T3 Promotes Glioma Cell Senescence and Apoptosis via THRA and THRB

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ABSTRACT: Glioma is one of the most common types of primary intracranial tumors. The relationship between triiodothyronine (T3) and glioma is not clear. This study aimed to investigate the effect of T3 on the proliferation of glioma cells and its mechanism. Cell viability was analyzed by cell counting kit 8 assay. Flow cytometry analysis was used to detect cell apoptosis and cell cycle. Thyroid hormone receptor α (THRA) and thyroid hormone receptor β (THRB) were silenced by transfecting si-THRA and si-THRB plasmids into HS683 and A172 glioma cells. Western blot was performed to assess the protein expressions. The results indicated that triiodothyronine (T3) affected the viability, apoptosis and cell cycle of HS683 and A172 glioma cells. Cell apoptosis was significantly inhibited in si-THRA and si-THRB experimental groups. Moreover, knockdown of THRA and THRB reversed the G_1 and G_2 phase arrest led by T3 and induced an up-regulation of cyclin D1 expression. The phosphorylated extracellular signal-regulated kinase (p-ERK), p-AKT, and phosphorylated signal transducer and activator of transcription (p-STAT3) proteins were markedly increased by inhibiting THRA and THRB in HS683 and A172 glioma cells. T3 affected apoptosis and cell cycle of glioma cells through regulating THRA and THRB expressions. THRA and THRB may affect glioma development through regulating, at least partially, the mitogen-activated protein kinase (MAPK)/ERK and phosphoinositide 3-kinase (PI3K)/Akt signaling pathways.

KEY WORDS: T3, THRA, THRB, proliferation, cell cycle, phosphorylation

I. INTRODUCTION

Glioma is a neuroepithelial tumor, accounting for about 31% of the primary brain tumors. Even with standardized surgery plus radiotherapy and chemotherapy, most patients survive for only 15–18 months, and the 5-year survival rate is less than 5%,[1] which imposes a heavy social and family burden. Therefore, studies on the pathogenesis and new therapies of glioma are urgently needed.

Triiodothyronine (T3), an active thyroid hormone, is widely involved in the body's physiological processes, but the role of T3 in tumors is controversial. Some studies have shown that T3 promotes tumor growth in breast cancer,[2] prostate cancer,[3] lung cancer,[4] ovarian cancer,[5] pancreatic cancer,[6] and colorectal cancer,[7] while some studies have shown that T3 inhibits tumor growth in the aforementioned tumors.[8–16] With regard to the relationship between T3 and glioma, some studies have shown that T3 can increase the proliferation and inhibit apoptosis of glioma cells.[8,17,18] And other studies have shown that T3 inhibits the proliferation of gliomas.[19] Moreover, the 5-year survival rate of glioma was reduced by preoperative low T3 status.[20]

Thyroid hormone receptor α (THRA) and thyroid hormone receptor β (THRB) are two nuclear receptors of T3.[21] Studies have shown that thyroid hormone receptors play an essential role in developing the nervous system, mainly in the migration and differentiation of nerve cells, synaptic formation and myelin formation.[22,23]

In this study, the effect of T3 on the proliferation of glioma cells was studied and its mechanism was further studied and analyzed.

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II. MATERIALS AND METHODS

A. Cell Culture

HS683 and A172 glioma cell lines and normal human glial cells (HEB) were purchased from Kunming Institute of Zoology, Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) complete culture medium containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin, and cultured in a 37°C, saturated humidity, and 5% CO_2 incubator, cell growth was observed under the light microscope every day. When the cells were covered with about 80% of the bottle's bottom, the cells were digested with 2.5 g/L trypsin and passaged at 1:3.

B. Cell Transfection

HS683 and A172 cells in the logarithmic phase were collected to prepare a cell suspension, which was inoculated into a 24-well plate at a density of 5×10^4/well. Then, the cells were cultured in 0.5 ml of DMEM containing fetal bovine serum and antibiotics and 20 pmol small interfering RNA (siRNA) was added to 50 μL of DMEM serum-free medium, and then mixed with lipofectamine reagent. The mixture was placed at room temperature for 20 min to form a siRNA/lipofectamine complex. The 100 μL siRNA/lipofectamine complex was added to the culture plate's hole containing cells and culture medium. The cell culture plate was gently shaken back and forth and placed in 5% CO_2, cultured in an incubator at 37°C. siRNA sequences were as follows: si-THRA: 5′-CCACAGCCACCTGCTTACTAGTTGT-3′; si-THRB: 5′-GGAATGTCGCTTTAAGAAA-3′.

C. Cell Counting Kit 8 (CCK-8) Assay

The experimental group was divided into two groups. The control group was un-treated cells, and the experimental group cells were treated by 10 ng/ml of T3. The HS683 and A172 cells in the logarithmic growth phase were digested with 0.25% trypsin and inoculated into a 96-well plate. Each group was inoculated with three holes. After 48 h, 10 μL of CCK-8 reagent was added to each well and incubated at room temperature for 2 h. The absorbance of 450 nm was measured by a microplate spectrophotometer. The cell survival rate was calculated as: survival rate = (absorbance value of experimental group – absorbance value of blank group)/(absorbance value of control group – absorbance value of blank group) × 100%.

D. Cell Apoptosis Assay

After transfection, cells were inoculated into six-well plates at a concentration of 5×10^4 cells /well and incubated in a constant temperature incubator (5% CO_2, 37°C) overnight. Cells were collected at 48 h, and Annexin V–fluorescein isothiocyanate and propidium iodide (PI) were added to each well and incubated for 20 min at room temperature, avoiding the light according to the product instructions. Finally, the apoptosis rate of cells was detected by C6 flow cytometry for data analysis. All of the above experiments were repeated three times.

E. Cell Cycle Assay

HS683 and A172 cells in the logarithmic phase were collected and made into cell suspension, which were fixed by 70% precooled ethanol overnight at 4°C. The cells were washed with phosphate-buffered saline (PBS) three times and collected by 1000 rpm/min centrifugation for 3 min. Then, the cells were resuspended with 500 μL of binding buffer. Next, 12.5 μL of PI and 10 μL of RnaseA were added to each tube to incubate 30 min in the dark at room temperature. Then the cell cycle was detected by flow cytometry.

F. Western Blot

Cells were centrifuged at 1000 rpm for 5 min. The cells were washed with pre-cooled PBS solution, added with cell lysate, shaken on a shaker for 50–60 min, and centrifuged at 12,000 rpm at 4°C for 20 min to collect the supernatant. The total protein concentration was determined using the bacterial artificial chromosome method. The corresponding concentration of gel was arranged according to the size of the detected molecular weight. The electrophoretic process of separating gel was 85 V, 20–30 min, and 140 V, 10 min for the stacking gel. The gel was then transferred

to polyvinylidene fluoride membrane, stained with Coomassie brilliant blue, and stained with Ponceau S dye for 5–10 min. The membrane was blocked with 5% skim milk for 2 h. Then, anti-extracellular signal-regulated kinase (anti-ERK), anti-AKT, anti-signal transducer and activator of transcription 3 (anti-STAT3), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibodies were added, incubated at 4°C overnight, and then washed with Tris-buffered saline and Tween 20 solution. Next, the membranes were incubated with a horseradish peroxidase–conjugated secondary antibody at room temperature for 2 h. Finally, protein bands were visualized using an enhanced chemiluminescence system.

G. Statistical Analysis

Data were analyzed with GraphPad Prism software (version 9.0.0.121, GraphPad Software Inc.). The data are presented as mean ± SD of three independent experiments. Student's *t*-test was performed for the two-group comparison, and analysis of variance (ANOVA) was used for the comparison among multiple groups. $p < 0.05$ suggested a significant difference.

III. RESULTS

A. T3 Inhibits Cell Viability of Glioma Cells

The chemical formula of T3 is shown in Fig. 1A. The results of CCK-8 assay showed that after 10 ng/ml T3 treatment for 48 h, compared with control groups, the survival rates of HS683 and A172 glioma cells were significantly decreased (Fig. 1B).

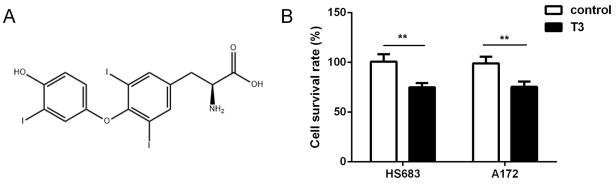

FIG. 1: T3 inhibited the cell viability of HS683 and A172 glioma cells. (A) Chemical formula of T3. (B) Survival rates of HS683 and A172 glioma cells (**$p < 0.01$ vs. the control group).

B. T3 Affects Apoptosis and Cell Cycle of Glioma Cells

After 10 ng/ml T3 treatment for 48 h, we found that apoptosis was increased in both HS683 and A172 glioma cells (Fig. 2A). Moreover, T3 led to cell G_1 and G_2 phase arrest and induced a down-regulation of cyclin D1 expression compared with the untreated group (Fig. 2B and 2C).

C. THRA and THRB are Abnormally Expressed in Gliomas

As shown in Fig. 3A, THRA and THRB were lower expressed in the glioma treatment group compared with the control group. Similarly, the protein expressions of THRA as well as THRB in HS683 and A172 cells were remarkably lower than that in HEB cells (Fig. 3B). The survival curves showed that patients with high expression of THRA and THRB had a good prognosis, whereas patients with low expression of THRA and THRB had poor survival (Fig. 3C).

D. T3 Inhibits the Expression of THRA and THRB and Phosphorylation of ERK, AKT, and STAT3 in Glioma Cells

Western blot was used to detect THRA and THRB protein expression in HS683 and A172 glioma cells before and after 10 ng T3 treatment. The results showed that the expressions of THRA and THRB protein were significantly increased after the T3 treatment in both HS683 and A172 glioma cells (Fig. 3D). In addition, to analyze the effect of T3 on the mitogen-activated protein kinase (MAPK)/ERK and phosphoinositide 3-kinase (PI3K)/Akt pathways, related proteins in these two pathways were detected in both HS683 and A172 glioma cells before and after 10 ng T3 treatment. The results indicated a statistically significant decrease in p-ERK, p-AKT, and p-STAT3 protein levels in T3-treated cells compared with untreated cells (Fig. 3D). Further, T3 declined the three protein levels by more than 50% in HS683 glioma cells.

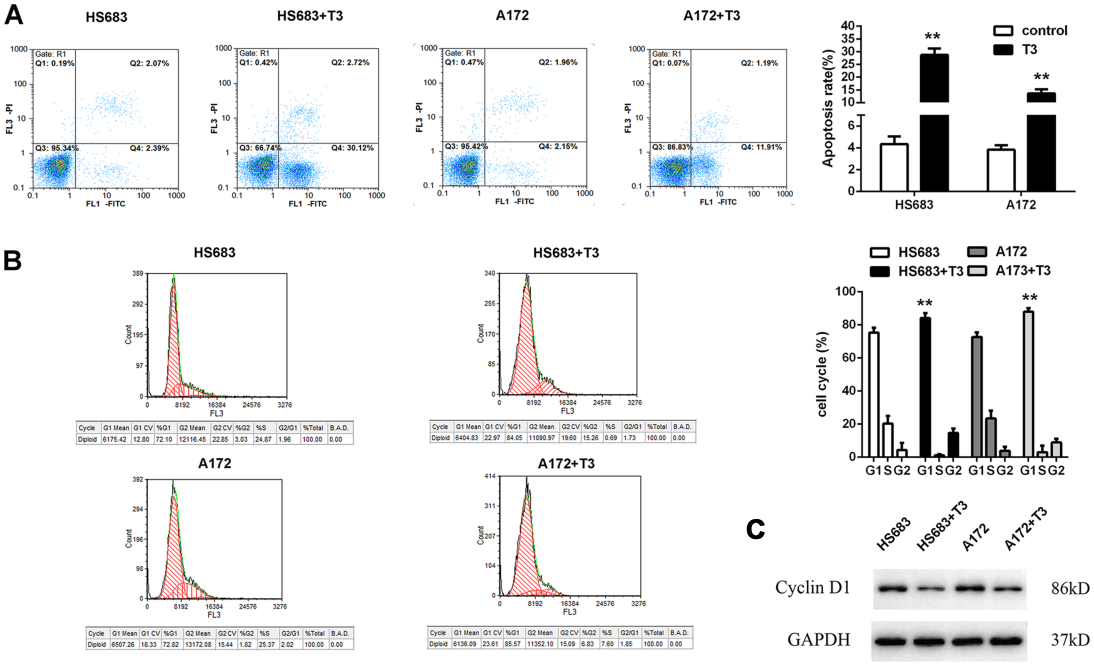

FIG. 2: T3 affects apoptosis and cell cycle of HS683 and A172 glioma cells. (A) Flow cytometry analysis was used to detect the apoptosis of HS683 and A172 glioma cells before and after 10 ng T3 treatment for 48 h (**$p < 0.01$ vs. control group). (B) Cell cycle analysis of HS683 and A172 glioma cells by flow cytometry assay (**$p < 0.01$ vs. the HS683 group and the A172 group). (C) Relative expression of cyclin D1 in HS683 and A172 glioma cells by Western blot.

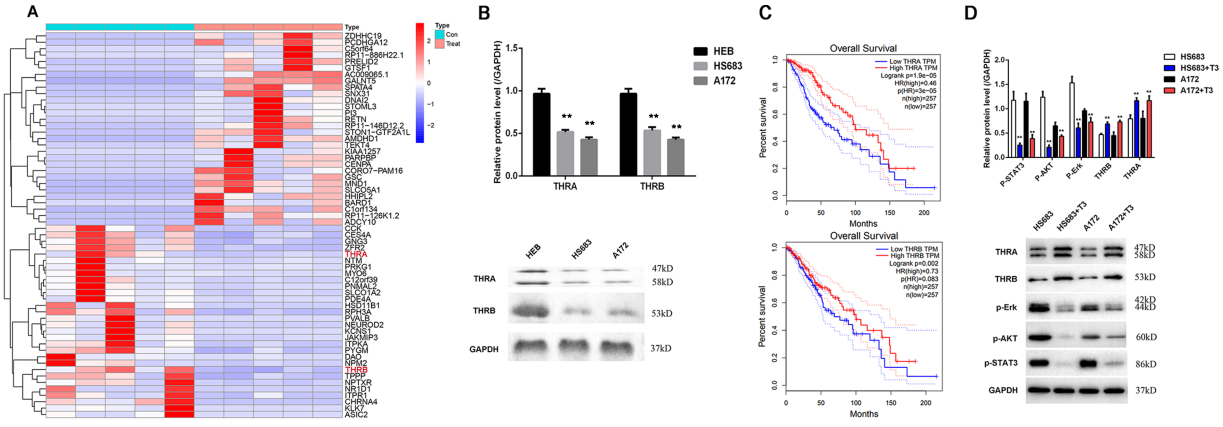

FIG. 3: T3 inhibits the expressions of THRA and THRB as well as phosphorylation of ERK, AKT and STAT3 in glioma cells. (A) THRA and THRB expressions in the glioma treatment group and control group. (B) THRA and THRB expressions in normal human glial cell (HEB) and glioma cells (**$p < 0.01$ vs. the HEB group). (C) The survival curves with different expression levels of THRA and THRB in gliomas. (D) Relative expression of p-STAT3, p-AKT, p-Erk, THRA, and THRB in HS683 and A172 glioma cells by Western blot. All data were normalized relative to the mRNA concentration for GAPDH and are presented as the mean ± SEM ($n = 3$) (**$p < 0.01$ vs. the HS683 group and the A172 group).

E. T3 Affects Cell Apoptosis and Cell Cycle of Glioma Cells through THRA or THRB

Next, to further explore the roles of THRA and THRB in gliomas, both HS683 and A172 glioma cells were transfected with si-THRA and si-THRB separately under T3 treatment. We found that cell apoptosis was prominently inhibited in the si-THRA and si-THRB experimental group (Fig. 4A and 4B). Moreover, interference with THRA and THRB

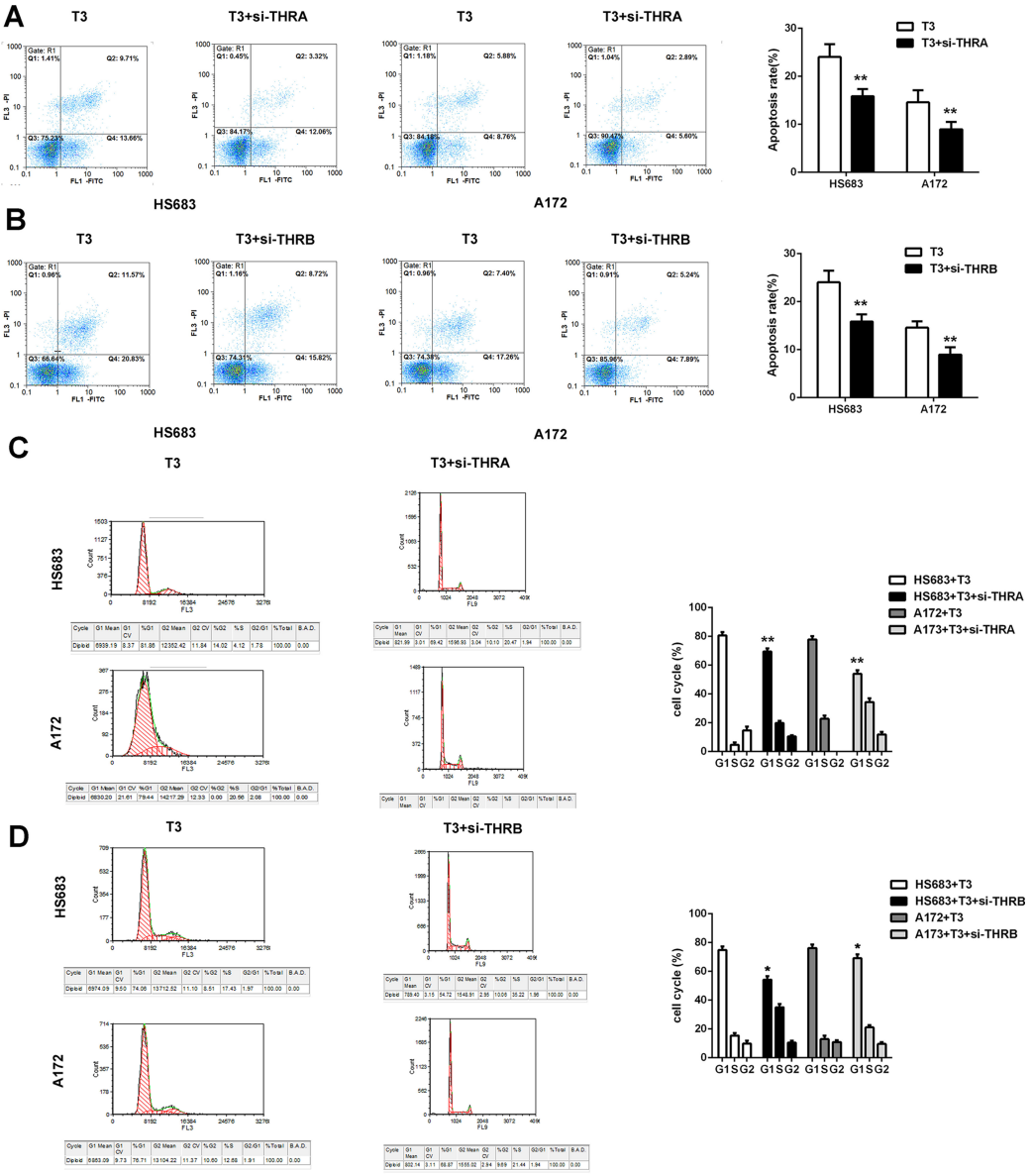

FIG. 4: T3 decreased apoptosis and cell cycle of HS683 and A172 glioma cells through THRA and THRB. (A and B) Flow cytometry analysis was used to detect the apoptosis of HS683 and A172 glioma cells after transfected with si-THRA or si-THRB under 10 ng T3 treatment for 48 h (**$p < 0.01$ vs. T3 group). (C and D) Cell cycle analysis of HS683 and A172 glioma cells after transfected with si-THRA or si-THRB by flow cytometry analysis (*$p < 0.05$ and **$p < 0.01$ vs. the HS683 + T3 group and the A172 + T3 group).

reversed the G_1 and G_2 phase arrest led by T3. The si-THRA and si-THRB experimental group showed an up-regulation in cyclin D1 expression G_1 phase arrest compared with the untransfected group (Fig. 4C and 4D).

F. T3 Inhibits the Phosphorylation of ERK, AKT, and STAT3 through THRA or THRB

To further explore the roles of THRA and THRB in MAPK/ERK and PI3K/Akt pathways, Western blot was used to demonstrate the effect after constructed THRA and THRB silencing expressed cells. The results indicated that, compared with the untransfected group, the p-ERK, p-AKT, and p-STAT3 proteins were markedly increased by inhibiting THRA and THRB in HS683 and A172 glioma cells (Fig. 5).

IV. DISCUSSION

Although the emergence of immunotherapy and tumor treatment field therapy has brought new treatments to glioma, the current primary treatment of glioma is still based on surgery plus radiotherapy and chemotherapy. The aggressive growth pattern leads to the incomplete resection of most tumors.[1] Due to the existence of the blood-brain barrier, it is necessary to use large doses of chemotherapy drugs in order to achieve effective concentrations in tumor tissues, often due to serious side effects, the required dosage cannot be followed, leading to poor efficacy.[1] T3 is a critical endocrine hormone in the human body, and its relationship with tumor proliferation is one of the hot spots in tumor research.[24] The results of the current work showed that the viability was inhibited while apoptosis was increased after T3 treatment of HS683 and A172 glioma cells. Moreover, T3 led to cell G_1 and G_2 phase arrest, which provided extra time for the cell to repair the damage, thereby reducing the occurrence of mutations and avoiding the formation of tumors,[25] indicating that T3 may inhibit cell viability by inducing cell apoptosis and cell cycle arrest. Our findings were consistent with the previous study by Song et al.[26]

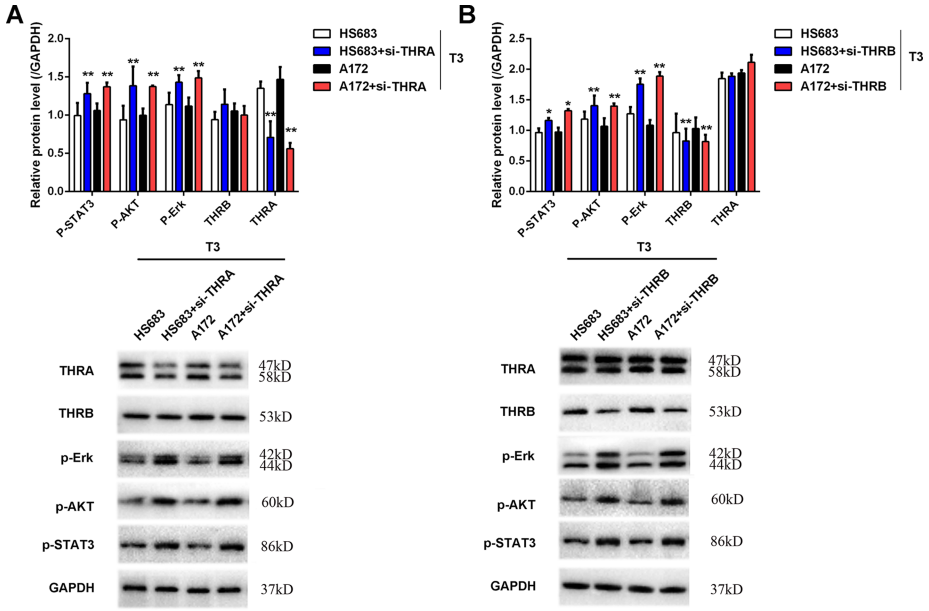

FIG. 5: T3 inhibits the phosphorylation of ERK, AKT, and STAT3 through THRA or THRB. (A and B) Relative expression of p-STAT3, p-AKT, p-Erk, THRA, and THRB in HS683 and A172 glioma cells transfected with si-THRA or si-THRB by Western blot. All data were normalized relative to the mRNA concentration for GAPDH and are presented as the mean ± SEM ($n = 3$) (*$p < 0.05$ and **$p < 0.01$ vs. the HS683 group and the A172 group).

In the study of nerve cells and tumors, T3 inhibited the neurons' proliferation in rats.[27] T3 can inhibit the proliferation of neuroblastomas.[28] In a study by Liappas et al., T3 inhibited the proliferation of high-grade glioma cell line U87 and promoted the proliferation of low-grade glioma cell line 1321N1.[19] The results of this study are consistent with those of Liappas et al., which suggests that the effect of T3 on cells may be related to the selection of experimental cells. Differential analysis of different experimental cells is needed to explore the mechanism of T3 on the proliferation of different cells.

THRA and THRB are two nuclear receptors of T3, which belong to steroid/thyroid hormone nuclear receptor superfamily, such as estrogen receptor, retinol receptor, retinoid X receptor, and vitamin D receptor.[21] Studies have shown that thyroid hormone receptors play an essential role in developing the nervous system, mainly in the migration and differentiation of nerve cells, synaptic formation and myelin formation.[22,23] In previous studies, the effects of thyroid hormone receptors were different in different types of tumors. THRA and THRB were regarded as tumor inhibitors in thyroid follicular cancer. Zhu et al.[29] used genetic engineering techniques to knock out THRA and THRB. As a result, the knockout mice formed spontaneously metastatic thyroid follicular cancer.[29] THRB was usually regarded as a tumor suppressor gene in colon cancer. In the study of colon cancer, the expression of THRB were the highest in normal colon mucosa, lower in adenoma and the lowest in cancer tissue, suggesting that THRB deletion plays a role in the occurrence of colon cancer.[30] García-Silva et al. found that T3 could inhibit the proliferation of N2a-β cell line, and both THRA and THRB played an inhibitory role.[28] In the study of glioma, the expression of THRB was positively correlated with the malignant degree of the tumors. In contrast, THRA was negatively correlated with the tumor's malignant degree, suggesting that THRA is a protective factor for gliomas.[31,32] In the study of TCGA, the expression of THRA and THRB in glioblastomas were lower than that in normal brain tissues.[33] Alexandros et al. studied T3 on glioma, in which THRA played a role in T3 inhibiting high-grade glioma cell line proliferation, but THRB did not.[19] In this study, the results indicated that THRA and THRB expressions were decreased in glioma and knockdown of THRA or THRB suppressed apoptosis and cell cycle, which was in line with previous studies in glioma.[19] In addition to normal THRA and THRB, mutations in the thyroid hormone receptor also play a role in forming and developing tumors. In the study of Park et al.,[34] four C-terminal frame transfer mutants PV, Mkar, MDBs, and AM were used to study THRB mutants' carcinogenesis. The results showed that THRB mutants induced tumor growth and activated PI3K-AKT-ERK/STAT3 signal in mouse tumor transplantation model.[34] In summary, this experiment's results show that both THRA and THRB play an important role in the inhibition of glioma proliferation by T3, but T3 signal transduction involved many signal pathways. Different types of cell signal transduction may interact with each other, which may be the reason for the inconsistency of many experimental results.

Numerous studies have shown that MAPK/ERK and PI3K/Akt signaling pathways are activated in tumors.[35–38] After the inhibition of these two signaling pathways, tumor cell proliferation is significantly inhibited.[35,37] MAPK/ERK and PI3K/Akt are also the two main signaling pathways for glioma.[39,40] Jin et al. found that PTPN1 promotes the progression of glioma by activating the MAPK/ERK and PI3K/AKT pathways.[39] Astragaloside IV, the major active triterpenoid in Radix Astragali, has been proved to inhibited the proliferation of glioma cells *in vitro* and attenuated tumor growth *in vivo* by regulating MAPK/ERK signaling pathway.[41] Furthermore, a clinical trial found that ACT001 directly binds PAI-1 to inhibit the PI3K/AKT pathway, which induces the inhibition of glioma cell proliferation, invasion and migration.[42] Current studies suggest that the MAPK/ERK and PI3K/Akt signaling pathways play an essential role in glioma development. However, T3 signal transduction involved many signal pathways, and different types of cell signal transduction may interact with each other, which may be the reason for the inconsistency of many experimental results. Therefore, related proteins in two pathways such as ERk and AKT were studied in this study. The results showed that T3 could inhibit the phosphorylation of ERK and AKT through THRA or THRB. The results verified that T3 could regulate multiple pathways in

glioma through THRA or THRB, which may be the next step to be investigated.

The limitations of this study are as follows: cells with different glioma tumor grades were not selected for the experiment *in vitro*, and the results of the *in vitro* experiment were not further verified *in vivo*.

V. CONCLUSION

T3 affects viability, apoptosis, and cell cycle of glioma cells by regulating THRA and THRB. THRA and THRB influence glioma development through hub genes related to the MAPK/ERK and PI3K/Akt signaling pathways.

FUNDING

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