S100A11 Promotes Glioma Cell Proliferation and Predicts Grade-Correlated Unfavorable Prognosis

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Abstract
The prognosis of glioma is significantly correlated with the pathological grades; however, the correlations between the prognostic biomarkers with pathological grades have not been elucidated. S100A11 is involved in a variety of malignant biological processes of tumor, whereas its biological and clinicopathological features on glioma remain unclear. In this study, the S100A11 expression and clinical information were obtained from the public databases (TCGA, GEPIA2) to analyze its correlations with the pathological grade and the prognosis of glioma patients. We then verified the expression of S100A11 by immunohistochemistry staining. The effects of S100A11 on the proliferation of glioma cells were confirmed by cytological function assays (CCK-8, Flow cytometry, Clone formation assay) in vitro, the role of S100A11 in regulation of glioma growth was determined by xenograft model assay. We observed that S100A11 expression positively correlated with the pathological grades, while negatively correlated with the survival time of patients. In cytological analysis, we found the proliferations of glioma cell lines were significantly inhibited in vitro (P < 0.05) after interfering S100A11 expression via shRNAs. The cell cycle was blocked at G0/G1 stage. The ability of clone formation was significantly decreased, and the tumorigenicity in vivo was weakened (P < 0.05). In summary, S100A11 was over-expressed in gliomas and positively correlated with the pathological grades. Interfering the expression of S100A11 significantly inhibited the proliferation of glioma in vitro and the tumorigenicity in vivo (P < 0.05). In conclusion, S100A11 might be considered as a potential biomarker in glioma.

Keywords
S100A11, glioma, proliferation, prognosis, biomarkers

Abbreviations
BCA, bicinchoninic acid; CNS, central nerve system; DAB, 3-diaminobenzidine; DMEM, Dulbecco’s modified Eagle’s medium; ECL, enhanced chemiluminescence; FIGO, International Federation of Gynecology and Obstetrics; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HGG, high-grade glioma; IHC, immunohistochemistry; LGG, low grade glioma; NC, negative control; OD, optical density; PVDF, polyvinylidene difluoride; qRT-PCR, Quantitative real-time PCR; RAGE, Receptor for Advanced Glycation End Products; RIPA, radioimmunoprecipitation; SD, standard deviation; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; TCGA, Cancer Genome Atlas; WB, western blotting; WHO, World Health Organization.

Introduction
Glioma is the most common malignant tumor in central nervous system (CNS) of adults,1 accounting for about 50% of primary intracranial tumors.2 The World Health Organization (WHO) has classified gliomas into 4 grades according to the histopathological characteristics (2007 World Health Organization classification). Among that, low-grade glioma (LGG),

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including WHO I-II grades, grows relatively slowly and with a relative favorable prognosis, while high-grade glioma (HGG), including WHO III-IV grades, was proved to have the characters of rapidly progression, high rates of recurrence and mortality, consequently leading to a poor prognosis. Furthermore, due to the diverse aims and methods about the treatments in different grades of gliomas, their clinical prognosis was also significantly different. Thus, it is particularly necessary to explore biomarkers and potential therapeutic targets associated with gliomas.

S100A11, also named S100C or calgizzarin, is a member of S100 protein family with classical EF hand motifs. The biological functions of S100A11 are exerted after the protein conformation is changed by interacting with Ca$^{2+}$, leading to the exposure of the hydrophobic area which combines with its target proteins and inverts Ca$^{2+}$-dependent cellular regulatory signals. Previous reports have been demonstrated that S100A11 protein played important roles in various biological processes, such as tumor cell proliferation, apoptosis, signal transduction, cell adhesion, extracellular matrix remodeling and cell migration.

The over-expressions of S100A11 have been found in a variety of tumors, including clear cell renal cell carcinoma, papillary thyroid carcinoma, and cervical cancer. Additionally, S100A11 antibody neutralization was reported to be an effective method in controlling the progression of malignant pleural mesothelioma. S100A11 overexpression was considered to be a novel prognostic factor for the high-grade serous ovarian cancer, and was positively correlated with International Federation of Gynecology and Obstetrics classification (FIGO) stage. However, the biological and clinicopathological characters of S100A11 protein on glioma have not been fully elucidated.

In this study, we screened the S100A11 protein expressions in different glioma grades and analyzed the correlations of its expressions with patients’ outcomes in the Cancer Genome Atlas (TCGA) data cohort. Then the S100A11 expressions in both tumor and adjacent tissues of glioma patients were verified by immunohistochemistry (IHC) tests. The biological functions of S100A11 in proliferation, cell cycle, clonal formation and tumorigenesis of glioma cells were further investigated in vivo and in vitro. The aim of the study was to investigate the correlations of S100A11 protein expression with pathological grades of gliomas, and further to explore its biological function in gliomas.

Materials and Methods

Cancer Genome Atlas (TCGA) Data

We used a cohort (n=538) from The Cancer Genome Atlas (TCGA) in this study. The gene expression data, clinical significance and follow-up information of the patients were obtained from TCGA data portal (http://tcga-data.nci.nih.gov/tcga/) to identify the differential expressions of S100A11 and its prognostic value. Different glioma subtypes were collected to analyze the expression profiles of S100A11 and its relationships with these glioma subtypes. The correlations of S100A11 expression with overall survival in both LGG and HGG patients in TCGA were obtained from Gene Expression Profiling Interactive Analysis (http://gepia.cancer-pku.cn).

Cell Lines

The human glioma cell lines U87, U251 and U118 were purchased from the Chinese Academy of Sciences, ShangHai Cell-bank (Shanghai, China). The human glial cell lines NHA and HA1880 were purchased from BeNa Culture Collection (BNCC, Beijing). The human glioma stem cell lines SU3 and G91, glioma cell lines U343, SNB19 and SHG44 were established and maintained from our lab. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Valencia, CA) supplemented with 10% fetal bovine serum (Invitrogen, Valencia, CA) in a humidified incubator with 5% CO$_2$ at 37°C, and were passaged twice a week. Glioma stem cell line G91 was cultured in DMEM/F12 medium (Gibco, USA) containing 20 ng/mL basic fibroblast growth factor (Gibco), 20 ng/mL epidermal growth factor (Gibco), B27 supplement (50X), 2 mM L-glutamine, MEM vitamin solution and 100 mM sodium pyruvate (100X) (Gibco).

Subcutaneous Xenograft Model

Balb/c nude mice were obtained from the Model Animal Research Center of Nanjing University and were raised in Experimental Animal Center of Anhui Medical University. Subcutaneous xenograft model of malignant glioma was established as previously described. In brief, 2×10$^6$ U87-shS100A11 and U87-shNC cells were injected subcutaneously into the right flanks of 6-week-old Balb/c nude mice, and 5 mice were used in each group. Tumor size was measured with vernier caliper once a week for 6 weeks by measuring the length (L) and width (W) of the subcutaneous tumor, and the volume (V) was calculated by $V=\frac{LW^2}{2}$. Mice were sacrificed by dislocation of vertebrae when the longest diameter of the largest xenograft of a group reached 1.5 cm, and tumor weights were determined. The animal procedures were approved by the Ethics Committee of Anhui Medical University and Animal Protection Institution of Anhui Medical University (Ethics number: 2020-12-07).

Clinical Specimens

Seventy-nine consecutive patients who were diagnosed with primary glioma (WHO II 27, III 14, IV 38) between September 2018 to September 2019 at Department of Neurosurgery, the First Affiliated of Anhui Medical University were collected in this study. No pretreatment radiation and chemical therapy were administered in any of these patients. Matched para-carcinoma tissues were obtained from areas away from the tumor, to avoid tumor field effect. Normal brain tissues were obtained from six brain injury patients. This study was
approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University and written informed consents were obtained from all the participants (Ethics number: 2020-12-07).

**Establishment of shRNA Stable Cell Lines**

A S100A11 short hairpin (sh) RNA vector was designed and synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China), with a target sequence of shRNA-1 (S100A11-Homo-192) sense (5’-3’) CUGGAAAGGAUGGUAAUAATT, anti-sense (5’-3’) UUUAAACCUCCUUUCAGTT, shRNA-2 (S100A11-Homo-257) sense (5’-3’) CUAGCUGC-CUUCACAAGATT, anti-sense (5’-3’) UCUUUGUGAAGG-CAGCUAGTT. A scramble shRNA vector was used as a negative control (NC). U87 and SHG44 cells were transfected with S100A11 or control shRNA using siRNA-mate reagent (GenePharma, Shanghai, China). Then the glioma cell lines which were transfected with shS100A11 (U87-shS100A11, SHG44-shS100A11) and with shNC (U87-shNC, SHG44-shNC) were established, respectively. The stability of transfected clones was identified by western blotting (WB). The stable cell clones were isolated using Geneticin® Selective Antibiotic (G418 Sulfate; Gibco Life Technologies, Beijing, China).

**RNA Extraction, Reverse Transcription, and qRT-PCR Analysis**

Cell lines and tissues from 79 human glioma patients and 18 normal brain samples were applied for total RNA extraction using the Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription of total RNA was conducted using HiScript II Q RT SuperMix for qPCR (Vazyme, Nanjing, China). Quantitative real-time PCR (qRT-PCR) was performed using SYBR® Green PCR Master Mix (Vazyme) on Roche instruments (Applied Biosystems). The primers used for qRT-PCR amplification of human S100A11 gene were AGGAGAGGCTCCAGACCCG and ACCGCTCAGTCTCTG-TAGGG, with a PCR product of 228 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control; and its primer was AGGTCGGTGTGAACGGATTTG and TGTAGACCATGTAGTTGAGGTCA. The 2−ΔΔCt method was used as a relative quantification measure for calculating the differential expressions.17 The program was as follows: an initial denaturation step at 95 °C for 5 minutes, followed by 40 cycles of denaturation at 60 °C for 15 seconds. A melting curve analysis of each sample was used to check the specificity of amplification, and each sample was assayed in triplicate.

**Tissue Microarray and HE Staining**

The glioma, para-carcinoma and normal brain tissues were used to fabricate tissue microarray for HE and IHC staining. Tissue samples embedded in paraffin were sectioned, deparaffinized, and subjected to antigen retrieval performed in citrate buffer (pH 6.0). Slides were incubated at 4 °C overnight with the S100A11 antibody (1:500; Cat. no. 0057513; Proteintech, Wuhan, China) and then with a secondary HRP-conjugated antibody (Proteintech) at 37 °C. Sections were stained with 3-diaminobenzidine (DAB) for 2 min. All pathological sections were scanned with high-resolution images via a full-field digital slice scanner (Pannoramic MIDI, Hungary).

**Cell Proliferation Assay**

The cell proliferation was tested by CCK8 kit (Beyotime, Shanghai, China). U87 and SHG44 cells were seeded in 96-well plate and were cultured to about 4000 cells per well. After removing the culture medium of original cells, 100μl CCK8 solution (containing 100μl culture solution and 10μl CCK8 reagent) was added to each well. The cells were incubated and then placed under 450 nm wavelength of the microplate reader after 1 hour of administration, testing the optical density (OD) value of each well and carrying out proliferation curve.

**Flow Cytometry**

The cell cycle was tested by cell cycle detection kit (BestBio, Shanghai, China). Cells were separated with trypsin and then centrifuged at 1000×g for 5 mins. The resulting pellet was washed twice with cold PBS, centrifuged again, fixed with 75% alcohol for a hour and washed twice with cold PBS. Then it was treated with RNase (Lot. BB19101; BestBio, Shanghai, China) for 30 mins at 37 °C, and incubated with PI (Lot. BB19101; BestBio, Shanghai, China) for 30 min at 4°C at dark area. The cell cycle was determined by flow cytometry.

**Clone Formation Assay**

The transfected cells were suspended in 10% serum media and were cultured in 6-well plates at a density of 400 cells per plate. The cells were then cultured until day 14. The culture medium was removed and cell clones were stained with crystal violet (EMD, USA) for 30 mins at 37°C, and incubated with PI (Lot. BB19101; BestBio, Shanghai, China) for 30 min at 4°C at dark area. The cell cycle was determined by flow cytometry.

**Western Blotting**

Total cellular proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer according to the manufacturer’s instructions. The concentrations of the protein samples were determined using the bicinchoninic acid (BCA) assay method, and 50μg protein from each sample was subjected to sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). After the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, the membranes were rinsed with TBST and blocked with skinned milk solution for 1 hour. After that, membranes were incubated overnight with S100A11 rabbit monoclonal antibody (1:1000; Cat. no.
0057513; Proteintech, Wuhan, China) or GAPDH rabbit polyclonal antibody (1:5000; Cat. no. 10494-1-AP; Proteintech, Wuhan, China) 4°C. After washing with TBST for 4 times, membranes were incubated with goat anti-rabbit secondary antibody (1:2,000; Cat. no.ZB-2301, ZSJQ-BIO) for 1.5 hours at room temperature. Following washing with TBST for 4 times, the blots were developed using the enhanced chemiluminescence (ECL) reagent (Vazyme, Nanjing, China) and the images were recorded in the Gel Imaging System. The relative S100A11 expressions were calculated by the Gel-Pro-Analyzer (Tanon, Shanghai, China).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 8.0.2 (GraphPad Software Inc, La Jolla, CA, USA). All enumeration data were given as the mean ± standard deviation (SD) and were analyzed by a Student’s t-test or one-way ANOVA analysis of variance analysis followed by a Bonferroni post hoc test. P < 0.05 was considered to be statistically significant.

Results

Elevated S100A11 Expression in Glioma Tissue Predicted Worse Prognosis

The expressions of S100A11 in LGG and HGG were analyzed in TCGA database. The results suggested that S100A11 showed elevated expressions in both LGG and HGG in comparison with that in the normal brain tissues (Figure 1A and B). The expression was relatively higher in HGG samples (Figure 1B and C). Furthermore, we analyzed the prognosis of patients and found that both LGG and HGG patients with high expressions of S100A11 performed poor overall survival rate (Figure 1D and E and 1F).

S100A11 Expression Positively Correlated With the Glioma Grades

To assess S100A11 expression in different glioma grades, we performed qPCR assay, WB tests and IHC staining in clinical samples. The results showed that the mRNA level of S100A11 positively correlated with the pathological grades (Figure 2A). WB results indicated that the tumor tissues showed higher
S100A11 expressions compared with that in the adjacent normal tissues. And the expressions positively correlated with the pathological grades (Figure 2B and C). The HE and IHC staining suggested that glioma tissues had evident atypia and deeper staining. The numbers of cytoplasmic positively marked cells positively correlated with the glioma grades (Figure 2D and E). Generally, the results suggested a positive correlation between S100A11 expressions in glioma tissues with glioma grades.

**Downregulation of S100A11 Expression Significantly Inhibited Glioma Cell Proliferation, Cell Cycle and Colony Formation Rate**

The mRNA levels of S100A11 in NHA, HA1800, SU3, U343, U118, U87, SNB19, U251, G91 and SHG44 cell lines were performed by qPCR. The results indicated that S100A11 expressions in glioma cell lines were significantly higher than that in normal glial cell lines (NHA and HA1800) \((P < 0.05)\). Significantly, the overexpressions of S100A11 mRNA were obviously found in two glioma cell lines U87 and SHG44 (Figure 3A) \((P < 0.05)\). Next, we transfected shRNA-targeted S100A11 mRNA into U87 and SHG44 cells (Figure 3B and C). CCK8 proliferation assay showed that knockdown of the S100A11 significantly inhibited the proliferation of SHG44 and U87 cells (Figure 3D and E) \((P < 0.05)\). The efficacy of shRNA interference with the expression of S100A11 in U87 cells was confirmed by WB, which showed interference effect of shRNA-1 was optimal (Figure 3F and G). Therefore, shRNA-1 was selected to further function study. Firstly, we investigated the relationship between cell-cycle distribution regulation and S100A11 expression in flow cytometry. The percentage of shS100A11 cells \((66.8\%)\) was higher than that of shNC cells \((35.9\%)\) at the G0/G1 phase. Secondly, the results of clone formation assay confirmed that the inhibition of S100A11 expression significantly decreased clone formation rate of U87 cells \((P < 0.05)\). The clone formation rate of U87 cells with shS100A11 was only 18.3\% compared with that in the control group \((43.8\%)\). The above results indicated that the downregulation of S100A11 expression significantly inhibited glioma cell proliferation, cell cycle and colony formation rate \((P < 0.05)\).

**Downregulation of S100A11 Expression Inhibited Tumor Formation In Vivo**

We further examined the role of downregulated S100A11 expression in glioma growth *in vivo* using a subcutaneous xenograft mouse model. Stable transfected U87-shS100A11 cells (Figure 4A) and corresponding control group U87-shNC cells (Figure 4B) were subcutaneously injected into right flanks of
The relative S100A11 mRNA expression in different glioma cell lines and knock-down of S100A11 gene showed a significant reduction in cell proliferation, cell cycle and colony formation rate. (A) The relative S100A11 mRNA expression in different glioma cell lines, glial cell NHA and HA1800 were served as control. (B) Knock-down of S100A11 mRNA expression in U87 and U251 human glioma cells were validated by qRT-PCR analysis. Two shRNA (shRNA-1 and shRNA-2) were used and shRNA-NC was served as negative control. (D and E) Cell proliferation assays after transfection were measured by CCK8 proliferation assay. (F) Western blot validation of downregulation by shRNAs showed interference effect of shRNA-1 was better. shRNA-NC was served as a Negative Control. (G) Quantification of downregulating S100A11 protein expression by shRNAs. (H, I and J) The cell cycle distribution of U87 glioma cells after knock-down of S100A11 expression. shRNA-NC was served as a negative control. (K) Clone formation assay was used to identify proliferation ability of knock-downing S100A11 expression. shRNA-NC was served as a Negative Control. *, *P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 3. The relative S100A11 mRNA expression in different glioma cell lines and knock-down of S100A11 gene showed a significant reduction in cell proliferation, cell cycle and colony formation rate. (A) The relative S100A11 mRNA expression in different glioma cell lines, glial cell NHA and HA1800 were served as control. (B) Knock-down of S100A11 mRNA expression in U87 and U251 human glioma cells were validated by qRT-PCR analysis. Two shRNA (shRNA-1 and shRNA-2) were used and shRNA-NC was served as negative control. (D and E) Cell proliferation assays after transfection were measured by CCK8 proliferation assay. (F) Western blot validation of downregulation by shRNAs showed interference effect of shRNA-1 was better. shRNA-NC was served as a Negative Control. (G) Quantification of downregulating S100A11 protein expression by shRNAs. (H, I and J) The cell cycle distribution of U87 glioma cells after knock-down of S100A11 expression. shRNA-NC was served as a negative control. (K) Clone formation assay was used to identify proliferation ability of knock-downing S100A11 expression. shRNA-NC was served as a Negative Control. *, *P < 0.05; **, P < 0.01; ***, P < 0.001.
Discussion

Glioma is a primary malignant tumor in CNS, which is a serious threat to the human’s life. with the characterization of diffuse growth, strong invasiveness and poor prognosis. Even received concurrent temozolomide with postoperative radiation, the median survival time of GBM patients is about 14.6 months.18 The prognosis of gliomas in different pathological grades varies significantly. GBM has the poorest overall survival, with <5% of patients surviving beyond 5 years after diagnosis,19 while the LGG, which occurs in the relatively younger patients, has a high treatment sensitivity and relatively favorable prognosis.20 Still, LGG patients have a median survival of 13 years even with aggressive treatments.4 Therefore, it is clinically urgent to explore novel therapeutic targets for glioma treatment and prognosis assessment.

S100A11 is a Ca\(^{2+}\) binding protein which can transfer cellular Ca\(^{2+}\) level changes into a variety of biological signals.21 According to the different cancer types and distributions, the biological functions of S100A11 are controversial. Several studies have confirmed that S100A11 was upregulated in cancers, such as pancreatic cancer, intrahepatic cholangiocarcinoma and ovarian cancer, involving in the progression of these cancers.22-24 However, individual study suggested the downregulated expression of S100A11 in bladder cancer predicted the worse prognosis, indicating that S100A11 could perform both tumor suppression and promotion effects to influence the proliferation of cancer cells.7 Whereas, the expression status and the potential role of S100A11 in different grades of glioma have not been fully elucidated.

Depending on the histologic subtypes, the same biomarker might show different prognostic values. Previous study demonstrated that S100A11 expression was extremely elevated in the high-grade serous ovarian cancer in comparison with the normal epithelial tissues and was positively correlated with FIGO stage.15 Similarly, S100A11 expression was positively correlated with the FIGO stage and lymph node metastasis in cervical squamous cell carcinoma patients.13 In colorectal cancer tissues, S100A11 level was increased following stage progression of the disease.25 In the present study, for the combinative consideration of the results from the bioinformatic analysis with the TCGA database and the IHC data in the clinical samples, we found that S100A11 was overexpressed in glioma tissues in comparison with that in normal brain tissues and positively correlated with the pathological grades. These results suggested that S100A11 might be a pathological grade-related oncogene in glioma. As mentioned earlier, the prognosis of glioma, which was significantly correlated with
the pathological grades and the treatment strategies of glioma, was obviously different. Hence, the detection of the pathological grades of gliomas seemed to be the key issue in clinical practice. Our findings might have potential implications for the individualized treatment of glioma patients.

It has been reported that S100A11 acted as a tumor promoter and played a role in affecting the proliferation of tumor. For instance, S100A11 was overexpressed in ovarian cancer cells, and knockdown of S100A11 expression suppressed the cell growth. In our study, the analysis of the relationship between S100A11 expression and proliferation ability in glioma cells indicated that knockdown of S100A11 expression could significantly inhibit the glioma cell proliferation. Xenograft model assay confirmed that knockdown of S100A11 expression could inhibit tumor growth in vivo. These results demonstrated that S100A11 performed tumor promotion effects in the proliferation of glioma. However, the underlying molecular mechanisms of S100A11 in regulating tumor proliferation have not yet been fully investigated. Recently, a new published research has proposed a novel site of S100A11 dimer which bound to Receptor for Advanced Glycation End Products (RAGE) V Domain and induced homodimerization of two adjacent RAGEs and autophosphorylation of their cytoplasmic domain. This result triggered a series of signaling cascades that resulted in cell proliferation and survival. A study has confirmed that extracellular S100A11, via RAGE, had a critical role in tumor progression of malignant pleural mesothelioma. Blocking S100A11-RAGE connection using either sRAGE (acting as a decoy to compete with S100A11) or S100A11 neutralizing antibody effectively inhibited cell growth of malignant pleural mesothelioma cells. However, whether the interaction occurs in glioma cells needs further investigations.

In conclusion, our study demonstrated the elevated S100A11 expressions in human glioma tissues and its positive correlation with the pathological grades. Knockdown of S100A11 expression significantly inhibited the proliferation of glioma cells and tumorigenic ability. The S100A11 might consider as a potential novel biomarker for evaluating the prognosis of glioma patients.

Authors’ Note
Our study involving human samples was carried out under the approval of the Ethics Committee of the First Affiliated Hospital of Anhui Medical University (Ethics number: 2020-12-07) and written informed consents were obtained from all the participants. The animal procedures were approved by the Ethics Committee of Anhui Medical University and Animal Protection Institution of Anhui Medical University (Ethics number: 2020-12-07). Significant efforts were made to minimize the number of animals and their pain.

Author Contributions
Haopeng Wang and Mengyuan Yin contributed equally to this work.

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.

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