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ORIGINAL PAPER

ZCCHC12, a potential molecular marker of papillary thyroid carcinoma: a preliminary study

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Abstract A gene expression profile analysis using an Affymetrix HG-U133 Plus 2.0 microarray with probes for 38,500 human full-length cDNAs was performed on a primary papillary thyroid carcinoma (PTC) and a nodular goiter (NG). ZCCHC12 was the gene with the most significant differential expression between PTC and NG, and this was verified using fluorescent quantitative PCR (FQ-PCR). A total of 9,485 genes were detected with a difference in transcription levels between PTC and NG. Of these, 2,098 were up-regulated with a signal log ratio (SLR) ≥ 1 and 1,714 were down-regulated with an SLR ≤ -1. Among these up-regulated and down-regulated genes, 12 genes were significantly up-regulated (SLR ≥ 5.0) and 6 genes were significantly down-regulated (SLR ≤ -5.0). The SLR of the ZCCHC12 gene was 8.8. The results of FQ-PCR showed that the medians of the log (ZCCHC12 RNA/GAPDH RNA) in PTC and NG were 0.73 and -1.68, respectively, and the difference between them was significant ($P < 0.05$). There were no significant correlations between the RNA levels of the ZCCHC12 gene and the clinicopathological and biochemical parameters of PTC in our pilot study. This study showed that a number of differentially expressed genes were discovered between PTC and NG. Significantly, the number of transcript copies of the ZCCHC12 gene in PTC was higher than in NG. The verified results of FQ-PCR were consistent with the microarray screening results. The ZCCHC12 gene may be a novel diagnostic molecular marker of PTC.

Keywords Papillary thyroid carcinoma · Nodular goiter · ZCCHC12 gene · Molecular marker · Microarray · Fluorescent quantitative PCR

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Introduction

Thyroid nodules are a very common problem in clinical practice. In the United States, 4–7% of the adult population has a palpable thyroid nodule [1]. The prevalence of thyroid nodules is much greater when nodules that are detected by ultrasonography or at autopsy are included [2]. High-resolution ultrasound (US) can detect thyroid nodules in 19–67% of randomly selected individuals, with higher frequencies in women and the elderly [3]. Regardless of the level of iodine in the diet, the prevalence of thyroid nodules in adults in Northern China is more than 10% by thyroid ultrasonography [4]. Today, the most common lesion of benign thyroid nodules is nodular goiter (NG), and the most common lesion of thyroid malignancy is papillary thyroid carcinoma (PTC); only approximately 1 in 20 clinically identified nodules is malignant [5]. In the United

States, approximately 44,670 new cases of thyroid cancer were diagnosed in 2010, and thyroid carcinomas in women ranked as the fifth most common type of cancer [6]. The management of a solitary thyroid nodule remains controversial, and distinguishing between benign and malignant thyroid nodules is a common diagnostic dilemma. The introduction of fine needle aspiration cytology (FNAC) in the 1970s simplified the evaluation of thyroid nodules and decreased the likelihood of surgery for a thyroid nodule by approximately 50% [7]. Despite the ability to identify PTC with some reliability by FNAC, clinicians must frequently make a decision regarding the management of patients with thyroid nodules on the basis of equivocal information.

Many attempts have been made to identify molecular markers for thyroid carcinoma that can preoperatively distinguish between benign and malignant lesions. None of the markers have proven to be ideal, including *LGALS3* (galectin-3), *KRT19* (keratin 19), *FN1* (fibronectin 1), *BRAF, RET/PTC, RAS, HBME-1*, *MET* (met pro-oncogene), *DPP4* (dipeptidyl-peptidase 4), *SERPINA1* (serpin peptidase inhibitor, clade A, member 1), *MUC1* (mucin 1), and *TIMP1* (tissue inhibitor of metallopeptidase 1) [8–16]. Microarray technology has become a powerful tool for analyzing the gene expression profiles of tens of thousands of genes simultaneously. Parallel analysis of gene expression profiles using the microarray technique offers a large-scale platform for screening novel markers for potential clinical applications. In general, the two main foci of microarray investigations are the improvement in the understanding of the molecular etiology of thyroid neoplasms and the development of improved techniques in the detection of genetic markers that could improve the differential diagnosis of thyroid nodules.

In the present study, we first screened the differential gene expression profiles of papillary thyroid carcinoma and nodular goiter with an oligonucleotide microarray. Next, we used FQ-PCR to validate the most significantly differentially expressed gene (ZCCHC12). Finally, we analyzed the correlation between the ZCCHC12 transcription levels and the clinicopathological and biochemical parameters of PTC.

Materials and methods

Tissue specimens

Two thyroid tissue samples, including one PTC and one NG, were obtained intraoperatively from two patients during primary thyroidectomy. The tissue specimens were placed in tissue tubes with RNA preservation solution and stored at −80°C. The histopathologic diagnosis was confirmed according to the 2004 World Health Organization (WHO) criteria [17].

RNA extraction

The total RNA of the tissue specimens was isolated using TRIzol (Invitrogen, Carlsbad, CA) and was purified with the RNeasy Kit (QIAGEN, Valencia, CA) according to the manufacturer's recommendations.

Oligonucleotide expression microarray analysis

The Affymetrix HG-U133 Plus 2.0 microarray (Affymetrix, Inc., Santa Clara, CA) was used for microarray analysis; this system includes 38,500 definite cDNA probes and more than 47,000 transcripts and variants. The samples were processed following the Affymetrix protocol. The procedure included the extraction and purification of RNA using TRIzol and QIAGEN's RNeasy Mini Kit; the synthesis and purification of cDNA; the synthesis of biotin-labeled cRNA; and the hybridization, washing, staining, and scanning of the chip using GeneChip Operating Software (GCOS) (Affymetrix, Inc., Sana Clara, CA) to read and manage data.

Fluorescent quantitative polymerase chain reaction (FQ-PCR)

The most significantly differentially expressed gene, ZCCHC12, was selected for validation by FQ-PCR (Table 1).

In total, 52 thyroid tissue samples were acquired intraoperatively from 52 patients during primary thyroidectomy. According to the 2004 WHO criteria, PTC was diagnosed in 28 patients and NG was diagnosed in 24 patients. The tissue specimens were collected in EP tubes with RNA preservation solution within half an hour after the tissue was removed from the body. The specimens were stored at −80°C until use.

Among the 28 PTC patients, there were 15 females and 13 males, aged 12–76 years. The patients were euthyroid before surgery. The classic variant of PTC was diagnosed in 21 cases, the follicular variant in 6 cases, and the column cell variant in 1 case. Regional lymph node metastases were diagnosed during primary surgery in 27 patients.

The primer and probe sequence data were attained from the Genbank sequence data bank. The probe of H-ZCCHC12 was FAM-ACCTCTGTCCTTGCTCCTTCT CCCTGC-TAMRA, the sense primer was GGATACCAG CACATTGGAGGG, and the antisense primer was TATA CCACTTTCACAAAGAATAAAGCTG. The FQ-PCR procedure included the extraction of tissue RNA using Trizol (Invitrogen, Carlsbad, CA), identification of the

Table 1 The ZCCHC12 gene was selected for FQ-PCR validation

Gene symbol	Gene title	Signal log ratio	Probe set ID	373T detection[a]	Change	392T detection[b]	Chromosomal location
ZCCHC12	Zinc finger, CCHC domain containing 12	8.8	228715_at	P	I	P	chrXq24

[a] 373T refers to PTC

[b] 392T refers to NG

RNA sample, reverse transcription of the RNA, preparation of a positive standard template, and FQ-PCR. The ZCCHC12 gene transcript was analyzed by real-time reverse transcription-PCR (ABI PRISM 7900 Sequence Detector apparatus, Applied Biosystems). First-strand cDNA was synthesized. Reactions were performed by using SYBR Green (SYBR Green PCR Core Reagent, Perkin-Elmer), and quantification was performed using ABI PRISM 7900 SDS software. The quantification of each gene's expression was calibrated using a standard reference curve obtained by serial dilutions of PCR product prepared from a mixture of cDNAs from the tissue samples that were handled separately but concomitantly with the clinical samples.

Ethical standards

The study had been approved by the ethics committee of Sun Yat-sen University Cancer Center and had therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The patients gave their informed consent prior to their inclusion in the study.

Statistical methods

The data were analyzed with the SPSS 13.0 software. A Mann–Whitney *U* rank sum test was used to compare the parameters between the two groups because of the skewed distribution of the data and the heterogeneity of variance.

Results

Microarray study

The differentially expressed genes between PTC and NG

There were 9,485 differentially expressed genes between PTC and NG. A total of 2,098 genes were up-regulated in PTC with an SLR ≥ 1, whereas 1,714 genes were down-regulated with an SLR ≤ -1 in PTC. Among the total number of differentially expressed genes between PTC and NG, 12 genes were significantly up-regulated with an SLR ≥ 5.0 (Table 2) and 6 genes were significantly down-regulated with an SLR ≤ -5.0 (Table 3). The ZCCHC12 gene was the most significantly differentially expressed gene between PTC and NG, with an SLR of 8.8.

The FQ-PCR of the ZCCHC12 gene in PTC and NG

The amplification of the ZCCHC12 gene in PTC and NG

The amplification of the ZCCHC12 gene in PTC and NG was shown in Figs. 1 and 2.

The amplification of the GAPDH gene (internal reference) in PTC and NG

The amplification of the GAPDH gene in PTC and NG was shown in Figs. 3 and 4.

Table 2 The 12 genes that were significantly up-regulated in PTC relative to NG (SLR ≥ 5.0)

Gene symbol	Gene title	PTC versus NG SLR	Probe set ID	Chromosomal location
IGFBP6	Insulin-like growth factor-binding protein 6	5.8	203851_at	chr12q13
PTGER4	Prostaglandin E receptor 4 (subtype EP4)	6.5	204897_at	chr5p13.1
PLAU	Plasminogen activator, urokinase	5.1	205479_s_at	chr10q24
ECM1	Extracellular matrix protein 1	6.9	209365_s_at	chr1q21
CHI3L1	Chitinase 3-like 1 (cartilage glycoprotein-39)	5.5	209395_at	chr1q32.1
CHI3L1	Chitinase 3-like 1 (cartilage glycoprotein-39)	5.4	209396_s_at	chr1q32.1
LRP4	Low-density lipoprotein receptor-related protein 4	5	212850_s_at	chr11p11.2-p12
SLIT1	Slit homolog 1 (Drosophila)	5.6	213601_at	chr10q23.3-q24
FLRT3	Fibronectin leucine-rich transmembrane protein 3	5	219250_s_at	chr20p11
–	CDNA clone IMAGE:4800096	5.4	228640_at	–
ZCCHC12	Zinc finger, CCHC domain containing 12	8.8	228715_at	chrXq24
C20orf133	Chromosome 20 open reading frame 133	5.8	235278_at	chr20p12.1

Table 3 The 6 genes that were significantly down-regulated in PTC relative to NG (SLR ≤ -5.0)

Gene symbol	Gene title	PTC versus NG SLR	Probe set ID	Chromosomal location
TFF3	Trefoil factor 3 (intestinal)	−5.2	204623_at	chr21q22.3
DIO1	Deiodinase, iodothyronine, type I	−5	206457_s_at	chr1p33-p32
DGKI	Diacylglycerol kinase, iota	−5	206806_at	chr7q32.3-q33
TPO	Thyroid peroxidase	−5.7	210342_s_at	chr2p25
SLC25A15	Solute carrier family 25 (mitochondrial carrier, ornithine transporter) member 15	−5.6	218653_at	chr13q14
IYD	Iodotyrosine deiodinase	−5.2	231070_at	chr6q25.1

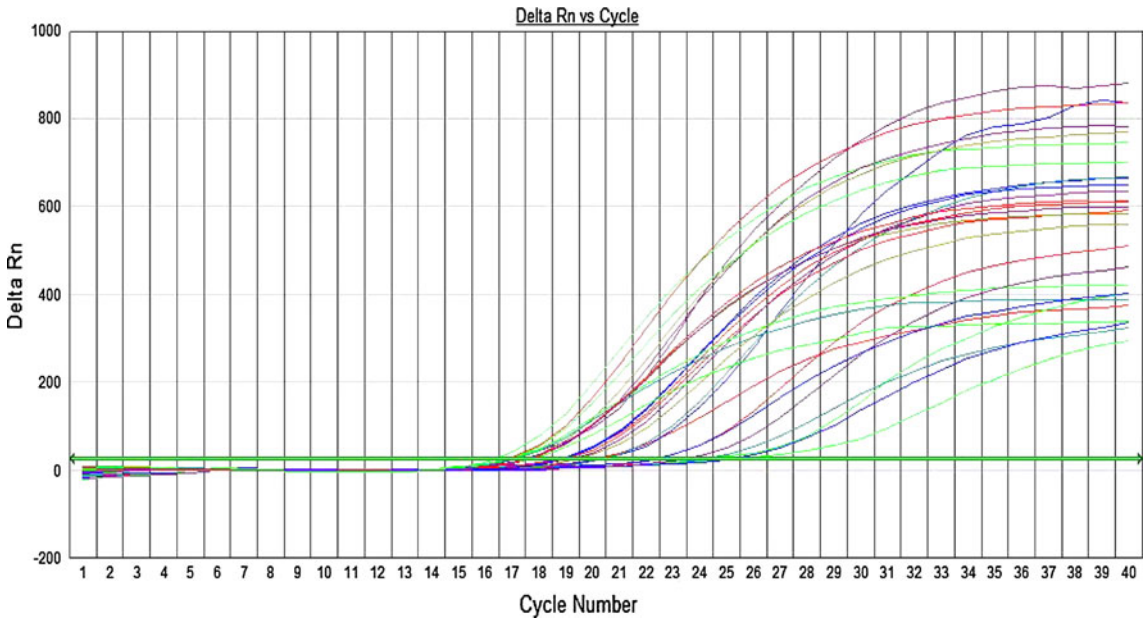

Fig. 1 The amplification curve of the ZCCHC12 gene in PTC

A comparison of the expression levels of the ZCCHC12 gene in PTC and NG

The number of amplification copies of the ZCCHC12 gene (RNA) in PTC and NG The number of amplification copies of the ZCCHC12 gene in PTC was higher than that in NG, but the values in Table 4 should not be compared directly; the numbers of amplification copies of the ZCCHC12 gene from the different tissues should be normalized to the internal reference gene first (Table 4).

A comparison of the ZCCHC12 gene expression level in PTC and NG After the numbers of amplification copies of the ZCCHC12 gene were compared with the numbers of copies of the internal reference gene (GAPDH), a series of ratio values were obtained. Because these ratio values showed a significantly skewed distribution, the common logarithm (log) of these ratios was calculated. The common logarithm of the ratios also showed a skewed distribution; so, they could not be analyzed statistically with a *t* test or ANOVA (analysis of variance). Therefore, the Mann–Whitney *U* rank sum test was chosen.

As shown in Table 5 and Fig. 5, the FQ-PCR showed that the medians of the log (ZCCHC12 RNA/GAPDH RNA) for PTC and NG were 0.73 and −1.68, respectively, and the difference between them was significant ($P < 0.05$). Therefore, the number of amplification copies of the ZCCHC12 gene in PTC was significantly higher than in NG.

The correlation between the amplification copy of the ZCCHC12 gene and the clinicopathological and biochemical parameters of PTC

We further analyzed the correlation between the numbers of amplification copies of the ZCCHC12 gene and the clinicopathological and biochemical parameters of PTC.

From Table 6, we conclude that the number of amplification copies of the ZCCHC12 gene in PTC had no

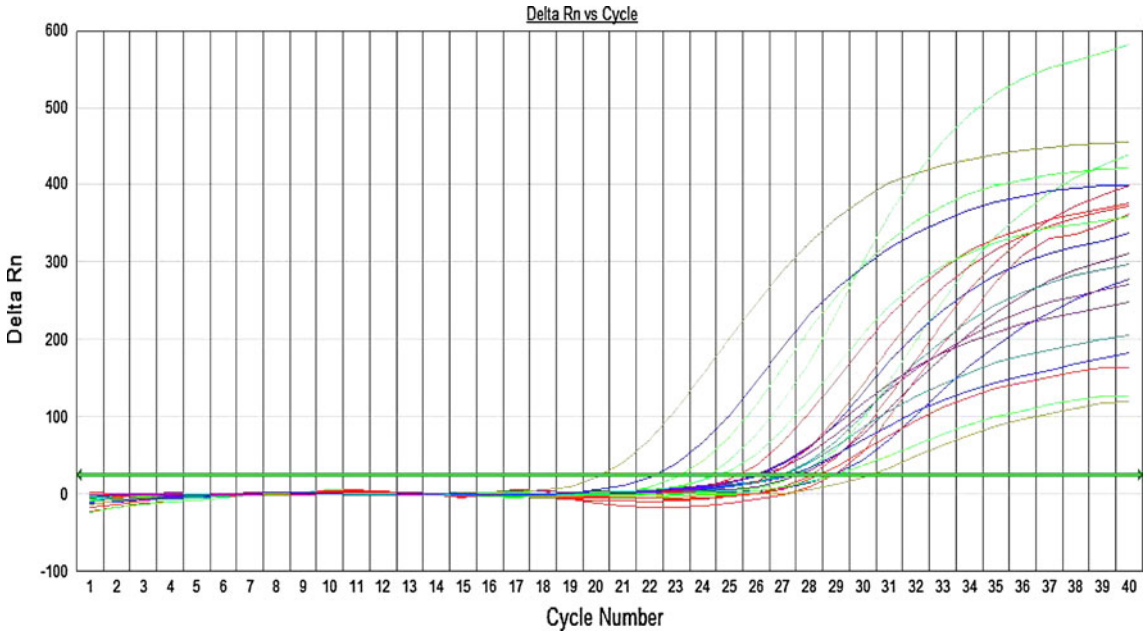

Fig. 2 The amplification curve of the ZCCHC12 gene in NG

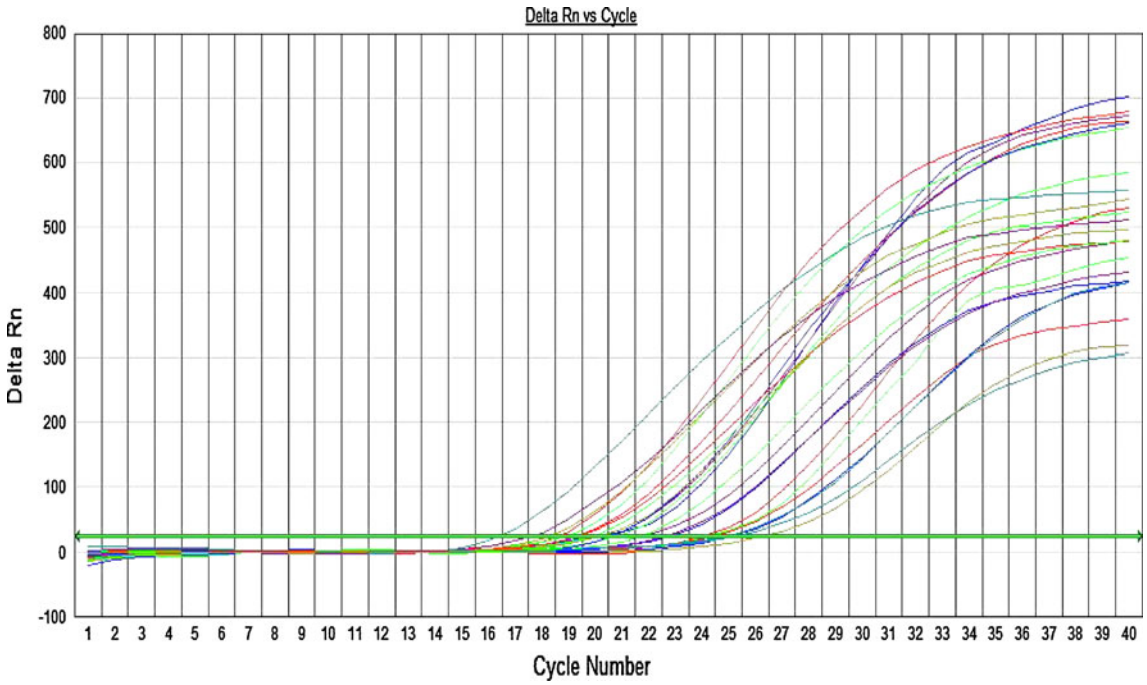

Fig. 3 The amplification curve of the GAPDH gene in PTC

correlation with the common clinicopathological and biochemical parameters of PTC ($P > 0.05$).

Discussion

Thyroid nodules are a very common problem in clinical practice. Most thyroid nodules are benign, and approximately 5% of all thyroid nodules are malignant. The key for the management of thyroid nodules is to discriminate malignant nodules from benign nodules. PTC accounts for approximately 80% of all incidences of thyroid cancer in the USA.

There have been many attempts to find a sensitive and specific molecular marker that can readily discriminate benign from malignant thyroid lesions, but the resultant

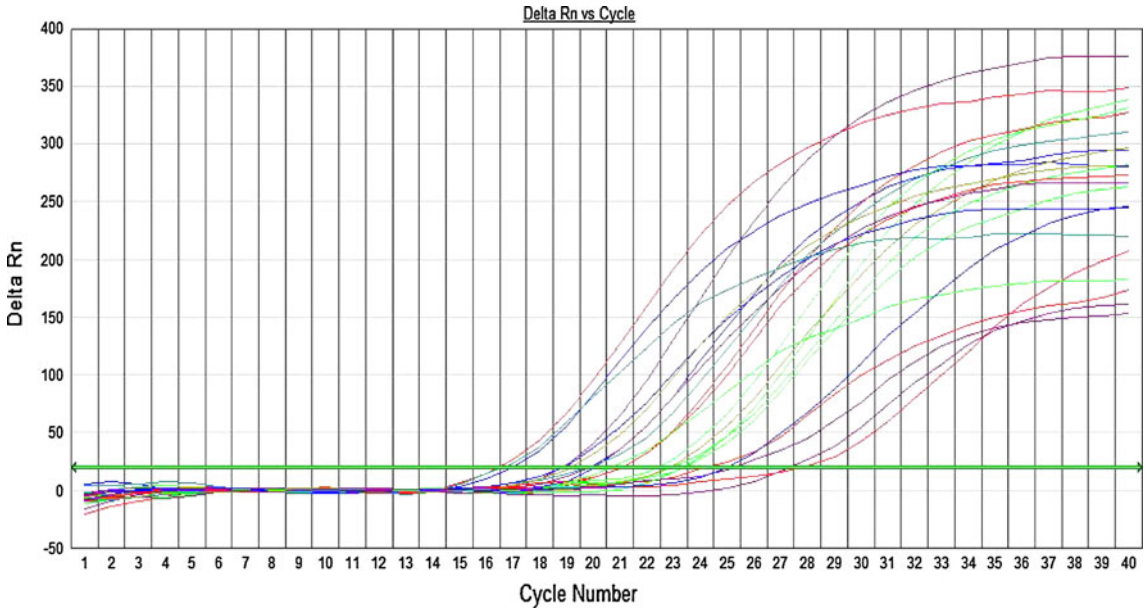

Fig. 4 The amplification curve of the GAPDH gene in NG

Table 4 The number of amplification copies of the ZCCHC12 gene (RNA) in PTC and NG

Gene	Group	Case	Median copies	Quantile copies range
ZCCHC12	PTC	28	2.09E + 08	32,604,893.75–496,578,309.1
	NG	24	5.42E + 06	1,298,218.202–8,825,644.841

Table 5 A comparison of the log (ZCCHC12 RNA/GAPDH RNA) between PTC and NG

Group	Case	log (ZCCHC12/GAPDH) median	log (ZCCHC12/GAPDH) quartile range	Rank sum	Mann–Whitney U	*P*
PTC	28	0.73	0.045–1.027	1,019	59.00	0.000
NG	24	−1.68	−2.708 to −0.907	359		

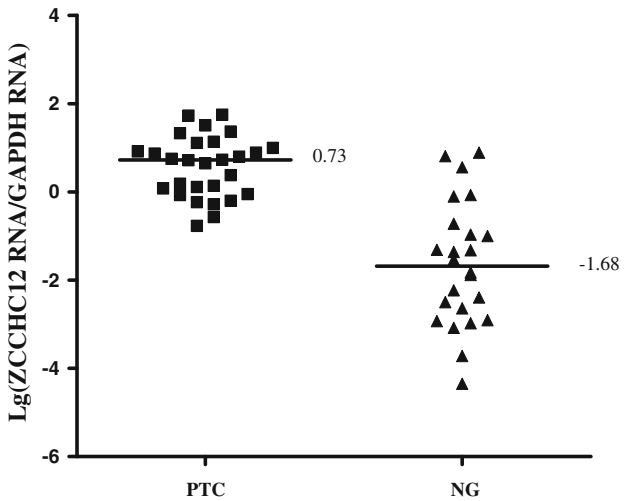

Fig. 5 A comparison of the number of copies of ZCCHC12 mRNA in PTC and NG

markers have all fallen short in some way. Two main genetic alternations have been found in PTC (*RET/PTC* rearrangement and *BRAF* mutations) [12, 13]. Many molecular markers have been investigated, and some were considered to be associated with PTC (such as *LGALS3*, *FN1*, *MET*, *DPP4*, *SERPINA1*, *KRT19*, *MUC1*, *TIMP1*, *CITED1*, and *DUSP6*), but none of them has proven to be sensitive enough or specific enough to be translated into a clinically ideal marker [18].

The first array investigation of PTC was performed by Huang et al. [14] using the Affymetrix U95A array that contains more than 12,000 transcripts. In their study of 8 PTC tissues, which were compared with the surrounding normal tissue from the same eight patients, Huang et al. specified fifty genes with the most distinct gene expression changes. Some of the genes were already known to be differentially expressed in PTC. These researchers also identified a number of additional PTC specific genes, and many of them associated with the cell cycle or mitogenic

Table 6 The correlation between the number of amplification copies of the ZCCHC12 gene and the clinicopathological and biochemical parameters of PTC

Parameter	Cases	log (ratio), median	Mean rank	Sum of ranks	Test[a]	*P*
Gender						
Male	13	0.72	14.54	189.00	97.00	0.982
Female	15	0.73	14.47	217.00		
Age						
<45 years	19	0.75	15.63	297.00	64.00	0.290
≥45 years	9	0.38	12.11	109.00		
T classification						
T1	7	0.14	11.43		1.617	0.656
T2	6	1.01	16.83			
T3	9	0.75	15.56			
T4	6	0.29	14.17			
Tumor size						
≤2 cm	12	0.43	13.08		1.00	0.607
>2 cm, ≤4 cm	12	0.74	14.83			
>4 cm	4	0.65	17.75			
Lesion number						
Single	18	0.52	13.67	246.00	75.00	0.472
Multiple	10	0.80	16.00	160.00		
Pathology variant						
Classic	21	0.72	14.05		0.661	0.719
Follicular	6	0.84	16.67			
Column	1	0.19	11.00			
TG level						
Normal	23	0.73	15.00	345.00	46.00	0.490
Increase	5	0.38	12.20	61.00		
Lymph node metastasis						
N0	1	1.37	25.00	25.00	3.00	0.194
N1	27	0.72	14.11	381.00		
N stage						
N0	1	1.37	25.00		2.34	0.310
N1a	6	0.57	16.50			
N1b	21	0.72	13.43			
M stage						
M0	26	0.74	15.04	391.00	12.00	0.212
M1	2	−0.02	7.50	15.00		

[a] For two variables, Mann–Whitney test was used, and for more than two variables, Kruskal–Wallis test was used

control. In subsequent studies, many genes identified in the study conducted by Huang et al. were confirmed to distinguish between PTC and normal or benign thyroid tissue, independent of the microarray platform or the analysis algorithms used [16, 19–22].

The Affymetrix Human Genome U133 Plus 2.0 microarray was used, which provides comprehensive coverage of the transcribed human genome on a single array covering over 47,000 transcripts represented by more than 1 million distinct oligonucleotide entities. Our study demonstrates that there are many differentially expressed genes between PTC and NG. A comparison of the PTC samples with the NG samples using the Affymetrix HG-U133 Plus 2.0 whole genome microarray produced a list of 9,485 genes that were differentially expressed. Although we used only two microarrays to screen for differentially expressed genes, we not only obtained several genes known to be related to PTC (such as *CITED1, SERPINA1, MET, TIMP1, TFF3, DPP4, FN1, LGALS3, TPO, EPS8, PROS1,* and *DIO1*), but we also identified some new PTC-related genes (such as

ZCCHC12 and *WIF1*). To validate the microarray results, we selected the most significantly differentially expressed gene between PTC and NG, the ZCCHC12 gene, for confirmation by FQ-PCR. This gene had not been implicated in thyroid carcinoma previously and was confirmed to be preferentially expressed in PTC by FQ-PCR. The results of the microarray and FQ-PCR were consistent and indicated that the ZCCHC12 gene may be a potential diagnostic molecular marker for PTC. The role of the ZCCHC12 gene in PTC requires further investigation. The preliminary analysis showed that the amplification copy number of the ZCCHC12 gene did not have a significant correlation with the clinicopathological and biochemical parameters of PTC.

Mouse ZCCHC12, or Sizn1 (Smad-interacting zinc finger protein 1), is a transcriptional coactivator in the bone morphogenic protein (BMP) signaling pathway, encoding a 402-amino acid protein with an N-terminal paraneoplastic antigen (MA)-homologous region, a single CCHC zinc finger motif, and a putative nuclear localization sequence [23]. Sizn1 positively modulates the BMP signal by interacting with Smad family members and the cAMP-responsive element-binding protein (CREB). Human ZCCHC12 shares 74% sequence identity with mouse ZCCHC12. Both mouse and human ZCCHC12 are located on chromosome X, contain a CCHC zinc finger motif at their C-terminus and have two putative nuclear localization signals (NLS). Four different sequence variants in human ZCCHC12 were characterized in 11 individuals with non-syndromic, X-linked mental retardation (NS-XLMR), implicating ZCCHC12 as a novel candidate gene for XLMR [24]. Cho et al. [25] reported that Sizn1 localized to promyelocytic leukemia protein nuclear bodies (PML-NBs) and showed that two SUMO interaction motifs (SIMs) in Sizn1 could bind to SUMO and govern SUMO conjugation to Sizn1 in the absence of the consensus motif for SUMO attachment. Li et al. [26] showed that human ZCCHC12 is a nuclear protein that is specifically expressed in human brain and mouse embryonic brain, functions as a positive modulator of activator protein 1 (AP-1) and the CREB signal and interacts with c-jun. Moreover, a novel nuclear localization signal that is necessary for the nuclear localization and punctate distribution was found in human ZCCHC12.

In conclusion, in the present study, we have demonstrated that a number of differentially expressed genes exist between PTC and NG. The number of transcript copies of the ZCCHC12 gene in PTC was significantly higher than in NG. The results verified by FQ-PCR were consistent with the microarray screening results. The ZCCHC12 gene may be a novel diagnostic molecular marker of PTC.

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