LncRNA TMPO-AS1 Promotes Proliferation and Invasion by Sponging miR-383-5p in Glioma Cells

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Purpose: Glioma is one of the most common malignant tumors affecting human health. Long non-coding RNA (lncRNA) TMPO-AS1 participates in the pathogenesis of various cancers. However, the role of lncRNA TMPO-AS1 in glioma remains largely unknown. This study aims to uncover the role of TMPO-AS1 and explore its potential mechanism in glioma.

Methods: Expression levels of TMPO-AS1 and miR-383-5p in glioma cell lines were measured by real-time quantitative PCR (RT-qPCR). CCK-8, colony formation, wound-healing, and Transwell assays were conducted to determine cell proliferation, migration and invasion abilities, respectively. Western blotting was applied to detect the expression of corresponding proteins. Immunofluorescence assay was performed to measure the expression of Ki67. The binding condition between TMPO-AS1 and miR-383-5p was verified by dual-luciferase reporter assay.

Results: We found that TMPO-AS1 was up-regulated while miR-383-5p was down-regulated in glioma cell lines, and knockdown of TMPO-AS1 significantly suppressed glioma cell proliferation, migration and invasion abilities. miR-383-5p was demonstrated to be a direct target of TMPO-AS1. Besides, inhibition of miR-383-5p abolished the effects of TMPO-AS1 knockdown on glioma cells.

Conclusion: In summary, our study revealed that inhibition of lncRNA TMPO-AS1 could suppress glioma progression through targeting miR-383-5p. TMPO-AS1 might be used as a therapeutic target for glioma treatment.

Keywords: lncRNA TMPO-AS1, miR-383-5p, glioma, proliferation

Introduction

Gliomas, derived from neuroepithelial cells, are one kind of the most common types of primary brain tumors, accounting for about 47% of all central nervous system (CNS) malignant tumors.¹ Despite great advances that have been achieved in surgery and chemoradiotherapy, the prognosis of patients with glioma still remains poor, and the median survival time was only 12–15 months.^{2,3} Thus, it is urgently required to study specific molecular mechanism underlying the physiology and pathology of glioma to find specific therapeutic targets for the treatment of glioma.

Long non-coding RNAs (lncRNAs), longer than 200 nt without protein-coding ability, play an important role in various diseases by regulating gene expression at multiple levels, such as transcription, translation, and epigenetic modulation.⁴ The function of lncRNA TMPO antisense RNA1 (TMPO-AS1) is widely recognized in various diseases, especially in human cancers. Previous studies have exhibited that lncRNA TMPO-AS1 is an oncogenic lncRNA in colorectal cancer, osteosarcoma,

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cervical cancer, non-small cell lung cancer, and so on.^{5–8} However, the role of lncRNA TMPO-AS1 in glioma remains unclear. In addition, miRNAs are small non-coding RNAs comprised about 22 nt that play critical roles in the occurrence and development of various cancers.⁹ In recent years, a large amount of miRNAs have been reported to be potential markers for the diagnosis, treatment, and prognosis of patients with cancers.¹⁰ By searching the Starbase (<http://starbase.sysu.edu.cn/>), we found a large amount of miRNAs that have potential binding site to lncRNA TMPO-AS1. Screening with the score of AgoExpNum (score $\geq$ 3) and literature research, the miRNAs which play the important role in the regulation of cancer development of the other malignant tumors but have not been investigated in glioma are taken into consideration for the further research. miR-383-5p is one of these miRNAs, and has been investigated in various cancers, such as gastric cancer, ovarian cancer, lung adenocarcinoma, and so on.^{11–13} However, the role of miR-383-5p and its biological function in glioma have not been explored. Given that lncRNAs are reported to work as a sponge by competitively binding to miRNAs, thus regulating their molecular function, we hypothesized that lncRNA TMPO-AS1 might be involved in tumor progression via targeting miR-383-5p.

In the present study, we found up-regulated lncRNA TMPO-AS1 and down-regulated miR-383-5p in glioma cells. Knockdown of lncRNA TMPO-AS1 suppressed glioma cell proliferation, migration, and invasion by targeting miR-383-5p.

Materials and Methods

Cell Culture

Normal human astrocytes (NHA) and human glioma cell lines (U251, A172, and LN229) were obtained from Ningbo Mingzhou Biological Technology Co., Ltd (Ningbo, Zhejiang, China). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

Cell Transfection

To interfere TMPO-AS1 expression in U251 and A172 cells, short hairpin (sh) RNA targeting TMPO-AS1 (shRNA-TMPO-AS1-1/2) and its negative control shRNA-NC were obtained from Shanghai GenePharma

(Roche, China). U251 and A172 cells were transfected with shRNA-TMPO-AS1-1/2, miR-383-5p inhibitor or corresponding negative control according to the instructions of Lipofetamine 3000 (Invitrogen, Carlsbad, CA, USA). 48 h after transfection, real-time quantitative PCR was used to examine transfection efficacy.

Cell Counting Kit-8 (CCK-8) Assay

U251 and A172 cells were seeded into 96-well plates, respectively. At different time points, 10 μ L of CCK-8 solution (Dojindo, Kumamoto, Japan) was added into each well, and cells were incubated in the incubator for another 3 h. Then, cell viability was determined by detecting the absorption at 450 nm using a microplate reader. Cell viability was obtained when cells were cultured for 24 h, 48 h, and 72 h.

Colony Formation Assay

U251 and A172 cells were seeded into 6-well plates, respectively, and were cultured for 10 days. During this period, culture medium was changed every 3 days. For visualization, cells were fixed in 4% paraformaldehyde for 30 min and stained with crystal violet for 30 min for visualization.

Real-Time Quantitative PCR (RT-qPCR)

Total RNA was extracted from U251 or A172 cells using Trizol (Invitrogen) following the manufacturer's protocol. After the concentration and purity of RNA were determined using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA), RNA was reversely transcribed into complementary DNA (cDNA) using PrimeScript RT Master Mix (Takara, Dalian, China), and real-time PCR was conducted to detect gene expression levels according to the instructions of the SYBR Green Premix Ex Taq II. The relative mRNA level was calculated using $2^{-\Delta\Delta C_t}$ method. The relative expression of TMPO-AS1 was normalized to GAPDH and the relative expression of miR-383-5p was normalized to U6.

Western Blotting

Total protein was extracted using RIPA lysis buffer with PMSF (Beyotime). After the concentration of protein was detected using a BCA protein Assay Kit (Generay, Shanghai, China), the same amount of protein (30 μ g/lane) was subjected to SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with non-fat milk for 2 h at room temperature and were incubated

with the primary antibodies at 4°C overnight. On the next day, membranes were incubated with secondary antibody solution (goat anti-mouse IgG). Chemiluminescent signals were detected using an ECL plus kit (Thermo Fisher Scientific), and were quantified with Image J software.

Immunofluorescence Assay

After transfection, U251 and A172 cells were fixed with 4% paraformaldehyde for 40 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS at room temperature. After being blocked with 5% bovine serum albumin, the primary antibody against Ki67 was added to cells for incubation at 4°C overnight. On the next day, cells were incubated with fluorescent secondary antibody for 1 h at room temperature in the dark, followed by staining with DAPI in the dark. The immunofluorescence images were acquired using a confocal laser microscope (Leica, TCS SP5, Germany)

Wound-Healing Assay

U251 and A172 cells were seeded into 6-well plates. When cells reached 100% confluence, a 200 µL pipette tip was used to draw a straight wound on the surface of cells. Then, cells were cultured with serum-free medium for 48 h. Cells were viewed and photographed at 0 h and 48 h under a light microscope, respectively.

Transwell Assay

U251 and A172 cells were suspended with serum-free medium and added to the upper chamber of Transwells (8 µm pore size) pre-coated with Matrigel. The complete medium with 10% FBS was added to the lower chamber. After incubation for 48 h, the cells failing to pass through the membranes were removed, and the invasive cells were fixed with 4% formaldehyde for 15 min and stained with 0.1% crystal violet for 20 min. The invasive cells were viewed, photographed, and counted under a light microscope.

Dual-Luciferase Reporter Assay

The putative binding site between TMPO-AS1 and miR-383-5p was predicted by Starbase 3.0. To verify this binding site, luciferase reporter assay was carried out. Briefly, the transcript 3'UTR sequence of TMPO-AS1 was cloned into the pGL3 vector containing the luciferase reporter gene to generate TMPO-AS1 WT, and mutant 3'UTR sequence of TMPO-AS1 was cloned into the pGL3 vector

to generate TMPO-AS1 WT. Cells were co-transfected with TMPO-AS1 WT or TMPO-AS1 MUT, and miR-383-5p mimic and mimic negative control (mimic-NC), respectively. At 48 h, cells were lysed, and the luciferase activity was detected using the Dual Luciferase Report Assay kit (Promega, Madison, MI).

RNA Pull Down Assay

The RNA pull down assay was performed using a Magnetic RNA-Protein Pull-down kit (Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. Sonicated U251 and A172 cells were incubated with the biotinylated-lncRNA TMPO-AS1 probe and magnetic beads at 4°C overnight. After elution of the magnetic beads, the bound RNA was extracted using Trizol kit, and the expression level of miR-383-5p was measured using RT-qPCR.

Statistical Analysis

All data were represented as mean $\pm$ standard deviation (SD) from at least three independent experiments. SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) was applied for data analysis. One-way ANOVA followed by Tukey's post hoc test was used for comparison among different groups. Differences were regarded as statistically significant when p value was less than 0.05.

Results

Knockdown of TMPO-AS1 Suppressed Glioma Cell Proliferation

We first detected the expression level of TMPO-AS1 in normal human astrocytes (NHA) and human glioma cell lines (U251, A172, and LN229). As shown in [Figure 1A](#), the expression level of TMPO-AS1 was greatly higher in glioma cell lines than that in NHA. Then, to investigate the role of TMPO-AS1 in glioma cells, U251 and A172 cells were transfected with shRNA-NC or shRNA-TMPO-AS1-1/2, and the expression of TMPO-AS1 was significantly reduced upon transfection with shRNA-TMPO-AS1-1/2 ([Figure 1B](#)). Due to its relatively high transfection efficacy, shRNA-TMPO-AS1-1 was used for the next experiments. CCK-8 assay exhibited that down-regulated TMPO-AS1 significantly inhibited cell viability at different time points in both U251 and A172 cells ([Figure 1C–D](#)). Down-regulated TMPO-AS1 also decreased cell colony formation ([Figure 1E](#)). Minichromosome maintenance (MCM), proliferating

Figure 1 Knockdown of TMPO-AS1 suppressed glioma cell proliferation. **(A)** The expression level of TMPO-AS1 in normal human astrocytes (NHA) and human glioma cell lines (U251, A172, and LN229) was detected using RT-qPCR. *** $p < 0.001$ vs NHA. **(B)** Both U251 and A172 cells were transfected with shRNA-NC and shRNA-TMPO-AS1-1/2, and the transfection efficacy was determined. **(C)** After transfection, CCK-8 assay was used to detect cell viability of U251 cells in different groups. **(D)** After transfection, CCK-8 assay was used to detect cell viability of A172 cells in different groups. **(E)** Cell colony formation assay was conducted to detect cell proliferation ability in U251 and A172 cells. **(F)** Protein expressions of MCM and PCNA were measured using Western blotting. **(G and H)** Immunofluorescence assay was performed to determine the expression of Ki67 in U251 and A172 cells, respectively. *, **, *** $p < 0.05, 0.01, 0.001$ vs shRNA-NC.

cell nuclear antigen (PCNA), and Ki67 are standard markers of proliferation which are commonly applied to evaluate the growth fraction of cell population.¹⁴ In both U251 and A172 cells, down-regulated TMPO-AS1 significantly decreased protein expressions of MCM and PCNA (Figure 1F). In addition, immunofluorescence assay exhibited that down-regulated TMPO-AS1 remarkably decreased the expression of Ki67 (Figure 1G–H). Taken together, down-regulated TMPO-AS1 could inhibit cell proliferation in U251 and A172 cells.

Knockdown of TMPO-AS1 Suppressed Glioma Cell Migration and Invasion

Then, wound-healing and Transwell assays were carried out to determine the effects of down-regulated TMPO-AS1 on cell migration and invasion abilities. As shown in Figure 2A–C, in both U251 and A172 cells, down-regulated TMPO-AS1 showed significant reduction in cell migration and invasion rates. Besides, the protein expressions of MMP2 and MMP9 were also decreased upon knockdown of TMPO-AS1 (Figure 2D). Taken

Figure 2 Knockdown of TMPO-AS1 suppressed glioma cell migration and invasion. **(A)** Wound-healing and Transwell assays were conducted to determine cell migration and invasion in U251 and A172 cells, respectively. **(B)** Cell migration rate was quantified. **(C)** Cell invasion rate was quantified. **(D)** Protein expression of MMP-2 and MMP-9 was detected using Western blotting. *** $p < 0.001$ vs shRNA-NC.

together, knockdown of TMPO-AS1 could inhibit cell migration and invasion.

miR-383-5p is a Direct Target of TMPO-AS1

The expression level of miR-383-5p was detected in NHA and glioma cell lines, and the results showed that the expression level of miR-383-5p was significantly decreased in glioma cell lines, compared to NHA (Figure 3A). By searching Starbase database (<http://starbase.sysu.edu.cn/>), we found that there was a potential binding site between lncRNA TMPO-AS1 and miR-383-5p (Figure 3B), and this binding condition was subsequently verified by luciferase reporter assay (Figure 3C). RNA pull down

assay further demonstrated the binding association between TMPO-AS1 and miR-383-5p (Figure 3D). In addition, we also found that the expression level of miR-383-5p was significantly up-regulated upon knockdown of TMPO-AS1 in U251 and A172 cells (Figure 3E). Taken together, miR-383-5p was a direct target of TMPO-AS1, and it was negatively regulated by TMPO-AS1.

TMPO-AS1 Exerted Functions in Cellular Activities by Regulating miR-383-5p

Next, we further discovered the regulatory connection underlying TMPO-AS1/miR-383-5p on cellular function in glioma. U251 cells were co-transfected with shRNA-TMPO-AS1 and inhibitor NC/miR-383-3p inhibitor. CCK-8 assay

Figure 3 MiR-383-5p is a direct target of TMPO-AS1. (A) The expression level of TMPO-AS1 in normal human astrocytes (NHA) and human glioma cell lines (U251, A172, and LN229) was detected using RT-qPCR. *** $p < 0.001$ vs NHA. (B) Starbase database (<http://starbase.sysu.edu.cn/>) predicted a potential binding site between lncRNA TMPO-AS1 and miR-383-5p. (C) Dual-luciferase reporter assay was performed to verify this binding condition. *** $p < 0.001$ vs mimic-NC. (D) The relationship that TMPO-AS1 bound to miR-383-5p was detected by RNA pull-down assay. *** $p < 0.001$ vs Oligo probe. (E) After transfected with shRNA-NC or shRNA-TMPO-AS1 in both U251 and A172 cells, the expression level of miR-383-5p was detected. **, *** $p < 0.01$, 0.001 vs shRNA-NC.

showed that knockdown of TMPO-AS1 decreased U251 cell viability, which was partly attenuated by inhibition of miR-383-5p (Figure 4A). In A172 cells, inhibition of miR-383-5p significantly weakened the suppressive effect of TMPO-AS1 on cell viability (Figure 4B). The protein expressions of Ki67 and PCAN in both U251 and A172 cells were decreased upon TMPO-AS1 knockdown, which were then reversed by co-transfection with shRNA-TMPO-AS1 and miR-383-5p inhibitor (Figure 4C). In addition, wound-healing and Transwell assays were also performed, and the inhibitory effects of TMPO-AS1 knockdown on cell migration and invasion rates were significantly reversed by inhibition of miR-383-5p (Figure 5A–C). The decreased protein expressions of MMP2 and MMP9 upon TMPO-AS1 knockdown were also increased when miR-383-5p was inhibited (Figure 5D). Taken together, the inhibitory effects of TMPO-AS1 knockdown on glioma cell proliferation, migration, and invasion could be reversed by inhibition of miR-383-5p, indicating that TMPO-AS1 might regulate glioma progression by targeting miR-383-5p.

Discussion

It has become extensively acknowledged that lncRNAs in a large part take the responsibility for regulating complex biological progression, which has attracted

much attention from many scholars. In this study, we first reported lncRNA TMPO-AS1 as an oncogene in glioma. The key findings of this study are that lncRNA TMPO-AS1 is up-regulated in glioma cell lines, and knockdown of lncRNA-TMPO-AS1 can inhibit glioma cell proliferation, migration and invasion, possibly by up-regulating miR-383-5p, thus hindering the progression of glioma.

As one of the important oncogenic lncRNAs in many types of cancers, lncRNA TMPO-AS1 is involved in different biological processes in tumorigenesis, including cell proliferation, apoptosis, invasiveness, and so on.^{15,16} Qin et al demonstrated that downregulation of TMPO-AS1 inhibited cell proliferation, colony formation, migration and invasion in vitro, and suppressed tumor growth in non-small cell lung cancer in vivo.⁶ Zhao et al reported that silence of TMPO-AS1 restrained the aggressiveness and the pro-angiogenic activity of ovarian cancer cells, blocking ovarian cancer development.¹⁷ In the present study, we first discovered the increase of lncRNA-TMPO-AS1 in glioma cell lines, and further cellular function assay disclosed that knockdown of TMPO-AS1 could suppress glioma cell proliferation, colony formation, migration and invasion, demonstrating the oncogenic activity of TMPO-AS1 in glioma.

Figure 4 The inhibitory effect of TMPO-AS1 knockdown on cell proliferation was reversed by inhibition of miR-383-5p. **(A and B)** U251 and A172 cells were co-transfected with shRNA-TMPO-AS1 and inhibitor NC/miR-383-3p inhibitor. CCK-8 assay was performed to detect cell viability. **(C)** Western blotting assay was applied to measure the protein expression of Ki67 and PCNA of U251 and A172 cells in different groups. *, **, ***p<0.05, 0.01, 0.001 vs shRNA-NC +inhibitor-NC; #, ###p<0.05, 0.001 vs shRNA-TMPO-AS1 +inhibitor-NC.

The crosstalk between lncRNAs and miRNAs is common in cancer biology. LncRNAs can act as competing endogenous RNAs (ceRNAs) to directly interact with miRNAs, thus regulating the expression and the activity of miRNAs.¹⁸ Accumulating evidence has shown that lncRNA TMPO-AS1 participates in the regulation of cancer progression by sponging various miRNAs. For

example, TMPO-AS1 serves as a ceRNA of miR-199a-5p to promote osteosarcoma initiation.⁷ TMPO-AS1 can promote cell proliferation of thyroid cancer via sponging miR-498.¹⁹ Consistently, after verifying the direct binding condition between TMPO-AS1 and miR-383-5p, a series of cellular function experiments exhibited that inhibition of miR-383-5p could reverse the low activities

Figure 5 The inhibitory effects of TMPO-AS1 knockdown on cell migration and invasion were reversed by inhibition of miR-383-5p. **(A)** U251 and A172 cells were co-transfected with shRNA-TMPO-AS1 and inhibitor NC/miR-383-3p inhibitor. Wound-healing and Transwell assays were conducted to determine cell migration and invasion in U251 and A172 cells, respectively. **(B)** Cell migration rate was quantified. **(C)** Cell invasion rate was quantified. **(D)** Protein expression of MMP-2 and MMP-9 was detected in U251 and A172 cells using Western blotting. *** $p < 0.001$ vs shRNA-NC +inhibitor-NC. ### $p < 0.001$ vs shRNA-TMPO-AS1 +inhibitor-NC.

of glioma cell proliferation, migration and invasion caused by TMPO-AS1 knockdown, indicating that TMPO-AS1 might sponge miR-383-5p to regulate cellular functions. The functionality of miR-383-5p has been characterized in several types of human cancers. miR-383-5p was down-regulated in gastric cancer tissues and cells, and overexpression of miR-383-5p suppressed gastric cancer cell proliferation and migration.¹¹ miR-383-5p was also down-regulated in ovarian cancer, and miR-383-5p exerted its anti-proliferation function to suppress tumor growth.¹² In our study, the down-regulated miR-383-5p was found in glioma cell lines, and inhibition of miR-383-5p promoted glioma cell proliferation, migration and invasion, reflecting that miR-383-5p also exerted its anti-cancer activity in glioma. Considering that

TMPO-AS1 could regulate the expression of miR-383-5p, the inhibitory effects of TMPO-AS1 knockdown on glioma progression was likely dependent on the activity of miR-383-5p.

However, there are still some limitations in the present study. Firstly, besides functioning as miRNA sponges, lncRNAs can also exert their functions by modulating mRNA translation or by associating with proteins, along with the associated signaling pathway; thus, the possible target mRNAs for lncRNA TMPO-AS1 and the related signaling pathways are deserved to be investigated to complete molecular network of TMPO-AS1 in glioma. Besides, in vivo experiments are needed to further demonstrate the anti-cancer function of TMPO-AS1 knockdown in glioma. Furthermore, clinical assays are also required to

verify the role of TMPO-AS1 in glioma and provide the possibility for the clinical application.

Conclusion

In summary, we clarified the role and molecular mechanism by which TMPO-AS1 regulated cell proliferation, migration and invasion in glioma cells. Particularly, knockdown of TMPO-AS1 suppressed cell proliferation, migration, and invasion by functioning as a sponge of miR-383-5p. The newly identified lncRNA TMPO-AS1/miR-383-5p axis provides a new insight into glioma tumorigenesis, and it represents a potential therapeutic target for glioma treatment.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest for this work.

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