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Article

Genetic trio of *BRAF* and *TERT* alterations and rs2853669TT in papillary thyroid cancer aggressiveness

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Abstract

Background: *BRAF* V600E and *TERT* promoter alterations are core components in current genetics-based risk assessment for precision management of papillary thyroid cancer. It remains unknown whether this approach could achieve even better precision through a widely recognized prognostic single-nucleotide variation (SNV, formerly SNP), rs2853669T>C, in the *TERT* promoter.

Methods: The genetic status of alterations and SNV were examined by sequencing genomic DNA from papillary thyroid cancer in 608 patients (427 women and 181 men) aged 47 years (interquartile range = 37-57), with a median follow-up time of 75 months (interquartile range = 36-123), and their relationship with clinical outcomes was analyzed. A luciferase reporter assay was performed to examine *TERT* promoter activities.

Results: *TERT* promoter alterations showed a strong association with papillary thyroid cancer recurrence in the presence of genotype TT of rs2853669 (adjusted hazard ratio [HR] = 2.12, 95% confidence interval [CI] = 1.10 to 4.12) but not TC/CC (adjusted HR = 1.17, 95% CI = 0.56 to 2.41). *TERT* and *BRAF* alterations commonly coexisted and synergistically promoted papillary thyroid cancer recurrence. With this genetic duet, TT of rs2853669 showed a robustly higher disease recurrence than TC/CC (adjusted HR = 14.26, 95% CI = 2.86 to 71.25). Patients with the genetic trio of *BRAF* V600E, *TERT* alteration, and TT of rs2853669 had a recurrence of 76.5% vs recurrence of 8.4% with neither variation and with TC/CC (HR = 13.48, 95% CI = 6.44 to 28.21). T allele of rs2853669 strongly increased *TERT* promoter activities, particularly the variant promoters.

Conclusions: The SNV rs2853669T>C dramatically refines the prognostic power of *BRAF* V600E and *TERT* promoter alterations to a higher precision, suggesting the need for including this SNV in the current genetics-based risk prognostication of papillary thyroid cancer.

Papillary thyroid cancer is common, accounting for nearly 90% of all thyroid cancers (1,2). Clinical prognosis of papillary thyroid cancer varies widely, with 10% to 15% of cases being inherently aggressive, with high recurrence and mortality, and the remainder of cases being generally indolent, making accurate risk assessment important to balance the benefits of treatment against the risks associated with them (3,4). This prognosis can be assisted by molecular-based risk assessment of thyroid cancer, particularly papillary thyroid cancer, in which genetics-based risk prognostication and precision management are becoming a reality (3,5-8). In this regard, 2 prominent prognostic genetic markers, *BRAF* V600E and *TERT* gene promoter variations, play a central role. *BRAF* V600E is the most common oncogenic alteration in papillary thyroid cancer, which exerts oncogenicity through constitutively activating the mitogen-activated protein kinase pathway (9). There are 2 common *TERT* promoter alterations: 1 295 228 C>T (C228T) and 1 295 250 C>T (C250T) (10,11). They generate consensus binding sites for the transcriptional GA binding protein complex to bind and oncogenically activate *TERT* (12,13). The *BRAF* and *TERT* alterations have been widely reported to be associated with tumor aggressiveness and poor clinical outcomes of papillary thyroid cancer (14-24). A notable phenomenon of *BRAF* V600E and *TERT* promoter alterations is their common coexistence to form a distinct genetic duet in papillary thyroid cancer, as initially reported in 2013 (17); this coexistence has consistently been found to be associated with tumor aggressiveness, disease recurrence, and patient mortality in papillary thyroid cancer (19,20,22,24-35). This genetic duet largely distinguishes the 10% to 15% of patients with aggressive papillary thyroid cancer from the majority with indolent disease (22,28,36). Therefore, *BRAF* V600E and *TERT* promoter alterations are currently the essential components of genetics-based risk prognostication strategies for papillary thyroid cancer, which are increasingly recognized and advocated for precision management of papillary thyroid cancer (3,7,10,11,37-39).

A common single-nucleotide variation (SNV, formerly SNP), rs2853669T>C, which is –245 base pairs from the translational start site in the core region of the *TERT* promoter, has been recognized as being capable of modifying the prognostic value of *TERT* promoter alterations for patient survival, particularly in glioblastoma and bladder cancer (40,41). When this SNV was initially identified more than a decade ago, it was found to disrupt the ETS proto-oncogene 2 (ETS2) binding site in the *TERT* promoter and consequently reduce *TERT* activities (42). Artificially induced

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point alterations in the ETS2 binding site similarly compromised *TERT* activities (43). We therefore hypothesized that SNV rs2853669T>C could differentiate the disease aggressiveness risk associated with *BRAF* V600E and *TERT* promoter alterations in papillary thyroid cancer and therefore refine their prognostic precision.

Methods

Patient and clinicopathological data

A total of 608 consecutive patients with papillary thyroid cancer who were treated and clinically followed up for papillary thyroid cancer at Johns Hopkins Hospital between January 1, 1990, and December 31, 2015, were included in the present study. Data were analyzed from January 30, 2019, to June 18, 2023. All patients received total or near-total thyroidectomy. Clinicopathological data were collected from medical records. The pathological diagnoses of papillary thyroid cancer were established according to World Health Organization criteria. Tumor stages were defined according to the American Joint Committee on Cancer Cancer Staging Manual, 8th edition, staging system for thyroid cancer (44). Tumor recurrence in this study was defined as recurrent or persistent structural tumor existence diagnosed by imaging and confirmed by radioactive iodine scanning, biopsy, or pathological examination. Patient follow-up time was defined as the interval from initial thyroidectomy to the most recent clinical contact date or, in the case of patients with papillary thyroid cancer recurrence, the date of discovery of disease recurrence. The study was approved by the institutional review board of Johns Hopkins University School of Medicine, and informed consent, when appropriate, was obtained from patients. This study followed the Strengthening the Reporting of Observational Studies in Epidemiology reporting guideline for cohort studies.

Mutational analyses

Genomic DNA was isolated from primary papillary thyroid cancer using the standard phenol-chloroform extraction and ethanol precipitation procedures. Exon 15 of the *BRAF* gene and the core region of the *TERT* promoter were amplified using polymerase chain reaction testing for BigDye reaction, followed by Sanger sequencing (22,41). The SNV and *TERT* promoter alterations were sequenced in the same polymerase chain reaction test.

Cell lines and cell culture

Human papillary thyroid cancer–derived cell line TPC1 (obtained from Dr Alan P. Dackiw, Johns Hopkins University) was used to test activities of introduced *TERT* promoter in the pGL3 luciferase reporter constructs under various genetic conditions. Cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Rochester, MN) at 37 °C in a humidified environment with 5% carbon dioxide.

Luciferase reporter activity assay for *TERT* promoter

The pGL3 luciferase reporter constructs containing allele C of rs2853669 in the wild-type, C228T, or C250T *TERT* promoter were generated as described previously (45). When desired, allele T of rs2853669 was generated in the above plasmids using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) with primers (forward: 5′-GCCACGTGGGAA GCGCGGTCCTGG-3′; reverse: 5′-CCAGGACCG CGCTTCCCACGTGGC-3′).

For the promoter activity assay, TPC1 cells were seeded in triplicate on a 24-well plate and transfected with 300 ng of pGL3 luciferase reporter plasmids containing the indicated *TERT* promoter variant together with 12 ng of thymidine kinase promoter Renilla luciferase plasmid (normalizing control) using the jetPRIME transfection reagent (Polyplus, Illkirch, France). At 24 hours after transfection, cells were lysed and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Three independent experiments were conducted, and each was performed in triplicate. Results were reported as relative luciferase activities by dividing firefly luciferase values with Renilla luciferase values.

Statistical analysis

Continuous data were summarized using medians with interquartile ranges or means with SDs, and categorical data were summarized using frequencies and percentages. Categorical variables were compared using the χ^2 test or, in the case of small samples, the Fisher exact test. The Wilcoxon-Mann-Whitney test was used for non-normally distributed continuous variables, and the independent t test was used for normally distributed continuous variables. Kaplan-Meier survival curves with log-rank tests were used to compare recurrence-free survival (RFS) by genetic status. Cox regression analyses were used to assess the associations of genetic variants with disease recurrence. The proportional hazards assumption was tested on the basis of Schoenfeld residuals. Potential confounding variables, including patient age, sex, multifocality, tumor size, extrathyroidal invasion, vascular invasion, and lymph node metastasis, were adjusted. The threshold for statistical significance was 2-tailed P less than .05. Analyses were performed using Stata/SE software, version 10.1 for Windows (StataCorp LLC, College Station, TX), and GraphPad Prism software, version 6.0 for Windows (GraphPad Software, La Jolla, CA).

Results

Association of SNV rs2853669T>C alone with the aggressiveness of papillary thyroid cancer

The clinicopathological characteristics of the 608 patients with papillary thyroid cancer included in the present study are presented in Supplementary Table 1 (available online). Overall, the median (interquartile range) age was 47 (37-57) years; 427 patients (70.2%) were women, and 181 (29.8%) were men. The prevalence of *BRAF* V600E was 31.7% (193 patients), and the prevalence of the *TERT* promoter variation was 11.5% (70 patients). The prevalence of the TT genotype of rs2853669 was 44.9% (273 patients), and the prevalence of the TC and CC (hereinafter, TC/CC) genotype was 55.1% (335 patients). All patients were followed up for a median (interquartile range) of 75 (36-123) months, representing 4232.25 person-years. The structural recurrence of papillary thyroid cancer was 18.1% (110 patients), with a recurrence rate of 25.99 cases per 1000 person-years (95% confidence interval [CI] = 21.56 to 31.33 cases per 1000 person-years). In the overall analysis of the entire cohort, there was generally no substantial difference in papillary thyroid cancer characteristics between the TT genotype and the TC/CC genotype of rs2853669, except for statistically larger tumor size and insignificantly higher prevalence of distant metastasis and structural tumor recurrence in the former.

In Kaplan-Meier analyses of all cases, SNV rs2853669T>C was not associated with overall RFS; patients with the TT genotype had a slightly higher rate of papillary thyroid cancer recurrence

than did those with the TC/CC genotype (Supplementary Figure 1, A, available online). In contrast, *BRAF* V600E and *TERT* promoter variation were each associated with an accelerated decline in RFS (Supplementary Figure 1, B and C, available online). Cox proportional hazards analyses also revealed that SNV rs2853669T>C was not associated with papillary thyroid cancer recurrence, with an adjusted hazard ratio (HR) of 0.85 (95% CI = 0.57 to 1.27; $P = .43$) for the TT vs TC/CC genotype (Supplementary Table 2, available online). *BRAF* V600E was associated with a statistically higher risk of papillary thyroid cancer recurrence, with an adjusted HR of 2.11 (95% CI = 1.37 to 3.26; $P = .001$). The *TERT* promoter alteration had a statistically significant unadjusted HR of 2.73 (95% CI = 1.77 to 4.20; $P < .001$) and insignificant adjusted HR of 1.52 (95% CI = 0.94 to 2.46; $P = .09$).

Association of *BRAF* V600E and *TERT* promoter variations with papillary thyroid cancer recurrence risk by SNV rs2853669T>C genotype status

When the entire cohort of patients was divided into TT and TC/CC genotypes of rs2853669 to examine the risk of papillary thyroid cancer recurrence, *BRAF* V600E had a statistically significant adjusted HR of 3.15 (95% CI = 1.70 to 5.81; $P < .001$) in the presence of the TT genotype and a statistically insignificant adjusted HR of 1.54 (95% CI = 0.79 to 3.02; $P = .21$) in the presence of the TC/CC genotype (Table 1). Similarly, the *TERT* promoter variation had a statistically significant adjusted HR of 2.12 (95% CI = 1.10 to 4.12; $P = .03$) in the presence of the TT genotype but an insignificant adjusted HR of 1.17 (95% CI = 0.56 to 2.41; $P = .68$) in the presence of the TC/CC genotype (Table 1). These results suggested that although rs2853669 alone had no association with tumor recurrence, it modified the associations of the alterations: The TT genotype robustly cooperated with *BRAF* V600E and *TERT* promoter alterations to increase the risk of papillary thyroid cancer recurrence, while the TC/CC genotype decreased or even eliminated the recurrence risk associated with the variations.

Consistent with previous findings (22), the present study found a robust synergism between *BRAF* V600E and *TERT* promoter variations in increasing the risk of papillary thyroid cancer recurrence (Figure 1, A; Supplementary Table 3, available online). Specifically, in the analysis of the entire patient cohort, recurrence rates were 64.5% (20 of 31 patients) in those harboring both variants vs 10.1% (38 of 376 patients) in those not harboring either variant, with an adjusted HR of 3.67 (95% CI = 1.75 to 7.70; $P = .001$). Correspondingly, the RFS curves revealed a moderate decline with *BRAF* V600E or *TERT* promoter variation alone but a sharp decline with the genetic duet of the 2 coexisting variations (Figure 1, A).

When dissecting *BRAF* V600E from the *TERT* promoter variations and examining the association of SNV rs2853669T>C with tumor recurrence in patients with each variation alone (without overlapping of the 2 variations), those with the TT vs TC/CC genotype had no statistically significant difference (Table 2). In contrast, among patients with the genetic duet of *BRAF* V600E and *TERT* promoter variations, the TT genotype of rs2853669 was associated with statistically higher papillary thyroid cancer recurrence than the TC/CC genotype, with an adjusted HR of 14.26 (95% CI = 2.86 to 71.25; $P = .001$). These results suggested that the TT genotype of rs2853669 required the presence of both *BRAF* V600E and *TERT* promoter variation to be implicated in the most aggressive forms of papillary thyroid cancer.

We further investigated the differentiating role of SNV rs2853669T>C in the prognostic precision of *BRAF* V600E and *TERT* promoter variations by dividing patients into 8 genotype groups according to the genetic status of *BRAF*, *TERT*, and SNV rs2853669T>C (Table 3 and Figure 1, B). The risk of recurrence with the TT genotype was highest when coexisting with the genetic duet of *BRAF* V600E and *TERT* promoter variations. The genetic trio of coexisting *BRAF* V600E, *TERT* promoter variation and TT genotype of rs2853669 was associated with a recurrence rate of 76.5% (13 of 17 patients) vs 8.4% (18 of 214 patients) in those with the TC/C genotype who were not harboring either alteration, with an unadjusted HR of 13.48 (95% CI = 6.44 to 28.21; $P < .001$); this hazard ratio remained statistically significant at 6.96 (95% CI = 2.39 to 20.27; $P < .001$) after multivariable adjustment (Table 3). In the presence of the TC/CC genotype of rs2853669, the genetic duet of *BRAF* V600E and *TERT* promoter variation had a substantially lower unadjusted HR of 5.65 (95% CI = 2.36 to 13.54), which became insignificant at 1.19 (95% CI = 0.29 to 4.86) after multivariable adjustment. It should be noted, however, that such adjustments are not entirely valid (46). From a biological perspective, if the clinicopathological characteristics (ie, tumor behaviors) are biologically promoted by the oncogenic variations, to adjust them may artificially nullify the consequences of the variations, resulting in a misleading underestimation of their biologically induced clinical impacts. Nevertheless, even after adjustment, the trio of coexisting *BRAF* V600E, *TERT* promoter variation and TT genotype of rs2853669

Table 1. Association of single-nucleotide variation rs2853669T>C genotype with the prognostic precision of *BRAF* V600E and *TERT* promoter variations

	Recurrence rate		1000 person-year recurrence		Hazard ratio (95% confidence interval)	
Genotype	n/N (%)	P	Rate	95% confidence interval	Unadjusted	Adjusted[a]
TT of rs2853669						
Without *BRAF* V600E	24/182 (13.2)	—	16.78	11.25 to 25.03	(Referent)	(Referent)
With *BRAF* V600E	34/91 (37.4)	<.001	63.19	45.15 to 88.43	3.41 (2.02 to 5.77)	3.15 (1.70 to 5.81)
TC/CC of rs2853669						
Without *BRAF* V600E	23/233 (9.9)	—	13.79	9.16 to 20.75	(Referent)	(Referent)
With *BRAF* V600E	29/102 (28.4)	<.001	48.65	33.81 to 70.01	3.21 (1.85 to 5.55)	1.54 (0.79 to 3.02)
TT of rs2853669						
Without *TERT* variation	41/236 (17.4)	—	23.65	17.41 to 32.12	(Referent)	(Referent)
With *TERT* variation	17/37 (45.9)	<.001	72.42	45.02 to 116.49	2.87 (1.63 to 5.05)	2.12 (1.10 to 4.12)
TC/CC of rs2853669						
Without *TERT* variation	40/302 (13.2)	—	19.69	14.44 to 26.84	(Referent)	(Referent)
With *TERT* variation	12/33 (36.4)	<.001	51.69	29.35 to 91.01	2.62 (1.38 to 5.00)	1.17 (0.56 to 2.41)

[a] Adjustment was made for patient age at diagnosis, sex, tumor multifocality, tumor size, extrathyroidal invasion, vascular invasion, and lymph node metastasis.

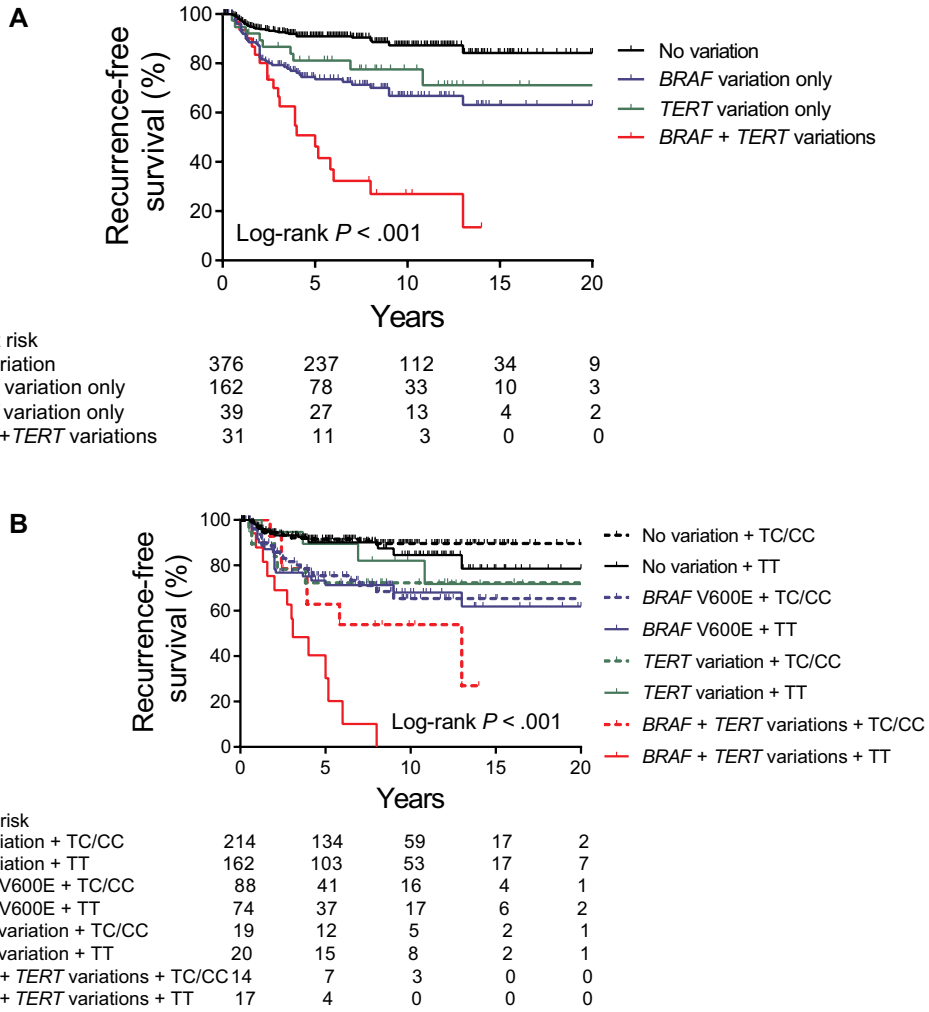

Figure 1. Kaplan-Meier analysis of the synergistic associations of genetic variants with disease-free survival of patients with papillary thyroid cancer. (**A**) Synergistic effects of *BRAF* V600E and *TERT* promoter variations. (**B**) Synergistic effects of *BRAF* V600E, *TERT* promoter variation, and genotype TT of rs2853669. The curves are truncated at 20 years of follow-up.

Table 2. Differentiating role of single-nucleotide variation rs2853669T>C genotype in the prognostic precision of the genetic duet of *BRAF* V600E and *TERT* promoter variation

Mutation status / Single-nucleotide variation status	Recurrence rate		1000 person-year recurrence		Hazard ratio (95% confidence interval)	
	n/N (%)	*P*	Rate	95% confidence interval	Unadjusted	Adjusted[a]
No variation						
TC/CC	18/214 (8.4)	—	11.80	7.43 to 18.72	(Referent)	(Referent)
TT	20/162 (12.3)	.21	16.00	10.32 to 24.80	1.42 (0.75 to 2.68)	1.01 (0.46 to 2.22)
BRAF variation only						
TC/CC	22/88 (25.0)	—	43.49	28.63 to 66.04	(Referent)	(Referent)
TT	21/74 (28.4)	.63	43.40	28.30 to 66.57	1.07 (0.59 to 1.95)	1.09 (0.58 to 2.06)
TERT variation only						
TC/CC	5/19 (26.3)	—	35.21	14.66 to 84.60	(Referent)	(Referent)
TT	4/20 (20.0)	.64	22.16	8.32 to 59.05	0.65 (0.17 to 2.44)	0.0009 (1.23e-06 to 0.60)
BRAF + *TERT* variation						
TC/CC	7/14 (50.0)	—	77.63	37.01 to 162.85	(Referent)	(Referent)
TT	13/17 (76.5)	.13	239.63	139.14 to 412.69	3.30 (1.22 to 8.92)	14.26 (2.86 to 71.25)

[a] Adjustment was made for patient age at diagnosis, sex, multifocality, tumor size, extrathyroidal invasion, vascular invasion, and lymph node metastasis.

still had a statistically significant HR of 6.96 (95% CI = 2.39 to 20.27), suggesting a strong tumor-promoting function of the TT genotype compared with the TC/CC genotype when cooperating with *BRAF* V600E and *TERT* promoter variations. Similar results were observed in conventional-variant papillary thyroid cancer (Supplementary Table 4 and Supplementary Figure 2, available online).

Modulation of *TERT* promoter activities by SNV rs2853669T>C

We used in vitro luciferase reporter assays to examine the relationship between SNV rs2853669T>C and *TERT* promoter activities with different genetic variants of the *TERT* promoter. The *TERT* promoter variation increased the promoter activities to 2 to

Table 3. Associations of *BRAF* V600E, *TERT* promoter variation, and single-nucleotide variation rs2853669T>C genotype with the recurrence of papillary thyroid cancer

Mutation/single-nucleotide variation status	Recurrence rate		1000 person-year recurrence		Hazard ratio (95% confidence interval)	
	n/N (%)	P	Rate	95% confidence interval	Unadjusted	Adjusted[a]
No variation + TC/CC	18/214 (8.4)	—	11.80	7.43 to 18.72	(Referent)	(Referent)
No variation + TT	20/162 (12.3)	.21	16.00	10.32 to 24.80	1.42 (0.75 to 2.68)	1.01 (0.46 to 2.22)
BRAF V600E + TC/CC	22/88 (25.0)	<.001	43.49	28.63 to 66.04	3.35 (1.80 to 6.25)	2.26 (1.09 to 4.72)
BRAF V600E + TT	21/74 (28.4)	<.001	43.40	28.30 to 66.57	3.56 (1.90 to 6.69)	2.27 (1.08 to 4.76)
TERT variation + TC/CC	5/19 (26.3)	.01	35.21	14.66 to 84.60	3.12 (1.16 to 8.40)	3.78 (1.05 to 13.61)
TERT variation + TT	4/20 (20.0)	.10	22.16	8.32 to 59.05	2.09 (0.71 to 6.19)	0.79 (0.17 to 3.64)
BRAF + *TERT* variations + TC/CC	7/14 (50.0)	<.001	77.63	37.01 to 162.85	5.65 (2.36 to 13.54)	1.19 (0.29 to 4.86)
BRAF + *TERT* variations + TT	13/17 (76.5)	<.001	239.63	139.14 to 412.69	13.48 (6.44 to 28.21)	6.96 (2.39 to 20.27)

[a] Adjustment was made for patient age at diagnosis, sex, multifocality, tumor size, extrathyroidal invasion, vascular invasion, and lymph node metastasis.

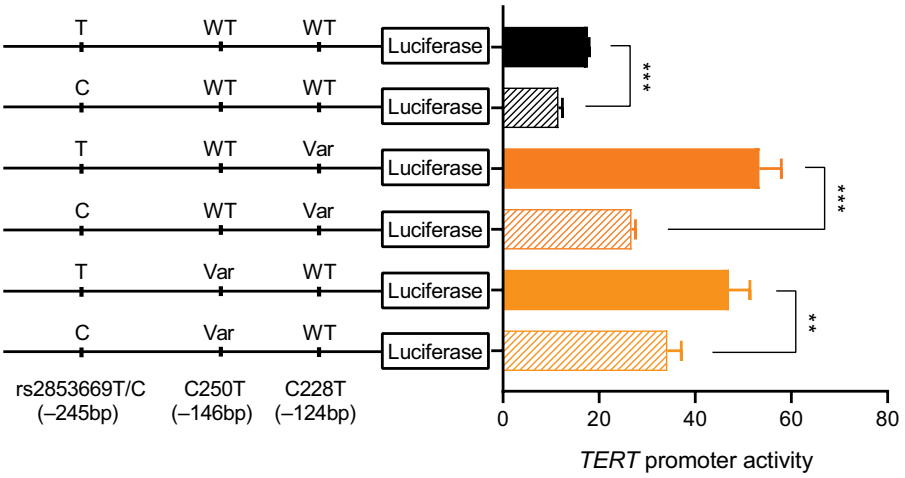

Figure 2. Luciferase report assay of activities of the *TERT* promoter with various genetic conditions. Luciferase reporter constructs containing various genetic variant combinations of the *TERT* promoter were transfected together with Renilla luciferase plasmid into papillary thyroid cancer cell–derived TPC1 cells for 24 hours, followed by measurement of the luciferase activities using the Dual-Luciferase Reporter Assay System. $^{**}P < .01$, $^{***}P < .001$ from the independent *t* test. bp = base pair; Var = variation; WT = wild type.

3 times those of the wild-type *TERT* promoter (Figure 2). Allele T of rs2853669 was associated with robustly higher *TERT* promoter activities compared with allele C; these higher activities were particularly prominent in the variant *TERT* promoter. These results were consistent with and explained the observations of the differentiating roles of rs2853669 in the prognostic precision of *BRAF* V600E and *TERT* promoter variations in estimating the risk of papillary thyroid cancer recurrence.

Discussion

This study demonstrated that the *BRAF* V600E and *TERT* promoter variation–centered molecular prognostic strategy for estimating papillary thyroid cancer outcomes could be refined to even higher precision by including SNV rs2853669T>C. Specifically, SNV rs2853669T>C could robustly differentiate the recurrence risk of papillary thyroid cancer associated with *BRAF* V600E and *TERT* promoter variations. In general, the TT genotype of rs2853669 synergized with the variations, while the TC/CC genotype decreased and even eliminated the consequences of the variations for papillary thyroid cancer aggressiveness. A large meta-analysis also revealed that the TT genotype of rs2853669 in coexistence with the *TERT* promoter variation was associated with poor clinical outcomes of some human cancers (40). SNV rs2853669T>C was found to particularly modify the prognostic precision of *TERT* promoter variations for estimating outcomes in bladder cancer (41), glioblastoma (47,48), clear cell renal cell carcinoma (49), and melanoma (50). Unlike the present study, these previous studies only examined the relationship of the SNV with the *TERT* promoter variation, not the genetic duet of *BRAF* and *TERT* variations. In fact, without the *BRAF* variation, the SNV had a limited role in the prognostic value of the *TERT* promoter variation.

The most notable finding in the present study was the association of SNV rs2853669T>C with the statistically greater prognostic precision of the genetic duet of *BRAF* V600E and *TERT* promoter variations; this prognostic refining by SNV rs2853669T>C was more robust for the genetic duet of the 2 variations than for the individual variation alone. Specifically, the genetic duet of *BRAF* V600E and *TERT* promoter variation was robustly associated with papillary thyroid cancer recurrence in the presence of the TT genotype of rs2853669; patients harboring the trio of *BRAF* V600E, *TERT* promoter variation, and TT genotype of rs2853669 had the worst clinical outcomes, while patients with the TC/CC genotype and neither variation had the best prognosis. These data had important new prognostic implications beyond the current knowledge and prognostic use of *BRAF* V600E and *TERT* promoter variations in papillary thyroid cancer. The genetic duet of *BRAF* V600E and *TERT* promoter variation and its association with tumor aggressiveness have been also reported in other cancers (51). The genetic trio of *BRAF* V600E, *TERT* promoter variation, and TT genotype of rs2853669 likely also has prognostic value in other cancers (52).

Because SNV rs2853669 can differentiate the aggressiveness of thyroid cancer, it is possible that it may differentiate the malignancy risk of indeterminate thyroid nodules, driving the malignancy risk toward 1 direction or the other on fine-needle aspiration biopsy. This will be an interesting study in the future.

The molecular mechanism underlying the interaction of SNV rs2853669T>C with *BRAF* V600E and *TERT* promoter variations in affecting the aggressiveness of papillary thyroid cancer remains to be elucidated but is likely associated with the regulatory machinery of the *TERT* promoter. SNV rs2853669T>C is in the ETS2 binding site; like the *TERT* variation sites, the ETS2 binding site is located within the proximal core promoter region of the *TERT* gene. The present study found that allele T of rs2853669 was associated with robustly increased *TERT* promoter activities compared with allele C; allele T was required to sustain the full activities of the *TERT* promoter, particularly the altered *TERT* promoter. Previous studies found that the ETS2 binding site in the *TERT* promoter was disrupted by allele C of rs2853669, resulting in the failure of ETS2 to bind the *TERT* promoter and hence the silencing of *TERT* expression (42,43). A prominent mechanism for the activation of the altered *TERT* promoter by *BRAF* V600E is the regulation of altered *TERT* by *BRAF* V600E/MAPK/FOS through the GA binding protein complex to act at the variation site in the *TERT* promoter to upregulate the *TERT* gene (45). It is then compelling to speculate that the ETS2-linked regulatory machinery on the *TERT* promoter may crosstalk with the regulatory system of *BRAF* V600E/MAPK/FOS/GABPB to cooperatively and oncogenically upregulate the altered *TERT* promoter. This crosstalk could explain the association of the trio of *BRAF* V600E, *TERT* promoter variation, and TT genotype of rs2853669 with the worst clinical outcomes of papillary thyroid cancer.

This study has limitations. We were unable to analyze the role of SNV rs2853669T>C in papillary thyroid cancer–associated mortality because of its low incidence rate in the current study. Further studies involving larger cohorts are needed to address this issue. Moreover, this is a single-center study without replication, and the findings need to be validated in other cohorts.

In conclusion, this study found that SNV rs2853669T>C, through modulating the *TERT* promoter activities, substantially refined the prognostic precision of *BRAF* V600E and *TERT* promoter variations, particularly that of the genetic duet, for estimating the risk of papillary thyroid cancer recurrence. The trio of *BRAF* V600E, *TERT* promoter variation, and TT genotype of rs2853669 was associated with the highest recurrence of papillary thyroid cancer, while the lack of both variations in the presence of the TC/CC genotype of rs2853669 was associated with the lowest recurrence. Combined use of these genetic variants of the *BRAF* and *TERT* genes may be a simple but precise genetics-based risk prognostication strategy for papillary thyroid cancer.

Data availability

All the main data and the supporting data are provided in the manuscript and the supplementary files.

Author contributions

Rengyun Liu, PhD (Data curation; Formal analysis; Investigation; Methodology; Writing—original draft; Writing—review & editing), Guangwu Zhu, MS (Data curation; Investigation; Methodology; Writing—original draft; Writing—review & editing), Jie Tan, MD, PhD (Data curation; Investigation; Methodology; Writing—original draft; Writing—review & editing), Xiaopei Shen, PhD (Data curation; Investigation; Methodology; Writing—original draft; Writing—review & editing), Mingzhao Xing, MD, PhD (Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Writing—original draft; Writing—review & editing).

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Conflicts of interest

Mingzhao Xing receives royalties as co-holder of a licensed US patent related to *BRAF* V600E variation in thyroid cancer. Other authors have no conflict of interest to disclose.

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