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ORIGINAL ARTICLE

BRAF^{T1799A} mutation in the primary tumor as a marker of risk, recurrence, or persistence of papillary thyroid carcinoma

Marisa Cañadas Garre[a,*], Martín López de la Torre Casares[b], Patricia Becerra Massare[c], Miguel Ángel López Nevot[d], Jesús Villar Del Moral[e], Nuria Muñoz Pérez[e], Ricardo Vílchez Joya[b], Rosa Montes Ramírez[f], José Manuel Llamas Elvira[a]

[a]Servicio de Medicina Nuclear, Hospital Universitario Virgen de las Nieves, Granada, Spain
[b]Servicio de Endocrinología, Hospital Universitario Virgen de las Nieves, Granada, Spain
[c]Servicio de Anatomía Patológica, Hospital Universitario Virgen de las Nieves, Granada, Spain
[d]Servicio de Análisis Clínicos, Hospital Universitario Virgen de las Nieves, Granada, Spain
[e]Servicio de Cirugía General y del Aparato Digestivo, Hospital Universitario Virgen de las Nieves, Granada, Spain
[f]Departamento de Fisiología, Universidad de Granada, Spain

Abstract
Background and objective: The BRAF^{T1799A} mutation is reported to be associated with aggressive, persistent, and recurrent tumor in patients with papillary thyroid carcinoma (PTC). The association of the BRAF^{T1799A} mutation in the primary tumor with the clinicopathological characteristics of PTC was analyzed.
Patients, materials, and methods: Ninety-seven PTC patients were followed up for a median of 64.1 months. The BRAF^{T1799A} mutation was analyzed in DNA from initial thyroidectomy biopsies by PCR amplification and restriction fragment length polymorphism using the TspRI enzyme. Positive results were confirmed by DNA sequencing. The statistical association of the BRAF^{T1799A} mutation and clinicopathological characteristics was analyzed using the relevant hypothesis tests and logistic regression.
Results: The BRAF^{T1799A} mutation was found in 46.4% of patients. Bivariate and multivariate analyses showed the mutation to be only associated with age over 60 years (odds ratio [OR] = 5.5; 95% confidence interval [CI],1.4-21.9; p = 0.019) and to a tumor size of 1 cm or greater (OR = 3.6, 95% CI, 1.2-10.3; p = 0.016). The mutation was not associated with histological subtype, metastasis, recurrence, more aggressive treatments (131I ablation therapy or other surgery), or PTC persistence at the end of follow-up.

*Corresponding author.
E-mail address:* marisacgarre@gmail.com (M. Cañadas Garre).

Conclusions: The BRAF[T1799A] mutation is associated with age over 60 and a tumor size of 1 cm or greater, but not with other clinicopathological characteristics, tumor recurrence, or PTC persistence.

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Presencia de la mutación BRAF[T1799A] en el tumor primario como indicador de riesgo, recidiva o persistencia de carcinoma papilar de tiroides

Resumen

Antecedentes y objetivo: La mutación BRAF[T1799A] se ha relacionado con características tumorales de más agresividad, recidiva tumoral y persistencia de carcinoma papilar de tiroides (CPT), aunque no todos los estudios apoyan esta asociación. En éste, se analiza la asociación entre la presencia de la mutación BRAF[T1799A] en el tumor primario de pacientes con CPT y las características clinicopatológicas de riesgo, recidiva y persistencia tumoral.

Pacientes, material y método: Hemos seguido a 97 pacientes intervenidos de CPT durante una mediana de 64,1 meses. La mutación BRAF[T1799A] se determinó en ácido desoxirribonucleico procedente de muestras de la tiroidectomía inicial mediante amplificación por PCR del exón 15 del gen braf y análisis de los fragmentos de restricción con la enzima TspRI. Los casos positivos fueron confirmados por secuenciación. La asociación estadística entre la mutación BRAF[T1799A] y las diferentes variables se estudió mediante los correspondientes tests de contraste de hipótesis más regresión logística.

Resultados: El 46,4% de los pacientes eran positivos para la mutación BRAF[T1799A]. Tras análisis bivariante y multivariante, la mutación BRAF[T1799A] sólo se asociaba con edad superior a 60 años (odds ratio [OR] = 5,5; intervalo de confianza [IC] del 95%, 1,4-21,9; p = 0,019) y tamaño de 1 cm o superior (OR = 3,6; IC del 95%, 1,2-10,3; p = 0,016). No se asociaba con subtipos histológicos, metástasis, recidiva, necesidad de nuevos tratamientos ablativos con I131 o de otras intervenciones quirúrgicas debidas a la aparición de metástasis o persistencia de enfermedad al final del seguimiento.

Conclusiones: La mutación BRAF[T1799A] está asociada a edad superior a 60 años y tamaño tumoral de 1 cm o mayor, pero no con otras características clinicopatológicas, recidiva tumoral o persistencia de enfermedad.

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Introduction

Thyroid cancer is a tumor with a low but increasing incidence worldwide (10.2 cases per 100,000 inhabitants/year), particularly due to the detection of small (less than 2 cm in size) papillary thyroid carcinomas (PTCs)[1,2]. PTC accounts for more than 70% of all malignant tumors derived from follicular cells[3].

Although PTC prognosis is usually good, one patient subgroup has a poorer prognosis, with a 12% death rate, and a greater proportion experiences morbidity due to tumor recurrence[4].

The risk of recurrence or death is related to several parameters, notably age, sex, tumor size, metastasis, multifocality, and extrathyroid, capsular, and vascular invasion[5]. Recognition and evaluation of these prognostic factors, combined with molecular factor data assessing the risk of recurrence, could be of help in stratifying patients and selecting specific treatment approaches.

PTCs often have mutations in genes encoding for proteins implicated in the MAPK (mitogen-activated protein [MAP] kinase) signalling pathway[6]. BRAF is one of the three members of the conserved family of RAF serine-threonine kinases (ARAF, BRAF, and CRAF), which are key effectors in the canonical MAPK pathway (RAF-MEK-ERK). Most point mutations (approximately 90%) in the BRAF gene are of a single type, involving the transversion of thymine in position 1799 of the gene sequence to an adenine (T1799A), which results in the replacement of the amino acid valine, located in position 600 of the protein, by a glutamate (V600E)[7,8]. The BRAF[T1799A] mutation is the main genetic change in PTC, with an approximate frequency of 45%, although the proportion reported ranges from 20% to 83% depending on the series[7].

Some studies have reported the BRAF[T1799A] mutation to be associated with more aggressive tumor characteristics, including more advanced age at diagnosis, male sex, extrathyroid extension, metastasis, advanced tumor stage at diagnosis, and tumor recurrence[9,10]. The mutation has been reported to be an independent predictor of tumor recurrence even in stage I-II patients[9]. It has also been associated with a decreased ^{131}I uptake capacity and with the failure of treatment for recurrence[9,11]. However, not all studies agree that the

BRAF^{T1799A} mutation is a marker of greater aggressiveness and poorer prognosis[9,10,12].

The objective of this study was to analyze the association of the presence of the BRAF^{T1799A} mutation in the primary tumor of patients with PTC with the high-risk clinicopathological characteristics relevant for PTC prognosis, and with tumor recurrence and persistence.

Materials and methods

Study subjects

The clinicopathological characteristics and the presence of the BRAF^{T1799A} mutation were studied in 97 patients diagnosed with PTC at Hospital Universitario Virgen de las Nieves from January 1st, 1998 to May 30th, 2007. Patients were followed up until September 1st, 2008 (median follow-up: 64.1 [28.9-91.1]; minimum: 15.8 and maximum: 129.8 months).

PTC recurrence was defined as cytological/histopathological confirmation of the tumor in surgical specimens either from the thyroid bed or distant lymph node metastases, or tumor demonstration using imaging procedures (thyroid scan, ultrasound [US], computed tomography [CT], magnetic resonance imaging [MRI], positron emission tomography-CT [PET-TC]), associated with increased thyroglobulin (Tg) levels and/or a positive whole body scan (WBS) from the second WBS with ^{131}I.

Only patients with at least 27 months of follow-up (74 patients) were included in the analysis of status at the end of follow-up. Patients whose serum Tg levels (as the most sensitive marker) remained negative (under 1 ng/ml) in the absence of interference from anti-Tg antibodies for the previous 24 months and in whom both consecutive WBS during that period were also negative were considered to be free of disease. In order to consider patients free of disease when the presence of anti-Tg antibodies might be interfering with negative Tg levels, additional imaging tests were performed (thyroid scan, US, TEP-CT, CT, MRI).

This study was approved by the ethics committee of Hospital Universitario Virgen de las Nieves. Informed consent for the study was obtained from all patients recruited.

Samples

Samples of paraffin-embedded tissue from the thyroidectomy specimen were used for genetic analysis. Five 7-μm sections were taken from the most representative area in histopathological diagnosis, selected by a pathologist expert in thyroid disease. For tumors ≤ 1 cm in size, tissue was extracted from the paraffin block using a 0.6-mm needle with the Manual Tissue Arrayer I (Beecher Instruments Inc, Sun Prairie, WI, USA).

DNA extraction

Deoxyribonucleic acid (DNA) was extracted using the QIAamp DNA Mini Kit (Qiagen GmBH, Hilden, Germany) following the manufacturer's instructions.

PCR amplification of exon 15 in the BRAF gene

Approximately 100 ng of total cell DNA were used for each PCR amplification. PCR was performed using the specific primers for exon 15 in BRAF reported by Davies y cols.[7] (forward primer: 5′-TCATAATGCTTGCTCTGATAGGA; reverse primer: 5′- GGCCAAAAATTTAATCAGTGGA) and the amplification protocol described by Cohen et al[13].

Analysis of the BRAF^{T1799A} mutation

BRAF^{T1799A} mutation status was first established by restriction fragment length polymorphism analysis using the restriction enzyme TspRI according to the protocol previously described by Cohen et al[14]. In positive samples, the presence of the mutation was confirmed by bidirectional sequencing using the same set of primers with reagents Big Dye terminator v1.1 (Applied Biosystems, Foster City, CA, USA); the amplification program was as follows: 94 °C for 3 min x 1 cycle; 96 °C for 10 sec, 58 °C for 5 sec and 60 °C for 4 min x 25 cycles. The sequence was read in an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

In the bivariate analysis, a Pearson's Chi-square test with continuity correction for 2 x 2 tables, or a Fisher's test when required, were used for qualitative variables. For continuous variables, a Student's t test or a non-parametric Mann-Whitney U test were used depending on whether variables were normally distributed or not.

For the multivariate analysis, a logistic regression model was fitted using the backward stepwise selection method.

A value of p < 0.05 was considered significant.

Data analysis was performed using SPSS 15.0 for Windows statistical software (SPSS Inc., Chicago, IL, USA).

Results

Prevalence of the BRAF^{T1799A} mutation in PTC

Forty-five of the 97 patients diagnosed with PTC (46.4%) were positive for the BRAF^{T1799A} mutation. Table 1 shows the distribution of the presence of BRAF^{T1799A} mutation in the different PTC subtypes. All tumors of the histological variant of tall and clear cells showed the BRAF^{T1799A} mutation. The classical (24/44; 54.5%) and follicular (8/14; 57.1%) variants were positive in a little over half of the patients. Finally, the mutation was found in 31.3% of papillary microcarcinomas.

Age and sex
While age and sex as continuous variables were not associated with the presence of the BRAF^{T1799A} mutation, this was more common in elderly patients. A statistical association was found between patients over 60 years of age and presence of the BRAF^{T1799A} mutation (Table 2).

Table 1 Prevalence of the BRAF^T1799A mutation in subtypes of papillary thyroid carcinoma (PTC)

Histological subtype	BRAF^T1799A			
	N	–	+	Percentage by histology
Classical PTC	44	20	24	54.5
MicroPTC	32	22	10	31.3
PTC-follicular variant	14	6	8	57.1
PTC-tall cells	2	0	2	100
PTC-clear cells	1	0	1	100
PTC-solid trabecular	1	1	0	0
PTC-diffuse sclerosing	1	1	0	0
PTC-diffuse follicular variant	2	2	0	0
Total	97	52	45	46.4

Tumor size

Median tumor size in patients negative and positive for BRAF^T1799A was 1.0 and 1.7 cm respectively (Table 2). Analysis of the distribution of BRAF^T1799A mutation status by tumor size in these patients revealed significant associations of the mutation with a greater tumor size (Table 2) ($p = 0.007$). In particular, the BRAF^T1799A mutation was more common in PTCs 1 cm or more in size ($p = 0.016$; Table 2), with a relative risk of 1.5 (95% confidence interval [CI], 1.1-2.0).

Stage

Most patients (65/97; 67.0%) had a stage I PTC according to the American Joint Committee on Cancer. Of these, 40.0% (26/65) were found to have the BRAF^T1799A mutation in their primary tumors. Although in more advanced stages the number of patients positive for the BRAF^T1799A mutation was always seen to be greater than that of negative patients in the corresponding stratum, no significant association was found between the presence of the BRAF^T1799A mutation in PTC tissue and any stage higher than stage I ($p = 0.086$; Table 2).

A multivariate analysis using logistic regression showed that age over 60 years (odds ratio [OR] = 5.5; 95% CI, 1.4-21.9; $p = 0.019$) and tumor size of 1 cm or more (OR = 3.6; 95% CI, 1.2-10.3; $p = 0.016$) were the only clinical variables statistically associated with the presence of the BRAF^T1799A mutation.

Histological subtypes

The BRAF^T1799A mutation was not found to be associated with any histological subtype, and stratification by histological variant in the analysis of the most significant clinicopathological characteristics (lymph node and distant metastases, perithyroid extension, multifocality, size) resulted in no significant difference in the distribution of the presence of the BRAF^T1799A mutation.

Metastasis

Regional or distant metastases occurred in BRAF^T1799A-positive patients at similar rates (40.0% and 2.2% respectively; Table 2) as compared to BRAF^T1799A-negative patients.

Other histopathological characteristics

No statistically significant differences were seen in the distribution of any other histopathological variables (multifocality, extrathyroid invasion, capsular invasion, vascular invasion, mitotic activity, tumor necrosis) in BRAF^T1799A-positive and BRAF^T1799A-negative patients.

Association of the presence of the BRAF^T1799A mutation with tumor recurrence and persistence

Median follow-up time was 64.1 (28.9-91.1) months. Twenty-two of the 97 patients (22.7%) experienced PTC recurrence. These 22 patients had a mean age of 48.3 ± 17.3 years and a male:female ratio of 4:18. Initial lesion size ranged from 0.3 to 8.0 cm (median: 2.3 [0.9-4.0] cm), and tumor size was greater than 1 cm in 70.6% of patients. Of these, 45.5% (10/22) had multifocal initial lesions, and 31.8% (7/22) showed invasion of perithyroid tissue by the PTC. Lymph node metastases were found at diagnosis in 63.6% of patients with recurrence (14/22), and distant metastases were also seen in 4.5% (1/22).

The BRAF^T1799A mutation was found in the primary tumor in 40.9% (9/22) of patients with subsequent recurrence. More than one dose of ^131I was required by 81.8% of patients (18/22), and repeat surgery was needed in 31.8% (7/22). At the end of follow-up, 50% (11/22) of these patients showed persistent thyroid disease.

Follow-up time was similar in PTC patients both positive and negative for the presence of the BRAF^T1799A mutation (medians: 59.8 [27.3-93.8] and 68.2 [29.5-94.5] months respectively) (Table 3).

No association was found between tumor recurrence and the presence of the BRAF^T1799A mutation in the primary tumor. Although a higher recurrence rate was seen in BRAF^T1799A-negative patients (13/52; 25.0%) as compared to BRAF^T1799A-positive patients (9/45; 20.0%), the difference was not statistically significant (Table 3). Stratification by histological variable did not result in any significant difference as regards distribution of the presence of the BRAF^T1799A mutation in patients with/without recurrence.

Table 2 Association between clinicopathological characteristics and BRAF^T1799A mutation status in patients with papillary thyroid carcinoma

	N (−/+)	BRAF^T1799A −	BRAF^T1799A +	RR	95% CI	p value
Age, years	97 (52/45)	44.4 ± 14.8	47.2 ± 16.7	–	–	0.395
Age over 60 years	97 (52/45)	6 (11.5%)	13 (28.9%)	2.5	(1.0-6.0)	0.041
Male sex	97 (52/45)	5 (9.6%)	8 (17.8%)	0.9	(0.8-1.1)	0.371
Tumor size, cm	84 (42/42)	1.0 [0.6-1.9]	1.7 [1.2-2.5]	–	–	0.007
Tumor size ≥ 1 cm	84 (42/42)	24 (57.1%)	35 (83.3%)	1.5	(1.1-2.0)	0.016
Multifocality	72 (40/32)	8 (20.0%)	13 (40.6%)	2.0	(1.0-4.3)	0.071
Extrathyroid invasion	97 (52/45)	6 (11.5%)	6 (13.3%)	1.2	(0.4-3.3)	1.000
Lymph node metastases	97 (52/45)	15 (28.8%)	18 (40.0%)	1.3	(0.8-2.0)	0.287
Distant metastasis	97 (52/45)	2 (3.8%)	1 (2.2%)	0.6	(0.1-6.2)	1.000
Tumor stage (AJCC)	97 (52/45)					
I		39 (75.0%)	26 (57.8%)			
II		6 (11.5%)	11 (24.4%)			
III		6 (11.5%)	7 (15.6%)			
IV		1 (1.9%)	1 (2.2%)			
Tumor stage II-III-IV	97 (52/45)	13 (25.0%)	19 (42.2%)	1.7	(0.9-3.0)	0.086
Capsular invasion	50 (24/26)	12 (50.0%)	11 (42.3%)	0.8	(0.5-1.5)	0.777
Vascular invasion	46 (22/26)	7 (31.8%)	6 (25.0%)	0.8	(0.3-2.0)	0.746
Tumor necrosis	52 (24/28)	3 (12.5%)	5 (17.9%)	1.4	(0.4-5.4)	0.711
Mitotic activity	49 (25/24)					
None		1 (4.0%)	1 (4.2%)			
Mild		20 (80.0%)	22 (91.6%)			
Moderate		3 (12.0%)	1 (4.2%)			
High		1 (4.0%)	0 (0.0%)			
Moderate/high	49 (25/24)	4 (16%)	1 (4.2%)	0.3	(0.0-2.2)	0.349

AJCC: American Joint Committee on Cancer; CI: confidence interval; RR. relative risk.
Normally distributed quantitative variables: mean ± standard deviation.
Non-normally distributed quantitative variables: median [25th-75th percentile].
Categorical variables: number (percentage of BRAF+/−).

The presence of the BRAF^T1799A mutation in the primary tumor of these patients was not associated either with the need for repeat ^131I ablation therapy or other surgical procedures due to the occurrence of metastases (Table 3). The relationship between the BRAF^T1799A mutation and the persistence of thyroid disease at study end was analyzed in 76 patients followed up for longer than 27 months, of whom 11 had thyroid disease. The BRAF^T1799A mutation was found in the primary tumor in 54.5% of patients with disease (6/11) (Table 3). However, no statistically significant association was found between the presence of the BRAF^T1799A mutation and the persistence of disease at the end of follow-up.

Association of the presence of the BRAF^T1799A mutation with variables related to 131I treatment in PTC

Thirteen patients received no ^131I ablation therapy for surgical remains. Of these, the BRAF^T1799A mutation in the primary tumor had been found in 46% (6/13) (Table 4). Nine of the 13 patients had papillary thyroid carcinoma; metastatic renal carcinoma, osteosarcoma, and a brain metastasis were each found in one patient; and a 17-year-old patient had a 1.2-cm tumor.

The therapeutic dose of ^131I in annual scan (subsequent to initial ablation) was required in 18 patients, of whom 7 had the BRAF^T1799A mutation in the primary tumor (38.9%), while 11 patients were negative (61,1%). Thus, a lower proportion of patients positive for the BRAF^T1799A mutation required more than one dose of treatment with ^131I as compared to negative patents (15.6% versus 21.2%), but the difference was not statistically significant (Table 4).

No significant differences were found either in the initial or total ^131I doses received by patients recruited into the study, depending on the presence of the BRAF^T1799A mutation in the primary tumor (Table 4).

In the 22 patients of the follow-up group who had experienced recurrence, the result of the last WBS was

Table 3 Association between variables related to recurrence of papillary thyroid carcinoma and persistence of the BRAF[T1799A] mutation

	N (−/+)	BRAF[T1799A] −	BRAF[T1799A] +	RR	95% CI	p
Tumor recurrence	97 (52/45)	13 (25.0%)	9 (20.0%)	0.8	(0.4-1.7)	0.631
More than one treatment with [131]I	97 (52/45)	11 (21.2%)	7 (15.6%)	0.7	(0.3-1.7)	0.603
Need for repeat surgery	97 (52/45)	2 (3.8%)	5 (11.1%)	2.9	(0.6-14.2)	0.244
With disease at the end of follow-up	76 (42/34)	5 (11.9%)	6 (17.6%)	1.5	(0.5-4.4)	0.527
Follow-up time, months	97 (52/45)	68.2 [29.5-94.5]	59.8 [27.3-93.8]	–	–	0.385

CI: confidence interval; RR. relative risk.
Non-normally distributed quantitative variables: median [25th-75th percentile].
Categorical variables: number (percentage of BRAF+/−).

analyzed based on the BRAF[T1799A] mutation status in the primary tumor. The WBS was positive in 3 patients, one of whom received one dose of [131]I. The remaining 2 patients underwent surgery. Histopathological examination after lesion excision showed lymphadenitis in one of the patients and confirmed metastasis in the other patient, the only one with the BRAF[T1799A] mutation in his primary tumor (Table 5).

Among the 19 patients with negative WBS, recurrence was suspected in 8 patients based on increased Tg levels or the occurrence of lesions in an imaging test (US/CT). TEP and histopathology were positive in 3 of these 8 patients, who also had the BRAF[T1799A] mutation in the primary tumor. In the fourth patient with negative WBS, increased Tg levels, and

lesions in TEP, histopathology after surgery revealed lymphadenitis, and recurrence was therefore not confirmed (Table 5). Distribution of the BRAF[T1799A] mutation among patients with positive and negative WBS or among patients with negative WBS and positive or negative TEP was not statistically different.

Thus, 5 patients in this group had a confirmed recurrence at the last WBS. Of the 4 patients positive for BRAF[T1799A], 3 (75%) had a negative WBS at the time of recurrence, a non-statistically significant result (Table 6).

To sum up, no statistically significant associations were seen between the presence of the BRAF[T1799A] mutation and the parameters related to ^{131}I treatment analyzed in this study.

Table 4 Association between variables related to 131I treatment and BRAF[T1799A] mutation status in the 97 patients with papillary thyroid carcinoma

	N (−/+)	BRAF[T1799A]		RR	95% CI	p value
		− (52)	+ (45)			
Number of treatments with [131]I	97 (52/45)					
0		7 (13.5%)	6 (13.3%)			
1		34 (65.4%)	32 (71.1%)			
2		9 (17.3%)	4 (8.9%)			
3		2 (3.8%)	3 (6.7%)			
More than one treatment with [131]I	97 (52/45)	11 (21.2%)	7 (15.6%)	0.7	(0.3-1.7)	0.603
Initial dose of ^{131}I greater than 100 mCi	84 (45/39)	8 (17.8%)	10 (25.6%)	1.4	(0.6-3.3)	0.432
Total dose of [131]I (mCi)	84 (45/39)					0.554
Up to 100 mCi		30 (66.7%)	25 (64.1%)			
100-200 mCi		9 (20.0%)	10 (25.6%)			
200-300 mCi		4 (8.9%)	4 (10.3%)			
More than 300 mCi		2 (4.4%)	0 (0.0%)			

CI: confidence interval; RR. relative risk.
Non-normally distributed quantitative variables: median [25th-75th percentile].
Categorical variables: number (percentage of BRAF+/−).

Table 5 BRAF[T1799A] mutation status in the 22 patients with tumor recurrence depending on the results of the last whole body scan

WBS	N	Tg/US/CT	N	TEP	N	BRAF[T1799A] −	BRAF[T1799A] +
Positive	3					2	1
Negative	19	Negative	11			8	3
		Positive	8	Negative	4	2	2
				Positive	4	1	3
						13	9

US: ultrasound; TEP: positron emission tomography; WBS: whole body scan; CT: computed tomography; Tg: Thyroglobulin > 1 ng/ml.

Table 6 Result of the last whole body scan (WBS) and BRAF[T1799A] status at the time of recurrence

WBS	BRAF[T1799A] −	BRAF[T1799A] +	Total
−	0	3	3
+	1	1	2
Total	1	4	5

Discussion

Although patients with PTC usually have a good prognosis, **approximately 10%-15% are not cured with initial treatment** consisting of total thyroidectomy and postoperative ablation of thyroid residues with [131]I. Many patients experience recurrence, and some even die from the disease[4].

Because of the increasing incidence of PTC, it would be very helpful to complement current risk stratification systems with molecular markers, as exemplified by the BRAF[T1799A] mutation, especially for taking surgical and clinical management decisions[9].

Many authors have investigated the relationship of the BRAF[T1799A] mutation with the clinicopathological characteristics of PTC[10]. While the results are not conclusive, most studies in patients of different ethnic and geographical origins show a significant association of the BRAF[T1799A] mutation with one or more clinicopathological characteristics of high-risk PTC[9,10,12], among which special mention should be made of extrathyroid invasion, lymph node metastases, and clinicopathological stages III/IV because they are more reliable predictors of the progression, recurrence, aggressiveness, and increased morbidity and mortality of PTC[9]. Many studies have shown the BRAF[T1799A] mutation to be **more frequently associated with these 3 risk predictors**[9,15-17], **reporting ORs of 2.50, 1.83, and 2.14 for extrathyroid invasion, nodal metastases, and advanced stages (III/IV)** respectively[9].

This study found no association between the presence of the BRAF[T1799A] mutation in the primary tumor of patients with PTC and any of these 3 prognostic factors, but an association of BRAF[T1799A] with older age and greater tumor size was documented, in agreement with other authors[10,17]. These are considered as poor prognostic factors in patients with PTC[5].

It has been suggested that discrepancies concerning the prognostic value of BRAF[T1799A] in the different studies could be due to the inclusion of different histological subtypes of thyroid cancer. Thus, the frequency of the BRAF[T1799A] mutation would be greater in PTCs showing a papillary architecture (classical PTC, tall cell PTC, Warthin-like PTC, microPTC), **especially high in tall cell PTC, the most aggressive variant (55%-100%)**[18,19], **and relatively low (7%-25%) in the follicular variant**[8,11,16-18,20,21]. In order to verify that the results obtained were not influenced by the histological subtype, the relationship between the presence of the BRAF[T1799A] mutation and the most important clinicopathological characteristics (nodal and distant metastases, perithyroid extension, multifocality, tumor size, recurrence, persistence) was examined by stratifying according to histological variant, but this did not result in significant differences regarding the distribution of the presence of the BRAF[T1799A] mutation in any of the variables analyzed. The reason for this may be that, in this group, the prevalence of the BRAF[T1799A] mutation was virtually the same in the classical and follicular subtypes (which accounted **together for 60% of the study patients).**

It has also been suggested that the lack of any statistical association in some studies may be due to the selection of too small a sample or a heterogeneous sample in terms of

treatment, diagnosis, or inadequate follow-up time. Ninety-seven patients with PTC were analyzed in our series. Since all the patients available during the study period were enrolled, the clinical and histopathological characteristics of the patients diagnosed with PTC at our hospital are accurately represented in the sample. The **distribution frequency of the pathological findings (tumor size, nodular metastases, stage, multifocality, etc.) agrees with other reported studies**[3,22]. In fact, mean age at diagnosis, male/female ratio, mean tumor size, multifocality, the prevalence of nodal metastases during follow-up and distant metastases at diagnosis or follow-up, recurrence, and tumor staging were within the ranges reported in the largest PTC series[3,22,23]. The number of processed samples by tumor, consistent clinical management of patients (managed by the same team), and **long-term follow-up (median follow-up of 64 months) argue** against asymmetrical deviation in clinicopathological data collection.

The association between the BRAF[T1799A] mutation and the occurrence of nodal metastases at diagnosis reported by many authors[10,17] and in a meta-analysis[15] was not found in our study. This lack of consistency probably reflects the fact that neck dissection may have been variable in the different patients and studies.

Since distant metastases from PTC are uncommon, particularly in adult patients, only a few studies have reported a sufficient number of cases for a significant association to be found[10,16]. This association was not found either in our study, where only 3 patients had distant metastases at diagnosis, and only one of them had the BRAF[T1799A] mutation (Table 2).

This study found no association of the BRAF[T1799A] mutation with histological characteristics (extrathyroid invasion, multifocality, capsular invasion, vascular invasion, tumor necrosis, or mitotic activity). Other researchers have reported an association between extrathyroid invasion and the BRAF[T1799A] mutation[10,11,15,17,19,20,24], but the association was lost for some[18,24] in a multivariate analysis stratified by histological subtype. Other authors found no association[10,16,25,26]. An association of the BRAF[T1799A] mutation with tumor multifocality and vascular invasion has been reported in a few studies[7,18,25].

Information about multifocality, vascular and capsular invasion, tumor necrosis, and mitotic activity could not be obtained for all of our patients because these data were not available. Because of this, the possibility that statistical significance was lost in associations of the BRAF[T1799A] mutation to those parameters cannot be ruled out.

Although most previously discussed studies support the existence of an association between the BRAF[T1799A] mutation and classical high-risk clinicopathological factors, a significant association was not found in some studies[10]. Again, there is no definitive explanation for these inconsistent results, although some potential contributing factors include the relatively low number of patients in some "negative" studies (some of which showed a non-statistically significant trend towards such an association), variations in disease extent at initial diagnosis (e.g. PTC in an early stage with a small tumor size is less likely to be associated with aggressive pathological characteristics), and the level of detail of pathological description and of

diagnostic criteria, particularly for defining the various PTC subtypes, used by different pathologists[9].

The value of the BRAF[T1799A] mutation for predicting PTC recurrence has been investigated in patients of various ethnic and geographic origins worldwide[10] and a clear association has been shown in some of them[16,19-21,24-26], with an OR for PTC persistence and recurrence ranging from 1.91 to 5.40[27]. Follow-up time is essential in order to observe the recurrence of slowly growing tumors. In this study, all 97 patients were followed up for at least 15 months, and **median follow-up was relatively long (64.1 months). This is** among the four studies with the longest follow-up periods reported in the literature[16,25,26,28]. No statistically significant association was found in our patients between tumor recurrence and the presence of the BRAF[T1799A] mutation in the primary tumor. In fact, there was a trend towards a lower recurrence rate in BRAF[T1799A]-positive patients (9/45; 20.0%) as compared to BRAF[T1799A]-negative patients (13/52; 25.0%) (Table 3). Stratification by histological subtype did not result in any significant differences as regards distribution of the presence of the BRAF[T1799A] mutation in patients with or without recurrence.

The presence of the BRAF[T1799A] mutation in the primary tumor of these patients was not associated with either the need for repeat ^{131}I ablation therapy or other surgical procedures due to the occurrence of metastases (Table 3).

To sum up, we did not find the presence of the BRAF[T1799A] mutation to be a predictor of PTC recurrence.

As regards the value of the BRAF[T1799A] mutation for predicting disease persistence, only a few studies with a relatively long follow-up time have analyzed the activation of the BRAF oncogene and the outcome of PTC patients. These were for 6 years[16,28], 7.3 years[26], 11.7 years[21], and 15 years[25] respectively, and had discrepant results, an association being found in three of them[16,25,26]. In the Elisei et al series[25], with a mean follow-up of 15 years, the longest to date, the BRAF[T1799A] mutation acted as an poor independent prognostic factor for PTC persistence and shorter survival. In the current study, no relationship was found between the BRAF[T1799A] mutation and patient outcome after a mean follow-up of 5.3 years. No patient died from PTC. Of the **76 patients followed up for longer than 27 months, 14.5% (11/76) had thyroid disease at the end of the study period** (Table 3). Although this proportion was similar to that reported in the Elisei et al study[25], where the disease persistence rate was much higher in BRAF-positive patients **(21.1%, as compared to 7.8% in BRAF-negative patients)**[25], the distribution of the BRAF[T1799A] mutation was more **balanced in our patient group (17.6% versus 11.9% in BRAF-positive and BRAF-positive patients respectively). In our study, patients undergoing total or almost total thyroidectomy and ablation of thyroid residues were** considered to be free of disease when they had no clinical or imaging evidence of tumor (detectable mainly in neck ultrasound or whole body scan during follow-up), and Tg levels were undetectable under TSH suppression and stimulation with no anti-Tg antibody levels interfering with **their assessment. Thus, 8 of the patients considered to be** on remission had positive levels of anti-Tg antibodies associated with negative ultrasound examinations, and were therefore considered to be free of disease. As a limitation of this study, the definition of absence of disease was related

to the sensitivity of the biochemical and imaging techniques used for the management of patients with differentiated thyroid cancer. Another limitation inherent in the design of the study itself, where patients were consecutively enrolled into the cohort, was that tumor persistence or recurrence was not detected in patients with a shorter follow-up, because it may have occurred after the study period.

Ablation therapy for residual tissue with ^{131}I is recommended in patients with distant metastases, extrathyroid disease, or size greater than 4 cm, and also in selected patients with smaller tumors which present characteristics of an increased risk[29]. Use of ablation therapy has become established because it allows for the early detection of recurrence, facilitates initial staging, identifies previously undiagnosed disease, and may be considered as an adjuvant therapy following surgery in patients at risk of recurrence or mortality. However, thyroid cancer may lose avidity for ^{131}I, which is a major cause of the failure of treatment with ^{131}I and its associated morbidity and mortality[30]. Several studies[8,11] have found the presence of the BRAF[T1799A] mutation in the primary tumor to be associated with a loss of avidity for ^{131}I in recurrent tumors. These studies reported more aggressive treatments for PTC recurrence, greater disease persistence, and the failure of treatment of tumor recurrence in patients with primary tumors positive for the BRAF[T1799A] mutation.

In this study, no association was found between BRAF[T1799A] status and tumor recurrence or the need for repeat ^{131}I ablation therapy or higher ^{131}I doses. A lower proportion of patients positive for the BRAF[T1799A] mutation required more than one dose of treatment with ^{131}I as compared to negative patents (15.6% versus 21.2%; Table 4), but the difference was not statistically significant. Nor were differences found either in the initial or total ^{131}I doses received by patients recruited into the study dependent on the presence of the BRAF[T1799A] mutation in the primary tumor.

As in the Riesco-Eizaguirre et al study[11], where 66.7% (6/9) of BRAF[T1799A]-positive patients had negative scans at the time of recurrence, 75% of our BRAF[T1799A]-positive patients (3/4; Table 6) had negative whole body scans, although the difference was not statistically significant, presumably due to the small number of cases.

The presence of the BRAF[T1799A] mutation was only analyzed in the primary tumor in this study, and we cannot therefore know whether patients with positive or negative scans had metastases which were positive or negative for BRAF[T1799A] mutation status. It would have been interesting to assess BRAF[T1799A] mutation status in tissue from metastases which did not take up ^{131}I in the whole body scan had they been excised.

Overall conclusions

Data from this study support the association between the BRAF[T1799A] mutation and some of the clinicopathological parameters found by other authors, namely tumor size, especially greater than 1 cm, and age over 60 years[10,11,16,18,19,26,28].

However, it cannot be inferred from our results that the BRAF[T1799A] mutation confers a more aggressive behavior or phenotype, reflected in histopathological characteristics involving a greater risk, such as multifocality, capsular/vascular invasion, extrathyroid extension, etc., and also in the occurrence of regional or distant metastases at the time of PTC diagnosis, or a greater risk of tumor recurrence or disease persistence in PTC patients. Thus, despite the body of opinion currently favoring an association of the BRAF[T1799A] mutation with a poorer prognosis of PTC, there are still discrepancies among the different studies, and decisions based on BRAF[T1799A] mutation status should therefore only be taken after careful consideration.

Conflict of interest

The authors state that they have no conflict of interest.

Acknowledgement

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