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*Original Article*

# CD300A inhibits tumor cell growth by downregulating AKT phosphorylation in human glioblastoma multiforme

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**Abstract:** Glioblastoma multiforme (GBM) is a primary malignant tumor of the central nervous system with the highest incidence and dismal prognosis. As a member of the CD300 glycoprotein family, CD300A plays a role in cell proliferation, apoptosis, differentiation, and immune response, but its role in solid tumors remains unknown. In this study, CD300A was observed to be overexpressed in human GBM samples using real-time PCR and western blotting. To investigate the role of CD300A in GBM, CCK8, transwell and flow cytometry analysis were performed to examine the proliferation, migration and apoptosis in GBM cell lines, respectively. From our results, knockdown of CD300A blocks cell proliferation and migration, and induces cell apoptosis in human GBM cells U251MG and U87MG. Further, we assessed AKT expression level in CD300A knockdown and negative control cells. The phosphorylation level of AKT was significantly suppressed in CD300A knockdown cells in comparison to negative control cells, suggesting that CD300A promoted tumor cell growth through the AKT pathway. In conclusion, our findings expand the knowledge of CD300A as an oncogene in solid tumor, and provide experimental and theoretical basis for further clinical application.

**Keywords:** CD300A, glioblastoma multiforme, proliferation, apoptosis

## Introduction

Glioblastoma multiforme (GBM) is the most malignant and common primary tumor in the central nervous system. GBM is characterized by infiltrating growth and is not clearly defined from the surrounding brain tissue, so it is rather difficult to cure [1]. In addition, most GBM patients inevitably relapse after receiving multimodal treatments, the 5-year survival rate of which is only 9.8% [2]. At present, the treatment of GBM is still based on surgical resection, supplemented by chemotherapy and radiotherapy [3]. In spite of the great progress of tumor treatment in human GBM, the overall prognosis of the patients has not been significantly improved. Accumulating evidence has shown that the investigation of the pathogenesis of GBM is useful to search for effective therapeutic targets, as a means to improve the cure rate of GBM [4, 5].

CD300A, also known as IRp60, is a member of the CD300 glycoprotein family, functioning as a transmembrane glycoprotein receptor type I located on the surface of the cell membrane [6, 7]. Abnormal expression of CD300A has been observed in various blood cells, such as NK cells, neutrophils, monocytes, T lymphocytes, and B lymphocytes [8-11]. Based on research on several blood diseases, CD300A is posited as a player in cell proliferation, apoptosis, differentiation and immune response [12, 13]. However, CD300A, as an immune receptor tyrosine-based inhibitory motif (ITIM), is less studied in tumors.

In the present study, we have found that CD300A was up-regulated in surgically resected GBM tissues, and predicted prognosis of glioma patients by bioinformatics analysis based on the TCGA dataset. Moreover, inhibition of CD300A blocked cell proliferation and migration, and induced apoptosis in human glioma

cells *in vitro*. Our findings highlight the positive role of CD300A on tumor growth and suggest that it can be a promising anticancer target in human GBM.

## Materials and methods

### *Patient characteristics and sample collection*

We retrospectively selected 31 patients (male: female = 17:14) with histologically confirmed GBM who underwent surgical resection at Shandong Provincial Hospital affiliated to Shandong University between September 2014 and October 2016. Normal brain tissue samples were obtained from 5 patients with spontaneous intracranial hemorrhage.

Written consent to use stored specimens was obtained from all living patients. The study was approved by the Research Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University. The surgical specimens were preserved at -80°C for later RNA and protein extraction.

### *Cell lines and cell culture*

Human GBM cell lines, U251-MG (U251) and U87-MG (U87), were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). U251 and U87 cells were cultured in DMEM medium with 10% fetal bovine serum, 100 units/mL streptomycin and 100 units/mL penicillin (Invitrogen Corp., Waltham, MA). U251 and U87 cells were incubated in a humidified atmosphere at 37°C with 5% $CO_2$, and transfected with CD300A specific siRNA (siCD300A) or negative control siRNA (NC) after reaching approximately 80% confluence.

### *Real-time quantitative PCR (qPCR)*

Total RNA was isolated from U251 and U87 after transfection with CD300A or NC siRNA for 48 h by Trizol reagent and Ultrapure RNA Kit (CWBIO, Beijing, China), followed by reverse transcribing by a Reverse Transcription Reaction Kit (CWBIO). mRNA levels for CD300A were evaluated by qPCR on an H-4800 Real-Time PCR System as per the manufacturer's instructions. Primers against CD300A (5'-GTTGCTGCTTCTGTTGGTGG-3' and 5'-CTTGGTGGTGCTGGCTTTTC-3') and GAPDH (5'-CGGAGTCAACGGATTTGGTCGTAT-3' and 5'-AGCCTTCTCCATGGTGGTGAAGAC-3') were synthesized by GENEWIZ (Beijing, China).

### *Western blot and antibodies*

After transfection for 24 h, U251 and U87 cells were collected and lysed using RIPA buffer (CWBIO). Forty micrograms of total protein from each sample was separated on 10% SDS-PAGE at 80 V for 50 min, followed by being transferred to a PVDF membrane at 110 V for 90 min. The blots were blocked with 5% fat-free milk in TBS for 1 h at room temperature, and then probed with primary antibodies (1:1000) for 1 h and then secondary antibodies (1:5000) for 1 hour. Primary antibodies, CD300A (Abcam, Cambridge, UK), AKT (Proteintech, Rosemont, IL), p-AKT (Proteintech), p70 (Proteintech), Active-Caspase3 (Proteintech), Bax (Proteintech) and GAPDH (Proteintech) were used for western blot. Densitometry was analyzed using Image J software v1.8.0 (National Institutes of Health, Bethesda, Maryland), and protein levels were normalized to GAPDH. All assays were performed in triplicate.

### *Proliferation detection using CCK8 assay*

Cell Counting Kit-8 (CCK-8) was used to assess proliferation of U251 and U87 cells. Cells transfected with CD300A or NC siRNA were seeded into a 96-well plate at a concentration of 2000 cells per well. The OD values were measured as follows: 10 μL CCK-8 solution (Beijing Solarbio Science & Technology, Beijing, China) (10 μL/well) was added to each well and maintained at 37°C for 2 h. Then the 96-well plate was read on a microplate reader to obtain the OD value. The OD values were measured every 24 hours, and each experiment was performed in triplicate.

### *Transwell assay*

Transwell assay was performed to assess cell migration ability in accordance with the manufacturer's instructions. Cells transfected with CD300A or NC siRNA were seeded at a density of $5\times10^4$ cells/well into the upper chamber (Merck Millipore, Darmstadt, Germany) in a 24-well plate. In addition, DMEM medium with 10% FBS was added into the lower chamber. After incubated for 48 h, cells were washed with PBS and stained with crystal violet for 10

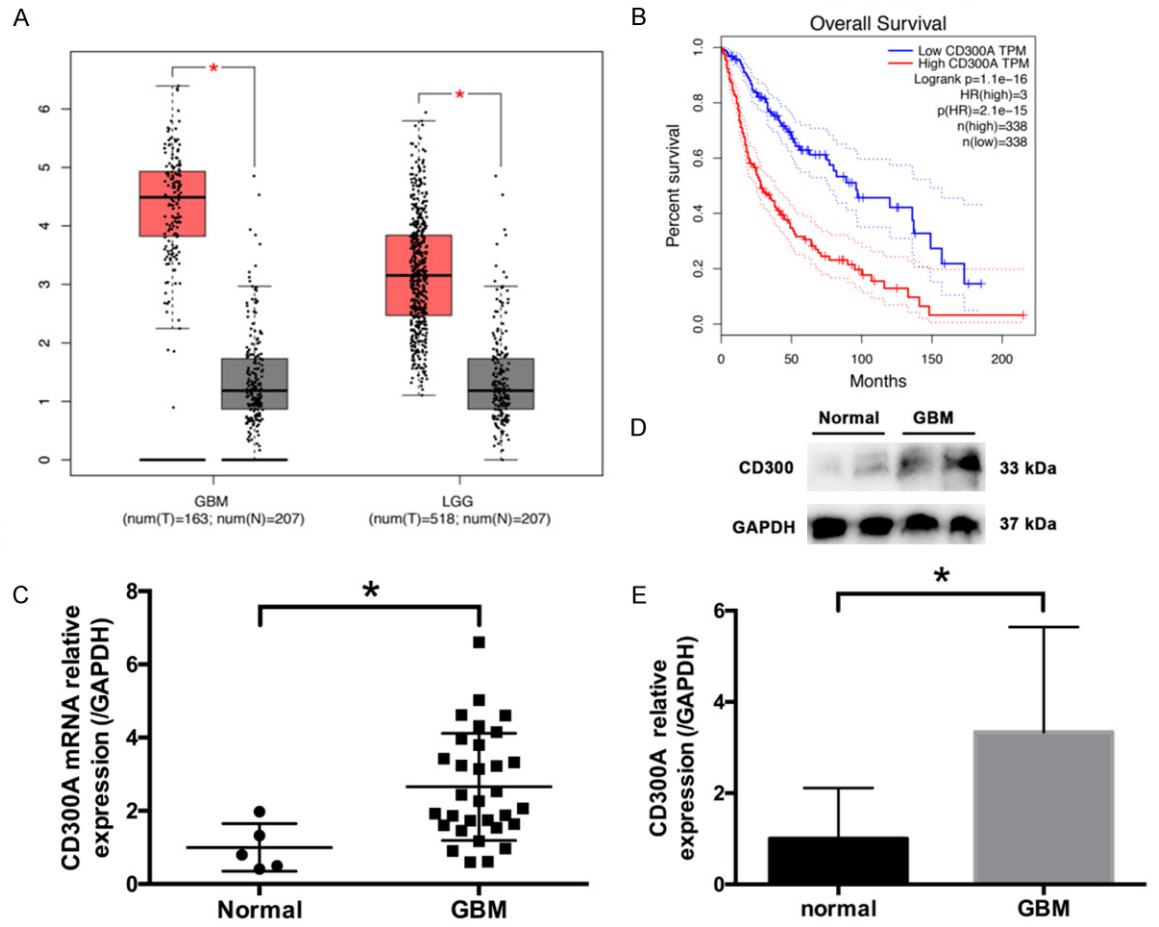


**Figure 1.** Analysis of CD300A expression and prognostic role in human GBM tissues. Bioinformatic analysis of CD300A was performed using TCGA database and GEPIA online dataset. A. The red and gray boxes represent tumor and normal tissues respectively. The genomics and clinical profiles were downloaded from Genotype-Tissue Expression (GTEx) and the Cancer Genome Atlas (TCGA). B. The survival curve was plotted in glioma patients, which were divided into a CD300A high expression group ($n$ = 338) and low expression group ($n$ = 338). CD300A mRNA and protein overexpression was also confirmed in frozen GBM tissues ($n$ = 31) compared with in normal brain tissues ($n$ = 5). C. Real-time PCR results showed that CD300A mRNA was upregulated in GBM tissues; D and E. Western blot indicated that CD300A protein expression in GBM was more than in normal brain tissues. $^{*}P < 0.05$.

min. Then photomicrographs were captured by a microscope, and migrated cells were calculated. Each experiment was performed in triplicate.

*Apoptosis detection using flow cytometry analysis*

Apoptosis was assessed by staining with annexin v-FITC and propidium iodide (PI) as per the manufacturer's instructions. After transfection for 48 h, U251 and U87 cells were starved for 24 h with being cultured in serum-free medium. Then cells were harvested and suspended at a concentration of 1-5×$10^6$ cells/mL, followed by incubation with Annexin V-FITC/PI for 5 min in the dark. Cell samples were measured using flow cytometry and analyzed using Flowjo software v10.4.2 (BD Biosciences, San Jose, CA). Each experiment was performed in triplicate.

*Statistical analyses*

Statistical analyses were performed using SPSS v19.0 (SPSS Inc., Chicago, IL, USA) and presented as mean ± SD. Student t test was used to compare the difference of CD300A mRNA and protein levels between normal brain tissues and GBM. The statistical significance of difference between NC and siCD300A group was calculated using the Student $t$-test. Results

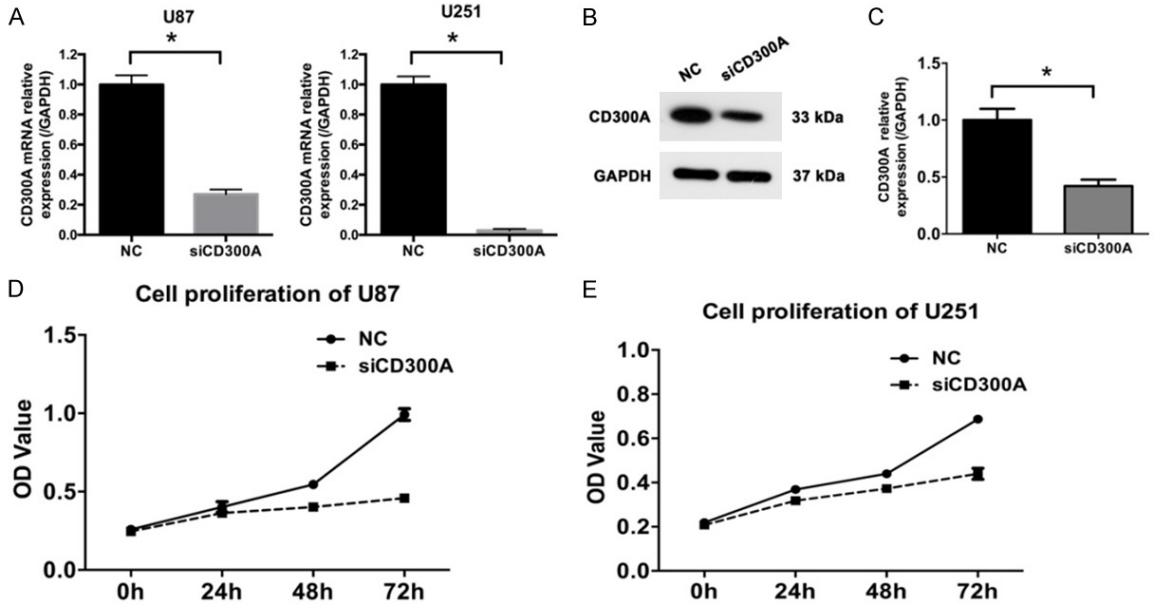


**Figure 2.** Inhibition of CD300A blocked cell proliferation in human GBM cell lines. CD300A-specific siRNA was transfected into U87 and U251 cells to build the knockdown cell line (siCD300A) for subsequent research. From results of qPCR (A) and western blot (B and C), CD300A expression was significantly suppressed in siCD300A cells in comparison to that in NC cells. CCK8 assay was performed to confirm proliferation of U251 (D) and U87 (E) cells. After transfection for 72 h, OD value of CD300A suppressed cells decreased statistically significantly, suggesting the inhibition of CD300A blocked proliferation of glioma cells *in vitro*. $^{*}P < 0.05$.

were reported as being statistically significant if $P < 0.05$.

## Results

### *Overexpression of CD300A was observed and associated with poor prognosis in human glioma through bioinformatics analysis*

With the development of sequencing technology, large-scale cancer genomics projects are performed, and bioinformatics analysis is becoming an effective means for oncology research. Gene Expression Profiling Interactive Analysis (GEPIA) is specifically designed to assess cancer and normal gene expression profiling and interactive analyses, including differential expression analysis and patient survival analysis [14]. In this study, we estimated overexpressed genes with unfavorable prognosis in GBM (tumor, 163 cases; normal, 207 cases) and brain low grade glioma (LGG) (tumor, 518 cases; normal, 207 cases), the genomics and clinical profiles of which were downloaded from Genotype-Tissue Expression (GTEx) and the Cancer Genome Atlas (TCGA) [15, 16]. From the data, we found that CD300A was upregulated both in GBM and LGG sample tissues (**Figure 1A**), and that high expression was associated with poor overall survival (**Figure 1B**) ($P < 0.05$).

Next, to confirm the CD300A expression in GBM tissues, we examined CD300A expression at transcriptional and translational levels in 31 GBM and 5 normal brain frozen samples using real-time PCR and western blotting. Real-time PCR results revealed that CD300A mRNA expression was upregulated in GBM tissues compared with normal brain tissues (2.66-fold, $t = 2.468$, $P = 0.019$, **Figure 1C**). Similarly, CD300A protein expression in GBM tissues was higher than in normal brain tissues (3.34-fold, $t = 2.208$, $P = 0.034$, **Figure 1D** and **1E**).

Thus, we predicted that CD300A may be involved in tumor progression of human glioma and worthy of further study.

### *Knockdown of CD300A inhibited proliferation of human glioma cells in vitro*

To elucidate the biological function of CD300A on glioma cells, we first built its knockdown cell lines of human glioma using U251 and U87 cells transfected with CD300A specific siRNA. **Figure 2A-C** show the interference efficiency

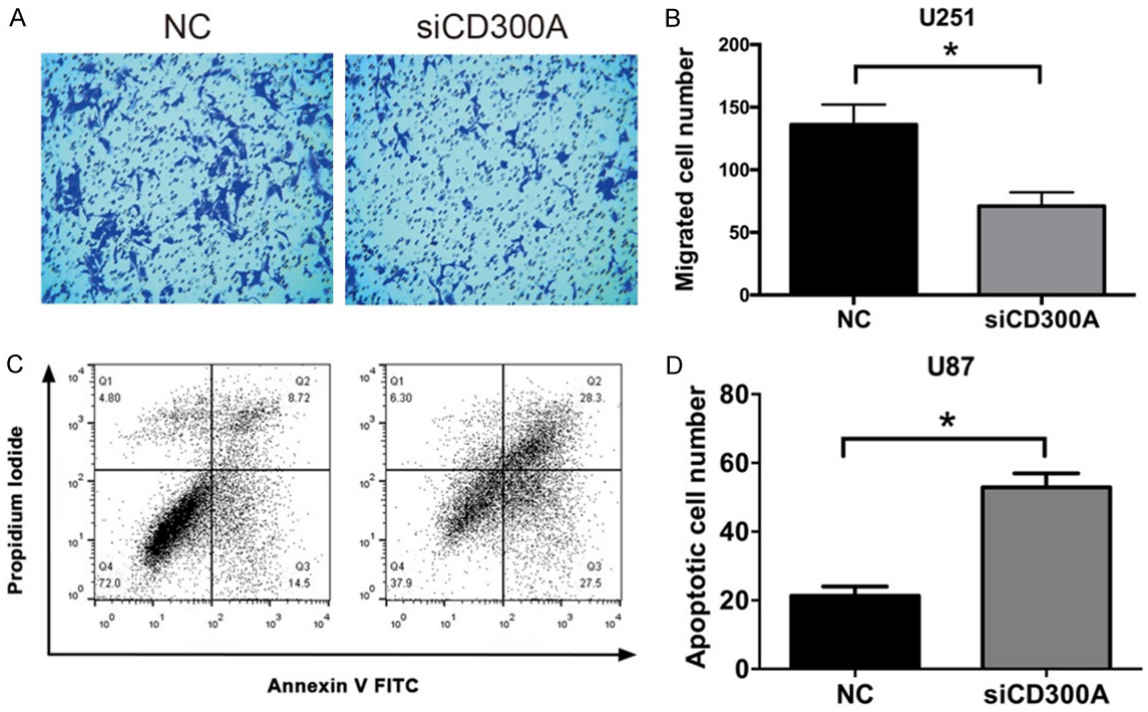


**Figure 3.** Inhibition of CD300A blocked cell migration and induced apoptosis in human GBM cell lines. A and B. Transwell assay was performed to detect cell migration ability, and migration cell number of the siCD300A group significantly decreased compared with the NC group; C and D. Cellular apoptosis was estimated using flow cytometry. Percentage of apoptotic cells in the siCD300A group increased to 52.91±2.89 in comparison to the NC group, 21.33±1.89. *$P < 0.05$.

confirmed by qPCR and western blot. We observed that CD300A expression was significantly suppressed in siCD300A cells in comparison to that in NC cells. These two groups were used for subsequent research. Then, we investigated the effect of CD300A on proliferation in human glioma cell lines, U251 and U87, using CCK8 assay. The results, as shown in **Figure 2D** and **2E**, indicated that after transfection for 72 h, the OD value of siCD300A cells decreased significantly compared with NC cells both in U251 and U87 cell lines, suggesting the inhibition of CD300A blocked proliferation of glioma cells *in vitro*.

*Knockdown of CD300A inhibited the migration of human glioma cells in vitro*

Next, we estimated the role of CD300A on migration of U251 cells using transwell assay. **Figure 3A** and **3B** show representative images and summary statistics for the migration assay, respectively. From these data, after transfection for 48 h, migrated cell number of siCD300A cells declined to 71±11, compared with 136±16 in NC cells. These results suggest that knockdown of CD300A suppressed the migration of glioma cells *in vitro*.

*Knockdown of CD300A induced apoptosis in human glioma cell lines*

Previous studies of CD300A have focused on its function of assisting tumor cells in immune escape through immune responses, but with less research on tumor cell apoptosis [17, 18]. In our study, we estimated cellular apoptosis of NC and siCD300A cells by using flow cytometry in the U87 cell line. In **Figure 3C** and **3D**, there was a clear trend of decreasing the percentage of apoptotic cells in the siCD300A group (52.91%±2.89%) in comparison to that in NC group (21.33%±1.89%) ($P < 0.05$). From these results, we predicted that inhibition of CD300A could promote cellular apoptosis in glioma cells. Moreover, we detected the expression of key apoptosis related proteins to further study the pathway by which CD300A mediates cell apoptosis. As shown in **Figure 4**, the expression of Active-Caspase3 significantly declined

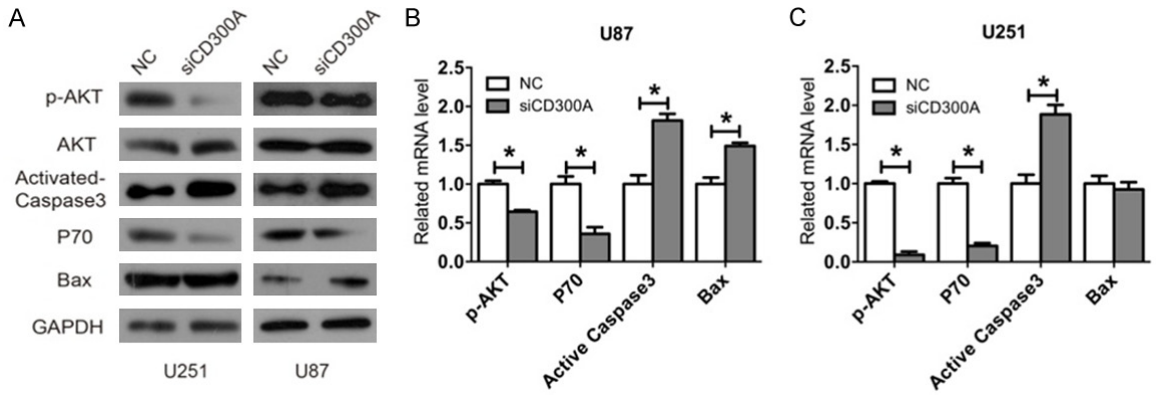


**Figure 4.** The mechanism by which CD300A contributes to tumor progression in human glioma. (A) Western blot was performed to confirm expression of apoptosis and signaling pathway-related genes. Knockdown of CD300A inhibited P70 expression and AKT phosphorylation, and promoted active-Caspase3 in U87 (B) and U251 (C) cells, suggesting that CD300A mediates biologic functions of human glioma cells through the AKT signaling pathway.

in CD300A-inhibited U251 and U87 cells, while Bax only declined in U87 cells compared with the NC group. The functional differences of CD300A between U251 and U87 cell lines suggested there might be a complex mechanism by which CD300A suppressed apoptosis, so more intensive study was needed.

*CD300A mediates biologic functions via the AKT signaling pathway in human glioma cells*

The next focus of this research was the signaling pathway by which CD300A was involved in cell growth regulation. AKT signaling pathway has a vital role in cell proliferation and apoptosis, and contributes to the origin and development of most malignant tumors [19]. So in this study, we examined the AKT phosphorylation level in siCD300A and NC cells by western blot. The result, as shown in **Figure 4**, was that knockdown of CD300A suppressed AKT phosphorylation in U251 and U87 cells, suggesting that CD300A mediates biologic functions of human glioma cells through the AKT signaling pathway.

## Discussion

The CD300 glycoprotein family is a transmembrane glycoprotein receptor on the cell surface, and contains 7 members [20]. The gene of the human CD300 glycoprotein family is located on 17q22-25 and named for its position on chromosome 17, CD300A-G [7]. CD300 family members share similar structure, containing an IgV-like domain outside the cell membrane, a transmembrane, and a section of cell membrane proximal extension sequence region, which is rich in proline, serine, and threonine. CD300A, as studied in our research is an important member of the CD300 family with a full length of 24 Kbp, including 8 exons, and encodes a protein of 60 Kda [6]. This member also shares the above structure. Compared with other members, CD300A has a long cytoplasmic tail region structure, and contains 4 immuno receptor tyrosine-based inhibitory motifs (ITIMs) domains, which can collect phosphatase SHIP, SHP-1 and SHP-2 after phosphorylation [21].

Much CD300A research has focused on identifying and evaluating its function on cells of the circulatory system. In view of related research, CD300A is expressed in mast cells, NK cells, neutrophils, eosinophils and basophils, and involved in the regulation of immune response. Abnormal activity of CD300A can cause a series of immune diseases, including infectious and autoimmune diseases [22, 23]. In recent years, the role of CD300A in blood system tumors has gradually been recognized. In diffuse large B-cell lymphoma (DLBCL), expression levels of CD300A were inversely correlated with prognosis. Decreasing levels of CD300A significantly suppressed proliferation of DLBCL cells and inhibited tumor formation in a xenograft animal model [13]. In two independent studies, CD300A overexpression was observed and it predicted prognosis in acute lymphoblastic leukemia (ALL) [24, 25].

In the present study we, for the first time, reported the role of CD300A in solid tumors. While studying prophase, we estimated overexpressed genes with unfavorable prognosis in human glioma samples using TCGA and GTEx database, and screened CD300A as a potential oncogene. The overexpression of CD300A in GBM at transcriptional and translational levels was confirmed using real-time PCR and western blot, compared with normal brain tissues.

Through the cell functional experiments, we confirmed that decreased expression of CD300A inhibited proliferation and migration, and induced apoptosis in U251 and U87 cells. Moreover, the decreased expression of CD300A suppressed the process of AKT phosphorylation, suggesting that CD300A mediated biologic functions through the AKT signaling pathway in human GBM cells. This result supported previous research that finds that the expression of p-AKT is significantly reduced while CD300A is down-regulated in DLBCL cells [13]. As a crucial molecule of the PI3K signaling pathway, AKT is observed to have continuous activation in a variety of tumors, including human GBM. Its activation has a vital role in promoting cell proliferation, migration and invasion, inhibiting cell apoptosis, inducing angiogenesis and resisting chemotherapy and radiotherapy [26, 27]. In gliomas, the PI3K/AKT pathway plays a significant role in tumor progression, metastasis, and drug resistance [28, 29]. In spite of a large volume of published studies describing the role of PI3K/AKT pathway, the mechanism of its abnormal activation in tumor remains unknown. Our study helps to improve the understanding of the activation mechanism of AKT pathway in tumors. In addition, although our data confirm CD300A as an oncogene in human GBM, but its function research in solid tumors is still in the primary stage, which deserves further study.

In conclusion, CD300A is overexpressed in human GBM samples, and its high expression predicts poor prognosis. Knockdown of CD300A blocks cell proliferation and migration, and induces cell apoptosis in human GBM cells by inhibiting the AKT signaling pathway, which is a classic antitumor pathway. Our research expands the understanding of CD300A functions in promoting solid tumor growth, and provides experimental and theoretical basis for further clinical application.

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## Disclosure of conflict of interest

None.

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