Association of TERT Promoter Mutation, But Not BRAF Mutation, With Increased Mortality in PTC

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Context: Papillary thyroid carcinoma (PTC) carrying the BRAF mutation has been reported to be associated with high recurrence and potentially increased mortality. PTC carrying the TERT promoter mutation has been associated with older age, recurrence, and aggressive disease.

Objective: The objective of this study was to determine the association of BRAF and TERT promoter gene alterations with recurrence and survival in a high-risk population.

Design: Genomic DNA was analyzed for the BRAF mutation from 256 persistent/recurrent PTC (p/rPTC; 202 new, 54 previously reported) and for the TERT promoter mutation and polymorphism (242 p/rPTC). Two-tailed Fisher exact tests or the Pearson χ^2 test were performed for the associations between mutations and other variables. Overall and disease-free survivals were compared by log rank tests on Kaplan-Meier plots and by Cox regression analysis. TERT promoter constructs were tested in PTC cell lines to determine their activities in these cells.

Results: BRAF V600E mutation was identified in 235 of 256 (91.8%), TERT promoter mutation at −124 was detected in 77 of 242 (31.8%), and TERT promoter polymorphism at −245 was found in 113 of 242 (46.7%) p/rPTC patients. A significant difference in survival was found in p/rPTC patients with the TERT promoter mutation, which also displayed increased activity in vitro as compared to the nonmutated promoter sequence. No association was noted between the BRAF mutation or TERT promoter polymorphism and recurrence or survival. A drawback of our study could be the limited number of patients with nonmutated BRAF (21 of 256 [8.2%]).

Conclusions: Mutation in the TERT promoter, but not in BRAF, was associated with decreased survival in 19 (24.7%) p/rPTC patients who died of disease and in 38 (49.4%) p/rPTC patients who died at last contact. The presence or absence of the BRAF mutation and TERT promoter polymorphism, however, was not significantly correlated with survival. **(*J Clin Endocrinol Metab* 100: E1550–E1559, 2015)**

Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer, accounting for 70–80% of cases. Its incidence is increasing across all demographic groups (1, 2). In most cases, PTC has an excellent prognosis, with 5-year survival rates approaching 98% for patients with local regional disease (3). Surgery with or without adjuvant radioactive iodine administration is the most common treatment for this malignancy and is curative in more than 85% of patients. However, nearly 15% of patients with PTC experience recurrence during the 10 years after their initial treatment (4, 5). The vast majority of these recurrences occur either locally or regionally in the

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Abbreviations: CI, confidence interval; DFS, disease-free survival; DM, distant metastasis; OS, overall survival; p/rPTC, persistent/recurrent PTC; PTC, papillary thyroid carcinoma; RR, risk ratio; TERT, telomerase reverse transcriptase.

lateral neck. Less commonly, PTC recurs as distant metastasis (DM) to the lung, bone, or brain.

Clinical and molecular investigations into the etiology of progressive, persistent/recurrent PTC (p/rPTC) are currently under way. One area of active investigation in p/rPTC is genetic alterations among candidate genes postulated to explain the biological behavior of more aggressive forms of PTC. In numerous studies, BRAF (V600E) mutation has been associated with negative prognostic factors, including larger tumor size, older age, male gender, soft tissue extension, tumor multifocality, lymph node metastasis, advanced TNM stage, and recurrence (6–11). A relationship between the BRAF mutation and survival has therefore been postulated (12), but no convincing association between the BRAF mutation and mortality has yet been found (13, 14).

The human telomerase reverse transcriptase (*TERT*) gene is the catalytic reverse transcriptase subunit of telomerase. Its function is to maintain the length of telomeres in DNA strands (15). Mutations in TERT promoter observed in many types of cancer (16), found at −124 and −146 from the start of the translational ATG site, have been shown to increase the expression of TERT mRNA (17, 18). The mutation at −146 is rare in PTC (19).

To investigate the genetic basis of p/rPTC, we previously analyzed the tumors of 54 patients (20). The mutational analysis showed that 68.5% of the patients had the BRAF V600E mutation. The high proportion of patients with the BRAF mutation suggested an association between BRAF and p/rPTC within this cohort. The objective of the current study was to determine whether an association existed in survival by comparatively evaluating the tumor genetic profile and survival patterns of patients from this cohort and 202 additional p/rPTC patients.

Patients and Methods

Patients with p/rPTC were enrolled from March 1, 1991 to July 1, 2010. Biomedical Institutional Review Board approval was granted for this study. Statistical analyses were performed using Statistica 12 and SPSS (version 21, IBM Corporation, Armonk, NY). Bonferroni adjusted levels of significance for correlations of multiple factors are shown in the relevant tables.

Results

Mutational analysis

Genomic DNA from patients with p/rPTC (Figure 1A) was analyzed using Sequenom (Supplemental Table 1) and next generation sequencing (Supplemental Table 2); the BRAF mutation (V600E) was detected in 235 (91.8%) patients (Supplemental Table 3).

TERT promoter in 242 of the 256 p/rPTC patients (14 patients had insufficient DNA for analysis) was sequenced, a single-nucleotide mutation from C to T at −124 (chr5:1,295,228C>T, C228T) in 77 patients (31.8%) and a single-nucleotide polymorphism from T to C at −245 (rs2853669) in 113 patients (46.7%, Supplemental Table 3) were detected.

Associations with demographic data

Patients were analyzed on the basis of BRAF mutation (total 256 patients) or TERT promoter mutation or polymorphism (total 242 patients). Among 77 patients with the TERT promoter mutation, 63.6% were > 45 years of age at their first surgery for primary PTC ($P = .00002$). No significant differences in age and gender were found in patients with the BRAF mutation or TERT promoter polymorphism. Ethnicity was not correlated with any mutations or polymorphism tested.

Patients were also analyzed by their disease status, recurrent or persistent; 93 were recurrent and 163 persistent for BRAF analysis, and 91 were recurrent and 151 persistent for TERT promoter. No significant differences between recurrent and persistent diseases were found in gender or ethnicity (Supplemental Table 4).

Associations with disease stage

Comparing TNM stages individually, the BRAF mutation, TERT promoter polymorphism, and TERT promoter mutation were not significant factors (Supplemental Table 5), nor was recurrent disease when compared to persistent disease (Supplemental Table 4). If grouping them together and using disease stages, a significant difference was found with the TERT promoter mutation when comparing stages III+IV vs stages I+II (61.9 vs 38.1%; $P = .00046$; Supplemental Table 5). In addition, a higher percentage of patients with TERT promoter mutation had stage III+IV tumors with recurrent disease than those with persistent disease (50.9 vs 32.5%; $P = .022$; Supplemental Table 4). More specifically, a higher percentage of stage III+IV patients with recurrent disease had TERT promoter mutation than those with nonmutated TERT promoter (79 vs 35.3%; $P = .0038$; data not shown). No significant differences were found in disease stages when BRAF mutation or TERT promoter polymorphism was examined.

Associations with disease recurrence

The median age at diagnosis of progressive p/rPTC in this study was 45.3 years. A higher percentage of patients with TERT promoter mutation was found to be older than 45 years when they underwent surgery for first p/rPTC (63.6%; $P = .00002$; Table 1). When comparing recurrent

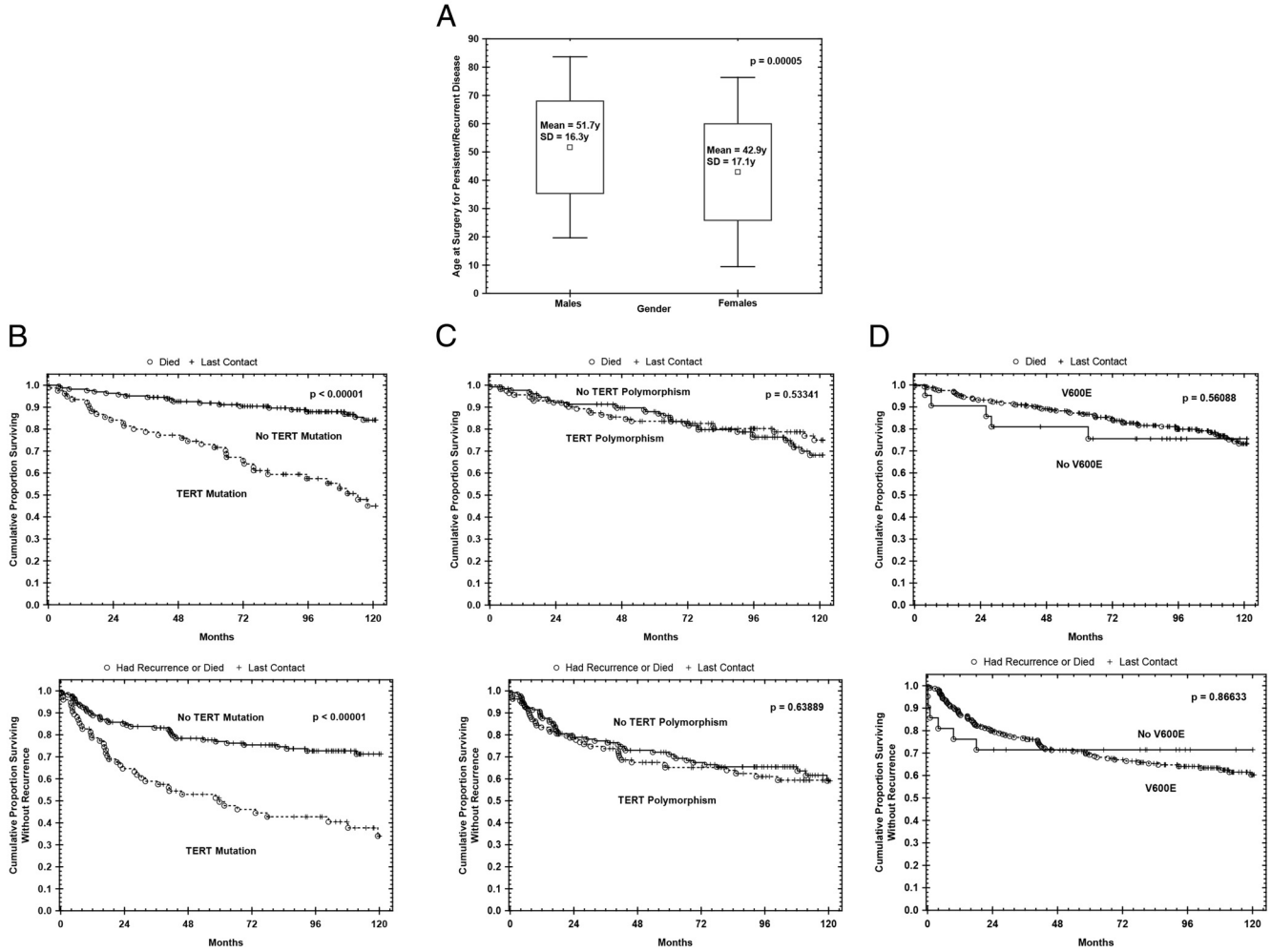

Figure 1. Kaplan-Meier analysis on mutations and/or polymorphism when analyzed alone. OS and DFS were compared by log rank tests on Kaplan-Meier plots and by Cox regression analysis. OS was measured in months from the date of surgery for the first recurrence to date of death or last contact, and DFS was measured in months from the end of the treatment for the first recurrence to the date of development of a second recurrence, death, or last contact. A, Patients' age and gender at the time of surgery for first p/rPTC surgery without analysis of mutations. B–D, OS (top) and DFS (bottom) truncated at 10 years. Curves describing OS and disease-specific survival were generated by the Kaplan-Meier product limit method. The statistical significance of differences between the actuarial curves was tested by the log rank test. OS was counted in months from the date of surgery for first recurrence to the date of death or last contact (top), and DFS was counted in months from the end of the treatment for the first recurrence without development of second recurrent tumor until the date of death or last contact (bottom). B, Kaplan-Meier analysis for patients with TERT promoter mutation vs nonmutated TERT promoter (No TERT mutation). C, Kaplan-Meier analysis for patients with TERT promoter polymorphism vs no TERT promoter polymorphism (No TERT polymorphism). D, Kaplan-Meier analysis for patients with the BRAF mutation (V600E) vs nonmutated BRAF (No V600E).

to persistent disease, the percentage of patients who were > 45 years of age at their first p/rPTC surgery was significantly higher in the recurrent group than in the persistent group (67.7 vs 41.7%; $P = .00006$), although the percentage was not significantly different at the time of surgery for their primary disease ($P = .11$; Supplemental Table 4). In addition, a trend suggested that a higher percentage of the patients with recurrent disease had TERT promoter mutation than those with persistent disease (39.6 vs 27.2%; $P = .045$; Supplemental Table 6). More specifically, more male patients with recurrent disease had TERT promoter mutation than those with nonmutated TERT promoter (58.3 vs 27.3%; $P = .0031$; data not shown). No correlation of BRAF mutation and TERT promoter polymorphism was detected between recurrent and persistent diseases ($P = .25$ and .35, respectively; Supplemental Table 6).

More patients with larger tumors (>1.5 cm; 62.8%; $P = .002$; Supplemental Table 5) at their first p/rPTC surgery had TERT promoter mutation than patients with nonmutated TERT promoter. There were no significant differences in tumor size or number of positive lymph nodes at first p/rPTC surgery between recurrent and persistent diseases (Supplemental Table 4). However, a significant difference in tumor size and number of positive lymph nodes at first p/rPTC surgery in patients with recurrent disease was detected to be associated with TERT promoter mutation than those with nonmutated TERT

Table 1. Demographic, Recurrence, and Last Contact When the BRAF Mutation (V600E Mutated), TERT Promoter Mutation (Mutated at −124), or TERT Promoter Polymorphism (Poly at −245) Was Analyzed Alone

	BRAF[a]			TERT Promoter[a]					
Category	V600E mutated, n (%)	Nonmutated, n (%)	*P* Value[b]	Mutated at −124, n (%)	Nonmutated, n (%)	*P* Value[b]	Poly at −245, n (%)	No Poly, n (%)	*P* Value[b]
Total patients	235 (91.8)	21 (8.2)		77 (31.8)	165 (68.2)		113 (46.7)	129 (53.3)	
Gender									
Male	103 (43.8)	6 (28.6)	.25	43 (55.8)	62 (37.6)	.0076	43 (38.1)	62 (48.1)	.12
Female	132 (56.2)	15 (71.4)		34 (44.2)	103 (62.4)		70 (61.9)	67 (51.9)	
Ethnicity									
Caucasian	167 (71.1)	15 (71.3)	.77	51 (66.2)	120 (72.7)	.83	81 (71.7)	90 (69.8)	.4
Black	3 (1.3)	1 (4.8)		1 (1.3)	3 (1.8)		1 (0.9)	3 (2.3)	
Hispanic	51 (21.7)	4 (19.1)		19 (24.7)	33 (20.0)		21 (18.6)	31 (24.0)	
Asian	11 (4.6)	1 (4.8)		5 (6.5)	7 (4.3)		8 (7.0)	4 (3.1)	
Filipino	3 (1.3)	0 (0.0)		1 (1.3)	2 (1.2)		2 (1.8)	1 (0.8)	
Age at surgery for primary disease, y									
<45	132 (56.2)	14 (66.7)	.49	28 (36.4)	108 (65.5)	.00002	63 (55.8)	73 (56.6)	.9
>45	103 (43.8)	7 (33.3)		49 (63.6)	57 (34.5)		50 (44.2)	56 (43.4)	
Age at surgery for first p/rPTC, y									
<45	111 (47.2)	14 (66.7)	.11	21 (27.3)	94 (57.0)	.00002	56 (49.6)	59 (45.7)	.55
>45	124 (52.8)	7 (33.3)		56 (72.7)	71 (43.0)		57 (50.4)	70 (54.3)	
Developed second recurrence									
No	164 (70.1)	13 (61.9)	.46	44 (57.9)	122 (73.9)	0.012	70 (62.5)	96 (74.4)	.046
Yes	70 (29.9)	8 (38.1)		32 (42.1)	43 (26.1)		42 (37.5)	33 (25.6)	
Overall DM									
No	178 (75.7)	16 (76.2)	1	42 (54.6)	141 (85.5)	<.00001	84 (74.3)	99 (76.7)	.66
Yes	57 (24.3)	5 (23.8)		35 (45.4)	24 (14.5)		29 (25.7)	30 (23.3)	
Died of their thyroid disease									
No	210 (89.4)	17 (81.0)	.27	58 (75.3)	155 (93.9)	.00003	97 (85.8)	116 (89.9)	.33
Yes	25 (10.6)	4 (19.0)		19 (24.7)	10 (6.1)		16 (14.2)	13 (10.1)	
Died at last contact									
No	175 (74.5)	16 (76.2)	1	39 (50.6)	138 (83.6)	<.00001	83 (73.5)	94 (72.9)	.92
Yes	60 (25.5)	5 (23.8)		38 (49.4)	27 (16.4)		30 (26.5)	35 (27.1)	

[a] Total number of patients was 256 for BRAF analysis and 242 for TERT promoter analysis. Tumor specimens of patients with p/rPTC were examined in 256 consecutive patients. Each patient was consecutively enrolled to permit genomic DNA analysis of their tumors using high-throughput mass array and next generation sequencing as described below. Formalin-fixed, paraffin-embedded archival specimens were retrieved from the Department of Pathology in accordance with protocols approved by the Biomedical Research Institutional Review Board at the University of Texas MD Anderson Cancer Center. Specimens were histologically reviewed. Those containing at least 70% tumor cellularity were selected for study. Histopathological classification of PTC was confirmed according to the most recent standards set by the World Health Organization. Genomic DNA was extracted using the Puregene Tissue Kit (QIAGEN) according to the manufacturer's protocol. Genomic DNA was tested for mutations by a Sequenom MALDI TOF MassArray system at the Characterized Cell Line Core Facility at the MD Anderson Cancer Center (MDACC). Thirty-seven frequent mutated genes were tested at 179 sites (Supplemental Table 1). Next generation sequencing was performed using a 46-gene panel platform for the detection of frequently reported point mutations in human malignancies by CLIA-certified molecular diagnostics laboratory at MDACC (Supplemental Table 2). A minimum of 250× coverage is required at a given base for the interpretation of a nonmutated or variant call. To confirm mutations, genomic DNA was amplified by PCR using advantage HF-2 (Clontech), QIAGEN Multiplex PCR Kit, or Kapa2G Robust HotStart ReadyMix (Kapa Biosystems). All PCR products were treated with ExoSAP-IT (Affymetrix) or gel purification before sequencing and were sequenced with both sense and antisense primers by the DNA Sequencing Core Facility at MDACC. Primers for BRAF and RAS genes (synthesized by Sigma-Aldrich) have been previously published (20). PCR primers for PIK3CA 5′-TTTCAGGAGATGTGTTACAAGGCTT-3′ (sense) and 5′-ATTAACAGTGCAGTGTGGAATCCAGAG-3′ (anti-sense) and for TERT promoter mutation were synthesized by Integrated DNA Technologies.

[b] Possible differences between groups for scaled parameters were assessed by one-way ANOVA testing with post hoc tests. Correlations between categorical variables were assessed by the Pearson χ^2 test or, when there are fewer than 10 subjects in any cell of a 2 × 2 grid, by the two-tailed Fisher exact test. The Bonferroni adjusted level of significance for this table and Supplemental Table 5 is $P = .00375$.

promoter (71.4 vs 34.2% with > 1.5 cm tumors, $P = .0076$; and 33.3 vs 7.9% with three or more positive lymph nodes, $P = .026$, respectively; data not shown). Tumor size and the number of positive lymph nodes at the first recurrence were not determining factors for patients with the BRAF mutation or TERT promoter polymorphism.

No significant difference was found in the development of a second recurrence between recurrent and persistent diseases (Supplemental Table 4) or in patients with any mutations or TERT promoter polymorphism (Table 1). However, a trend suggested that more persistent patients with TERT promoter mutation developed a second recurrence than those with nonmutated

Table 2. Demographic, Recurrence, and Last Contact When Overall DM Was Compared With Those Without DM

Overall DM	No, n (%)	Yes, n (%)	*P* Value[a]
Total patients	194 (75.8)	62 (24.2)	
Gender			
Male	72 (37.1)	37 (59.7)	.0018
Female	122 (62.9)	25 (40.3)	
Age at surgery for primary disease, y			
<45	131 (67.5)	15 (24.2)	<.00001
>45	63 (32.5)	47 (75.8)	
Age at surgery for first p/rPTC, y			
<45	116 (59.8)	9 (14.5)	<.00001
>45	78 (40.2)	53 (85.5)	
Developed second recurrence			
No	152 (78.4)	25 (41.0)	<.00001
Yes	42 (21.6)	36 (59.0)	
Died at last contact			
No	175 (90.2)	25 (40.3)	<.00001
Yes	19 (9.8)	37 (59.7)	

[a] Possible differences between groups for scaled parameters were assessed by one-way ANOVA testing with post hoc tests. Correlations between categorical variables were assessed by the Pearson χ^2 test or, when there are fewer than 10 subjects in any cell of a 2×2 grid, by the two-tailed Fisher exact test. Bonferroni adjusted level of significance is $P = .01$.

TERT promoter (42.5 vs 24.6%; $P = .033$; data not shown).

Associations with DM and survival

Although quite rare in PTC patients, DM was associated with TERT promoter mutation. The overall DM was higher in patients with TERT promoter mutation than in those who had nonmutated TERT promoter (42.7 vs 17.4%; $P = .00004$; Table 1). DM to the lung or bone was significantly higher in patients with TERT promoter mutation than in those with nonmutated TERT promoter (data not shown). No significant differences in DM were detected in patients with the BRAF mutation or TERT promoter polymorphism or between patients with recurrent and persistent diseases (Supplemental Table 4). However, a higher percentage of patients having more chance of developing overall DM was found to have TERT promoter mutation than those with nonmutated TERT promoter in both recurrent and persistent diseases (41.7 vs 14.6%, $P = .006$, for recurrent disease; and 48.8 vs 14.6%, $P = .00001$, for persistent disease; data not shown).

Overall DM was analyzed further in 256 p/rPTC patients despite their mutation and/or polymorphism status (Table 2). We found that male patients (59.7 vs 37.1%; $P = .0018$), patients aged 45 years or older at the time of primary (75.8 vs 32.5%; $P < .00001$) or first p/rPTC surgery (85.5 vs 40.2%; $P < .00001$), patients who developed second recurrence (59 vs 21.6%; $P < .00001$), or patients who were dead at last contact (59.7 vs 9.8%; $P < .00001$; Table 2) were more likely to develop DM than those without DM.

In this cohort of 256 p/rPTC patients undergoing surgery with a median follow-up time of 112 months, overall survival (OS) was 86.7% at 5 years, and disease-free survival (DFS) was 69.5% at 5 years (Supplemental Figure 1). For the cohort of 242 p/rPTC patients with or without TERT promoter mutation, OS and DFS were significantly better in patients without mutation than those with mutation (risk ratio [RR], 4.46; 95% confidence interval [CI], 2.59–7.67; $P < .00001$; and RR, 2.83; 95% CI, 1.85–4.34; $P < .00001$, respectively; Table 3 and Figure 1B). In addition, we found that a higher percentage of patients with TERT promoter mutation died of their thyroid disease or had died at last contact than did the patients with nonmutated TERT promoter (24.7 vs 6.1%; $P = .00003$; and 49.4 vs 16.4%; $P < .00001$, respectively; Table 1). No significant differences were found for OS and DFS in p/rPTC patients with TERT promoter polymorphism (Table 3 and Figure 1C) or the BRAF mutation (Table 3 and Figure 1D). No significant differences in OS and DFS were detected when recurrent disease was compared with persistent disease (Supplemental Figure 2A and Supplemental Table 6). TERT promoter mutation significantly affected OS and DFS in both recurrent and persistent diseases when compared to nonmutated TERT promoter ($P < .00001$ for OS, $P = .00016$ for DFS; Supplemental Figure 2B and Supplemental Table 6). A higher percentage of patients with TERT promoter mutation died of their thyroid disease or had died at last contact than did those patients with nonmutated TERT promoter in recurrent disease (22.2 vs 5.5%, $P = .023$; and 44.4 vs 20%, $P = .013$, respectively; data not shown), as well as in persistent disease (26.8 vs 6.4%, $P = .0013$; and 53.7 vs 14.6%, $P < .00001$, respectively; data not shown). When comparing recurrent with persistent disease, no significant differences in OS and DFS were detected with TERT promoter polymorphism (Supplemental Figure 2C) or BRAF mutation (Supplemental Figure 2D) when compared to no TERT promoter polymorphism or nonmutated BRAF (Supplemental Table 6).

In addition to determining OS and DFS for the BRAF mutation, TERT promoter polymorphism, or TERT promoter mutation alone, OS and DFS were also determined when the BRAF mutation, TERT promoter polymorphism, and TERT promoter mutation were present in combination. A significant difference in DFS was detected when patients with nonmutated BRAF, nonmutated

Table 3. Survival When the TERT Promoter Mutation, BRAF Mutation, or TERT Promoter Polymorphism Was Analyzed Alone or in Combination[a]

Category	No. of Patients/Total	% in Group	% at 3 Years	% at 5 Years	Log Rank *P* Value	Cox Regression RR	95% CI
OS							
Total patients	256/256	100.0	90.5	86.7	n/a	n/a	n/a
TERT mutation	77/242	31.8	78.6	73.1	<.00001	4.46	2.59 to 7.67
V600E	235/256	91.8	91.3	87.2	.56	0.74	0.30 to 1.87
TERT polymorphism	113/242	46.7	88.3	83.6	.57	1.18	0.70 to 2.01
Nonmutated	10/87	11.5	100.0	100.0	.062	1.72	0.89 to 3.34
TERT mutation ± V600E ± TERT polymorphism	77/87	88.5	78.6	73.4			
V600E ± TERT polymorphism	147/224	65.6	97.9	92.2	<.00001	2.18	1.64 to 2.90
TERT mutation ± V600E ± TERT polymorphism	77/224	34.4	78.6	73.1			
TERT polymorphism ± V600E	75/152	49.3	95.9	93.2	<.00001	7.28	3.06 to 17.32
TERT mutation ± V600E ± TERT polymorphism	77/152	50.7	78.6	73.1			
DFS							
Total patients	254/254	100.0	76.2	69.3	n/a	n/a	n/a
TERT mutation	75/240	31.3	58.9	49.6	<.00001	2.83	1.85 to 4.34
V600E	233/254	91.7	76.7	69.3	.87	1.07	0.47 to 2.47
TERT polymorphism	111/240	46.3	73.7	65.1	.64	1.11	0.72 to 1.70
Nonmutated	10/85	11.8	90.0	90.0	.011	1.95	1.01 to 3.77
TERT mutation ± V600E ± TERT polymorphism	75/85	88.2	58.9	49.6			
V600E ± TERT polymorphism	147/222	66.2	83.9	76.9	<.00001	1.68	1.35 to 2.10
TERT mutation ± V600E ± TERT polymorphism	75/222	33.8	58.9	49.6			
TERT polymorphism ± V600E	75/150	50.0	79.1	72.9	.00076	2.37	1.42 to 3.97
TERT mutation ± V600E ± TERT polymorphism	75/150	50.0	58.9	49.6			

Abbreviation: n/a, Not applicable.

[a] Kaplan-Meier plots shown in Figure 2 were compared by the log rank test. OS was measured from the date of surgery for first recurrence to death or last contact. DFS was measured from the end of treatment for first recurrence to second recurrence, death, or last contact. RRs were assessed using the Cox proportional hazard model. Bonferroni adjusted level of significance is $P = .00417$.

TERT promoter, or no TERT promoter polymorphism were compared with patients with TERT promoter mutation in the presence or absence of TERT promoter polymorphism and the BRAF mutation (RR, 1.95; 95% CI, 1.01–3.77; $P = .011$; Table 3 and Figure 2A). No difference was found in OS in this case ($P = .062$). OS and DFS in patients with the BRAF mutation in the presence or absence of TERT promoter polymorphism (RR, 2.18; 95% CI, 1.64–2.90; $P < .00001$, for OS; RR, 1.68; 95% CI, 1.35–2.10; $P < .00001$, for DFS; respectively; Table 3 and Figure 2B) or in patients with TERT promoter polymorphism in the presence or absence of the BRAF mutation were significantly higher than in patients with the TERT promoter mutation in the presence or absence of TERT promoter polymorphism and the BRAF mutation (RR, 7.28; 95% CI, 3.06–17.32; $P < .00001$, for OS; and RR, 2.37; 95% CI, 1.42–3.97; $P = .00076$, for DFS, respectively; Table 3 and Figure 2C). These data suggested that the TERT promoter mutation, but not the BRAF mutation or TERT promoter polymorphism, contributed significantly to determining OS and DFS.

Significance of the TERT promoter mutation/polymorphism in PTC cells

Because a mutation in the TERT promoter and TERT promoter polymorphism was found in p/rPTC patients, we sought to determine whether this mutation would induce the expression of TERT in PTC cell lines. Two BRAF-mutated PTC cell lines, BCPAP (Figure 3A) and K2 (Figure 3B), were used because most p/rPTC patients carry the BRAF mutation (91.8%) and some patients carry both the BRAF and PI3K mutations (0.8%) as present in the K2 cells. We found in both cell lines that TERT promoter mutation significantly increased promoter activity when compared with nonmutated TERT promoter ($P < .001$; Figure 3, A and B). TERT promoter polymorphism also increased the expression of TERT promoter in both cell lines, but to a lesser extent when compared with nonmutated TERT promoter ($P = .05$ and $P = .011$, respectively; Figure 3, A and B). These data suggested that both alterations to the TERT promoter may affect expression of TERT in PTC cells.

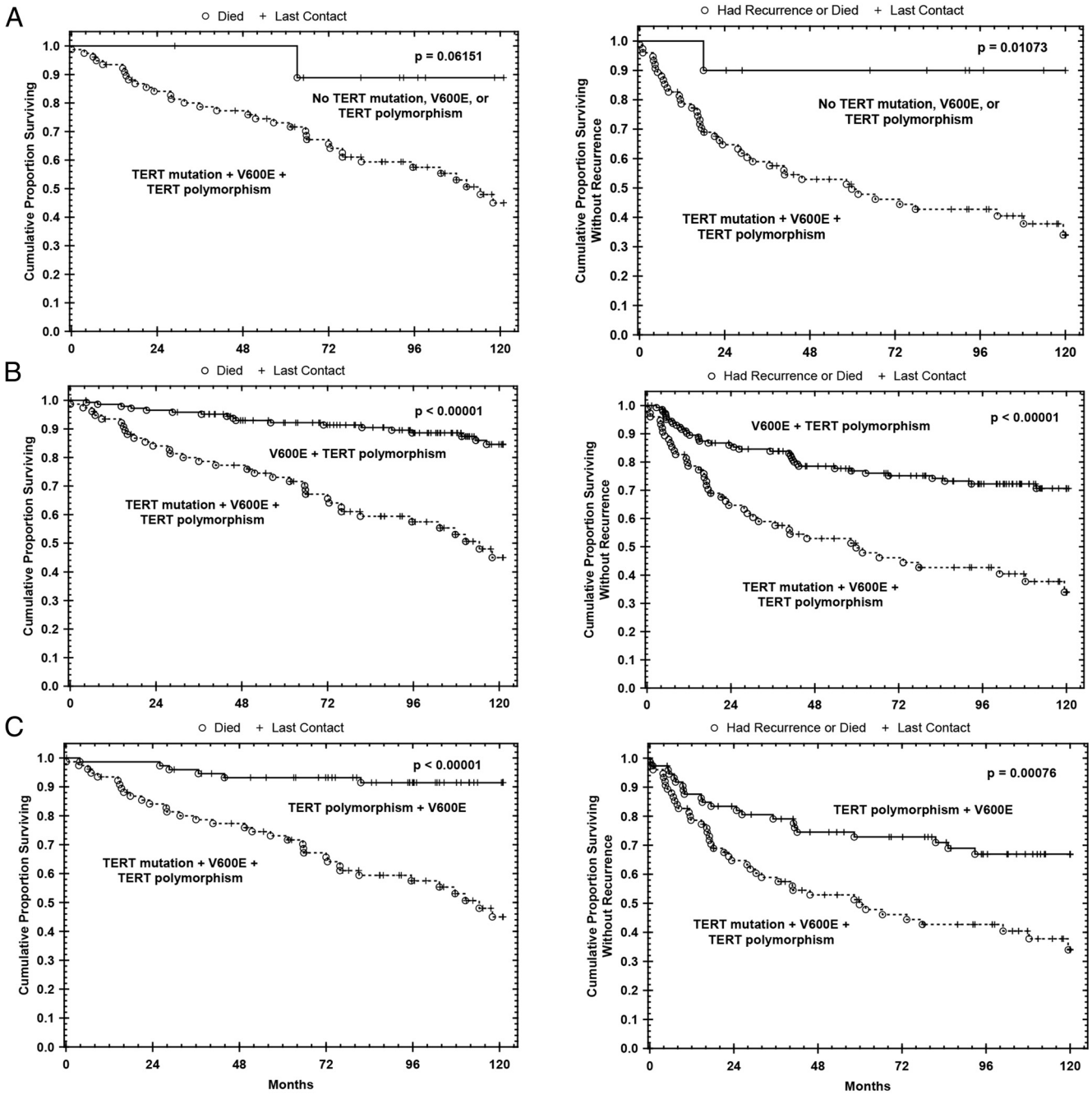

Figure 2. Kaplan-Meier analysis on combined mutations and/or polymorphism. Descriptive statistics for scaled values and frequencies of study patients within the categories for each of the parameters of interest were enumerated with the assistance of commercial statistical software. Correlations between parameters and endpoints were assessed by Pearson's χ^2 or, where there are fewer than 10 subjects in any cell of a 2 × 2 grid, by the two-tailed Fisher exact test. Curves describing overall (left) and disease-free (right) survival were generated by the Kaplan-Meier product limit method. The statistical significance of differences between the actuarial curves was assessed by the log rank test. Risk ratios were assessed using the Cox proportional hazard model. A, Patients with nonmutated BRAF, nonmutated TERT promoter, or no TERT promoter polymorphism (no TERT mutation, V600E, or TERT polymorphism); B, patients with the BRAF V600E mutation in the presence or absence of TERT promoter polymorphism (V600E ± TERT polymorphism); or C, patients with TERT promoter polymorphism in the presence or absence of the BRAF V600E mutation (TERT polymorphism ± V600E) were compared with patients with TERT promoter mutation in the presence or absence of the BRAF V600E mutation and TERT promoter polymorphism (TERT mutation ± V600E ± TERT polymorphism). Correlative details are presented in Table 3.

Discussion

We examined the BRAF mutation, TERT promoter mutation, and TERT promoter polymorphism in our p/rPTC cohort and on the basis of the patient's disease status, recurrent or persistent. TERT promoter polymorphism was first reported in bladder cancer (18) and has not been reported in PTC.

In this cohort of p/rPTC patients undergoing surgery with a median follow-up time of 112 months, we found

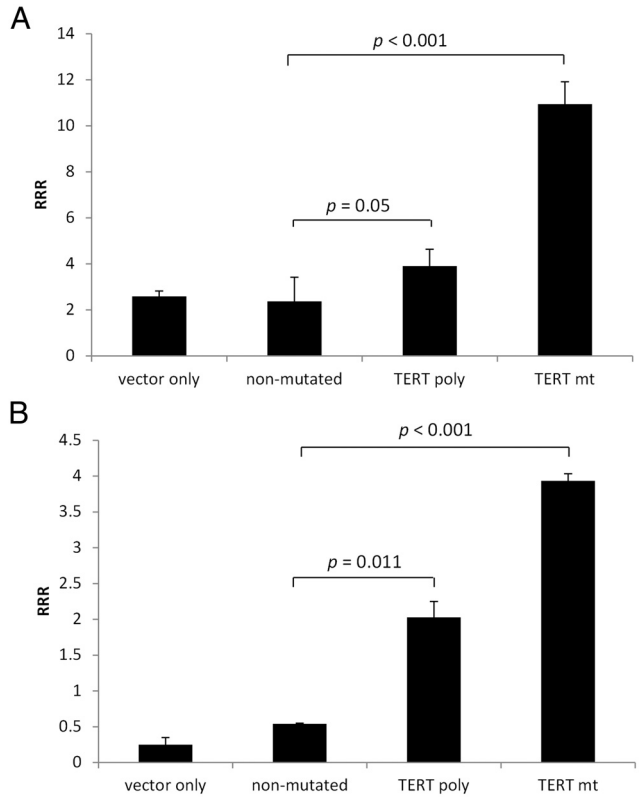

Figure 3. TERT promoter activity in BCPAP (A) and K2 (B) cells. K2 cells (PTC cell line with BRAF and PI3K mutations kindly provided by Dr. D. Wynford-Thomas from Cardiff University, Cardiff, United Kingdom) were maintained in DMEM/F12 medium (Sigma-Aldrich) containing 10% fetal bovine serum and 2 mM L-glutamine. BCPAP cells (PTC cell line with a BRAF mutation only, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) were maintained in RPMI 1640 medium containing 10% fetal bovine serum (Sigma-Aldrich) and 2 mM L-glutamine. BCPAP and K2 cells were plated in a 24-well plate in triplicate at 4–5 $\times$ 10^4 cells per well at 37°C overnight and transfected with TERT promoter constructs in pGL4.10 (kindly provided by Dr. Rajiv Kumar from the German Cancer Research Center, Heidelberg, Germany) and Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. The TERT promoter constructs are nonmutated, polymorphism at −245 (TERT poly), and mutation at −124 (TERT mt). pRL-CMV, pGL4.10 vector (vector only), and pGL3 control vectors were used as controls. Cells were lysed 48 hours after transfection by a Dual-Glo Luciferase Assay System (Promega), and luciferase activity was measured by a Berthold Detection System. The experimental sample ratio was determined by the value of firefly luciferase divided by the value of *Renilla* luciferase in each well. A relative response ratio (RRR) was then calculated using the following formula: RRR = [(experimental sample ratio) − (negative control ratio)]/[(positive control ratio) − (negative control ratio)]. Each cell line was repeated in at least three independent experiments.

that patients aged 45 years or older and with a larger tumor at first p/rPTC surgery were significantly associated with TERT promoter mutation, especially in patients with recurrent disease. This is in agreement with the study of Liu et al (21), where older patients were associated with TERT promoter mutation. Male patients, especially those with recurrent disease, were associated with TERT promoter mutation in our study when the TERT promoter was analyzed alone, but gender was not a significant factor in the study of Liu et al ($P = .259$).

TNM staging, if analyzed individually, was not a significant factor when the BRAF mutation, TERT promoter polymorphism, or TERT promoter mutation was examined or when recurrent disease was compared to persistent disease in this cohort. However, when grouping them together as disease stages, stages III+IV were significantly associated with TERT promoter mutation, especially in patients with recurrent disease. This is not surprising because older age is associated with TERT promoter mutation, and stages III+IV are representing patients with older ages. This was in agreement with what has been reported by others to be associated with the TERT promoter mutation for primary PTC compared with nonmutated TERT promoter (21–23). The percentages of patients with the BRAF mutation that was detected in large cohorts and reported recently were 42.8% in conventional PTC and 38.3% in overall PTC from a cohort of 507 patients (22), and 56.1% in conventional PTC and 48.5% in overall PTC in a cohort of 2099 PTC patients (24). The BRAF mutation was found to be associated with advanced disease, lymph node metastasis, and recurrence in primary PTC ($P < .001$) (22). In our previously published study where only 54 p/rPTC were evaluated, TERT promoter was not analyzed (20). Our conclusion from that smaller cohort was that p/rPTC was significantly associated with a predominant BRAF mutation, based on the information available at the time. However, no analysis was done in that study on the association of the BRAF mutation with recurrence, DM, or survival. The percentage of BRAF mutation was much higher in our current p/rPTC cohort (91.8%) than it was in primary PTC. To our surprise, we did not find that tumor size, number of positive lymph nodes, advanced disease stages, or first and second recurrences were associated with BRAF mutation in this p/rPTC cohort, although a drawback of our study could be the relatively small number of patients with nonmutated BRAF (8.2%). Our finding confirmed those of Gouveia et al (1), who showed that the BRAF mutation was not associated with tumor multicentricity, lymphovascular invasion, extranodal extension, or advanced (III or IV) stage in a cohort of 430 patients with PTC.

The BRAF mutation has been associated with PTC recurrence when considering recurrence from primary disease (24, 25). This was demonstrated by the study conducted by Xing et al (24), which included 2099 PTC patients. However, Henke et al (26) indicated that the BRAF mutation has no predictive value for recurrence in primary PTC in a cohort of 508 patients. Our study agreed with the findings by Henke et al (26) and suggested that the

BRAF mutation is not a factor when patients with first recurrence developed another recurrence.

DM and survival (including OS and DFS) were closely related to the TERT promoter mutation in our p/rPTC cohort. We found significant differences in lung and bone metastases in patients with TERT promoter mutation compared with those in patients with nonmutated TERT promoter. The overall DM was also significantly associated with male gender, an age of 45 years or older at primary and first p/rPTC surgeries, recurrence, and mortality. The association of older age and DM has been well documented by others, and this led to a higher mortality rate in these patients (27). OS and DFS were also associated with the TERT promoter mutation. Patients with nonmutated TERT promoter exhibited significantly improved survival compared to those with the TERT promoter mutation. This was confirmed in recurrent disease as well as in persistent disease. The association of DM and survival with the TERT promoter mutation has been reported by others in primary PTC patients (22, 23). In contrast, the BRAF mutation was not associated with DM, OS, or DFS in this study. This is in agreement with the study by Henke et al (26). When combining the BRAF mutation, TERT promoter mutation, and TERT promoter polymorphism, we found that p/rPTC patients with the TERT promoter mutation with or without the BRAF mutation and TERT promoter polymorphism had worse OS and DFS than do those without any mutations in the presence or absence of TERT polymorphism, with BRAF mutation in the presence or absence of TERT promoter polymorphism, or with TERT polymorphism in the presence or absence of BRAF mutation. Our results do not agree with the study by Xing et al (28) in which the BRAF mutation alone was associated with DFS ($P < .001$). The different conclusion obtained from the Xing et al (28) study (median, 33 mo from the date of surgery for primary PTC), compared with ours and the study of Henke et al (26), may be explained by the length of the follow-up. The follow-up times were 8 years for Henke et al (26) and 9.3 years for our group from the date of first recurrent surgery, although we detected no significant differences in either OS or DFS at our shortest follow-up period (3 y). Because PTC is a slow-growing tumor, longer follow-up time may be needed to draw conclusions regarding this disease. The other difference between the studies was a primary PTC cohort for Xing et al (28) and Henke et al (26) vs our p/rPTC cohort, although we do not currently know the significance of this difference.

To understand the molecular mechanisms of TERT promoter mutation and TERT promoter polymorphism in association with the TERT expression, we analyzed the activity of TERT promoter in two PTC cell lines. Our in vitro studies demonstrated that the TERT promoter mutation significantly increased the luciferase activity, and TERT promoter polymorphism increased to a lesser extent in both PTC cell lines. These findings suggested that TERT promoter mutation was important for regulating the expression of TERT. Because TERT expression has been indicated in promoting cell growth and tumorigenesis (29), it is not surprising to find a link between TERT promoter mutation and DM and recurrence in PTC, which played important roles in patient survival. Our findings were in agreement with those in bladder cancer cell lines where TERT promoter mutation and TERT polymorphism increased luciferase activity in vitro; and TERT promoter mutation decreased patient survival in bladder cancer patients, whereas TERT promoter polymorphism did not affect patient survival (18).

In summary, these data support the growing body of evidence showing that the BRAF mutation and TERT promoter polymorphism do not engender poor survival outcomes. Mutation in the TERT promoter, found most often in male patients 45 years or older and with recurrent disease, resulted in larger tumor size at first p/rPTC surgery, DM, and decreased OS and DFS compared to patients with a nonmutated TERT promoter. Patients with persistent disease and TERT promoter mutation have increased risk of developing further recurrence and DM than those with nonmutated TERT promoter. All of these clinicopathological parameters in p/rPTC patients were associated with TERT promoter mutation only and were not affected by the presence or absence of the BRAF mutation and TERT promoter polymorphism when the BRAF mutation, TERT promoter mutation, and TERT promoter polymorphism were analyzed together. This information may lead to a better understanding of the fundamental molecular basis for recurrence and aggressive biological behavior in PTC and perhaps to the development of better treatment options for these patients in the future.

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