Original Paper

Enhanced Expression of TGFBI Promotes the Proliferation and Migration of Glioma Cells

Shi-Kun Guo Ming-Feng Shen Hong-Wei Yao Yong-Sheng Liu

Neurosurgery, First People's Hospital of Shangqiu, Shangqiu, China

Key Words

TGFBI • Glioma • Proliferation • Migration • PI3K/Akt signaling pathway

Abstract

Background/Aims: Transforming growth factor beta-induced protein (TGFBI) is an extracellular matrix protein induced by TGF- β . Previous studies have reported that the abnormal expression of TGFBI is related to the occurrence and development of some types of cancers, while the role of TGFBI in glioma is uncertain. **Methods:** The association between TGFBI expression and the prognosis of patients with glioma was analyzed based on data obtained from The Cancer Genome Atlas database. TGFBI expression was analyzed in 3 normal human brains and 57 cases of human gliomas by immunohistochemistry followed by an evaluation of the relationships between TGFBI expression and clinic-pathological features. Furthermore, the RNA interference plasmid pSUPER-shTGFBI was constructed and transfected into U87 and U251 cells to explore the effect of short hairpin RNA against TGFBI (shTGFBI) on cell proliferation, migration, invasion and apoptosis. Western blot analysis was performed to examine the expression of proteins related to apoptosis and proteins in the PI3K/Akt signaling pathway. **Results:** High TGFBI expression was found to be associated with poor prognosis in patients with glioblastoma multiforme. Immunohistochemistry showed that TGFBI expression was significantly higher in glioma tissue than in normal human brain tissues. The expression level of TGFBI showed no significant correlation with age, sex, lymph-node metastasis, or pathological grade. sh-TGFBI could inhibit proliferation, invasion and migration and induce apoptosis in U87 and U251 cells *in vitro*. Furthermore, the phosphorylation levels of AKT and mTOR declined significantly in sh-TGFBI transfected U87 and U251 cells when compared with control. **Conclusion:** TGFBI was up-regulated in glioma cells and played a promoting role in the growth and motility of U87 and U251 cells. These results suggested that TGFBI has the potential to be a diagnostic marker and to serve as a target for the treatment of gliomas.

© 2018 The Author(s)
Published by S. Karger AG, Basel

Introduction

Glioma is the most common tumor of the primary central nervous system and has a high mortality rate [1]. Glioblastoma multiforme (GBM) (World Health Organization [WHO] grade IV astrocytoma) is the most aggressive subtype among malignant gliomas and is often associated with a poor prognosis [2, 3]. Lower grade gliomas (LGGs, WHO grade II/III) are a heterogeneous group of relatively slowly growing primary tumors that originate from astrocytes and/or oligodendrocytes [2, 4]. Although LGGs are less aggressive than GBM, the prognosis of patients with LGG varies widely [5]. At present, the clinical treatment for glioma is mainly surgery combined with radiotherapy and chemotherapy [6]. Although great progress has been made in the efficacy of the current treatment regimen, GBM patients still have a poor prognosis [7, 8]. Therefore, there is an urgent need to study the molecular biological mechanism of glioma development and explore new approaches and drugs to diagnose and treat gliomas [9].

Transforming growth factor beta-induced protein (TGFBI), also known as betaig-3, is an extracellular matrix protein induced by TGF- β [10]. This protein contains 4 fasciclin-1 domains and has been reported to inhibit tumor growth and promote apoptosis [11, 12]. TGFBI has been demonstrated to have a variety of physiological functions, with most reports describing its role in corneal dystrophy [13]. However, its precise role is still unclear. TGFBI expression is diverse in different cancer tissues. In ovary carcinoma [14], neuroblastoma [15], lung [16] and breast cancers [17], decreased TGFBI expression has been reported, while TGFBI expression is up-regulated in pancreas [18], colon [19], kidney [20], and glioma [21, 22] tumors. These observations suggest that TGFBI may play an anti- or pro-tumor role in a tissue dependent manner. To date, only a few studies have examined the effect of TGFBI protein in glioma cells and its mechanism is obscure.

In the present study, we investigated TGFBI expression in gliomas by bioinformatics analysis and immunohistochemistry. Furthermore, the effects of TGFBI on the proliferation, migration, invasion and apoptosis of U87 and U251 glioma cells were explored by RNA interference. Finally, the potential mechanism of its action was explored by western blot analysis. We found that TGFBI expression was up-regulated in glioma and short hairpin RNA against TGFBI (shTGFBI) could inhibit the growth and motility of U87 and U251 glioma cells by inhibiting the PI3K/Akt signaling pathway.

Materials and Methods

Database and Human tissue samples

The expression data of TGFBI were collected from The Cancer Genome Atlas (TCGA) database (<http://tcga-data.nci.nih.gov>), including 207 normal human brain tissue samples, 163 GBM cancer tissue samples and 518 LGG cancer tissue samples. The process of data collection complies with all laws and regulations.

The brain glioma tissue microarray used in this study was purchased from Shanghai Outdo Biotech Company. The tissue microarray contains 60 samples, including 3 normal human brains and 57 cases of human gliomas (Astrocytoma and Glioblastoma). All samples obtained were approved by local ethics committee.

Immunocytochemistry

In order to observe the expression of TGFBI in glioma tissues and normal human brain tissues, immunocytochemistry was performed using EliVision TM plus kit (KIT-9902). At first the samples were dewaxed and hydrated. Then the slices were immersed in the antigen repair solution and heated to boiling by microwave (98°C -100°C-) for two times. Subsequently, 3% hydrogen peroxide was added to the slices and incubated at room temperature for 10 min followed by washing with ddH₂O and incubating with tris buffered saline (TBS) for 3 times (5 min each). Then they were blocked by 5% normal goat serum in TBS-Tween for 1h at room temperature. Sections were incubated with the primary antibody for 1h at room temperature and washed by TBS for 3 times. The sections were added with reagent A and incubated at

room temperature for 20 min followed by washing with PBS for 3×5 min. Then sections were incubated with reagent B for 20 min and washed with PBS for 3×5 min. Next, DAB reagent was added dropwise and observed under a microscope at room temperature for 5 min followed by washing with ddH₂O. Then hematoxylin re-dyeing, tap water rinsing, TBS back to blue, ethanol dehydration, xylene transparent, neutral resin seal were performed successively. Normal human brain was used as control. The results were observed under a microscope (OPTIKA B-150, ITALY).

In order to quantify immunohistochemical staining, the slides were reviewed by two independent pathologists using a microscope. Immunohistochemical positive staining of TGFBI was calculated as color intensity. The staining intensity was graded as “0” (absent), “1” (weak), “2” (moderate), and “3” (strong). The expression of TGFBI was assessed by staining index (SI). SI = proportion score × intensity score. SI of 0 was categorized as negative (–), 1–2 as low expression, 3 as high expression.

Cell culture

Cerebral glioma cell lines, U87 and U251, were obtained from the Cell Bank, Chinese Academy of Science, Shanghai, China. They were cultured in Dulbecco's modified Eagle's medium (DMEM) medium (Hyclone) at 37°C with 5% CO₂. When the cells entered logarithmic growth phase, single cell suspensions were prepared and planted into 6-well plates for subsequent experiments.

Construction of pSUPER - shTGFBI plasmid

TGFBI interference sequence 5'GGTAACGGCCAGTACACGC3' was synthesized. Bgl II and Hind III cleavage site were added at the forward and afterword of this sequence, respectively. Then the genes were digested with Bgl II and Hind III and ligated into pSUPER plasmid. The constructed pSUPER - shTGFBI plasmid was verified by double enzyme digestion and sequencing. The scrambled shRNA (5'-GCGTGTACTGGCCGTTACC-3') was used as the negative control (shNC).

Transfection

When the cells in the six-well plate grew to logarithmic period, the media was replaced with fresh complete culture medium two hours before transfection. Cells were transfected with pSUPER-shTGFBI or pSUPER-shNC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following manufacturer's description. Then the cells were cultured at standard conditions for 6 hours. Subsequently, the culture medium was replaced with fresh complete culture medium again and continued to incubate for 48 hours for subsequent experiments.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA of U87 cells and U251 cells was extracted using Ultrapure RNA kit (CwBio, Beijing, China) according to manufacturer's description. Reverse transcription was performed to form cDNA using HiFiScript cDNA Synthesis Kit (CwBio, Beijing, China) in accordance with manufacturer's instructions. Real-time PCR was performed using an ABI 7500 system (Applied Biosystems, Carlsbad, CA, USA). TGFBI was amplified using the SYBR Premix Ex Taq II kit (TAKARA Bio Inc.) according to manufacturer's protocol. 5'TGCTCCACAAATGAAGCCT3' and 5'GCCTCCGCTAACCAGGATT3' (Genewiz Beijing, China) were used as the forward and reverse primers, respectively. The amplification was performed as the following procedure: 95 °C for 30 sec, then for 40 cycles of 5 sec at 95 °C, 34 sec at 60 °C. The relative expression level of TGFBI was calculated by 2^{-ΔΔC} method. β-actin was used as the internal reference gene. All the experiments were performed in triplicates and repeated for 6 independent times.

Western blotting

The cells were placed on ice after transfected for 48 h, and lysed by RIPA lysate (plus protease inhibitor) (Com Win Biotech Co, Ltd, Beijing, China) for protein extracting. The concentration of the protein was measured using BCA Protein Assay Kit (Com Win Biotech Co, Ltd, Beijing, China). The proteins (20 μg) were heated at 95°C for 5 min and separated by 10% SDS polyacrylamide gel electrophoresis. Subsequently, the separated proteins were transferred to a PVDF membrane (Millipore, Bedford, MA), blocked by 5% skimmed milk for 1h. Subsequently, the membranes were incubated with the rabbit anti-human primary antibody (anti-TGFBI, anti-AKT, anti-p-AKT, anti-mTOR, anti-p-mTOR, anti-Bcl2, anti-bax, anti-active-caspase3, anti-p70 and anti-GAPDH) overnight at 4°C. All the primary anti-bodies were diluted at 1:1000,

except anti- GAPDH (1:5000). After washing with TBST for 3 times $\times$ 5min, the membranes were incubated with secondary antibody (goat anti-rabbit, 1:5000) at room temperature for 1 h and washed by TBST again. Finally, signals detection was done by using enhanced chemiluminescence (ECL) plus detection kit (Thermo Fisher Scientific, Inc.).

Proliferation assays and colony formation assays

Cell counting kit-8 (CCK8) was used to analyze the proliferation of U87 and U251 cells. After 24 h of transfection, the cells were digested and cell suspensions were prepared. One hundred microliters of cell suspensions (1000 cells each well) were seeded into 96-well plates and cultured under standard conditions. Cell viability was measured at 24 h intervals. The optical density (OD) at 450 nm was detected by microplate reader after adding 10 μ l CCK8 chemical (Solarbio, Beijing, China) to each well and incubating at 37 °C for 1.5 h. Graph Pad Prism 5 was used for drawing the proliferation curves.

For colony formation assays, 500 cells were added into a 60mm dish containing 5 ml pre-incubated medium. The medium was replaced every 3 days. After culturing for a week at 37°C, the cells were fixed with 4% paraformaldehyde and stained using 0.1% crystal violet dye. Finally, the visible colonies were counted directly. All the experiments were repeated for 6 independent times.

Invasion and migration assay

Transwell invasion assays were performed as the follows. Firstly, 100 μ l overnight melted matrigel (diluted with serum-free medium at a ratio of 1: 6) (BD Biosciences, Franklin Lakes, NJ, USA) was added to the upper transwell chamber (Millipore, Bedford, MA) and incubated at 37°C for 4 h. Secondly, the liquid in the chamber was removed and the upper and lower chamber were added with 100 μ l or 600 μ l serum-free 1640 medium, respectively. Afterwards, the chamber was kept static at 37°C for 30min and sipping up the medium suction medium. Thirdly, suspensions of cells transfected for 24 h were prepared and added to the upper chamber (1×10^4 cells/well). The lower chambers were added with 600 μ l culture medium containing 10% FBS. Subsequently, the chamber was incubated at 37°C for 24 h and the cells remaining on the upper chamber were wiped by cotton swab. The invaded cells were fixed with formaldehyde and stained by 0.1% crystal violet dye. Finally, cells were counted under a light microscope. Migration assay was similar to invasion assay, but matrigel is not required. A number of 5000 cells were added to each well. All the invasion and migration assays were performed in triplicate and repeated for 5 independent times.

Flow apoptotic assay

After transfection for 24 h, the cells were digested by trypsin without EDTA and collected through centrifuge (1000rpm, 5min) followed by washing with precooled PBS and collected again. The cells were resuspended with binding buffer to a cell density of $1-5 \times 10^6$ cells /ml. Annexin V-FITC/PI kit was used to perform the Annexin V-FITC and PI double staining and quantifying the number apoptotic cells. Following staining, the samples were analyzed by flow cytometry immediately.

Statistical analysis

The values are shown as Mean $\pm$ SD. Student's t-test was performed to compare experimental group with control group. It was considered as statistically significant when $P < 0.05$. Pearson's chi-square test was used to analyze the association between TGFBI expression in glioma and clinic-pathological features. The online Kaplan-Meier plotter was used to assess the effect of TGFBI expression on the survival of glioma patients. Log-rank test was performed to test the significant differences between high-expression and low-expression groups. Correlation between TGFBI and TGFBR2 expression was analyzed by spearman-correlation test based on the data from TCGA database.

Results

TGFBI expression is up-regulated in glioma and correlated with poor prognosis in patients with GBM based on TCGA derived data

TCGA was used to analyze TGFBI expression. As shown in Fig. 1A, TGFBI expression levels were significantly higher in both GBM and LGG cancer tissues compared with normal brain tissue ($p < 0.05$). This result indicated that TGFBI might play a promoting role in the formation of glioma.

The prognostic values of TGFBI expression in GBM and LGG patients was explored, and survival curves were drafted based on data obtained from TCGA. Patients were dichotomized into high TGFBI and low TGFBI groups by the median expression level of TGFBI. GBM patients with high TGFBI expression had the worst overall survival ($p = 0.0079$) (Fig. 2A), indicating that high TGFBI expression was associated with poor prognosis in patients with GBM. No significant correlation was observed between high TGFBI expression and disease free survival in GBM ($p = 0.2$) (Fig. 2B). While in LGG patients, TGFBI expression was not significantly correlated with overall survival ($p = 0.086$) (Fig. 2C) or disease-free survival ($p = 0.86$) (Fig. 2D). These results further suggest that TGFBI may play a role in promoting the development of GBM.

Expression of TGFBI in glioma patients and the association between TGFBI expression and clinicopathological features

Immunohistochemical staining was performed to analyze TGFBI expression in glioma tissues obtained from the Shanghai Outdo Biotech Company. As shown in Fig. 1B and -C, TGFBI expression was significantly higher in glioma tissue than in normal human brains ($p < 0.05$).

The correlation between TGFBI expression and clinical parameters was analyzed based on microarray data. However, there was no significant correlation between TGFBI expression and the clinic-pathological features of glioma patients, including age, sex, lymph-node metastasis, and pathological grade (Table 1).

Suppression of TGFBI inhibits the proliferation of U87 and U251 cells

To explore further the effect of TGFBI in glioma, the RNA interference plasmid pSUPER-shTGFBI was constructed and transfected into glioma cell lines (U87 and U251 cells). According to qPCR, pSUPER-shTGFBI #4# was found to induce the strongest inhibition of

Fig. 1. The expression of TGFBI in Glioma. (A) Analysis of TGFBI expression based on TCGA dataset. The red and gray boxes represent normal and cancer tissues respectively. GBM, Glioblastoma multiforme; LGG, Low Grade Glioma. (B) Immunohistochemical staining for TGFBI protein in normal human brain tissues and gliomas. (C) Quantification of immunohistology. Columns, mean ($n=57$); bars, SD. ** $P < 0.01$, vs. non-tumor tissues.

Fig. 2. Prognostic value of TGFBI in glioma. Overall survival curves (A, C) and Disease free survival curves (B, D) for GBM ($n=162$) and LGG ($n=512$) patients were drafted, respectively. The median of TGFBI expression was used as the cutoff point of high TGFBI and low TGFBI. * $p<0.05$.

TGFBI expression in U251 cells, so it was selected for the following experiments (Fig 3A). Moreover, TGFBI knockdown by pSUPER – shTGFBI #4# was also demonstrated at the protein level by western blot analysis (Fig. 3B). The effects of shTGFBI on the proliferation of U87 and U251 cells were tested using a Cell Counting Kit-8. The OD_{450} values of both U87 and U251 cells transfected with shTGFBI were decreased significantly compared with their corresponding controls at 48 h and 72 h ($p<0.05$), suggesting that shTGFBI showed a suppressive effect on the proliferation of U87 and U251 cells (Fig. 3C, D). Subsequent colony formation assays showed that the number of colonies was greatly decreased after depleting TGFBI expression in U87 and U251 cells ($p < 0.05$, Fig. 3E, F). These data indicated that TGFBI play a promoting role in the proliferation of glioma cells.

shTGFBI inhibits the invasion and migration of U87 and U251 cells

Cell invasion and migration were investigated by Transwell invasion and migration assays with or without Matrigel. The results showed that the numbers of crystal violet staining positive cells in the shTGFBI-treated groups (both U87 and U251 cells) was significantly decreased compared with the corresponding shNC group ($p < 0.05$, Fig. 4). These data suggested that shTGFBI can suppress the invasion and migration of U87 and U251 cells.

shTGFBI induces apoptosis in U87 and U251 cells

We explored whether shTGFBI could induce apoptosis in U87 and U251 cells by an annexin V-FITC and PI double staining method.

Table 1. Relationship between TGFBI expression and clinicopathological features of glioma patients. Staining index (SI) of 0 was categorized as negative (-), 1–2 as low expression, 3 as high expression. $p < 0.05$ was considered as statistically significant. *In some cases, the clinical pathological information is not complete, so the total cases of Grades, Pathological Classification and lymph-node metastasis were less than 60 cases

Clinicopathologic feature	Cases (n)	TGFBI level		P
		High-expression (n)	Low-expression (n)	
Age				
≤60	18	8	10	0.3001
> 60	15	10	5	
Sex				
Male	19	9	10	0.654
Female	14	7	7	
Lymph-node metastasis				
With	13	8	5	0.8548
Without	10	4	6	
Pathological grade				
I - II	11	9	2	0.1333
III	12	5	7	

Fig. 3. shTGFBI inhibits the proliferation of U87 and U251 cells. (A) TGFBI mRNA expression levels in shTGFBI transfected U251 cells. (B) Western blot analysis confirmed the down-regulation of TGFBI after transfected with shTGFBI in U251 cells. (C, D) Proliferation of U87 and U251 cells transfected with shTGFBI were determined using cell counting kit-8. Cell proliferation was tested at 24 h intervals. (E, F) Colony formation assays were performed to further explore the proliferation of transfected U87 and U251 cells. The data are presented as means $\pm$ SD ($n=6$). * $P < 0.05$, vs. negative control group (shNC).

As shown in Fig. 5A, the percentage of U87 cells undergoing apoptosis (including the early and late stages) in the shTGFBI group (13.84%) was significantly increased compared with the shNC group (4.58%) ($p < 0.05$). Similarly, the ratio of U251 cells undergoing apoptosis (including the early and late stages) in the shTGFBI group was 14.03%, which was also significantly increased compared with the shNC group (5.00%) ($p < 0.05$) (Fig. 5B). These observations demonstrated that shTGFBI could promote apoptosis in U87 and U251 glioma cells and proved the inhibitory effect of shTGFBI on the proliferation of glioma cells.

To better understand the mechanism of shTGFBI induced apoptosis, the expression of the apoptosis-related proteins Bcl2, Bax and active caspase-3 were investigated by western

Fig. 4. shTGFBI inhibit U87 and U251 cell invasion and migration. (A, B) Transwell invasion and migration images of U87 and U251 cells, (10×10), respectively. (C, D) Quantitative analysis of the invaded and migrated cells. Columns, mean ($n = 5$); bars, SD. * $P < 0.05$, vs. negative control group (shNC). * $P < 0.05$, vs. negative control group (shNC).

Fig. 5. shTGFBI induced apoptosis in U87 and U251 cells. Fluorescence-activated cell sorting (FACS) analysis of U87 (A) and U251 (B) cells transfected with shTGFBI or shNC. (C) Western blot analysis of apoptosis-related proteins in shTGFBI transfected U87 and U251 cells. (D, E) Quantification of Bcl-2, Bax and Active-Caspase3 expression levels. Columns, mean ($n = 6$); bars, SD. * $P < 0.05$, vs. negative control group (shNC).

blot analysis. As observed in Fig. 5C-E, the level of the anti-apoptotic protein Bcl2 was down-regulated significantly in both U87 and U251 cells transfected with shTGFBI. On the other hand, the expression of the pro-apoptotic proteins Bax and caspase-3 was increased significantly in U87 and U251 cells transfected with shTGFBI compared with controls. Hence, shTGFBI promoted the apoptosis of U87 and U251 cells possibly by down-regulating the expression of Bcl2 and up-regulating Bax and caspase 3 expression.

shTGFBI suppresses the activation of the PI3K signaling pathway in glioma cells

The PI3K/Akt signaling pathway, as an important cellular signal transduction pathway, plays a key role in inhibiting apoptosis and promoting proliferation by influencing the activity of downstream effector molecules. Hence, we explored whether the PI3K/Akt signaling pathway was involved in shTGFBI induced apoptosis by western blot analysis. The expression levels of AKT and mTOR were not significantly changed in U87 and U251 cells transfected with shTGFBI compared with controls, while the levels of p-AKT and p-mTOR were significantly reduced. Moreover, the expression of the downstream proteins p70 and cyclin-D1 in the PI3K/Akt signaling pathway was also down-regulated accordingly (Fig. 6). These observations indicated that shTGFBI induced apoptosis in glioma cells by suppressing the activation of the PI3K signaling pathway.

TGFBR2 expression in glioma

Transforming growth factor beta receptor II (TGFBR2) is a transmembrane serine/threonine protein kinase receptor in the TGF- β signaling pathway [23]. We analyzed the correlation between TGFBI and TGFBR2. The correlation coefficient between TGFBI and TGFBR2 was 0.51 and 0.36 in GBM and LGG, respectively (Fig. 7A). TGFBR2 expression was significantly higher in both GBM and LGG tissues compared with normal brain tissues ($p < 0.05$, Fig. 7B), which was consistent with the expression of TGFBI. High TGFBR2 expression showed no significant correlation with overall survival ($p = 0.96$) or disease-free survival ($p = 0.058$) in GBM (Fig. 7C). While in LGG patients, high TGFBR2 expression was found to be associated with a worse prognosis (Fig. 7D).

Fig. 6. Effect of shTGFBI on the level of AKT, p-AKT, mTOR, p-mTOR and p70. (A, C) Western blot analysis of the expression levels of AKT, p-AKT, mTOR, p-mTOR and p70 in U87 and U251 cells, respectively. (B, D) Quantification of p-AKT and p-mTOR expression levels in U87 and U251 cells, respectively. Columns, mean ($n = 6$); bars, SD. * $P < 0.05$, vs. negative control group (shNC). The data are presented as means $\pm$ SD.

Fig. 7. (A) Correlation between TGFBI and TGFBR2 expression was analyzed by spearman-correlation test. (B) Analysis of TGFBR2 expression based on TCGA dataset. The red and gray boxes represent normal and cancer tissues respectively. (C, D) Prognostic value of TGFBR2 in GBM (n=162) and LGG (n=514), respectively. GBM, Glioblastoma multiforme; LGG, Low Grade Glioma.

Discussion

TGFBI was first identified in a cDNA library of TGF- β -treated human A549 lung ADC cells. It is a secretory protein induced by TGF- β that mediates cell anchoring to the extracellular matrix [24]. Studies have shown that the absence or low expression of TGFBI in some tissues promotes tumorigenesis, while in some tissues, its overexpression promotes cell proliferation and lead to tumorigenesis [25]. WC Lam [26] demonstrated that p110 δ knock-down leads to the downregulation of TGFBI expression in U87 GBM cells, indicating that TGFBI may involve in the invasion and migration of U87 GBM cells. However, until now, there was only limited information on the role of TGFBI in glioma cells and its mechanism of action is still unclear.

In this study, we found that TGFBI expression was up-regulated significantly in both GBM and LGG based on the analysis of expression data from TCGA. Furthermore, through immunocytochemistry, we confirmed the up-regulation of TGFBI in glioma cells. The expression level of TGFBI showed no significant correlation with age, sex, lymph- node metastasis, or pathological grade (Table 1). Furthermore, high TGFBI expression was found to be associated with a worse prognosis in GBM patients. However, no significant correlation between TGFBI expression and overall survival was observed in LGG patients. We found that sh-TGFBI could inhibit the proliferation, invasion and migration and induce apoptosis in U87 and U251 cells by suppressing the PI3K/Akt signaling pathway *in vitro*.

Lin et al. [27] found that TGFBI expression was increased at the RNA and protein levels in GBM tissues compared with normal tissues. Our bioinformatics analysis and immunohistochemistry staining results further confirmed the higher expression of TGFBI in GBM and LGG tissues than in normal tissues. Ivanov [20] found the up-regulation of TGFBI expression in renal clear cell carcinoma and Schneider [18] observed that the mRNA level of TGFBI was clearly increased in pancreatic cancer cells. Our results are consistent with these previous findings and provide clinical information to support them.

A major cause of death in cancer patients is metastasis, a complex process involving cell adherence, migration and invasion [28]. Our study found that U87 and U251 cells transfected with shTGFBI both showed a decrease in cell migration and invasion compared with control

cells. Shang [29] found that TGFBI could effectively elevate the ability of renal carcinoma cells to adhere, migrate, and invade. Collectively, these data suggested that TGFBI may play a promoting role in the metastasis of cancer cells.

Many studies have demonstrated that the PI3K-Akt signaling pathway is often disturbed in human tumors [30, 31], suggesting that this pathway is closely related to the development of tumors. Thus, it has been considered as an appealing target for the development of novel anti-tumor agents [32]. In the present study, we found that the levels of p-AKT and p-mTOR were decreased significantly in U87 and U251 cells transfected with shTGFBI compared with controls. These results demonstrated that shTGFBI may suppress the proliferation and induce apoptosis in U87 and U251 cells by inhibiting the PI3K/Akt signaling pathway. Furthermore, inhibiting TGFBI expression might lead to the suppression of the PI3K/Akt signaling pathway.

As an important member of the TGF- β signaling pathway, TGFBR2 is often associated with many cancers [33]. By analyzing the correlation between TGFBI and TGFBR2, we found they had a certain correlation and both were overexpressed in GBM and LGG (Fig 6). However, their relationships with prognosis were different. The detailed relationship between both of these genes need further exploration.

Conclusion

TGFBI was overexpressed in both GBM and LGG, and high TGFBI expression was often associated with poor prognosis in GBM. Through an RNA interference approach, we found that shTGFBI inhibited proliferation, migration, and invasion and promoted apoptosis by suppressing the PI3K/AKT signaling pathway in U87 and U251 cells, demonstrating that TGFBI plays a promoting role in the growth and motility of U87 and U251 cells. Our research indicated that TGFBI has the potential to serve as a target for the treatment of gliomas.

Acknowledgements

Shi-Kun Guo, Hong-Wei Yao and Yong-Sheng Liu assisted in carrying out the laboratory work. Shi-Kun Guo also contributed in writing and revising the manuscript and legends of the biological results. Ming-Feng Shen contributed in suggesting the point of the research and modifying the paper.

Disclosure Statement

The authors declare to have no competing interests.

References

- 1 Kim YW, Liu TJ, Koul D, Tiao N, Feroze AH, Wang J, Powis G, Yung WK: Identification of novel synergistic targets for rational drug combinations with PI3 kinase inhibitors using siRNA synthetic lethality screening against GBM. *Neuro Oncol* 2011;13:367-375.
- 2 Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW, Kleihues P: The 2007 WHO Classification of Tumours of the Central Nervous System. *Acta Neuropathol* 2007;114:97-109.
- 3 Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL: An integrated genomic analysis of human glioblastoma multiforme. *Science* 2016;321: 1807-1812.
- 4 Reon BJ, Anaya J, Zhang Y, Mandell J, Puro B, Abounader R, Dutta A: Expression of lncRNAs in Low-Grade Gliomas and Glioblastoma Multiforme: An In Silico Analysis. *PLoS Med* 2016;13:e1002192.

- 5 Stupp R, Baumert BG: Promises and controversies in the management of low-grade glioma. *Ann Oncol* 2003;14:1695-1696.
- 6 Zhang J, Zhang J, Zhang J, Qiu W, Xu S, Yu Q, Liu C, Wang Y, Lu A, Zhang J, Lu X: MicroRNA-625 inhibits the proliferation and increases the chemosensitivity of glioma by directly targeting AKT2. *Am J Cancer Res* 2017;7:1835-1849.
- 7 Wang Y, Jiang T: Understanding high grade glioma: molecular mechanism, therapy and comprehensive management. *Cancer Lett* 2013;331:139-146.
- 8 Bush NA, Chang SM, Berger MS: Current and future strategies for treatment of glioma. *Neurosurg Rev* 2017;40:1-14.
- 9 Sui A, Xu Y, Li Y, Hu Q, Wang Z, Zhang H, Yang J, Guo X, Zhao W: The pharmacological role of histone demethylase JMJD3 inhibitor GSK-J4 on glioma cells. *Oncotarget* 2017;8:68591-68598.
- 10 Thapa N, Lee BH, Kim IS: TGFBIp/betaig-h3 protein: a versatile matrix molecule induced by TGF-beta. *Int J Biochem Cell B* 2007;39:2183.
- 11 Zhao YL, Piao CQ, Hei TK: Overexpression of Betaig-h3 gene downregulates integrin alpha5beta1 and suppresses tumorigenicity in radiation-induced tumorigenic human bronchial epithelial cells. *Brit J Cancer* 2002;86:1923-1928.
- 12 Skonier J, Neubauer M, Madisen L, Bennett K, Plowman GD, Purchio AF: cDNA cloning and sequence analysis of beta ig-h3, a novel gene induced in a human adenocarcinoma cell line after treatment with transforming growth factor-beta. *DNA Cell Biol* 1992;11:511-522.
- 13 Runager K, Enghild JJ, Klintworth GK: Focus on molecules: Transforming growth factor beta induced protein (TGFBIp). *Exp Eye Res* 2008;87:298-299.
- 14 Ahmed AA, Mills AD, Ibrahim AE, Temple J, Blenkiron C, Vias M, Massie CE, Iyer NG, McGeoch A, Crawford R, Nicke B, Downward J, Swanton C, Bell SD, Earl HM, Laskey RA, Caldas C, Brenton JD: The extracellular matrix protein TGFBI induces microtubule stabilization and sensitizes ovarian cancers to paclitaxel. *Cancer Cell* 2007;12:514-527.
- 15 Becker J, Erdlenbruch B, Noskova I, Schramm A, Aumailley M, Schorderet DF, Schweigerer L: Keratopithelin suppresses the progression of experimental human neuroblastomas. *Cancer Res* 2006;66:5314-5321.
- 16 Zhao Y, Elgabry M, Hei TK: Loss of Betaig-h3 protein is frequent in primary lung carcinoma and related to tumorigenic phenotype in lung cancer cells. *Molecular Carcinogen* 2006;45:84-92.
- 17 Wen G, Partridge MA, Li B, Hong M, Liao W, Cheng SK, Zhao Y, Calaf GM, Liu T, Zhou J, Zhang Z, Hei TK: TGFBI expression reduces *in vitro* and *in vivo* metastatic potential of lung and breast tumor cells. *Cancer Lett* 2011;308:23-32.
- 18 Schneider D, Kleeff J, Berberat PO, Zhu Z, Korc M, Friess H, Buchler MW: Induction and expression of betaig-h3 in pancreatic cancer cells. *Biochim Biophys Acta* 2002;1588:1-6.
- 19 Kitahara O, Furukawa Y, Tanaka T, Kihara C, Ono K, Yanagawa R, Nita ME, Takagi T, Nakamura Y, Tsunoda T: Alterations of gene expression during colorectal carcinogenesis revealed by cDNA microarrays after laser-capture microdissection of tumor tissues and normal epithelia. *Cancer Res* 2001;61:3544-3549.
- 20 Ivanov SV, Ivanova AV, Salnikow K, Timofeeva O, Subramaniam M, Lerman MI: Two novel VHL targets, TGFBI (BIGH3) and its transactivator KLF10, are up-regulated in renal clear cell carcinoma and other tumors. *Biochem Biophys Res Commun* 2008;370:536-540.
- 21 Golembieski WA, Rempel SA: cDNA array analysis of SPARC-modulated changes in glioma gene expression. *J Neuro-Oncol* 2002;60:213-226.
- 22 Colin C, Baeza N, Bartoli C, Fina F, Eudes N, Nanni I, Martin P, Ouafik L, Figarellabranger D: Identification of genes differentially expressed in glioblastoma versus pilocytic astrocytoma using Suppression Subtractive Hybridization. *Oncogene* 2006;25:2818.
- 23 Lei J, Rudolph A, Moysich KB, Rafiq S, Behrens S, Changclaude J: Assessment of variation in immunosuppressive pathway genes reveals TGFBR2 to be associated with prognosis of estrogen receptor-negative breast cancer after chemotherapy. *Breast Cancer Res* 2015;17:18.
- 24 Zhu J, Chen X, Liao Z, He C, Hu X: TGFBI protein high expression predicts poor prognosis in colorectal cancer patients. *Int J Clin Exp Pathol* 2015;8:702-710.
- 25 Pajares MJ, Agorreta J, Salvo E, Behrens C, Wistuba II, Montuenga LM, Pio R, Rouzaut A: TGFBI expression is an independent predictor of survival in adjuvant-treated lung squamous cell carcinoma patients. *Brit J Cancer* 2014;110:1545.

- 26 Lam WC: Validation of gene expression TGFBI & TIMP1 induced by p110 δ isoform of class IA phosphoinositide 3-kinase (PI3K) in glioblastoma multiforme. 2013.
- 27 Lin B, Madan A, Yoon JG, Fang X, Yan X, Kim TK, Hwang D, Hood L, Foltz G: Massively parallel signature sequencing and bioinformatics analysis identifies up-regulation of TGFBI and SOX4 in human glioblastoma. PLoS One 2010;5:e10210.
- 28 Sternlicht MD, Werb Z: How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev B 2001;17:463.
- 29 Shang D, Liu Y, Yang P, Chen Y, Tian Y: TGFBI-promoted adhesion, migration and invasion of human renal cell carcinoma depends on inactivation of von Hippel-Lindau tumor suppressor. Urology 2012;79:961-967.
- 30 Porta C, Paglino C, Mosca A: Targeting PI3K/Akt/mTOR Signaling in Cancer. Front Oncol 2014;4:64.
- 31 Fangjun L, Zhijia Y: Tumor suppressive roles of eugenol in human lung cancer cells. Thorac Cancer 2018;9:25-29.
- 32 Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB: Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov 2005;4:988-1004.
- 33 Sivadas V P, Kannan S: TGFBR2 (Transforming Growth Factor, Beta Receptor II (70/80kDa)). Atlas Genet Cytogenet Oncol Haematol 2014;18:737-745.