

## Overexpression of LACTB, a Mitochondrial Protein That Inhibits Proliferation and Invasion in Glioma Cells

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LACTB, a mitochondrial protein, was ubiquitously expressed in different mammalian tissues, such as liver, heart, and skeletal muscle. It has been shown that LACTB is downexpressed in breast cancers, and it suppresses the proliferation and promotes the apoptosis of breast cancers. However, its role in the progression and prognosis of glioma remains unknown. In this study, we analyzed the clinicopathological features and outcomes of LACTB expression in 98 glioma patients and investigated the effects of LACTB overexpression on the proliferation, invasion, and angiogenesis of glioma cells in vitro. We observed a significant decrease in LACTB expression in glioma, and downexpression of LACTB is correlated with a poor prognosis of glioma patients. Moreover, Cox regression analysis reveals that the LACTB is an independent prognostic indicator for glioma patients. Overexpression of LACTB could suppress the proliferation, invasion, and angiogenesis of glioma cells. In addition, overexpression of LACTB could inhibit the expression of PCNA, MMP2, MMP9, and VEGF. Taken together, these data indicate that LACTB may serve as a promising therapeutic target for gliomas.

**Key words:** LACTB; Glioma; Proliferation; Invasion; Angiogenesis; Prognosis

### INTRODUCTION

Gliomas, accounting for 80% of all malignant brain tumors and resulting in poor prognosis, are the most common brain tumor<sup>1-3</sup>. Despite the great advances in neurosurgery, radiotherapy, and chemotherapies that have been made during the past decades, the median survival duration for patients with glioma is less than 12–15 months<sup>4-6</sup>. Considering that glioma is a heterogeneous disease, with the abnormalities of oncogenes and tumor suppressor genes<sup>7</sup>, there is an urgent need to identify novel diagnostic and prognostic markers during the development of gliomas to help in providing an effective treatment method and enhancing the prognosis of glioma patients.

LACTB (lactamase ) is a mitochondrial protein that is widely expressed in various mammalian tissues<sup>8-12</sup>. Previous research reported that LACTB could promote intramitochondrial membrane organization and regulate complex I of the mitochondrial electron transport chain and cellular metabolic processes<sup>13-15</sup>. Overexpression of LACTB in mouse resulted in an obese phenotype<sup>14</sup>. More recently, it was reported that LACTB positively contributed to the apoptosis and suppression of the proliferation

of breast cancer cells<sup>16</sup>. However, up to now, the expression of LACTB in gliomas and its clinicopathological significance have not been elucidated. Therefore, to explore the role of LACTB in gliomas is greatly significant.

In this study, we demonstrated the downregulation of LACTB in gliomas and analyzed the relationship between clinicopathological characteristics and glioma patients' survival. Next, we then investigated the effects of overexpressing LACTB on the proliferation, invasion, and angiogenesis of glioma cells in vitro, which suggested that LACTB might play a critical role in glioma progression and might function as a novel prognostic factor and a promising treatment target for glioma.

### MATERIALS AND METHODS

#### *Cell Culture and Reagents*

Human glioma cell lines were purchased from Type Culture Collection of the Chinese Academy of Sciences. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 mg/ml streptomycin, and 100 U/ml penicillin. The cells were cultured at 37°C and 5% CO<sub>2</sub> in a

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humidified atmosphere. Every 2–3 days, the cells were subcultured in 1 mM EDTA and 0.25% trypsin. The cells were routinely screened and were found to be free of mycoplasma. Antibodies against LACTB were obtained from Abcam (Cambridge, MA, USA); PCNA, MMP2, MMP9, VEGF, p-AKT, t-AKT, p-STAT3, and t-STAT3 were obtained from Cell Signaling Technology (Danvers, MA, USA). GAPDH was purchased from Beyotime (Shanghai, P.R. China).

#### *Patients and Tissue Preparation*

Specimens of 98 gliomas and 10 nontumor brains were obtained from The First People's Hospital of Chongqing Liang Jiang New Area between 2009 and 2015. Dissected samples were preserved in formalin immediately after surgery and stored at room temperature. Approval from The First People's Hospital of Chongqing Liang Jiang New Area Ethics Committee was obtained before the research. All experimental protocols were carried out in accordance with the guidelines approved by The First People's Hospital of Chongqing Liang Jiang New Area Ethics Committee. Informed consent was obtained from all of the patients in this study.

#### *Immunohistochemistry*

Immunohistochemistry (IHC) was performed as previously described<sup>17</sup>. The IHC results were independently evaluated in a blinded manner by two pathologists. The signal was assessed by a semiquantitative scoring system, which represented the percentage of positive tumor cells and the intensity of staining. The intensity of staining of cells was scored and graded as follows: 0 (negative), 1 (faint yellow), 2 (yellow or deep yellow), and 3 (tan or brown). The proportion score according to the percentage of positively stained cells was as follows: 0 (0%–25%), 1 (26%–50%), 2 (51%–75%), and 3 (76%–100%). The expression of LACTB was measured by multiplying the staining intensity by the percentage of positively stained cells. The samples with a final score  $\geq 5$  were defined as high expression, and those with a final score  $< 5$  were defined as low expression.

#### *Cell Transfections*

LACTB lentivirus used for LACTB overexpression, corresponding negative control lentivirus, lentiviral shRNA, and matched empty lentivirus were purchased from GeneChem Co. (Shanghai, P.R. China). For cell infection, viral supernatants and 8  $\mu\text{g}/\text{ml}$  polybrene were added to the culture medium, and the medium was incubated for 24 h. The target sequence of sh-LACTB was 5'-GATTACTGATTTCCCATTTAA-3'. Green fluorescence was detected 96 h later, and the expression of LACTB was verified by Western blot and real-time reverse transcription quantitative PCR (RT-qPCR).

#### *Real-Time PCR*

Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to cDNA using a Revert-Aid<sup>TM</sup> First-Strand cDNA synthesis kit (Fermentas, Waltham, MA, USA) following the supplier's instructions. The primer sequence and product size for LACTB were 5'-C ACGTTCTCAACATCAGCA-3' (forward) and 5'-GC ACAGGATCAAGGATGAGG-3' (reverse), 179 base pairs (bp), and for GAPDH, which was used as a standard, was 5'-ACATCAAGAAGGTGGTGA AG-3' (forward) and 5'-ATACCAGGAAATGAGCTTGA-3' (reverse), 173 bp. All primers were synthesized by Takara (Dalian, P.R. China).

#### *Western Blot*

Total proteins were extracted from tissues and cells. From each sample, 30  $\mu\text{g}$  of total protein lysates was loaded onto a 10% acrylamide gel, and SDS-PAGE was performed, before being transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membrane was blocked in 5% nonfat milk and incubated with primary antibody overnight at 4°C. The membranes were washed three times with TBST buffer, followed by incubation with secondary antibody. Then the membranes were washed three times with TBST buffer and quantified using the Quantity One 4.6 computer software.

#### *Cell Proliferation, Invasion, and Tube Formation Assay*

The proliferation, invasion, and tube formation were determined as previously described<sup>17,18</sup>.

#### *ELISA Assay*

Concentration of VEGF in supernatants of glioma cells was tested by VEGF ELISA kit according to the manufacturer's instructions (R&D, Minneapolis, MN, USA).

#### *The Gene Expression Omnibus and The Cancer Genome Atlas Analysis*

Data of patients with glioma were downloaded from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) data portal website (<http://cancer.genome.nih.gov>). Clinical data, including survival time, age, gender, grade, and Karnofsky performance score (KPS), were also obtained from TCGA. Patients with incomplete clinical data were excluded from this analysis.

#### *Statistical Analysis*

All of the statistical analyses were performed using the SPSS 17.0 software package. Statistical differences among groups were analyzed by ANOVA, Kruskal–Wallis, *t*-test, or chi-square test. The prognostic significance analysis was performed using the Kaplan–Meier method and log

rank tests. Cox's proportional hazards regression model was used for multivariate survival analysis. A value of  $p < 0.05$  was considered significant. All data are presented as mean  $\pm$  standard deviation (SD).

## RESULTS

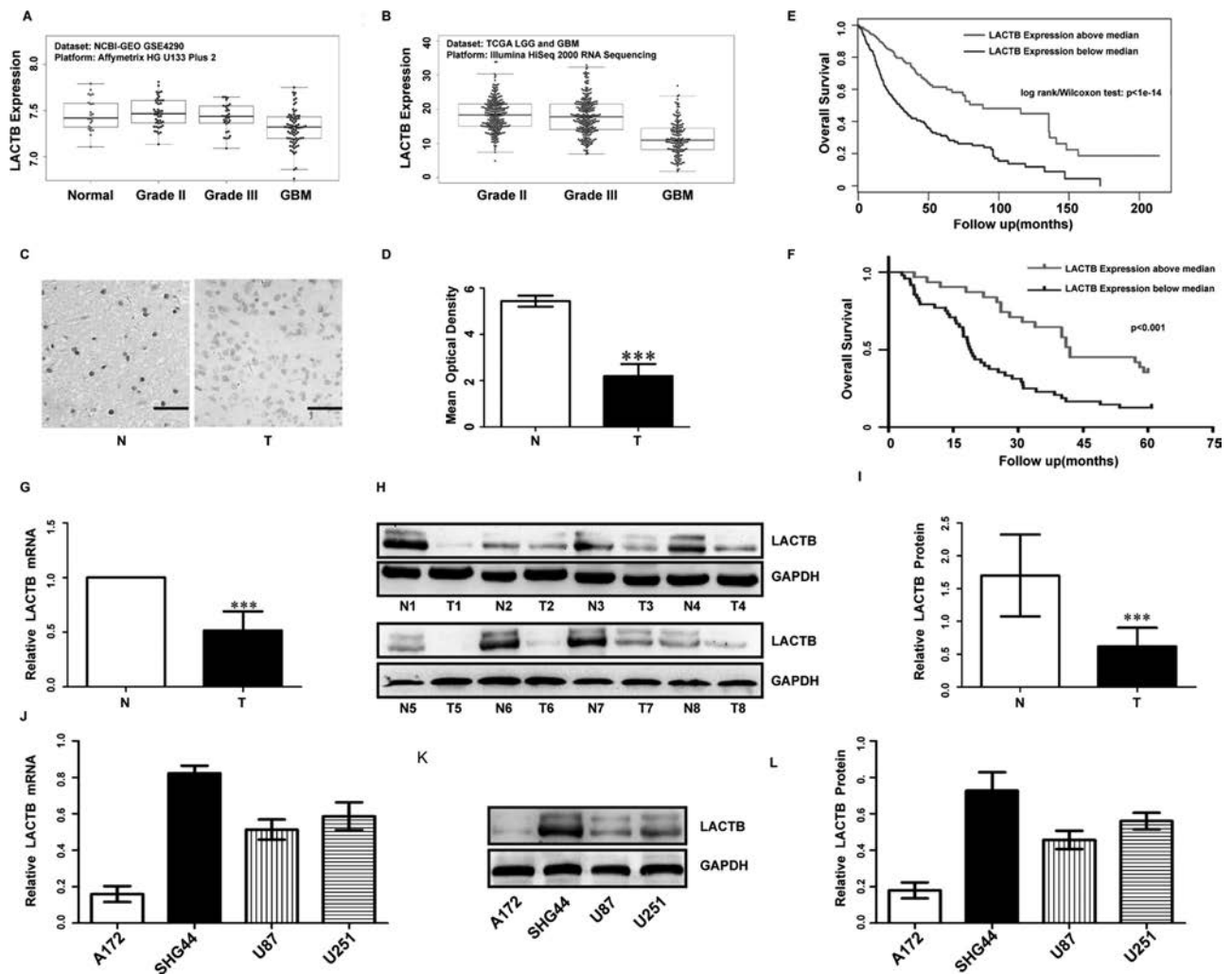
### Downexpression of LACTB in Gliomas

We analyzed the data from the GEO and the glioma cohort of TCGA, and we found that expression of LACTB was markedly decreased in glioma tissues compared with normal tissues, and it was lower with the increase in grade of glioma (Fig. 1A and B). Then we also tested LACTB

expression in 98 glioma specimens and 10 normal brain tissues by IHC. The expression level of LACTB in the gliomas was significantly decreased compared with that in the normal brain tissues (Fig. 1C and D), which is in accordance with the result of RT-PCR (Fig. 1G) and Western blot (Fig. 1H and I).

### LACTB Expression Related to Glioma Patients' Survival

The relationship between LACTB expression levels and the clinicopathologic features in 98 glioma patients is shown in Table 1. Chi-square test revealed that the decreased expression of LACTB was significantly correlated



**Figure 1.** Low lactamase (LACTB) expression predicted a worse prognosis for patients with glioma. LACTB expression in glioma tissues and normal brain tissues from the Gene Expression Omnibus (GEO) (A) and The Cancer Genome Atlas (TCGA) (B) data sets. LACTB expression in 98 glioma tissues and 10 normal brain tissues was tested by immunohistochemistry (IHC; scale bars: 50  $\mu$ m, 400  $\times$ ) (C) and statistical quantification of the mean optical density (D) in normal brain tissues and glioma tissues ( $*p < 0.001$ ). Survival analysis of patients with glioma from the TCGA data sets (E) and our data (F). Real-time reverse transcription quantitative PCR (RT-qPCR) (G) and Western blot analysis (H, I) of LACTB in eight glioma tissues and paired adjacent nontumor tissues.  $***p < 0.001$ . RT-qPCR (J) and Western blot analysis (K, L) of LACTB in four glioma cell lines.

**Table 1.** Association of LACTB Expression With the Clinical Parameters

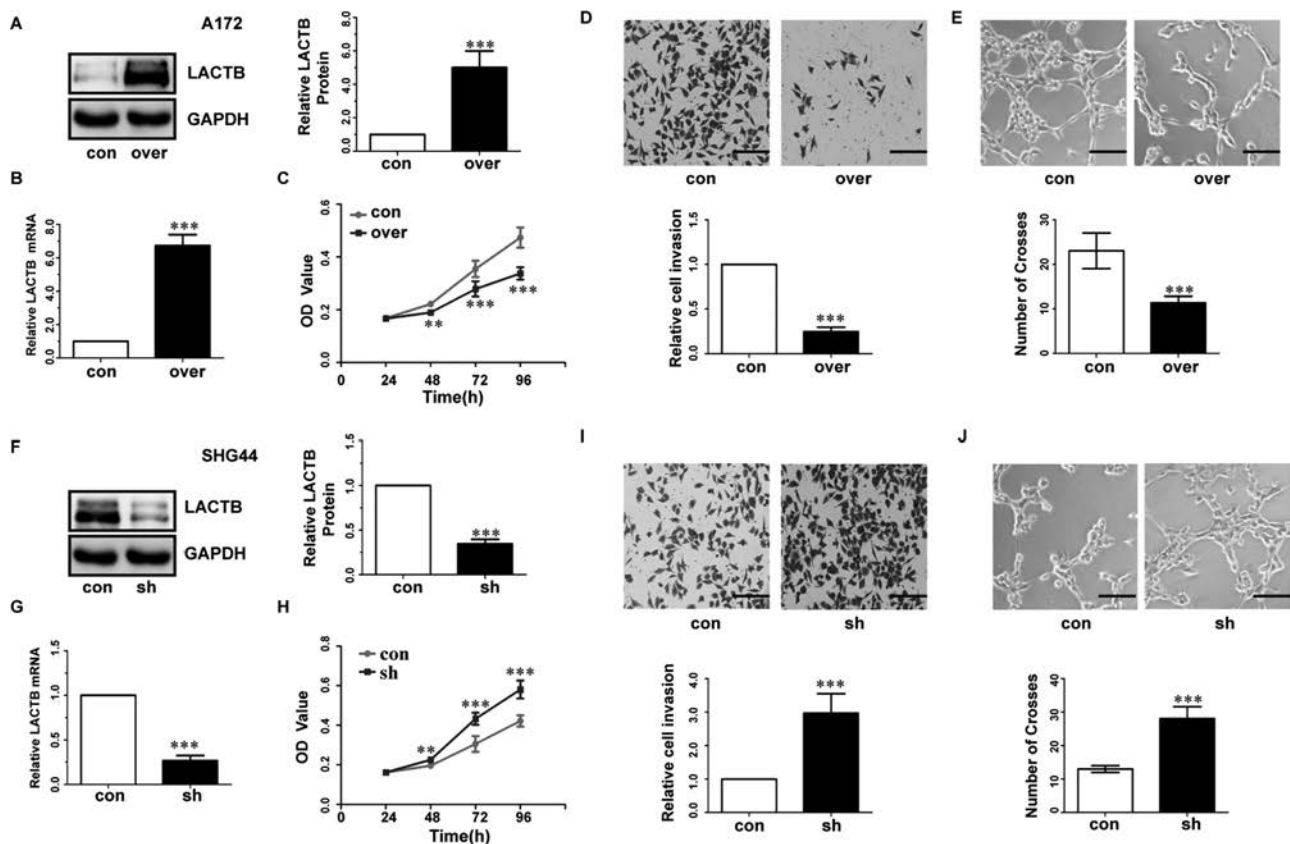
Groups	Total	LACTB Expression		<i>p</i>
		Low	High	
Gender				0.18
Male	56	15	39	
Female	42	16	26	
Age (years)				0.58
60	49	14	35	
>60	49	17	32	
WHO grade				<0.001
I–II	40	18	22	
III–IV	58	13	45	
KPS				0.005
70	49	27	22	
>70	49	4	45	

WHO, World Health Organization; KPS, Karnofsky performance score.

with KPS and World Health Organization (WHO) grade. There were no statistically significant differences in gender or age according to the staining results (data not shown). Kaplan–Meier survival curves demonstrated that downexpression of LACTB was closely related to poor overall survival (Fig. 1E and F). Furthermore, univariate and multivariate analyses indicated that KPS and LACTB expression were two prognostic factors of gliomas (data not shown).

#### LACTB Inhibits the Proliferation of Glioma Cells

Next, Western blot and RT-qPCR were performed to examine LACTB expression levels in glioma cell lines (Fig. 1J–L). To further investigate the functions of the LACTB in glioma cells, the SHG44 and A172 cell lines were selected for further experimentation. We introduced the LACTB gene into the A172 cells using LACTB lentivirus expression vector, and sh-LACTB was transfected into SHG44 cells, as described in the Materials



**Figure 2.** LACTB inhibited proliferation, invasion, and angiogenesis of glioma cells. (A, B, F, G) RT-qPCR and Western blot were conducted to determine the mRNA levels and protein of LACTB in A172 and SHG44 cells. (C, H) CCK-8 assay tested cell proliferation in A172 and SHG44 cells. (D, I) Representative fields of A172 and SHG44 invasive cells (scale bar: 100  $\mu$ m, 200  $\mu$ m) (up), average number of invasive cells per field from three independent experiments (down). con, transfected with empty vectors or control shRNA; over, transfected with LACTB vectors; sh, transfected with LACTB shRNA. (E, J) Representative fields of tube formation of HUVECs (scale bars: 100  $\mu$ m, 200  $\mu$ m) (up) and number of crosses of tube formation by HUVECs. Data are based on at least three independent experiments and shown as the means  $\pm$  standard deviation (SD). \*\**p* < 0.01, \*\*\**p* < 0.001, compared with con.



and Methods section. RT-PCR and Western blot results revealed that LACTB expression was increased in A172 cells and effectively silenced in SHG44 cells, compared with control cells (Fig. 2A, B, F, and G). CCK-8 cell proliferation assay was utilized to determine cell proliferation. The growth curves revealed that LACTB overexpressing significantly inhibited cell proliferation in A172 cells compared with the control group (Fig. 2C). Meanwhile, depletion of LACTB promoted cell proliferation in SHG44 lines (Fig. 2H).

#### LACTB Suppresses Invasion and Angiogenesis of Glioma Cells

Transwell assay was performed to determine the effect of LACTB on the invasiveness of glioma cells. As a result, LACTB-overexpressing A172 cells showed a significantly decreased invasiveness compared with the control A172 cells (Fig. 2D). Conversely, the invasiveness of LACTB-overexpressing SHG44 cells was significantly weaker than that of the control cells (Fig. 2I).

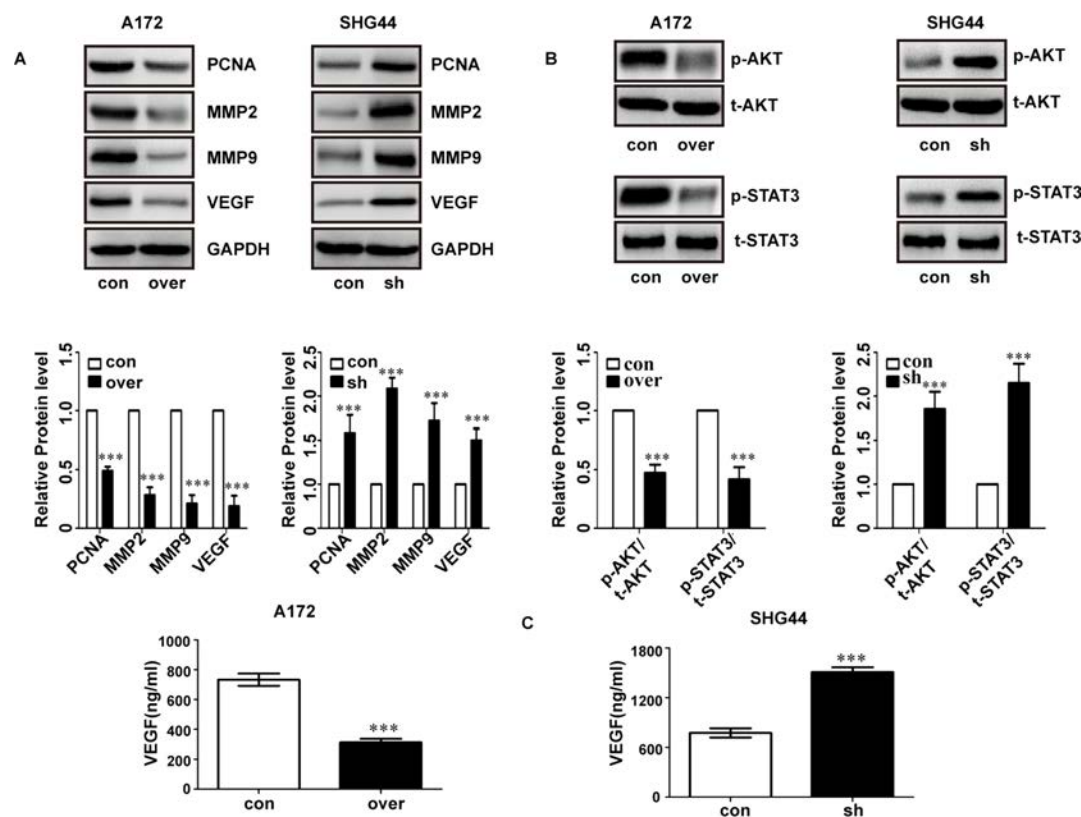
To determine whether LACTB could affect the angiogenesis ability of glioma cells, we first performed the tube formation assay. LACTB overexpression in A172 cells elicited a significant reduction in the number of crosses

(Fig. 2E) compared with control groups. Conversely, the number of crosses was significantly enhanced upon depletion of LACTB in SHG44 cells (Fig. 2J).

#### LACTB Inhibits Expression of PCNA, MMP9, MMP2, and VEGF in Glioma Cells

Next, we tested the expression of PCNA, MMP9, MMP2, and VEGF and found they were suppressed in LACTB-overexpressing A172 cells (Fig. 3A, left). Conversely, depletion of LACTB in SHG44 cells promoted expression of these molecules (Fig. 3A, right). We also tested the expression of p-AKT and p-STAT3, which was closely related to the invasion and proliferation. The results showed that p-AKT and p-STAT3 expression was significantly lower in LACTB-overexpressing A172 cells compared with the control cells (Fig. 3B, left). Conversely, depletion of LACTB in SHG44 cells significantly promoted the expression of p-AKT and p-STAT3 (Fig. 3B, right).

In addition, an ELISA assay was used to detect expression of VEGF in the culture supernatants of the glioma cells. Our results showed that VEGF expression was markedly lower in LACTB-overexpressing A172 cells compared with the control cells (Fig. 3C, left). In contrast,



**Figure 3.** LACTB suppressed PCNA, MMP9, MMP2, and VEGF in glioma cells. (A) LACTB overexpression inhibits the expression of PCNA, MMP9, MMP2, and VEGF in A172 cells. (B) LACTB downexpression promotes the expression of PCNA, MMP9, MMP2, and VEGF in SHG44 cells. (C) The level of secreted VEGF in A172 and SHG44 cells was detected by ELISA. \*\*\* $p < 0.001$ .

depletion of LACTB in SHG44 cells significantly promoted VEGF expression (Fig. 3C, right).

### DISCUSSION

LACTB is a mitochondrial protein that is related with cellular metabolism<sup>18</sup>. It is a newly found tumor suppressor gene. However, it remains unknown whether LACTB has important biological functions in gliomas. In this study, we investigated the expression level and biological function of LACTB in glioma tissues. We found that LACTB was frequently downexpressed in the glioma tissues, which is in accordance with the data from TCGA and GEO. We hypothesized that LACTB may function as a tumor suppressor gene, and down-expression of LACTB may promote the development of glioma. In order to test our hypothesis, we analyzed the relationship between the expression level of LACTB and the clinicopathologic features of gliomas, and investigated the biological role of its expression in glioma cells. The results demonstrated that LACTB expression levels were inversely associated with KPS and WHO grade. In addition, the Kaplan–Meier survival curve data revealed that LACTB downexpression predicted poor survival of glioma, and univariate and multivariate analyses demonstrated that LACTB was an independent prognostic factor for the overall survival of patients with glioma.

Recent studies suggested that downexpression of LACTB promoted cell proliferation and inhibited apoptosis in breast cancer cells<sup>16</sup>. However, to the best of our knowledge, there is no research to study the biological role of LACTB in glioma cells. Thus, we determined the effects of LACTB on the proliferation, invasion, and angiogenesis of glioma cells. Consequently, we found that suppression of LACTB resulted in a significantly promoted proliferation, invasion, and angiogenesis of glioma cells. Furthermore, we observed that overexpression of LACTB significantly decreased the protein levels of PCNA, MMP2, MMP9, and VEGF, which are considered to be important in glioma cell proliferation, invasion, and angiogenesis<sup>19–25</sup>. The results proved that LACTB plays an important function in the progression of the glioma cells.

In conclusion, we found for the first time that LACTB was downexpressed in gliomas. Downregulation of LACTB predicted poor survival of glioma and promoted cell proliferation, invasion, and angiogenesis of gliomas. Our data demonstrated that LACTB may serve as a promising therapeutic target of glioma treatment.

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