## Original Article A primary investigation for the function of UbcH10, a cancer-promoting gene, in TPC-1 thyroid cancer cells

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**Abstract:** Thyroid cancer is the most common and highly aggressive endocrine malignancy. Recently, UbcH10 was correlated with this disease, however, the exact role of UbcH10 in the development of thyroid cancer remains unknown. In this study, the effect of UbcH10 knockdown by siUbcH10 on apoptosis, proliferation, and other cellular functions of thyroid cancer cell line TPC-1 was evaluated. The results showed that UbcH10 knockdown induced apoptosis and inhibition of proliferation. The outcomes of the cells cycle detection showed that the percent of G2/M phase of TPC-1 cells transfected with siUbcH10 enhanced from 5.0% to 20.68%. In addition, siRNA induced UbcH10 knockdown also resulted in a reduction in migration and invasion of TPC-1 cells. Moreover, transfection with siUbcH10 suppressed the activation of PI3K/Akt pathway, evidenced by the decreased levels of phosphorylated PI3K and Akt. In conclusion, UbcH10 may be a tumor promoter and a potential therapeutic target for the clinical management of thyroid cancer.

Keywords: UbcH10, thyroid cancer, PI3K/Akt pathway, TPC-1 cells

#### Introduction

Thyroid cancer, known as the most common endocrine malignancy, drew a great deal of attention for its increasing morbidity. In the United States, the incidence of thyroid cancer increased 4.99 folds form 1989 to 2012, and about 56,460 patients have been diagnosed with thyroid cancer up to 2012 [1]. More than 62,980 new cases and 1,890 deaths were estimated in 2014 [2]. In addition, the incidence rate of thyroid cancer in Korea has also increased sharply, and this disease has already become a unneglectable type of cancer in the country [3]. Similarly, the tendency of increasing morbidity has occurred in Beijing where about 1,099 cases of thyroid cancer were diagnosed in 2010 and the incidence rate is 8.78/100,000. In comparison with the incidence rate in 2001 when the rate was 2.70/100,000, the morbidity has grimly increased by 225.2% during the past 9 years accompanied with an annual growth rate of 14.2% [4]. Thyroid cancer has naturally become the most common cancer among all the malignancies.

The main origins of thyroid cancer are parafollicular cells (medullary) and follicular cells (nonmedullary), which account for over 95% of all thyroid cancer cases. Simultaneously these two kinds of cancers are further categorized into four histological types such as follicular thyroid cancer (FTC), papillary thyroid cancer (PTC), anaplastic thyroid cancer (ATC), and Hürthle cell carcinoma (HCC) [5, 6]. FTC which accounts for 5% of thyroid cancer is an aggressive malignancy, and patients suffered with it only have median survival of 6 months after treatment [7]. PTC is found to be the most common type (comprising ~80% of thyroid cancer compared to 10-15% for follicular) accounting for the majority in the increasing rate of thyroid cancer overall [8], and reports further indicate that PTC is more likely to produce distant metastases, leading to a poorer prognosis [9]. PTC is also the fifth leading malignancy in females. Although prognosis for PTC is generally good and patients with PTC

Start	GC%	Sequence
275	47	Sense: 5'-ATTTGCCCTGGTTATATCA-3'
		Anti-sense: 5'-AUAUGUGUGUAUCCGACUGTT-3'
345	45	Sense: 5'-TG GTCTGTGCCCTTCAA GA-3'
		Anti-sense: 5'-UUGAAGGUGAGGAUAACGCTT-3'
465	45	Sense: 5'-TGGAAGGAATGAAGGAATG-3'
		Anti-sense: 5'-GAGAGUGGGUUUCCAGU AUTT-3'
Negative control	46	Sense: 5'-UUCUCCG AACGUGUC ACGUTT-3'
		Anti-sense: 5'-ACGUGAC ACGUUCGGAGAATT-3'

 Table 1. The sequences of siRNAs

In addition, transfection with siUbcH10 also suppressed migration and invasion of TPC-1 cells, and the inactivation of PI3K/Akt signal pathway. In conclusion, UbcH10 may play an important in the occurrence and progress of thyroid cancer, and may be a potential therapeutic target for the clinical management of thyroid cancer.

## Materials and methods

## Cell lines and cell culture

The TPC-1 cell line was purchased from Shanghai Bioleaf Biotech Co., Ltd (Shanghai, China). The cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere containing 5%  $CO_2$ .

## Design and synthesis of siRNA

The complete sequence of UbcH10 was obtained from GenBank corresponded to the gene ID NC\_000020.11 (GI: 568815578). The rule of Tuseh1 was used to design the sequence of siRNA, BLAST was applied to confirm the uniqueness for siRNA by comparison of the selected sequence and relevant sequence in Genome Database. Finally, three sequences of siRNA were obtained (**Table 1**).

## Plasmid construction and cell transfection

Small interfering RNA (siRNA) for UbcH10 was carried out using the target sequence: (UbcH10 5'-AACCTGCAAGAAACCTACTCA-3'), and twostep method of siRNA expression cassettes was used to amplify the siRNA under the conditions of 94°C 30 s, 72°C 90 s respectively. Then T4 DNA ligase was utilized to combine products of PCR with pRNAT U6.1 for 24 h at 16°C. Glycerin bacteria processed by Blue-White Selection and endonuclease cutting was proceeded to perform gene sequencing. The plasmids with correct sequence of siUbcH10 were prepared in quantity. DNA in these plasmids was extracted and purified for spare. The plasmids of siRNA-UbcH10 were transfected into TPC-1 cells by using Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Thereafter, cells were cultured in McCoy's medium (GIBCO, Carlsbad, CA)

can be treated by surgery, it is difficult to decide whether total thyroidectomy, hemithyroidectomy or prophylactic central neck dissection should be performed in patients without preoperative or intraoperative evidence correlated with metastatic lymph nodes [10]. In spite of small proportion, HCC has been recently suggested that it may be a unique thyroid cancer distinct from PTC and FTC by usage of genomewide analysis for mutation genotyping. However, whatever type of thyroid cancer patients have got, the disease is urgent for treatment in time. Therefore, a new marker suited for detection and prediction is particularly important for doctors who are able to take effective approaches to keep patients alive.

UbcH10 (also known as E2C or UBE2C) is a cellcycle-related protein which was involved in mitosis completion [11]. UbcH10 plays an important role during the phase from G2/M to early G1 through exerting ubiquitin-conjugating enzymatic activity (E2). UbcH10 together with ubiquitin ligase (E3) deliver ubiquitin to the mitotic cyclins promoting their degradation by the proteosome. Once mitotic cyclins ubiquitination occurred, UbcH10 subsequently triggers its own destruction [11]. This event marks mitotic completion and provides the molecular switch which allows cells to bring cell division to an end, and a new round of DNA duplication will be processed [12]. Thus, UbcH10 is essential for cell cycle progression, and mutation of its active-site cysteine will confer a dominant-negative phenotype [13]. UbcH10 is up-regulated in thyroid cancer cell lines [14], and a prognostic marker for thyroid, ovarian [15] and breast carcinomas [16].

In this study, we found that UbcH10 knockdown using siRNA induced apoptosis, proliferation inhibition and cell cycle arrest in TPC-1 cells.

supplemented with 10% FBS and 1% penicillin/ streptomycin solution (GIBCO, Carlsbad, CA) for 10-14 days in the presence of 500  $\mu$ g/mL of G418 (Promega, Madison, WI).

# Quantitative reverse transcription polymerase chain reaction (qRT-PCR analysis)

Cells were harvested and RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Then 2 µg total RNA was reverse transcribed in a volume of 20 µl for cDNA synthesis using TaqMan Reverse Transcription (Applied Biosystems, USA) following the manufacturer's protocol. The products were amplified for PCR. Quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) in the Bio-Rad PTC-200 Detection System (Bio-Rad, Hercules, CA). Primers used in PCR were as follows: UbcH10, GCC-CGTAAAGGAGCTGAG (sense) and GGGAAGGC-AGAAATCCCT (antisense); β-actin, CATTAAGGA-GAAGCTGTGCT (sense) and GTTGAAGGTAGTTT CGTGGA (antisense). The reaction system consisted of 2 µL of sense primer, 2 µL of antisense primer, 12.5 µL of SYBR Green PCR Master mix (2×concentration, Applied Biosystems Incorporation, USA), 1 µL of template, supplemented with double-distilled water to a final volume 25 µL. The PCR protocol consisted of the following: 95°C for 10 min, 40 cycles of 95°C for 10 sec, 60°C for 20 sec, and 72°C for 30 sec, and then 4°C for 5 min.

## Western blot analysis

Whole TPC-1 cells lysates were obtained by using RIPA lysis buffer (150 mM Tris-HCl, 50 mM NaCl, 1% NP-40, 0.1% Tween-20), and protein content was measured using the Bradford assay (Bio-Rad, Hercules, CA). Protein extracts (30 µg) were separated by SDS-PAGE (6 or 7.5% wt/vol) and transferred electrophoretically to nitrocellulose membranes. After blocking with non-dry fat milk (5% wt/vol), membranes were probed with primary antibodies including anti-procaspase-3, anti-procaspase-9, anti-Bcl-xl, anti-PI3K and anti-Akt overnight at 4°C and then incubated with corresponding secondary antibody (Beyotime, Shanghai, China). Immunoreactive bands were quantified using ImageQuant (Bio-Rad) and normalized to GAPDH.

## Determination of cell proliferation

Treated and untreated TPC-1 cells were seeded in 96-well plates and incubated for 24, 48, and 72 h. CCK-8 Assay (Dojindo Lab, Kumamoto, Japan) was performed by adding 10% CCK-8 solution (v/v) to each well for 1 h at 37°C. Optical density values (OD) of each well was measured at wavelength 450 nm by a microplate reader (Bio-Rad). All experiments were run in triplicates and repeated at least three times.

## Cell apoptosis assay

Apoptosis was analyzed by the annexin V-FITC plus PI staining and flow cytometry measurements according to manufacturer's instructions. After transfection and cultured for 48 h, cells were harvested by trypsinization, washed with ice-cold PBS, and fixed with ice-cold 70% ethanol for at least 2 h at -20°C. The fixed cells were then washed with PBS and incubated with ribonuclease A (Sigma) and PI (0.05 mg/mL, Sigma) at room temperature for 30 min. Cells were then double-labeled with Annexin V-FITC and PI and analyzed by flow cytometry. At least  $2 \times 10^4$  cells were performed in triplicate.

## Cell cycle analysis by flow cytometry

The flow cytometry was used to analyze the effects of UbcH10 siRNA on the cell cycle distribution of TPC-1 cells. TPC-1 cells were digested were digested with 0.25% trypsin 48 h after transfected with siRNAs and collected by centrifugation at 200 g for 5 min at 4°C. The cells were washed with ice-cold phosphate-buffered saline (PBS) 3 times and fixed in 70% ethanol for 24 h. The samples were stained with PBS containing 50  $\mu$ g/mL of propidium iodide, 10  $\mu$ g/mL RNase A, 0.1% sodium citrate and 0.1% Triton X-100. At last, the cell cycle distribution was detected by the flow cytometer (Millipore Guava).

#### The detection of cell migration and invasion

TPC-1 cells were cultured in serum free medium for 24 h and digested. The cell density was adjusted to  $3 \times 10^4$ /ml with serum free medium. Cell suspension with adjusted cell density (100 µL) was added into the upper chamber, culture medium (500 µL) containing 10% FBS was



**Figure 1.** The expression of UbcH10 in TPC-1 was detected using qRT-PCR and western blot. A. Relative mRNA expression of UbcH10 in TPC-1 after transfection with Scramble siRNA or siUbcH10-1, 2, 3 measured using qRT-PCR. B. Representative bands of western blot assay for UbcH10 protein level and the results of quantitative analysis of gray level. R is expressed as the mean  $\pm$  SD, \*\*P < 0.01 (vs. Scramble).

added into the lower chamber. Transwell chambers were covered and incubated. The upper chamber was removed to 24-well Transwell plates, marked, covered and then incubated. The culture medium in the upper chamber was removed and 4% formaldehyde (600 µL) was added into the 24-well Transwell plates. The upper chamber was then moved to wells containing 4% formaldehyde, and anhydrous methanol (150 µL) was added. The upper chamber was marked, the anhydrous methanol in the upper chamber was removed after 15 min standing, and the upper chamber was inverted on covers of the 24-well Transwell plates. The lower surface of the upper chamber was airdried naturally in a ventilated kitchen. Giemsa reagent 1 was added into the upper chamber, standing for 1 min, then Giemsa reagent 2 was added into the upper chamber for 5 min standing. After this, the upper chamber was washed with PBS (30 min/3 times), results were observed and photographed under the inverted microscope, and were further observed and photographed with a microscope image acquisition system. Five-fold counts were randomly selected. In invasion test, the number of cells in each group passing through the Matrigel was used as an index for evaluating the ability of invasion, and the migration test without adding the Matrigel shared the same steps with the invasion test.

#### Statistical analysis

All experiments were repeated in three times. The data were analyzed using SPSS 16.0 software (IBM, Armonk, NY, USA) and presented as the mean  $\pm$  standard deviation (SD). Comparisons within groups and between groups

were analyzed using Student's t test and AN-OVA-Duncan's Test. P < 0.05 was considered statistically significant.

#### Results

#### UbcH10 expression was reduced after transfection with siUbcH10

After transfection with scramble siRNA or siUbcH10-1, 2, 3, the expression level of UbcH10 was determined using qRT-PCR and western blot. As shown in **Figure 1**, transfection with siUbcH10 significantly reduced the mRNA and protein levels of UbcH10.

SiUbcH10 in TPC-1 cells and the establishment of stable sub-clone cell lines with siRNA were used to investigate the role of UbcH10 in mesangial cells. We compared changes in the cellular activity of TPC-1 cells transfected with siUbcH10-1/2/3 vectors, as shown in Figure 1. Transfection efficiency was confirmed using RT-PCR to test the mRNA level of siUbcH10-1/2/3 in TPC-1 cells respectively. The expression levels of mRNA in three siUbcH10 groups were found to be significantly reduced in comparison with scramble group. Of these, siUbc-H10-1 with obvious inhibition on the expression of mRNA is up to 79.32%, and the result indicated that the expression of siUbcH10 has been significantly inhibited (P < 0.01).

## SiUbcH10 promotes the apoptosis of TPC-1 cells

In order to examine whether UbcH10 is related to the apoptosis of TPC-1 cells, we then evaluated the apoptotic function of siUbcH10 in



**Figure 2.** The influence of siUbcH10 transfection on TPC-1 cell apoptosis and proliferation. A and B. Apoptosis in TPC-1 cells was detected by flow cytometric assay. C. Growth curves of TPC-1 cells after siUbcH10 treatment. TPC-1 cells were transfected with siUbcH10 and the relative number of viable cells was determined by CCK8 assay. Cells transfected with a scrambled and untransfected cells were used as negative controls. Absorbance was read at 570 nm and the data are the mean of triplicates. All data are presented as mean  $\pm$  SD, n = 6. \*\*P < 0.01 vs. control group.

TPC-1 cells by the annexin V-FITC/PI assay. Annexin V-FITC/PI double stain was used to make a quantitative evaluation of apoptosis. As shown in **Figure 2A** and **2B**, significantly statistical difference in cell apoptosis between siUbcH10 and scramble groups was found.

#### The proliferation of TCP-1 cells was significantly inhibited by siUbcH10

To study the effect of siUbcH10 on cell proliferation in TPC-1 cells, we monitored the growth curve of siUbcH10 and control group. The result showed that the proliferation of cells combined with the scrambled siRNA was significantly reduced. As shown in **Figure 2C**, cell growth was significantly decreased from third day to fifth day compared with scramble siRNA. Furthermore, the CCK-8 assay showed that the proliferation of cells transfected with siUbcH10 was attenuated in a time-dependent manner comparing with control group.

#### SiUbcH10 induced cell cycle arrest at G2/M phase

To investigate the role of UbcH10 in regulation of the cell cycle, cell cycle distribution was ana-

lyzed by the flow cytometry. The results shown in Figure 3A indicated that the percentage of cells in G2/M increased, the percentage of cells in S phase decreased, and the proportion of G1-phase cells was found no significant alteration. TPC-1 cells were detected to accumulate in G2/M phase from 5% to 20.68% after 48 h incubation, and significant difference was detected in the siUbcH10-1/2/3 groups (as shown in Figure 3B). In addition, compared with un-transfected and scramble group, the cell population in the S phase were decreased to  $(62.08 \pm 3.0)\%$ ,  $(65.29 \pm 2.4)\%$  and  $(63.8 \pm$ 1.7)%, which were corresponded to siUbcH10-1/2/3 groups. There was no significant difference among each group in the G1 phase where the cells accumulation of siUbcH10-1/2/3 were  $(17.23 \pm 2.3)\%$ ,  $(20.8 \pm 1.4)\%$  and  $(17.59 \pm$ 2.3)% in comparison with un-transfected and scramble group.

#### SiUbcH10 resulted in the inhibition of invasiveness and migration in TPC-1 cells

To further explore the other biological significance of UbcH10 in thyroid cancer, we investigated whether UbcH10 could inhibit cell migra-

## An investigation of UbcH10 function in TPC-1 thyroid cancer cells



Figure 3. UbcH10 knockdown induced TPC-1 cell cycle arrest at G2 phase. A. Cell cycle distribution detected by flow cytometry in the control cells and the cells treated with siUbcH10 for 48 h. B. Quantitative analysis of cell cycle distribution. \*\*P < 0.01 (vs. Scramble).



Figure 4. siUbcH10 negatively regulated the migration and invasion of TPC-1 cells *in vitro*. A. The representative migration and invasion images of TPC-1 cells transfected with siUbcH10. B and C. The relative ratios of TPC-1 cell migration and invasion were counted in five random fields.

tion and invasion in thyroid cancer. As shown in **Figure 4A** and **4B**, transfection with siUbcH10 significantly reduced the number of migrating cells. UbcH10 knockdown also suppressed TPC-1 cells invasion (**Figure 4A**, **4C**).

## SiUbcH10 inhibits the activation of PI3K/Akt pathway in TPC-1

To investigate the mechanism of how UbcH10 influences TPC-1 cellular functions, we examined the activation of PI3K and Akt in TPC-1 cells. As shown in Figure 5, compared with scramble group, the phosphorylation level of p-PI3K and p-Akt was significantly inhibited by siUbcH10, P < 0.01, suggested UbcH10 contributed to the activation of PI3K/Akt pathway in TPC-1. To further define the relationship between UbcH10 and apoptosis, the protein levels of caspase-3, 9 and Bcl-xl were also detected. The results showed UbcH10 knockdown using siUbcH10 significantly promoted the activation of caspase-3 and -9 (Figure 5). At the same time, siUbcH10 also possessed the ability of reducing the expression of anti-apoptotic gene Bcl-xL as came into view in Figure 5.

#### Discussion

Thyroid cancer keeps the steadily rising tendency of morbidity over the past few decades, UbcH10 was found to appear greatly increased in all of the thyroid carcinoma cell lines of previous reports. UbcH10 was previously identified as a human homologue of the cyclin-selective E2 (E2-c), which is required for the destruction of mitotic cyclins by the ubiquitination pathway. The UbcH10 gene belongs to the E2 gene family and codes for a protein of 19.6 kDa that is involved in the ubiquitin-dependent proteolysis. More precisely, the ubiquitin-conjugating enzyme (E2) transfers activated ubiquitin by ubiquitin-activating enzyme (E1) to a lysine residue of the target proteins in cooperation with the ubiquitin-ligase (E3). Poly-ubiquitinated proteins are then recognized by the 26S proteasome and rapidly degraded [17].

In our study, significant different expression of UbcH10 was observed in TPC-1. A great reduction of expression was observed in experiments of siUbcH10 compared with control, and the declining proliferation and increasing apoptosis



Figure 5. SiUbcH10 induces inactivation of PI3K/Akt pathway and activation of caspase-3 and -9. After transfection with siUbcH10 and cultured for 48 h, the protein expression of PI3K, p-PI3K, Akt, p-Akt, caspase-3, caspase-9 and Bcl-2 were detected via western blotting assay and normalized to GAPDH. \*P < 0.05 (vs. Control), \*\*P < 0.01 (vs. Control).

of TPC-1 cells were subsequently found in the related experiment of siUbcH10. These data strongly indicated that UbcH10 could be associated with thyroid cancer progression, which might be able to deteriorate the process of thyroid cancer. Migration and invasion assay further emphasize the role of UbcH10 in thyroid cancer. The invasion and migration abilities of TPC-1 have been significantly impaired by siUbcH10. The results indicate that UbcH10 associated closely with invasion and migration of thyroid cancer cells, suggesting that UbcH10 might seriously influence the aggravation of thyroid cancer by some mechanism of signal pathway existing in thyroid cancer cells.

In order to further investigate the mechanism of how UbcH10 regulating thyroid cancer, we detected the expression level of PI3K and Akt, which are downstream proteins correlating with UbcH10. In comparison with control groups, siUbcH10 was detected to induce the reducing expression level of p-PI3K and p-Akt. PI3K/Akt signaling pathway is known to play a fundamental role in the regulation of various cellular and molecular functions, including glucose uptake, cell growth and proliferation, cell motility and survival [18-22]. Our results indicate that siUbcH10 has influenced the expression of p-PI3K and p-Akt, in other words UbcH10 somehow is capable to promote the activation of p-PI3K/ Akt, this key point should be further explored in our future research. Here, we conclude that UbcH10 might be able to induce the generation of ATP which serves as the essential key to

activate kinases like PI3K, and UbcH10 might utilized indirectly this characteristic of PI3K/Akt facilitating cell growth and proliferation to accelerate the production of thyroid cancer cell then promote the migration and invasion. Hence, even though the mechanisms by which UbcH10 contributes to the thyroid cancer remains uncertainty, we can assume that it leads to aggravation of thyroid cancer. These results are also quite consistent with previous published data showing that UbcH10 was expressed at high levels in primary tumours derived from the lung, stomach, uterus, and bladder as compared with their corresponding normal tissues. these evidences suggest that UbcH10 is involved in tumorigenesis or cancer progression. Therefore, we pay more attention to UbcH10 which is considered as a target to perform detection for thyroid cancer in order to aware of cancer ahead of time.

In addition, there no significant difference between siUbcH10 group and control in the expression correlated with pro-caspase 3/9 which play a key role in mediating apoptotic signaling events [23]. This result can further confirm that PI3K/Akt pathway is the specific way belonged to UbcH10 to regulate the proliferation and apoptosis of cancer cells, and no matter expression or inhibition of procaspases, this not the approach of UbcH10 functioning to aggravate the disease. Simultaneously siUbcH10 has been found to reduce the expressive quantity of Bcl-xL which is an anti-apoptotic gene. In other words, overexpression of UbcH10 in thyroid cancer might promote the expression of Bcl-xL to induce proliferation of cancer cell, and this method has aggravated the pathogenetic condition.

The result further reminds us that UbcH10 might be a nonnegligible gene in the development of thyroid cancer. According to our results, we investigated the behavior of siUbcH10 in TPC-1 cells, and demonstrated that UbcH10 is able to increase the proliferation of thyroid cancer cell, and the apoptosis of these cells can be inhibited, meanwhile the migration/ invasion is induced by UbcH10 in thyroid cancer cells. Probably, PI3K/Akt signal pathway might play essential role of aggravating thyroid cancer, but this should be further investigated in depth. We hope that this study will provide the basis for recognizing UbcH10 as a potential therapeutic target in thyroid cancer.

#### Disclosure of conflict of interest

None.

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