RNA interference-mediated knockdown of translationally controlled tumor protein induces apoptosis, and inhibits growth and invasion in glioma cells

HUA JIN1,2, XUEXIN ZHANG2, JUN SU2, YUEQIU TENG3, HUAN REN1 and LI ZHUANG YANG4

1Department of Immunology, Harbin Medical University, Harbin, Heilongjiang 150081; 2Department of Neurosurgery, The Third Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang 150040; 3Stem Cell Research Institute, The First Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang 150001; 4Department of Neurosurgery, The Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang 150081, P.R. China

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Abstract. Translationally controlled tumor protein (TCTP) is a highly conserved, growth-associated and small molecule protein, which is highly expressed in various types of tumor cell. TCTP can promote the growth and suppress apoptosis of tumor cells. However, few studies have reported the effects of TCTP in gliomas. In the present study, a glioma cell line was established, which was stably transfected with TCTP short hairpin ribonucleic acid (shRNA), to investigate the impact of downregulated expression of TCTP on the proliferation, apoptosis and invasion of glioma cells. Western blot and reverse transcription-quantitative polymerase chain reaction analyses demonstrated that TCTP shRNA effectively reduced the expression of TCTP in the U251 glioma cell line. MTT and colony formation assays revealed that downregulated expression of TCTP significantly inhibited glioma cell proliferation. Cell cycle analysis using flow cytometry revealed that the cells in the pRNA-H1.1-TCTP group were arrested in the G0/G1 phase of the cell cycle. Western blot analysis detected downregulated expression levels of cyclins, including Cyclin D1, Cyclin E and Cyclin B. Annexin V-fluorescein isothiocyanate/propidium iodide and Hoechst staining demonstrated that the apoptotic rate of the cells in the pRNA-H1.1-TCTP group was significantly higher than that of the cells in the pRNA-H1.1-control group, with upregulated expression levels of B-cell-associated X protein and cleaved-caspase-3 and downregulated expression of B-cell lymphoma-2 in the apoptotic process. Wound healing and Transwell assays revealed that downregulated expression of TCTP significantly inhibited the migration and invasiveness of the glioma cells; and the expression levels and activities of matrix metalloproteinase (MMP)-2 and MMP-9 were also significantly affected. In conclusion, the present study demonstrated that downregulated expression of TCTP significantly inhibited proliferation and invasion, and induced apoptosis in the glioma cells. These results suggested that TCTP may be important in glioma development and metastasis. Therefore, TCTP is expected to become an effective target for glioma gene therapy.

Introduction

Glioma is the most common type of malignancy in the central nervous system (1), accounting for more than half of all intracranial tumors, with an incidence rate of 6.5/100,000 individuals (2) and an average survival duration of 14 months (3). The five-year survival rate of malignant glioma is <5% (4). Glioma is characterized by rapid growth, high levels of invasiveness, frequent postoperative recurrence and high mortality rates. Although the range of comprehensive treatments for glioma, including surgical resection, radiotherapy and chemotherapy, have been improving, the therapeutic effect remains inadequate (5,6). Therefore, clarifying the mechanisms of glioma invasion and metastasis, and determining effective treatment methods, remains a focus of investigation.

Translationally controlled tumor protein (TCTP) is a highly conserved hydrophilic protein, which was identified in 1989 (7). The expression levels of TCTP in a variety of tumor cells are markedly higher, compared with those in normal tissues (8-11). Previous studies have indicated that TCTP is important in the regulation of cell proliferation, cell cycle, malignant metastasis and anti-apoptotic effects (12-16). Additionally, TCTP is associated with the development and progression of glioma (17). In vitro and in vivo experiments have demonstrated that abnormally high expression levels of TCTP in glioma cells can promote cell proliferation, and
that this promotion of proliferation can be eliminated by downregulation of the expression of TCTP expression (18).

The expression of TCTP is also closely associated with tumor deterioration and the sensitivity of tumor cells to drugs (19). The over-expression of TCTP in cells has been observed to significantly inhibit 5-fluorouracil (5-Fu)-induced apoptosis of ovarian cancer and osteosarcoma cells. Following silencing of the expression of TCTP using an antisense oligonucleotide, the sensitivity of U2OS osteosarcoma cells to 5-Fu is enhanced, and the apoptotic rate is significantly increased (20,21). However, the role of TCTP in the occurrence and development of glioma remains to be fully elucidated and further investigation is required.

In the present study, the expression of TCTP in glioma cells was downregulated using RNAi to investigate its effects on the proliferation, apoptosis, metastasis and invasion of the glioma cells, and to examine the associated mechanisms. This investigation suggested that TCTP may be a potential target for the treatment of glioma.

Materials and methods

Cell lines. The U251, A172, U87-MG and SHG-44 human glioma cell lines were purchased from the Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The U373 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The U251, U373, A172 and U87-MG cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, GE Healthcare, Logan, UT, USA) at 37°C and 5% CO₂. The SHG-44 cells were cultured in RPMI-1640 medium (Gibco Life Technologies) containing 15% FBS (GE Healthcare) at 37°C and 5% CO₂.

Construction of a TCTP short hairpin (sh)RNA eukaryotic expression plasmid and screening for a stably transfected cell line. The TCTP interfering sequences were designed, according to the TCTP mRNA sequence in GenBank using shRNA designing software, as shown in Table I. The obtained interfering sequences (Wanleibio, Shenyang, China) were ligated into the pRNA-H1.1 eukaryotic expression vector (GenScript, Nanjing, China) using the restriction sites of HindIII and BamHI, and the resulting plasmid was termed pRNA-H1.1-TCTP. U251 cells in the logarithmic growth phase were seeded into 6-well plates. pRNA-H1.1-TCTP was transfected into the U251 cells using Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). After 24 h, complete DMEM, containing 400 µg/ml G418 (Invitrogen Life Technologies) was added into each well for screening for 7-14 days, and the clones exhibiting positive expression of TCTP were selected and identified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting. In the process of the experiment, an untransfected group (parental) and an empty vector-transfected group (pRNA-H1.1-control) were set up as the controls.

Western blot analysis. Following lysis of the cells using NP-40 lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China), the total cellular proteins were extracted, and the protein concentrations were determined using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). Equal quantities of proteins (40 µg) were subjected to 10 or 12% SDS-PAGE, and the proteins were then transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Bedford, MA, USA), followed by incubation overnight at 37°C with rabbit anti-human poly clonal antibodies against TCTP antibody (1:200; cat. no. sc-133131; Santa Cruz biotechnology Inc., Santa Cruz, CA, USA), cyclin D1 antibody (1:1,000; cat. no. WL0205), cyclin E antibody (1:1,000; cat. no. WL0055), cyclin B antibody (1:1,000; cat. no. WL0023), Bax polyclonal antibody (1:1,000; cat. no. WL0101), Bcl-2 antibody (1:1,000; cat. no.WL0104), cleaved-caspase-3 antibody (1:1,000; cat. no. WL0146), MMP-2 antibody (1:1,000; cat. no. WL0657), MMP-9 antibody (1:1,000; cat. no. WL0884) or β-actin antibody (1:1,000; all from Wanleibio, Shenyang, China) at 4°C. Subsequently, the membrane was incubated with the corresponding horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody at a 1:5,000 dilution (Beyotime Institute of Biotechnology) for 45 min at 37°C. Signals were detected using enhanced chemiluminescence (ECL) solution (Qihai Biotec, Shanghai, China) and the band intensities were normalized against those of β-actin using Gel-Pro-Analyzer version 4.0 software (Media Cybernetics, Inc., Bethesda, MD, USA).

RT-qPCR. Total cellular RNA was extracted from the cells in each group, strictly according to the instructions of the total RNA extraction kit (Tiangen Biotech, Co., Ltd., Beijing, China). The cDNA (20 µl) was obtained by RT using M-MLV Reverse Transcriptase (BioTeke, Beijing, China), and quantitative fluorescence analysis was performed using SYBR Green MasterMix (10 µl; Solarbio, Beijing, China) was used to fix the cells for proliferation, apoptosis, metastasis and invasion of the glioma cells, and to examine the associated mechanisms. This investigation suggested that TCTP may be a potential target for the treatment of glioma.

MTT assay for cell proliferation. Cells in the logarithmic growth phase were harvested and seeded into 96-well plates at a density of 3x10⁴ cells/well. Following culture of the cells for 12, 24, 48, 72 and 96 h, MTT solution (final concentration, 0.2 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added into the each well. The cells were continuously cultured at 37°C for 4 h, and the supernatant was then discarded. The purple crystals were dissolved using 200 µl dimethyl sulfoxide (Sigma-Aldrich), and the optical density (OD₅₇₀) values were measured using a microplate reader (ELX-800; Bio-Tek Instruments Inc, Winooski, VT, USA). A cell growth curve was plotted and statistical analysis was performed.

Colon formation assay. The cells in each group were seeded (200 cells/dish) into a 35 mm petri-dish for culture. Subsequent to the formation of colonies, 4% paraformaldehyde (Guoyao, Shenyang, China) was used to fix the cells for 20 min, followed
Table I. shRNA sequences.

| Primer          | Sequence (5’-3’)                              |
|-----------------|-----------------------------------------------|
| **TCTP shRNA**  |                                               |
| Forward         | GATCCCCGATGTCAGTACGAAGCATGATCTGCTAATGACCATCTTT |
| Reverse         | AGCTAAAAAGATGGCTAGTAGCAAGATCTCTTGAATTCTGAGCT |
| **Control shRNA**|                                              |
| Forward         | GATCCCCCTTCCTCCGAACGTCAGTTCAGTTCTAAGAGAACGG |
| Reverse         | AGCTAAAAATTCCTCCGAACGTCAGTTCCTGACAGAAGAGG |

TCTP, translationally controlled tumor protein; shRNA, short hairpin ribonucleic acid.

Table II. Sequences of the primers used for quantitative polymerase chain reaction.

| Primer          | Sequence (5’-3’)                              |
|-----------------|-----------------------------------------------|
| **TCTP**        |                                               |
| Forward         | GCCGTGCCTGCTCTCCCTCTCA                       |
| Reverse         | ACCGGTCGGCGATCTCCCCT                        |
| **β-actin**     |                                               |
| Forward         | CTTAGTTGCGTTAACCCTCTTTG                     |
| Reverse         | CTGTACACCTTTCACCACGTCCGTT                    |

by Wright-Giemsa staining (Jiancheng Bioengineering Institute, Nanjing, China) for 5-8 min. The number of colonies, defined as a cell group with ≥50 cells, were counted and recorded under an inverted microscope (AE31; Motic Electric, Xiamen, China). The colony formation rate was then calculated according to the following formula: Colony formation rate = (number of colonies / number of seeded cells) x 100%.

Detection of the cell cycle using flow cytometry. The cells were cultured in DMEM medium and were harvested on reaching ~90% confluence. The cell cycle was detected using a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA, USA) using a Cell Cycle Analysis kit (Beyotime Institute of Biotechnology). In brief, the cells were harvested, washed with phosphate-buffered saline (PBS), and fixed with pre-cooled 70% ethanol at 4°C for 2 h. The fixed cells were then resuspended in 500 µl binding buffer, containing 25 µl propidium iodide (PI) and 10 µl RNase A, at 37°C in the dark for 30 min, and detected using flow cytometry.

Hoechst staining. A Hoechst staining kit (Beyotime Institute of Biotechnology) was used to observe the apoptotic nuclear morphology of the cells. Briefly, cells in the logarithmic growth phase were seeded onto cell slides in 12-well plates, at a density of 1x10^5 cells per well, and were cultured for 24 h. Then, the supernatant was discarded. The cells were fixed with 0.5 ml fixing solution at room temperature for 20 min and were then treated with Hoechst staining solution for 5 min. Anti-fluorescence quenching mounting solution (Beyotime Institute of Biotechnology) was added dropwise onto the slide, which was then covered with the coverslip containing the cells. The cells were visualized and images were captured under a fluorescence microscope (Olympus BX61; Olympus Corporation, Tokyo, Japan).

Detection of apoptosis using an Annexin V-FITC/PI assay. The cells were seeded into T25 cell culture flasks. When they had reached 90% confluence, the cells were harvested. The cells were resuspended in 500 µl Binding Buffer, according to the instructions of the apoptosis detection kit (KeyGEN Biotech, Co., Ltd., Nanjing, China). 5 µl Annexin V-FITC and 5 µl PI were immediately added with thorough mixing. Following 15 min incubation at 37°C in the dark, the cells were detected using flow cytometry (FACSCalibur; BD Biosciences).

Wound healing assay. The cells in each group were seeded into 6-well plates for culture. At a confluence of 80-90%, the culture medium was discarded. Subsequently, a scratch was created on the cell layer using a sterile 200 µl pipette tip. Images of the cells in each group were captured (AE31; Motic Electric) following being washed twice with serum-free medium. After 12 and 24 h of continuous culture, the migration distances of the cells were measured, and the cell migration rates were calculated using the following formula: Cell migration rate = (1 - distance following healing / distance prior to healing) x 100%. Each experiment was repeated three times.

Transwell assay. Following harvesting, 200 µl cell suspension containing 2x10^4 cells (resuspended in serum-free culture medium) were seeded in upper Transwell chambers (Corning Incorporated Life Sciences, Tewksbury, MA, USA), which were coated with Matrigel (BD Biosciences), while 800 µl DMEM medium containing 30% FBS was added to the lower chamber. Following culture for 24 h, the uninjured cells on the upper side of the microporous membrane were removed using a cotton swab. The remaining cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet staining solution (Amresco, Solon, OH, USA). The numbers of invaded cells were recorded under an inverted microscope (AE31; Motic Electric) in five randomly-selected fields (magnification, x200), to obtain the average.
Figure 1. Establishment of a glioma cell line stably transfected with TCTP shRNA. (A) Expression levels of TCTP in the U373, U251, A172, U87-MG and SHG-44 glioma cell lines were compared using western blot analysis, with β-actin as the internal control. Each experiment was repeated three times. *P<0.01 for U251 cells compared with U373 cells. (B) Protein expression of TCTP was detected using western blot analysis, with grayscale analysis using β-actin as an internal control. (C) mRNA expression of TCTP was detected using reverse transcription-quantitative polymerase chain reaction analysis. The data are presented as the mean ± standard deviations. **P<0.01, compared with the pRNA-H1.1-control group. TCTP, translationally controlled tumor protein; shRNA, short hairpin ribonucleic acid.

Statistical analysis. The data are presented as the mean ± standard deviation. One-way analysis of variance was used for comparisons between groups, and Bonferroni's post hoc-test was used for the comparison of multiple variables. The image and data were processed using Graphpad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Establishing and identifying the glioma cell line stably transfected with TCTP shRNA. In the present study, total proteins were extracted from the U373, U251, A172, U87-MG and SHG-44 cells to analyze the expression of TCTP in these cells. The results of the western blotting revealed that the expression levels of TCTP in the A172, U87-MG and SHG-44 cells were essentially the same as that in the U373 cells (Fig. 1A; P>0.05), while the level of expression in the U251 cells was significantly higher than that in the U373 cells (P<0.01). Thus, U251 cells with high expression levels of TCTP were selected to investigate TCTP gene function. The TCTP shRNA eukaryotic expression plasmid, pRNA-H1.1-TCTP, was subsequently transfected into the U251 cells. Western blot and RT-qPCR analyses revealed that the protein and mRNA expression levels of TCTP in the pRNA-H1.1-TCTP group were significantly decreased, and were 0.28-fold (Fig. 1B; P<0.01) and 0.18-fold (Fig. 1C; P<0.01) lower than that in the pRNA-H1.1-control group, respectively. Thus, a U251 glioma cell line stably transfected with TCTP shRNA was successfully established.

Downregulation of the expression of TCTP suppresses glioma cell proliferation. The impact of the downregulated expression of TCTP on glioma cell proliferation was investigated. The MTT results demonstrated that at 48, 72 and 96 h, cell proliferation in the pRNA-H1.1-TCTP group was significantly lower, compared with that in the pRNA-H1.1-control group (Fig. 2A; P<0.05). The effect of downregulated expression of TCTP on the clonogenic capacity of glioma cells, detected using a colony formation assay, is shown in Fig. 2B. The colony formation rate of the cells in the pRNA-H1.1-TCTP group was significantly lower than that in the pRNA-H1.1-control group (32.0±4.9, vs. 60.3±6.9%, respectively; P<0.01). The present study further detected the cell cycle distribution using flow cytometry. The percentage of G2/M phase cells in the pRNA-H1.1-TCTP group was significantly reduced, compared with that in the pRNA-H1.1-control group (0.56±0.08, vs. 8.97±0.94%), respectively; Fig. 2C; P<0.01). The percentage of S phase cells was essentially unchanged (26.17±2.48, vs. 27.00±2.23%), while the percentage of G0/G1 phase cells was significantly increased (73.27±2.47, vs. 63.03±2.11%; P<0.01). Western blotting revealed that the expression levels of Cyclin D1, Cyclin E and Cyclin B in the pRNA-H1.1-TCTP cells were significantly reduced (Fig. 2D; P<0.01). These results suggested that downregulation of the expression of TCTP caused cell cycle arrest in the G0/G1 phase and ultimately inhibited glioma cell proliferation.

Downregulation of the expression of TCTP effectively induces the apoptosis of brain glioma cells. The apoptosis
expression levels of apoptosis-associated factors were analyzed using flow cytometry and Hoechst staining. The Hoechst staining revealed that the nuclei of the cells in the parental group and pRNA-H1.1-control group were homogeneous blue. Typical apoptotic morphological changes, including condensed chromatin and shrunken, crumpled and condensed nuclei, were observed in the pRNA-H1.1-TCTP group. The apoptotic rate of the cells in the pRNA-H1.1-TCTP group was significantly higher than that in the pRNA-H1.1-control group (29.6±3.03, vs. 6.36±0.79%, respectively; Fig. 3A; P<0.01). Flow cytometry consistently indicated that the apoptotic rate of the cells in the pRNA-H1.1-TCTP group was significantly higher than that in the pRNA-H1.1-control group (24.60±2.68, vs. 6.88±0.86%, respectively; Fig. 3B; P<0.01). Western blot analysis demonstrated that the expression levels of Bax and cleaved-caspase-3 in the cells in the pRNA-H1.1-TCTP group were significantly increased (Fig. 3C; P<0.01), whereas the expression of Bcl-2 was significantly decreased (P<0.01). These results suggested that downregulation of the expression of TCTP effectively induced apoptosis in the glioma cells.

**Downregulation of the expression of TCTP inhibits the migration and invasion of glioma cells.** The present study investigated the impact of downregulated expression of TCTP on glioma cell migration. The cell migration rate of the cells was examined using a wound healing assay, the results of which revealed that the migration rate of the cells in the pRNA-H1.1-TCTP group 12 h (Fig. 4A; P<0.01) and 24 h (P<0.05) post-wounding were significantly lower than those in the pRNA-H1.1-control group. The effect of TCTP shRNA on the invasiveness of the glioma cells was further detected using a Transwell assay, the results of which revealed that the number of invaded cells in the pRNA-H1.1-TCTP group was significantly lower than that in the pRNA-H1.1-control group (32.40±6.07, vs. 73.40±9.04), respectively; Fig. 4B; P<0.01). Western blot analysis revealed that the protein expression levels of MMP-2 and MMP-9 in the cells in the pRNA-H1.1-TCTP group were significantly lower than those in the pRNA-H1.1-control group (Fig. 4C; P<0.01). Gelatin zymography revealed bands at 72 and 92 kDa (corresponding to MMP-2 and MMP-9, respectively) in the cells of all groups. Additionally, the activities of MMP-2 and MMP-9 in the cells of the pRNA-H1.1-TCTP group were significantly lower than those in the pRNA-H1.1-control group (Fig. 4D; P<0.01).
decreased (Fig. 4D; P<0.01). These results demonstrated that downregulation of the expression of TCTP inhibited glioma cell migration and invasion.

**Discussion**

TCTP is widely expressed in several types of tumor cell and is important in cell cycle regulation, malignant metastasis and anti-apoptosis (23). However, its role in the malignant metastasis of gliomas remains to be fully elucidated. In the present study, the expression levels of TCTP in various glioma cell lines were detected using western blotting. To investigate the role of TCTP on glioma cell proliferation, cell cycle regulation, apoptosis and invasion, and the associated mechanism, high expression levels of TCTP in the U251 cells was downregulated using shRNA.

An important difference between cancer cells and normal cells is that the growth and division of cancer cells is out of control, and the mechanism of programmed cell death is lacking. The overexpression and downregulation of TCTP can affect tumor cell proliferation (24,25). Studies have reported that hepatoma cell proliferation is significantly inhibited and cell cycle is arrested in the G0/G1 phase following silencing of TCTP using an antisense oligonucleotide (26), and the arrest
of the cell cycle is considered to be a predominant reason for the inhibition of tumor cell growth (27-29). The present study investigated the impact of downregulated expression of TCTP on glioma cell proliferation and the cell cycle. The results demonstrated significantly decreased cell proliferation following downregulation of TCTP. Flow cytometric analysis
determined that the cell cycle was arrested in the G0/G1 phase and that the expression levels of Cyclin D1, Cyclin E and Cyclin B in the cells were significantly lower, compared with those in the control group. This indicated that TCTP regulated the cell cycle by regulating the expression of cell cycle proteins, thereby promoting glioma cell proliferation, which is consistent with the results of previous studies (24-26).

It has been demonstrated that adenovirus vector-driven overexpression of TCTP can effectively resist apoptosis in HeLa cells through the mitochondrial pathway, induced by the chemotherapy drug etoposide, while the activities of cytochrome c, caspase-3 and caspase-9 are simultaneously inhibited (30). TCTP can anchor to the mitochondrial membrane to inhibit Bax dimerization, thereby exerting an anti-apoptotic effect through the inhibition of mitochondrial injury (31). Following silencing of the expression of TCTP using a small interfering RNA in breast cancer, squamous cell carcinoma, prostate cancer and lung cancer cells, tumor cell proliferative capability has been observed to decrease, and apoptosis significantly increase (20,32-34). The results of the present study also demonstrated that, following downregulation of the expression of TCTP in glioma cells, the expression levels of Bax and cleaved-caspase-3 increased, while the expression of Bel-2 decreased, suggesting that high expression levels of TCTP in glioma cells may exert an anti-apoptotic role by inhibiting Bax and cleaved-caspase-3, and activating Bel-2, thereby promoting glioma onset and progression.

The metastasis and invasion of tumors is closely associated with their degree of malignancy. MMP-2 and MMP-9 can degrade a variety of extracellular matrices to function in tumor metastasis and invasion (35), which are important in tumor metastasis (36). Previous studies have demonstrated that, following downregulation of the expression of TCTP in colon cancer cells, the abilities of cell proliferation, migration and invasiveness also decrease (37,38). The present study also observed that TCTP is closely associated with glioma cell migration and invasion, as downregulation of TCTP significantly inhibited glioma cell migration and invasion. In addition, the expression and activity levels of MMP-2 and MMP-9 were significantly decreased. These results indicated that TCTP may facilitate glioma cell migration and invasion through MMP-2 and MMP-9.

In conclusion, shRNA-mediated downregulation of the expression of TCTP in brain glioma cells effectively inhibited glioma cell proliferation, promoted apoptosis and inhibited cell migration and invasion. These findings demonstrate the importance of TCTP in the regulation of proliferation, cell cycle, apoptosis and invasion of glioma cells, and offer preliminary evidence for the mechanism underlying its interaction with cell cycle proteins, MMPs and downstream apoptosis-associated factors, and provide a theoretical basis and experimental evidence for using TCTP as a target for gene therapy in glioma.

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