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BRAF and *TERT* mutations in papillary thyroid cancer patients of Latino ancestry

Ana P Estrada-Flórez[1,2,3], Mabel E Bohórquez[2], Alejandro Vélez[4,5], Carlos S Duque[5], Jorge H Donado[5], Gilbert Mateus[6], Cesar Panqueba-Tarazona[7], Guadalupe Polanco-Echeverry[1], Ruta Sahasrabudhe[1], Magdalena Echeverry[2] and **Luis G Carvajal-Carmona[1,4,8,9]**

[1]Genome Center and Department of Biochemistry and Molecular Medicine, School of Medicine, University of California, Davis, California, USA
[2]Grupo de Citogenética, Filogenia y Evolución de Poblaciones, Facultad de Ciencias y Facultad de Ciencias de la Salud, Universidad del Tolima, Ibagué, Tolima, Colombia
[3]Facultad de Ciencias para la Salud, Universidad de Caldas, Manizales, Caldas, Colombia
[4]Dinamica IPS, Medellín, Antioquia, Colombia
[5]Hospital Pablo Tobón Uribe, Medellín, Antioquia, Colombia
[6]Hospital Federico Lleras Acosta, Ibagué, Tolima, Colombia
[7]Universidad Surcolombiana, Neiva, Huila, Colombia
[8]University of California Davis Comprehensive Cancer Center, Sacramento, California, USA
[9]Fundación de Genética y Genómica, Medellín, Antioquia, Colombia

Correspondence should be addressed to L G Carvajal-Carmona: lgcarvajal@ucdavis.edu

Abstract

Papillary thyroid cancer (PTC) is the second most commonly diagnosed malignancy in U.S. Latinas and in Colombian women. Studies in non-Latinos indicate that *BRAF* and *TERT* mutations are PTC prognostic markers. This study aimed to determine the prevalence and clinical associations of *BRAF* and *TERT* mutations in PTC Latino patients from Colombia. We analyzed mutations of *BRAF* (V600E) and *TERT* promoter (C228T, C250T) in tumor DNA from 141 patients (75 with classical variant PTC, CVPTC; 66 with follicular variant PTC, FVPTC) recruited through a multi-center study. Associations between mutations and clinical variables were evaluated with Fisher exact tests. Survival was evaluated with Kaplan–Meier plots. Double-mutant tumors (*BRAF*+/*TERT*+, $n = 14$ patients) were more common in CVPTC ($P = 0.02$). Relative to patients without mutations ($n = 48$), double mutations were more common in patients with large tumors ($P = 0.03$), lymph node metastasis ($P = 0.01$), extra-thyroid extension ($P = 0.03$), and advanced stage ($P = 6.0 \times 10^{-5}$). In older patients, *TERT* mutations were more frequent (mean age 51 years vs 45 years for wild type *TERT*, $P = 0.04$) and survival was lower (HR = 1.20; $P = 0.017$); however, given the small sample size, the decrease in survival was not statically significant between genotypes. Comparisons with published data in US whites revealed that Colombian patients had a higher prevalence of severe pathological features and of double-mutant tumors (10 vs 6%, $P = 0.001$). Mutations in both oncogenes show prognostic associations in Latinos from Colombia. Our study is important to advance Latino PTC precision medicine and replicates previous prognostic associations between *BRAF* and *TERT* in this population.

Key Words
- cancer risk factors
- somatic mutations
- Hispanics
- BRAF
- TERT

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Introduction

In the United States and several other countries, the incidence of papillary thyroid cancer (PTC) has significantly increased in recent decades (1). PTC is now the second most commonly diagnosed malignancy among US Latinas (2, 3). In Colombia, the country with the second largest Latino population in Latin America (4, 5),

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incidence has also been increasing and PTC is now the third most common female cancer with an age-standardized rate of 14.5 per 100,000 people (4, 5, 6). We have previously shown that Colombian PTC patients have a higher prevalence of indicators of severity and aggressive tumor behavior, such as large size, extra-thyroid extension, and lymph node and distant metastasis, than reported in developed countries (7). In contrast to over-diagnosis driven increases in incidence, which is common in developed countries, a smaller fraction of incidental diagnoses occurs in Colombia. This represents a unique opportunity to investigate the role of molecular makers in PTC etiology and prognosis.

Given the significant worldwide increase in PTC incidence (8), there is a great need to identify prognosis biomarkers that allow for effective patient stratification and management. The *BRAF* V600E mutation has been associated with tumorigenesis in a wide range of human malignancies (9) and represents the most common PTC mutation. *BRAF* V600E has been associated with clinicopathological features, such as lymph node metastasis and advanced disease stage (1, 10, 11), although the evidence is not consistent (12, 13, 14). Hence, *BRAF* V600E on its own has limited utility as a prognosis PTC biomarker. More recently, two *TERT* promoter mutations, C228T and C250T, were found in ~10% of PTC patients (15, 16, 17, 18, 19, 20) and have been associated with a higher risk of developing the classical variant of PTC (CVPTC) (15) and with disease severity (15, 18, 19, 20, 21, 22, 23, 24, 25).

Given the high prevalence of *BRAF* and *TERT* mutations in PTC and their prognostic associations, there have been several studies showing the coexistence and cooperative role of these mutations in aggressive disease (15, 16, 17, 18, 19, 21, 23, 24, 26, 27), likely because the acquisition of a *TERT* mutation could extend the lifespan of *BRAF*- or *RAS*-driven clones and enable accumulation of additional genetic defects leading to more advanced disease. Double mutants (i.e., carrying both *TERT* promoter and *BRAF* mutations) are associated with older age at diagnosis, CVPTC (18, 19), large tumors (18, 22), extra-thyroid extension (18, 22), lymph node (18) and distant metastasis (18, 19), advanced (18, 19, 22, 26), recurrence (18), and mortality (18, 23). Given the importance of *BRAF* and *TERT* mutations in PTC and the fact that these changes have not been examined in patients of Latino ancestry, we investigated the role of these mutations in clinical manifestations and the survival of patients recruited in a multi-center study in Colombia.

Materials and methods

Study population

The research protocol used in the study adhered to the Declaration of Helsinki and was approved by the Ethics Committees from University of Tolima (Ibague), Hospital Federico Lleras Acosta (Ibague), Clínica Tolima (Ibague), Hospital Hernando Moncaleano (Neiva), and Hospital Pablo Tobón Uribe (Medellin). These institutions are among the largest cancer hospitals in their corresponding cities. A total of 149 incident and histologically verified PTC patients, 81 with classical variant PTC (CVPTC) and 68 with follicular variant PTC (FVPTC), were recruited in between 2006 and 2016. All patients provided written informed consent, were interviewed in person by trained research nurses, and authorized access to pathology reports, clinical records, and to retrieve archival tumor samples for molecular analyses. We collected information on age of onset, gender, tumor size, focality, laterality, capsular or vascular invasion, lymph node metastases, extra-thyroid extension, distant metastasis, stage (AJCC), vital status, and cause of death.

Mutation status

A pathologist (MEB) demarcated tumor regions with >80% tumor cells on hematoxylin and eosin (H&E)-stained slides. We isolated the DNA from the demarcated regions using Qiagen DNeasy Blood & Tissue Kit and protocol. *BRAF* exon 15 and the *TERT* promoter region were amplified using previously reported primers (21, 28) and Sanger sequenced. The PCR amplification failed in three samples for *BRAF* and eight for *TERT* (including those three samples that failed for *BRAF*) and were excluded from all subsequent analyses. The mutation status in *BRAF* (V600E) and *TERT* promoter (C228T, C250T) was inspected in electropherograms with 4 Peaks v. 1.7 (Nucleobytes B.V. 2004–2015) by two experienced independent researchers (APE and GPE). Mutation calling concordance was 100%.

Statistical analyses

Statistical analysis was performed with R (https://www.r-project.org/). We stratified clinicopathological variables by histology (CVPTC and FVPTC) and

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compared the histological subtypes using chi-square (for dichotomous variables) and Student's *t* tests (for continuous variables, for which we verified that they were normally distributed). The association between mutation status and various characteristics, such as gender, age at diagnosis, histopathological subtype, tumor size, lymph node metastasis, and tumor stage, were determined by calculation of odds ratios; statistical significance was considered when two-sided *P* values were <0.05. Comparisons of mutation prevalence were carried out using a Student's t test and data from the Cancer Genome Atlas (TCGA) (29) and the Johns Hopkins Hospital PTC cohort (23). Survival curves, stratified by mutational status, were calculated with the Kaplan–Meier method and compared with log-rank test using survival v2.41-3 (https://cran.r-project.org/package=survival). Vital status (alive or death) was determined by investigating databases affiliated to the Colombian health system (Base de Datos Unica de Afiliados del Sistema General de Seguridad Social en Salud) and the National Civil Registry (Registraduria Nacional del Estado Civil) dataset. The last vital status assessment in all patients was carried out in December 2017, which resulted in a mean follow-up time of 74.5 months/patient (standard deviation (S.D.): 29.8).

Results

Characteristics of the study population

The characteristics of Colombian patients are shown in Table 1. In total, 80% (113 of 141) of these patients were women. The mean age of diagnosis was 45.9 years (S.D. = 13.7), large tumors (>2 cm) were diagnosed in 40% of the patients, 38% had multifocal disease, 20% had bilateral tumors, 30% had capsular invasion, 38% had vascular invasion, 36% had lymph node metastases, 26% had extra-thyroid extension, 6% had distant metastasis, and 30% of patients were classified with stage III/IV. At the final follow up, 94% of patients were alive. No statistical differences were found in tumor features between the two histopathological subtypes (Table 1).

Comparisons of clinical data in Colombian (Latino) patients with that in non-Latinos from TCGA and from the Johns Hopkins Hospital cohort are shown in Supplementary Table 1 (see section on supplementary data given at the end of this article). Relative to TCGA, we found a higher prevalence of FVPTC (47 vs 15%, $P = 1.74 \times 10^{-9}$) in Colombia. Comparisons with the Johns Hopkins Hospital cohort (23) revealed that Colombians had a higher prevalence of FVPTC (47 vs 25%, $P = 2.4 \times 10^{-8}$), large tumors (mean tumor size

Table 1 Clinical and histological characteristics of the 141 Colombian PTC patients analyzed in the study, stratified by histologic subtype.

Clinical feature	Number of patients (%)			*P* value
	All (*n* = 141)	CVPTC (*n* = 75)	FVPTC (*n* = 66)	
Male gender	28 (19.9)	18	10	0.189
Mean age in years	45.9	45.1	46.7	0.506
<45 years	57 (40.4)	33	24	
≥45 years	84 (59.6)	42	42	0.357
Mean tumor size	2.35	2.37	2.32	0.858
Large tumors, >2 cm	54 (40.3)	26	28	0.436
Multifocal tumors	51 (37.5)	24	27	0.352
Bilateral tumors	25 (19.7)	14	11	0.653
Capsular invasion	38 (30.2)	24	14	0.052
Vascular invasion	47 (38.2)	24	23	0.978
LNM	46 (36.2)	29	17	0.080
ETT	34 (26.2)	19	15	0.555
Distant metastasis	8 (5.7)	6	2	0.203
Stage III–IV[a]	42 (30.4)	25	17	0.302
Vital status[b]				
Alive	129 (94.2)	66	63	
Dead	8 (5.8)	5	3	0.719
Cancer-related death	4 (2.9)	3	1	0.620
Mean follow-up in months	74.5	73.5	75.6	0.669

[a]Based on American Joint Committee on Cancer (AJCC) protocol of classification but including two patients diagnosed before 45 years with metastasis.
[b]Vital status was unknown in four patients.
CVPTC, classical variant of PTC (papillary thyroid carcinoma); FVPTC, follicular variant of PTC; LNM, lymph node metastasis; ETT, extra-thyroid extension.

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2.35 cm vs 1.5 cm, $P=5.8\times10^{-51}$), vascular invasion (38 vs 15%, $P=1.5\times10^{-10}$), extra-thyroid extension (26 vs 18%, $P=0.016$), and advanced stage (30 vs 20%, $P=0.004$). These comparisons with US patients suggest that PTC diagnoses in Colombia are belated, resulting in a higher prevalence of advanced tumors.

Individual effects of *BRAF* and *TERT* mutations

The prevalence of *BRAF* V600E mutations in our study was 60% (49 of 75 CVPTCs, and 35 of 66 FVPTCs had V600E), which was lower than in TCGA data (70%, $P=0.058$, Supplementary Table 1) but higher than in the Johns Hopkins Hospital cohort (34%, $P=2.0\times10^{-5}$, Supplementary Table 1). We found that *BRAF* V600E was more frequent in patients with lymph node metastases (34 of 84 vs 12 of 57; $P=0.04$, Supplementary Table 2). As reported in other populations, CVPTC patients with advanced stage tumors had a higher prevalence of *BRAF* mutation (21 of 25 vs 28 of 48 of early stage CVPTCs; $P=0.03$ not shown) (1, 10, 11).

The presence of high-risk clinicopathological characteristics did not show sex differences although most of aggressive features had a higher frequency in men (Supplementary Table 3). This was also observed when patients were stratified by *BRAF* mutation status, where men had a higher risk of prevalence of *BRAF* wild type tumors with lymph node metastasis (5 of 12 men and 7 of 45 women who were *BRAF* wild type had lymph node metastases, $P=0.03$). The latter finding is consistent with previous reports suggesting that male sex is a risk factor for aggressive disease (30, 31).

The two mutations in the *TERT* promoter were mutually exclusive in our study and were identified in 16% of the patients (14 patients had tumors with the *TERT* C228T mutation and nine with *TERT* C250T). This *TERT* mutation prevalence in Colombia was two-fold higher than in TCGA (8%, $P=0.017$; Supplementary Table 1) (23, 29). Consistent with previous reports (21), we found that *TERT* mutations were associated with a late age of diagnosis (mean age at diagnosis 51.3 years for *TERT* mutant tumors vs 44.8 years for *TERT* wild type tumors, $P=0.04$). *TERT* mutations were also more frequent in patients with extra-thyroid extension (9 of 23 vs 25 of 118, $P=0.007$), and with advanced stage tumors (13 of 25 vs 29 of 118, $P=0.001$) (Supplementary Table 2). These associations between *TERT* mutation status and aggressive clinical manifestations are consistent with previous reports in other populations (15, 18, 22, 23, 24).

The combined effects of *BRAF* V600E and *TERT* promoter mutations

We found that 10% ($n=14$) of our patients had mutations in both *BRAF* and *TERT* (herein referred to as double-mutants), a prevalence that is higher than in the Johns Hopkins Hospital cohort (6%, $P=0.001$, Supplementary Table 1). The comparisons between the clinicopathological characteristics of double-mutants with those in wild type patients for both genes (double-wild types, $n=48$ patients) are shown in Table 2. Comparisons between all four mutation subgroups are shown in Supplementary Table 4. Compared to double-wild types, double-mutants were diagnosed at older age (56 years vs 45 years, $P=0.003$) and had a higher prevalence of CVPTC (11 of 14 vs 21 of 48, $P=0.022$), large tumors (8 of 14 vs 16 of 48, $P=0.026$), lymph node metastasis (7 of 14 vs 9 of 48, $P=0.009$), extra-thyroid extension (6 of 14 vs 11 of 48, $P=0.028$), and tumors with advanced stage (11 of 14 vs 10 of 48, $P=6.0\times10^{-5}$). Therefore, our study in Latinos replicates previously reported associations of double mutants in white/Caucasian patients with CVPTC (18, 19), age of diagnosis (18, 19, 22), tumor size (18, 22), extra-thyroid extension (18, 22), lymph node metastasis (18), and advanced disease stage (18, 19, 22, 26).

Exploratory analyses of survival

In our study, the overall mortality (i.e., by any cause) was 6% (8 of 137 patients in available vital status; Table 1). Of the eight deceased patients, four died of cancer and four of unknown reasons. Cancer-specific mortality was associated with older age at diagnosis (HR = 1.20; $P=0.017$) and patients with *TERT* promoter mutations generally had a lower chance of survival (HR = 3.9; $P=0.17$ and Fig. 1). This association with mortality in our study replicates the prognostic value of *TERT* mutations in an independent population (21, 22, 24, 25). Given the relatively small sample size of our study, we were unable to determine the statistical support of potential prognostic factors, such as *BRAF* mutations, *TERT* mutations, older age, advance stage, and CVPTC histology, to survival. Future studies should include a larger sample size to further investigate factors related to survival.

Discussion

The increment of PTC incidence in developed countries might be explained by the over-diagnosis of small

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Table 2 Comparison of clinical characteristics between wild-type tumors versus those with both *BRAF* V600E and *TERT* promoter mutations (double mutants).

Clinical feature	Number of patients (%)		OR (95% CI)	*P* value[a]
	No mutation (*n* = 48)	Double mutants (*n* = 14)		
Male gender	10	4	1.52 (0.39–5.88)	0.542
Mean age (s.d.)	44.7 (14.6)	55.5 (9.33)	NA	**0.003**
<45 years	20	3		
≥45 years	28	11	2.62 (0.65–10.62)	0.168
CVPTC subtype	21	11	4.71 (1.16–19.08)	**0.022**
Large tumors (>2 cm)	16	8	4.83 (1.12–20.82)	**0.026**
Multifocal tumors	17	4	0.97 (0.25–3.82)	0.971
Bilateral tumors	5	2	1.56 (0.26–9.37)	0.628
Capsular invasion	12	5	3.33 (0.76–14.54)	0.098
Vascular invasion	16	5	2.92 (0.61–13.85)	0.166
LNM	9	7	6.03 (1.43–25.32)	**0.009**
ETT	11	6	4.64 (1.10–19.50)	**0.028**
Distant metastasis	3	2	2.50 (0.37–16.70)	0.331
Stage III-IV[b]	10	11	19.80 (3.76–104.3)	$\mathbf{6.0 \times 10^{-5}}$
Vital status				
Alive	43	13	Reference	
Dead	4	1	0.83 (0.08–8.06)	0.870
Cancer-related death	1	1	3.31 (0.19–56.64)	0.428

[a]Statistically significant two-tailed *P* values are shown in bold. [b]Based on American Joint Committee on Cancer (AJCC) protocol of classification but including two patients diagnosed before 45 years with metastasis.
CVPTC, classical variant of PTC (papillary thyroid carcinoma); ETT, extra-thyroid extension; FVPTC, follicular variant of PTC; LNM: lymph node metastasis; s.d., standard deviation.

incidental tumors (32). However, few studies have also noted that the prevalence of large (33, 34) and aggressive tumors is also increasing (33, 35), suggesting that factors other than overdiagnosis might be affecting the increase of PTC incidence (36). Relative to reports in white patients from the U.S. (23), we found a higher prevalence of aggressive disease. A total of 30% of Colombian patients had stage III/IV tumors, 40% had large tumors >2 cm, and 6% had metastases to lungs and medulla, which support the notion of belated diagnosis rather than of incidental over-diagnosis. Therefore, the absence of confounding factors resulting from over-diagnosis indicates that factors influencing incidence and disease aggressiveness can be better studied in populations like Colombia.

Thyroid cancer is now the third most diagnosed cancer in Colombian women (6) and the second in US Latinas. A recent report from the American Cancer Society found that 9% of the newly diagnosed cancer patients in US Latinas (2, 6) and 5% in US white women are now PTC patients (6). The high incidence of PTC in Latinas is puzzling and could be explained, in part, by high rates of obesity in both US Latinas (where overweight/obesity rates are ~two-fold higher than in white women) (37) and Colombians (where obesity rates increased by 18% between 2005 and 2010) (38, 39, 40). Other population-specific factors, such as American Indian ancestry, which influences cancer patters in the region (41, 42, 43) or other unidentified etiological factors may mediate the risk of PTC in the population.

Even though recent studies suggest that CVPTCs tend to have more aggressive clinical manifestations

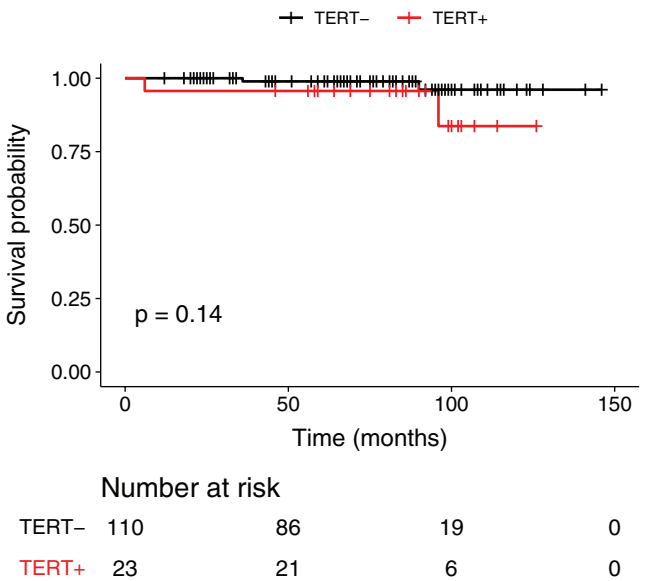

Figure 1
Effects of *TERT* promoter mutations on cancer-survival of PTC patients (log-rank test = 2.16, df = 1, *P* = 0.14).

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and worse outcomes (44), our study failed to detect statistically significant differences between the CVPTC and FVPTC subtypes (Table 1). However, we found a trend where CVPTCs, relative to FVPTCs, had a higher frequency of high-risk features, such as vascular invasion (32 vs 21%, $P=0.052$, Table 1) or lymph node metastasis (39 vs 23%, $P=0.08$, Table 1), which is consistent with a previous report in 1293 patients (44). Consistent with this observation, we found that most double mutants (11 out 14, Table 2) were CVPTCs, which may indicate that this histological subtype could be more aggressive than FVPTCs. We acknowledge that out failure to detect differences in clinical manifestations between the two histological subtypes is likely the result of limited power given our small sample size.

We found *TERT* mutations in 16% of the patients and these mutations were associated with a two-fold increment of the risk of extra-thyroid extension and of advanced stage. When *BRAF* and *TERT* promoter mutations were analyzed together, double-mutants compared to double-wild types had a six-fold higher risk of lymph node metastasis and a 20-fold higher risk of advanced tumors. The coexistence of *TERT* and *BRAF* mutations was also significantly associated with older age (Table 2). These clinical features have been associated with mortality in several studies (16, 22, 24, 25, 26, 45), demonstrating that *TERT* mutations, rather than *BRAF-V600E*, were restricted to PTC patients >45 years. This highlights the specific role of the age of patients in the mutational event (21). Hence, our study provides further support for the prognostic importance of *TERT* mutations in PTC.

PTC survival is mainly affected by tumor stage, with patients with stage IV tumors having the lowest survival rates (46). Survival up to 10 years in our sample was 89%, which is similar to the survival rate found by a recent report from the Colombian National Cancer Institute (47) but lower than reports from US patients (i.e., ~95%) (48). This observation further suggests that relative to US white patients, Colombians are more likely to have severe PTC. Future studies involving US Latinos and other US minorities are therefore warranted to assess whether survival and clinical manifestations are more severe, relative to white patients.

In the Johns Hopkins Hospital cohort, the analyses of the combined effects of *BRAF-TERT* mutations (i.e., double-mutants) revealed a significant association with mortality, which remained strong after multivariate adjustment for all of the conventional clinicopathological characteristics, demonstrating the independent role of double-mutant stats in PTC-related mortality. Exploratory analyses in our cohort suggested that *TERT* promoter mutations and a late age of onset appear to be stronger predictors than *BRAF* mutations. We acknowledge that our sample size is small and hence, under-powered to draw stronger conclusions on the combined role of *BRAF/TERT* mutation status on PTC mortality. Additionally, the clinic-based setting of our study may not reflect the characteristics of the patients of the general population and may have introduced some biases. However, population-based studies in Colombia (and in most of Latin America) are unfeasible due to the lack of country-wide cancer registries. Here, we made an effort to recruit patients from the largest cancer hospitals in their corresponding cities. Nonetheless, we believe that the multi-site nature of the study is a close reflection of the characteristics of the general population.

In summary, we found a high fraction of Colombian patients with large and advanced tumors and with distant metastasis, suggesting that most patients were not the result of incidental findings. To our knowledge, this is the first study of *BRAF* and *TERT* promoter mutations in Colombia and in Latinos. We found strong associations between *BRAF* and *TERT* promoter mutations and PTC prognosis, suggesting that these mutations could be a factor explaining the aggressiveness of the disease in this study. We believe that this report represents an important initial step to develop precision medicine for PTC in Latinos from the Americas.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/EC-19-0376.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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