Original Research

LncRNA TUG1 acts as a tumor suppressor in human glioma by promoting cell apoptosis

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Abstract

Previous studies have revealed multiple functional roles of long non-coding RNA taurine upregulated gene 1 in different types of malignant tumors, except for human glioma. Here, it was designed to study the potential function of taurine upregulated gene 1 in glioma pathogenesis focusing on its regulation on cell apoptosis. The expression of taurine upregulated gene 1 in glioma tissues was detected by quantitative RT-PCR and compared with that in adjacent normal tissues. Further correlation analysis was conducted to show the relationship between taurine upregulated gene 1 expression and different clinicopathologic parameters. Functional studies were performed to investigate the influence of taurine upregulated gene 1 on apoptosis and cell proliferation by using Annexin V/PI staining and cell counting kit-8 assays, respectively. And, caspase activation and Bcl-2 expression were analyzed to explore taurine upregulated gene 1-induced mechanism. taurine upregulated gene 1 expression was significantly inhibited in glioma and showed significant correlation with WHO Grade, tumor size and overall survival. Further experiments revealed that the dysregulation of taurine upregulated gene 1 affected the apoptosis and cell proliferation of glioma cells. Moreover, taurine upregulated gene 1 could induce the activation of caspase-3 and-9, with inhibited expression of Bcl-2, implying the mechanism in taurine upregulated gene 1-induced apoptosis. taurine upregulated gene 1 promoted cell apoptosis of glioma cells by activating caspase-3 and -9-mediated intrinsic pathways and inhibiting Bcl-2-mediated anti-apoptotic pathways, acting as a tumor suppressor in human glioma. This study provided new insights for the function of taurine upregulated gene 1 in cancer biology, and suggested a potent application of taurine upregulated gene 1 overexpression for glioma therapy.

Keywords: Taurine upregulated gene 1, human glioma, cell apoptosis, caspase, Bcl-2

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Introduction

Human glioma is one common malignancy in the central nervous system of brain, accounting for up to 10%,[1] and of which glioblastoma (GBM) is the most aggressive subtype with less than 5% of five-year survival rates.[2] Currently, some advances have been achieved in multimodal treatments, including surgical extirpation, local irradiation and conventional chemotherapy; however, the fatality rate in patients within two years of diagnosis is still high according to recent reports.[3,4] Mounting efforts have been made to explore the complex gene interaction and molecular modulation network involved in the development of glioma for reliable diagnostic markers and effective therapeutic targets.[5,6] However, the mechanism is still far from clearly understood, and the identification of genes or proteins that show potential effect on glioma development is still imperative.

Long non-coding RNAs (LncRNAs), a new class of non-coding RNAs with more than 200 bases have been reported to function in various biologcal process due to their regulation on gene expression.[7] Emerging evidence suggests that LncRNAs show close correlation with diverse malignancies, and act as oncogenes or tumor suppressors in different types.[8] LncRNA taurine upregulated gene 1 (TUG1), with a length of 7.1 kb, was initially detected in a genomic screening study for upregulated genes in retinal cells under taurine treatment.[9] More studies show that p53, one common tumor suppressor, can induce the expression of TUG1, while TUG1 can bind to the PRC2 complex, acting as a repressor for specific genes involved in cell-cycle regulation.[10] And, TUG1 downregulation has been detected in the non-small cell lung carcinoma tissues, showing positive association with advanced tumor development and poor overall survival.[11] These studies imply a role of TUG1 as one potential tumor suppressor by inhibiting cell proliferation and promoting apoptosis. Conversely, however, significantly higher TUG1 expression is reported in osteosarcoma, indicating another potential role of TUG1 as one oncogene,[12] while similar function of TUG1 as cell growth promoter has been also reported in esophageal

squamous cell carcinoma,[13] as well as mouse pancreatic β cells.[14]

Although one latest research has reported the downregulation of TUG1 in doxorubicin-induced necrosis of human glioma cells,[15] the functional role of TUG1 in glioma pathogenesis is still unrevealed. In this study, TUG1 expression was detected and analyzed in glioma tissues with different clinicopathologic parameters. Further *in vitro* studies were performed to identify the functional role of TUG1 as one potential oncogene or tumor suppressor, involving the affected apoptosis and cell proliferation of human glioma cell lines.

Materials and methods

Tissue collection

A total of 120 paired primary glioma tissues and adjacent normal ones were obtained from patients who had undergone surgical treatment at the Affiliated Xuzhou Hospital of College of Medicine of Dongnan University between 2013 and 2014, with written informed consent. And, all patients were free from chemotherapy or radiotherapy before the surgery. All procedures were approved by the Ethics Committee of the Affiliated Xuzhou Hospital.

Cell culture

U251 and SHG-44, two human glioma cell lines, were purchased from Chinese Academy of Science Cell Bank (Shanghai, China). These cells were incubated in the DMEM (Invitrogen, Shanghai, China), supplemented with 10% FBS (Invitrogen), 2 mM glutamine, and 100 U/ml penicillin, at 37°C with 5% CO_2.

Plasmids, small interference RNA, and cell transfection

The full length of human TUG1 cDNA was synthesized and sub cloned into a pCDNA3.1 (Invitrogen) vector, resulting in TUG1-pCDNA for its overexpression. For small interfering RNAs (siRNAs) analysis, three TUG1 siRNAs and negative control siRNA (si-NC) were provided by Invitrogen, and the siRNA sequences targeting the sequence of TUG1 transcript were as follows: siTUG1-1# 5′-GGGAUAUAG CCAGAGAACAAUUCUA-3′; siTUG1-2# 5′-GCUUGGCU UCUAUUCUGAAUCCUUU-3′; siTUG1-3# 5′-CAGCUGU UACCAUUCAACUUCUUAA-3′. Cells were pre-incubated to 40–60% confluence on a six-well plate and then transfected by incubation with plasmids or siRNAs with Lipofectamine 2000 (Invitrogen) following the manufacturer's protocols. At indicated time point post the transfection, cells were harvested for further analysis.

Quantitative real-time PCR

TRIzol reagent (Invitrogen, Grand Island, NY, USA) was used to isolate total RNA from tissues or cultured cells, which was then transcribed into cDNA by the PrimeScript RT Reagent Kit (Takara, Dalian, China) with provided random primers, according to the manufacturers' protocol. Quantitative PCR was performed on ABI 7500 real-time PCR system (Applied Biosystems; Foster, CA, USA) by using the SYBR PrimeScript RT-PCR kit (Takara). The gene expression was quantified by calculating the ΔCT value, and results were normalized to the expression of GAPDH. The sequences of primers used here were as follows: TUG1-F 5′-TAGCAGTTCCCCAATCCTTG-3′, TUG1-R 5′-CACAAATTCCCATCATTCCC-3′; GAPDH-F 5′-GTGT CTGAGCGATGTGGCT-3′, GAPDH-R 5′-GGATTTGGTCG TATTGGGC-3′.

Cell apoptosis analysis

Annexin V-FITC apoptosis detection kit (BD Biosciences; San Jose, CA, USA) was used to analyze cell apoptosis, following the manufacturer's protocols. In brief, cells were collected after the dissociation with EDTA-free trypsin, and then washed with cold phosphate-buffered saline (PBS). Then, cells were resuspended in the binding buffer with the addition of Annexin V-FITC and PI for an incubation of 15 min in the darkness. Finally, flow cytometry analysis was performed immediately on the BD FACSCalibur (BD Biosciences).

Cell proliferation assay

Glioma cells from different groups were seeded at 3×10^4 cells/well and grown in 150 μL cultured medium. Cell viability was assayed by using cell counting kit (CCK)-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's protocols. Briefly, CCK8 reagents were added into corresponding wells at indicated time points and incubated at 37°C for 2 h. The absorbance at 450 nm was recorded with a microwell plate reader (HynergyTM HT, BIO-TEK).

Caspases analysis

Since caspases activation is an important indicator for apoptotic process, the activities of caspase- 3, and -9 were analyzed to explore the pathways during TUG1-induced apoptosis, by using corresponding caspase Colorimetric Assay Kit (KeyGen, China) following the manufacturer's protocols. Briefly, cultured cells at 72 h post-transfection were lysed at 4°C for 30 min, and protein concentration in the supernatant was determined. Samples with equal protein concentration were incubated with provided reaction buffer and respective substrate at 37°C in the darkness for 4 h. The absorbance was measured at 405 nm, and caspase activity was expressed as the ratio of $OD_{405\ nm}$ of transfected cells in that of parental cells.

Statistical analysis

All data were expressed as the mean ± SD or means ± SEM. Student's *t*-test or one-way ANOVA analysis was performed to calculate the statistical significance of the means. The unpaired *t*-test was used to compare the TUG1 expression in groups showing different clinical parameters (WHO grading or tumor size). Survival rate was analyzed with the Kaplan–Meier method, and log-rank tests were used to test the difference between stratified survival groups. *P* value of less than 0.05 was considered as significant difference.

Results

TUG1 downregulation was observed in human glioma tissues and correlated with advanced development and poor prognosis

The expression levels of TUG1 were determined using qRT-PCR in glioma tissues and adjacent normal tissues from 120 glioma patients. The clinical features of these patients are provided in Table 1. In cancerous tissues, the average expression level of TUG1 was 0.036 after normalized to GAPDH, and lower than the value of 0.051 in normal specimens (Figure 1). Further correlation analysis showed that TUG1 downregulation was closely related to advanced pathological stage and larger tumor size (Figure 2(a) and (b)).

The association of TUG1 expression and glioma patient prognosis was also detected following Kaplan–Meier survival analysis and log-rank tests by using patient postoperative survival. On the basis of the mean ratio of relative TUG1 expression in different tumor tissues, total 120 glioma patients were classified into two groups: high TUG1 expression group ($n = 60$, TUG1 expression ratio ≥ 0.036) and low TUG1 expression group ($n = 60$, TUG1 expression ratio ≤ 0.036). According to the results of overall survival, significantly poorer prognosis was indicated in patients with lower TUG1 expression than those with higher TUG1 expression ($P < 0.001$, log-rank tests) (Figure 2(c)). Taken together, TUG1 downregulation might play important roles in human glioma.

TUG1 overexpression contributed to the cell apoptosis of human glioma cells

Since the significant decrease of TUG1 expression in glioma tissues showed positive correlation with poor prognosis, the possible biological significance of TUG1 in tumor genesis was investigated through gain- and loss-of-function studies *in vitro*. Enforced TUG1 overexpression was performed using the TUG1-pCDNA transfection in U251 and SHG-44 cells, with expression level change confirmed by qRT-PCR analysis at 24 h post-transfection. Around 15-fold increase of TUG1 expression was achieved in TUG1 overexpressing cells (Figure 3(a)). Subsequently, Annexin V/PI staining analysis was performed to determine TUG1-induced apoptosis at 48 h post-transfection. And, the percentage of apoptotic cells significantly increased in TUG1 overexpressing cells when compared to respective controls (Figure 3(b) and (c)). These results indicated that TUG1 can induce glioma cell apoptosis *in vitro*.

Table 1 Characteristic of individuals

Variable	Patients
Number of cases	120
Sex	
Male/female	79/41
Age at diagnosis (mean)	49.5
Clinical pathology features	
Extent of resection	
Subtotal	49
Total	71
Radiographic pattern	
Solitary lesion	75
Invasive and multifocal lesions	45
WHO grading	
I–II	57
III–IV	63
Tumor size	
≥3 cm	60
<3 cm	60
KPS score	
≥80	79
<80	41

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

TUG1 inhibition improved the cell proliferation of human glioma cells

To further examine whether TUG1 inhibition could promote glioma cell proliferation, TUG1 knockdown with siRNA transfection was performed in U251 and SHG-44 cells. The expression levels of TUG1 strongly decreased in cells with si-TUG1s transfection compared with si-NC-transfected cells (Figure 3(d)). After transfection for 48 h, the same number of U251 and SHG-44 cells from each group (si-TUG1-1, si-TUG1-2, si-TUG1-3, and si-NC group) was inoculated and undergone CCK8 analysis at indicated time points. The results revealed that inhibition of TUG1 promoted cell proliferation of both U251 and SHG-44 cells (Figure 3(e) and (f)). These data showed that downregulated TUG1 can promote glioma cell proliferation.

TUG1-induced caspases activation, with inhibited BCL-2 expression

In order to further investigate the mechanism underlying TUG1 regulation on glioma cell apoptosis, caspases pathways, involving caspase-3 and -9 activation, were tested in TUG1 overexpressing cells. As shown in Figure 4(a) and (b), significant activation of both caspase-3 and -9, key executors of cell apoptosis, was observed in U251 and SHG-44 cells

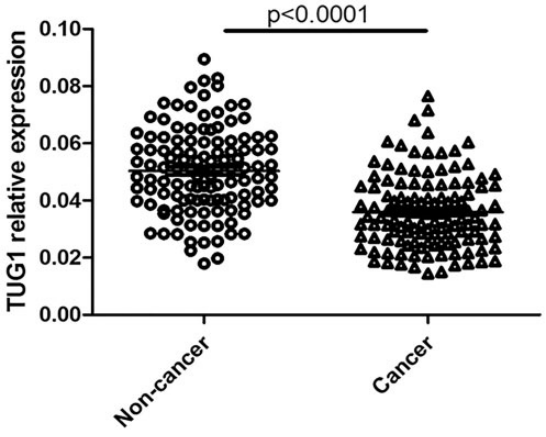

Figure 1 Downregulated TUG1 expression in human glioma tissues. TUG1 expression was analyzed in 120 pairs of human glioma tissues by real-time PCR. The levels of TUG1 in glioma tissues (Cancer) were significantly lower than those in non-tumorous tissues (non-cancer) ($P < 0.0001$)

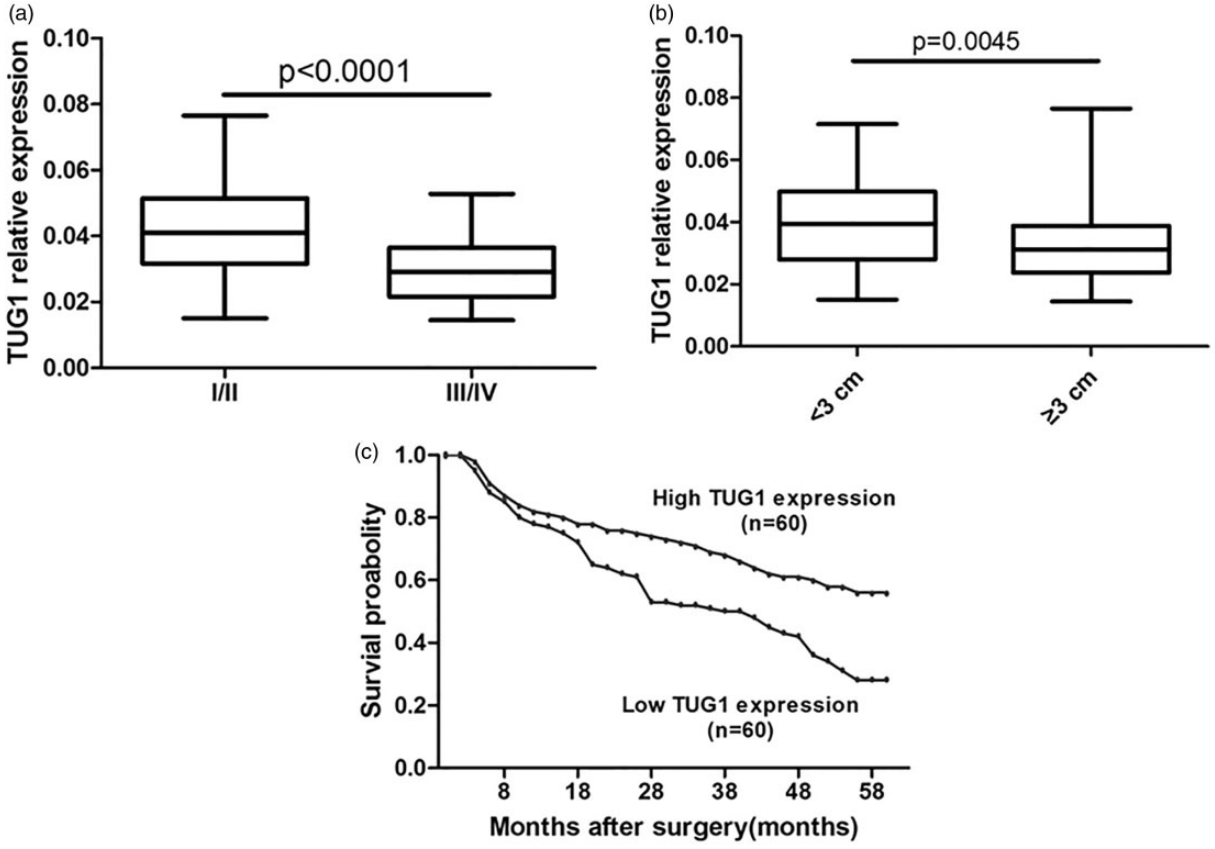

Figure 2 Analysis for TUG1 expression in the clinical parameters of human glioma. The levels of TUG1 were compared in human glioma tissues according to the WHO grading (I/II vs. III/IV; (a) or tumor size (>3 cm vs. <3 cm; (b) The values are reported means ± SEM. TUG1 expression was significantly lower in patients with a higher pathological stage ($P < 0.001$) and big tumor size ($P = 0.0045$). (c) Patients with low levels of TUG1 expression showed reduced survival probability by Kaplan–Meier method, compared with those with high levels of TUG1 expression

72 h after transfection with TUG1-pcDNA, as compared to respective controls. These results suggested that the upregulated expression of TUG1 promoted the apoptosis of glioma cells via the activation of caspase-3 and -9 pathway. Moreover, to examine involvement of Bcl-2, a key anti-apoptotic protein, in TUG1-induced apoptosis, changes in its protein levels was also examined in TUG1 overexpressing cells with western blotting analysis. Significant downregulation of Bcl-2 was achieved under TUG1 overexpression 48 h after transfection (Figure 4(c)). Taken together, it was concluded that the mechanism of TUG1 induced glioma cell apoptosis involves caspase activation and Bcl-2 downregulation, showing anti-tumor like potential.

Discussion

Glioma is the most common malignant brain tumor with remarkable characters like vascular proliferation, aggressive invasion, and poor prognosis.[16] It is difficult to be cured for most glioma patients due to the resistance to conventional therapies, and the median survival time remains approximately 14 months.[17] The dysregulation of TUG1 expression has been reported to influence tumor cell proliferation, apoptosis and invasion in urothelial carcinoma of the bladder, osteosarcoma, non-small cell lung carcinoma and esophageal squamous cell carcinoma,[11–13,18] indicating its potential role as diagnostic marker or therapeutic target. But the study of TUG1 in glioma is very less, let alone its mechanism in glioma tumorigenesis. Of note, a latest research has reported the downregulation of TUG1 in doxorubicin-induced necrosis of human glioma cell lines.[15] Thus, the purpose of this study was to identify whether TUG1 expression was disordered in glioma tissues, or involved in glioma incidence and proliferation.

Firstly, the expression profiles of TUG1 were detected in human glioma tissues and compared normal tissues through qRT-PCR analysis, and results showed decreasing tendency of TUG1 expression in cancer tissues. It was also found that decrease of TUG1 expression showed significant correlation with Grade, tumor size, and KPS scores of glioma, indicating the important role of TUG1 expression in glioma tumorigenesis. Therefore, TUG1 was speculated to negatively regulate glioma cell proliferation, showing anti-tumor like effects. To further explore the functional role of TUG1 in glioma cells, ectopic expression of TUG1 was executed in both U251 and SHG-44 glioma cells, as well as the introduction of three TUG1 siRNAs. The results showed that TUG1 overexpression could promote cell apoptosis, with significant increased apoptotic cells, while downregulated TUG1 expression resulted in increased proliferation of U251 and SHG-44 cells.

Generally, apoptosis can be stimulated by extrinsic pathway, intrinsic or mitochondrial pathway and granzyme B pathway, while the three different pathways will converge

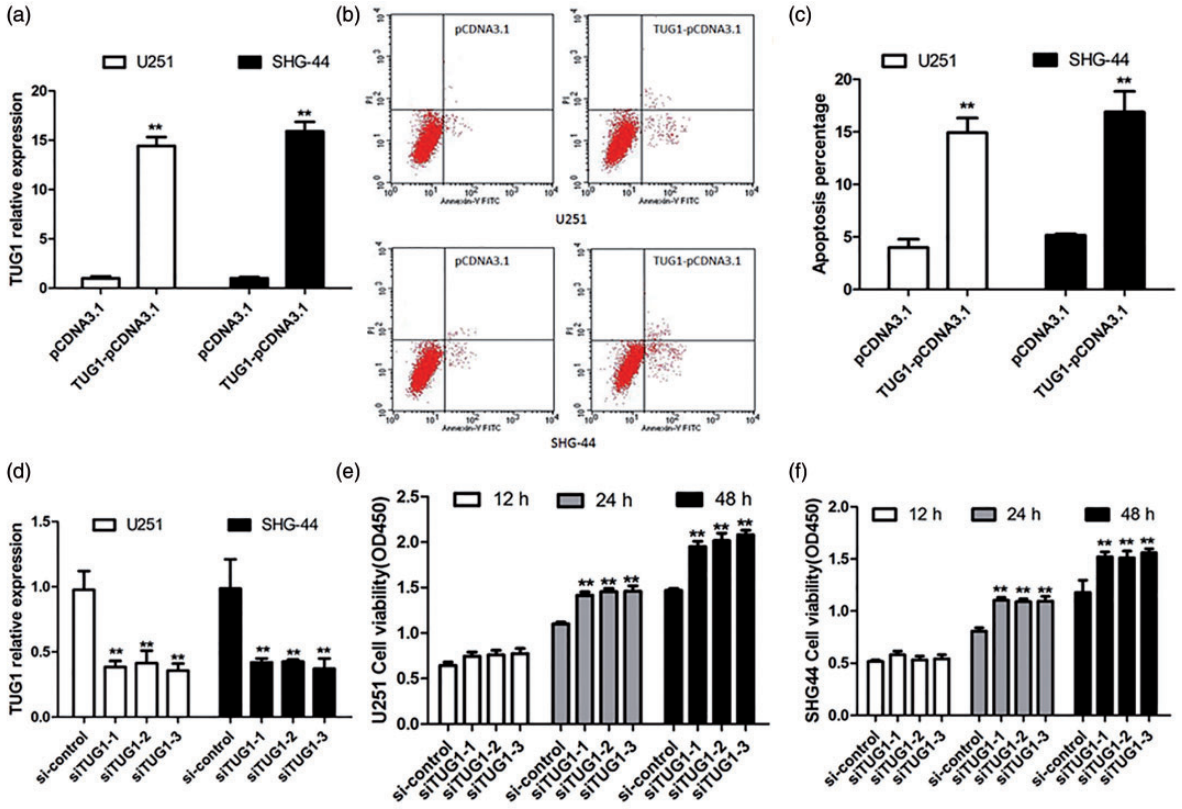

Figure 3 TUG1 affected cell viability of human glioma cells. Induced or inhibited TUG1 expression was conducted in U251 and SHG-44 cells through TUG1 overexpression plasmid (TUG1-pCDNA3.1) or siRNAs (siTUG1) transfection, respectively. Dysregulated TUG1 expression was confirmed by real-time PCR (a) and (d). TUG1-induced cell apoptosis were detected by flow cytometric analysis. The Annexin V-FITC positive cells in the top (PI positive) and bottom (PI negative) right quadrants were induced by TUG1 overexpression (b), and the number of apoptotic cells was also shown here (c). Besides, increased cell viability was detected in TUG1 inhibiting cells after transfection for 12, 24, or 48 h through CCK8 analysis (e) and (f). $^{**}P < 0.01$. (A color version of this figure is available in the online journal.)

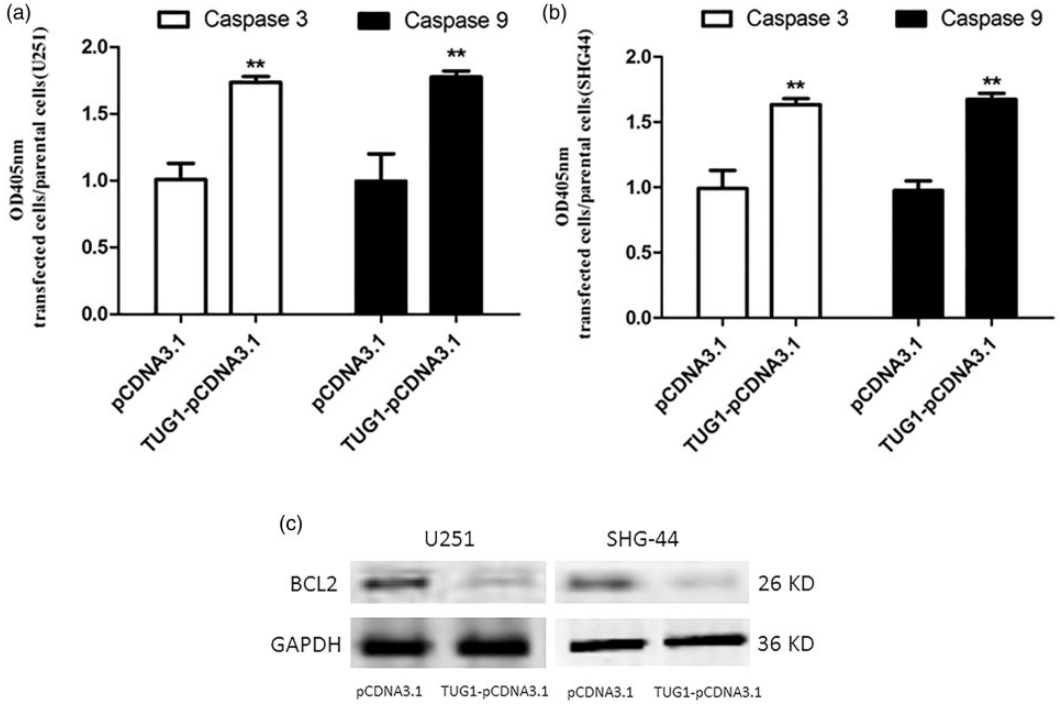

Figure 4 TUG1 promoted the caspases activation with inhibited Bcl-2 expression in human glioma cells. The activation of caspases, including caspase-3 and caspase-9, was detected using colorimetric method in TUG1 overexpressing cells after TUG1-pCDNA3.1 transfection, with normal parental cells as control (a) and (b). And, Bcl-2 expression was analyzed at the same time through western blot analysis. (c) Results showed significantly activated caspases, as well as decrease of Bcl-2 expression, by TUG1 overexpression. $^{**}P < 0.01$

to a same execution phase that needs the caspases-3 and/or -7 activation.[19,20] Through cascade reactions and/or death receptors function, cytochrome c is released from mitochondria, leading to cell death. Thus, caspase activation was also investigated here to get insights for TUG1-induced apoptosis. And, obvious activation of both caspase-3 and -9 was observed in TUG1 overexpressing glioma cells, suggesting that TUG1 promoted glioma cell apoptosis via intrinsic pathways mediated by caspase-3 and -9.[21] Besides, the expression level of Bcl-2, one anti-apoptotic Bcl-2 family member, was also detected by western blotting, and data showed significant decrease of Bcl-2 level in cells with TUG1 overexpression. Therefore, it was speculated that TUG1 promoted glioma cell apoptosis via the caspase-3 and -9-mediated intrinsic pathway, involving inhibited anti-apoptotic pathways that mediated by Bcl-2.[22] The precise mechanisms will require further study and elucidation.

In conclusion, TUG1 expression was significantly repressed in human glioma tissues, while this inhibition showed significant correlation with WHO Grade, tumor size, and overall survival, implying the important role of TUG1 in glioma tumorigenesis. Further gain- and loss-of-function studies revealed that the dysregulation of TUG1 affected apoptosis and cell proliferation in glioma cells. Moreover, mechanism studies for TUG1 induced apoptosis revealed the involvement of caspase 3- and -9-mediated intrinsic pathway, as well as Bcl-2-mediated anti-apoptotic pathways. This study provided new insight for TUG1 function in carcinogenesis, and suggested a potent therapeutic tool in preventing glioma by TUG1 overexpression. Further research could be performed to explore the precise mechanism between TUG1 and cell proliferation, as well as invasion, in glioma development.

Authors' contribution: LJ was responsible for the main conceive of the study and the draft of the manuscript, and carried out most molecular genetic studies. ZM carried out the immunoassays and participated in the sequence alignment. AG helped to design the study and performed the statistical analysis. MQ helped to revise the manuscript and participated in its design. All authors have read and approved the final manuscript.

DECLARATION OF CONFLICTING INTERESTS

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