European Review for Medical and Pharmacological Sciences 2019; 23: 3440-3446

Correlations of ultrasound and pathological features of thyroid carcinoma with TC-1 mRNA and protein expression

C.-L. ZHANG[1], C.-H. GENG[2], J.-J. JIAN[2], M. HE[3], H.-W. ZHAO[2], H.-Y. LI[2]

[1]Department of Ultrasound, Daqing Oilfield General Hospital, Daqing, Heilongjiang, China
[2]Department of General Surgery, the Fifth Affiliated Hospital of Harbin Medical University, Daqing, Heilongjiang, China
[3]Department of Pathology, Daqing Oilfleral General Hospital, Daqing, Heilongjiang, China

Abstract. – OBJECTIVE: To investigate the correlations of ultrasound and pathological characteristics of thyroid carcinoma through evaluating the messenger ribonucleic acid (mRNA) level and protein expression of thyroid cancer-1 (TC-1).

PATIENTS AND METHODS: The patients with papillary thyroid carcinoma (PTC) hospitalized in our hospital were enrolled. Then, real-time fluorescence quantitative polymerase chain reaction (qPCR) and immunohistochemistry (IHC) streptavidin-peroxidase (SP) technique were applied to measure the mRNA and protein expression levels of TC-1 in PTC and corresponding adjacent tissues (NCE) of 50 patients. The relations with clinicopathological and ultrasound characteristics were analyzed.

RESULTS: The expression of TC-1 mRNA in PTC tissues was statistically higher than that in corresponding adjacent tissues and significantly correlated with tumor-node-metastasis (TNM) stage, pathological grade, and lymph node metastasis of PTC ($p<0.05$). According to IHC, TC-1 positive expression was mainly found in the cytoplasm in PTC samples, which was statistically increased compared to adjacent tissues ($p<0.05$). Western blotting results revealed that the relative protein expression of TC-1 in PTC tissues was 2.646±195, which was significantly higher than that in corresponding adjacent tissues (892±76) ($p<0.05$). The TC-1 protein expression also showed significant associations with TNM stage, pathological grade, and lymph node metastasis of patients ($p<0.05$). The level of TC-1 mRNA in PTC tissues with micro-calcification detected by ultrasound (87.46±49.55) was higher than that in those without micro-calcification (38.46±29.15) ($p<0.05$).

CONCLUSIONS: The expression of TC-1 plays an important role in the occurrence and development of PTC. Ultrasound characteristics reflect the expression of TC-1 in PTC tissues to some extent, providing a certain value in evaluating the prognosis of PTC.

Key Words:
Thyroid carcinoma, TC-1, Ultrasound characteristics, Clinical pathology.

Introduction

Papillary thyroid carcinoma (PTC), as the most common type of thyroid malignancy, is detected in approximately 90% of all diagnosed cases[1]. It is considered as one of the most common and clinically-worrying malignancies of the endocrine system. Usually, thyroid carcinoma is manifested as nodules and accounts for about 1% of all the newly-diagnosed cancer cases. Notably, the incidence rate has been increasing recently among various types of cancers. High-dose radioactive iodine treatment and surgical resection of the lesion are mainly used for the therapy of thyroid carcinoma. Although the incidence and mortality rates are remarkably reduced owing to the treatment, poor prognosis remains a crucial issue in some patients. Currently, the most reliable method for thyroid nodules diagnosis is fine-needle aspiration (FNA) biopsy combined with subsequent cytological analysis. FNA maintains a high specificity but is characterized with unsatisfactory sensitivity. In fact, 10-40% of the analyzed nodules are proved as indeterminate lesions, impeding the optimal treatment towards patients. In addition, only 10-30% of the surgically-removed indeterminate thyroid nodules are further identified as malignant nodules. BRAF (V600E) mutation is one of the most commonly used markers in the diagnosis of PTC. The genetic characteristics of BRAF dramatically increase the preoperative diagnostic accuracy by 20-30%, but it still leads to the diagnosis of suspected PTC (SPTC) and in-

Corresponding Author: Changhui Geng, MM; email: ChanghuiGeng7sh@163.com

determinate follicular hyperplasia (IFP). Therefore, diagnostic markers with high specificity are continuously needed to facilitate the management of patients with indeterminate thyroid nodules. Presently, the understanding of molecular events regarding the occurrence and development of thyroid carcinoma remains limited. Existing studies have found that the genes leading to the formation of thyroid carcinoma include RET and RAS oncogenes, tumor suppressor gene p53 that activates BRAF mutation, Pax8/peroxidase, etc. However, the exact causative genes of thyroid carcinoma still cannot be identified. Previous researches have discovered that thyroid cancer-1 (TC-1) (C8orf4) is overexpressed in PTC. TC-1 sequence shows no homology to those of any known genes and the function is still elusive. TC-1 is widely expressed in extensive human tissues and displays a high homology to mouse, bovine and chicken. Conserved sequence and widespread expression indicate that TC-1 may play a crucial role in cell biology. The copy-number variations of TC-1 are found related to acute myelogenous leukemia and other hematological malignancies[2-4]. Moreover, it is reported that TC-1 is associated with various cancers, for TC-1 is highly expressed in thyroid carcinoma, gastric cancer, and breast cancer, through activating the Wnt/β-catenin signaling pathway in these tumors[5,6]. However, TC-1 is reduced in colon cancer[7]. In this work, both protein and messenger ribonucleic acid (mRNA) levels of TC-1 in patients with thyroid carcinoma were measured. Their associations with ultrasound and pathological characteristics of thyroid carcinoma were analyzed to evaluate their clinical values in thyroid carcinoma.

Patients and Methods

Clinical Data of Patients

The tissue specimens and data of 112 patients with thyroid carcinoma admitted to our hospital from January 1, 2014, to December 31, 2016, were enrolled. At the same time, postoperative carcinoma tissues and adjacent normal tissues of thyroid carcinoma patients hospitalized in our hospital from January 1, 2017, to January 1, 2018, were collected. Paired carcinoma and adjacent tissues of 50 patients were finally obtained. Freshly-collected tissue specimens were divided into three groups, quickly placed in liquid nitrogen, and stored in an ultra-low temperature refrigerator at -80°C. Specimens prepared into frozen sections were subjected to hematoxylin-eosin (HE) staining. Paraffin sections were subjected to immunohistochemistry (IHC) to analyze the semi-quantitative expression of TC-1 protein, and remaining specimens were used for RNA extraction and qPCR.

All human tissues were collected according to the human subject protocol approved by the Review Committee of Daqing Oilfield General Hospital. Tumor and corresponding non-tumor tissues were obtained after patients signed the written informed consent.

Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The tissue was grinded in liquid nitrogen and treated with TRIzol (100 mg tissue: 1 ml TRIzol). Then, the solution was moved to an Ep tube and added with 200 μl chloroform. After vibrated for 15 s, the upper aqueous phase was added with 500 μl isopropanol for 10 min. After centrifuged at 12000 g for 10 min, the precipitation was added with 1 ml ethanol (75%). After centrifuged at 4°C and 7500 g for 5 min, the supernatant was removed and the tube was dried for 10 min. Next, the RNA was solved in DEPC water and qualified by 1.2% agarose gel electrophoresis. RNA content and purity were determined by ultraviolet spectrophotometer. Reaction solution was prepared according to the instruction, including 2 μg total RNA, 1 μl oligo primer (50 μM), 1 μl dNTP mix (10 μM), and ddH2O. The solution was predegenerated at 65°C for 5 min. Then, cDNA first chain synthesis reaction system was prepared, including 2 μl 10×RT buffer, 4 μl $MgCl_2$ (25 μM), 2 μl DTT (0.1 M), l μl RNAase OUT (40 U/μl), 1 μl PrimeScript (TaKaRa Bio Inc., Otsu, Shiga, Japan), and ddH_2O. The reaction condition was composed by 50°C for 50 min and 85°C for 5 min. The complementary deoxyribonucleic acid (cDNA) was synthesized. The gene sequences of the target gene and the internal reference β-actin were obtained from GenBank (Bethesda, MD, USA), and primers were designed using Primer-Blast (Bethesda, MD, USA), a primer design software on the NCBI website. The sequences of primers (TC-1 forward, 5'-GGACATTCAAAGACCAAGTGAG-3', TC-1 reverse, 5'-GTAACCAGTCTGTTCAGTCCTG-3', β-actin forward, 5'-ACATCCGCAAAGACCTGTAC-3', β-actin reverse, 5'-TGATCTTCATTGTGCTGGGTG-3') were synthesized by Sangon Biotech (Shanghai, China). The reaction system was 10 μL. Reaction conditions are as follows: pre-dena-

turation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s and sequential at 60°C for 60 s. DNA synthesizes a single strand that is complementary to the template strand after undergoing several stages including template denaturation, annealing, and primer extension. The semi-reserved replication pattern amplifies the gene to be amplified by several million times. After the reaction, the dissolution curve was a single-peak curve indicating the specificity of the qPCR product, and the amplification curve reached the platform stage when the gene was completely amplified. Relative mRNA expression was calculated via $2^{-\Delta Ct}$ [Δ cycle threshold (Ct) = Ct (target gene)-Ct (β-actin)], the fold change between different treatments was calculated by $2^{-\Delta\Delta CT}$, where ΔΔCT = ΔCT (experimental group) – ΔCT (control group).

IHC

Thyroid carcinoma and corresponding adjacent normal tissues were fixed with formaldehyde and embedded in paraffin, followed by staining with TC-1 polyclonal antibody (1:500, Abcam, Cambridge, MA, USA) using IHC streptavidin-peroxidase (SP) technique. Positive control: Strongly-positive expression was detected in tissues known to contain test antigens according to the official website of Abcam. Negative control: The primary antibody was replaced with phosphate-buffered solution (PBS) solution, and the results were all negative. Positive signals are yellow, brownish-yellow or tan signals. Five fields (10×40) were randomly observed under an electron microscope, and the proportion of positive cells showing yellow, brownish-yellow or tan signals and the intensity of these signals were used for determination.

Western Blotting (WB)

The protein concentration was determined using a BCA protein concentration assay kit (Beyotime, Beijing, China). 40 μg total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. Then, primary antibody (1:2000, Abcam, Cambridge, MA, USA) was added for incubation at 4°C, followed by incubation with the corresponding horseradish peroxidase-labeled antibody (1:1000, Beyotime, Beijing, China) at room temperature for 1 h. Luminescence solution (200 μL) (Bio-Rad, Hercules, CA, USA) was added uniformly and dropwise, followed by development using a chemiluminescence imaging analysis system. Image J (NIH, Bethesda, MD, USA) and other image analysis software were utilized for gray value calculation and statistical analysis of the band on the developed images.

Statistical Analysis

SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Chi-square test was adopted to analyze the correlations of TC-1 expression level with clinicopathological features. *T*-test was used for comparisons of measurement data between the two groups, and one-way ANOVA, with the Tukey's post hoc test, was employed for comparison among groups. A statistical significance was defined when $p<0.05$.

Results

Expression Level of TC-1 in Thyroid Carcinoma and Adjacent Tissues

The mRNA and protein expression levels of TC-1 in 50 pairs of thyroid carcinoma and adjacent tissues were detected by real-time PCR and WB, respectively. The results showed that the level of TC-1 in carcinoma tissues was significantly higher than those in adjacent tissues ($p<0.05$) (Figure 1, Table I). The mRNA expression of TC-1 was significantly related to the protein expression ($p<0.05$) (Table II).

Expression Level of TC-1 in Thyroid Carcinoma and Adjacent Tissues Detected Via IHC

The expression of TC-1 protein in different stages of thyroid carcinoma and adjacent tissues was detected through IHC. TC-1 protein was positively expressed in the cytoplasm (Figure 2). Positive expression presented brownish yellow or dark brown on IHC. The positive expression rate of TC-1 protein in thyroid carcinoma tissues was significantly higher than that in adjacent tissues, and it was increased with the advance of tumor stage ($p<0.05$) (Table III).

Table I. Protein expression level of TC-1 in carcinoma and adjacent tissues.

Group	TC-1	Actin
Carcinoma tissue	2646±195	1645±113
Adjacent tissue	892±76[a]	1723±98

Note: [a]$p<0.05$

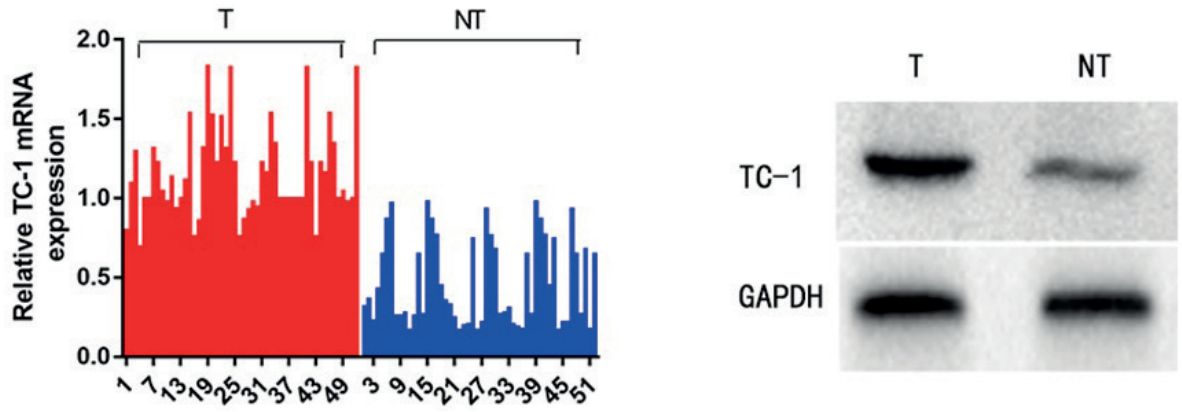

Figure 1. Expression level of TC-1 in thyroid carcinoma (T) and adjacent tissues (NT) measured by PCR and WB.

Table II. Relation between mRNA expression and protein expression of TC-1.

	qPCR	
TC-1 protein	Positive	Negative
Carcinoma tissue	2646±195	1645±113
Adjacent tissue	892±76[a]	1723±98

Correlations of TC-1 Expression with Clinicopathological Features

Analyses on the correlations of TC-1 expression with clinicopathological features revealed that TC-1 expression was associated with stage, tumor pathological grade and lymph node metastasis ($p<0.05$), but had no significant relations with gender and age. The positive expression rate of TC-1 in stage III-IV tumors (80.95%) was higher than that in stage I (42.11%) and stage II (34.38%) tumors ($p<0.01$). The positive rate in those with lymph node metastasis was also higher than that in those without lymph node metastasis (63.89% *vs.* 24.0%, $p<0.01$) (Table IV).

Table III. Expression of TC-1 in thyroid carcinoma and adjacent tissues.

	TC-1 protein expression		
	Negative (n)	Positive (n)	*p*
Carcinoma tissue in stage I-II	42	28	0.01
Carcinoma tissue in stage III-IV	10	32	
Adjacent tissue	98	14	3

Table IV. Correlations of TC-1 protein expression with clinicopathological features.

Feature	N.	TC-1 negative	TC-1 positive	*p*
Gender				
Male	81	48	33	0.456
Female	31	18	13	
Age				
>60	54	35	19	0.623
≤60	58	31	27	
TNM stage				
I	38	22	16	
II	32	21	11	0.003
III-IV	42	8	34	
Pathological grade				
I	45	20	25	
II	67	12	55	0.000
Lymph node metastasis				
No	50	38	12	0.007
Yes	72	26	46	

Table V. Relationship between TC-1 mRNA expression level and B-ultrasound characteristics.

B-ultrasound characteristics	TC-1 mRNA	Actin
Micro-calcification observed	87.46±49.55	97.46±22.55
No micro-calcification displayed	38.46±29.15	89.46±43.95
p	0.032	0.26

Different B-Ultrasound Characteristics

The relationship between TC-1 expression and B-ultrasound characteristics was analyzed, and ultrasound detection that TC-1 mRNA in PTC tissues with micro-calcification was 87.46±49.55. This was significantly elevated compared with that in those without micro-calcification (38.46±29.15) ($p<0.05$) (Table V).

Discussion

TC-1, first discovered in PTC and its surrounding normal thyroid tissues[8,9], has multiple functions and is considered to play an important role in cell cycle control, transcriptional control, and translational regulation[10]. TC-1 (C8orf4) is a novel gene upregulated in certain cancers, which is associated with metastasis and poor prognosis. TC-1 is deemed as a positive regulator of the Wnt signaling pathway. In addition, TC-1 is also identified as a novel heat shock response regulator[11]. Heat shock and various cellular stresses induce the expression of TC-1. TC-1 is involved in the mitogen-activated ERK1/2 signaling pathway and promotes the G1-s phase transition in the cell cycle. Recently, a study[12] reported that TC-1 served as a hematopoietic regulator in mice. It has been manifested that TC-1 up-regulates the expression of downstream genes and promotes the proliferation and invasion of cancer cells[13]. Interestingly, TC-1 upregulates IL-1β and TNF-α and facilitates the proliferation of follicular dendritic cells[14,15]. This is critical for the development of B cells, suggesting its involvement in various biological regulation, inflammation and immune regulation under various stimuli.

Previously Zhang et al[16] indicates that the apoptosis rate of cells transfected with TC-1 is reduced. TC-1 is an over-expressed gene in PTC, and the Wnt/β-catenin signal transduction pathway plays a crucial role in its occurrence and development. The activation of Wnt/β-catenin pathway will also lead to high expression of target genes, resulting in the proliferation and abnormal differentiation of cells and thereby facilitating tumorigenesis[17,18].

TC-1 may play a key role in the malignant transformation of normal thyroid cells[13]. Compared the expression of TC-1 in normal thyroid tissues, an evident difference in TC-1 expression between benign and malignant thyroid lesions was found in nodular goiter and PTC, indicating that TC-1 can be used to differentiate benign and

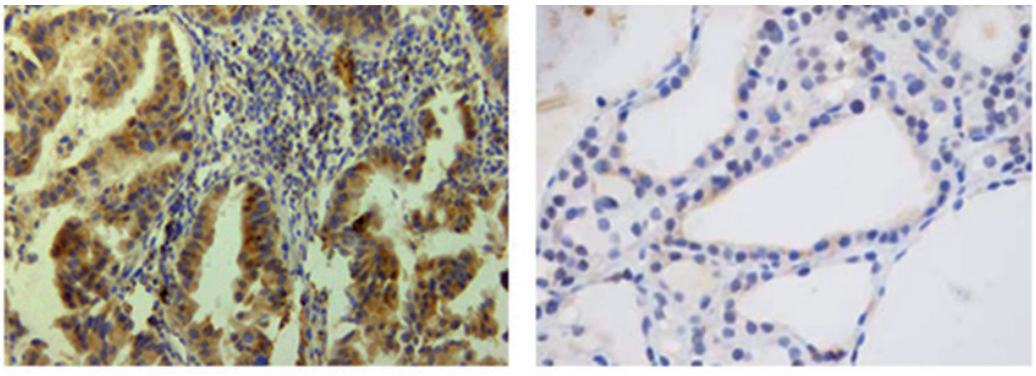

Figure 2. Expression of TC-1 detected via IHC: positive expression in carcinoma tissues (T) and negative expression in adjacent tissues (NT).

malignant thyroid carcinoma[19-20]. This finding is of certain significance for the diagnosis of benign and malignant thyroid lesions.

In this study, qPCR and WB were conducted to detect the mRNA and protein expression of TC-1 in PTC and corresponding adjacent tissues from 50 patients. The results suggested that the expression of TC-1 in PTC tissues was overtly higher than those in adjacent tissues, further proving an oncogene profile of TC-1. This implies that highly-expressed TC-1 may be involved in the occurrence and development of thyroid carcinoma. Moreover, a statistical difference was detected between two groups with different pathological grade and lymph node metastasis ($p<0.05$), suggesting that the expression of TC-1 is related to the malignancy of PTC. To further clarify the role of TC-1 gene expression in the progression of PTC, IHC was utilized to retrospectively analyze the protein expression of TC-1 in 112 patients with thyroid carcinoma. The results also showed that the protein expression of TC-1 was significantly associated with tumor-node-metastasis (TNM) stage, pathological grade and lymph node metastasis. Further in-depth investigations are needed to clarify the specific mechanism of TC-1 in regulating the occurrence and development of PTC. The cocktail strategy of prognostic factors, such as TC-1 combined with miR-791, still needs further evaluation in the diagnosis of thyroid carcinoma[21].

Conclusions

We demonstrated that TC-1 was highly expressed in PTC and had some value in diagnosing and staging PTC. It provides academic support for the early diagnosis of PTC in the future.

References

1) Cobin RH, Gharib H, Bergman DA, Clark OH, Cooper DS, Daniels GH, Dickey RA, Duick DS, Garber JR, Hay ID, Kukora JS, Lando HM, Schorr AB, Zeiger MA, Thyroid Carcinoma Task F. AACE/AAES medical/surgical guidelines for clinical practice: management of thyroid carcinoma. American Association of Clinical Endocrinologists. American College of Endocrinology. Endocr Pract 2001; 7: 202-220.
2) Zhang J, Gao Y, Zhao X, Guan M, Zhang W, Wan J, Yu B. Investigation of copy-number variations of C8orf4 in hematological malignancies. Med Oncol 2011; 28 Suppl 1: S647-652.
3) Walter MJ, Payton JE, Ries RE, Shannon WD, Deshmukh H, Zhao Y, Baty J, Heath S, Westervelt P, Watson MA, Tomasson MH, Nagarajan R, O'Gara BP, Bloomfield CD, Mrozek K, Selzer RR, Richmond TA, Kitzman J, Geoghegan J, Eis PS, Maupin R, Fulton RS, McLellan M, Wilson RK, Mardis ER, Link DC, Graubert TA, DiPersio JF, Ley TJ. Acquired copy number alterations in adult acute myeloid leukemia genomes. Proc Natl Acad Sci U S A 2009; 106: 12950-12955.
4) Jung Y, Bang S, Choi K, Kim E, Kim Y, Kim J, Park J, Koo H, Moon RT, Song K, Lee I. TC1 (C8orf4) enhances the Wnt/beta-catenin pathway by relieving antagonistic activity of Chibby. Cancer Res 2006; 66: 723-728.
5) Kim B, Koo H, Yang S, Bang S, Jung Y, Kim Y, Kim J, Park J, Moon RT, Song K, Lee I. TC1(C8orf4) correlates with Wnt/beta-catenin target genes and aggressive biological behavior in gastric cancer. Clin Cancer Res 2006; 12: 3541-3548.
6) Friedman JB, Brunschwig EB, Platzer P, Wilson K, Markowitz SD. C8orf4 is a transforming growth factor B induced transcript downregulated in metastatic colon cancer. Int J Cancer 2004; 111: 72-75.
7) Sunde M, McGrath KC, Young L, Matthews JM, Chua EL, Mackay JP, Death AK. TC-1 is a novel tumorigenic and natively disordered protein associated with thyroid cancer. Cancer Res 2004; 64: 2766-2773.
8) Chua EL, Young L, Wu WM, Turtle JR, Dong Q. Cloning of TC-1 (C8orf4), a novel gene found to be overexpressed in thyroid cancer. Genomics 2000; 69: 342-347.
9) Kim Y, Kim J, Park J, Bang S, Jung Y, Choe J, Song K, Lee I. TC1(C8orf4) is upregulated by IL-1beta/TNF-alpha and enhances proliferation of human follicular dendritic cells. FEBS Lett 2006; 580: 3519-3524.
10) Park J, Jung Y, Kim J, Kim KY, Ahn SG, Song K, Lee I. TC1 (C8orf4) is upregulated by cellular stress and mediates heat shock response. Biochem Biophys Res Commun 2007; 360: 447-452.
11) Jung Y, Kim M, Soh H, Lee S, Kim J, Park S, Song K, Lee I. TC1(C8orf4) regulates hematopoietic stem/progenitor cells and hematopoiesis. PLoS One 2014; 9: e100311.
12) Lee S, Choi K, Ahn H, Song K, Choe J, Lee I. TuJ1 (class III beta-tubulin) expression suggests dynamic redistribution of follicular dendritic cells in lymphoid tissue. Eur J Cell Biol 2005; 84: 453-459.
13) Tan SH, Barker N. Wnt signaling in adult epithelial stem cells and cancer. Prog Mol Biol Transl Sci 2018; 153: 21-79.
14) de Melo Martins PC, Parise Junior O, Pereira Hors C, Villela Miguel RE, da Costa Andrade VC, Garicochea B. C8orf4/TC-1 (thyroid cancer-1) gene expression in thyroid cancer and goiter. ORL J Otorhinolaryngol Relat Spec 2007; 69: 127-130.
15) Kim J, Kim Y, Kim HT, Kim DW, Ha Y, Kim J, Kim CH, Lee I, Song K. TC1(C8orf4) is a novel endothelial inflammatory regulator enhancing NF-kappaB activity. J Immunol 2009; 183: 3996-4002.

16) Zhang P, Cao HY, Bai LL, Li WN, Wang Y, Chen SY, Zhang L, Yang LH, Xu HT, Wang EH. The high expression of TC1 (C8orf4) was correlated with the expression of beta-catenin and cyclin D1 and the progression of squamous cell carcinomas of the tongue. Tumour Biol 2015; 36: 7061-7067.

17) Jang H, Kim M, Lee S, Kim J, Woo DC, Kim KW, Song K, Lee I. Adipose tissue hyperplasia with enhanced adipocyte-derived stem cell activity in Tc1(C8orf4)-deleted mice. Sci Rep 2016; 6: 35884.

18) Uversky VN. Natively unfolded proteins: a point where biology waits for physics. Protein Sci 2002; 11: 739-756.

19) Yang ZQ, Streicher KL, Ray ME, Abrams J, Ethier SP. Multiple interacting oncogenes on the 8p11-p12 amplicon in human breast cancer. Cancer Res 2006; 66: 11632-11643.

20) Lee S, Choi K, Ahn H, Song K, Choe J, Lee I. TuJ1 (class III beta-tubulin) expression suggests dynamic redistribution of follicular dendritic cells in lymphoid tissue. Eur J Cell Biol 2005; 84: 453-459.

21) Gao XB, Chen CL, Tian ZL, Yuan FK, Jia GL. MicroRNA-791 is an independent prognostic factor of papillary thyroid carcinoma and inhibits the proliferation of PTC cells. Eur Rev Med Pharmacol Sci 2018; 22: 5562-5568.