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ORIGINAL ARTICLE

BRAF V600E/TERT promoter mutations and NIS/TSHR expression in differentiated thyroid carcinoma and their clinical significance*

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Abstract

Objective Telomerase reverse transcriptase (TERT) promoter mutations have recently been described in thyroid carcinoma. The purpose of this study was to investigate the clinical significance of (v-raf murine sarcoma viral oncogene homolog B1) BRAF V600E and TERT promoter mutations in differentiated thyroid carcinoma (DTC). The relationship between the two mutations and NIS/TSHR expression was also analyzed.

Methods We have detected BRAF V600E and TERT promoter mutations by direct sequencing and NIS/TSHR expression by immunohistochemistry in 229 cases of DTC, 52 cases of benign nodular goiter, and 31 cases of normal thyroid tissue.

Results The BRAF V600E mutation was detected in 142 (62.0%) of 229 cases of DTC [141 cases of papillary thyroid carcinoma (PTC) and 1 case of follicular thyroid carcinoma (FTC)]. TERT promoter mutations were detected in 18 (7.9%) of 229 cases of DTC (14 cases of PTC and 4 cases of FTC), including the mutations C228T (0.9%) and C250T (7.0%), which were mutually exclusive. Moreover, 11 (61.1%) cases also harbored the BRAF V600E mutation, which was not associated with gender, age, tumor size, lymph node metastasis, and recurrence risk stratification (P >0.05). The rate of TERT promoter mutation was higher in males, age ≥45, and in the middle/high-risk group (P <0.05), and the rate of simultaneous BRAF V600E and TERT promoter mutations were higher in the middle/high-risk group (P <0.05). In addition, NIS positive rate in the concurrent BRAF V600E and TERT promoter mutation group (45.5 %) was lower than in other groups (that is, the DTC group with BRAF V600E or TERT promoter mutations (55.1%), the DTC group with no BRAF V600E or TERT promoter mutation (57.5%), the nodules and normal group (75.9%); $|r| = 0.171$, $P = 0.002$).

Conclusion TERT promoter mutations were lower in patients with DTC, with the C250T mutation being the most common. The detection of BRAF V600E mutation combined with TERT promoter mutations was instructive for the prognosis assessment and treatment of DTC.

Key words: differentiated thyroid carcinoma (DTC); BRAF V600E; TERT promoter mutations; sodium iodide symporter; thyroid stimulating hormone receptor

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Thyroid cancer is a common endocrine malignancy, and differentiated thyroid carcinoma (DTC), which includes papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC), is the most common. In recent years, the global incidence of thyroid cancer has gradually increased. In China, thyroid cancer has the fastest growing incidence. Surgically, radioactive ^{131}I and thyroid-stimulating hormone (TSH) suppression therapies have been used for the treatment of DTC, with a 10-year survival rate over 95% [1]; however, the recurrence rate of DTC is about 30% [2]. Therefore, it is necessary to control the recurrence of DTC, but there is no precise biological

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indicator to monitor the recurrence of DTC.

The BRAF V600E mutation is the most important genetic alteration in DTC and plays an important role in the tumor oncogenic mechanism. With the improvement of molecular biology techniques, the BRAF V600E mutation detection rate gradually increases in DTC. However, the association between the BRAF V600E mutation and the prognosis of DTC is a controversial subject [3]. Telomerase reverse transcriptase (TERT) is the catalytic subunit of the telomerase and also its active part, and the TERT promoter is the regulatory region of TERT. TERT promoter mutations have been found in a variety of tumors [4], including thyroid carcinomas, and they have become a hot topic in the prognostic assessment of thyroid cancer. Recently, some studies suggested that the BRAF V600E mutation teams up with TERT promoter mutations to enhance the aggressiveness of DTC [5].

Radioactive ^{131}I therapy has become an important treatment for DTC, but some patients with DTC have shown iodine resistance and poor response to radioactive ^{131}I therapy. Some studies revealed that this might be related to the sodium iodide symporter (NIS) and TSH receptor (TSHR), which participate in iodine metabolism. Most studies have found that NIS expression in thyroid cancers is reduced or absent [6]. Moreover, irregular expression of NIS in thyroid cancer may be associated with the BRAF V600E mutation [7]. Previous studies demonstrated that TSHR is involved in the NIS expression level and transport to the cell membrane [8].

In China, the DTC incidence has gradually increased, especially in the western region with a poor environment. Therefore, we have detected BRAF V600E and TERT promoter mutations, as well as NIS and TSHR expression, in the hope of clarifying the prognosis assessment of DTC through multiple molecular markers and providing a theoretical basis for the molecular diagnosis and treatment of DTC.

Materials and methods

Thyroid surgical samples

We studied 229 surgically removed thyroid tumors from patients with DTC (163 females and 66 males) of 46 ± 12.8 years of age (mean ± SD), including 216 patients with PTC and 13 patients with FTC, as well as 52 patients with benign nodular goiter and 31 normal thyroid tissue. All thyroid tumors and nodular goiters were histologically verified. Based on the recurrence risk stratification standards described in 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer [9], 99 cases were assigned to the low-risk group and 130 cases to the middle/high-risk group. All surgical samples were collected from October 2014 to January 2016 at the Department of Head and Neck Surgery in Gansu Province Tumor Hospital. The Ethics Committee of Gansu Province Tumor Hospital approved this study, and all surgical sampling has received consent from the patients and their families.

DNA extraction

Neutral formalin-fixed, paraffin-embedded specimens were cut into 5–10 μm thick slices, and submitted for routine HE staining and identification of tumor cell content by two pathologists. DNA was extracted with the FFPE DNA Kit (Omega, USA), according to the step-by-step manufacturer's instructions, and the concentration and purity of the product was measured with ultrafine spectrophotometer ND-2000, the OD260/280 ratio in the range of 1.8 to 2.0. The extracted DNA was stored in a refrigerator (–20°C).

BRAF V600E and TERT promoter mutation analysis

PCR assays were carried out using PrimeSTAR® HS DNA Polymerase (TaKaRa, Japan). The PCR reaction system was as follows: 10 μL of 5× PrimeSTAR Buffer, 4 μL of deoxy-ribonucleoside triphosphate (dNTP) (2.5 mM), 1 μL of forward/reverse primer, < 200 ng of DNA template, 0.5 μL of polymerase, and water to 50 μL. The PCR conditions were set according to the TM value of synthetic primers, and a preliminary experiment determined the number of cycles and the annealing temperature. BRAF V600E was amplified by PCR using the following primers: 5′-ACATTTCAAGC CCCAAAAATCTT-3′, 5′-CATCTCAGGGCCAAAAATTTAATC-3′. The PCR conditions included an initial denaturation step at 95°C for 5 min, followed by 40 denaturation cycles at 95°C for 30 s, annealing at 59°C for 30 s, elongation at 72°C for 30 s, a final elongation step at 72°C for 10 min, and cooling down to 4°C . The TERT promoter was amplified by PCR using the following primers: 5′-ACCCGTCCTGCCCCTTCA-3′, 5′-GGCAGCACCTCGCGGTAGT-3′. The PCR conditions included an initial denaturation step at 95°C for 5 min, followed by 40 denaturation cycles at 95°C for 30 s, annealing at 53°C for 30 s, elongation at 72°C for 30 s, a final elongation step at 72°C for 10 min, and cooling down to 4°C. Agarose gel electrophoresis was used to detect the amplification quality of PCR products (ChemiDoc™ XRS+), and the high-quality PCR product was subjected to DNA sequencing (Jinzhiwei Biotechnology, China).

Immunohistochemistry

Neutral formalin-fixed, paraffin-embedded specimens were cut into 4-μm thick slices, and sections were baked at 60°C overnight. Sections were deparaffinized, and antigen retrieval (high pressure) was performed by using Antigen Unmasking Solution (citrate buffer, Zhongshan

Goldbridge Biotechnology, China). After 3 washes in Phosphate buffer saline (PBS), the sections were blocked using an animal serum for 15 min, followed by sequential incubations in 1:200 rabbit anti-human NIS/TSHR antibody (Zhongshan Goldbridge Biotechnology, China) at 37°C for 2 h and further washes in PBS. Sections were then incubated with a biotinylated secondary antibody for 15 min and washed with PBS. The sections were incubated with a horseradish peroxidase complex for 15 min and washed with PBS. Finally, the peroxidase substrate diaminobenzidine (DAB) was added to stain the sections, followed by washing with tap water. A semi-quantitative analysis was used to evaluate the immunohistochemistry results [10].

Statistical analysis

IBM SPSS Statistics 22.0 was used to analyze the data. Continuous data were summarized as mean ± SD or range. Categorical data were expressed as numbers and percentages or ratios. Pearson chi-square tests or Fisher exact test were used for the significance analysis. P value <0.05 was considered to be significant, and Spearman rank correlation analysis was used to determine the positive rate of NIS/TSHR between groups, $|r| \in [0.1,1]$, $P<0.05$.

Results

BRAF V600E and TERT promoter mutations in DTC

The BRAF V600E mutation was detected in 142 (62.0%) of 229 cases with DTC, including 141 cases of PTC and 1 case of FTC. TERT promoter mutations were detected in 18 (7.9%) of 229 cases of DTC (14 cases of PTC and 4 cases of FTC), with the mutation C250T in 16 cases (7.0%) vs. C228T in 2 cases (0.9%), and both mutations were mutually exclusive. Moreover, 11 (61.1%) cases also harbored the BRAF V600E mutation out of the 18 cases with TERT promoter mutation (Fig. 1). None of the 52 benign nodular goiters and 31 normal thyroid tissue harbored BRAF V600E and TERT promoter mutations.

Relationship of BRAF V600E and TERT promoter mutations with DTC clinicopathologic features

The BRAF V600E mutation was not associated with gender (P = 0.781), age (P = 0.786), tumor size (P = 0.461), lymph node metastasis (P = 0.408), and recurrence risk stratification (P = 0.123). TERT promoter mutations were associated with gender (females 15.2% vs. males 4.9%, P = 0.009), age (older age 11.6% vs. younger age 3.0%, P = 0.016), and recurrence risk stratification (middle/high-risk group 11.5% vs. low-risk group 3.0%, P = 0.018). Concurrent BRAF V600E and TERT promoter mutations were associated with lymph node metastasis (N1 8.3% vs. N0 1.7%, P = 0.018) and recurrence risk stratification (middle/high-risk group 7.7% vs. low-risk group 1.0%, P = 0.019; Table 1).

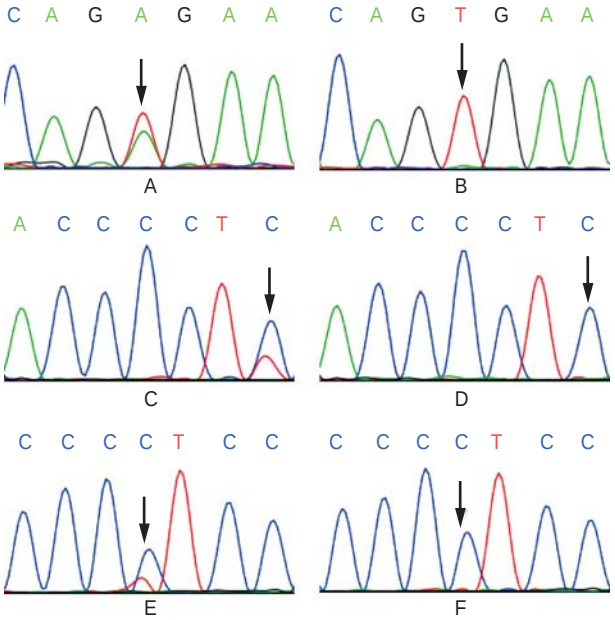

Fig. 1 BRAF V600E and TERT promoter mutations Sequencing. (a) BRAF V600E mutation; (b) BRAF V600E Wild-Type; (c) TERT promoter C228T mutation; (d)TERT promoter C228T Wild-Type; (e) TERT promoter C250T mutation; (f) TERT promoter C250T Wild-Type

Relationship of BRAF V600E and TERT C228T mutations with NIS/TSHR expression

NIS/TSHR immunohistochemical staining (in brown) was located in the cytoplasm and/or the cell membrane (Fig. 2). NIS positive rates of the DTC group with concurrent BRAF V600E and TERT promoter mutations, the DTC group with BRAF V600E or TERT promoter mutations, the DTC group with no BRAF V600E or TERT promoter mutation, the benign nodule goiter group, and the normal thyroid tissue group were 45.5%, 55.1%, 57.5%, and 75.9%, respectively. Moreover, in the concurrent BRAF V600E and TERT promoter mutation group, NIS positive rate was lower than in the other three groups ($|r|$ = 0.171, P = 0.002; Table 2). However, there was no correlation between TSHR expression and the two mutations.

Discussion

More than one gene was involved in the occurrence and development of DTC. The BRAF V600E mutation is one of the most common mutations in DTC (29%–83%), and it did not occur in benign nodules [11]. The BRAF V600E mutation was also the first genetic mutation detected in

Table 1 Relationship of BRAF V600E and TERT promoter Mutations With Clinicopathologic features of DTC

Features	BRAF V600E (*n*, %)		*P*	TERT promoter (*n*, %)		*P*	BRAF + TERT (*n*, %)		*P*
	Mutation *n* = 142	Wide-Type *n* = 87		Mutation *n* = 18	Wide-Type *n* = 211		Mutation *n* = 11	Wide-Type *n* = 218	
Gender			0.781			0.009*			0.054
Male	40 (60.6)	26 (39.4)		10 (15.2)	56 (84.8)		6 (9.1)	60 (90.9)	
Female	102 (62.6)	61 (37.4)		8 (4.9)	155 (95.1)		5 (3.1)	158 (96.9)	
Age (years)			0.786			0.016*			0.261
<45	63 (63.0)	37 (37.0)		3 (3.0)	97 (97.0)		3 (3.0)	97 (97.0)	
≥45	79 (61.2)	50 (38.8)		15 (11.6)	114 (88.4)		8 (6.2)	121 (93.8)	
Size (cm)			0.461			0.546			0.790
< 2	16 (76.2)	5 (23.8)		2 (9.5)	19 (90.5)		1 (4.8)	20 (95.2)	
2–4	62 (62.0)	38 (38.0)		6 (6.0)	94 (94.0)		4 (4.0)	96 (96.0)	
≥ 4	64 (65.3)	34 (34.7)		10 (10.2)	88 (89.8)		6 (6.1)	92 (93.9)	
LN metastasis			0.408			0.217			0.018*
N0	72 (59.5)	49 (40.5)		7 (5.8)	114 (94.2)		2 (1.7)	119 (98.3)	
N1	70 (64.8)	38 (35.2)		11 (10.2)	97 (89.8)		9 (8.3)	99 (91.7)	
Recurrence risk stratification			0.123			0.018*			0.019*
Low	67 (67.7)	32 (32.3)		3 (3.0)	96 (97.0)		1 (1.0)	98 (99.0)	
Middle/high	75 (57.7)	55 (42.3)		15 (11.5)	115 (88.5)		10 (7.7)	120 (92.3)	

Note: * $P < 0.05$

Table 2 NIS、TSHR expression in groups

Groups	NIS (*n*, %)		*r*	*P*	TSHR (*n*, %)		*r*	*P*
	Positive	Negative			Positive	Negative		
N	63 (75.9)	20 (24.1)			38 (45.8)	45 (54.2)		
Mutation 0	46 (57.5)	34 (42.5)			22 (27.5)	58 (72.5)		
Mutation 1	76 (55.1)	62 (44.9)			68 (49.3)	70 (50.7)		
Mutation 2	5 (45.5)	6 (54.5)	–0.171*	0.002*	3 (27.3)	8 (72.3)	0.042	0.462

Note: $|r| \in [0.1,1]$, * $P < 0.05$; N: Benign nodular goiter and Normal thyroid tissue; Mutation 0: None of BRAF V600E and TERT promoter mutation; Mutation 1: BRAF V600E or TERT promoter mutations; Mutation 2: Both BRAF V600E and TERT promoter mutations

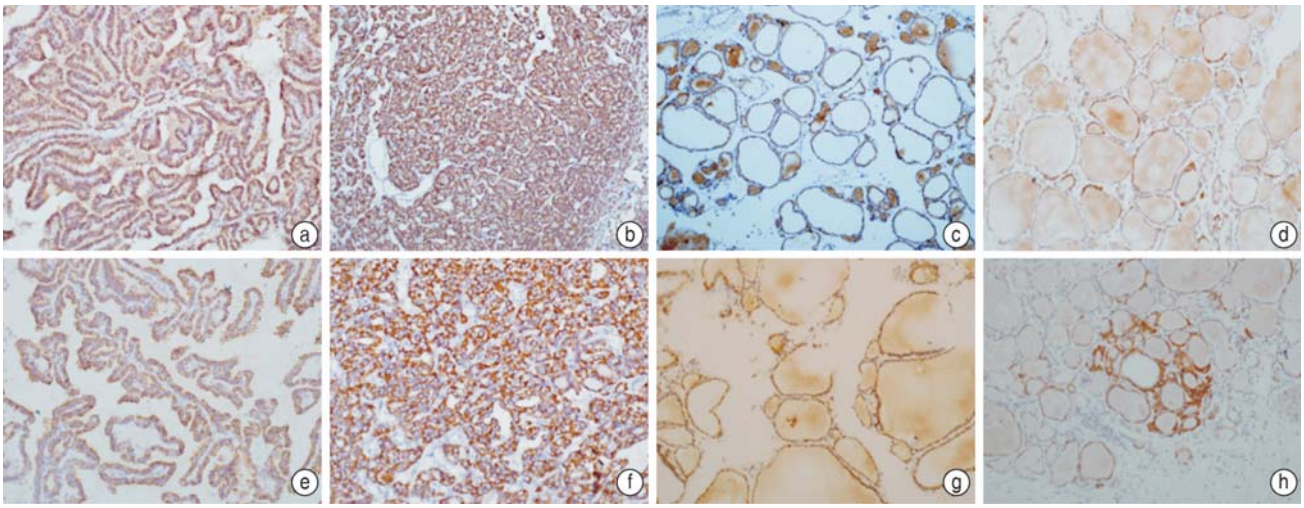

Fig. 2 NIS, TSHR Immunohistochemistry (SP × 200). (a) PTC showing NIS immunostaining; (b) FTC showing NIS immunostaining; (c) Benign nodular goiter showing NIS immunostaining; (d) Normal thyroid tissue showing NIS immunostaining; (e) PTC showing TSHR immunostaining; (f) FTC showing TSHR immunostaining; (g) Benign nodular goiter showing TSHR immunostaining; (h) Normal thyroid tissue showing TSHR immunostaining

DTC, and most of the researchers working on the prognosis of patients with DTC. Some studies suggested that BRAF V600E mutations were associated with extrathyroidal invasion, lymph node metastasis, and other characteristics related to invasiveness [12–13]. However, recent studies suggested that BRAF V600E mutations were associated with recurrence [14]. Currently, there is still a controversy on BRAF V600E mutation and the prognosis of DTC. TERT promoter mutations have recently been reported in human cancer, are considered a new genetic mechanism, and are involved in the occurrence and development of DTC. Other studies have reported that TERT promoter mutations occurred for 10–13% of the mutations in DTC, and are associated with tumor size, vascular invasion, high TNM stage (and), recurrence, and distant metastasis [15–17]. Specifically, the coexistence of TERT promoter and BRAF V600E mutations presented a more aggressive cancer and higher recurrence rate [18]. However, Melo *at al* [19] found that the concurrence or coexistence of TERT and BRAF V600E mutations was not associated with increased aggressiveness or worse outcome in comparison with the presence of TERT mutations alone. These conflicting results might also reflect the role of the tumor microenvironment.

Our data show that BRAF V600E mutation rate was 62.0% in DTC (142/229), including 65.3% (141/216) in PTC and 7.7% (1/13) in FTC. The mutation rate of TERT promoter was 7.9% (18/229): C250T was 7.0% (16/229), and C228T was 0.9% (2/229), with both mutations mutually exclusive. Surprisingly, the C250T mutation was more common than C228T, contrary to a previous report [15]. The low frequency of TERT promoter mutations in our study might be one of the reasons behind C250T mutation prevalence. The second possible reason is the geographic/ethnic difference.

Our study found that the BRAF V600E mutation was not associated with gender, age, tumor size, lymph node metastasis, and recurrence risk stratification. Kim *et al* [20] studies have also failed to establish a relationship between BRAF V600E mutation and the prognostic of patients with DTC. However, TERT promoter mutations were associated with gender (males), age (≥45), recurrence risk stratification (middle/high-risk group), and concurrent BRAF V600E and TERT promoter mutations were associated with lymph node metastasis (N1) and recurrence risk stratification (middle/high-risk group). In addition, we found that the positive rate of NIS in the concurrent BRAF V600E and TERT promoter mutation group was lower. This might be due to the BRAF gene involvement in mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK) signaling pathways. The BRAF V600E gene mutation can lead to protein kinase activation and activation of ERK, and the mitotic signal is transmitted downstream of MAPK signaling pathway, resulting in the formation of tumors and further malignant transformation; TERT promoter mutations may aggravate this process by prolonging the life of cancer cells. Therefore, we speculate that DTC is more aggressive in patients with concurrent BRAF V600E and TERT promoter mutations, with a reduced degree of differentiation, increased degree of malignancy, reduced expression of some proteins (NIS), and loss of function ("nitrogen pump"). Unfortunately, we failed to find the relationship between TSHR expression and the two mutations. Perhaps, TSHR expression involved in carcinogenic mechanism differs from NIS expression in DTC.

BRAF V600E and TERT promoter mutations occur only in cancer tissue; they do not occur in benign nodular goiter and normal thyroid tissue. Detection of BRAF V600E and TERT promoter mutations will help to improve the pathological diagnosis of thyroid nodules that cannot be determined as benign or malignant by fine-needle aspiration biopsy. For DTC with a higher recurrence, it is necessary to access the prognosis (recurrence) of patients with DTC. Therefore, detecting BRAF V600E and TERT promoter mutations rather than the BRAF V600E mutation alone would help in prognosis prediction and development of individualized treatment programs. TERT promoter mutations or concurrent BRAF V600E and TERT promoter mutations were more common in the middle/high-risk group. For patients with TERT promoter mutations or concurrent BRAF V600E and TERT promoter mutations, which present an increased risk of recurrence, it will help to determine the risk of recurrence at an early stage through long-term follow-up and monitoring of patients that have been cured. In patients with concurrent BRAF V600E and TERT promoter mutations, NIS expression is also lower, when carrying out ^{131}I radiotherapy; therefore, we may need to take other therapeutic measures. Liu *et al* [21] found that radiofrequency-mediated ^{131}I radiotherapy shows obvious effects on DTC. Another study found that BRAF V600E mutations had an impact on NIS expression, and thyroid cancer cells increased iodine intake by silencing the BRAF gene and adding TSH [7]. However, our study has also failed to establish the specific mechanisms underlying NIS abnormal expression in DTC with concurrent BRAF V600E and TERT promoter mutations. There are maybe other unknown factors that affect NIS expression, and how to restore iodine uptake in DTC will become a major research focus in the future.

In summary, our results show that TERT promoter mutations were lower in patients with DTC, and the TERT C250T mutation was more common; the BRAF V600E mutation was not associated with gender, age, tumor size, lymph node metastasis, or recurrence risk stratification. TERT promoter mutations were associated with gender,

age, and recurrence risk stratification, and concurrent BRAF V600E and TERT promoter mutations were associated with lymph node metastasis, recurrence risk stratification, and a lower positive rate of NIS. Therefore, the detection of BRAF V600E mutation combined with TERT promoter mutation was instructive to prognosis assessment and treatment of DTC.

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Conflicts of interest

No conflict of interest exits in the submission of this manuscript, which has been approved by all authors for publication.

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