cases | 1.48 ± 0.34 | p = 0.013 |
| II Grade | 32    | 36–58| 17 cases/15 cases | 1.67 ± 0.45 | p = 0.053 |
| III Grade| 28    | 50–74| 16 cases/12 cases | 1.79 ± 0.41 | p < 0.001 |
| IV Grade | 8     | 60–74| 4 cases/4 cases | 1.94 ± 0.31 | p < 0.001 |

**Figure 2.** CD47 promotes the growth and migration of U-87 MG and U251 cells. Cell growth was analyzed by a modified MTT assay (a and b). The migration of U-87 MG and U251 cells was analyzed with the wound healing assay (c and d). Briefly, the CD47 plasmid and control plasmid were used to transfect U-87 MG and U251 cells. After 48h, cell growth and migration were measured and the width of the healed zones were assessed by microscopy. Expression of the transfected CD47 plasmid was detected with anti-his tag antibody (e).

**Anti-CD47 antibody inhibits the proliferation of glioma cells.** We used MTT assays to assess whether anti-CD47 antibody inhibits the proliferation of glioma cells in vitro. The proliferation of U251 and...
U-87 MG cells was not significantly inhibited after 48 h of incubation compared to control cells (Figure 4(a) and (b)). However the U251 cells were sensitive to the culture environment such that the addition of more anti-CD47 antibody or control Ig G slowed their growth compared to PBS group (Figure 4(b)). We also assessed the anti-tumor activity of anti-CD47 antibody against U251 cells in vivo. U251 cell suspensions were transplanted into the right armpits of BALB/c nude mice. After 8 days, the mice received intraperitoneal injections (0.2 mL) of 40 mg/kg Ig G control or anti-CD47 antibody every 48 h. After 10 days of treatment, tumor volumes were lower in the anti-CD47 group than in the Ig G control group (Figure 4(c)). After the last injection, the tumors were isolated and weighed, and the weight of the anti-CD47-treated tumors was 30% lower than that of the control group (Figure 4(d) and (e)). It is likely that the anti-CD47 antibody inhibits glioma cell growth in vivo but not in vitro because the antibody exerts its anti-tumor effect by blocking the “don’t eat me” signal of the tumor cells.

**Anti-CD47 antibody enhances the anti-tumor effect of Taxol on glioma cells.** The growth inhibitory effects of PTX (Taxol) and anti-CD47 antibody on U251 cells and U-87 MG cells were analyzed by MTT assay. Cells were incubated with different concentrations of PTX or anti-CD47 alone for 48 h. PTX inhibited the growth of the U251 and U-87 MG cells in a dose-dependent manner, while the anti-CD47 antibody had no obvious effect on the growth of these cells at these concentrations; however the combination of PTX and anti-CD47 antibody inhibited the growth of these cells significantly more than PTX alone (Figure 5).

**Discussion**

CD47 is widely expressed in normal tissues and highly expressed in various cancers. The pathological effect of CD47 is related to the escape of tumors from immune surveillance and CD47 over-expression is associated with a poor prognosis in cancer patients. Until now, although the role of CD47 has been studied in a number of cancers, the association between clinicopathological stage and CD47 expression has not been examined in gliomas. In the present study, we collected all gliomas specimens in our hospital between 2013 and 2018 year, but 75% gliomas patients in our hospital were with grade II or III clinical diagnoses and relatively few cases were with grade I and grade IV clinical diagnoses. It is a limitation to explore the association between clinicopathological stage and CD47 expression, but the results indicated the expression of CD47 increased with increasing grade of glioma, and to some extent they were associated with clinicopathological stage.

Up-regulation of CD47 protein expression was associated with clinicopathological stage in glioma patients’ specimens. We also examined the influence of CD47 protein over-expression on the glioma cell lines. CD47 protein over-expression significantly promotes the growth and migration of glioma cells.

It seems possible that when anti-CD47 monoclonal antibody blocks CD47 binding to SIRPα, the “do-not-eat-me” signal is disrupted, and solid tumors are cleared by phagocytosis. Over-expression of CD47 protein in gliomas showed it was possible...
to develop anti-CD47 therapeutic antibody for gliomas therapy. Numerous pharmaceutical companies are researching and developing anti-CD47 blocking antibodies with this possibility in mind. Sharareh et al. have reported that a humanized anti-CD47 antibody caused a significant reduction in growth of gliomas and Kim et al. observed that an anti-CD47 monoclonal antibody significantly inhibited the growth of human myeloma cells. In this study, we showed that a new mouse anti-CD47 monoclonal antibody clearly inhibited the growth of glioma cells, but volumes or weights of the gliomas in the group receiving anti-CD47 antibody were reduced by only 30%-40% compared to the IgG₁ isotype controls. The reason for this is that we treated the mice for only 10 days using 100 μg anti-CD47 monoclonal antibody every 48 h. In addition, the anti-CD47 monoclonal antibody was obtained and...
purified from the mouse ascites, and some mouse antibodies are present in the mouse ascites. To enhance glioma inhibition, we should express and purify the monoclonal antibody in CHO cells, and administer a larger dose.

The targeting effect of the anti-CD47 monoclonal antibody was also detected. The antibody obviously accumulated in the tumors, but the fluorescence intensity was relative weak. The reason of this is that we injected less Cy7-labeled anti-CD47 antibody. The fluorescence intensity could be improved by injecting more Cy7-labeled antibody or labeling more Cy7 into the antibody.

We detected an additive effect of anti-CD47 antibody in combination with PTX (Taxol) on the growth of U-87 MG and U251 cells over 48 h: anti-CD47 antibody significantly enhanced the inhibitory effect of PTX. We also observed that anti-CD47 antibody accumulated at U-87 MG tumor sites. Gliomas are located in the brain, and complete antibody molecules cannot easily cross the blood brain barrier. Therefore targeting CD47 with anti-CD47 mini-antibody in combination with Taxol or other chemotherapeutic drug to form Antibody Drug Conjugates (ADC) or nanomedicines could be a promising therapeutic strategy for treating gliomas.18,28

Conclusions
This investigation demonstrated that CD47 plays a critical role in the progression of gliomas from stage I to IV. CD47 could be a reliable biomarker for diagnosing and assessing the prognosis of gliomas and might be a useful therapeutic target. The anti-CD47 monoclonal antibody used was developed in this laboratory and its therapeutic potential against gliomas will be further evaluated.

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Animal welfare
The present study followed international, national, and institutional guidelines for humane animal treatment and complied with relevant legislation. The animals of the present study were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. These animals had been demonstrated with a high standard of veterinary care and involved informed client consent.

Availability of data and materials
The data used and analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval and consent to participate
The study was approved by the Ethics Committee of the People’s Hospital of Lan Ling, Shandong. All participants had signed the informed consent.

Ethics approval
Ethical approval for this study was obtained from Beijing Normal University (CLS-EAW-2020-023).

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Informed consent
Written informed consent was obtained from all subjects before the study. The tissue samples of all subjects were from the surgical specimens resected as gliomas. After the surgery had performed, the patients have agreed their specimens could be used as scientific studies in their case files. In the study, we informed the patients or their relatives again and re-signed the informed consent on a piece of paper.

Patient consent for publication
All patients had allowed to publish the study.

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Supplemental material
Supplemental material for this article is available online.

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