Reactivity of thyroid papillary carcinoma cells to thyroid stimulating hormone-dominated endocrine therapy

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Abstract. This study investigated the effect of thyroid stimulating hormone (TSH) on the proliferation of papillary thyroid carcinoma (PTC) cells and the therapeutic effect of levothyroxine sodium (TH). PTC cells (TPC-1) were cultured using 0.1, 1.0 and 10 U/l TSH and 10^{-2}, 10^{-4} and 10^{-6} mol/l TH. After the appropriate concentration was screened, TPC-1 cells were further divided into control group, TSH group, TH group and TSH+TH group. The cell proliferation was detected via methyl thiazolyl tetrazolium (MTT) method, TPC-1 cell cycle was detected via flow cytometer, and the mRNA and protein expression of cyclin D1 were detected via real-time polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). Compared with control group, TSH significantly promoted the proliferation of TPC-1 cells ($P<0.05$ or $P<0.01$), obviously promoted the transition of TPC-1 cells from G1 phase to S phase ($P<0.01$) and remarkably increased the mRNA and protein expression of cyclin D1 ($P<0.01$); but TH had a significant inhibitory effect on these results of TSH ($P<0.05$ or $P<0.01$). TSH can promote the proliferation of PTC cells, and the appropriate complement of TH can inhibit its proliferation.

Introduction

Thyroid cancer is a kind of malignant endocrine system tumor with the highest incidence rate (1), which consists of 4 different pathological types: papillary thyroid carcinoma (PTC), medullary carcinoma, follicular carcinoma and undifferentiated carcinoma. Approximately 60-89% of thyroid cancer is PTC (2) that is highly differentiated (3) and often occurs in children or female patients aged 20-50 years. The pathogenesis of PTC is very complex, which is closely related to the heredity, environment and endocrine levels; moreover, the radioactive substances, Hashimoto thyroiditis and iodine deficiency can lead to the occurrence and development of PTC (4). In recent years, the incidence rate of PTC has risen continuously (5,6), and it has aroused great concern of scholars world-wide. The combined application of treatment methods, such as surgery and nuclide, can significantly improve the survival of PTC patients, and the 10-year survival rate of 70% patients can be up to 10 years (7). Thyroid stimulating hormone (TSH) receptor exists in PTC cells, and the PTC cell growth and differentiation depend on TSH secreted by pituitary gland to a certain extent. Therefore, the routine application of levothyroxine (TH) for TSH inhibition therapy after PTC operation can reduce the postoperative recurrence of patients and obtain a better prognosis (8). At the same time, the serum TSH level can be used as a clinical index of predicting PTC in patients with thyroid nodules (9,10). In this study, the *in vitro* cell experiment was performed to observe the effects of TSH and TH intervention on the proliferation of PTC cells, and the role of TSH in the pathogenesis of PTC and the therapeutic effect of TH were investigated from the cytological level, so as to provide an experimental basis for the clinical prevention and treatment of PTC.

Materials and methods

Experimental materials and reagents. Human PTC cells (TPC-1) (BNCC, Beijing, China); cattle TSH and TH (Sigma, San Francisco, CA, USA); methyl thiazolyl tetrazolium (MTT) (Sigma); RPMI-1640 medium and fetal calf serum (Gibco, Grand Island, NY, USA); cell cycle assay kit (Beyotime, Shanghai, China); TRIzol RNA extraction reagent (Takara, Shiga, Japan); reverse transcription kit (Toyobo, Osaka, Japan); human cyclin D1 and β-actin primers (Shanghai Sangon Biomedical Engineering Co., Ltd., Shanghai, China); SYBR Green PCR Master mix (Takara); cyclin D1 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, MN, USA).

Laboratory equipment. CO_2 incubator (Thermo Fisher Scientific, Inc., Waltham, MA, USA); clean bench (Suzhou Purification Equipment Co., Ltd., Suzhou, China); inverted fluorescence microscope (Nikon, Tokyo, Japan); flow

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Table I. Primer sequences.

Detection gene	Amplification length	Primer sequence
Cyclin D1	146 bp	Forward primer: 5'-CAATGACCCCGCACGATTTC-3' Reverse primer: 5'-CATGGAGGGCGGATTGGAA-3'
β-actin	200 bp	Forward primer: 5'-CGCACCACTGGCATTGTCAT-3' Reverse primer: 5'-TTCTCCTTGATGTCACGCAC-3'

cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA); continuous-wavelength multi-function microplate reader (Tecan Austria, Grodig, Austria); real-time fluorescence quantitative polymerase chain reaction (PCR) instrument (Eppendorf, Hamburg, Germany).

TPC-1 cell culture. TPC-1 cells were cultured in RPMI-1640 medium containing 100 μg/ml streptomycin, 100 U/ml penicillin and 10% fetal bovine serum, and the liquid was replaced every other day, followed by passage after cells grew to 80% confluence. The 3-5-generation cells in the logarithmic growth phase were used for experiments.

Experimental grouping. 1) Single application of 0.1, 1.0 and 10 U/l TSH; 2) single application of 10^{-2}, 10^{-4} and 10^{-6} mol/l TH; according to the appropriate concentration 1) and 2) screened in the early experiment, cells were further grouped and used for experiment: 1) Normal group: RPMI-1640 medium + 10% fetal calf serum; 2) TSH group: 10U/l TSH intervention; 3) TH group: 10^{-2} mol/l TH intervention; 4) combined group: (TSH+TH group): 10 U/l TSH+10^{-2} mol/l TH intervention. TPC-1 cells were treated in different culture solution for 48 h. The study was approved by the Ethics Committee of Shandong Institute Hospital. Signed written informed consents were obtained from all participants before the study.

Detection of cell proliferation via MTT assay. TPC-1 cells were inoculated onto a 96-well plate with 10^4 cells in each well. After the normal culture for 24 h, the cells were synchronously incubated in the serum-free culture solution for 24 h, and then 200 μl different media were added for intervention for 48 h according to the above grouping. At the same time, the blank control group that was only added with complete medium without cells was set for zero setting. Six control wells were set for each group. After 48 h, the original culture solution was removed from each well, and then 100 μl 0.5 mg/ml MTT solution was added into each well for continuous incubation at 37˚C for 4 h. Then the MTT solution was absorbed, and 100 μl dimethyl sulfoxide was added into each well, followed by vibration on the shaking table to dissolve the crystal. The continuous-wavelength multi-function microplate reader was used to measure the optical density (OD) value at 492 nm.

Detection of TPC-1 cell proliferation cycle. TPC-1 cells were inoculated onto a 6-well plate with 10^5 cells in each well. After normal culture for 24 h, the control group, TSH group, TH group and TSH+TH group received drug intervention for 48 h. Then cells were collected, pre-cooled using 75% alcohol at -20˚C, and fixed overnight. Then cells were washed with phosphate buffered saline (PBS) 3 times, and then RNase and PI dye were added successively. Finally, the cell cycle in each group was measured using the flow cytometer, and the proportions of cells in G1 phase and S phase in each group were observed.

Total RNA extraction and reverse transcription. TPC-1 cells in control group, TSH group, TH group and TSH+TH group received the drug intervention for 48 h. Then the cells were collected and added with appropriate amount of TRIzol reagent. The total RNA was extracted according to the instructions of TRIzol kit, and its concentration was determined using the ultraviolet spectrophotometer. Both RNA concentration and purity reached the experimental requirements. The cDNA was prepared via RNA reverse transcription according to the instructions and stored at -80˚C.

Detection of mRNA expression level of cyclin D1 in each group via real-time PCR. The primer sequences of cyclin D1 and β-actin gene are shown in Table I. The reaction system was as follows: 2 μl cDNA, 12.5 μl 2X SYBR Green PCR master mixes, 0.5 μl forward primer and 0.5 μl reverse primer; finally ultra-pure water was added until the total volume was 25 μl. Amplification procedure: 95˚C for 30 sec, 95˚C for 5 sec and 60˚C for 30 sec, a total of 40 cycles. After the amplification, the amplification curve and Ct value were read. With β-actin as a reference, the relative quantitative $2^{-\Delta\Delta Ct}$ method was used to compare the expression difference of each gene.

Detection of cyclin D1 in TPC-1 cells. After the drug intervention for TPC-1 cells according to the above grouping for 48 h, the supernatant was collected and centrifuged at 3,627 x g for 10 min at 4˚C. The supernatant was separated for ELISA. Sample loading and treatment were performed strictly according to the instructions of the kit and the OD value was measured at 490 nm using the continuous-wavelength multi-function microplate reader. Besides, the standard curve was drawn and the content of cyclin D1 in each sample was calculated.

Statistical analysis. Experimental results are presented as mean ± SD, and SPSS 20.0 software (IBM SPSS, Armonk, NY, USA) was used for statistical analysis of data. Independent sample t-test was used for the comparison between the two groups, one-way analysis of variance was used for the comparison among groups, and LSD method was used for the pairwise comparison. $P<0.05$ is considered to indicate a statistically significant difference.

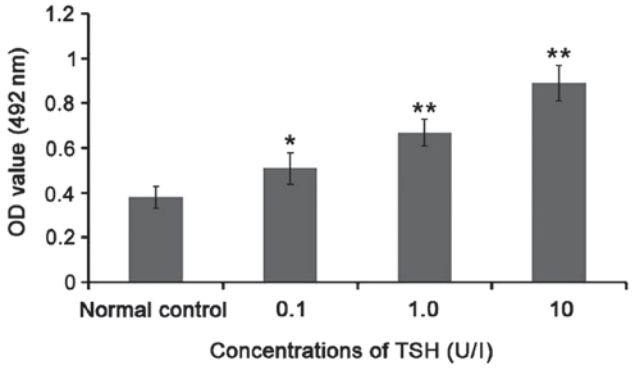

Figure 1. Effect of TSH on the proliferation of TPC-1 cells. MTT assay is used to detect the effects of different concentrations of TSH on the proliferation of TPC-1 cells, and the results show that the proliferation rate of TPC-1 cells is increased significantly with the increase in TSH concentration, compared with that in control group (P<0.05 or P<0.01). *P<0.05, **P<0.01 compared with normal group.

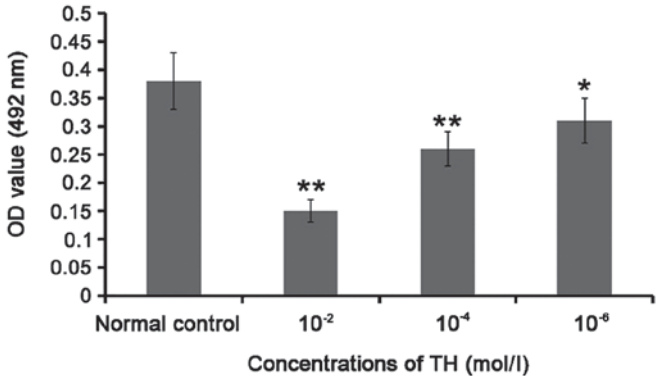

Figure 2. Effect of TH on the proliferation of TPC-1 cells. MTT assay is used to detect the effects of different concentrations of TH on the proliferation of TPC-1 cells, and the results show that the inhibition rate of TPC-1 cells is increased significantly with the increase in TH concentration, compared with that in control group (P<0.05 or P<0.01). *P<0.05, **P<0.01 compared with normal group.

Results

Effect of TSH on the proliferation of TPC-1 cells. Compared with that in normal control group, the OD value was significantly increased (P<0.05 or P<0.01) after 0.1, 1.0 and 10 U/l TSH acted on TPC-1 cells for 48 h; with the increase in TSH concentration, the OD value was significantly increased, suggesting that TSH can significantly promote the TPC-1 cell proliferation, and its proliferation reached the peak at 10 U/l. Thus, 10 U/l TSH was selected for the subsequent experiments (Fig. 1).

Effect of TH on the proliferation of TPC-1 cells. Compared with that in normal control group, the OD value was significantly decreased (P<0.05 or P<0.01) after 10^{-2}, 10^{-4} and 10^{-6} mol/l TH acted on TPC-1 cells for 48 h; with the increase in TH concentration, the OD value was significantly decreased, suggesting that TH can significantly inhibit the TPC-1 cell proliferation, and its proliferation reached the bottom at 10^{-2} mol/l. Thus, 10^{-2} mol/l TH was selected for the subsequent experiments (Fig. 2).

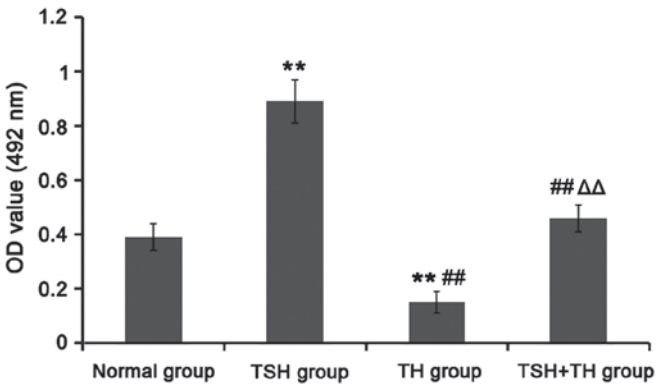

Figure 3. Effect of TH combined with TSH on the proliferation of TPC-1 cells. The cell proliferation in different groups was detected via MTT assay. The results show that TSH significantly promotes the proliferation of TPC-1 cells (P<0.01), while TH significantly inhibits the proliferation of TPC-1 cells, compared with control group (P<0.01). TH can significantly reduce the proliferation of TPC-1 cells (P<0.01). **P<0.01 compared with normal group; ##P<0.01 compared with TSH group; ΔΔP<0.01 compared with TH group.

Table II. Effects of TH, TSH and combined application on the mRNA and protein expression levels of cyclin D1.

Group	mRNA	Protein (ng/l)
Normal group	1.02±0.11	1.89±0.25
TSH group	2.18±0.34[b]	3.07±0.38[b]
TH group	0.63±0.20[a,d]	1.42±0.27[a,d]
TSH+TH group	1.32±0.32[c,e]	2.34±0.36[c,e]

[a]P<0.05, [b]P<0.01 compared with normal group; [c]P<0.05, [d]P<0.01 compared with TSH group; [e]P<0.05, compared with TH group.

Effect of TH combined with TSH on the proliferation of TPC-1 cells. Compared with that in normal group, the OD value was significantly increased in TSH group (P<0.01), but significantly decreased in TH group (P<0.01). Compared with that in TSH group, the OD values in TH group and TSH+TH group were significantly decreased (P<0.01). The OD value in TSH+TH group was significantly increased compared with that in TH group (P<0.01). There was no significant difference between TSH+TH group and normal group (P>0.05). It can be seen that TH can significantly inhibit the proliferation of TPC-1 cells (Fig. 3).

Effects of TH, TSH and combined application on the TPC-1 cell cycle. Compared with those in normal group, the proportion of cells in G1 phase in TSH group was significantly decreased, but that in S phase was significantly increased (P<0.01); the proportion of cells in G1 phase in TH group was significantly increased, but that in S phase was significantly decreased (P<0.05). Compared with those in TSH group, the proportions of cells in G1 phase in TH group and TSH group were significantly increased, but those in S phase were significantly decreased (P<0.05 or P<0.01). Compared with those in TH group, the proportion of cells in G1 phase in TSH+TH group was significantly decreased, but that in S phase was significantly increased (P<0.05 or P<0.01). There was no

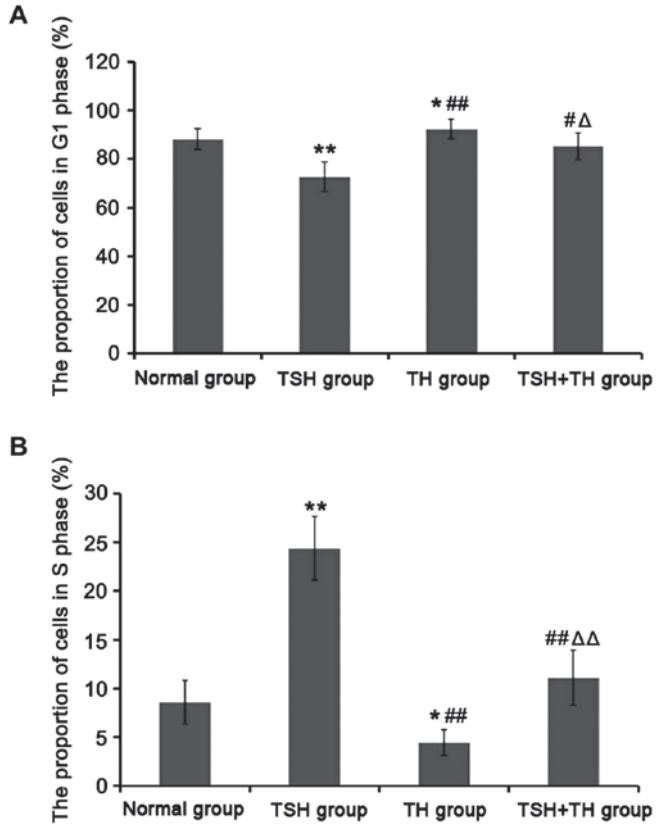

Figure 4. Effects of TH, TSH and combined application on the TPC-1 cell cycle. Flow cytometer was used to detect the cell cycle in each group. The results show that TSH significantly promotes the transition of TPC-1 cells from G1 phase to S phase (P<0.01), TH significantly inhibits the transition of TPC-1 cells from (A) G1 phase to (B) S phase (P<0.05) and TH can significantly inhibit TSH from promoting the transition of TPC-1 cells from G1 phase to S phase (P<0.05 or P<0.01). *P<0.05, **P<0.01 compared with normal group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 compared with TSH group; $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 compared with TH group.

significant difference between TSH+TH group and normal group (P>0.05) (Fig. 4).

Effects of TH, TSH and combined application on the mRNA and protein expression levels of cyclin D1 in TPC-1 cells. Compared with those in normal group, the mRNA and protein expression levels of cyclin D1 in TSH group were significantly increased (P<0.01), and those in TH group were significantly decreased (P<0.05). Compared with those in TSH group, the mRNA and protein expression levels of cyclin D1 in TH group and TSH+TH group were significantly decreased (P<0.05 or P<0.01). Compared with those in TH group, the mRNA and protein expression levels of cyclin D1 in TSH+TH group were significantly increased (P<0.05). There was no significant difference between TSH+TH group and normal group (P>0.05) (Table II).

Discussion

The largest endocrine gland in human body is the thyroid gland, and hypothalamus-anterior pituitary system regulates the growth, development and functions of thyroid gland (11). TSH is secreted by the anterior pituitary, its function is to promote the synthesis of thyroid hormone. Human TSH is a kind of glycoprotein in the body, mainly composed of 211 amino acids, 15% of which in the whole molecule is the sugar. Moreover, the entire TSH molecule consists of two peptide chains: α and β chains. Moreover, the secretion of TSH has significant rhythm: TSH gradually increases at 2 h after sleep and its level in serum reach the peak at 2-4 o'clock in the morning (12,13). TSH receptor belongs to the G-protein-coupled receptor, mainly expressed on the thyroid follicular epithelial cell membrane. The encoding gene of the TSH receptor is located on chromosome 14 and approximately 60 kb in length, which consists of 10 exons (14). TSH binds to the extracellular amino terminal of its receptor to regulate the thyroid function by enhancing the iodine pump activity and tyrosine iodination, promoting the thyroglobulin synthesis and enhancing the peroxidase activity (15).

In recent years, a large number of basic and clinical studies world-wide have found that when the thyroid hormone is lacking in the body, it can secrete a large amount of TSH into the blood, leading to high TSH and increasing the risk of thyroid tumor (16). Under the long-term stimulation of TSH, thyroid tissues will suffer from diffuse enlargement, and the thyroid nodules are gradually formed with the course of disease, ultimately developing into thyroid cancer without timely treatment (17). In patients with thyroid nodules, the probability of suffering from thyroid cancer increases with the increase in serum TSH level (9). In animal experiments, it is found that the appropriate supplement of thyroxine can reduce the incidence of thyroid cancer (18).

PTC belongs to the thyroid cancer with a higher degree of differentiation. Compared with that in normal thyroid follicle cells, the TSH receptor transcription in PTC cells is reduced, but some functions are still retained. When TSH binds to its receptor, it can regulate the expression of thyroid peroxidase antibody, sodium/iodide symporter and other thyroid-specific genes through the adenosine cyclophosphate and other signaling pathways, while it can also regulate the thyroid cell proliferation and differentiation, ultimately accelerating the deterioration of PTC (19). Experimental studies have confirmed that the appropriate amount of TSH can promote the growth of thyroglobulin and thyroid cell proliferation (1), but the excessive increase in TSH can induce the PTC progression (20). After the PTC operation, the routine supplement of thyroxine can effectively inhibit the serum TSH level in the body and significantly reduce the recurrence and mortality rates of patients (21). It can be seen that the serum TSH level is closely related to the occurrence and progression of PTC, so reducing the serum TSH level can lower the incidence rate of PTC. In this study, the cell experiment proved that TSH can significantly promote the proliferation of PTC cells cultured *in vitro*, and the appropriate supplement of TH can inhibit its proliferation.

Cyclin D1 is a kind of important regulatory protein in G1 phase, and its encoded D1 protein plays an important role in regulating the transition from G1 phase to S phase. After D1 protein binds to cyclin-dependent kinase 4P6 (CDK4P6), the transcription and expression of a series of cell cycle-related genes are induced, thus promoting the proliferation and differentiation of the cell cycle from G1 phase to S phase, which accelerates the whole cell cycle (22). Cyclin D1 is over-expressed in many malignant tumors of human, such as breast

cancer and thyroid cancer (22,23). In this study, it was also found that TSH can promote the mRNA and protein expressions of cyclin D1 in PTC cells, thus promoting the transition of cell cycle from G1 phase to S phase, which can be inhibited by the supplement of TH.

In conclusion, it was confirmed in this study through *in vitro* cell experiments that TSH can promote the proliferation of PTC cells, and the appropriate supplement of TH can inhibit its proliferation, providing an experimental basis for clinical medication. The occurrence and progression of PTC is a complex pathological process involving a variety of cell signaling pathways, and its relevant pathways need to be further studied.

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