TMC01 expression Promotes Cell Proliferation and Induces Epithelial-mesenchymal Transformation in Human Gliomas

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Abstract

Transmembrane and coiled-coil domains 1 (TMCO1) is a recently discovered transmembrane protein of endoplasmic reticulum (ER), which plays a critical role in maintaining calcium homeostasis. TMCO1 dysfunction has been proved to be closely related to a variety of human diseases, including glaucoma, deformities, mental retardation and tumorigenesis. However, the role of TMCO1 in gliomas remains unclear. The purpose of this study was to detect the role of TMCO1 in the pathogenesis and progression of gliomas. This study demonstrated that TMCO1 was up-regulated in gliomas and its overexpression predicted poor prognosis. We also revealed that the expression of TMCO1 was associated with the World Health Organization (WHO) grade of gliomas. Knockdown of TMCO1 inhibited the proliferation and induced apoptosis of U87 and U251 cells. In addition, TMCO1 induced GBM cell migration and invasion by promoting epithelial-mesenchymal transition (EMT). These dates collectively proved the crucial role of TMCO1 as a novel prognostic factor and underlying therapeutic target for glioma patients.

Introduction

Glioma is the most common primary malignant tumor in the brain, accounting for 35.2%~61.0% of intracranial tumors. It’s notorious for its high morbidity, high recurrence, high mortality and low cure rate. Glioma is a heterogeneous central nervous system tumor with distinct biological and clinical characteristics[1, 2]. Despite advances in surgical techniques and postoperative radiotherapy and chemotherapy, the treatment of glioma is still challenging, and the cure rate and overall survival time are still upsetting[3]. Therefore, it is urgent to explore the tumorigenesis mechanism of gliomas in order to obtain new therapeutic strategies.

TMCO1 is a type of highly conserved protein in species. The loss of TMCO1 will lead to overload of the endoplasmic reticulum (ER) Ca\(^{2+}\) storage and the incorrect processing of Ca\(^{2+}\) signal. When Ca\(^{2+}\) in the ER is overloaded, TMCO1 will assemble into a Ca\(^{2+}\) selective channel to expel excess Ca\(^{2+}\) in the ER[4]. As a regulator of cellular calcium, TMCO1 has been demonstrated to affect a variety of growth factors and participate in the regulation of a variety of biological functions[5]. Recent studies have found that TMCO1 is closely related to the occurrence of tumors. TMCO1 has been proved to be a tumor suppressor of urinary bladder urocarcinoma (UBUC), which inhibites cell proliferation, invasion and metastasis through Akt signaling pathway[6]. However, the exact role of TMCO1 in glioma is still unknown. In this study, we are dedicated to investigate the relationship between TMCO1 and glioma.

In this study, we found that TMCO1 expression in gliomas was higher than that in normal brain tissues. Higher TMCO1 expression was associated with shorter overall survival time of glioma patients. Additional assays revealed that TMCO1 promoted cell proliferation and EMT-mediated cell invasion and metastasis in GBM cells. These new findings contribute to an in-depth understanding of the pathogenesis of glioma and may help to the development of more effective anti-glioma therapies.

Materials And Methods
Patients and samples

Glioma tissues and normal brain tissues were collected from the Department of Neurosurgery in Renmin Hospital of Wuhan University from July 2015 to July 2018. Normal brain tissues were obtained during the operation of severe traumatic brain injury with the informed consent of the patients requiring surgery. A total of 92 paraffin-embedded glioma tissues were used for immunohistochemical staining. For Western blot, 8 frozen glioma tissues and 5 frozen normal brain tissues stored at −80 °C was evaluated. None of the patients received any chemotherapy or radiation therapy before surgery. All patients signed an informed consent form, and the study was subject to approval by the Ethics Committee of Renmin Hospital of Wuhan University (approval number: 2012LKSZ (010) H).

Antibodies

TMCO1 (ARP49429_P050) antibody was purchased from Aviva Systems Biology. E-cadherin (20874-1-AP), N-cadherin (22018-1-AP), β-actin (66009-1-Ig), Vimentin (10366-1-AP), Snai1 (13099-1-AP), caspase3 (19677-1-AP), MMP2 (10373-2-AP), and Bcl2 (12789-1-AP) antibodies were purchased from Proteintech.

Bioinformatics analysis

In order to evaluated the expression and prognostic role of TMCO1 in gliomas, we download normalized RSEM gene-level RNAseq and corresponding clinical data of TCGA, CGGA, Gill and Rembrandt datasets from Gliovis database (http://gliovis.biinfo.cnio.es/). Gliovis website is an important data visualization and analysis platform for studying brain tumors[7].

Immunohistochemical (IHC) staining

A total of 92 glioma tissues and 10 normal brain tissues were fixed with 4% paraformaldehyde and embedded in paraffin. The slices were hydrated in xylene and ethanol with different concentrations (100%, 95%, 75%). Afterwards, 3% H₂O₂ was added and incubated for 10 minutes after washing three times with PBS buffer. Then, the sections were subjected to antigen retrieval in 10 mM sodium citrate (pH, 6.0) at 95°C for 10 mins and cooled naturally. Subsequently, the sections were blocked with 1% bovine serum albumin (BSA) for 30 minutes, and incubated with primary antibody at 4 °C overnight. The next day, the sections were incubated with HRP-labelled secondary antibody (Antgene, China) for 1 hour and stained with DAB kit and hematoxylin. Finally, images were obtained using an Olympus BX51 microscope (Olympus, Japan). The intensity of IHC was divided into: 0, 1, 2, 3 points, which indicated background staining, faint staining, moderate staining and strong staining respectively. Two independent pathologists examined and scored. If they have different opinions, a third pathologist will be added for scoring. IHC
score of 0-1 was defined as low expression group, and score of 2-3 was divided into high expression group.

**Cell culture and transfection**

U251 and U87 cell lines were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured at 37 °C in a humid atmosphere of 5% CO₂ using Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA). TMCO1-shRNA was purchased from Genechem (Shanghai, China). Cells were transfected with 2.0 ug plasmid per well in 6-well plates using transfected with lipo3000 (Invitrogen, USA) according to the manufacturer’s instructions. Fresh DMEM supplemented with 10% FBS was added to 6-well plates after 24 hours of transfection, and the cells were harvested for subsequent experiments after 48 hours of transfection.

**Western blot**

U87 and U251 were lysed on ice with modified RIPA buffer (Beyotime, China) for about 30 minutes, and then centrifuged at 12,000 rpm at 4°C for 15 minutes. For cryopreserved glioma tissues, we added 1.5ml of modified RIPA buffer per 100 mg of tissue. The sample concentration was quantitatively determined by BCA protein assay. The lysate was mixed with loading buffer (Beyotime, China) and heated at 100°C for 5 minutes. An equal amount of protein was loaded into 10 or 12% SDS-PAGE, and then transferred to a PVDF membrane (Millipore, Germany). Next, the PVDF membrane was blocked in 5% skimmed milk for 1 hour, and incubated with the primary antibody at 4°C overnight. Then, the membrane was incubated with a secondary antibody (Antgene, Chian, 1:3000) at room temperature for 1 hour, and then visualized using ChemiDoc™ Touch Imaging System (BIO RAD, China).

**Wound healing and transwell assay**

Cells were seeded in a 6-well plate and cultured for a certain time to reach 80% confluence. A sterile pipette tip was used to scratch a linear wound and washed away floating cells with PBS buffer, and then serum free DMEM was added for further cultivating. Wound healing images were captured at certain time using an inverted microscope (Olympus BX51, Japan) and ImageJ software was used in the subsequent analysis. For transwell assay, U87 and U251 cells were seeded into the upper chambers (Corning, USA) precoated with Matrigel (R&D, USA) after transfection for 48 hours, then 200 ul serum-free DMEM medium was added. The lower chamber was filled with 600 μl of DMEM containing 10% FBS. Transwell chambers were placed in an incubator (37 °C, 5% CO2) for 24 h. Cells were fixed with 4% paraformaldehyde for 30mins, stained with 0.2% crystal violet for 15mins and counted under an inverted microscope (Olympus BX51, Japan).
Clone formation and cell counting kit-8 (CCK8) assay

After transfection for 48 hours, 500 glioma cells per well were seeded in 6-well plates and cultured in complete medium until there was obvious single-cell colony formation. Subsequently, cells were fixed with 4% paraformaldehyde for 15mins and stained with 0.2 crystal violet for 15mins. For CCK8 assay, 4000 cells were resuspended in 100 μl complete medium and then seeded to a 96 well plate. The proliferation capacity of the cells was assessed at specific times according to the instructions of the CCK8 kit (Dojindo, Japan).

Flow cytometric analysis

Annexin V-PE/7-ADD kit (Becton Dickinson, USA) were used to measure the apoptosis of glioma cells. According to the manufacturer’s instruction, cells were harvested after transfection for 48 hours and washed three times with PBS buffer. The apoptosis of samples was measured by FACS Calibur flow cytometer (BD Biosciences, USA) after cells were stained with PE and 7-ADD for 15 mins under dark conditions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0.2. software. All datas were presented as mean ± standard deviation (SD). Student’s t-test was used to analyze the differences between two groups. One-way analysis of variance (ANOVA) was used for the comparison among three or more groups, and Tukey’s multiple comparisons test was performed to test differences between groups if analysis of variance was significant. Patients were divided into high and low groups according to the 50% cutoff point of TMCO1 expression and Kaplan–Meier survival analysis was used to analyzed significance between groups. The p value less than 0.05 was considered significant.

Results

TMCO1 is overexpressed in gliomas

We acquired comprehensive genetic alteration of TMCO1 by using Gliovis and Oncomine (www.oncomine.org)[8]. As showed in Fig. 1A, we found increased TMCO1 expression in 4 of the 4 studies in brain and CNS cancer. We also revealed that mRNA expression of TMCO1 was more enriched in glioma tissue than in normal brain tissue using RNAseq data from four public datasets including TCGA, Rambrandt, Gravendell and Gill datasets (Fig. 1B). Moreover, TMCO1 protein level in glioma tissue was further elevated compared with normal brain tissues detected by IHC staining and Western blot analysis (Fig. 1C-D). These dates confirmed that TMCO1 was overexpressed in gliomas.
The expression of TMCO1 is associated with WHO grade and prognosis of glioma

After careful analysis of the data from two datasets including TCGA and Rembrandt datasets, we could conclude that the mRNA expression of TMCO1 increase in sequence in WHO II, WHO III, and WHO IV gliomas. In the CGGA datasets, although there was no significant difference in mRNA expression of TMCO1 between WHO II and WHO III gliomas, it was still significantly lower than WHO IV gliomas (Fig. 2A). Moreover, TMCO1 also increased successively from WHO II, WHO III to WHO IV gliomas by IHC staining. (Fig. 2B). Correlation analysis showed that there was no correlation between TMCO1 and age, gender, Karnofsky Performance Scale (KPS) or tumor location (Table 1). We analyzed the expression of TMCO1 in 92 glioma tissues, the results showed that TMCO1 expression was extremely higher in grade V (Fig. 2C). Then we used the public datasets to explore the impact of TMCO1 on the prognosis of glioma patients, and divided glioma patients into two groups based on the 50% cutoff point of TMCO1 expression. The results showed that patients with high TMCO1 expression had relatively short survival time compared with patients with low TMCO1 expression. (Fig. 2D). These evidences indicated that TMCO1 was a novel prognostic marker in gliomas.

Table 1

| Correlation between TMCO1 and clinical features |
| Variables       | Number | Low | High | Chi-square value | P value |
|-----------------|--------|-----|------|------------------|---------|
| Age (years)     |        |     |      | 1.360            | 0.507   |
| <60             | 68     | 32  | 36   |                  |         |
| ≥60             | 24     | 8   | 16   |                  |         |
| Gender          |        |     |      | 0.124            | 0.724   |
| Female          | 41     | 20  | 21   |                  |         |
| Male            | 51     | 23  | 28   |                  |         |
| Location        |        |     |      | 0.554            | 0.457   |
| Supratentorial  | 82     | 38  | 44   |                  |         |
| Subtentorial    | 10     | 3   | 7    |                  |         |
| KPS             |        |     |      | 0.229            | 0.632   |
| ≥80             | 66     | 29  | 37   |                  |         |
| <80             | 26     | 10  | 16   | 5.849            | 0.016   |
| Grade           |        |     |      |                  |         |
| II+III          | 51     | 25  | 26   |                  |         |
| V               | 41     | 10  | 31   |                  |         |

Low TMCO1: 0-1 (IHC score). High TMCO1: 2-3 (IHC score).

Knockdown of TMCO1 inhibited the proliferation of glioma cells

ShRNA was used to specifically knockdown TMCO1 expression in U87 and U251 cells. As shown in Fig. 3A-B, Western blot showed that TMCO1 was significantly knocked down in U87 and U251 cells. Next, we further investigated whether TMCO1 knockdown could affect glioma cells in vitro. Firstly, CCK-8 assay was performed to detect the proliferation rate of U87 and U251 cells in the control group and TMCO1 knockdown group. The results showed that knockdown of TMCO1 inhibited cell proliferation of U87 and U251 cells (Fig. 3C-D). We also performed cell clone formation assays on U87 cells and U251 cells, and found that the cell clone formation of TMCO1 knockdown group was lesser and smaller than that of control group (Fig. 3E-F). Edu staining assay was also carried out to assess the role of TMCO1 in cell proliferation, and the EdU positive cells meant that the cells were in the S phase of mitosis. The results showed that the percentage of EdU positive cells in TMCO1 knockdown group was significantly lower...
Knockdown of TMCO1 inhibits the EMT in glioma cells

Epithelial-to mesenchymal transition (EMT) is considered to be a key event for cancer cells to obtain an aggressive phenotype by activating specific transcription factors and signaling pathways[9]. Metalloproteinases were also activated during the EMT process, and promoted cell migration and invasion in various cancers by degrading membrane substrate and extracellular matrix components[10]. Therefore, we speculated that TMCO1 may affect the EMT processes of glioma cells. Transwell and wound healing assays were performed on U87 cells and U251 cells, and we found that TMCO1 knockdown glioma cells showed lower migration and invasion capabilities than that in the control group (Fig. 4A-F). As showed in Figure 4G-J, the gap distance of cells in the control group was reduced faster than that in the TMCO1 knockdown group, and the wound healing percentage obviously lower in TMCO1 knockdown group. Snail is recognized to be one of the regulators of EMT execution, which promotes the transcription of normally expressed genes in mesenchymal cells, such as N-cadherin and vimentin[11]. We found that TMCO1 expression was positively correlated with Vimentin and Snai1 in the CGGA dataset (Fig. 4K). In order to further explore how TMCO1 gene affects the EMT of gliomas, we investigated the pivotal proteins involved in EMT by Western blot. The results indicated that the expression of Vimentin, N-cadherin MMP2 and Snai1 were decreased, but E-cadherin was up-regulated in TMCO1 knockdown group (Fig. 4L-N). These results proved that TMCO1 promoted EMT-mediated cell invasion and metastasis in U87 and U251 cells.

Knockdown of TMCO1 induced apoptosis of glioma cells in vitro

We further explored the role of TMCO1 in apoptosis by flow cytometry. TMCO1 knockdown cells showed a higher percentage of apoptotic cells than that in control group. (Fig. 5A-D). Then we conducted Tunel staining in U87 and U251 cells, and the results showed that the Tunel positive proportion in control group was higher than that in TMCO1 knockdown group (Fig. 5E-F). In CGGA dataset, TMCO1 and Bcl2 were found to be positively correlated (Fig.5G). Whereafter, we investigated the key protein involved in apoptosis. Western blot analysis indicated that Bcl2 was remarkably decreased, and cleaved -caspase3 was increased in TMCO1 knockdown group. However, there was no significant difference in caspase-3 between the two groups. (Fig. 5H-J). These results demonstrated that TMCO1 inhibited cell apoptosis in U87 and U251 cells.

Discussion
It is reported that TMCO1 plays a crucial role in Ca\textsuperscript{2+} signaling and key cell functions by maintaining the homeostasis of Ca\textsuperscript{2+} storage in the ER \cite{4}. Studies have found that TMCO1 mediated Ca\textsuperscript{2+} leakage is firmly related to the function of osteoblasts\cite{12}. Another study found that TMCO1 plays an important role in open-angle glaucoma \cite{5}. However, TMCO1 was poorly understood in tumors, except that the only cancer-related research found that TMCO1 is a new tumor suppressor in UBUCs, which mainly regulates abnormal cell cycle processes by inhibiting the AKT pathway \cite{6}. So far, no study has ever found and discussed the possible relationship between TMCO1 and glioma. Our study provided key evidence that TMCO1 was an underlying prognostic marker and therapeutic target for glioma.

Our study revealed that both the mRNA and protein levels of TMCO1 were upregulated in glioma tissues, and elevated TMCO1 expression reduced the overall survival time of glioma patients. GBM is the most common aggressive phenotype of glioma, which has obvious rapid proliferation and anti-apoptosis characteristics\cite{13}. Our study proved that knockdown of TMCO1 inhibited the malignancy of glioma by inducing apoptosis and suppressing cell proliferation in U87 and U251 cells.

EMT has been studied for decades, and it is generally believed that EMT plays an important role in cancer progression and metastasis, and has a significant impact on the prognosis of various cancers\cite{14, 15}. Activation of EMT is believed to be essential to tumor invasion and metastasis, which is the leading cause of death in cancer patients\cite{16}. We confirmed that TMCO1 contributed to migration and invasion by performing Transwell and wound healing assays. Subsequent Western blot analysis indicated TMCO1 knockdown increased the expression of the epithelial marker E-cadherin and reduced the expression of the mesenchymal marker's vimentin and N-cadherin. The loss of E-cadherin protein was considered to be the basis for the occurrence of EMT\cite{17}. Moreover, MMP2 was decreased in TMCO1 knockdown group. This may lead to a decrease in the tumor's ability to degrade membrane substrates and extracellular matrix components, thereby inhibiting cell invasion and migration. These results suggested that TMCO1 promotes cell migration and invasion in glioma by inducing EMT.

In conclusion, our current study proves for the first time that TMCO1 is overexpression in GBM tissues, and reducing the expression of TMCO1 helps to improve the survival time of glioma patients. Moreover, we verified that TMCO1 promotes cell proliferation and induces cell apoptosis in U87 and U251 cells. We also demonstrated that silencing TMCO1 inhibits EMT mediated cell invasion and metastasis. Therefore, TMCO1 may be a potential and valuable therapeutic target for glioma.

**Declarations**

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

LG, J-HL, GD and Q-XC conceived and designed the study. LG, J-HL, and S-AT performed experiments. LG, ZY, J-AY, J-YC, Y-XW analyzed data. LG and ZY wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Figures
Figure 1

TMCO1 was upregulated in gliomas. (A) The expression of TMCO1 in different types of cancers in Oncomine. (B) Four public datasets confirmed TMCO1 mRNA was increased in gliomas. (C-D) Western blot and IHC analysis demonstrated that TMCO1 was overexpression in gliomas. ** P<0.01 **** P<0.0001. NBT, non-tumor brain tissues.
Figure 2

TMCO1 was associated with WHO grade and prognosis of gliomas (A) Three public datasets were utilized to explore TMCO1 expression in different grades of gliomas. (B) Represented images of IHC staining of TMCO1 protein in distinct grades of gliomas. (C) TMCO1 expression was extremely higher in grade V. (D) Three public datasets confirmed that TMCO1 was prognostic marker of gliomas. *P<0.05, **P<0.01, ****P<0.0001, ns, no significance. Low TMCO1: 0-1 (IHC score); High TMCO1: 2-3 (IHC score).
Figure 3

Knockdown TMCO1 inhibited cell proliferation in U87 and U251 cells. (A, B) The knockdown of TMCO1 protein level was shown. (C, D) Cell viability in TMCO1 knockdown group and control group was measured at characteristic time. (E, F) Knockdown TMCO1 inhibited U87 and U251 cells colony formation and results were statistically analyzed. (G-J) Edu assay was performed to assess DNA replication. *P<0.05, **P<0.01, ***P<0.001
Figure 4

Knockdown TMCO1 suppressed EMT and metastatic potential in GBM Cells. (A-F) Knockdown TMCO1 inhibited cell migration and invasion in U87 and U251 cells. (G-J) Wound healing assays were performed to evaluate cell metastatic capacity. (K) Correlation analysis of TMCO1 and VIM/snai1 based CGGA database. (L-N) Key proteins involved in EMT were investigated by western blot analysis. *P<0.05, **P<0.01, ***P<0.001
Knockdown TMCO1 induced cell apoptosis in U87 and U251 cells. (A-D) Cell apoptosis rate was enhanced in knockdown TMCO1 group. (E, F) U251 cell death were detected by TUNEL staining. (G) Correlation analysis of TMCO1 and bcl2 based CGGA database. (H-J) Apoptosis related protein was investigated by western blot analysis. (H) *P<0.05, **P<0.01, ***P<0.001, ns: non significances.