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Original Article

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YB-1, a new biomarker of glioma progression, is associated with the prognosis of glioma patients

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Abstract

Y box protein 1 (YB-1) is a multifunctional cellular protein expressed in various cancers, and is a potential target in cancer therapy. Although there is evidence showing that YB-1 plays a role in human cancers, the clinical significance of YB-1 expression in glioma has not been established. In the present study, we investigated the YB-1 level in glioma tumors and analyzed the relationship between the YB-1 level and the grade of malignant glioma, with the aim of providing new ideas for the diagnosis and treatment of gliomas in clinical and basic research settings. A total of 108 patients, comprising 14, 31, 30, and 33 with gliomas of Grades I, II, III, and IV, respectively, were included in this study. The mRNA and protein levels of YB-1 were found to be significantly different between Grade IV and lower-grade tumors. The YB-1 levels in cerebrospinal fluid were significantly higher in Grades III and IV glioma patients than in Grades I and II patients. Immunofluorescence staining was used to detect the levels of YB-1 in the cytoplasm and the nucleus, and results indicated that the intracellular distribution was significantly associated with the pathological grade of glioma. A higher level of YB-1 was associated with shortened survival, suggesting that YB-1 plays a role in the progression of human glioma.

Key words: YB-1, glioma, biomarker, prognosis

Introduction

Y box protein 1 (YB-1) is a 42-kDa multifunctional cellular protein that is expressed in various cancers [1]. It is localized in the cytoplasm as well as in the nucleus, but its expression in the nuclear compartment increases under cellular stress, such as radiation, drug treatment, hyperthermia, or viral infection [2]. YB-1 can act as a transcription factor within the nucleus, and it is involved in the regulation of important cancer-associated genes.

In previous studies, we screened the potential neuroblastoma-associated antigens from the sera of co-stimulatory molecule-engineered AGN2a-immunized mice and AGN2a-bearing mice using the SEREX technique, and identified YB-1 as one of these antigens [3–5]. We also found that YB-1 immunization combined with Treg depletion could induce specific T-cell responses against neuroblastomas and might be a potential strategy for the prevention and treatment of neuroblastomas at the early stage [6].

Gliomas represent ~30% of all central nervous system (CNS) tumors, of which 80% are malignant brain tumors [7]. With the exception of pilocytic astrocytomas, the prognosis of glioma patients is poor. The 5-year survival rate after diagnosis is <10%, with the age at diagnosis and the score on the preoperative Karnofsky Performance scale being well-documented predictors of survival [8]. Over the past

30 years, the standard treatment for glioma has evolved to maximally safe surgical resection, radiation therapy, and temozolomide chemotherapy. While the median survival time of patients with gliomas has improved from 6 to 14.6 months, the tumors are still lethal for the vast majority of patients [9–11].

Although much progress has been made in the understanding of the molecular biology of glioma, it is still difficult to translate the current knowledge into effective treatment. Nearly all patients will experience tumor recurrence, yet to date, very few therapies have established efficacy as salvage regimens [11]. It is therefore important to study the pathogenesis of glioma at the protein level in order to explore new diagnostic and treatment modalities.

Both gliomas and neuroblastomas are derived from cells originating embryologically from the neural crest. It was reported that neuroectodermal antigens are commonly expressed in melanomas, gliomas, and neuroblastomas [12,13]. Using the SEREX technique, we discovered that YB-1 is one of the tumor-associated antigens in neuroblastomas. However, YB-1 expression in glioma and its correlations with clinical and pathological parameters have not been studied previously. We supposed that YB-1 could also play a role in human gliomas. In this study, we evaluated the expression characteristics of YB-1 in glioma of different grades in order to provide reference data for clinical diagnosis and clarify their prognostic value in glioma patients.

Materials and Methods

Patients and tissue samples

The study was approved by the Ethics Committee of Biomedicine Research, the First Affiliated Hospital of Xi'an Jiaotong University. A total of 108 patients who were diagnosed with glioma and treated between June 2005 and June 2015 at the First Affiliated Hospital of Xi'an Jiaotong University were enrolled in this study with written consent. All patients were treatment-naïve initial cases, and were not preoperatively treated with chemotherapy or radiotherapy. The cases were classified according to the 2007 WHO classification for histological grading of CNS tumors, as listed in Table 1. All pathological diagnoses were confirmed by at least two experienced pathologists simultaneously, and brain tissue samples from 10 patients with craniocerebral injury or cerebral hemorrhage after decompression were used as control samples.

Cerebrospinal fluid (CSF) samples were obtained in the Department of Pathology, the First Affiliated Hospital of Xi'an Jiaotong University. CSF was collected by lumbar puncture between 09:00 a.m. and 12:00 a.m. from control subjects (patients with craniocerebral trauma; $n = 6$) and glioma patients ($n = 31$; 10 females and 21 males; age range 60–87 years). Subjects were fasted from midnight to the morning of CSF collection. Subjects were maintained in a supine position for 1 h before performing the lumbar puncture, and then placed in the lateral decubitus position. The L3–4 or L4–5 interspace was infiltrated with 1% lidocaine to provide local anesthesia. CSF was collected with a 25-gauge Quincke spinal needle into polypropylene tubes in 1-ml aliquots, frozen immediately on dry ice, and stored at −75°C until further analyses. The samples were assessed for blood contamination, by measuring the total protein, albumin, glucose, and apoB levels, as well as the total cell and red blood cell counts, to insure that the blood–brain barrier was not compromised in any of the tested subjects.

Table 1. Patient characteristics

Description	Number of patients (%)
Male	64 (59.3)
Female	44 (40.7)
<40-year-old	34 (31.5)
>40-year-old	74 (68.5)
Grade I	14 (13.0)
Grade II	31 (28.7)
Grade III	30 (27.8)
Grade IV	33 (30.5)

RT-PCR analysis of YB-1

Total RNA from glioma tissues was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA) and transcribed using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Otsu, Japan) according to the standard protocols. cDNA amplification was monitored using BRYT Green chemistry using Go Taq qPCR Master Mix (Promega, Madison, USA) on an ABI 7500 fast real-time PCR system (Applied Biosystems, Waltham, USA). The primer sequences for YB-1 were 5′-GAGAAGTGATGGAGGGTGCT-3′ (forward) and 5′-TTAGGG TTTTCTGGGCGTCT-3′ (reverse), whereas 5′-GACTACCTCATG AAGATCCTCACC-3′ (forward) and 5′-TCTCCTTAATGTCACG CACGATT-3′ (reverse) primer sequences for β-actin served as an endogenous control. Primers were synthesized by AuGCT DNA-SYN Biotechnology Synthesis Lab (Beijing, China). After 40 cycles, data reduction was performed with Sequence Detection System software (Applied Biosystems). The relative changes in expression were calculated using the standard $2^{-\Delta\Delta Ct}$ quantification method.

Protein isolation

Proteins were isolated using the Nuclear and Cytoplasmic Extraction Reagent Kit (NE-PER; Thermo Fisher Scientific, Waltham, USA), in the presence of HALT phosphatase and protease inhibitor cocktails (Thermo Fisher Scientific) [14].

Western blot analysis

YB-1 in CSF and brain-tissue homogenates from control and glioma patients were detected by western blot analysis. Samples were lysed on ice for 30 min with radioimmunoprecipitation assay lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, and 0.5% sodium deoxycholate) and then centrifuged (12,000 *g*) for 20 min at 4°C. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). Samples were resolved by SDS-PAGE on Criterion XT 4%–12% gels, and transferred to nitrocellulose membranes (Bio-Rad, Hercules, USA). Rabbit monoclonal anti-YB-1 antibody (1:1000; Abcam, Cambridge, UK) was used as the primary antibody. Mouse monoclonal anti-β-actin antibody (1:5000; Boster, Wuhan, China) was used as a loading control. Membranes were washed three times with TBST buffer before incubating with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:10,000; Santa Cruz, Dallas, USA) or goat anti-mouse IgG secondary antibody (1:10,000; Santa Cruz) 1 h at room temperature, then washed three times and developed with the Pro-light Horseradish Peroxidase Chemiluminescent Kit (Tiangen Biotech, Beijing, China) and exposed to X-ray film. The levels of YB-1 protein in glioma tissue were expressed as the ratio of the optical intensity of the band relative to that of β-actin, and the protein levels in CSF were expressed as the ratio of the optical intensity of the band relative to that of the control. Densitometry analyses were performed using ImageJ software (http://rsb.info.nih.gov/ij/), and data were analyzed and compared by using

Variance software (SPSS, version 17.0; Chicago, USA). A probability value of $P < 0.05$ was considered statistically significant.

Immunohistochemistry

Sections (4-μm thick) were obtained from formalin-fixed, paraffin-embedded samples, mounted on slides, and routinely deparaffinized and rehydrated in xylene and ethanol series. Endogenous peroxidase activity was quenched by 20 min of treatment with 3% H_2O_2 in a standard immunohistochemistry (IHC) staining protocol, followed by antigen retrieval by heating the samples under pressure in an EDTA solution (Bio SB, Santa Barbara, USA) for 8 min. Samples were cooled for 15 min, and the tissues were incubated with anti-YB-1 antibody (10 μg/ml) diluted in antibody dilution buffer (1:150; Abcam) for 2 h at room temperature in a humidified chamber, followed by 1 h of incubation with biotinylated anti-rabbit IgG secondary antibody (1:200; Santa Cruz). Finally, samples were stained for 30 min using the VECTASTAIN ABC Elite kit (1:200; Vector Labs, Burlingame, USA) according to the manufacturer's instructions. The samples were also stained using the DAB kit (Vector Labs), counterstained with Mayer's hematoxylin, dehydrated, and then coverslipped. The slides were observed under a BX51 microscope (Olympus Optical, Tokyo, Japan), and micrographs were obtained using a Mark EOS 5D camera (Kodak, Rochester, USA) and EOS utility software (Kodak). Densitometry analyses were performed using ImageJ software (http://rsb.info.nih.gov/ij/) and Image Pro Plus software (http://www.mediacy.com/index.aspx?page=IPP).

Immunofluorescence staining

Paraffin sections (4 μm) were prepared and de-paraffinized in xylene and re-hydrated through graded concentrations of alcohol. Epitope retrieval was carried out by cooking the samples under pressure in the EDTA solution for 8 min. Subsequently, cooled samples were incubated with primary antibodies (anti-YB-1; 10 μg/ml, 1:100) overnight at 4°C, followed by goat anti-rabbit secondary antibodies (1:200, Alexa Fluor 594; Abcam) for 30 min at room temperature. Slides were mounted using anti-Fade mounting medium with DAPI, and micrographs obtained using a microscope (TiE; Nikon, Tokyo, Japan) and camera (Coolsnap ES2) were merged using NIS Elements software (Nikon).

IHC evaluation

All IHC slides were independently and blindly assessed and scored by two pathologists, with consensus reached where necessary. The staining scores for YB-1 were assessed semiquantitatively according to the percentage of positive staining and the staining intensity. An unequivocal positive reaction was defined as a brown signal in the cytoplasm. The staining intensity was scored as 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellow-brown), or 3 (strong staining, brown). The percentage of positive staining was scored as 1 (≤10%), 2 (11%–50%), 3 (51%–80%), or 4 (≥81%). The positive grade score (range 1–12) was calculated as the product of the staining intensity and percentage score, and scored as 0 (−), 1–4 (+), 5–8(++), 9–12(+++). The positive grade score was the average of the two scores assessed by the pathologists. For statistical analysis, the YB-1 expression was classified as negative or weak expression when the staining score was <4, and as positive or strong expression when the staining score was ≥4.

Statistical analysis

Data were analyzed with SPSS (version 17.0; SPSS, Chicago, USA) and GraphPad Prism (version 5.0; GraphPad, La Jolla, USA). Differences in YB-1 mRNA expression levels were assessed by the Mann–Whitney test. One-way analysis of variance was used for comparing protein expression based on the histological grade of glioma. Survival curves were plotted using the Kaplan–Meier method and assessed using the log-rank test. The threshold for statistical significance was set at $P < 0.05$.

Results

mRNA and protein levels of YB-1 in glioma tissues of different grades

To determine whether YB-1 expression varied with the glioma grade, the mRNA levels of YB-1 were initially examined by real-time RT-PCR. The expression levels of YB-1 in all grades of gliomas were markedly stronger than those in normal tissues. Furthermore, the levels of YB-1 mRNA were significantly higher in Grade IV tissues than in Grade III tissues and low-grade tissues (including Grades I and II) (**Fig. 1A**, $P < 0.01$). Additionally, western blot analysis showed that stronger YB-1 protein expression was associated with a higher pathological grade. The levels of YB-1 were significantly higher in Grade IV tissues than in tissues of Grade III, II, or I (**Fig. 1B**, $P < 0.05$), and did not differ among Grades III, II, and I. Notably, the YB-1 mRNA expression exhibited a significant positive correlation with YB-1 protein expression ($r = 0.8$, $P = 0.01$).

YB-1 levels in CSF of patients with glioma of different grades

The results of western blot analysis of YB-1 expression in the CSF of patients with glioma of different grades are shown in **Fig. 1C**. Only a significant difference was found between the control and Grades III and IV glioma groups ($P < 0.05$), whereas no marked differences were found between the control and Grades I and II glioma groups. However, the expression of YB-1 protein was significantly higher in the CSF of Grades III and IV glioma patients than that of Grades I and II samples ($P < 0.05$), and no significant differences were found in the CSF of patients with Grades III and IV tumors.

Expression characteristics of YB-1 in glioma of different grades

Morphology analysis was performed to detect the cellular localization and expression of YB-1 protein in glioma tissue samples of the 108 patients. Immunofluorescence staining revealed strong YB-1 expression in the cytoplasm and nucleus of tumor cells, but weak YB-1 expression in the control (**Fig. 2A,B**, $P < 0.05$). In Grades I and II, YB-1-positive staining was found mainly in the cytoplasm of glioma cells, with little staining found in the nucleus. However, in Grades III and IV, YB-1 was abundantly distributed in both the nucleus and the cytoplasm of tumor cells.

We further evaluated the intensity of YB-1 staining by immunohisochemical analysis, and results showed that the intensity was significantly higher in Grades III and IV glioma cells than that in Grades I and II (**Fig. 3A,B**, $P < 0.05$). Furthermore, the intensity was much higher in Grade IV glioma than in the other tumor groups ($P < 0.01$).

Positive grade of YB-1 in gliomas of different stages

The positive grade of YB-1 expression was assessed semiquantitatively according to the percentage of positive staining and the staining intensity (**Supplementary Fig. S1**). The YB-1 positive cell percentages in all grades of gliomas were higher than those in normal tissues. Furthermore, the scores were significantly higher in Grades III and IV tissues than in Grades I and II tissues (**Supplementary Fig. S1B**, $P < 0.01$).

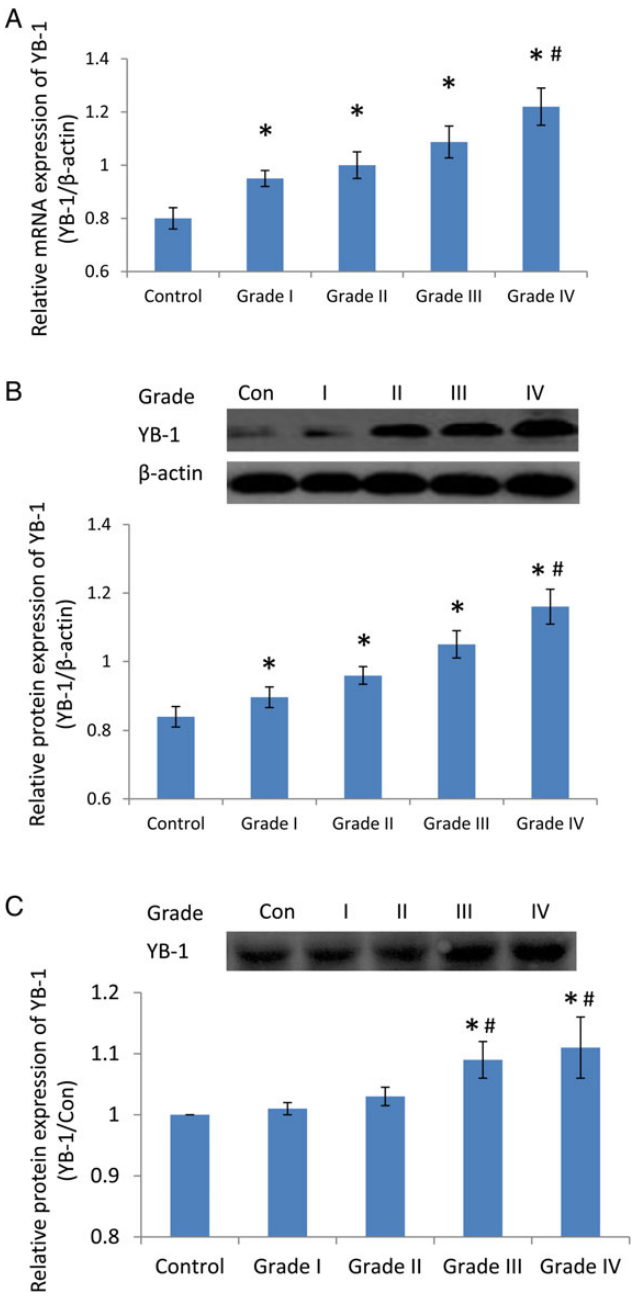

Figure 1. Expression of YB-1 mRNA and protein in glioma tissues of different grades The expression of YB-1 mRNA and protein in tissues of different grades was detected by real-time qPCR assay and western blot analysis, respectively. (A) Quantitative analysis of mRNA levels of YB-1 in 60 glioma samples normalized to those of β-actin. $^{*}P<0.01$ vs. control; $^{\#}P<0.01$ vs. Grades I, II, and III. (B) YB-1 protein levels in glioma tissues detected by western blotting. $^{*}P<0.01$ vs. control; $^{\#}P<0.01$ vs. Grades I, II, and III. (C) YB-1 protein levels in the CSF of glioma patients detected by western blotting. $^{*}P<0.01$ vs. control; $^{\#}P<0.05$ vs. Grades I and II.

For the staining intensity of YB-1, the markedly differences were only found between Grades II, III and IV and normal tissues, and the scores were significantly higher in Grade IV tissues than in Grades II and III tissues (**Supplementary Fig. S1C**, $P<0.01$). The positive grades of YB-1 expression were calculated as the product of the staining intensity and percentage score. As shown in **Supplementary Fig. S1D**, the positive grades of YB-1 were significantly higher in all gliomas stage. Notably, the scores of Grades III and IV exhibited a statistical difference when compared with those of Grades I and II ($P<0.01$).

Relationship between YB-1 level and clinical diagnosis

To evaluate how YB-1 expression was associated with various clinical and pathological characteristics of human glioma, the protein levels of YB-1 in the cytoplasm and nucleus from tumor cells of 108 patients were analyzed and compared (Table 2). The protein levels of YB-1 in the cytoplasm and nucleus were found to be significantly associated with the pathological grade of glioma ($P=0.005$). Glioma tissue samples with high expression levels of YB-1 protein tended to be of higher pathological grade. There was a significant positive correlation between the YB-1-positive cell rates and glioma clinical pathological grades ($r=0.901$, $P<0.05$). The average positive expression rate of YB-1 was 20.33% in Grade I tumors ($n=14$) and 23.94% in Grade II tumors ($n=31$). No statistically significant differences were found between the expression rates of YB-1 in Grades I and II tumors ($P=0.834$). However, in Grade III tumors ($n=30$) and Grade IV tumors ($n=33$), the average positive expression rates of YB-1 were 47.52 and 49.87%, respectively, and the rates were statistically different when compared with other types of gliomas ($P<0.001$).

Correlation between YB-1 expression and prognosis

The prognostic values of YB-1 expression levels in gliomas on the overall survival were finally evaluated in our study cohort. The patients were separated into a weak-expression group (Grades I and II) and a strong-expression group (Grades III and IV) according to the expression levels of YB-1. The Kaplan–Meier analysis and log-rank test showed that patients with strong YB-1 expression had a significantly worse overall survival than those with weak expression (**Fig. 4**). These results indicate that the increased YB-1 expression is associated with shortened survival time, suggesting that YB-1 may play a role in the progression of human glioma.

Discussion

This is the first report showing that YB-1 expression is dramatically stronger in human anaplastic astrocytoma (Grade III) and glioblastoma (Grade IV) than in low-grade gliomas (pilocytic astrocytoma and diffuse astrocytoma) at both mRNA and protein levels. The expression of YB-1 mRNA was strongly correlated with that of YB-1 protein, suggesting that the upregulation of YB-1 in gliomas may be primarily caused by transcriptional activation.

It is well known that YB-1 protein is a multifunctional cellular protein that is expressed in various cancers, including breast [15], lung [16], colorectal [17], prostate [18], and ovarian [19] cancers. It has also been demonstrated that YB-1 is strongly expressed in different glioma cell lines, such as U373-MG and U87-MG [20]. YB-1 was also found in 94.6% of neuroblastoma cases and suggested to be a potential novel tumor marker for neuroblastoma [21]. YB-1 also triggers the expression of Her-2 and estrogen receptor alpha (ER-α) in breast cancer [22]. In some cancers, such as nasopharyngeal cancer, YB-1 is a promising predictive marker of radioresistance and chemoradioresistance [23]. It was also found that inhibition of YB-1 could slow the growth of glioblastoma multiforme and sensitize to temozolomide-independent O6-methylguanine-DNA methyltransferase [24].

YB-1 has recently been immunolocalized in the developing and adult human brain. With the exception of a small population of hypothalamic astrocytes, YB-1 in the brain is predominantly distributed to multiple neurons in the mature human CNS [25].

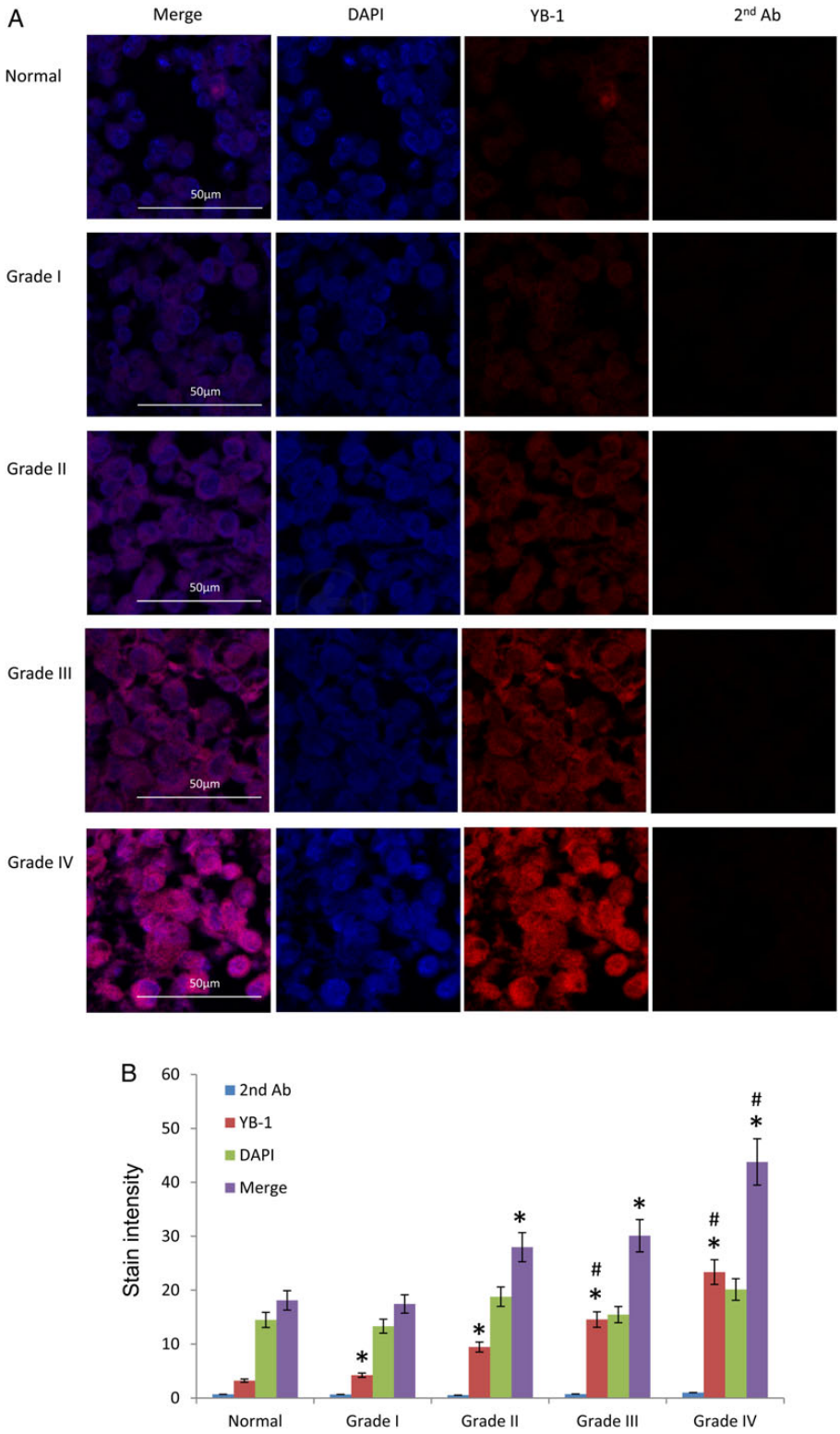

Figure 2. Immunofluorescence staining of YB-1 in gliomas of different stages (A) YB-1 in glioma tissues of Grades I, II, III, and IV, showing positive Alexa Fluor 594 staining for YB-1. DAPI (blue fluorescence) was used to stain the nucleus (×400). Scale bar: 50 μm. (B) Measurement of YB-1 immunofluorescence staining intensity in glioma of different grades. $^{*}P<0.01$ vs. normal control; $^{\#}P<0.01$ vs. Grades I and II.

In the present study, we found that YB-1 is strongly expressed in both the nucleus and cytoplasm of tumor cells in Grades III and IV gliomas, with the YB-1 protein expression levels being significantly lower in the nuclei in controls and in Grades I and II gliomas. These data suggest that the changes in the intracellular distribution and levels of YB-1 in different cell compartments may be related to the

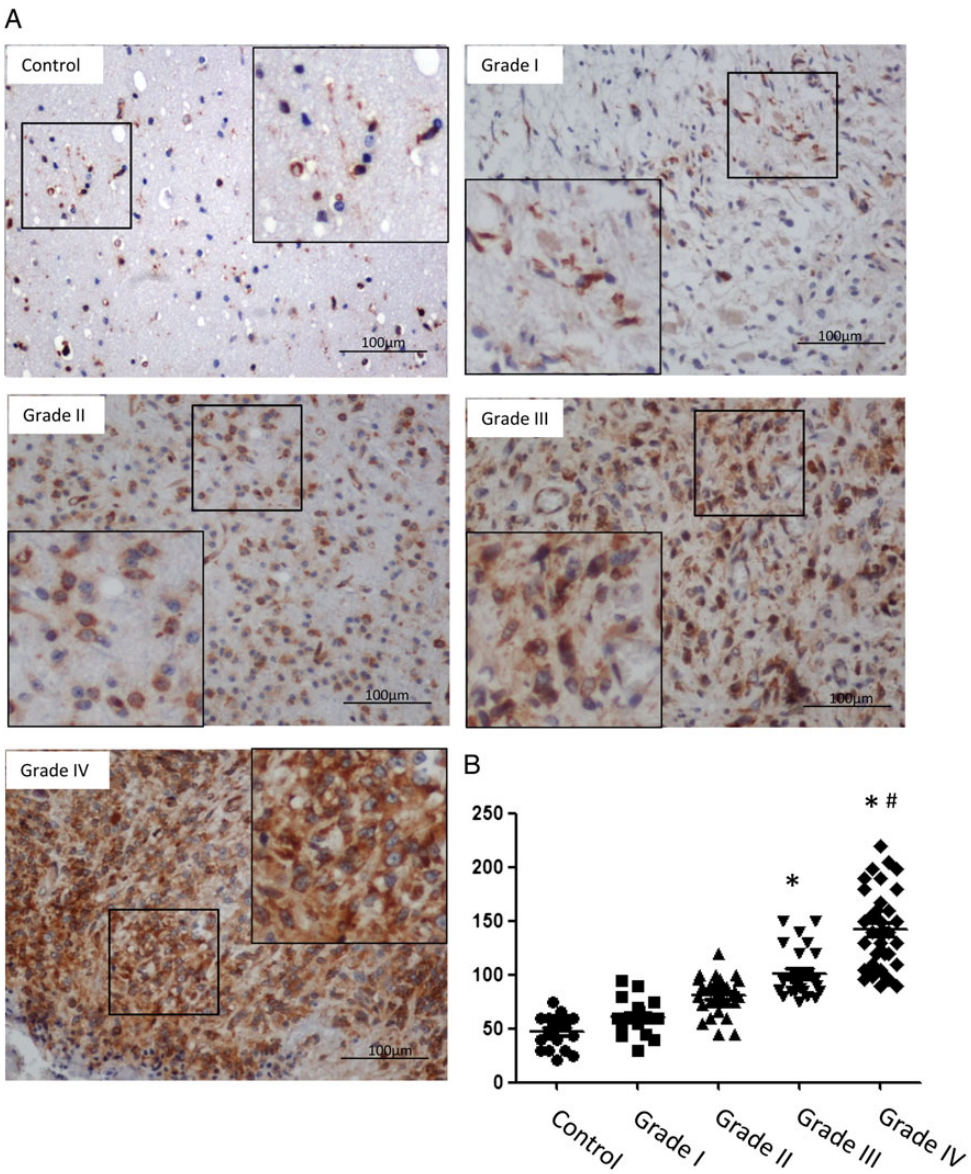

Figure 3. Immunohistochemistry analysis of YB-1 in gliomas (A) YB-1 expression in normal brain tissues and glioma tissues (200×). (B) Measurement of YB-1 staining intensity in glioma of different grades. $^{*}P<0.01$ vs. Grades I and II; $^{\#}P<0.05$ vs. Grades I, II, and III. There are significantly more YB-1-positive cells in Grade IV than in Grades I, II, and III. Scale bar: 100 μm.

progression of glioma. A previous study revealed that the nuclear expression of YB-1 was closely associated with proliferative activity [26]. Furthermore, other studies investigating breast cancer, ovarian cancer, non-small-cell lung cancer, and head and neck cancer specimens have also showed concomitant nuclear YB-1 expression and cytoplasmic localization of YB-1 [15,16,19,27], which is similar to our data.

We demonstrated that YB-1 overexpression in the nucleus and cytoplasm was associated with poor overall survival of patients after surgery. Moreover, we also found that a high intracellular YB-1 level was strongly correlated with glioma malignancy, and that the prevalence of YB-1 in the nucleus was significantly increased in higher-grade gliomas. Therefore, it is reasonable to suggest that the nuclear and cytoplasmic YB-1 proteins have both specific and distinct biological functions.

In the present study, we also found that YB-1 levels were significantly higher in CSF samples obtained from glioma patients than in those from controls. YB-1 protein expression was significantly stronger in the CSF of Grades III and IV glioma patients than in the Grades I and II samples, indicating that YB-1 may be a good CSF biomarker for distinguishing malignant gliomas.

Recently, it has also been found that YB-1 can regulate the cell-cycle progression at G1/S and regulate tumor growth in human osteosarcoma cells both *in vitro* and *in vivo*. The nuclear expression of YB-1 was closely associated with the prognosis of osteosarcoma, suggesting that YB-1 could simultaneously be a potential molecular target and prognostic biomarker for osteosarcoma and associated with clinical outcomes of osteosarcoma [28]. This is consistent with our findings that glioma tissue samples with strong protein expression of YB-1 tended to be of higher pathological grade, and that the average positive expression rates of YB-1 increased gradually up to 47.52 and 49.87% for glioma Grades III and IV, respectively. The Kaplan–Meier analysis and log-rank test showed that patients with Grades III and IV gliomas

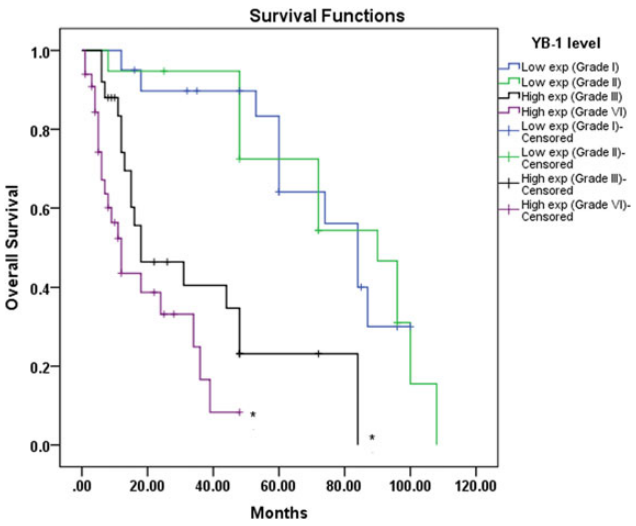

Figure 4. Kaplan–Meier survival curves of glioma patients with strong and weak YB-1 expression The overall survival was significantly worse in patients with high YB-1 levels than in those with low YB-1 levels. *$P<0.05$.

Table 2. Relationship of YB-1 level and clinical diagnosis

Clinic data	Cases	YB-1				Positive rate (%)	*P* value
		(−)	(+)	(++)	(+++)		
Gender							0.064
Male	64	0	23	36	3	41.25	
Female	44	0	25	19	2	34.12	
Age							0.271
<40	34	0	12	12	10	35.69	
≥40	74	0	23	27	24	39.98	
Histopathologic grading							
I	14	0	10	4	0	20.33	Reference
II	31	0	24	7	0	23.94	0.834
III	30	0	8	20	2	47.52	0.0001
IV	33	0	6	24	3	49.87	0.0001

with strong YB-1 expression had a significantly worse overall survival than those of Grades I and II with weak YB-1 expression.

To our knowledge, this is the first study demonstrating the expression patterns and clinical significance of YB-1 at the transcriptional and translational levels in a large number of glioma patients. These findings indicate that YB-1 may be a valuable biomarker for diagnosing malignant gliomas and evaluating the patient prognosis following surgery since increased YB-1 expression in glioma tissues was associated with shorter survival time in patients.

Supplementary Data

Supplementary data is available at *ABBS* online.

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