Regulatory SNP of TERT promoter accompanied by C228T and *BRAF*^{V600E} is an exacerbating factor of papillary thyroid carcinoma

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Abstract. Despite the increased incidence of thyroid cancer due to enhanced precision of ultrasound technology and extensive utilization of puncture aspiration cytology, the mortality rate remains low, raising concerns about overdiagnosis. Papillary thyroid carcinoma (PTC) is the most common type, primarily diagnosed through cell nuclei examination. Recent advancements in identifying genetic mutations and tumor classification have refined diagnostic methods. Point mutations in the telomerase reverse transcriptase promoter (*TERTp*), specifically -124 C >T (C228T) and -146 C >T (C250T), and the regulatory single nucleotide polymorphism -245 T >C, C allele of rs2853669 (TrSNP) are potential thyroid cancer biomarkers. The present study tested the hypothesis that the coexistence of *BRAF* mutations in driver genes upstream of the MAPK pathway and late mutations unrelated to signaling, such as point mutations in *TERTp*, increases tumor virulence. A total of 133 patients with PTC who underwent surgery between January 2014 and November 2021 were included in the study. Blood and tumor tissue samples were collected, and DNA was extracted for genetic mutation analysis using PCR and Sanger sequencing. The TrSNP analysis of blood and surgical tissue samples showed a 97.7% agreement rate. TrSNP was detected in 70 of 133 patients (52.6%) and was significantly associated with tumor size, particularly in tumors >2.0 cm. *TERTp* point mutations were identified in 29 of 133 patients (21.8%), with C228T strongly associated with tumor size, particularly in tumors >4.0 cm, and extraglandular invasion. *BRAF*^{V600E} was detected in 82 patients (61.7%) but showed no significant association with clinicopathological parameters. However, the coexistence of *BRAF*^{V600E} with C228T and TrSNP affected tumor size and progression. The findings indicated that TrSNPs, along with C228T and *BRAF*^{V600E}, may serve as potential molecular markers to predict PTC growth or exacerbation. Notably, coexistence of C228T and TrSNP is a preoperative indicator of disease progression.

Introduction

The incidence of thyroid cancer has increased worldwide over the past several decades. In 2020, the age-standardized incidence rate of thyroid cancer was 10-1 per 100,000 women and 3-1 per 100,000 men, and the age-standardized mortality rate was 0-5 per 100,000 women and 0-3 per 100,000 men (1). Advancements in the accuracy of ultrasound equipment and the widespread use of fine-needle aspiration cytology have led to the increased detection of early-stage cancers. Despite this trend, the mortality rate for thyroid cancer has remained stable. A global assessment of thyroid cancer in 2020 showed a rise in incidence rates across many countries, whereas the mortality rate remained low (1,2). Some reports attribute this phenomenon to overdiagnosis (3). The early detection of papillary thyroid carcinoma (PTC) could benefit patients considered for surgery by correctly identifying tumor grade, for instance, if it were feasible to identify an indicator of malignancy in differentiated thyroid cancer, such as the expression of cancer driver genes or the impact of late-stage genetic mutations prior to treatment. If patients are willing to accept a diagnosis of PTC with slow disease progression,

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Abbreviations: FFPE, formalin-fixed paraffin-embedded; PTC, papillary thyroid carcinoma; SNP, single nucleotide polymorphism; *TERTp*, telomerase reverse transcriptase gene promoter; TrSNP, C allele of rs2853669; WHO, World Health Organization

Key words: TrSNP, PTC, *TERTp*, C228T, C250T

the need for aggressive therapeutic intervention may be eliminated.

In the field of cervical cancer, persistent infection with the human papillomavirus (HPV) is a driver of cervical carcinogenesis, with the HPV type playing a key role in determining the grade and prognosis of cervical lesions (4). Therefore, genetic testing for HPV is recommended for all patients with cervical cancer (5). In thyroid cancer, various genetic mutations have been implicated in carcinogenesis and described in the fifth edition of the World Health Organization (WHO) classification (6). As the preoperative diagnosis of thyroid cancer is based on cytology, determining the nature of the tumor by genetic testing on cytological specimens is in the best interest of the patient.

PTC is pathologically diagnosed based on cytological nuclear findings. Advances in ultrasound-guided puncture aspiration cytology have led to the early detection and increased treatment of the disease. In Japan, PTC is defined as low-risk thyroid carcinoma without lymph node or distant metastasis, invasion of the recurrent nerve or trachea, or high-grade histological findings (tall cell variant or poorly differentiated components). To minimize overdiagnosis, various studies recommend the treatment of PTC <1 cm as micropapillary carcinoma without the need for surgery (7,8). Although PTC has a better prognosis than other types of cancer, 5-10% of PTC have a high proliferative tendency and recur after surgery, with some cases being difficult to treat and resulting in death (9).

Various genetic abnormalities are involved in the development of thyroid tumors, which are expected to be clinically applicable. These include mutually exclusive driver mutations of PTC, such as the *BRAF*^{V600E} mutation and *RET*/*PTC* translocation. Theoretically, the activation of telomerase reverse transcriptase (*TERT*), which elongates telomere DNA, is believed to be involved in the infinite proliferative capacity of various tumors. Recently, mutations in the human *TERT* promoter (*TERTp*) have been reported as attractive prognostic factors for thyroid carcinoma. Point mutations of *TERTp* in thyroid carcinoma were first reported in 2013, with -124 C >T (Chr5:1295228; C228T) and -146 C >T (Chr5:1295250; C250T) mutations located 124 and 146 bp upstream of the translational initiation site, respectively (10,11) (Fig. 1). A few studies have demonstrated that these two mutations are molecular markers associated with thyroid tumor grade. These mutations are strongly correlated with high-risk clinicopathologic features, including age, tumor size, extraglandular invasion and distant metastasis. The frequencies of mutations in PTC, follicular thyroid carcinoma, and follicular adenoma are 5-24%, 10-35%, and 2-8%, respectively (12,13). The fifth edition of the WHO classification, published in 2023 describes C228T and C250T as late mutations in thyroid carcinoma that are added to early mutations in driver genes, such as *BRAF* and *NRAS* (6). Additionally, *TERT* mutations are synergistic with the *BRAF*^{V600E} mutation. The coexistence of *TERTp* point and *BRAF*^{V600E} mutations worsens the prognosis of PTC (14-19).

A recent study revealed that mutations in the core *TERTp* create a new binding site for the E-twenty-six transcription factor, which is believed to be the cancer-specific *TERTp* reactivation mechanism (20). Two-step mechanisms have been proposed for the involvement of *TERTp* mutations in tumorigenesis based on *in vitro* experiments. These experiments suggest that *TERTp* mutations contribute to tumorigenesis through two steps: immortalization and promotion of genomic instability. *TERTp* mutations elevate telomerase activity but do not prevent the wear and tear of telomers, and instead repair short telomeres to extend cell life. When telomeres are critically short and increase in number, the genome becomes unstable, prompting further upregulation in *TERT* expression to maintain cell proliferation (21).

A regulatory single nucleotide polymorphism (SNP) of the *TERTp*, C allele of rs2853669 (Chr5:1295234), -245 T>C (TrSNP), manifests an allelic change from thymine (T) to cytosine (C). It has been identified in the proximity of the two *TERTp* mutations.

The C allele of rs2853669 (TrSNP) is not an acquired mutation, such as C228T or C250T; however, it is a familial pleomorphism in the germ cell lineage. It is present in all cells of the body; therefore, it can be easily detected preoperatively using a blood sample. TrSNP alters the expression levels of wild-type and mutant (C228T and C250T) *TERT in vitro*, potentially impacting the cancer phenotype (22). TrSNP is associated with malignancy in glioblastoma, lung carcinoma, acute leukemia, and hepatocellular carcinoma (22-28). Moreover, the homozygous TrSNP (CC) genotype affects the prognosis of neuroglioma. Notably, the overall survival periods of patients who additionally carry the C228T or C250T mutations are markedly short. Notably, the homozygous TrSNP genotype was significantly higher (41%) in South Indian women with cervical cancer (29). In a further report on 144 cases of oral squamous cell carcinoma, it has been demonstrated that the C228T mutation and the TrSNP wild-type TrSNP genotype are independent prognostic biomarkers (30).

Our previous study using cultured thyroid cells demonstrated that TrSNP and C228T mutations enhance the transcriptional activity of *TERTp* in thyroid tumors. Specifically, our *TERTp* luciferase assay revealed that TrSNP increased the promoter activity, with the coexistence of TrSNP and C228T further enhancing *TERTp* activity (31). In our previous study, we also showed that TrSNP was associated with tumor size in PTC and follicular neoplasm surgical specimens. In particular, thyroid tumors with the C228T mutation and TrSNP were found to be larger in size compared to tumors with either variant alone (32,33). The C allele of the TrSNP of *TERTp* showed a statistically significant correlation with thyroid tumor size, similar to C228T, indicating that it influences thyroid tumor growth via regulating the expression of *TERT*. However, no statistical association with tumor grade, such as invasion or lymph node metastasis, was observed, probably owing to the small number of cases included in the previous study. Notably, these analyses were performed using surgical specimens and not blood samples, which are easily obtained clinically. Additionally, the small number of cases hindered comprehensive analysis of the detailed association between TrSNP and clinical outcomes or the effect of the coexistence of the C allele of TrSNP with *TERTp* point mutations on tumor progression or exacerbation.

The *BRAF*^{V600E} mutation, a known driver gene for papillary thyroid carcinoma, is a common mutation in the MAPK

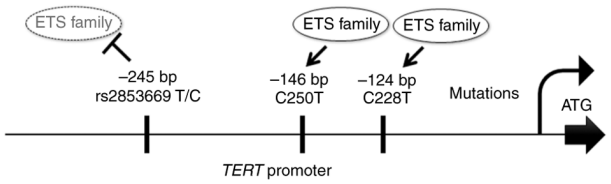

Figure 1. TERT promoter mutation variants. The point mutations C228T and C250T are located 124 and 146 bp upstream of the translation start site, respectively. In addition, a regulatory single nucleotide polymorphism of the TERT promoter, C allele of rs2853669 (TrSNP), is present in the nearby region (-245 bp). This TrSNP has been shown to form a novel ETS binding sequence and transcription factor binding site and to increase promoter activity. ETS, E-twenty six; *TERT*, telomerase reverse transcriptase; TrSNP, regulatory single nucleotide polymorphism in the TERT promoter (C allele of rs2853669).

pathway that results in uncontrolled cell growth and proliferation. According to the AACR's Precision Medicine GENIE tumor prognostic database (version 13.0), the most common concomitant BRAF ^{V600E} mutations and the most frequent concomitant mutations were *TERT* and *TP53* (34). The objective of this study was to test the hypothesis that the coexistence of *BRAF* mutations in driver genes upstream of the MAPK pathway and late mutations unrelated to signal transduction, such as point mutations in the *TERT p*, would result in increased tumor virulence. In this study, the allelic genotype of *TERTp* rs2853669 was analyzed in detail in patient blood samples with the aim of evaluating the potential utility of *TERTp* rs2853669 in the preoperative evaluation of PTC. The objective of this study is to elucidate whether point mutations and single nucleotide polymorphisms (SNPs) within *TERTp* can serve as molecular markers to determine preoperative indicators of PTC malignancy.

Materials and methods

Patients. We selected 144 patients with PTC who had undergone surgery at Kyorin University Hospital between January 2014 and November 2021 and who provided written consent to participate in the study. Blood samples were used for TrSNP analysis; therefore, we excluded patients whose blood samples could not be collected owing to the difficulty of visiting the hospital postoperatively or resulted in inadequate DNA extraction owing to demineralization during sample preparation. The final number of examined patients was 133.

Patient backgrounds are presented in Table I. The men/women ratio was 35/98, with a median age of 54 (18-90) years. The patients were categorized by clinicopathological factors according to the Japanese thyroid cancer handling, and their distributions were statistically evaluated (35). The mean tumor diameter was 21.1 (2-115) mm.

Methods. To examine the C allele of rs2853669 and C228T and C250T mutations in *TERTp*, we extracted DNA from formalin-fixed paraffin-embedded (FFPE) surgical tumor tissue specimens (5-10 slices of 5 μm thickness) and blood samples (1 cc) collected in blood collection tubes (PAXgene^{R}, PreAnalytiX, Hombrechtikon, Switzerland). Following deparaffinization with xylene and alcohol and protease treatment, DNA was extracted from FFPE samples using the QIAamp DNA FFPE Tissue Kit (Qiagen) with the spin column method. DNA extraction from whole blood samples was performed using the QIAamp DNA Mini Kit (Qiagen). After quantifying the amount of extracted DNA using NanoDrop (Thermo Fisher Scientific, Wilmington, DE, USA), 200 ng DNA was amplified using Taq polymerase (GoTaq Green Master Mix, Promega Corporation, Madison, WI, USA) under the following PCR (36) cycling conditions: 95˚C for 10 min, followed by 40 cycles of 95˚C for 30 sec, 68˚C for 30 sec, and 72˚C for 30 sec, with a final extension at 72˚C for 4 min (31). DNA extracted from blood samples was also subjected to PCR to confirm that C228T, C250T, and *BRAF*^{V600E} were somatic mutations. Following confirmation of specific amplification using gel agarose electrophoresis, the PCR products were purified using the FastGene Gel/PCR Extraction Kit (FastGene, Tokyo, Japan). Finally, the nucleotide sequence of the PCR products was determined using Sanger sequencing analysis (Macrogen, Tokyo, Japan) with reverse primers. The primers used are listed in Table II.

Table I. Characteristics of patients with papillary thyroid carcinoma (n=133).

Clinicopathological features	Value
Sex, n (%)	
Male	35 (26.3)
Female	98 (73.7)
Age, years	
Median	54
Range	18-90
Tumor size, mm	
Mean ± SD	21.86±17.38
Range	2-115
Age, n (%)	
<55 years	66 (49.6)
≥55 years	67 (50.4)
Size, n (%)	
<2.0 cm	78 (58.6)
≥2.0 cm	55 (41.4)
<4.0 cm	117 (88.0)
≥4.0 cm	16 (12.0)
Pathological T factor, n (%)	
≤pT2	81 (61.0)
≥pT3	52 (39.1)
Lymph node metastasis, n (%)	
0	79 (59.4)
≥1	54 (40.6)
Intrathyroidal spread, n (%)	
Presence	60 (45.1)
Absence	73 (54.9)
Extraglandular invasion n (%)	
Presence	70 (52.6)
Absence	63 (47.4)

pT, pathological T factor.

Table II. Primers used in the present study.

Target	Primer sequences (5'-3')	Ta, ˚C	Amplicon, bp
TERT promoter	F: ACGCCCAGGACCGCGCT R: CCCACGTGCGCAGCAGG	68	236
BRAF	F: GCTTGCTCTGATAGGAAAATGAG R: GTAACTCAGCAGCATCTCAGG	58	237

F, forward; R, reverse; Ta, annealing temperature; *TERT*, telomerase reverse transcriptase.

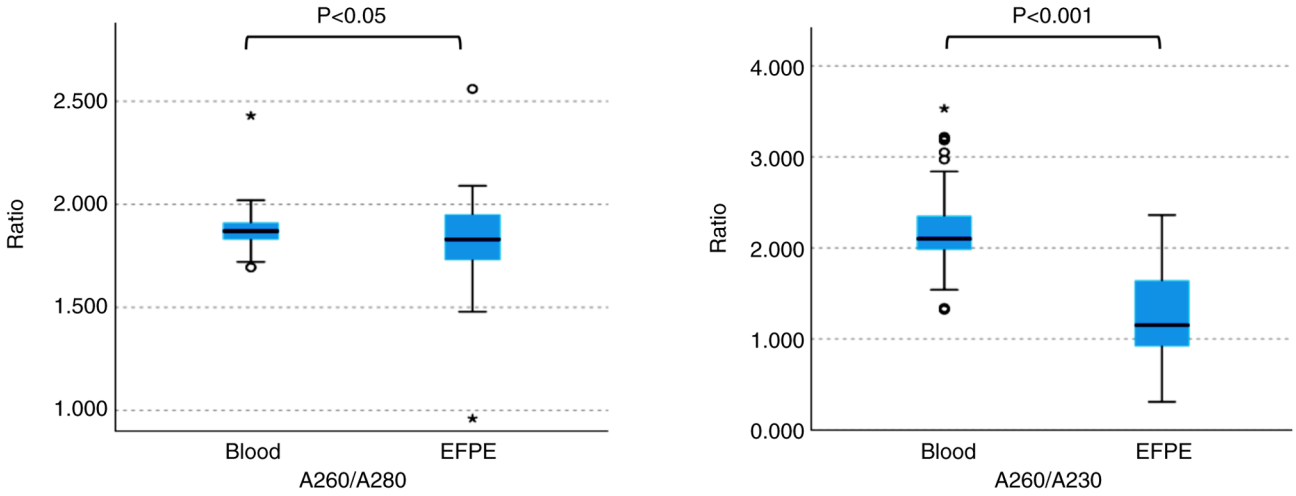

Figure 2. Comparison of absorbance ratios A260/A280 and A260/A230 (indicators of DNA purity) for blood and FFPE samples analyzed in the present study. The test results showed that the blood samples were more stable than the FFPE samples, with less variation. Extreme outliers are marked with an asterisk. Mild outliers are marked with a circle. The P-value was calculated using the paired samples t-test. FFPE, formalin-fixed paraffin-embedded.

Statistical analysis. The cases in which the height of peaks in the sequence waveform reached 25% of the normal peak were considered point-mutation or heterozygous TrSNP variants. For the C allele of rs2853669, cases with 100% overlapping peaks were considered homozygous variants. Sanger sequencing images are shown in Fig. S1. The results were categorized by clinicopathological parameters, including sex, age, tumor diameter (size), pT classification, lymph node metastasis, extraglandular infiltration, and intrathyroidal spread (as per the WHO and American Joint Committee on Cancer guidelines), and statistically analyzed. Tumors located at a distance of at least 5 mm from the primary tumor were defined as intrathyroidal spread. In the comparative analysis of the abnormality of each gene and clinicopathological parameters, the Mann-Whitney test was used for continuous data (age and tumor diameter), whereas the χ^2 and Fisher's exact tests were used for categorical data. The absorbance ratios A260/A280 and A260/A230 (a measure of DNA purity) of blood and FFPE samples obtained from the same patients were compared using the paired sample t-test. Furthermore, multiple comparisons of combinations of genetic mutations were performed for each group using the Fisher's exact test followed by Bonferroni's post hoc test and the Kruskal-Wallis test followed by Bonferroni's post hoc test. All analyses were performed using IBM SPSS software ver. 28 (IBM, Chicago, IL, USA). Statistical significance was set at P≤0.05.

Results

Concordance between FFPE and blood samples. In a quality assurance, the concordance rate between FFPE and blood samples for TrSNP polymorphisms was an impressive 97.7%. In order to evaluate DNA purity, we compared the A260/A280 and A260/A230 absorbance ratios of samples. The average A260/A280 ratio was 1.88±0.08 for blood samples and 1.82±0.18 for FFPE samples, whereas the A260/A230 ratio was 2.17±0.39 for blood samples and 1.27±0.49 for FFPE samples. Hence, blood samples demonstrated better stability and less variability than FFPE samples (P<0.05, P<0.001) (Fig. 2).

Genetic analysis of TrSNP (C allele of rs2853669) in PTC patients. Table III shows the results of the genetic analysis of the C allele of rs2853669 (TrSNP), *TERTp* point mutations C228T and C250T, and *BRAF*^{V600E} in 133 cases of PTC and their relationship with clinicopathological parameters. TrSNP was detected in 70 (52.6%) out of 133 patients. We found that 51 women (52.0%) and 19 men (54.3%) carried TrSNP. Among them, 31 (47.0%) patients with PTC were aged ≤55 years, whereas 39 (58.2%) were ≥55 years of age. We did not detect any significant difference in the presence or absence of TrSNP by sex or age.

Clinicopathological analysis showed a significant association between TrSNP and PTC size (P<0.05), with larger tumor diameters observed in patients with TrSNP. When categorized

Table III. Clinicopathological parameters and frequency of TrSNP, C228T, C250T and $BRAF^{V600E}$ in 133 patients with papillary thyroid carcinoma.

	TERT promoter			
Parameters	TrSNP, % (n)	C228T, % (n)	C250T, % (n)	$BRAF^{V600E}$, % (n)
Positive cases (n=133)	52.6 (70)	17.3 (23)	8.3 (11)	61.7 (82)
Sex				
Female (n=98)	52.0 (51)	17.3 (17)	9.2 (9)	60.2 (59)
Male (n=35)	54.3 (19)	17.1 (6)	5.7 (2)	65.7 (23)
Age, years				
<55 (n=66)	47.0 (31)	13.6 (9)	13.6 (9)[d]	65.2 (43)
≥55 (n=67)	58.2 (39)	20.9 (14)	3.0 (2)[d]	58.2 (39)
Size, cm				
≤2.0 (n=78)	44.9 (35)	11.5 (9)[a]	6.4 (5)	64.1 (50)
>2.0 (n=55)	63.6 (35)	25.5 (14)[a]	10.9 (6)	58.2 (32)
≤4.0 (n=117)	51.3 (60)	14.5 (17)[b]	7.7 (9)	60.7 (71)
>4.0 (n=16)	62.5 (10)	37.5 (6)[b]	1.3 (2)	68.8 (11)
Pathological T factor				
≤pT2 (n=81)	49.4 (40)	12.3 (10)	7.4 (6)	55.6 (45)
≥pT3 (n=52)	57.7 (30)	25.0 (13)	9.6 (5)	71.2 (37)
Lymph node metastasis				
0 (n=79)	46.8 (37)	12.7 (10)	3.8 (3)[e]	59.5 (47)
≥1 (n=54)	61.1 (33)	24.1 (13)	14.8 (8)[e]	64.8 (35)
Extraglandular invasion				
0 (n=70)	45.7 (32)	8.6 (6)[c]	7.1 (5)	58.6 (41)
≥1 (n=63)	60.3 (38)	27.0 (17)[c]	9.5 (6)	65.1 (41)
Intrathyroidal spread				
Presence (n=60)	55.0 (33)	21.7 (13)	13.3 (8)	65.0 (39)
Absence (n=73)	50.7 (37)	13.7 (10)	4.1 (3)	58.9 (43)

[a]P=0.037; [b]P=0.023; [c]P=0.005; [d]P=0.026; [e]P=0.05. P-values for the comparison of presence vs. absence of each mutation in groups based on the different parameters. P-values were calculated using the χ^2 test and Fisher's exact test. pT, pathological T factor; TERT, telomerase reverse transcriptase; TrSNP, regulatory single nucleotide polymorphism in the *TERT* promoter (rs2853669).

by tumor diameter, 35 (44.9%) of the 78 cases of PTC tumors <2.0 cm and 35 (63.6%) of the 55 cases of tumors >2.0 cm had TrSNP, indicating a significant association of TrSNP with PTC tumors >2.0 cm (P<0.05). However, when we used a cutoff of 4.0 cm, we did not observe any significant difference between the occurrence of TrSNP and PTC tumor size.

Interestingly, we did not detect any significant association between the presence or absence of TrSNP and other clinicopathological parameters, which was consistent with the results of previous studies.

Genotype distribution and tumor size. We detected the homozygous genotype (CC) of TrSNP in 14 cases (10.5%), the heterozygous genotype (CT) in 56 cases (42.1%), and the wild type (TT) in 63 cases (47.4%). The frequency of the C allele was 31.6% (84/266 genes), with the minor allele frequency (MAF) being 0.316. The goodness-of-fit test verified that the P-value of the Hardy-Weinberg equilibrium was 0.95.

The tumor diameter was significantly greater in cases with the C allele than in those carrying the wild type (P<0.05). The tumor size was significantly larger in the heterozygous group than in the wild-type group (P<0.05) (Fig. 3). However, no significant difference was detected in tumor diameter between the wild-type and homozygous group.

Clinicopathological implications of the TERT promoter mutations (C228T and C250T). We found *TERTp* mutations in 29 of 133 patients (21.8%): more specifically, 23 (17.3%) and 11 (8.3%) had C228T and C250T point mutations, respectively, whereas five (3.8%) had both. However, no somatic C228T or C250T mutations were detected in the blood samples. In addition, the C228T mutation was significantly correlated with tumor size (mm) (P<0.001), indicating a higher detection rate of C228T in larger tumors, whereas no correlation was observed between the C250T mutation and tumor size (Fig. 4).

In categorical analysis, we detected the C228T mutation in 9 (11.5%) of 78 PTC tumors measuring ≤2.0 cm and in 14 (25.5%) of 55 tumors measuring >2.0 cm, indicating that C228T significantly correlated (P<0.05) with PTC tumors >2.0 cm in size. When a cutoff of 4.0 cm was used, the C228T

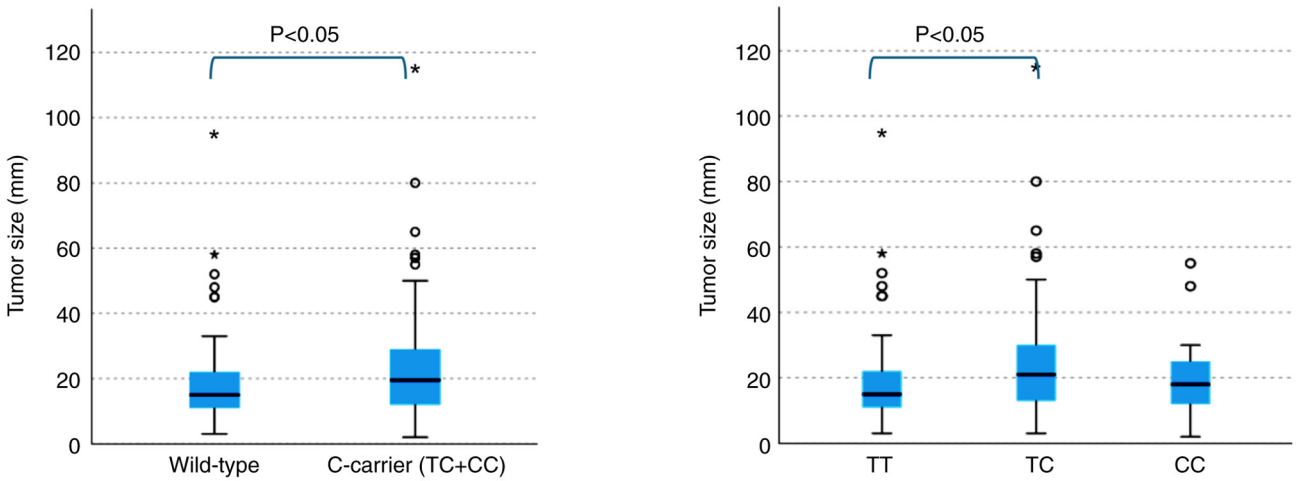

Figure 3. Relationship between tumor diameter and wild-type and C carriers of the TrSNP and the junction type of the TrSNP. Compared with the wild-type group, the tumor diameter was significantly larger in cases with the C allele. The comparison of the three zygotypes showed that tumor diameters were significantly larger in the heterozygous group compared with the wild-type group, but there was no significant difference between the wild-type group and the homozygous group. Extreme outliers are marked with an asterisk. Mild outliers are marked with a circle. The P-value was calculated using the Mann-Whitney test for results showing comparisons between wild-type and C carriers, followed by the Kruskal-Wallis test and the Bonferroni correction for comparisons between different genotypes. TrSNP, regulatory single nucleotide polymorphism in the *TERT* promoter (C allele of rs2853669).

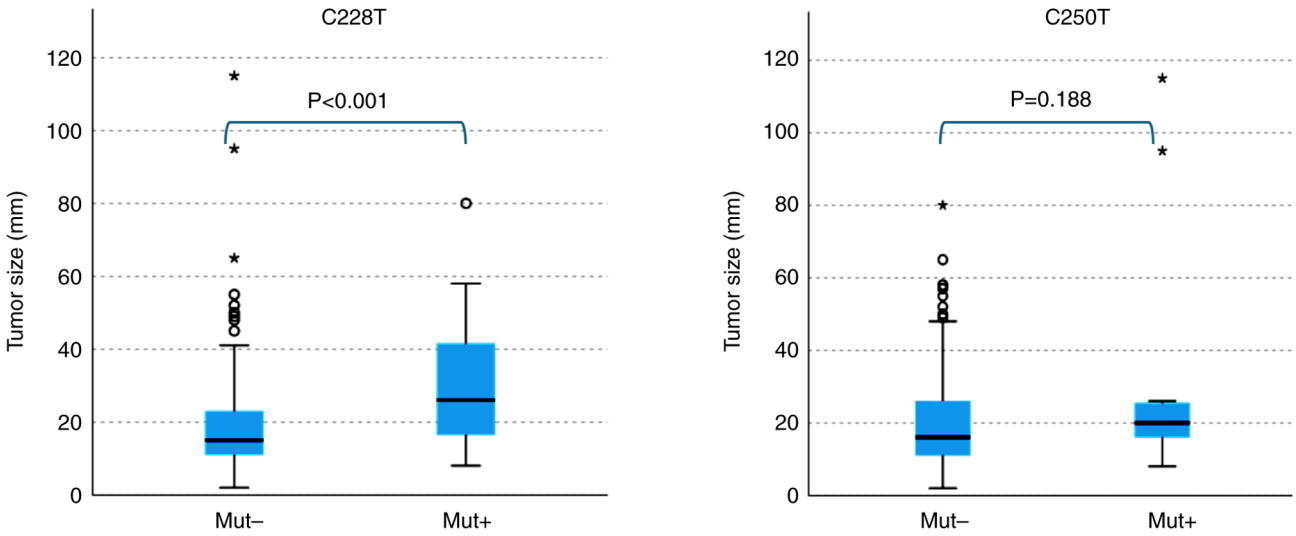

Figure 4. Relationship between papillary carcinoma tumor size and groups with and without point mutations in the telomerase reverse transcriptase promoter. Tumors of cases with the C228T mutation were significantly larger, but there was no significant association between the C250T mutation and tumor size. Extreme outliers are marked with an asterisk. Mild outliers are marked with a circle. The P-value was calculated using the Mann-Whitney test for results showing comparisons of size between tumors with and without point mutations in the telomerase reverse transcriptase promoter.

mutation was detected in 17 (14.5%) of 117 patients with tumors ≤4.0 cm and in 6 (37.5%) of 16 patients with tumors >4.0 cm, further confirming the significant correlation of C228T with PTC tumor size (P<0.05). Regarding other clinicopathological parameters, we found the C228T mutation in 17 (27.0%) of 63 patients with extraglandular invasion and in 6 (8.6%) of 70 patients without invasion, indicating a significant association of C228T with the presence of extraglandular invasion (P<0.05). Additionally, 13 of the 54 patients (24.1%) with lymph node metastasis and 10 of the 79 patients (12.7%) without metastasis had the C228T mutation; however, the difference was not statistically significant. We did not observe any significant association of the C228T mutation with other factors. In contrast to C228T, we observed that the C250T mutation was not associated with tumor size; however, we noticed that C250T was positively correlated with a younger age (P<0.05). Categorical analysis showed that the C250T mutation tended to be more frequent in patients younger than 55 years (P<0.05). Regarding the other clinicopathological parameters, we found that eight (14.8%) of 54 patients with lymph node metastasis and three (3.8%) of 79 patients without lymph node metastasis had the C250T mutation, indicating that C250T was significantly associated with lymph node metastasis (P<0.05) (Table III).

Co-occurrence of TrSNP and TERT mutations. We detected TrSNP in 56.5% (13/23) of the cases with the C228T mutation and in 45.5% (5/11) of the cases with the C250T mutation.

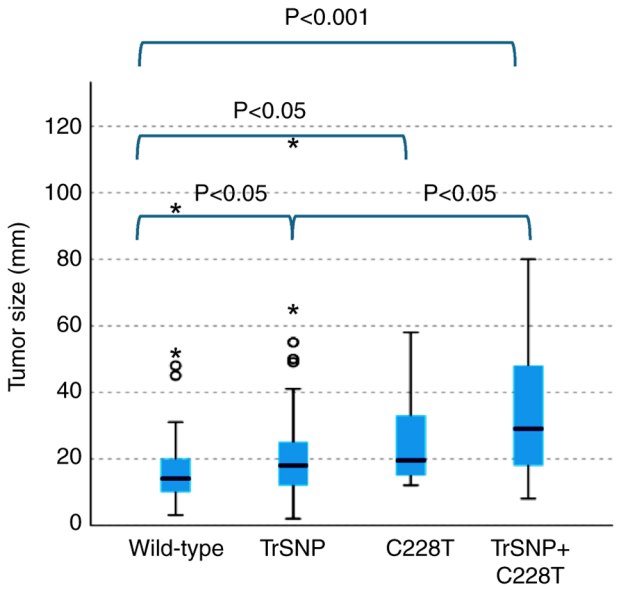

Figure 5. Relationship between telomerase reverse transcriptase: TrSNP (rs2853669) and C228T point mutation and tumor diameter in papillary carcinoma. To analyze the relationship between the C allele of TrSNP and the C228T mutation, statistical analysis of the tumor diameter in the four groups was performed. The group with both the C allele of TrSNP and the C228T mutation had significantly larger tumor diameters than the groups with the wild-type or only the C allele of TrSNP. Extreme outliers are marked with an asterisk. Mild outliers are marked with a circle. The P-value was calculated using the Kruskal-Wallis test, followed by Bonferroni correction. TrSNP, regulatory single nucleotide polymorphism in the *TERT* promoter (rs2853669).

In particular, three patients had TrSNP and both mutations (C228T and C250T). Subsequently, we divided the patients into four groups according to TrSNP and the C228T point mutation and analyzed the relationship between TrSNP and C228T with tumor size and grade. The group with both C228T and TrSNP had larger tumors than those in the group with only the wild-type and TrSNP (Fig. 5).

In categorical analysis, we observed significant associations between the variants and tumor diameter (2.0 cm, 4.0 cm), extraglandular invasion, and lymph node metastasis, with increased numbers of higher grade and larger tumors in the group with the C228T and TrSNP (P<0.05, P<0.05; P<0.05; P<0.05). Additionally, the incidence of extraglandular invasion was higher in the group with both C228T and TrSNP than in the group with TrSNP alone (P<0.05) (Table IV).

Clinicopathological implications of the BRAF^{V600E} mutation. We also detected the *BRAF*^{V600E} mutation in 82 (61.7%) patients. The *BRAF*^{V600E} mutation alone was not significantly associated with any clinicopathologic parameters. Of the 82 patients, 43 (52.4%) carried TrSNP. Additionally, 15 (18.2%) had the C228T mutation, and 5 (6.0%) had the C250T mutation. Eight patients had TrSNP and C228T mutations along with the *BRAF*^{V600E} mutation. An analysis of the association among the *BRAF*^{V600E} mutation, TrSNP and the C228T *TERT* mutation revealed no significant difference in tumor size between the wild-type group and the group with only the *BRAF*^{V600E} mutation. However, when the groups with wild-type and two mutations (*BRAF*^{V600E} and C228T) and the groups with wild-type and three mutations (*BRAF*^{V600E}, C228T and TrSNP) were compared, a significant trend towards larger tumors was observed (P<0.05; Fig. 6). Furthermore, a notable increase in tumor size was observed in the groups with two mutations (*BRAF*^{V600E} and C228T, and *BRAF*^{V600E} and TrSNP) and the group with three mutations (*BRA*F^{V600E}, C228T and TrSNP) compared with that in the group with only the *BRAF*^{V600E} mutation (P<0.05; Fig. 6). Moreover, the group with three mutations (*BRA*F^{V600E}, C228T and TrSNP) exhibited significantly increased tumor progression, including extraglandular invasion, compared with the group with only the *BRAF*^{V600E} mutation (P<0.05; Table V).

Discussion

In recent years, the discovery of oncogenes has prompted a cross-organ approach through the use of molecular targeting therapies. Immune checkpoint inhibitors have emerged as a pioneering approach in this field. For tumors with high PD-1 or PD-L1 expression determined by immunostaining, immune checkpoint inhibitors such as pembrolizumab have been used to suppress tumor growth (37). Response to pembrolizumab has been documented in solid tumors exhibiting high microsatellite instability (MSI) and a high tumor mutation burden (TMB) (38,39). However, in thyroid cancer, the prevalence of high MSI and high TMB is approximately 2% each, thereby restricting the potential for utilizing immune inhibitors in treatment (40).

Thyroid cancer is associated with a small number of actionable genetic abnormalities that have implications for clinical decision-making. The *BRAF*^{V600E} gene has a mutation upstream of the MAPK pathway and the *TERTp* mutations that are unrelated to signaling, C228T and C250T, are of particular interest in PTC, due to their potential correlation with tumor progression. C228T and C250T have been implicated in several multi-organ cancers, including other thyroid carcinomas, hepatocellular carcinoma, bladder cancer, renal pelvic cancer, and glioblastoma. In general, cases with C228T and C250T show higher clinicopathologic malignancy regardless of the organ. As mentioned previously, rs2853669 functions as a regulatory SNP of the *TERTp* located in the proximity of C228T and C250T. It manifests an allelic change from thymine (T) to cytosine (C). Our previous *in vitro* study demonstrated that the C allele of rs2853669 (TrSNP) increases the activity of *TERTp* in thyroid cancer cells and C228T and C250T (31). Some reports have also indicated that TrSNP may interact with C228T and C250T (41). We attempted to detect TrSNP in a simple and accurate blood sample for clinical application due to its status as a germline polymorphism.

The use of blood samples has technical advantages in the extraction of nucleic acids. It increases the accuracy of the genetic analysis and enables preoperative assessment for therapeutic decision-making. In the genetic analysis of blood and FFPE samples, 2.3% of the results were discordant. A lower protein content is indicated by an A260/280 absorbance ratio closer to 1.8-2.0. A lower A260/A230 ratio indicates contamination by organic compounds, such as chaotropic agents that absorb light at 230 nm.

A previous study of 58 cases reported that the TrSNP was found in 58.6% of patients and was significantly

Table IV. Relationship between telomerase reverse transcriptase: TrSNP (rs2853669) and C228T point mutation in papillary carcinoma and tumor size based on category and the presence of extraglandular invasion and lymph node metastasis.

						P-value					
Variable	TrSNP/C228T	-/-	-/+	+/-	+/+	-/- vs. -/+	-/- vs. +/-	-/- vs. +/+	-/+ vs. +/-	-/+ vs. +/+	+/- vs. +/+
Tumor size	<2.0 cm, n	29	4	33	0	0.271	0.780	0.021	0.835	0.666	0.340
	≥2.0 cm, n	5	2	6	6						
	<4.0 cm, n	49	2	27	4	0.228	0.527	0.029	0.596	0.646	0.067
	≥4.0 cm, n	4	8	41	9						
Extraglandular invasion	EX0, n	34	4	30	2	0.297	0.444	0.009	0.505	0.348	0.026
	EX1,2, n	19	6	27	11						
Lymph node metastasis	N0, n	36	6	33	4	0.733	0.558	0.054	0.949	0.391	0.124
	>N1a, n	17	4	24	9						

The P-value was calculated using Fisher's exact test followed by Bonferroni's post hoc test. EX, extraglandular invasion; TrSNP, regulatory single nucleotide polymorphism in the *TERT* promoter (rs2853669).

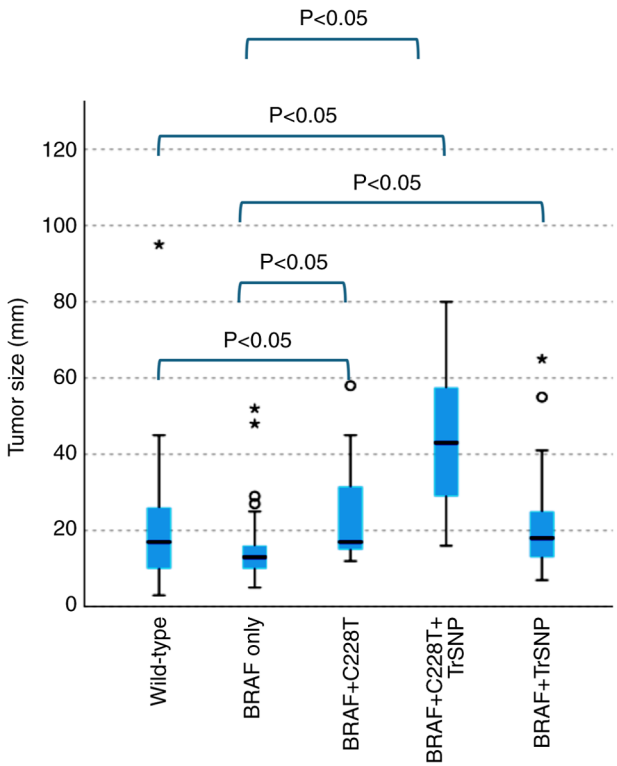

Figure 6. Association between C228T, TrSNP and *BRAF*^{V600E} mutations and tumor size in papillary thyroid cancer. There was no difference in tumour size between the wild-type group and the *BRAF*^{V600E}-only group. However, tumours in the groups with two (*BRAF*^{V600E} + C228T or *BRAF*^{V600E} + TrSNP) or three mutations (*BRAF*^{V600E} + C228T + TrSNP) were significantly larger compared with those in the *BRAF*^{V600E}-only group. Extreme outliers are marked with an asterisk. Mild outliers are marked with a circle. The P-value was calculated using the Kruskal-Wallis test, followed by Bonferroni correction. TrSNP, regulatory single nucleotide polymorphism in the *TERT* promoter (rs2853669).

associated with the size of PTC tumors (33). In the present study, we examined more cases and found that TrSNP was found in 52.6% of 133 patients with PTC and was significantly associated with tumor size (P<0.05), similar to the findings of previous studies. The frequency of TrSNP as a genetic polymorphism was previously reported by Muzza *et al* (42) in an Italian cohort of differentiated thyroid cancer. In the present study, TrSNP was found in 44.4% of 254 patients; a higher rate than that previously detected. This discrepancy suggested that such polymorphisms may differ among ethnic groups (43). Although we did not detect any homozygous forms of TrSNP in patients with PTC in our previous studies, in the present study, we clearly showed that the rate of the homozygous genotype of the C allele (CC) in PTC cases was 10.5%, that of the heterozygous genotype (CT) was 42.1%, whereas that of the wild type (TT) was 47.4%. The frequency of the C allele was 31.6% (84/266 genes), which we believe was slightly higher than the 29.3% reported in a previous study owing to the increased number of cases, increased accuracy of the analysis, and improved waveform definitions. A significant difference in tumor size was observed between wild-type and heterozygous samples; however, no significant correlation was observed for homozygous samples owing to the small number of cases. Hence, this required further evaluation in future studies using larger sample sizes. The allele C frequency of rs2853669 in the population of Tokyo is 24.5% (44). A meta-analysis to determine the association between TrSNP and lung cancer risk shows that the frequency of the wild type in healthy Asians was 10.4%, that of heterozygous was 43.1%, and that of homozygous 46.5%, with the C allele frequency being 32.0% (1237/3984 genes) (22). The authors reported that the homozygous form of TrSNP was significantly associated with lung cancer risk (22). In the present study, the frequency of the C allele in PTC cases was 31.6%, similar to the reported statistics for healthy Asians. Additionally, homozygosity for TrSNP alone did not confer a significant risk for PTC. This suggested that TrSNP is not involved in the development of PTC. In a 2015 study, TrSNP was associated with malignancy but not with the risk of developing glioblastoma (45). Despite the differences in the effects of TrSNP depending on the type of cancer, the individual effect of TrSNP on cancer

Table V. Association of C228T, TrSNP and BRAF with grade in papillary thyroid cancer.

Variable	BRAF(+) only, n (%) (n=33)	BRAF(+) C228T(+) TrSNP(+), n (%) (n=8)	P-value
Extraglandular invasion			0.015
Absent	19 (57.6)	1 (12.5)	
Present	14 (42.4)	7 (87.5)	
Lymph node metastasis			0.070
Absent	20 (60.6)	2 (25.0)	
Present	13 (39.4)	6 (75.0)	

The P-value was calculated using Fisher's exact test. TrSNP, regulatory single nucleotide polymorphism in the *TERT* promoter (rs2853669).

development has not been determined (23). The correlation of TrSNP with tumor size shown in previous studies of PTC was more clearly confirmed in the present study with a larger number of cases (P<0.05). Furthermore, the coexistence of TrSNP with the C228T point mutation in the *TERTp* was associated with tumor size and cancer progression. The frequencies of both variants in previous studies ranged from 4.7 to 25.5%, and the frequencies in the present study did not differ from those previously reported (14,46,47). Additionally, in the present study, both C228T and C250T mutations were found in 3.8% of cases, which is a rare finding. Notably, these were cases of patients with advanced cancer with lymph node metastasis. This was consistent with the fact that point mutations in *TERTp* (in addition to tumor size) are associated with PTC grade, including extraglandular invasion, lymph node metastasis, and recurrence. This study also showed that C228T was significantly associated with tumor size and tumor grade, such as extraglandular invasion. Additionally, C250T was associated with lymph node metastasis, although the number of cases was small. Moreover, the coexistence of TrSNP with the C228T mutation was more strongly associated with cancer progression, including extraglandular invasion and lymph node metastasis, as well as with tumor size compared with the single presence of the C228T mutation. Overall, TrSNP in *TERTp* may increase the risk of tumor growth and progression associated with the C228T point mutation.

In this study, the frequency of the *BRAF*^{V600E} mutation in PTC was 61.7%, which was at the upper limit of the 38-62% range previously reported (48,49). There is a divergence of opinions regarding the impact of the *BRAF*^{V600E} mutation alone on the grade and prognosis of PTC. Some claim that there is no association, whereas others have reported that *BRAF*^{V600E} in PTC is associated with extraglandular invasion (50) and lymph node metastasis (51). No significant association with clinicopathologic parameters was observed in the present study in cases with *BRAF*^{V600E} mutation only. However, cases with *BRAF*^{V600E} accompanied by *TERTp* mutations were correlated with tumor size and extraglandular invasion, similar to C228T mutations. The prognosis cannot be discussed in this study due to the short observation period. Muzza *et al* (42) reported that *TERTp* mutations (but not *BRAF*^{V600E}) affect the prognosis of thyroid cancer. Liu *et al* (14) also observed that the hazard ratio (HR) for PTC-specific mortality in patients with *BRAF* ^{V600E} mutations was 3.08 (95% CI, 0.87-10.84) compared to patients without mutations, while the HR for *BRAF* ^{V600E} alone was 8.18 (95% CI, 2.04-32.5). The specific mortality for cases with both mutations remained significant after adjustment for clinicopathologic factors (HR, 9.34; 95% CI, 2.53-34.48), indicating that the coexistence of the *TERT* and *BRAF* genes is more strongly associated with mortality than either mutation alone (14). Similarly, Moon *et al* (15) reported that mortality associated with PTC was significantly higher in patients with coexisting mutations than in those with *BRAF*^{V600E} alone. Chung (17) also concluded that the coexistence of *BRAF* and *TERTp* mutations was associated with increased relapse and mortality and worse survival. Another meta-analysis of 3911 patients with PTC showed that tumors with concurrent *BRAF*^{V600E} and *TERTp* mutations were more aggressive than those with *BRAF*^{V600E} or *TERTp* mutations alone (48). Another study of 653 patients with PTC also reported that the coexistence of *BRAF*^{V600E} and *TERTp* mutations was associated with extraglandular invasion, and it was concluded that certain clinicopathologic features increased the risk of extraglandular invasion compared with that in the group without either mutation (52). As shown in the present study, extraglandular invasion was significantly more common when the *BRAF*^{V600E} mutation coexisted with a *TERT* point mutation. In addition, when TrSNP was present, the tumor was larger and there was a higher incidence of extraglandular invasion. These findings suggested that the coexistence of *BRAF*^{V600E}, *TERT* point mutations, and TrSNP may increase the risk of PTC growth and progression. Therefore, careful follow-up is required for carriers of TrSNP regardless of their tumor size and grade. Furthermore, the presence or absence of C228T and *BRAF*^{V600E} mutations in cytology specimens can be utilized to infer the grade of malignancy exhibited by individual papillary carcinomas. This information is particularly valuable in the context of patient treatment planning. Nevertheless, inconsistencies have been noted between the genetic variants identified in cytology and FFPE specimens (53). This discrepancy must be validated in a subsequent step.

Molecularly targeted therapies are being developed based on the specific gene expression profile of the patient. The ATA guidelines recommend using the *BRAF* inhibitor dabrafenib for patients carrying *BRAF* ^{V600E} mutations (54,55). Further research can validate *TERTp* mutations as a novel therapeutic target. Although treatment with eribulin is an option for malignant meningiomas and soft tissue sarcomas, it is still in the early stages of development (56,57).

This study has some limitations. It was a retrospective study conducted at a single institution. Prospective studies conducted at multiple institutions should be considered for further validation with a larger number of cases. The prognosis of thyroid cancer is favorable; therefore, a prolonged period of observation is necessary to evaluate the risk of recurrence and the overall prognosis.

In conclusion, based on the analysis of 133 patients with PTC, TrSNP in the *TERTp* region and the C228T mutation were significantly correlated with the PTC tumor size and grade. In particular, the TrSNP and *TERTp* mutations, particularly C228T increased the *TERTp* activity. In this study, we elucidated the involvement of TrSNP and C228T in the adverse biology of PTC. The coexistence of the *BRAF*^{V600E} mutation with TERTp mutations and TrSNP was significantly associated with tumor size as well as extraglandular invasion indicating a higher malignant potential. Therefore, the TrSNP polymorphism accompanied by the C228T point mutation and the *BRAF*^{V600E} mutation could serve as potential molecular markers for tumor growth or exacerbation of PTC. As the diagnosis of thyroid cancer is based on cytologic analysis, it is in the patient's best interest to confirm the nature of the tumor by oncogene testing of a preoperative cytologic specimen. Preoperative genetic testing of the tumor and assessing the risk of progression can assist in decision-making, including the decision to proceed with surgery. Therefore, it is recommended that TrSNP carriers undergo careful follow-up regardless of tumor size or grade. It would be beneficial to consider the possibility of testing cytology specimens for the C228T and *BRAF*^{V600E} mutations.

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Availability of data and materials

The data generated in the present study are not publicly available due to the institution's policy but may be requested from the corresponding author.

Authors' contributions

YN and HKa designed the study, wrote the first draft of the manuscript and confirm the authenticity of all the raw data. KH and RT contributed to data analysis and interpretation and assisted in drafting the manuscript. TC and MF contributed to data analysis. YA, TH, TM and HKo contributed to data collection and interpretation, and critically reviewed the manuscript. All authors agree to take responsibility for all aspects of the work to ensure that questions about the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Review Committee of the Faculty of Medicine of Kyorin University on April 28, 2023 (No. R01-002_759-02; Mitaka, Tokyo, Japan). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Use of artificial intelligence tools

During the preparation of this work, artificial intelligence tools were used to improve the readability and language of the manuscript or to generate images, and subsequently, the authors revised and edited the content produced by the artificial intelligence tools as necessary, taking full responsibility for the ultimate content of the present manuscript.

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