VOLUME 32 · NUMBER 25 · SEPTEMBER 1 2014

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

BRAF V600E and *TERT* Promoter Mutations Cooperatively Identify the Most Aggressive Papillary Thyroid Cancer With Highest Recurrence

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Published online ahead of print at www.jco.org on July 14, 2014.

Supported by US National Institutes of Health Grants No. RO1CA113507 and R01CA134225 (M.X.).

Disclaimer: The content of this article is solely the responsibility of the authors and does not necessarily reflect the official views of the funding agency (US National Institutes of Health). The funding organizations had no role in the design or conduct of the study; the collection, management, analysis, or interpretation of the data; or the preparation, review, or approval of the manuscript.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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0732-183X/14/3225w-2718w/$20.00

DOI: 10.1200/JCO.2014.55.5094

ABSTRACT

Purpose
To investigate the prognostic value of the *BRAF* V600E mutation and the recently identified *TERT* promoter mutation chr5:1,295,228C>T (C228T), individually and in their coexistence, in papillary thyroid cancer (PTC).

Patients and Methods
We performed a retrospective study of the relationship of *BRAF* and *TERT* C228T mutations with clinicopathologic outcomes of PTC in 507 patients (365 women and 142 men) age 45.9 ± 14.0 years (mean ± SD) with a median follow-up of 24 months (interquartile range, 8 to 78 months).

Results
Coexisting *BRAF* V600E and *TERT* C228T mutations were more commonly associated with high-risk clinicopathologic characteristics of PTC than they were individually. Tumor recurrence rates were 25.8% (50 of 194;77.60 recurrences per 1,000 person-years; 95% CI, 58.81 to 102.38) versus 9.6% (30 of 313; 22.88 recurrences per 1,000 person-years; 95% CI, 16.00 to 32.72) in *BRAF* mutation–positive versus –negative patients (hazard ratio [HR], 3.22; 95% CI, 2.05 to 5.07) and 47.5% (29 of 61; 108.55 recurrences per 1,000 person-years; 95% CI, 75.43 to 156.20) versus 11.4% (51 of 446; 30.21 recurrences per 1,000 person-years; 95% CI, 22.96 to 39.74) in *TERT* mutation–positive versus –negative patients (HR, 3.46; 95% CI, 2.19 to 5.45). Recurrence rates were 68.6% (24 of 35; 211.76 recurrences per 1,000 person-years; 95% CI, 141.94 to 315.94) versus 8.7% (25 of 287; 21.60 recurrences per 1,000 person-years; 95% CI, 14.59 to 31.97) in patients harboring both mutations versus patients harboring neither mutation (HR, 8.51; 95% CI, 4.84 to 14.97), which remained significant after clinicopathologic cofactor adjustments. Disease-free patient survival curves displayed a moderate decline with *BRAF* V600E or *TERT* C228T alone but a sharp decline with two coexisting mutations.

Conclusion
Coexisting *BRAF* V600E and *TERT* C228T mutations form a novel genetic background that defines PTC with the worst clinicopathologic outcomes, providing unique prognostic and therapeutic implications.

J Clin Oncol 32:2718-2726. © 2014 by American Society of Clinical Oncology

INTRODUCTION

Papillary thyroid cancer (PTC) is a common endocrine malignancy that accounts for 80% to 85% of thyroid malignancies.[1,2] It can be classified further as conventional variant (CPTC), follicular variant (FVPTC), tall-cell variant (TCPTC), or one of a few other rare variants, among which CPTC is the most common. Although PTC is highly curable in general, approximately 10% of patients are destined for a progressive disease course with aggressive tumor behaviors and high disease recurrence and mortality rates.[3-5] This wide spectrum of disease behaviors often creates dilemmas in clinical risk stratification and decision making for the management of PTC. The aggressive group of PTCs poses a particularly difficult prognostic and therapeutic challenge. It has been suggested that novel molecular-based management would help tackle this challenge,[6] but the molecular mechanisms, particularly the genetic backgrounds, for the aggressiveness of this special group of PTCs remain to be better defined.

Molecular-based risk stratification of PTC using *BRAF* V600E mutation has been proposed in

recent years.[6,7] This is based on the association of *BRAF* mutation with poor clinicopathologic outcomes of PTC.[8-11] *BRAF* V600E is the most common oncogene in PTC, with an average prevalence of 45%,[12] and it promotes PTC tumorigenesis through constitutively activating the mitogen-activated protein kinase pathway and other mechanisms.[13] We recently reported for the first time common mutations in the promoter of the gene for telomerase reverse transcriptase (TERT) in thyroid cancers,[14] particularly the chr5:1,295,228C>T mutation (C228T), which represents the nucleotide change of −124 C>T from the ATG translation start site of the *TERT* gene. We also found that *TERT* C228T was particularly prevalent in aggressive types of thyroid cancer, such as anaplastic thyroid cancer and poorly differentiated thyroid cancer, as well as *BRAF* V600E mutation–positive PTC. These findings prompted us to propose and test in this study our hypothesis that *BRAF* V600E and *TERT* C228T mutations may cooperatively form a unique genetic background that identifies the most aggressive type of PTC and has important prognostic and therapeutic implications.

PATIENTS AND METHODS

Patients and Clinicopathologic Data

This study included 507 patients (365 women and 142 men) age 45.9 ± 14.0 years (mean ± SD) who were treated for PTC with total thyroidectomy and clinically observed between 1990 and 2012 at Johns Hopkins Hospital; the overall median follow-up time was 24 months (interquartile range, 8 to 78 months) after the initial treatments. Therapeutic neck dissection and radioiodine ablation were pursued following standard indications and criteria, as previously presented.[11] The demographic data are listed in Table 1. After institutional review board approval and informed patient consenting, we obtained thyroid tumor specimens for genetic analysis and retrospectively collected clinicopathologic data. The pathologic diagnoses of PTC in our patients were formally established.[11] Disease stages of PTC were defined on the basis of the American Joint Committee on Cancer staging system. Tumor recurrence was defined by the existence of histologically/cytologically/radioiodine radiographically confirmed recurrent/persistent PTC tumor. Follow-up time was defined as the time interval from the initial thyroidectomy to the discovery of disease recurrence or, in cases without disease recurrence, to the most recent clinical follow-up visit. All mutational analyses were performed after the surgical and radioiodine treatments of patients, and the genetic results had no influence on the treatment decision making.

Mutational Analyses

Genomic DNA was isolated from primary PTC tumors by standard phenol-chloroform extraction and ethanol precipitation procedures and subjected to classical Sanger sequencing for the detection of *BRAF* V600E and *TERT* C228T mutations. For *BRAF* V600E, the polymerase chain reaction (PCR) protocol and conditions described previously[11] were used to amplify exon 15 of the *BRAF* gene containing the mutation hot spot, followed by a Big Dye (Applied Biosystems, Foster City, CA) reaction for Sanger sequencing. For *TERT* C228T, our recently described PCR conditions were used to amplify a fragment of the *TERT* promoter containing the C228T hot spot.[14] *BRAF* V600E and *TERT* C228T were recognized on sequencing electropherograms.

Statistical Analyses

Categorical data were summarized with frequencies and percentages. Continuous data were summarized with means ± standard deviations (if normally distributed) or medians and interquartile ranges (if not normally distributed). Comparisons of categorical variables were performed using the χ^2 test or, for small cell sizes, Fisher's exact test. The independent *t* and Wilcoxon-Mann-Whitney tests were used for normally and non-normally distributed continuous variables, respectively. Kaplan-Meier survival curves with log-rank tests and Cox proportional hazards regression analyses, censoring patients at the time of recurrence or, if no recurrence, at the time of last follow-up visit, were used to compare recurrence-free survival rates by mutation status. Independent associations of mutations with PTC recurrence were examined by Cox regression analyses. All *P* values were two sided, and a *P* value of <.05 was treated as statistically significant. The analyses were performed using Stata (Stata/SE version 10.1 for windows; Stata, College Station, TX) and GraphPad Prism (version 6 for Windows; GraphPad Software, San Diego, CA).

RESULTS

BRAF *V600E and* TERT *C228T Mutations in PTC*

We examined *BRAF* V600E and *TERT* C228T mutations in 507 cases of PTC that consisted of several variants (Appendix Table A1, online only). *BRAF* V600E was found in 164 of 383 (42.8%) CPTCs, 15 of 103 (14.6%) FVPTCs, 14 of 19 (73.7%) TCPTCs, and one of two (50%) columnar PTCs, with an overall prevalence of 38.3% (194 of 507). *TERT* C228T was found in 47 of 383 (12.3%) CPTCs, eight of 103 (7.8%) FVPTCs, five of 19 (26.3%) TCPTCs, and one of two (50.0%) columnar PTCs, with an overall prevalence of 12.0% (61 of 507). A significant association of *TERT* C228T with the *BRAF* mutation was observed (Appendix Table A2, online only). Specifically, on the overall analysis of all PTCs, *TERT* C228T was found in 26 of 313 (8.3%) *BRAF* mutation–negative cases versus 35 of 194 (18.0%) *BRAF* mutation–positive cases, and conversely, the *BRAF* mutation was found in 159 of 446 (35.7%) *TERT* mutation–negative cases versus 35 of 61 (57.4%) *TERT* mutation–positive cases (odds ratio [OR], 2.43; 95% CI, 1.40 to 4.21; $P = .001$). A significant association of the two mutations was similarly observed in CPTC (Appendix Table A2). Coexistence of *BRAF* and *TERT* mutations was found in 35 of 507 (6.9%) PTCs and 28 of 383 (7.3%) CPTCs (Appendix Table A1).

Relationship of BRAF *V600E and* TERT *C228T Mutations With Clinicopathologic Outcomes of PTC*

In the overall analysis of 507 PTCs (Table 1), the *BRAF* V600E mutation was found to be significantly associated with several high-risk clinicopathologic characteristics, including male sex of the patient, larger tumor size, extrathyroidal invasion, vascular invasion, lymph node metastasis, and stage III/IV. Tumor recurrence was 30 of 313 (9.6%; 22.88 recurrences per 1,000 person-years; 95% CI, 16.00 to 32.72) in *BRAF* mutation–negative patients versus 50 of 194 (25.8%; 77.60 recurrences per 1,000 person-years; 95% CI, 58.81 to 102.38) in *BRAF* mutation–positive patients (hazard ratio [HR], 3.22; 95% CI, 2.05 to 5.07; $P < .001$; Appendix Table A3, online only). Similarly, *TERT* C228T was significantly associated with these clinicopathologic characteristics in addition to older patient age and distant metastatic recurrence (Table 1). Tumor recurrence was 51 of 446 (11.4%; 30.21 recurrences per 1,000 person-years; 95% CI, 22.96 to 39.74) in *TERT* mutation–negative cases versus 29 of 61 (47.5%; 108.55 recurrences per 1,000 person-years; 95% CI, 75.43 to 156.20) in *TERT* mutation–positive cases (HR, 3.46; 95% CI, 2.19 to 5.45; $P < .001$; Appendix Table A3). The HRs of *BRAF* V600E and *TERT* C228T for tumor recurrence were all highly significant, which remained significant after adjustment for patient age and sex and, as may not be unexpected (see Discussion), they lost significance with the 95% CI marginally crossing 1.0 after additional adjustment for aggressive tumor behaviors (Appendix Table A3).

Similar results were obtained when analyses were performed only on CPTCs (Table 1; Appendix Table A3). For example, *BRAF* V600E

Table 1. Relationship of *BRAF* V600E and *TERT* C228T Mutations With Clinicopathologic Outcomes of PTC

PTC Type and Clinicopathologic Outcomes	*BRAF* Status							*TERT* Status						
	BRAF V600E			Wild-Type *BRAF*				*TERT* C228T			Wild-Type *TERT*			
	No.	%	No. of Missing Cases	No.	%	No. of Missing Cases	*P*	No.	%	No. of Missing Cases	No.	%	No. of Missing Cases	*P*
All PTC														
Total No. of cases	194			313				61			446			
Age at diagnosis, years*	47.1 ± 14.4			45.2 ± 13.8			.138	51.7 ± 15.7			45.1 ± 13.6			< .001
Sex, male	69	35.6		73	23.3		.003	32	52.5		110	24.7		< .001
Tumor size, cm			12			9	.003			6			15	.048
Median	2.0			1.7				2.3			1.8			
Interquartile range	1.3-3.0			1.0-2.8				1.2-3.5			1.1-3.0			
Multifocality	68	36.4	7	122	39.1	1	.542	20	33.9	2	170	38.6	6	.482
Extrathyroidal invasion	58	31.3	9	35	11.2	1	< .001	27	46.5	3	66	15.0	7	< .001
Vascular invasion	36	20.0	14	41	13.2	2	.045	14	25.9	7	63	14.4	9	.028
Lymph node metastasis	85	46.2	10	68	21.7		< .001	31	52.5	2	122	27.8	8	< .001
Distant metastatic recurrence	12	6.2		10	3.2		.108	12	19.7		10	2.2		< .001
Disease stage			6										6	
I	114	60.6		237	75.7			26	42.6		325	73.9		
II	13	6.9		31	9.9			6	9.8		38	8.6		
III	39	20.7		35	11.2			12	19.7		62	14.1		
IV	22	11.7		10	3.2		< .001	17	27.9		15	3.4		< .001
III + IV	61	32.4		45	14.4		< .001	29	47.5		77	17.5		< .001
Tumor recurrence	50	25.8		30	9.6		< .001	29	47.5		51	11.4		< .001
Total ^{131}I dose, mCi			16			7	.004			7			16	.001
Median	87.7			74.9				100			75			
Interquartile range	0-100			0-100				29.9-105			0-100			
Total follow-up, months							.027							.056
Median	18			31				30			24			
Interquartile range	7-53			8-87				12-78			6-76			
CPTC														
Total No. of cases	164			219				47			336			
Age at diagnosis, years*	46.7 ± 13.7			45.8 ± 13.9			.511	51.6 ± 16.0			45.4 ± 13.4			.004
Sex, male	60	36.6		53	24.2		.009	25	53.2		88	26.2		< .001
Tumor size, cm			12			9	< .001			6			15	.009
Median	2			1.5				2.3			1.6			
Interquartile range	1.3-3			0.8-2.3				1.2-3.5			1-2.5			
Multifocality	54	34.4	7	87	39.9	1	.277	16	35.6	2	125	37.9	6	.763
Extrathyroidal invasion	48	31.0	9	29	13.3	1	< .001	23	52.3	3	54	16.4	7	< .001
Vascular invasion	27	18.0	14	27	14.4	2	.140	10	25.0	7	44	13.5	9	.052
Lymph node metastasis	76	49.0	9	58	26.5		< .001	27	60.0	2	107	32.5	7	< .001
Distant metastatic recurrence	7	4.3		8	3.6		.759	8	17.0		7	2.1		< .001
Disease stage			6										6	
I	98	62.0		172	78.5			19	40.3		251	76.1		
II	10	6.3		15	6.9			3	6.4		22	6.7		
III	32	20.2		23	10.5			10	21.3		45	13.6		
IV	18	11.4		9	4.1		.001	15	31.9		12	3.6		< .001
III + IV	50	31.6		32	14.6		< .001	25	53.2		57	17.3		< .001
Tumor recurrence	42	25.6		22	10.0		< .001	24	51.1		40	11.9		< .001
Total ^{131}I dose, mCi			15			5	.043			7				< .001
Median	75			51.7				100			75			
Interquartile range	0-100			0-100				75-104			0-100			
Total follow-up, months							.026							.026
Median	19			32				48			24			
Interquartile range	6.5-52			9-80				12-95			6-70			

Abbreviations: CPTC, conventional papillary thyroid cancer; PTC, papillary thyroid cancer.
*Data were summarized with means ± standard deviations.

Table 2. Impact of *BRAF* V600E or *TERT* C228T or Their Coexistence on Clinicopathologic Outcomes of PTC

PTC Type and Clinicopathologic Outcomes	No Mutation			*BRAF* Mutation Only				*TERT* Mutation Only				*BRAF* + *TERT* Mutation			
	No.	%	No. of Missing Cases	No.	%	No. of Missing Cases	*P*	No.	%	No. of Missing Cases	*P*	No.	%	No. of Missing Cases	*P**
All PTC															
Total No. of cases	287			159				26				35			
Age at diagnosis, years†	45.3 ± 13.7			44.8 ± 13.5			.724	44.0 ± 14.6			.651	57.4 ± 14.1			< .001
Sex, male	65	22.6		45	28.3		.185	8	30.8		.348	24	68.6		< .001
Tumor size, cm			7			8	.044			2	.885			4	.002
Median	1.7			2.0				1.8				2.7			
Interquartile range	1.0-3.0			1.3-3.0				1.1-2.5				1.3-4.0			
Multifocality	114	39.7		56	36.6	6	.522	8	32.0	1	.448	12	35.3	1	.617
Extrathyroidal invasion	31	10.8		35	23.0	7	.001	4	16.0	1	.503	23	69.7	2	< .001
Vascular invasion, n (%)	35	12.2	1	28	18.5	8	.074	6	24.0	1	.096	8	27.6	6	.022
Lymph node metastasis	58	20.2		64	42.4	8	< .001	10	38.5		.031	21	63.6	2	< .001
Distant metastatic recurrence	8	2.8		2	1.3		.506	2	7.7		.198	10	28.6		< .001
Disease stage						6									
I	219	76.3		106	69.3			18	69.2			8	22.9		
II	27	9.4		11	7.2			4	15.4			2	5.7		
III	33	11.5		29	18.9			2	7.7			10	28.6		
IV	8	2.8		7	4.6		.106	2	7.7		.373	15	42.9		< .001
III+IV	41	14.3		36	23.5		.015	4	15.4		.776	25	71.4		< .001
Tumor recurrence	25	8.7		26	16.3		.015	5	19.2		.081	24	68.6		< .001
Total ^{131}I dose, mCi			5			11	.084			2	.560			5	< .001
Median	74.6			75.4				77				100			
Interquartile range	0-100			0-100				0-100				98-136			
Total follow-up, months							.048				.030				.864
Median	28			17				66				24			
Interquartile range	6-85			3-52				12-116				12-60			
CPTC															
Total No. of cases	200			136				19				28			
Age at diagnosis, years†	46.0 ± 13.7			44.7 ± 12.8			.398	44.2 ± 16.1			.603	56.7 ± 14.2			< .001
Sex, male	47	23.5		41	30.1		.174	6	31.6		.432	19	67.9		< .001
Tumor size, cm			7			8	< .001			2	.349			4	< .001
Median	1.5			2				1.5				2.8			
Interquartile range	0.8-2.3			1.3-2.5				1.0-2.3				1.7-3.5			
Multifocality	81	40.5		44	33.8	6	.223	6	33.3	1	.552	10	37.0	1	.730
Extrathyroidal invasion	25	12.5		29	22.5	7	.017	4	22.2	1	.272	19	73.1	2	< .001
Vascular invasion	21	10.5	1	23	18.0	8	.055	6	33.3	1	.005	4	18.2	6	.287
Lymph node metastasis	49	24.5		58	45.0	7	< .001	9	47.4		.031	18	69.2	2	< .001
Distant metastatic recurrence	6	3.0		1	0.7		.248	2	10.5		.146	6	21.4		< .001
Disease stage						6									
I	159	79.5		92	70.8			13	68.4			6	21.4		
II	13	6.5		9	6.9			2	10.5			1	3.6		
III	21	10.5		24	18.5			2	10.5			8	28.6		
IV	7	3.5		5	3.8		.212	2	10.5		.429	13	46.4		< .001
III+IV	28	14.0		29	22.3		.051	4	21.0		.492	21	75.0		< .001
Tumor recurrence	18	9.0		22	16.2		.046	4	21.0		.107	20	71.4		< .001
Total ^{131}I dose, mCi			3			10	.193			2	.110			5	< .001
Median	50.9			75				100				100			
Interquartile range	0-100			0-100				0-103				75-131.5			
Total follow-up, months							.025				.067				.686
Median	30.5			17				73				35.5			
Interquartile range	8-79			2-48.5				12-108				12-61.5			

Abbreviations: CPTC, conventional papillary thyroid cancer; PTC, papillary thyroid cancer.
**P* values are from the comparison of the indicated genetic group in the column immediately left of the *P* value column with the no mutation group.
†Data were summarized with means ± standard deviations.

Table 3. Hazard Ratios of *BRAF* V600E or *TERT* C228T or Their Coexistence for the Recurrence of PTC

Type of PTC	Mutations	Recurrence	%	Recurrence per 1,000 Person-Years	95% CI	Unadjusted		Adjustment 1*		Adjustment 2†	
						Hazard Ratios	95% CI	Hazard Ratios	95% CI	Hazard Ratios	95% CI
All PTC	No mutation	25 of 287	8.7	21.60	14.59 to 31.97	1.00				1.00	
	BRAF mutation only	26 of 159	16.3	48.96	33.34 to 71.91	2.24	1.29 to 3.88	2.16	1.24 to 3.75	1.17	0.62 to 2.20
	TERT mutation only	5 of 26	19.2	32.50	13.53 to 78.09	1.69	0.65 to 4.43	1.60	0.60 to 4.25	0.87	0.27 to 2.76
	BRAF + *TERT* mutations	24 of 35	68.6	211.76	141.94 to 315.94	8.51	4.84 to 14.97	8.41	4.44 to 15.94	3.10	1.24 to 7.75
CPTC	No mutation	18 of 200	9.0	22.23	14.00 to 35.28	1.00				1.00	
	BRAF mutation only	22 of 136	16.2	50.25	33.08 to 76.31	2.20	1.18 to 4.11	2.06	1.10 to 3.86	1.03	0.49 to 2.15
	TERT mutation only	4 of 19	21.0	35.22	13.22 to 93.83	1.82	0.61 to 5.38	1.71	0.56 to 5.22	0.50	0.12 to 2.00
	BRAF + *TERT* mutations	20 of 28	71.4	191.85	123.77 to 297.36	7.73	4.07 to 14.67	7.50	3.71 to 15.17	4.39	1.42 to 13.54

NOTE. Hazard ratios and 95% CIs were calculated using Cox regression for the comparison of the indicated mutation group with the group harboring neither mutation.
Abbreviations: CPTC, conventional papillary thyroid cancer; PTC, papillary thyroid cancer.
*Adjustment 1 was made for patient age at diagnosis and sex.
†Adjustment 2 was made for patient age at diagnosis, sex, multifocality, tumor size, extrathyroidal invasion, vascular invasion, and lymph node metastasis.

and *TERT* C228T mutations were each associated with several high-risk clinicopathologic characteristics. Higher tumor recurrence rates and the number of recurrences per 1,000 person-years were associated with *BRAF* V600E or *TERT* C228T mutations. The HR of *BRAF* V600E for tumor recurrence was 3.10 (95% CI, 1.85 to 5.20; $P < .001$), and the HR of *TERT* C228T for PTC recurrence was 3.32 (95% CI, 2.00 to 5.52; $P < .001$).

Impacts of BRAF *V600E or* TERT *C228T Alone or Their Coexistence on Clinicopathologic Outcomes of PTC*

In the analysis of all PTCs (Table 2), in comparison with the group negative for either mutation, *BRAF* V600E alone was significantly associated with larger tumor size, extrathyroidal invasion, lymph node metastasis, disease stage III/IV, and tumor recurrences. *TERT* C228T alone was significantly associated with lymph node metastasis, and there was an insignificant association with other clinicopathologic characteristics. In contrast, the coexistence of *BRAF* V600E and *TERT* C228T was strongly associated with virtually all the classical high-risk characteristics as well as distant metastatic recurrence. Patients harboring both *BRAF* and *TERT* mutations had the highest recurrence rate as well, which was 24 of 35 (68.6%; 211.76 recurrences per 1,000 person-years; 95% CI, 141.94 to 315.94) versus only 25 of 287 (8.7%; 21.60 recurrences per 1,000 person-years; 95% CI, 14.59 to 31.97) in patients harboring neither mutation (HR, 8.51; 95% CI, 4.84 to 14.97; $P < .001$; Table 3).

Similar individual impacts of *BRAF* V600E and *TERT* C288T mutations on clinicopathologic outcomes were observed in CPTC (Table 2). In comparison with the group negative for either mutation, *BRAF* V600E was significantly associated with several high-risk clinicopathologic characteristics as well as tumor recurrences. The impacts of *TERT* C228T alone on clinicopathologic outcomes were significant for vascular invasion and lymph node metastasis and short of statistical significance for other parameters. In contrast, the coexistence of *BRAF* V600E and *TERT* C228T was highly associated with virtually all the high-risk clinicopathologic characteristics. Tumor recurrence was 20 of 28 (71.4%; 191.85 recurrences per 1,000 person-years; 95% CI, 123.77 to 297.36) in patients harboring both mutations versus 18 of 200 (9.0%; 22.23 recurrences per 1,000 person-years; 95% CI, 14.00 to 35.28) in

Table 4. Comparison of PTC Recurrence Between the *BRAF* V600E + *TERT* C228 Mutations Group and the *BRAF* V600E–Only or *TERT* C288T–Only Group

PTC Type and Recurrence	*BRAF* V600E Only (A)	*TERT* C228T Only (B)	*BRAF* + *TERT* Mutations (C)	Comparison of C With A			Comparison of C With B		
				HR	95% CI	*P*	HR	95% CI	*P*
All PTC									
Recurrence									
No.	26 of 159	5 of 26	24 of 35						
%	16.3	19.2	68.6						
Recurrence per 1,000 person-years	48.96	32.5	211.76	3.62	2.07 to 6.33	< .001	6.16	2.29 to 16.61	< .001
95% CI	33.34 to 71.91	13.53 to 78.09	141.94 to 315.94						
CPTC									
Recurrence									
No.	22 of 136	4 of 19	20 of 28						
%	16.2	21.0	71.4						
Recurrence per 1,000 person-years	50.25	35.22	191.85	3.30	1.79 to 6.06	< .001	5.28	1.76 to 15.83	.003
95% CI	33.08 to 76.31	13.22 to 93.83	123.77 to 297.36						

Abbreviations: CPTC, conventional variant papillary thyroid cancer; HR, hazard ratio; PTC, papillary thyroid cancer.

patients harboring neither mutation (HR, 7.73; 95% CI, 4.07 to 14.67; $P < .001$; Table 3).

There was an incremental impact of coexisting *BRAF* and *TERT* C228T mutations on PTC recurrence over either mutation alone (Table 4). Specifically, in the analysis of all PTCs, tumor recurrence was 24 of 35 (68.6%; 211.76 recurrences per 1,000 person-years; 95% CI, 141.94 to 315.94) in patients harboring both mutations versus 26 of 159 (16.3%; 48.96 recurrences per 1,000 person-years; 95% CI, 33.34 to 71.91) in patients harboring only the *BRAF* mutation (HR, 3.62; 95% CI, 2.07 to 6.33; $P < .001$) and five of 26 (19.2%; 32.5 recurrences per 1,000 person-years; 95% CI, 13.53 to 78.09) in patients harboring only *TERT* mutation (HR, 6.16; 95% CI, 2.29 to 16.61; $P < .001$). In fact, PTC recurrence associated with coexisting *BRAF* and *TERT* mutations was dramatically higher than the sum of those associated with the two mutations individually, demonstrating a synergistic effect of the two mutations on PTC recurrence. Similar results were also obtained in CPTC (Table 4).

Impacts of BRAF *V600E and* TERT *C228T Mutations on Disease-Free Survival of Patients With PTC*

We performed Kaplan-Meier and log-rank analyses of disease-free survival rates of patients by genotype. In analyses of all PTCs (Fig 1A and 1B), tumor recurrence-free survival curves had a modest decline in patients negative for *BRAF* V600E (Fig 1A) or *TERT* C228T (Fig 1B). They declined further with either the *BRAF* mutation (Fig 1A) or the *TERT* mutation (Fig 1B). Similar results were obtained in the analyses of CPTCs (Figs 1C and 1D).

Figure 2A shows the impacts of individual *BRAF* V600E or *TERT* C228T mutations or their coexistence on tumor recurrence-free survival curves of all patients with PTC. There was an increasing decline in recurrence-free survival curves from patients with neither mutation to patients with the *TERT* mutation alone, those with the *BRAF* mutation alone, and those with both mutations. The curve decline with *TERT* mutation alone was modest, consistent with the modest effects of the *TERT* mutation alone on other clinicopathologic outcomes

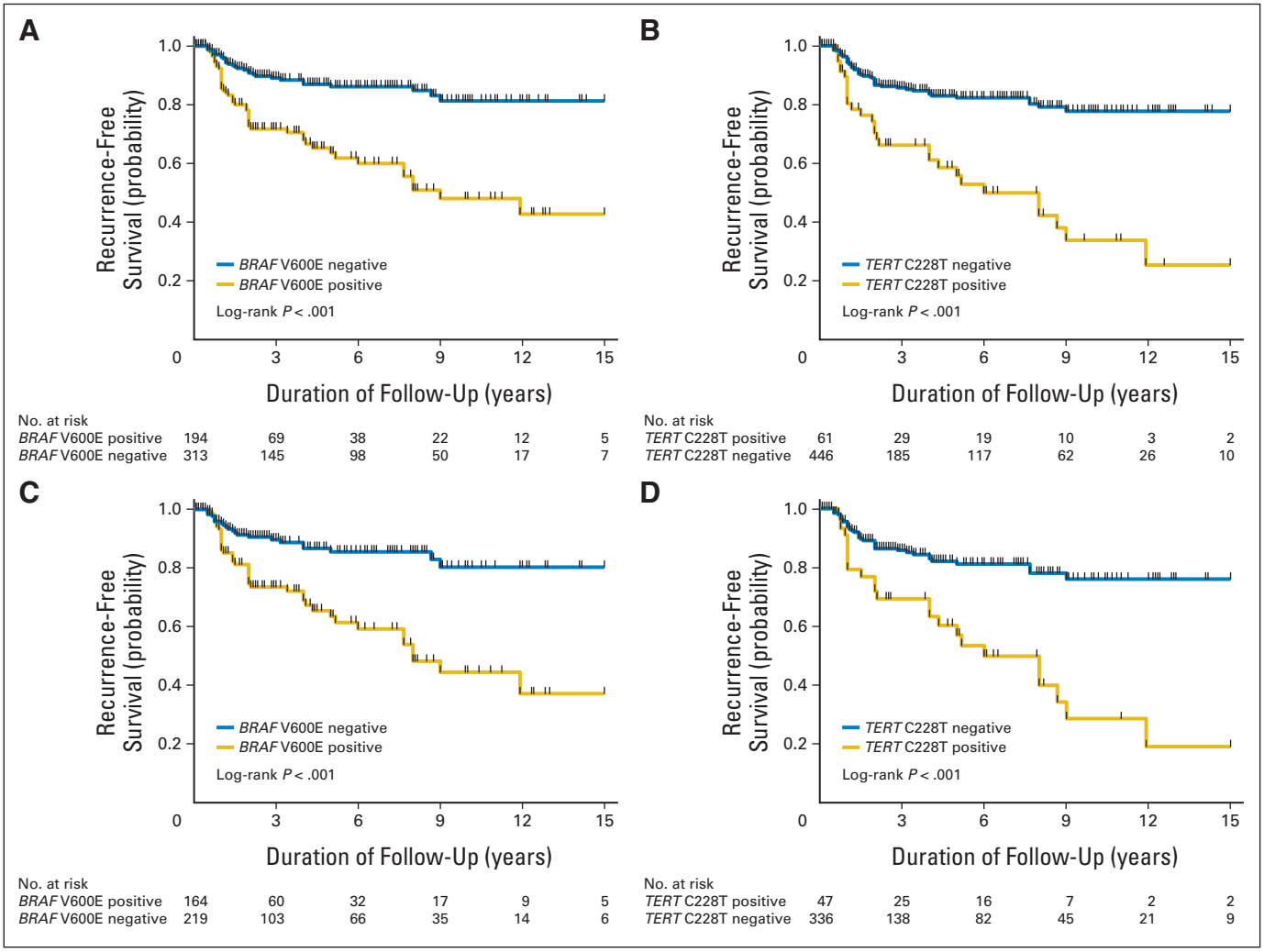

Fig 1. Kaplan-Meier analyses of the impacts of *BRAF* V600E and *TERT* C228T mutations on disease-free survival of patients with papillary thyroid cancer (PTC). (A, B) Results of the analyses of patients with PTC of all types. (C, D) Results of the analyses of conventional variant PTC only. (A, C) Effects of the *BRAF* V600E mutation on tumor recurrence-free survival. (B, D) Effects of the *TERT* C228T mutation on tumor recurrence-free survival. Blue lines represent patients negative for the indicated mutation. Gold lines represent patients positive for the indicated mutation.

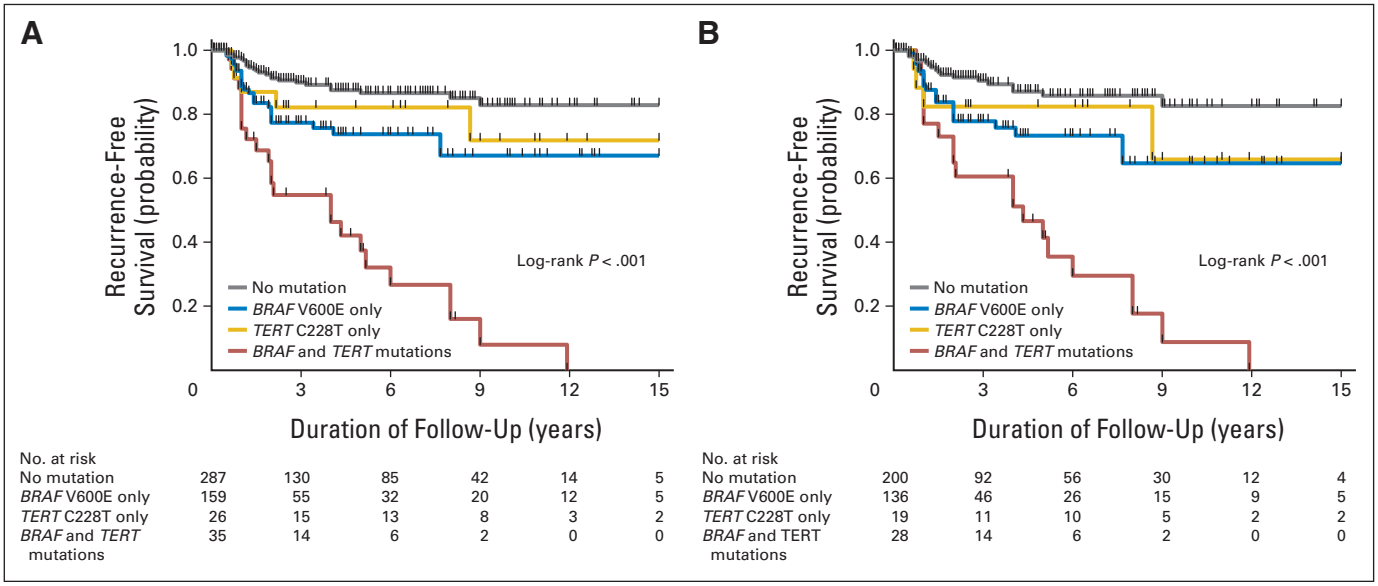

Fig 2. Kaplan-Meier analyses of the impacts of *BRAF* V600E or *TERT* C288T alone or their coexistence on disease-free survival of patients with papillary thyroid cancer (PTC). (A) Results of the analyses of patients with PTC of all types. (B) Results of the analyses of conventional variant PTC only. Four groups of patients are indicated in A and B, including patients with neither mutation (gray lines), *TERT* C228T mutation only (gold lines), *BRAF* V600E mutation only (blue lines), and coexistence of the two mutations (red lines).

(Table 2). The curve decline with coexisting *BRAF* and *TERT* mutations was sharp and dramatic, and the curve decline with the *BRAF* mutation alone was intermediate. Virtually identical results were obtained in patients with CPTC (Fig 2B).

Table 3 summarizes the impacts of *BRAF* V600E, *TERT* C228T, and their coexistence on PTC recurrence after adjustment for classical clinicopathologic risk factors. The HR of *BRAF* mutation alone for tumor recurrence in all PTCs was 2.24 (95% CI, 1.29 to 3.88), and it remained significant at 2.16 (95% CI, 1.24 to 3.75) after the first adjustment for patient age at diagnosis and sex. This significance was lost after an additional adjustment for aggressive tumor behaviors, including tumor size, multifocality, extrathyroidal invasion, vascular invasion, and lymph node metastasis. The HR of *TERT* C228T alone for tumor recurrence was not significant, with the 95% CIs all crossing 1.0. In striking contrast, the HRs of coexisting *BRAF* and *TERT* mutations for tumor recurrence in all PTCs was 8.51 (95% CI, 4.84 to 14.97), and it remained significant at 8.41 (95% CI, 4.44 to 15.94) after the adjustment for patient age and sex and was still significant at 3.10 (95% CI, 1.24 to 7.75) after the additional adjustment for tumor behaviors.

We obtained similar HR results for tumor recurrence in CPTCs (Table 3). For example, the HRs of coexistence of the two mutations for tumor recurrence of CPTC—unadjusted, adjusted for the first level, and adjusted for the second level—were significant at 7.73 (95% CI, 4.07 to 14.67), 7.50 (95% CI, 3.71 to 15.17), and 4.39 (95% CI, 1.42 to 13.54), respectively.

DISCUSSION

We have identified a novel genetic background—coexistence of *BRAF* V600E and *TERT* C228T mutations—which defines the most aggressive subgroup of PTCs. The combined effects of the two mutations on recurrence compared with no mutation remained significant even on multivariable adjustments for the classical clinicopathologic risk factors. The PTC recurrence rate for patients with coexisting *BRAF* and *TERT* mutations was also significantly higher than that associated with either mutation alone or the sum of the recurrences associated with the two mutations individually, demonstrating an incremental and synergistic effect of the coexisting two mutations. These results were found both in the overall analyses of all PTCs and of the CPTC variant, establishing coexistence of the two mutations as an important novel genetic background for the worst aggressiveness of PTC.

This cooperative effect of *BRAF* and *TERT* promoter mutations can be explained at a molecular level. TERT maintains the length of chromosomes by adding telomeres to them, thus increasing the immortality of cells, and promotes cell proliferation and decreases apoptosis.[15-17] Transgenic mouse models overexpressing TERT showed increased tumor development and malignant transformation.[18,19] Consistent with this oncogenic role of TERT is its common overexpression in human cancers,[15-17] including thyroid cancer.[20,21] *TERT* C228T confers increased transcriptional activities of the *TERT* promoter by creating consensus binding motifs (GGA[A>T] or CCGGAA) for E-twenty-six (ETS)/ternary complex transcription factors.[22,23] As activation of the mitogen-activated protein kinase pathway upregulates the ETS system,[24-26] the coexistence of *BRAF* V600E and *TERT* C228T forms a unique mechanism upregulating the expression of *TERT*. Indeed, coexistence of the two mutations was associated with increased expression of the TERT mRNA in PTC.[27] This oncogenic cooperation of TERT with *BRAF* mutation is interestingly similar to the finding in a transgenic mouse model in which *p53* mutation and induced overexpression of TERT cooperatively promoted cancer development.[18] Consistent with the role of *TERT* C228T in poor clinicopathologic outcomes of PTC were recent reports of the association of *TERT* promoter mutations with brain tumor-associated patient mortality,[28] bladder cancer recurrence,[29] and poor survival of patients with laryngeal cancer.[30]

Many studies have demonstrated a role of *BRAF* V600E in tumor aggressiveness[8-10] and even patient mortality[31] in PTC, but some studies failed to do so. In the present study, HRs for tumor recurrence remained significant on the multivariable adjustment for patient age and sex but fell short of significance when aggressive tumor pathologic behaviors were adjusted. This statistical result should not be interpreted as the lack of a role of *BRAF* mutation in the aggressiveness of PTC. Biologically, *BRAF* mutation uses various molecular mechanisms to promote the aggressive tumor behaviors.[13] Because some of these tumor behaviors, particularly lymph node metastases, are the main source of PTC recurrence, it is not surprising that statistical adjustment for them could artificially (and misleadingly) diminish or even null the effect of *BRAF* mutation on recurrence. This study represents the largest uniform series of PTC to examine this role of the *BRAF* mutation, but perhaps an even larger study is needed to show an independent role of *BRAF* mutation. The persistently significant effects of coexisting *BRAF* and *TERT* mutations on PTC recurrence after multivariable adjustments for the classical clinicopathologic factors suggest that coexisting *BRAF* and *TERT* mutations have a more profound impact.

The effects of *TERT* C228T mutation fell short of significance when it was separated from the *BRAF* mutation and examined alone, suggesting that *TERT* mutation needs additional genetic alterations to cooperate to promote the aggressiveness of PTC. We previously reported a particularly high prevalence of *TERT* C228T in anaplastic thyroid cancer, poorly differentiated thyroid cancer, and thyroid cancer cell lines,[14] which were confirmed in several subsequent publications.[27,32,33] Thyroid cancer cell lines are usually undifferentiated[34] and commonly harbor multiple genetic alterations, including the *BRAF* mutation.[35-37] Thus, it is likely that their aggressiveness is cooperatively driven by coexisting *TERT* promoter mutations and other genetic alterations, similar to their cooperation found in this study. Our results in this American cohort of patients are consistent with our recent findings of the impact of coexisting *BRAF* V600E and *TERT* promoter mutations on aggressive behaviors of PTC in a Chinese cohort of patients.[38]

The follow-up time of patients in this study was relatively short. However, patients with PTC usually present recurrence within the first few years. Therefore, a median of 2 years should have captured the majority of recurrence events of PTC. The disease-free survival curves (Figs 1 and 2) show that, as time progresses, the separation of the mutation–positive and –negative curves becomes even more prominent, suggesting that in later follow-up years the impact of the mutations on PTC recurrence is even more profound. Therefore, if anything, a median follow-up time of 2 years likely caused an underestimate of the impacts of the *BRAF* and *TERT* mutations on PTC recurrence. The follow-up times were different among some groups. However, this variation was corrected by the Cox proportional and regression analyses, because these standard statistical methods take the time as a variable into the model. To further correct the time variations, we also additionally report recurrences per 1,000 person years.

In summary, this study identified coexisting *BRAF* V600E and *TERT* C228T mutations as a novel genetic background for the most aggressive subgroup of PTC, whereas the two mutations alone have relatively less impact on the aggressiveness of PTC. These genetic patterns, by separating patients with PTC into different risk groups and particularly by defining the group with the most aggressive disease, have important prognostic and therapeutic implications.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.
Employment or Leadership Position: None **Consultant or Advisory Role:** None **Stock Ownership:** None **Honoraria:** None **Research Funding:** None **Expert Testimony:** None **Patents, Royalties, and Licenses:** Mingzhao Xing, *BRAF* mutation in thyroid carcinoma, USA patent 7,378,233 **Other Remuneration:** None

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Final approval of manuscript: All authors

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Appendix

Table A1. Prevalence of *BRAF* V600E and *TERT* C228T Mutations in Various Variants of PTC

PTC Type	*BRAF* V600E Mutation		*TERT* C228T Mutation		*BRAF* + *TERT* mutations	
	No.	%	No.	%	No.	%
CPTC	164 of 383	42.8	47 of 383	12.3	28 of 383	7.3
FVPTC	15 of 103	14.6	8 of 103	7.8	1 of 103	1.0
TCPTC	14 of 19	73.7	5 of 19	26.3	5 of 19	26.3
Columnar PTC	1 of 2	50.0	1 of 2	50.0	1 of 2	50.0
All PTC	194 of 507	38.3	61 of 507	12.0	35 of 507	6.9

Abbreviations: CPTC, conventional variant papillary thyroid cancer; FVPTC, follicular variant papillary thyroid cancer; PTC, papillary thyroid cancer; TCPTC, tall-cell variant papillary thyroid cancer.

Table A2. Association of *TERT* Promoter C228T Mutation With *BRAF* V600E Mutation in PTC

Tumor Type	*TERT* C228T Mutation				*BRAF* V600E Mutation						
	BRAF−		*BRAF*+		*TERT*−		*TERT*+				
	No.	%	No.	%	No.	%	No.	%	OR	95% CI	*P*
All PTC	26 of 313	8.3	35 of 194	18.0	159 of 446	35.7	35 of 61	57.4	2.43	1.40 to 4.21	.001
CPTC	19 of 219	8.7	28 of 164	17.1	136 of 336	40.5	28 of 47	59.6	2.17	1.16 to 4.06	.013

Abbreviations: CPTC, conventional papillary thyroid cancer; PTC, papillary thyroid cancer.

Table A3. Association of *BRAF* or *TERT* C228T Promoter Mutation With PTC Recurrence

Tumor Type and Mutation Status	Recurrence			Recurrence per 1,000 Person-Years	95% CI	Unadjusted		Adjusted*		Adjusted†	
	n	No.	%			Hazard Ratios	95% CI	Hazard Ratios	95% CI	Hazard Ratios	95% CI
All PTC											
BRAF V600E											
Negative	30	313	9.6	22.88	16.00 to 32.72	1.00		1.00		1.00	
Positive	50	194	25.8	77.60	58.81 to 102.38	3.22	2.05 to 5.07	3.02	1.91 to 4.77	1.51	0.87 to 2.60
TERT C228T											
Negative	51	446	11.4	30.21	22.96 to 39.74	1.00		1.00		1.00	
Positive	29	61	47.5	108.55	75.43 to 156.20	3.46	2.19 to 5.45	3.21	2.02 to 5.09	1.78	0.97 to 3.25
CPTC											
BRAF V600E											
Negative	22	219	10.0	23.82	15.69 to 36.18	1.00		1.00		1.00	
Positive	42	164	25.6	77.48	57.26 to 104.84	3.10	1.85 to 5.20	2.88	1.71 to 4.86	1.46	0.77 to 2.75
TERT C228T											
Negative	40	336	11.9	32.06	23.52 to 43.71	1.00		1.00		1.00	
Positive	24	47	51.1	110.18	73.85 to 164.38	3.32	2.00 to 5.52	3.15	1.89 to 5.24	1.54	0.76 to 3.12

NOTE. Hazard ratios and 95% CIs were calculated with Cox regression.
Abbreviations: CPTC, conventional papillary thyroid cancer; PTC, papillary thyroid cancer.
*Adjustment was made for patient age at diagnosis and sex.
†Adjustment was made for patient age at diagnosis, sex, multifocality, tumor size, extrathyroidal invasion, vascular invasion, and lymph node metastasis.