RESEARCH ARTICLE

CCNG2 Suppressor Biological Effects on Thyroid Cancer Cell through Promotion of CDK2 Degradation

Wei-Juan Li, Ge-Ling Liu*, Fang Yu, Xiu-Xiu Xiang, Yi-Fang Lu, Hong-Zhen Xiao, Yan-Ping Shi

Abstract

This study aimed to analyze the expression and clinical significance of cyclin G2 (CCNG2) in thyroid carcinoma and the biological effects of CCNG2 overexpression in a cell line. Immunohistochemistry and Western blotting were used to analyze CCNG2 protein expression in 63 cases of thyroid cancer and normal tissues to allow the relationship with clinical factors to be assessed. CCNG2 lentiviral and empty vectors were transfected into the thyroid cancer K1 cell line. Reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting were applied to detect the mRNA and protein levels of CCNG2. MTT assay and cell cycle were also conducted to assess the influence of up-regulated expression of CCNG2 on K1 cell biology. The level of CCNG2 protein expression was found to be significantly lower in thyroid cancer tissue than normal tissues (P < 0.05). Western blot: The relative amount of CCNG2 protein in thyroid cancer tissue was respectively found to be significantly lower than in normal tissues (P<0.05), correlating with lymph node metastasis, clinic stage and histological grade (P<0.05), but not gender, age or tumor size (P>0.05). Loss of CCNG2 expression correlated significantly with poor overall survival time on Kaplan-Meier analysis (P < 0.05). The results for biological functions showed that K1 cell transfected CCNG2 had a lower survival fraction, a greater percentage in the G0/G1 phases, and lower cyclin-dependent kinase 2 (CDK2) protein expression compared with K1 cells non-transfected with CCNG2 (P<0.05). CCNG2 expression decreased in thyroid cancer and correlated significantly lymph node metastasis, clinic stage, histological grade and poor overall survival, suggesting that CCNG2 may play important roles as a negative regulator in thyroid cancer K1 cells by promoting degradation of CDK2.

Keywords: CCNG2 - thyroid carcinoma - overall survival - metastasis

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Introduction

Thyroid carcinoma is the most common endocrine malignancy in human. The incidence of thyroid carcinoma is continuously rising worldwide. Approximately 80% of thyroid cancer is papillary carcinoma and 15% of thyroid cancer is follicular carcinoma, both of which are derived from thyroid follicular epithelial cells. These two types of thyroid carcinoma can give rise to poorly differentiated thyroid carcinoma, and if they are not properly treated, fatalities will occur (DeLellis et al., 2004; Nikiforov et al., 2009). Although significant progression has been made in both basic and clinical research in thyroid cancer, the molecular mechanism responsible for development of thyroid carcinoma, like most other cancers, remains to be determined, and a number of gene alterations may contribute to thyroid carcinogenesis (Kimura et al., 2003; Frattini et al., 2004). Thus, further studies will clarify the pathogenesis of thyroid cancer and provide the molecular targets for effective treatment of thyroid cancer.

which has a close relationship with cell carcinogenesis. Cyclin G is a new member of cyclin family, which includes cyclin G1 and cyclin G2. Previous data (Ahmed et al., 2012) showed that as a key negative regulator of cell cycle progression, this gene may contribute to the regulation of cell proliferation, apoptosis, cell death signaling, even and carcinogenesis. However the role of CCNG2 is still unknown, even there still are few reports about the function and mechanism of CCNG2 in thyroid cancer cell. In this study, we detect CCNG2 expression in thyroid cancer tissue and transfected CCNG2 into K1 cell to clarify the role of CCNG2 expression on the cell proliferation in vitro.

Materials and Methods

Main Reagents

Mouse anti-human CCNG2 monoclonal antibody (Amcam Inc.). GAPDH mouse anti-human monoclonal antibody (Santa Cruz). immunohistochemistry kit (Zhongshan Goldenbridge Biotechnology Co., Ltd.). RNA

Cell cycle regulation is the core part in cell proliferation,

Department of Endocrinology (Section 1), Tangshan Workers Hospital, Tangshan, China *For correspondence: weijuan_li2013@163.com

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Trizol Extraction Kit (Beijing Solarbio). Superscript III reverse transcriptase kit (Invitrogen Corporation). TaqDNA polymerase, OligodT, dNTPs, reverse transcriptase, RNases inhibitor (Fermentas Inc.).

Clinical Data

From January 2000 to January 2003, 63 patients with thyroid cancer cancer underwent resection in Tangshan work's Hospital. There were 18 males and 45 females, aged from 27 to 76 years old, with a median age of 39 years. Of the 63 cases of thyroid cancer: 25 of them with lesion ≤ 4 cm and 38 with lesion > 4cm. The grades of differentiation were 18 with Grade III (poorly differentiated) and 45 with Grade I or II (moderately to well differentiated). Meanwhile, 20 patients demonstrated no lymph node metastasis (N0), whereas 43 with identified lymph nodes involvement (N+). As for the clinic stages, 22 cases had I~II stages and 41 had a III~IV stages. All of the patients did not have any neoadjuvant therapies. The fresh specimens of tumor tissue or adjacent normal epithelium 5 cm apart from the tumor edge were immediately taken after the surgery, one was fixed in 4% paraformaldehyde solution, then embedded in paraffin for immunohistochemistry, and the other one was stored in liquid nitrogen for western blot assay.

Cell culture and gene transfection

Human PTC K1 cells were maintained in DMEM: Ham's F12: MCDB105 (2:1:1) (Sigma, USA) supplemented with 10% fetal calf serum (FCS) (Gibco, USA) at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. The plenti6/V5-DEST vector (Invitrogen, CA, USA) was used to harbor CCNG2 cDNA through cloning of CCNG2 cDNA sequences into the BamH I and Asc I sites of the plenti6/V5-DEST vector. After amplification and DNA sequence confirmation, this vector was used to induce CCNG2 expression in K1 cell. Briefly, K1 cell was grown and stably transfected with pLenti6-CCNG2 or plenti6/V5-DEST vector using Lipofectamine 2000 and grown in antibiotic Blastidicin (5 μ g/ml)-containing DMEM for selection of stable sublines.

Immunohistochemistry

A 4 µm section was prepared from paraffin-embedded block and dehydrated, then incubated in 3% hydrogen peroxide for 10 min to block endogenous peroxidase, followd by using trypsin for repair of 20 min; 10% goat serum was introduced at room temperature for closure of 20 min, and CCNG2 antibody (1: 100) was left in the wet box at 4°C refrigerator for overnight. Then the secondary and third antibodies were dropped into the wet box at room temperature for incubation of 20 min, respectively; DAB staining was again visualized by the hematoxylin stain, and then came to normal dehydration with the coverslip sealed. Results evaluation: two pathologists without knowing patients' information were responsible for assessing the results. Regarding cell counting under microscope, 5 fields were randomly selected, and 3 slides for each specimen were counted. CCNG2 expression was determined based on the percentage of positive cells,

combined with the staining intensity. The percentage of positive cells was divided into four levels: 0 point: $\leq 5\%$ of positive cells, 1 point: $5\% \sim 25\%$, 2 points: $25\% \sim 50\%$, and 3 points: > 50% of positive cells. The intensity of staining was classfied as: 0 point: no staining, 1 point: weak staining (light yellow); 2 points: moderate staining (brown); and 3 points: strong staining (yellowish-brown). The final score of CCNG2 expression was the product of the CCNG2 expression rate and intensity, graded as 0 for negative, + for 1-3 points, ++ for 4-6 points, and +++ for 7-9 points. As for the negative control, the primary antibody was replaced with PBS.

Semi-Quantitative RT-PCR

Total RNA extraction: specimen was removed from the liquid nitrogen, and the total RNA was extracted according to the instructions on Trizol reagent. 2ug total RNA was taken for synthesis of cDNA according to the operating requirements of Superscript III Reverse Transcriptase Kit Instructions in 20ul reaction system; Reaction conditions: denaturation at 65°C for 5min, and RT at 50°C for 50 min. PCR amplification of CCNG2 gene upstream primer: 5'-CTTTGGGCATTATTAGGA-3' downstream primer: 5'-GAGGAGGAAACAGTAGCAG-3', GAPDH gene upstream primer: 5'-CCGACCTGCCCTACGACTA-3', downstream primer: 5'-CTGGGCTGT AACATCTCCCTT-3'. PCR reaction 50ul include: 5 ul 10 × PCR buffer, 1 ul 10 mmol dNTP, 0.5 ul TaqDNA polymerase, the upstream and downstream primers 2 ul, respectively, 2 ul template cDNA, plus ddH2O, was complemented to 50ul. Reaction conditions: predenaturation at 95°C for 3 min, denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 45 s, a total of 35 cycles, placed at -20°C. PCR product detection and its semi-quantitative analysis: after the product underwent gel electrophoresis in 1.5% agarose, the gel imaging system combined with Multi Gauge V 3.1 was used for optical density analysis of the results.

Western Blot

All operations were completed on the ice. At 4°C, 12000 r/min centrifugation lasted for 20 min, and then the supernatant was taken for backup at -20°C. After the detection of protein concentration with BCA Protein Assay Kit, each hole was given a sample amount of 50ug for SDS-PAGE electrophoresis. Regulator power for ice bath was transferred to nitrocellulose membrane, followed by closure for 2 h with 5% skim milk. Subsequent to Anti-1 overnight incubation at 4°C (CCNG2 1:1000, GAPDH 1:3000). The latter was from Sigma Chemical Company, St. Louis, MO, USA) in 5% nonfat dry milk for 1 h at room temperature. After washing, the membrane was incubated with goat anti-rabbit fluorescent secondary antibody (IRDye700, 1:20,000 dilution; the DyLight Fluor conjugated to goat anti-rabbit IgG was obtained from LI-COR Bioscience, Inc., Lincoln, Nebraska, USA) in the dark for 1 h at room temperature. The blots were then scanned and analyzed using the Odyssey Infrared Imaging System (LI-COR Bioscience). Western blot data were quantified by normalizing the signal intensity of each sample to that of GAPDH.

Table 1. Expressions of CCNG2 in 7	Thyroid (Cancer
Tissue and in Normal Thyroid Tissue	9	

			v					
Group	Case	F	Expression of CCNG2 Protein					
		-	+	++	+++	χ^2	Р	
Cancer Tissue	63	42	5	9	7	56.189	0	
Normal Tissue	63	2	12	24	25			
	A CAR						X	

Figure 1. Expressions of CCNG2 Protein in Thyroid Carcinoma and Normal Tissue. A thyroid carcinoma (SP×400), B normal tissue (SP×400)

MTT assay

Cell viability was determined by using the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were plated into 96well culture plates at an optimal density of 5×10^3 cells/ ml in 200 µl of culture medium per well. After 24–96 h of culture, 20 µl of 5 mg/ml MTT was added to each well and incubated at 37°C for 4 h. The medium was then gently aspirated and 150 µl of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals. The optical density of each sample was immediately measured using a microplate reader (BioRad) at 570 nm.

Flow cytometry assay

For cell cycle distribution, PI staining and the flow cytometry assay were performed as described previously. Approximately 1×10^6 cells were fixed in 70% ethanol and resuspended in 1 ml of a solution containing 3.8 mM sodium citrate, 50 µg/ml propidium iodide, and 0.5 µg of RNase A, and analyzed with a flow cytometer (Becton-Dickinson). The data were evaluated using the ModFit software program (Verity Software House, Topsham, ME, USA).

Statistical analyses

All statistical analyses were performed using SPSS16.0 software. For the clinicopathologic features, P values were calculated using the χ^2 test. CCNG2 mRNA expression was shown in mean \pm SD. Student t-test was used to analyze the difference between groups. A 5% or lower *P*-value was considered to be statistically significant.

Results

CCNG2 mRNA and protein expression in thyroid cancer and normal tissue

In thyroid cancer tissues, CCNG2 staining was negative or weak. In normal thyroid tissues, CCNG2 staining ranged from light yellow to brown. Statistically, CCNG2 was expressed in 33.3% (21/63) of thyroid cancer tissues, which was lower than the 96.8% (61/63) in normal

Group	Case	Exp	ression of C	CNG2 Pr	otein	
		_	+~+++	χ^2	Р	-
Sex						-
Male	18	10	8	1.4	0.237	
Female	45	32	13			
Age (year)						
≤ 40	47	30	17	0.67	0.413	
> 40	16	12	4		-	00 0
Tumor lengt	h (cm)				1	100.0
≤4	25	16	9	0.133	0.716	
> 4	38	26	12			
Clinic stages	8					75 0
I~II	22	11	11	4.226	0.04	/ 5.0
III~IV	41	31	10			
Histological	grade					
I	18	8	10	5.6	0.018	50.0
II~III	45	34	11			50.0
Lymph node	metastasis					
NO	20	8	12	9.377	0.002	
N+	43	34	9			25.0
						-
A	+ -				CCNG2	
в —					CCNG2	0
		2 3		-		
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Table 2. Relation Between CCNG2 Expression and **Clinic Characteristics in Thyroid Cancer Tissue**

Thyroid cancer Figure 2. Expressions of CCNG2 Protein in Thyroid Carcinoma and Normal Tissue. A, thyroid carcinoma B normal tissue. *p < 0.05 compared to the normal tissue

Normal tissue

tissues. The difference was statistically significant (P <0.05) (Table 1, Figure 1). While Western blot showed that the relative expression of CCNG2 protein presented volume between cancer lesion and adjacent normal tissue were 0.343 ± 0.033 and 0.713 ± 0.062 , showing the difference with statistical significance (P < 0.05) (Figure 2). The expression of CCNG2 was correlated with clinical stages, lymph node metastasis and pathological differentiation (P < 0.05), regardless of age, gender, and tumor size (P > 0.05) (Table 2).

CCNG2 expression and prognosis

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Survival analysis was performed in all the patients and follow-up data were collected. All patient follow-ups ended in 2010 after a revisit time of 120 months. Among all cases, 28 were still alive at this time and 35 were dead. Patients were divided into two groups according

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Figure 3. Relation Between CCNG2 Expression and Overall Survival in Thyroid Carcinoma by Kaplan-Meier Analysis



Figure 4. Expression and identification of the CCNG2 gene. A RT-PCT, B Western blot, 1. LeCCNG2 cell, 2. LeEmpty cell. *p < 0.05 compared to the LeEmpty cell

to CCNG2 expression level. There were 21 individuals with high levels of CCNG2 expression, among whom 15 were still alive and 6 were dead. The survival rate was 71.4%. There were 42 individuals with negative levels of CCNG2 expression, among whom 13 were still alive and 29 were dead. The survival rate was 30.9%. Patients with high levels of CCNG2 expression had significantly



Figure 5. The Effects of CCNG2 Overexpression on the Cell Proliferation. *p < 0.05 compared to the LeEmpty cell



Figure 6. The Effects of CCNG2 Overexpression on the Cell Cycle. A. LeEmpty cell, B. LeCCNG2cell.*p < 0.05 compared to the LeEmpty cell

higher 10-year survival rates than those with low levels of CCNG2 expression group (P < 0.05) (Figure 3).

Stable transfection of CCNG2 cDNA in thyroid cancer K1 cells

In this study, we first stably transfected CCNG2 cDNA into K1 cells and obtained overexpressed CCNG2 K1 sublines (named as LeCCNG2 cell) and empty vectortransfected K1 cell (named as LeEmpty cell) as the control. RT-PCR data showed that the CCNG2 mRNA expression level was 0.289±0.022 in empty vector-transfected cells. In contrast, the amount of CCNG2 mRNA in the CCNG2-transfected cell lines was 0.782±0.038 (Figure 4A). Furthermore, western blot analysis showed that the control cells had approximately equal amounts of immunoreactive protein (0.321±0.032). In contrast, the amount of CCNG2 protein in the CCNG2-transfected cell lines was 0.632±0.065 (Figure 4B).



Figure 7. The Effects of CCNG2 Overexpression on CNK2. 1. LeCCNG2 cell, 2. LeEmpty cell.*p < 0.05 compared to the LeEmpty cell

Effects of CCNG2 overexpression on effect of thyroid cancer cell

Next, we assessed the effect of CCNG2 expression on the regulation of thyroid cancer cell viability. MTT assay showed that relative proliferative capacity of the LeCCNG2 cell relative grew slower at 24, 48, 72 h and 96 h compared with the parental LeEmpty cell (Figure 5). Moreover, cell cycle analysis showed that the G0/G1 and S phases of the cell cycle were significantly different in LeCCNG2 cell compared to the control cell lines (60.6 \pm 3.5 and $12.1 \pm 0.7\%$ vs. 45.3 ± 2.3 and $29.3 \pm 1.9\%$; Figure 6). To further identify the mechanisms by which CCNG2 inhibited thyroid cancer cell proliferation and changed cell cycle, we analyzed the expression level of cyclindependent kinase 2 (CDK2) due to their critical roles in cell proliferation and apoptosis. Western blot analysis revealed that CCNG2 significantly reduced CDK2 protein expression in K1 cell compared to controls cells (Figure 7).

Discussion

Similar to cell-cycle Gl protein, CCNG2 belongs to the cell cycle 'G' family. This protein was initially identified in 1996. The gene encoded by CCNG2 locates on human chromosome 4q21.1. Despite the cDNA sequence and the structure of amino acid residue seems to be similar to that of cell-cycle protein A, the biological function of CCNG2 is obviously different from the conventional cell-cycle proteins (Bates et al., 1996). CCNG2 negatively regulates cell cycle progression. DNA damage and other growth inhibition signals may upregulate the activity of CCNG2 gene. After activation, CCNG2 interacts with protein phosphatase 2A (PP2A), and the CCNG2-PP2A complex stops cell-cycle progression and regulates cell proliferation by inhibiting the cyclin-dependent kinase 2 (CDK2) and binding with centrosomes (Horne et al., 1996). Recent

studies reveal that the level of CCNG2 is significantly downregulated in tumor tissues, including oral squamous cell carcinoma (Kim et al., 2004), laryngeal squamous cell carcinoma (Cui et al. 2009), bladder metastatic carcinoma (Shan et al., 2009), and gastric adenocarcinoma (Choi et al., 2009), as compared with normal control tissues. These evidences suggest that CCNG2 gene might be an important tumor suppressor gene and participate in the tumorigenesis and progression. Nevertheless, the role of CCNG2 in thyroid carcinoma remains poorly known. Therefore, investigating the expression of CCNG2 in thyroid carcinoma and the CCNG2-mediated molecular mechanism of tumorigenesis, progression, and malignant transformation may help to better understand the biological activity of thyroid carcinoma. Besides, CCNG2 may be a novel biomarker for the clinical therapy and prognosis of thyroid carcinoma.

Our study compared the distribution and expression of CCNG2 protein in thyroid carcinoma tissues and normal thyroid tissues using immunohistochemistry, RT-PCR and Western blotting techniques. Our results showed that the protein levels of CCNG2 were greatly downregulated in thyroid carcinoma as compared with normal thyroid tissues. These findings are consistent with previous reports, which show the decreased CCNG2 level in gastric carcinoma (Choi et al., 2009), bladder carcinoma (Shan et al., 2009), and oral squamous cell carcinoma (Kim et al., 2004) compared to healthy tissues. Univariate analysis results showed that the mRNA and protein expressions of CCNG2 were associated with lymph node metastasis, clinical stage and tumor differentiation, but were not correlated with the gender, age, tumor size of patients with thyroid carcinoma. Chinese researchers demonstrate that CCNG2 are highly expressed in the laryngeal carcinoma tissues without lymph node metastasis or with high differentiation, whereas its expression is reduced in laryngeal carcinoma tissues with lymph node metastasis or with poor differentiation (Shi et al., 2011). Furthermore, survival analysis shows that the ten-year overall survival rate for patients with CCNG2 positive expression is greatly higher than those CCNG2-negative patients. In addition, some Chinese researchers find that the five-year overall survival rate for gastric adenocarcinoma patients with CCNG2 negative expression is obviously lower than those CCNG2-positive patient (Song et al., 2006). These data imply that the CCNG2 level might be associated with the tumorigenesis and progression of thyroid carcinoma, and may have a potential tumor-suppression role. Nonetheless, the underlying mechanism is not yet fully addressed. CCNG2 contains a PTB domain which is the binding site for Sbc signal protein. Besides, CCNG2 also has two phosphorylated tyrosine residues, suggesting CCNG2 protein may be involved in the regulation of growth signal transduction (Song et al., 2006). Inhibitors of JNK, PI3K, mTOR/p70S6K or PTEN signal pathways have been shown to elevate the CCNG2 expression (Le et al., 2007). Overexpression of the p110 α catalytic subunit of PI3K, can decrease the CCNG2 expression. Inhibition of PI3K or activation of FoxO3 α and FoxO1 both contribute to the upregulation of CCNG2 expression (Martinez-Gac et al., 2004; Chen et al., 2006). PI3K-AKT negatively regulates

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the activities of FoxO 1 α , FoxO 3 α and FoxO4 subfamily members. FoxO 1 α and FoxO 3 α activate the CCNG2 gene transcription and increase the mRNA expression of CCNG2 by binding to the promoter of CCNG2 (Martinez-Gac et al., 2004). CCNG2 is degradated mainly through ubiquitin-proteasome pathway, and SKP2 plays a key role in the regulation of CCNG2 level (Xu et al., 2008). It has been revealed that knocking down of SKP2 using siRNA increases the CCNG2 expression, whereas overexpression of SKP2 decreases the CCNG2 level (Xu et al., 2008). It is likely that CCNG2 may interact with SKP1, SKP2, which contributes to the decreased CCNG2 expression following transfection (Xu et al., 2008).

In vitro study for the first time shows that thyroid carcinoma K1 cells overexpressing CCNG2 exhibit decreased proliferation capability. The proportion of cells in G0+G1 stage is increased, while the proportions of cells in G2+M or S stages are reduced in K1 cells. Additionally, the expression of CDK2 protein is obviously downregulated in these cells. Choi MG et al. conducted tissue chip analysis using 166 cases of patients with gastric carcinoma, and found that CCNG2 negatively regulated the cell cycle of human gastric carcinoma cells and thus closely associated with the proliferation of these cells (Choi et al., 2009). WEB2170 can induce cell apoptosis of acute myeloid leukemia cells. During WEB2170-induced NB4 cell death, the CCNG2 and PTEN gene expressions are upregulated while CCND level is downregulated (Cellai et al., 2009). PTEN-induced G1phase cell cycle arrest in prostate cancer LNcap cells is linked to the AKT-dependent CCNG2 level alternation. Despite the underlying mechanism of CCNG2-mediated cell cycle regulation remains unclear, it is suggested that following pathways might be involved in the CCNG2induced cell growth inhibition. CCNG2 directly blocks the activity of CDK by replacement of cell cycle proteins in Cyclin-CDK complex (Horne et al., 2007). Bennin et al. shows that inhibitory role of CCNG2 on cell cycle is correlated with protein phosphatase 2A (PP2A). After binding to the B subunit of PP2A, CCNG2-PP2A complex exerts its cell cycle suppression activity [21]. Bennin et al. also demonstrates that the CCNG2-PP2A complex may inactivate CDK2 through Rb or P107 dephosphorylation. Secondly, this complex may directly induce the dephosphorlation of CDK2 and result in the G1phase cell cycle arrest. Thirdly, CCNG2-PP2A complex may indirectly inactivate CDK2 and inhibit the cdc25 dephosphorlation, which has the phosphatase activity (Bennin et al., 2002). Arachchige et al. finds that CCNG2 encodes a nucleocytoplasmic shuttling protein which is regulated by CRMI. Using immunostaining, 100 amino acids in C-terminal region of CyclinG2 are found to be colocalized with centrosomes, and induce TP53-dependent cell cycle suppression (Arachchige Don et al., 2006).

Our present study shows that the protein expressions of CCNG2 are downregulated in thyroid carcinoma tissues. The downregulation of CCNG2 is associated with lymph node metastasis, clinical stage and tumor differentiation. Hence, CCNG2 actively participates in the cell proliferation cells and cell cycle of thyroid carcinoma by regulation CDK2 protein expression. However, the malignant transformation of normal cells is a complicated process, involving the alternations of cell division, signal transduction and many proto-oncogenes and tumor suppressor genes (Gao et al., 2012; Malz et al., 2012). A great number of cytokines and pathways may contribute to the malignant transformation.

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The author(s) declare that they have no competing interests.

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