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ORIGINAL ARTICLE

Effects of rearranged during transfection mutation on calcitonin and procalcitonin expression in sporadic medullary thyroid carcinoma*

Yaqiong Ni, Wei Yao, Yunsheng Wang, Hui Wang, Qinjiang Liu (✉)

Department of Head and Neck Surgery, Gansu Provincial Cancer Hospital, Lanzhou 730050, China

Abstract

Objective The aim of this study was to investigate the effects of rearranged during transfection (*RET*) mutation on the expressions of calcitonin (CTn) and procalcitonin (PCT) in sporadic medullary thyroid carcinoma (SMTC).

Methods *RET* mutation was detected by polymerase chain reaction direct sequencing in 64 cases of SMTC, and the expression levels of CTn and PCT in SMTC tissues were detected using the immunohistochemical streptavidin-perosidase (SP) method. The effect of *RET* mutations on the expression of CTn and PCT along with its relationship with clinicopathological parameters were analyzed.

Results The expression rates of CTn and PCT in SMTC tissues were 90.6% (58/64) and 67.2% (43/64), respectively. CTn and PCT expression were found to be associated with tumor size and lymph node metastasis ($P < 0.05$) but not with gender, age, or tumor capsule invasion ($P > 0.05$). There was a significant correlation between CTn and PCT expression ($r = 0.269$, $P = 0.041$), and the intensity of positive CTn expression was positively correlated with *RET* mutation ($r = 0.507$, $P = 0.000$). However, PCT expression was not associated with *RET* mutation ($r = 0.188$, $P = 0.136$).

Conclusion High expression of CTn and PCT was associated with the progression of medullary carcinoma, and the intensity of CTn expression was associated with *RET* mutation. PCT may provide valuable information for the diagnosis and prognosis of SMTC.

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Medullary thyroid carcinoma (MTC) is derived from thyroid parafollicular cells and occurs in two forms, namely sporadic (SMTC) and hereditary (HMTC). MTC accounts for approximately 5%–10% of thyroid cancer cases [1], with a high degree of malignancy characterized by high invasiveness and high risks of metastasis, recurrence, and poor prognosis. Thus, early diagnosis is extremely important. The most common tumor marker used for MTC diagnosis and monitoring is calcitonin (CTn) [2–3]. Procalcitonin (PCT) is a precursor of CTn and has been used as a diagnostic marker for bacterial or fungal infections and sepsis [4–5], but recent studies have shown that serum PCT detection is also useful in MTC diagnosis and follow-up [6]. In this study, rearranged during transfection (*RET*) mutations and their effects on the expression of CTn and PCT in patients with SMTC were analyzed. The results were then comprehensively evaluated in reference to clinicopathological parameters to provide valuable information for SMTC diagnosis and treatment.

Materials and methods

Subjects

Data on 64 patients with pathologically confirmed SMTC who were treated at the Department of Head and Neck Surgery, Gansu Cancer Hospital, from October 2007 to January 2018 were collected. Of these, 30 were males and 34 were females, with a median age of 49.8 ± 12.6 years. Thirty-seven patients had lymph node metastasis, while 27 were without lymph node metastasis. All histopathological diagnoses were reconfirmed by two senior pathologists. No patient received treatment for SMTC before being treated at our hospital, and informed

✉ Correspondence to: Qinjiang Liu. Email: liuqj99@126.com
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Table 1 PCR amplification primers for *RET* mutation

Gene	Sequence (5'→3')	Annealing Temperature
RET ex8	Forward TGCTGCCCTGGGTCTGTC	59℃
	Reverse ACCTTCCCAAGTCCAGAGTGAATC	
RET ex10	Forward CATGGCTTCAGAAAGGCACTG	59℃
	Reverse CCTTGTTGGGACCTCAGATGTG	
RET ex11	Forward CATGCTCGATGGGGTGTTC	60℃
	Reverse GACCCTCACCAGGATCTTGAA	
RET ex13	Forward CTGCTCTGTGCTGCATTTCA	58℃
	Reverse GCCCCTCTGATGGAAAGTGAC	
RET ex14	Forward GGAGGCAGAGAGCAAGTGGTT	58℃
	Reverse CCATATGCACGCACCTTCATCT	
RET ex15	Forward CGACTCGTGCTATTTTTCCTC	60℃
	Reverse AGGCTGAGCGGAGTTCTAAT	
RET ex16	Forward CTCCAGCCCCTTCAAAGATGT	60℃
	Reverse CCATTTGCCTCACGAACACA	

consent was obtained from all patients and their families.

Reagents and methods

DNA extraction and purification E.Z.N.A.™ FFPE DNA Kit was purchased from Omega Bio-tek (USA), and Green Tag Mix was purchased from Nanjing Vazyme Biotech Co., Ltd. (China). Mouse anti-human PCT monoclonal antibody was purchased from Novus (USA), and CTn antibody, SP immunohistochemical kit, DAB chromogenic kit, phosphate-buffered solution (PBS), and citric acid repair solution were purchased from Beijing Zhongshan Jinqiao Biotechnology Company (China). The primers corresponding to the target fragment of the *RET* gene to be amplified were designed using Primer 5.0 and Oligo6 software (Table 1) and synthesized by Suzhou Jinweizhi Biotechnology Company (China).

All specimens were fixed in 4% neutral formaldehyde, dehydrated, embedded in paraffin, and cut into 4 μm-thick sections. Immunohistochemical staining was performed after reexamination of routine hematoxylin and eosin (HE)-stained sections to verify and determine the diagnosis. Known sections were used as positive controls, and PBS, instead of primary antibody, was used as negative controls. All steps were performed in strict accordance with the instructions provided by the manusfacturer of the SP immunohistochemical kit. After immunohistochemical staining, additional – three to eight sections (4–10 μm thick) were processed with conventional xylene and absolute ethanol, after which DNA was extracted and purified using an E.Z.N.A.™ FFPE DNA Kit according to the manufacturer's instructions. The concentration and purity of the extracted DNA were determined using an ultramicro spectrophotometer. Amplified gene fragments were then determined according to the complete *RET* gene sequence published in the GenBank database of the National Center for Biotechnology Information. Qualified genomic DNA was amplified using polymerase chain reaction, and the products were then sequenced. The sequencing results were compared with the normal *RET* gene sequence to determine whether mutation had occurred. All samples were divided into the mutant and wild-type *RET* groups.

Determination of results

CTn- and PCT-positive products were localized in the cytoplasm or nucleus and were brown and brownish-yellow in color. The results of pathological staining were determined by two professional pathologists with reference to the literature (7). The following scores were given according to the degree of positive staining of the cells: 0, no staining; 1, yellow (+); 2, brownish-yellow (++); and 3, brown (+++). Meanwhile, the following scores were given according to the number of positive cells: 0, positive cell count below 5%; 1, positive cell count below 25% (+); 2, positive cell count ≥ 25% but < 50% (++); and3, positive cell count ≥ 50% (+). Five fields were randomly selected, and 100 cells were counted by light microscopy at 200× magnification, and the average was calculated. The staining degree score and the percentage of stained cells in each slide were multiplied, and a final score of 0 represents a negative while ≥ 1 is a positive.

Statistical analysis

Data analysis was conducted using SPSS 22.0 (SPSS Inc., USA). The correlation of CTn and PCT expressions with clinicopathological parameters was determined using Pearson chi-square (χ^2) test, while that with their *RET* mutations were analyzed by non-parametric Spearman correlation analysis. $|r| \in [0.1, 1]$ was considered correlated, and $P < 0.05$ was considered statistically significant.

Results

CTn and PCT expression and correlation

The positive rates of CTn and PCT in SMTC were 90.6% (58/64) and 67.2% (43/64), respectively. The expressions of CTn and PCT in SMTC tissues were positively correlated ($r = 0.269$, $P = 0.041$) and significantly different ($P < 0.05$; Table 2 and Fig. 1).

Relationship between CTn or PCT expression and clinicopathologic parameters

CTn e xpression was not associated with gender, age, or capsular invasion ($P > 0.05$) but was significantly associated with tumor size ($P = 0.001$) and lymph node metastasis ($P = 0.032$). Meanwhile, PCT expression was not associated with gender, age, tumor size, or capsular invasion but was associated with lymph node metastasis ($P = 0.026$; Table 3)

Table 2 Relationship between CTn and PCT expression in SMTC tissues [*n* (%)]

PCT	CTn			*r*	*P*
	+	++	+++		
<1 (–)	11 (64.7)	5 (29.4)	1 (5.9)	0.269	0.041
≥1 (+)	16 (39.0)	14 (34.1)	11 (26.8)		

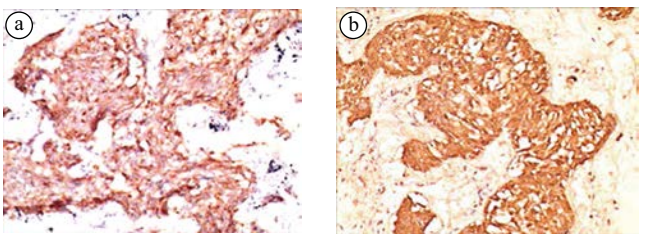

Fig. 1 Immunohistochemical staining of CTn and PCT in SMTC tissues (SP × 200). (a) CTn was positively expressed in SMTC tissues; (b) PCT was positively expressed in SMTC tissues

Relationship between CTn or PCT expression and RET gene mutation

The *RET* mutation rate in SMTC tissues was 37.5% (24/64), with 24 cases in the mutation group and 40 cases in the wild-type group.

Among the *RET* mutants, the proportion of moderate and strong positive CTn expression were 54.2% (13/24) and 33.3% (8/24), respectively, which were higher than the 15.0% (6/40) and 10.0% (4/40) observed in the wild-type. The intensity of positive CTn expression was significantly and positively correlated with *RET* mutation ($r = 0.507$, $P = 0.000$).

The positive rate of PCT in mutant *RET* was 75.0% (18/24), which was higher than the 62.5% (25/40) observed in the wild-type, although the difference was not statistically significant ($P > 0.05$). In addition, no correlation was found between PCT expression and *RET* mutation ($r = 0.188$, $P = 0.136$; Fig. 2 and Table 4).

Discussion

With the development of tumor molecular biology research in recent years, significant achievements have been made in the study of the molecular biology of MTC, particularly for serial studies related to the *RET* gene. *RET* gene mutation detection is of great value in MTC diagnosis, treatment, and prognostic evaluation [8–9].

Table 3 Relationship between CTn or PCT expression and clinicopathological parameters [*n* (%)]

Variables	CTn positive	χ^2	*P*	PCT positive	χ^2	*P*
Gender		0.488	0.485		0.007	0.934
male	28 (93.3)			20 (66.7)		
female	30 (88.2)			23 (67.6)		
Age (years)		0.717	0.397		2.430	0.119
< 55	39 (92.9)			31 (73.8)		
≥ 55	19 (86.4)			12 (54.5)		
Tumor size (cm)		13.094	0.001		0.345	0.842
< 2	6 (60.0)			6 (60.0)		
2–4	23 (95.8)			16 (66.7)		
≥ 4	29 (96.7)			21 (70.0)		
Lymph node metastasis		4.596	0.032		4.982	0.026
N0	22 (81.5)			14 (51.9)		
N1	36 (97.3)			29 (78.4)		
Capsular invasion		0.003	0.955		0.015	0.902
Yes	38 (90.5)			28 (66.7)		
No	20 (90.9)			15 (68.2)		

MTC has high malignancy, poor prognosis, and is prone to metastasis and recurrence; thus, its early diagnosis is crucial. This highlights the importance of valuable molecular markers that may help advance early diagnosis and prognostic evaluation of MTC.

CTn, a 32-amino acid short peptide hormone synthesized and secreted by thyroid C cells, is the product of the CALC1 gene and the main tumor marker of MTC. It is currently widely used for MTC diagnosis and follow-up [10–11]. While most of the CTn tests in clinical work use serological test values to assess disease progression, postoperative follow-up, and prognosis, they can also be used to guide the choice of surgical approach for MTC [12]. The results of this study showed that the positive rate of CTn in SMTC tissues was 90.6% and that its expression was related to tumor size and lymph node metastasis, similar to the serum detection results of CTn values [13–14]. The expression of CTn in tumor tissues also showed an increasing trend with the progression of the disease, and the probability of cervical lymph node metastasis increased upon CTn expression. Although the level of CTn expression in MTC tissues is similar to that of serum CTn, the latter is affected by hypercalcemia, hypergastrinemia, and other chronic autoimmune diseases [15]. The expression status of CTn in tumor tissues

Table 4 Relationship between CTn or PCT expression and *RET* mutation [*n* (%)]

RET	CTn expression				χ^2	*P*	*r*	*P*	PCT expression				χ^2	*P*	*r*	*P*
	+++	++	+	-					+++	++	+	–				
mutant	8 (33.3)	13 (54.2)	1 (4.2)	2 (8.3)	25.309	0.000	0.507	0.000	14 (58.3)	4 (16.7)	0	6 (25.0)	2.627	0.269	0.188	0.136
wild-type	4 (10.0)	6 (15.0)	26 (65.0)	4 (10.0)					15 (37.5)	10 (25.0)	0	15 (37.5)				

can directly reflect the synthesis of CTn by tumors, which can in turn reflect tumor growth. This study showed that the intensity of positive CTn expression intensity was positively correlated with *RET* mutation and that the proportions of moderate and strong positive CTn in the mutant *RET* group were significantly higher than those in the wild-type *RET* group ($P = 0.000$). Mutation of *RET* led to abnormal structure and function of some proteins or enzymes in the body, resulting in uncontrolled cell growth and tumorigenesis while increasing CTn mRNA, followed by increased CTn synthesis and secretion. A stronger CTn expression indicated a higher degree of malignancy and poor prognosis of the tumor, while the clinical manifestations of the patients were characterized by rapid disease progression, poor therapeutic efficacy, and poor prognosis.

PCT is a polypeptide containing 116 amino acids composed of CTn and a product of the CALC1 gene. In thyroid C cells, transcription and synthesis generate pre-mRNA, which is then translated by polypeptidase to form PCT [16]. PCT, as a specific indicator for the differential diagnosis between infectious and noninfectious diseases, has also been reported to be expressed at increased levels in MTC and is considered a valuable tumor marker for MTC diagnosis and follow-up [17–18]. Therefore, serum PCT testing has been recommended as a routine test for MTC; however, there are many influencing factors of PCT in the serum, and its clinical value is limited [19]. This study showed that the positive expression level of PCT in SMTC tissues was associated with lymph node metastasis, similar to that reported in serum PCT studies [6, 18], reinforcing the value of PCT in the diagnosis and prognostic evaluation of MTC. Previous studies have also suggested a correlation between PCT and CTn levels in the serum of patients with MTC. PCT is relatively stable compared with serum CTn under *in vitro* conditions and in some cases of patients with MTC and negative serum CTn or low CTn levels. A low PCT to CTn ratio may indicate that it is related to C cell proliferation, while a high ratio is related to MTC metastasis [20–21]. In this study, we found a significant correlation between PCT and CTn expression in SMTC tissues ($P = 0.041$), whereas no correlation was observed between PCT expression and *RET* mutation ($r = 0.188$, $P = 0.136$). This suggests that PCT expression is relatively stable in SMTC tissues and may be a valuable and reliable indicator for MTC diagnosis.

To conclude, CTn and PCT were highly expressed in SMTC tissues and were correlated with tumor size and lymph node metastasis. Their expression was significantly correlated, suggesting that they are associated with tumor occurrence and development. We believe that PCT expression in MTC tissues may be used as a diagnostic indicator and as a reference indicator to evaluate the degree of malignancy and prognosis of MTC. However, the relationship between PCT expression in MTC tissues and serum PCT levels and the significance of changes in serum PCT require further exploration.

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Conflicts of interest
The authors indicated no potential conflicts of interest.

Author contributions
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