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Original Article

TMP21 modulates cell growth in papillary thyroid cancer cells by inducing autophagy through activation of the AMPK/mTOR pathway

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Abstract: Objective: To investigate the role of transmembrane protein (TMP) 21 in human thyroid cancer. Methods: The recombinant expression vector pcDNA3.1 (+)-TMP21 and specific small interfering RNAs (siRNA) against TMP21 were transfected into a papillary thyroid cancer cell line (TPC1). After transfection, the expression of TMP21 was confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting. Moreover, cell viability and apoptosis rate were respectively determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay and flow cytometry (FCM). Additionally, Western blotting was performed to analyze the adenosine monophosphate (AMP)-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathways associated protein (P-AMPKαThr172, P-mTORSer2448, light chain (LC)-II/LC3-I, and P-S6KThr389) after pre-treatment with AMPK inhibitor, compound C (Com C) and siTMP21. Results: The TMP21 protein level and cell viability were significantly higher, but apoptotic rate was significantly lower by transfection with pcDNA3.1-TMP21 than those in control group ($P < 0.05$), and reverse results were obtained by transfection with siTMP21. However, qRT-PCR showed different results due to the feedback inhibition of mRNA. Besides, silencing of TMP21 significantly reduced the levels of P-mTORSer2448 and P-S6KThr389 ($P < 0.05$), but significantly increased the levels of P-AMPKαThr172 and LC3-II/LC3-I compared with the control group ($P < 0.01$). Whereas, the levels of P-AMPKαThr172 and LC3-II/LC3-I were significantly decreased by Com C compared with the control group ($P < 0.05$). Conclusion: TMP21 modulates cell growth in TPC1 cells by inducing autophagy, which may be associated with activation of AMPK/mTOR pathway.

Keywords: Thyroid cancer, transmembrane protein 21, autophagy, AMPK/mTOR

Introduction

Thyroid cancer is a relatively rare malignancy, accounting for approximately 1% of all tumors with female dominance (female-to-male ratio 3:1) [1]. However, it is the most frequent endocrine cancer worldwide [2]. It has been reported that thyroid cancer is the tenth common malignancy in urban areas in China, and the age-standardized incidence rate is $6.56/10^5$ [3]. Despite the rare incidence, a fast worldwide increase in the past few decades has been reported [4, 5]. In the U.S., it is the most rapidly increasing tumor among all the malignancies for both men and women [6]. Although much progress has been made in molecular pathogenesis in thyroid cancer, the exact mechanism still remains unclear.

Transmembrane protein (TMP) 21 (also known as p23 protein) is an important member of the p24 protein families, which plays significant roles in maintaining the integrity of the secretory pathway in mammals [7]. It has been reported that TMP21 is broadly expressed at different levels in normal adult human tissues, such as heart, liver, lung, kidney, brain, pancreas, skeletal muscle, thyroid gland and etc. [8]. Recently, Wang et al. found that TMP21 was involved in protein kinase C (PKC) δ-mediated apoptosis and signaling in prostate cancer. However, little information is available regarding the function of TMP21 on thyroid cancer.

In addition, the role of cellular metabolism in tumor progression has been paid attention recently [9]. Adenosine monophosphate (AMP)-

activated protein kinase (AMPK) is a regulatory protein of energy utilization, which plays an important role in modulating normal cell metabolism [10, 11]. But it is now considered as a key cellular energy sensor in cancers, and critically associated with cell sensitivity to anticancer agents [12]. The mechanism responsible for inhibition of tumor cell growth by AMPK is thought to be associated with activation of the tumor suppressor tuberous sclerosis complex 2 (TSC2) [13]. Then the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) signaling is effectively inhibited by controlling of translation initiation and protein synthesis [14]. Additionally, mTOR signaling pathway has been reported to play an important role in autophagy. Activation of this pathway suppresses of autophagy and enhances cell growth and proliferation in both normal and tumor cells [15, 16]. Previous studies have investigated the role of AMPK in thyroid disorder, such as nodular goiter and thyroid tumorigenesis, suggesting that activation of AMPK could be beneficial for thyroid diseases [17, 18]. For example, a study conducted by Choi *et al.* suggested that treatment with a direct AMPK activator leads to apoptosis and S-phase cell cycle arrest in BRAFV600E-mutant thyroid tumor cell lines though down-regulation of ERK and mTOR/p70S6K signaling [19]. However, whether TMP21 induces autophagy regulated by AMPK/mTOR in thyroid cancer is unclear.

Hence, the purpose of our study was to explore the role of TMP21 on thyroid cancer, as well as the associated mechanism. Our results may benefit on helping in the design and guidance of future studies concentrated on the function of TMP21 on thyroid cancer.

Material and methods

Cell lines and cultures

In our study, papillary thyroid cancer cell line (TPC1) purchased from Shanghai Cell Biochemical Institute, China Academy of Science was used. These cells were cultured in Dulbecco Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS; Santa Ana, CA), 100 units/mL penicillin G sodium (Santa Ana, CA), 100 μg/mL streptomycin sulfate (Santa Ana, CA) in a humidified incubator containing 5% CO_2 at 37°C.

Vector construction and transfection

A TMP21 expression vector (pcDNA3.1-TMP21) was provided by Vector Co., USA. It was constructed by sub-cloning the full-length cDNA for wild-type TMP21 into pcDNA3.1 (+) and confirmed by sequencing. As a control, an empty construct pcDNA3.1 was transfected into TPC1 cell. The target sequence for TMP21-specific siRNA was synthesized according to Wang *et al.* [20]. Cell transfections were performed using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instruction. Normal media was added after incubation of the transfected cells for 4 h. After 48 h incubation, the cells were collected for further analysis.

Cell proliferation assay

The cell proliferative capacity was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay. Briefly, TPC1 cells (5×10^4 per mL) were placed in 96-well plates and were maintained at 37°C in 5% CO_2 incubator for 24 h. After that, MTT (20 μl; 5 g/l) was put into each well and the plates were maintained at 37°C for another 4 h. A microplate reader (Model Benchmark) was used to determine the cell vitality at 570 nm. Experiments were carried out at least 3 times.

Apoptosis assay

Flow cytometry (FCM) detection was used to measure the apoptosis rate using Annexin V-fluorescein-5-isothiocyanate (Annexin V-FITC) apoptosis detection kit (Sigma, St Louis, MO, USA). Briefly, cells were collected at 24, 48, 72, and 96 hours, washed by phosphate buffer saline (PBS), and re-suspended in binding buffer at a density of 10^6 cells/ml. Then 10 μL Annexin V-FITC (20 μg/ml) was added and incubation at room temperature in the dark for 15 min, following with mixture with 5 μL (50 μg/ml) propidium iodide (PI). The cells were immediately analyzed using FCM (Becton Dickinson, San Jose, CA, USA) (excitation wavelength 488 nm and emission wavelength 635 nm). Results were analyzed by CELLQuest 3.0 software (Becton Dickinson, San Jose, CA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Expression of TMP21 mRNA in TPC-1 cells treated with pcDNA3.1-TMP21 and siTMP21

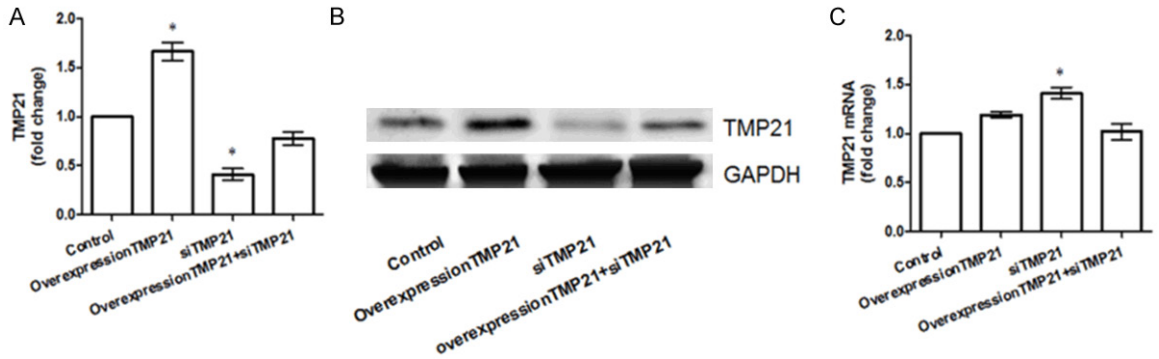

Figure 1. The influence on expression of TMP21 after transfection. A and B. Expression protein level of TMP21 after transfection; C. Expression mRNA level of TMP21 after transfection. TMP, transmembrane protein; siTMP21, small interfering against TMP21; $^{*}P < 0.05$ compared with control group.

was analyzed using qRT-PCR. Total mRNA was isolated from TPC-1 cells using Trizol RNA Isolation Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendation. Complementary DNA (cDNA) was synthesized using the Reverse Transcription System (Promega, Madison, WI). Expression levels of TMP21 genes were analyzed by SYBR green-based qRT-PCR (Thermo Scientific Inc., USA). β-actin gene was used as a reference. PCR amplification was carried out using LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics, Meylan, France). PCR conditions consisted of 1 predenaturation cycle of 10 min at 95°C, 40 denaturation cycles of 30 s at 95°C, 60°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. The mRNA levels were presented with threshold cycle (CT). The amount of target gene was assessed using $2^{-\Delta\Delta}$CT method. Reactions were carried out in triplicate.

The primers sequences used were as follows: TMP21, forward primer: 5'-CGGGA TCCGCCATGTCTGGTTTGTCTGGCCCAC-3', reverse primer: 5'-GGAATTCCTC AATCAATTTCTTGGCCTTG-3'; β-actin, forward primer: 5'-CGAGGATCCGGA CTTCGAGCAAGAGATGG-3', reverse primer: 5'-CAGTCTAGAGAAGCATTTGC GGTGGACG-3'.

Western blotting for signaling pathway analysis

For Western blotting analysis, TPC1 cells were incubated for 24 h. After pre-treatment with Compound C (Com C, 5 μM, an AMPK inhibitor) and siRNA for 24 h, the cells were harvested for protein extraction and concentration determination. The protein concentration was determined using BCA assay kit (Pierce Piscataway, NJ, USA) according to the manufacturer's instruction. Proteins samples were resolved with a 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride (PVDF) membranes (Bedford, MA, USA), blocked in 5% defatted milk powder for 2 h at room temperature and probed with the following primary antibodies overnight at 4°C: anti-TMP21 (1:1000, Santa Cruz Biotech, Dallas, Texas), anti-P-AMPKα^{Thr172} (1:1000, Cell Signaling Technology Inc., Beverly, MA), anti-P-mTOR^{Ser2448} (1:1000, Cell Signaling Technology Inc., Beverly, MA), anti-light chain (LC) 3-II/LC3-I (1:1000, Santa Cruz Biotech, Dallas, Texas), anti-P-S6K^{Thr389} (1:1000, Cell Signaling Technology Inc., Beverly, MA), and anti-GAPDH (1:1000, Santa Cruz Biotech, Dallas, Texas). After 2 h incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature, enhanced chemiluminescence and densitometric analysis was performed.

Statistical analysis

The data were presented as the mean ± standard deviation (SD). Statistical analyses were performed using statistic package for social science (SPSS, version 19.0, Chicago, IL) software. One-way analysis of variance (ANOVA) was performed to calculate the *P*-values. A *P*-value of < 0.05 was considered to be statistically significant result.

Results

The influence on expression of TMP21 after transfection

To examine the influence on expression of TMP21 after transfection with pcDNA3.1-

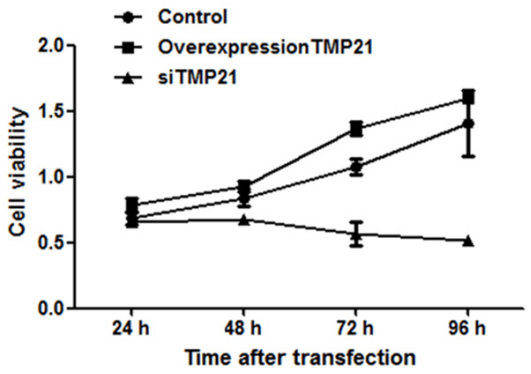

Figure 2. Silencing of TMP21 to TPC1 cells decreases cell viability. TMP, transmembrane protein; siTMP21, small interfering against TMP21.

TMP21 and target sequence for TMP21-specific siRNA into TPC1 cells, we performed qRT-PCR and Western blotting to confirm the mRNA and protein levels of TMP21, respectively. The Western blotting results showed that the TMP21 protein level transfected by pcDNA3.1-TMP21 was significantly higher than that in control group ($P < 0.05$), while the TMP21 protein level in siTMP21 group was markedly reduced than the control group ($P < 0.05$). However, there were no significant differences in TMP21 protein level between the control group and combined transfection with pcDNA3.1-TMP21 and siTMP21 group (**Figure 1A** and **1B**).

The results of qRT-PCR showed different TMP21 protein levels. We found that TMP21 mRNA level in siTMP21 group showed the highest level. There were significances in TMP21 mRNA level between the siTMP21 group and the control group (**Figure 1C**).

Silencing of TMP21 to TPC1 cells decreases cell viability and increases apoptotic rate

To investigate the impact of silencing of TMP21 to TPC1 cells, cell viability and apoptotic rate were examined using MTT and FCM in TPC1 cells, respectively. We found that silencing of TMP21 could significantly reduce the cell viability (**Figure 2**) and upregulate the apoptotic rate compared with the control group ($P < 0.05$). The cell viability was significantly increased in overexpression TMP21 group than that in control group. Besides, FCM analysis revealed that there were no significant differences among the control group, overexpression TMP21 group, and the combined transfection with pcDNA3.1-TMP21 and siTMP21 group (**Figure 3A** and **3B**).

Silencing of TMP21 to TPC1 cells activates AMPK/mTOR pathways

To examine the role of TMP21 induced AMPK/mTOR pathway activation in TPC1 cells; we measured AMPK/mTOR pathway associated protein (P-AMPKα^{Thr172}, P-mTOR^{Ser2448}, LC3-II/LC3-I, and P-S6K^{Thr389}) after transfection with siTMP21 and pre-treated with Com C, an AMPK inhibitor. The results demonstrated that silencing of TMP21 significantly decreased the levels of P-mTOR^{Ser2448} and P-S6K^{Thr389} ($P < 0.05$), but significantly increased the levels of P-AMPKα^{Thr172} and LC3-II/LC3-I compared with control group ($P < 0.01$). However, the levels of P-AMPKα^{Thr172} and LC3-II/LC3-I were significantly decreased by Com C ($P < 0.05$). No significant differences in levels of P-mTOR^{Ser2448} and P-S6K^{Thr389} after treatment with Com C were found (**Figure 4A** and **4B**).

Discussion

In the present study, the function of TMP21 protein on the proliferation of human thyroid cancer cells (TPC1 cell line) and whether the function was induced by activation of AMPK/mTOR pathway were explored. We found that silencing of TMP21 significantly decreased TPC1 cells viability and increased apoptotic rate, while overexpression of TMP21 obtained reverse results. In addition, silencing of TMP21 modulated the expression of the associated protein of AMPK/mTOR pathway. These results indicated that TMP21 plays significant roles in thyroid cancer, and silencing of TMP21 could inhibit cancer growth by inducing autophagy through activation of the AMPK/mTOR pathway.

TMP21, one key member of the p24 protein families, is recognized as an important and new member of the presenilin-associated complex and a candidate mammalian cargo receptor [21-23]. It has been reported that TMP21 regulates γ-secretase and ε-secretase cleavage activities. The expression of TMP21 is regulated by nuclear factor of activated T cells (NFAT), and the degradation is mediated by the ubiquitin-proteasome pathway [24]. Besides, TMP21 plays significant roles in maintaining the integrity of the secretory pathway in mammals [7]. Recently, TMP21 is found to be a PKCδ-

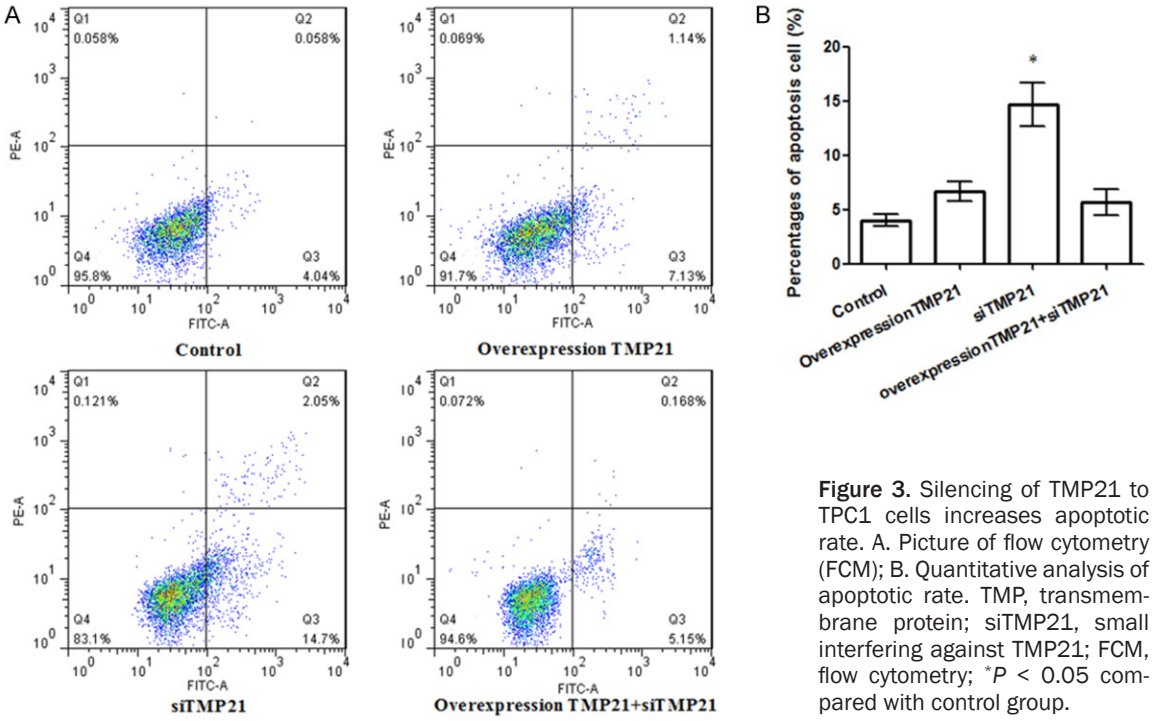

Figure 3. Silencing of TMP21 to TPC1 cells increases apoptotic rate. A. Picture of flow cytometry (FCM); B. Quantitative analysis of apoptotic rate. TMP, transmembrane protein; siTMP21, small interfering against TMP21; FCM, flow cytometry; $^{*}P < 0.05$ compared with control group.

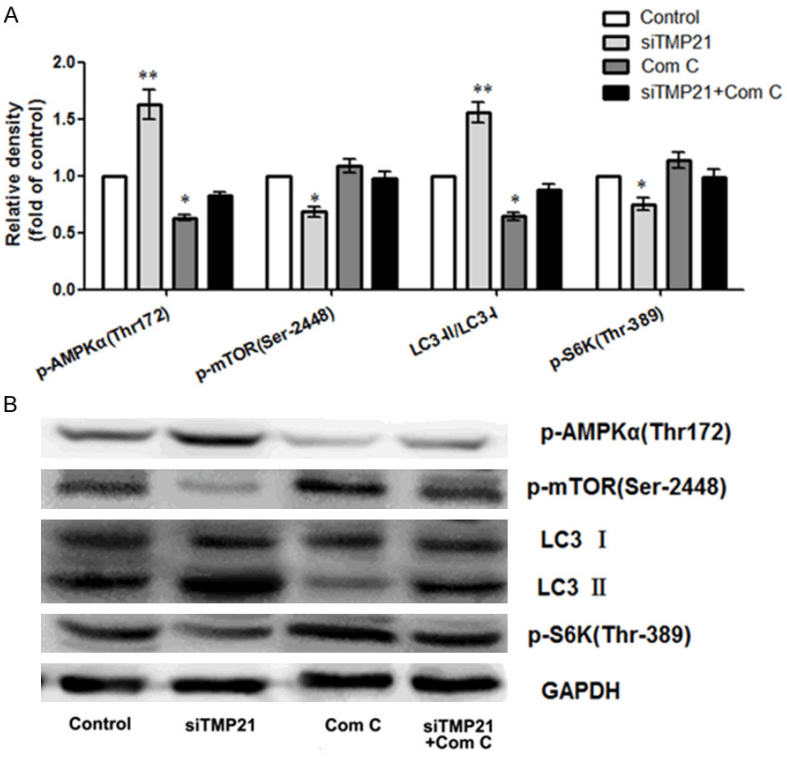

Figure 4. Silencing of TMP21 to TPC1 cells activates AMPK/mTOR pathways. A. Quantitative analysis of P-AMPKα^{Thr172}, P-mTOR^{Ser2448}, LC3-II/LC3-I and P-S6K-^{Thr389}; B. Picture of Western blotting. TMP, transmembrane protein; siTMP21, small interfering against TMP21; AMPK, adenosine monophosphate activated protein kinase; mTOR, mammalian target of rapamycin; LC, light chain; Com C, Compound C; $^{*}P < 0.05$ compared with control group; $^{**}P < 0.01$ compared with control group.

interacting protein. The silencing of TMP21 results in increased PKCδ translocation to the plasma membrane [20]. Besides, Xie *et al.* investigated the distribution of TMP21 in normal human tissues [8]. The results showed that TMP21 is broadly and varyingly distributed in 19 normal human tissues, such as thyroid gland (moderate levels). Additionally, animal experiments showed that TMP21 ubiquitously distributes in endocrine cells, such as pancreatic acinar cells, among the mouse tissues [23, 25]. The distribution and location of TMP21 indicates that it might be associated with some diseases in varied organs, such as Alzheimer's disease (AD), diabetes, kidney disorders [8, 20, 25, 26]. However, the function of TMP21 in thyroid disorders is still unclear. In addition,

the role of AMPK/mTOR pathway in thyroid cancer has been investigated [13, 27, 28]. Activation of AMPK but inhibition of mTOR has been regarded as a good target in certain types of thyroid cancer. Therefore, in our study, we speculated TMP21 might be involved with thyroid cancer, and the autophagy induced by activation of AMPK/mTOR pathway may be the possible mechanism responsible.

To confirm the speculation, we firstly transfected the recombinant expression vector pcDNA3.1 (+)-TMP21 and siRNA into the papillary thyroid cancer cell line (TPC1). The expression of TMP21 was assessed by qRT-PCR and Western blotting after transfection. However, the protein and mRNA levels of TMP21 were different from each other. As expected, the TMP21 protein level was significantly higher after transfection with pcDNA3.1-TMP21 than that in control group, while the TMP21 protein level was significantly lower after transfection with siTMP21. But the mRNA level of TMP21 showed different results. The TMP21 mRNA level in the siTMP21 group showed the highest level, and there were significances between the siTMP21 group and the control group. The possible reason was that there was some feedback inhibition of mRNA processing occurring. Moreover, we determined the cell viability and apoptosis rate. The results showed that the cell viability was significantly decreased by transfection with siTMP21 compared with control group, but was obviously increased by transfection with pcDNA3.1-TMP21, while the apoptotic rate presented with reverse results. These results demonstrated that silencing of TMP21 could effectively inhibit the growth of thyroid cancer cells.

To determine whether these above effects caused by TMP21 were through AMPK/mTOR pathways, we detected the associated protein of the pathway (P-AMPKα^{Thr172}, P-mTOR^{Ser2448}, LC3-II/LC3-I, and P-S6K^{Thr389}) using Western blotting after pre-treatment with Com C and siTMP21. Com C is an inhibitor of AMPK, and while mTOR is a main negative regulator of autophagy [29, 30]. P-S6K^{Thr389}, phosphorylation of S6K, is responsible for the increased mTOR activity [31], and P-S6K^{Thr389} could phosphorylate mTOR on Ser-2448 (p-mTOR^{Ser2448}) [32]. LC3-II/LC3-I is an established indicator of autophagy, and P-AMPKα^{Thr172} is known to activate AMPK [33]. Our results showed that silencing of TMP21 could significantly reduce the protein levels of P-mTOR^{Ser2448} and P-S6K^{Thr389}, but significantly increased the protein levels of P-AMPKα^{Thr172} and LC3-II/LC3-I compared with the control group, indicating that silencing of TMP21 induced autophagy by activation of the AMPK but inhibition of the mTOR. These results were similarly with a previous study, in which OSU-53 (regarded as a novel dual AMPK activator/mTOR inhibitor) effectively inhibited various thyroid cancer cell lines growth [13]. However, the levels of P-AMPKαThr^{172} and LC3-II/LC3-I were significantly decreased by Com C compared with the control group, indicating that Com C effectively inhibit the activation of AMPK and induced autophagy.

In conclusion, our study demonstrated for the first time that TMP21 plays significant roles in thyroid cancer. Silencing of TMP21 inhibits cancer cell growth by inducing autophagy though activation of AMPK/mTOR pathway.

Disclosure of conflict of interest

None.

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