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ORIGINAL ARTICLE

Highly prevalent *BRAF* V600E and low-frequency *TERT* promoter mutations underlie papillary thyroid carcinoma in Koreans

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Background: The presence of telomerase reverse transcriptase (*TERT*) promoter mutations have been associated with a poor prognosis in patients with papillary thyroid carcinomas (PTC). The frequency of *TERT* promoter mutations varies widely depending on the population and the nature of the study. **Methods:** Data were prospectively collected in 724 consecutive patients who underwent thyroidectomy for PTC from 2018 to 2019. Molecular testing for *BRAF* V600E and *TERT* promoter mutations was performed in all cases. **Results:** *TERT* promoter alterations in two hotspots (C228T and C250T) and C216T were found in 16 (2.2%) and 4 (0.6%) of all PTCs, respectively. The hotspot mutations were significantly associated with older age at diagnosis, larger tumor size, extrathyroidal extension, higher pathologic T category, lateral lymph node metastasis, and higher American Thyroid Association recurrence risk. The patients with C216T variant were younger and had a lower American Thyroid Association recurrence risk than those with hotspot mutations. Concurrent *BRAF* V600E was found in 19 of 20 cases with *TERT* promoter mutations. Of 518 microcarcinomas measuring ≤1.0 cm in size, hotspot mutations and C216T variants were detected in five (1.0%) and three (0.6%) cases, respectively. **Conclusions:** Our study indicates a low frequency of *TERT* promoter mutations in Korean patients with PTC and supports previous findings that *TERT* promoter mutations are more common in older patients with unfavorable clinicopathologic features and *BRAF* V600E. *TERT* promoter mutations in patients with microcarcinoma are uncommon and may have a limited role in risk stratification. The C216T variant seems to have no clinicopathologic effect on PTC.

Key Words: Papillary thyroid carcinoma; *TERT* promoter; *BRAF*; Molecular typing; Mutation rate

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The incidence of thyroid cancer has dramatically increased over the past several decades [1,2]. The highest incidence of thyroid cancer in the world has been observed in Korea [3,4]. The increase in the incidence of thyroid cancer was responsible for the increase in papillary thyroid carcinoma (PTC), which accounts for over 95% of all thyroid cancer cases in Korea [3,5,6]. Despite the increased incidence of thyroid cancer, the thyroid cancer mortality rate has not changed significantly over the last three decades [3,5]. A multicenter cohort study reported a disease-specific 10-year survival rate of 98% in Korean patients with well-differentiated thyroid carcinoma [6].

Independent prognostic factors related to survival in patients with PTC include elements of cancer staging, such as patient age at diagnosis, tumor size, extensive extrathyroidal extension, and distant metastasis [7]. There have been many studies demonstrating the prognostic value of molecular markers for tumor recurrence and survival. Telomerase reverse transcriptase (*TERT*) promoter mutation is one of the most evident molecular factors related to poor prognosis of patients with PTC [8-11]. The cancer-specific *TERT* promoter mutations occur in two mutually exclusive hotspots in chromosome 5, g.1 295 228 C > T (C228T) and g.1 295 250 C > T (C250T) which correspond to 124 bp (c.–124C > T) and 146 bp (c.–146C > T), upstream from the translation start codon of the *TERT* gene promoter sequence [10-16]. The pooled prevalence of *TERT* promoter mutations in PTC was 11.3% (95% confidence interval, 9.3 to 13.5) in a

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previous meta-analysis of 13 studies [14]. However, the retrospective data may overestimate the mutation frequency because of potential patient selection bias. Patients with microcarcinoma ≤ 1.0 cm were more easily excluded from the molecular studies [8-10,15,17-19]. Furthermore, old archival paraffin blocks may have suboptimal DNA quality that results in molecular test failures and analytical errors.

The *BRAF* V600E mutation is the most common genetic alteration in PTC and remains controversial as an independent prognostic factor [7]. The coexistence of *BRAF* V600E and *TERT* promoter mutations, however, could more accurately indicate the highest mortality risk for patients with PTC [8,19].

The present study aimed to evaluate the real-world frequency of *TERT* promoter mutations in prospectively-collected consecutive cases of PTC and assess the relationship between *TERT* promoter mutations and clinicopathological features in Korean patients with PTC and a high frequency of *BRAF* V600E mutations.

MATERIALS AND METHODS

Patients

We reviewed the prospectively collected data from 724 consecutive patients who underwent thyroidectomy for PTC and molecular testing at Seoul St. Mary's Hospital of the Catholic University of Korea from 2018 to 2019. Molecular tests for *BRAF* and *TERT* promoter mutations were performed in all patients who agreed to allow molecular analysis of their surgical specimens. In cases of multifocal PTCs, the largest tumor was defined as the primary lesion and was chosen for evaluation. The histologic variants of PTC were classified following the diagnostic criteria and terminology of the World Health Organization [7]. The tall cell variant was defined using 30% of tall cell area as a criterion. The PTCs were further classified as classic PTC with tall cell features if it harbored between 10%–30% tall cells and as classic PTC if it contained less than 10% of tall cell area and any well-formed papillae. Cancer staging was done using the 8th edition of the American Joint Committee on Cancer (AJCC) staging system [20]. Minimal extrathyroidal extension was defined as extrathyroidal invasion that was restricted to the perithyroidal soft tissues detected only on microscopic examination (including microscopic strap muscle invasion). When strap muscle invasion was found on preoperative imaging and/or at the time of surgery, the case was considered as gross extrathyroidal extension. Risk stratification of patients for tumor recurrence was done using the 2015 American Thyroid Association (ATA) guidelines [21].

Mutational analyses for *TERT* promoter and *BRAF* V600E mutations

Genomic DNA was extracted from 10 μm-thick formalin-fixed paraffin-embedded (FFPE) tissue blocks using a Maxwell 16 FFPE Tissue LEV Purification Kit (Promega, Fitchburg, WI, USA). Tumor areas were manually dissected with a scalpel under a microscope.

The *TERT* promoter was amplified using the nested polymerase chain reaction (PCR) method. The first-round 235-bp PCR amplicon was amplified using forward 5'-AGTGGATTCGCGGGCACAGA-3' and reverse 5'-CAGCGCTGCCTGAAACTC-3' primers. Then, the second-round 163-bp PCR amplicon was amplified using forward 5'-GTCCTGCCCCTTCACCTT-3' and reverse 5'-CAGCGCTGCCTGAAACTC-3' primers. Bidirectional Sanger sequencing was performed in both directions using the same primers that were used for the second-round PCR. *BRAF* V600E mutation was analyzed using the real-time PCR clamping technology of PNAClampTM BRAF kit (Panagene, Daejeon, Korea) [22]. Each test had a positive control of mutation-holding human genomic DNA and a negative control of distilled water.

Statistical analysis

Categorical variables were analyzed using the Pearson's chi-square, Fisher exact test, or linear-by-linear association when appropriate. Continuous variables were compared using the Student's t-test or Mann-Whitney test when appropriate. The statistical significance threshold was defined as a p-value less than 0.05. All statistical analyses were done using SPSS Statistics program, ver. 21.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Demographic and clinicopathologic characteristics

Table 1 summarizes the baseline clinicopathologic characteristics of the 724 patients with PTC. The median age of the patients at the time of diagnosis was 46 years (interquartile range [IQR], 36 to 56 years). The female to male ratio was 2.7:1. The median tumor size was 0.7 cm (IQR, 0.5 to 1.1 cm). The proportion of microcarcinomas (≤1.0 cm in size) was 71.5% (518/724). Lobectomy was done in 504 (69.6%) and total thyroidectomy in 191 patients (26.4%). The numbers of patients with minimal and gross extrathyroidal extension were 405 (55.9%) and 41 (5.7%), respectively. Cervical lymph node metastases were found in 409 patients (56.5%).

Table 1. Baseline characteristics

Characteristic	No. (%) (n = 724)
Age at diagnosis (yr)	45.9 ± 13.0
< 55	531 (73.3)
≥ 55	193 (26.7)
Sex	
Female	528 (72.9)
Male	196 (27.1)
Tumor size (cm)	
≤ 1.0	518 (71.5)
> 1.0	206 (28.5)
Surgical procedure	
Lobectomy	504 (69.6)
Total thyroidectomy	191 (26.4)
Isthmusectomy	29 (4.0)
Histologic types	
Classic	490 (67.7)
Classic with tall cell features	83 (11.5)
Classic encapsulated	46 (6.4)
Tall cell variant	49 (6.8)
Warthin-like variant	15 (2.1)
Infiltrative follicular variant	10 (1.4)
Invasive encapsulated follicular variant	6 (0.8)
Diffuse sclerosing variant	8 (1.1)
Oncocytic variant	8 (1.1)
Solid variant	5 (0.7)
Hobnail variant	3 (0.4)
Cribriform-morular variant	1 (0.1)
Extrathyroidal extension[a]	
Absent	278 (38.4)
Minimal (microscopic)	405 (55.9)
Gross (strap muscle invasion, pT3b)	30 (4.1)
Gross (tracheal, esophageal or recurrent laryngeal nerve invasion, pT4a)	11 (1.5)
Pathologic T category[a]	
pT1	651 (89.9)
pT2	30 (4.1)
pT3	32 (4.4)
pT4	11 (1.5)
Lymph node metastasis[a]	
Absent (pN0)	315 (43.5)
Central lymph node (pN1a)	346 (47.8)
Lateral lymph node (pN1b)	63 (8.7)
ATA recurrence risk	
Low risk	241 (33.3)
Intermediate risk	358 (49.4)
High risk	125 (17.3)
AJCC cancer staging[a]	
Stage I	623 (86.0)
Stage II	98 (13.5)
Stage III	3 (0.4)
Stage IV	0
BRAF V600E mutation	
Absent	108 (14.9)
Present	616 (85.1)
TERT promoter mutation	
Wild	704 (97.2)
C228T mutation	14 (1.9)
C250T mutation	2 (0.3)
C216T variant	4 (0.6)

ATA, American Thyroid Association; *TERT*, telomerase reverse transcriptase.
[a]All TNM categorization and staging were done according to the 8th American Joint Committee on Cancer (AJCC).

Frequency of *TERT* promoter and *BRAF* V600E mutations

Hotspot-point mutations (C228T and C250T) in the *TERT* promoter were found in 16 (2.2%) patients: 14 with C228T and two with C250T (Table 2, Fig. 1). Four cases had the *TERT* promoter variant of g.1 295 216 C > T (c.–112C > T) (hereafter C216T) (Fig. 1). The *BRAF* V600E mutation was found in 616 (85.1%) patients. Of 20 PTCs with *TERT* promoter aberrations, 19 had coexisting *BRAF* V600E (Table 3, Fig. 2). Fig. 2 summarizes the distribution of histologic variants of PTC and mutational profiles according to the variants. There was no correlation between *TERT* promoter mutations and histologic variants.

Clinicopathologic features of patients with *TERT* promoter mutation

Hotspot mutations in the *TERT* promoter were significantly associated with age ≥ 55 years ($p < .001$), tumor size > 1.0 cm ($p = .001$), extrathyroidal extension ($p = .032$), lateral lymph node metastasis ($p = .041$), and higher ATA recurrence risk ($p < .001$) (Table 2). Compared with patients with hotspot mutations, those with C216T variant were younger ($p = .032$) and had a lower rate of high ATA recurrence risk ($p = .014$). There were no significant differences in the clinicopathologic features between the patients with wild-type *TERT* promoter mutations and those with C216T variant of the *TERT* promoter (Table 2). Table 3 shows the detailed clinicopathologic features of the patients with a *TERT* promoter mutation.

DISCUSSION

The strength of this study stems from the prospectively collected data encompassing all consecutive patients treated for PTC with thyroid surgery. In our study, almost three-quarters of patients with PTC underwent thyroid surgery before the age of 55 years (73.3%) and had microcarcinomas (71.5%). Gross

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Table 2. Association between *TERT* promoter alterations and clinicopathologic features in 724 consecutive patients with papillary thyroid carcinoma

Variable	*TERT* promoter alteration, n (%)			p-value		
	Wild-type (A)	C228T, C250T (B)	C216T (C)	A vs. B	B vs. C	A vs. C
Age at diagnosis (yr)				<.001	.032	>.99
<55	526 (99.1)	2 (0.4)	3 (0.6)			
≥55	178 (92.2)	14 (7.3)	1 (0.5)			
Sex				.776	.587	.295
Female	515 (97.5)	11 (2.1)	2 (0.4)			
Male	189 (96.4)	5 (2.6)	2 (1.0)			
Tumor size (cm)				.001	.255	>.99
≤1.0	510 (98.5)	5 (1.0)	3 (0.6)			
>1.0	194 (94.2)	11 (5.3)	1 (0.5)			
Histologic variant				.313	.214	.405
Classic[a]	603 (97.4)	12 (1.9)	4 (0.6)			
Classic with TCF	78 (94.0)	5 (6.0)	0			
Tall cell variant	46 (93.9)	3 (6.1)	0			
Follicular variant[b]	16 (100)	0	0			
Other[c]	39 (97.5)	1 (2.5)	0			
Extrathyroidal extension				.032	.162	.645
Absent	274 (98.6)	2 (0.7)	2 (0.7)			
Present[d]	430 (96.4)	14 (3.1)	2 (0.4)			
Pathologic T category				<.001	.267	>.99
pT1-2	667 (97.9)	10 (1.5)	4 (0.6)			
pT3-4	37 (86.0)	6 (14.0)	0			
Pathologic N category				.297	.619	.322
pN0	304 (96.2)	9 (2.8)	3 (0.9)			
pN1	400 (98.0)	7 (1.7)	1 (0.2)			
Lateral lymph node metastasis				.041	>.99	.294
Absent	646 (97.7)	12 (1.8)	3 (0.5)			
Present	58 (92.1)	4 (6.3)	1 (1.6)			
ATA recurrence risk				<.001	.014	.344
Low risk	237 (98.3)	2 (0.8)	2 (0.8)			
Intermediate risk	354 (98.9)	2 (0.6)	2 (0.6)			
High risk	113 (90.4)	12 (9.6)	0			
AJCC cancer staging, 8th edition				.065	>.99	>.99
Stage I/II	702 (97.4)	15 (2.1)	4 (0.6)			
Stage III/IV	2 (66.7)	1 (33.3)	0			
BRAF V600E mutation				.489	>.99	>.99
Absent	107 (99.1)	1 (0.9)	0			
Present	597 (96.9)	15 (2.4)	4 (0.6)			

TERT, telomerase reverse transcriptase; TCF, tall cell features; ATA, American Thyroid Association; AJCC, American Joint Committee on Cancer.
[a]Classic papillary thyroid carcinoma (PTC) included classic PTC (n=490), classic PTC with tall cell features (n=83) and encapsulated classic PTC (n=46); [b]Follicular variant included infiltrative follicular variant (n=10) and invasive encapsulated follicular variant (n=6); [c]Other variants included 15 Warthin-like variant, 8 diffuse sclerosing variant, 8 oncocytic variant, 5 solid variant, 3 hobnail variant, and 1 cribriform-morular variant; [d]Included both microscopic and gross extrathyroidal extension.

extrathyroidal extension was found in only 41 patients (5.7%). No case developed synchronous distant metastasis. Therefore, it stands to reason that the vast majority (86.0%) of patients with PTC were assigned to stage I by the 8th edition of the AJCC staging system. Although *BRAF* V600E mutations were highly prevalent in our study cohort, the frequency of hotspot mutations in the *TERT* promoter was 2.2%, which is far lower than that reported in previous studies for PTC (pooled mean prevalence of 11.3%) [14,23]. These results indicate that most PTC tumors in the current study should have indolent behavior.

In our study, additional benefits were gained by including microcarcinomas in the molecular analysis. Hotspot mutations of the *TERT* promoter were found in five of 518 papillary microcarcinomas (1.0%). Minimal extrathyroidal extension, found in three of the five patients with hotspot mutations, did not affect the pathologic T category. Patient age ranged from 39 to 84

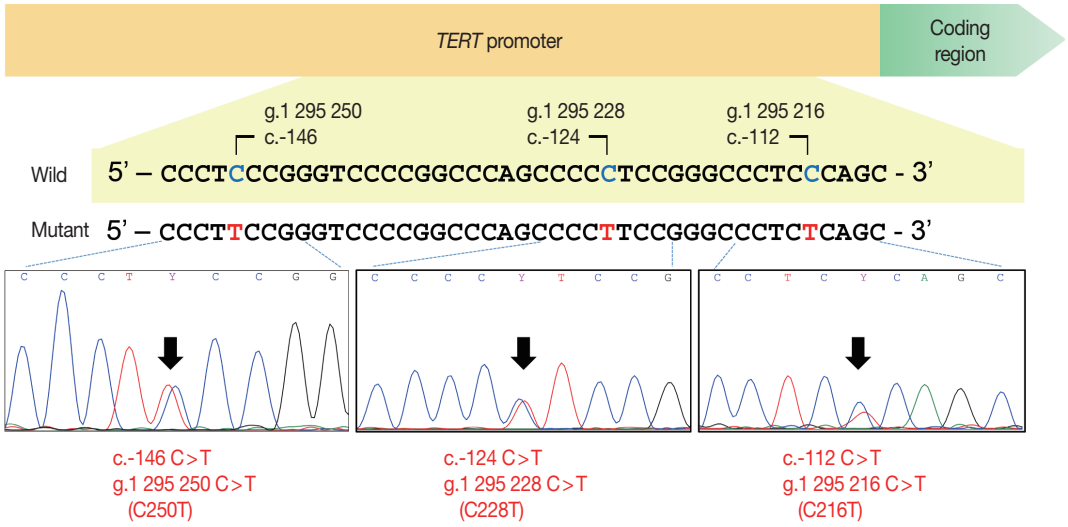

Fig. 1. Schematic figure of the telomerase reverse transcriptase (*TERT*) promoter region and sequencing electropherograms of two hotspot mutations (C228T and C250T) and a C216T variant in the *TERT* promoter. The hotspot mutations resulted from a cytosine-to-thymine transition at genomic loci Chr5:1,295,228 (C228T) and 1,295,250 (C250T), respectively. The C216T variant is a cytosine-to-thymine transition at the 1,295,216 position of Chr5.

Table 3. Clinicopathologic features of papillary thyroid carcinoma patients with *TERT* promoter alterations

Case No.	Age (yr)	Sex	Surgery	Tumor size (cm)	Variant	Multi-focality	ETE	pT	pN	M	Stage	*TERT* promoter	*BRAF*
1	77	F	Isthmusectomy	0.3	Classic	N	Absent	1a	0	0	1	C250T	Wild
2	60	F	Total lobectomy	0.5	Classic, encapsulated	Y	Absent	1a	0	0	1	C250T	V600E
3	55	F	Lobectomy	0.8	Classic	Y	Microscopic	1a	0	0	1	C228T	V600E
4	46	F	Total lobectomy	1.5	Classic	Y	Microscopic	1b	0	0	1	C228T	V600E
5	66	F	Total lobectomy	2.0	Classic	Y	Strap muscle invasion	3b	0	0	2	C228T	V600E
6	57	M	Total lobectomy	2.6	Classic	N	Strap muscle invasion	3b	1b	0	2	C228T	V600E
7	68	M	Lobectomy	2.8	Classic	Y	Microscopic	2	0	0	1	C228T	V600E
8	60	F	Lobectomy	0.7	Classic with TCF	Y	Microscopic	1a	0	0	1	C228T	V600E
9	76	F	Total lobectomy	1.3	Classic with TCF	Y	Microscopic	1b	1a	0	2	C228T	V600E
10	59	F	Total lobectomy	1.6	Classic with TCF	N	Microscopic	1b	0	0	1	C228T	V600E
11	39	F	Total lobectomy	2.1	Classic with TCF	N	Microscopic	2	1b	0	1	C228T	V600E
12	65	M	Total lobectomy	3.2	Classic with TCF	Y	Strap muscle invasion	3b	1a	0	2	C228T	V600E
13	75	F	Total lobectomy	2.0	Tall cell	N	Strap muscle invasion	3b	1a	0	2	C228T	V600E
14	74	M	Lobectomy	2.7	Tall cell	N	Strap muscle invasion	3b	0	0	2	C228T	V600E
15	84	F	Total lobectomy	5.5	Tall cell	N	Esophagus invasion	4a	1b	0	3	C228T	V600E
16	64	M	Total lobectomy	0.7	Oncocytic	Y	Microscopic	1a	1b	0	2	C228T	V600E
17	44	F	Lobectomy	0.4	Classic	N	Absent	1a	0	0	1	C216T	V600E
18	55	F	Total lobectomy	0.5	Classic	N	Absent	1a	0	0	1	C216T	V600E
19	29	M	Total lobectomy	1.0	Classic	Y	Microscopic	1a	1b	0	1	C216T	V600E
20	54	M	Lobectomy	1.2	Classic	N	Microscopic	1b	0	0	1	C216T	V600E

TERT, telomerase reverse transcriptase; ETE, extrathyroidal extension; F, female; M, male; Y, yes; N, no; TCF, tall cell features.

years. Although the frequency of *TERT* promoter mutations is lower than that of previous studies, these findings are in line with a previous Italian study showing no correlation with unfavorable outcomes [24]. The Italian study showed that *TERT* promoter mutations were found in 4.7% of papillary microcarcinomas and were not associated with poor clinical features [24]. As active surveillance is one of the treatment options for low-risk papillary microcarcinomas, the identification of *TERT* promoter mutations may facilitate decision-making on appropriate candidates for active surveillance [21,25]. One Japanese study reported that no *TERT* promoter mutations were found in 25 patients selected from 1,252 patients with low-risk papillary microcarcinoma who were managed with active surveillance [25]. These results, however, need to be validated in further larger studies.

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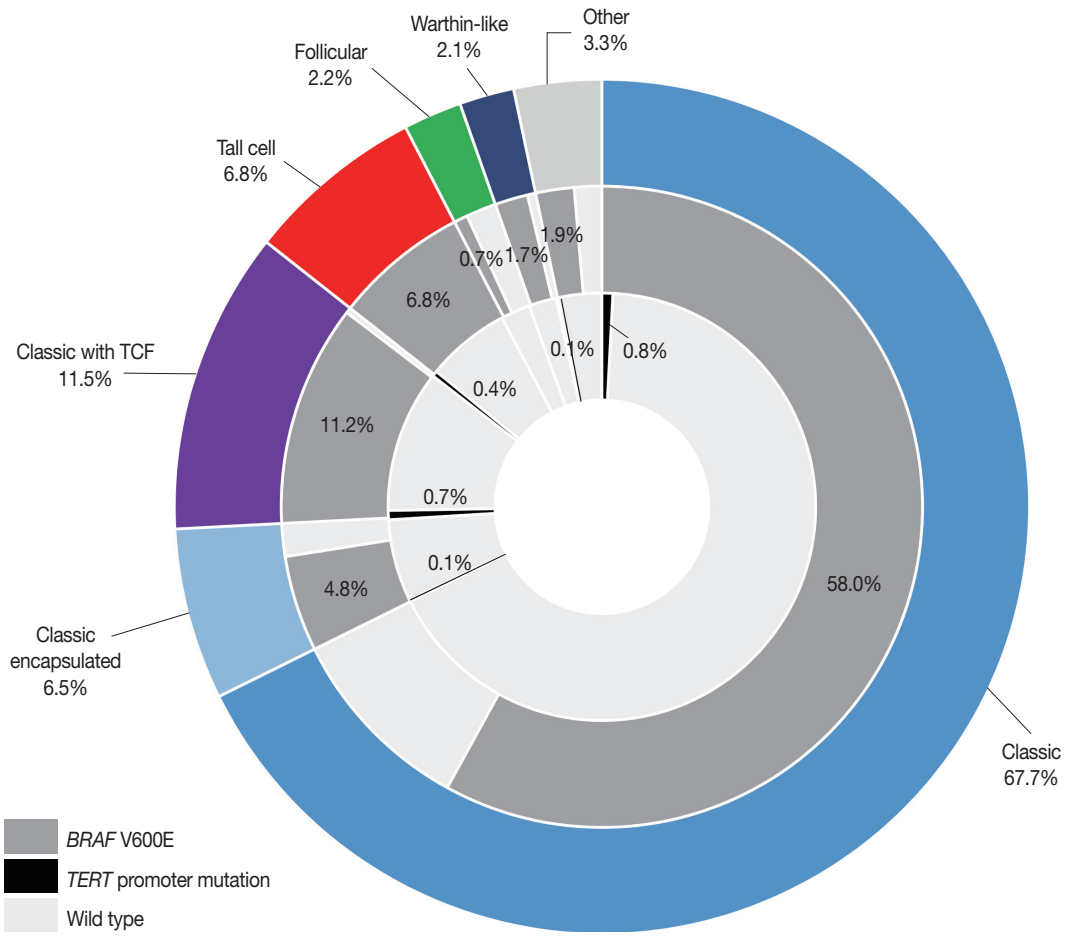

Fig. 2. A pie chart depicting a portion of *BRAF* V600E and telomerase reverse transcriptase (*TERT*) promoter mutations (C228T and C250T) in relation to histologic variants of papillary thyroid carcinoma (n=724). The middle and inner circles show the frequency of *BRAF* V600E and *TERT* promoter mutations, respectively. The other variants included eight diffuse sclerosing, eight oncocytic, five solid, three hobnail, and one cribriform-morular variant. TCF, tall cell features.

Since most studies reported only pathogenic hotspot mutations, little is known about the prevalence and functional role of the *TERT* promoter C216T variant in human cancers. The C216T variant was found in four cases of our study cohort and has been previously reported in two lung adenocarcinomas [26] and one esophageal squamous cell carcinoma [27]. In our study, all four patients with the C216T were younger (range, 29 to 55 years) than those with hotspot mutations and had no unfavorable clinicopathologic features. Therefore, we suggest that the *TERT* promoter C216T variant may be a non-pathogenic DNA polymorphism in PTC.

Many studies have shown synergistic effects of concurrent *BRAF* V600E and *TERT* promoter mutations on the poor prognosis and mortality risk of patients with PTC [8,11,17-19,23]. The C228T and C250T mutations of the *TERT* promoter generate an 11-bp binding motif (5'-CCCCTTCCGGG-3') for E-twenty-six (ETS) transcription factors [13]. Mitogen-activated protein kinase pathway activation by the *BRAF* V600E mutation upregulates ETS transcription factors, which results in increased *TERT* mRNA expression by the binding of the mutated *TERT* promoter to ETS [28]. In our study, all 14 patients with the *TERT* promoter C228T mutation had a concurrent *BRAF* V600E mutation. In Korean patients with PTC and a high prevalence of the *BRAF* V600E, further studies are needed to validate the prognostic utility of risk stratification of patients with PTC by combining *BRAF* V600E and *TERT* promoter mutations.

In summary, this study demonstrated that the *TERT* promoter mutation frequency was 2.2% in prospectively collected patients, and the presently reported frequency is lower than that reported in previous studies. *TERT* promoter mutations were more common in older patients with unfavorable clinicopathologic fea-

tures and a *BRAF* V600E mutation. Although they were observed less frequently than in those with larger tumors, *TERT* promoter mutations also occurred in patients with microcarcinoma and low-risk clinicopathologic features. The C216T variant was found in 0.6% of all PTCs and may be a non-pathogenic DNA polymorphism.

Ethics Statement

This study was approved by the Institutional Review Board of Seoul St. Mary's Hospital, the Catholic University of Korea (KC16SISI0709). Informed consent was obtained from each patient.

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Conflicts of Interest

C.K.J. is the editor-in-chief of the *Journal of Pathology and Translational Medicine* and was not involved in the editorial evaluation or decision to publish this article. All remaining authors declare that they have no potential conflicts of interest.

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