TERT Promoter Mutations and Their Association with *BRAF* V600E Mutation and Aggressive Clinicopathological Characteristics of Thyroid Cancer

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Context: Promoter mutations chr5:1,295,228C>T and chr5:1,295,250C>T (termed C228T and C250T, respectively) in the gene for telomerase reverse transcriptase (TERT) have been reported in various cancers and need to be further investigated in thyroid cancer.

Objective: The aim of the study was to explore *TERT* promoter mutations in various thyroid tumors and examine their relationship with *BRAF* V600E mutation, iodine intake, and clinicopathological behaviors of thyroid cancer.

Design: *TERT* promoter and *BRAF* mutations were identified by sequencing genomic DNA of primary thyroid tumors from normal- and high-iodine regions in China, and clinicopathological correlation was analyzed.

Results: The C228T mutation was found in 9.6% (39 of 408) of papillary thyroid cancer (PTC), C250T was found in 1.7% (7 of 408) of PTC, and they were collectively found in 11.3% (46 of 408) of PTC. C228T was found in 31.8% (7 of 22) and C250T in 4.6% (1 of 22) of follicular thyroid cancer (FTC), and they were collectively found in 36.4% (8 of 22) of FTC. No *TERT* mutation was found in 44 benign thyroid tumors. The two mutations occurred in 3.8% (6 of 158) of *BRAF* mutation-negative PTC vs 16.0% (40 of 250) of *BRAF* mutation-positive PTC ($P = 5.87 \times 10^{-4}$), demonstrating their association. Unlike *BRAF* mutation, *TERT* promoter mutations were not associated with high iodine intake, but they were associated with older patient age, larger tumor size, extrathyroidal invasion, and advanced stages III/IV of PTC. Coexisting *TERT* and *BRAF* mutations were even more commonly and more significantly associated with clinicopathological aggressiveness.

Conclusions: In this large cohort, we found *TERT* promoter mutations to be common, particularly in FTC and *BRAF* mutation-positive PTC, and associated with aggressive clinicopathological characteristics. **(*J Clin Endocrinol Metab* 99: E1130–E1136, 2014)**

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Abbreviations: ATC, anaplastic thyroid cancer; FTC, follicular thyroid cancer; PTC, papillary thyroid cancer; TERT, telomerase reverse transcriptase.

Telomerase reverse transcriptase (TERT) is the catalytic subunit of telomerase, a ribonucleoprotein complex that plays a key role in cellular immortality by maintaining telomere length at the end of chromosomes (1, 2). TERT has long been known to be overexpressed in many human cancers, suggesting an important role of this protein in human tumorigenesis (3). This role is directly supported by the demonstration that in transgenic mouse models, induced expression of TERT led to increased development of tumors (4, 5). Two interesting somatic mutations, chr5:1,295,228C>T and chr5:1,295,250C>T (termed here as C228T and C250T, respectively), in the promoter of the *TERT* gene have been identified in melanoma, which represent nucleotide changes of −124 C>T and −146 C>T from the ATG translation start site of the *TERT* gene, respectively (6, 7). These mutations confer *TERT* increased transcriptional activities by creating binding sites for ETS transcription factors in the *TERT* promoter, providing a mechanism for the overexpression of TERT observed in human cancers. The two *TERT* promoter mutations, particularly the C228T mutation, have also been demonstrated in other cancers, including bladder cancer and glioblastoma, as well as many other human cancers (8, 9), suggesting a wide role of *TERT* promoter mutations in human tumorigenesis.

Follicular cell-derived thyroid cancer is the most common endocrine malignancy, with a rapidly rising incidence in recent years (10, 11). This cancer can be classified into several histological types, including papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), and anaplastic thyroid cancer (ATC) (12). PTC and FTC are differentiated thyroid cancers, and ATC is a deadly undifferentiated thyroid cancer. PTC is the most common type of thyroid cancer, accounting for 85–90% of all thyroid malignancies. Thyroid cancer, like other human cancers, is a genetically driven malignancy. The most common genetic alteration in PTC is the *BRAF* V600E mutation, which, through constitutively activating the MAPK pathway, plays an important role in the tumorigenesis of PTC (13, 14). Recently, we for the first time reported common occurrence of the C228T and C250T *TERT* promoter mutations, particularly the C228T mutation, in thyroid cancers in an American cohort of patients (15). Interestingly, in this study we found the C228T mutation to be associated with the *BRAF* V600E mutation and to be particularly highly prevalent in aggressive types of thyroid cancer, such as poorly differentiated thyroid cancer and ATC. In the present study, we explored *TERT* promoter mutations and their characteristics in a Chinese cohort of thyroid cancer patients to further examine the role of *TERT* promoter mutations in human thyroid tumorigenesis.

Materials and Methods

Tumor samples and DNA isolation

The study included 44 benign thyroid tumors, 22 classical FTC, and 408 classical PTC. To try to be representative of the general Chinese population, we obtained paraffin-embedded surgical primary PTC specimens from five regions in China, spanning from the south to the north and including Shanghai, Shenyang, Qingdao, Heza, and Binzhou. Clinicopathological data were obtained from the medical records of the patients. As reported previously (16), these regions had different iodine content levels in natural drinking water, ranging from the normal levels of 10–21 μg/L in Shanghai, Shenyang, and Qingdao to a high level of 104–287 μg/L in Heza and Binzhou. Urinary iodine levels in individuals living in these regions were previously documented to be correspondingly normal or high (16). The study was approved by related institutional review boards or ethical committees. Patient consent was obtained where required.

Tissues dissected from paraffin-embedded specimens were treated for 8 hours at room temperature with xylene to remove paraffin. This was followed by digestion with 1% sodium dodecyl sulfate and 0.5 mg/mL proteinase K at 48°C for 48 hours. Mid-interval additions of a spiking aliquot of concentrated sodium dodecyl sulfate-proteinase K were added to the samples to facilitate the digestion. DNA was isolated from the digested tissues by standard phenol-chloroform extraction and ethanol precipitation procedures.

Identification of mutations by genomic DNA sequencing

Identification of the *BRAF* V600E mutation on tumor genomic DNA was accomplished by amplifying exon 15 of the *BRAF* gene using the primers and PCR conditions that we established previously (16). *TERT* promoter C228T and C250T mutations were identified on genomic tumor DNA as we recently described (15). Briefly, a 235-bp region of the *TERT* promoter containing the hotspots of C228T and C250T mutations was PCR-amplified using primers 5′-AGTGGATTCGCGGGCACAGA-3′ (sense) and 5′-CAGCGCTGCCTGAAACTC-3′ (antisense) and 40–50 ng of genomic DNA. The efficiency of this PCR was enhanced by the use of the GC-RICH PCR System (Roche Applied Science) according to the manufacturer's instructions. After gel electrophoresis to confirm the quality of the PCR products, sequencing PCR was performed using a Big Dye terminator version 3.1 cycle sequencing ready reaction kit (Applied Biosystems), and DNA sequence was analyzed on an ABI PRISM 3730 automated genetic analyzer (Applied Biosystems). When a mutation was identified, an independent PCR amplification/sequencing, both in forward and reverse directions, was performed to confirm the result.

Statistical analysis

Categorical data were summarized using frequencies and percentiles. Comparison of two groups of categorical variables was performed using the Pearson χ^2 test or Fisher's exact test if the number was < 5. Comparison of two groups of continuous variables was performed using Wilcoxon-Mann-Whitney test. All reported P values were two-sided. $P \leq .05$ was considered to be statistically significant. Analysis was performed using SPSS software version 11.5 (SPSS Inc).

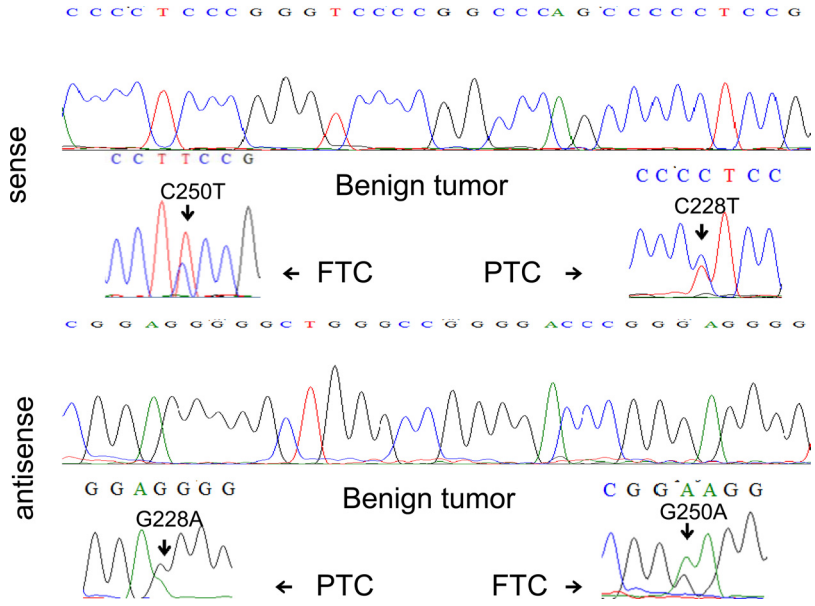

Figure 1. Representative electropherograms of the two *TERT* promoter mutations. Shown are C228T and C250T in a PTC tumor and a FTC tumor, respectively, which were detected both by sense (upper panel) and antisense (lower panel) primers. Shown at the top of each panel is also the wild-type allele of the *TERT* promoter in a benign thyroid tumor.

Results

Common *TERT* promoter mutations in thyroid cancer in a Chinese cohort

Figure 1 illustrates representative electropherograms of the two *TERT* promoter mutations, C228T and C250T, in PTC and FTC, respectively, detected by sense (upper panel) and antisense (lower panel) primers. Shown at the top of each panel is also the wild-type allele of the *TERT* promoter from a benign thyroid tumor. The C228T mutation was far more common than C250T in both PTC and FTC. As summarized in Table 1, we found C228T in 9.6% (39 of 408) of PTC and C250T in 1.7% (7 of 408) of PTC. The two mutations were collectively found in 11.3% (46 of 408) of PTC. In FTC, C228T was found in 31.8% (7 of 22) and C250T in 4.6% (1 of 22) of samples, and they were collectively found in 36.4% (8 of 22) of FTC. The two mutations were mutually exclusive in both FTC and PTC, and they were more prevalent in the former than the latter in this Chinese cohort (36.4 vs 11.3%; $P = .00054$). No *TERT* promoter mutation of any type was found in 44 benign thyroid tumors. All the *TERT* promoter mutations found in this Chinese cohort of thyroid cancers were heterozygous. The germline A>C (T>G on the opposite strand) mutation at −57 bp from the ATG translation start site of the *TERT* gene previously reported in familial melanoma (6) was not found in thyroid tumors in the present study.

Association of *TERT* promoter mutations with *BRAF* V600E mutation in PTC

As summarized in Table 2, *TERT* mutation C228T was found in 3.2% (5 of 158) of *BRAF* V600E mutation-negative PTC vs 13.6% (34 of 250) of *BRAF* mutation-positive PTC, revealing a significantly higher prevalence of C228T in the *BRAF* mutation-positive PTC ($P = 2.95 \times 10^{-3}$). There was a higher trend of *TERT* C250T mutation in the *BRAF* mutation-positive PTC, but this was not statistically significant, probably due to the small number of *TERT* C250T mutation events (Table 2). The two *TERT* promoter mutations were collectively found in 3.8% (6 of 158) of *BRAF* V600E mutation-negative PTC vs 16.0% (40 of 250) *BRAF* mutation-positive PTC, again showing a significantly higher prevalence of *TERT* promoter mutations in the *BRAF* mutation-positive PTC ($P = 5.87 \times 10^{-4}$). Thus, these data demonstrate a significant association of *TERT* promoter mutations with the *BRAF* V600E mutation in this Chinese cohort of PTC.

Lack of association of *TERT* promoter mutations with iodine intake in PTC

Among the 408 cases of PTC, 206 cases were from normal-iodine regions, and 202 cases were from high-io-

Table 1. *TERT* Promoter Mutations in Thyroid Tumors in a Chinese Cohort

Samples	Mutation C228T	Mutation C250T	Collective Mutations
Benign tumor	0/44 (0)	0/44 (0)	0/44 (0)
PTC	39/408 (9.6)	7/408 (1.7)	46/408 (11.3)
FTC	7/22 (31.8)	1/22 (4.6)	8/22 (36.4)

Data are expressed as number of mutations/number of tumors (percentage).

Table 2. Association of *TERT* Promoter Mutations with *BRAF* V600E Mutation in PTC

TERT C228T		*TERT* C250T		Collective TERT mutations	
BRAF−	BRAF+	BRAF−	BRAF+	BRAF−	BRAF+
5/158 (3.2)	34/250 (13.6)	1/158 (0.6)	6/250 (2.4)	6/158 (3.8)	40/250 (16)
$P = 2.95 \times 10^{-3}$		$P = .26$		$P = 5.87 \times 10^{-4}$	

Data are expressed as number of mutations/number of tumors (percentage).

Table 3. Relationship of Mutations in PTC with Iodine Intake

Mutation Type	Normal Iodine Intake	High Iodine Intake	*P* Value
TERT promoter mutations (both types)	22/206 (10.7)	24/202 (11.9)	.73
BRAF V600E	102/206 (49.5)	148/202 (73.3)	8.46×10^{-7}

Data are expressed as number of mutations/number of tumors (percentage).

dine regions as described in *Materials and Methods*. Because *BRAF* V600E mutation was previously shown to be associated with high iodine intake (16), we were curious about the relationship of *TERT* promoter mutations with iodine intake. We explored this issue by taking advantage of our PTC tumors available from both normal-iodine and high-iodine regions. As summarized in Table 3, the two *TERT* promoter mutations were collectively found in 10.7% (22 of 206) of PTC from normal-iodine regions vs 11.9% (24 of 202) of PTC from high-iodine regions ($P = .73$), revealing no significant association of *TERT* promoter mutations with high iodine intake in patients. In contrast, the *BRAF* V600E mutation was found in 49.5% (102 of 206) of PTC from normal-iodine regions vs 73.3% (148 of 202) of PTC from high-iodine regions ($P = 8.46 \times 10^{-7}$). This shows a significant association of *BRAF* V600E mutation with high iodine intake in patients, consistent with our previous findings (16).

Association of *TERT* promoter mutations with aggressive clinicopathological characteristics of PTC

We next examined the relationship of *TERT* promoter mutations with the classical clinicopathological characteristics of PTC. As shown in Table 4, patient age at the diagnosis of PTC was significantly older in the *TERT* promoter mutation-positive group than the mutation-negative group, being 53.40 ± 16.14 years in the former vs 43.66 ± 12.91 years in the latter ($P = 1.08 \times 10^{-5}$). There was no significant difference in the occurrence rate of *TERT* promoter mutations between male and female sexes, although there was a trend of higher prevalence of *TERT* promoter mutations in male patients. Tumor size was significantly bigger in the *TERT* promoter mutation-positive group than the mutation-negative group, being 3.14 ± 1.62 cm in the former vs 2.48 ± 1.58 cm in the latter ($P = .029$). Extrathyroidal extension of PTC was also more common in the *TERT* promoter mutation-positive group than the mutation-negative group, being 28.1% (9 of 32) in the former vs 8.2% (17 of 207) in the latter ($P = .00076$). We did not find a significant difference in neck lymph node metastasis rate between the *TERT* promoter mutation-positive and mutation-negative groups in this cohort of patients. Stage III/IV disease was significantly more common in the *TERT* promoter mutation-positive group than the mutation-negative group ($P = .05$). Thus, overall, *TERT* promoter mutations were associated with aggressive clinicopathological characteristics of PTC.

We also analyzed the individual effects of *BRAF* V600E alone, *TERT* promoter mutation alone, and coexistence of *BRAF* V600E and *TERT* promoter mutations on clinicopathological outcomes of PTC (Table 5). Interestingly, whereas the effects of *BRAF* alone became weaker and the effects of *TERT* promoter mutations alone were lost, coexistence of *BRAF* and *TERT* mutations was more commonly and more significantly associated with the aggressive clinicopathological characteristics of PTC. Specifically, tumor size was 3.32 ± 1.67 cm vs 2.15 ± 1.62 cm ($P = .001$) in the patients with coexisting *BRAF* V600E and *TERT* promoter mutations vs the patients with neither mutation, demonstrating larger PTC tumors associated with the coexistence of the two types of mutations.

Table 4. Relationship of *TERT* Promoter Mutations With Clinicopathological Characteristics of PTC

Clinicopathological Characteristics	TERT Mutations	TERT Wild-Type	*P* Value
Age at diagnosis in y, mean ± SD (n)	53.40 ± 16.14 (42)	43.66 ± 12.91 (325)	1.08×10^{-5}
Gender			.259
Male	12/42 (28.6)	68/325 (20.9)	
Female	30/42 (71.4)	257/325 (79.1)	
Tumor size in cm, mean ± SD (n)	3.14 ± 1.62 (31)	2.48 ± 1.58 (291)	.029
Extrathyroidal extension	9/32 (28.1)	17/207 (8.2)	.00076
Lymph node metastasis	7/26 (26.92)	68/213 (31.9)	.604
Cancer stages			.05
Stages I/II	15/26 (57.69)	161/213 (75.59)	
Stages III/IV	11/26 (42.31)	52/213 (24.41)	

Data are expressed as number of mutations/number of tumors (percentage), unless otherwise indicated.

Table 5. Impact of *BRAF* V600E or *TERT* Promoter Mutations or Their Coexistence on Clinicopathological Outcomes of PTC

Clinicopathological Outcomes	No Mutation	BRAF Mutation Only	*P* Value	TERT Mutation Only	*P* Value	BRAF + TERT Mutation	*P* Value
Age at diagnosis in y, mean ± SD (n)	43.73 ± 13.23 (141)	43.61 ± 12.69 (184)	.936	44.5 ± 13.97 (6)	.889	54.89 ± 16.17 (36)	<.0001
Gender, no. of males/total (%)	28/141 (19.86)	40/184 (21.74)	.679	0/6 (0)	.225	12/36 (33.33)	.084
Tumor size in cm, mean ± SD (n)	2.15 ± 1.62 (129)	2.74 ± 1.50 (162)	.0014	2.2 ± 1.01 (5)	.946	3.32 ± 1.67 (26)	.0011
Extrathyroidal invasion	5/69 (7.25)	18/137 (13.14)	.205	0/3 (0)	1	9/23 (39.13)	.00023
Lymph node metastasis	20/69 (28.99)	49/137 (35.77)	.33	0/3 (0)	.555	7/23 (30.43)	.895
Disease stage							
I + II	52/69 (75.36)	102/137 (74.45)		3/3 (100)		12/23 (52.17)	
III + IV	17/69 (24.64)	35/137 (25.55)	.887	0/3 (0)	1	11/23 (47.83)	.036

Data are expressed as number of mutations/number of tumors (percentage), unless otherwise indicated. *P* values are from the comparison of the indicated genetic group in the column immediately left to the *P* value column with the "No mutation" group.

Extrathyroidal invasion was 39.13% (9 of 23) vs 7.25% (5 of 69) ($P = .0002$) in patients with coexisting *BRAF* V600E and *TERT* promoter mutations vs the patients with neither mutation. Stage III/IV disease was 47.83% (11 of 23) vs 24.64% (17 of 69) ($P = .036$) in patients with coexisting *BRAF* V600E and *TERT* promoter mutations vs the patients with neither mutation. The age at the diagnosis of thyroid cancer was older in patients with coexisting *BRAF* V600E and *TERT* promoter mutations than the patients with neither mutation, being 54.89 ± 16.17 years vs 43.73 ± 13.23 years ($P < .0001$) in the former vs the latter. These results demonstrated a unique role of the coexisting *BRAF* V600E and *TERT* promoter mutations in the tumorigenesis and aggressiveness of PTC.

Discussion

Our previous exciting documentation of *TERT* promoter mutations in thyroid cancer in an American cohort of patients (15) prompted us to extend the studies to this large Chinese cohort of thyroid cancer patients. The present study, together with several other recent studies on *TERT* promoter mutations in thyroid cancer from different ethnic populations (17–19), now firmly establishes the wide occurrence of this novel genetic alteration in human thyroid cancers and provides definitive evidence to support its important role in thyroid tumorigenesis. The prevalence of *TERT* promoter mutations in PTC in this Chinese cohort is very similar to that in our initial report on *TERT* promoter mutations in a large American cohort (15), which was around 11%. It is also similar to that in a large Portuguese cohort recently reported by Soares' group (18). Interestingly, in the Chinese cohort in the present study, the prevalence of *TERT* promoter mutations in FTC was 36%, which was significantly higher than that in PTC. It is also higher than that in FTC reported in the American cohort (15) and in the Portuguese cohort (18). It thus seems that *TERT* promoter mutations may play a more extensive role in the tumorigenesis of FTC in Chinese patients, but this needs to be confirmed in a larger cohort of patients. Unlike *BRAF* V600E mutation, which has been shown to be associated with high iodine intake, a presumptive high risk factor for thyroid cancer (20, 21), the present study showed no association of *TERT* promoter mutations with high iodine intake, suggesting that iodine may not be a risk factor for the occurrence of *TERT* promoter mutations.

In our initial report on *TERT* promoter mutations in a American cohort of 257 PTC patients (15), we observed a significant association of *TERT* promoter mutations with the *BRAF* V600E mutation. A similar finding was also reported in the study of Soares' group (18) on a Portuguese cohort of 169 PTC patients. In contrast, Fagin's group (17) reported a significantly inverse relationship between the *TERT* promoter mutations and the *BRAF* mutation in PTC; we cannot explain this discrepancy, but the small size of the study could be an explanation. Our present study on a Chinese cohort of 408 PTC patients represents the largest single study to address the issue of the relationship of *TERT* promoter mutations with the *BRAF* mutation. The demonstration of a significant association of *TERT* promoter mutations with *BRAF* V600E mutation in this large study on Chinese patients confirms our initial finding of this interesting phenomenon in the American patients (15); these large studies from different ethnic backgrounds, taken together, strongly support the existence of

an associative relationship of the two types of genetic events in PTC. We have previously speculated and proposed that this association of *TERT* promoter mutations with *BRAF* V600E mutation potentially has important biological relevance and may confer to thyroid cancer a unique survival advantage (15). The C228T and C250T promoter mutations create binding sites for ETS transcription factors (6, 7), and ETS factors are targets of the MAPK signaling pathway (22–24). Thus, coexisting *TERT* promoter mutations and *BRAF* V600E mutation forms a unique mechanism in which BRAF V600E-activated MAPK pathway promotes the up-regulation of the *TERT* gene through generating and enhancing the interaction of ETS factors with the *TERT* promoter. In fact, increased TERT expression was observed in PTC tumors harboring both the *TERT* promoter and *BRAF* V600E mutations (18). This may be an important mechanism in promoting thyroid tumorigenesis and aggressiveness of thyroid cancer. Indeed, we have recently demonstrated in an American cohort of PTC that coexistence of *TERT* promoter mutations and *BRAF* V600E mutation was most commonly and significantly associated with clinicopathological aggressiveness of PTC (25), similar to the findings that coexisting *BRAF* V600E and *TERT* promoter mutations were more commonly associated with aggressive clinicopathological characteristics of PTC in the present study. These results are consistent with an interesting observation in our previous study that several PTC tumors that harbored both *TERT* promoter and BRAF V600E mutations also contained anaplastic features (15), raising the possibility that coexistence of *TERT* promoter and *BRAF* mutations may be a genetic mechanism driving conversion of PTC to ATC. The molecular mechanisms in the process of this aggressive progression of thyroid cancer are likely complex and multifaceted, and one speculative mechanism could involve pathological changes in the microenvironments of thyroid cancer that drive thyroid cancer progression (26, 27). It is important to note that our present study demonstrated a significant association of *TERT* promoter mutations with several conventional high-risk factors for poor prognosis of PTC, including older patient age, large tumor size, extrathyroidal extension, and advanced disease stages III/IV. The lack of significant association of *TERT* promoter mutations with lymph node metastasis in the present study likely reflects the possibility that patients studied here mostly did not have, or only had limited, surgical neck dissection at their thyroidectomy. In our initial study on *TERT* promoter mutations in thyroid cancer, a striking finding was the association of such mutations with aggressive types of thyroid cancers, such as tall-cell PTC variant, poorly differentiated thyroid cancer, and ATC (15). Based on this finding, we proposed that *TERT* promoter mutations play an important role in thyroid cancer aggressiveness. This was further supported by subsequent reports that also reported association of *TERT* promoter mutations with aggressive types or aggressive features of thyroid cancer (17–19). Thus, our present study, together with other recent studies, strongly suggests that *TERT* promoter mutations play an important role in the aggressiveness of thyroid cancer and thus represent potential novel prognostic molecular markers that may be useful in assisting risk stratification of thyroid cancer patients. This role of *TERT* promoter mutations, however, seems to require and cooperate with additional genetic alterations, such as *BRAF* V600E mutation, which aberrantly activate other tumor-promoting signaling pathways in promoting the tumorigenesis and development of progression and aggressiveness of PTC. This concept is supported by the fact that, when separated from *BRAF* V600E mutation, *TERT* promoter mutations alone showed a limited effect on clinicopathological outcomes of PTC. This needs to be further investigated, given the small number of cases positive only for *TERT* promoter mutations.

In summary, we present here a study with the largest cohort of PTC patients to investigate *TERT* promoter mutations and demonstrate common occurrence of this novel genetic alteration. The study also demonstrates a significant association of *TERT* promoter mutations with *BRAF* V600E mutation and several aggressive clinicopathological characteristics of PTC. This study, together with other recent studies, unequivocally establishes an important role of *TERT* promoter mutations in the tumorigenesis of human thyroid cancers.

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