BRAF V600E and decreased *NIS* and *TPO* expression are associated with aggressiveness of a subgroup of papillary thyroid microcarcinoma

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Abstract

Background: Thyroid cancer incidence has dramatically increased worldwide over the last two decades. The rise is mostly due to an increased detection of small papillary thyroid carcinomas (PTCs) (≤ 20 mm), predominantly microPTC (≤ 10 mm).

Although small tumors generally have an excellent outcome, a considerable percentage may have a more aggressive disease and worse prognosis. The clinical challenge is to preoperatively identify those tumors that are more likely to recur.

Aim: To improve risk stratification and patient management, we sought to determine the prognostic value of BRAF V600E, NRAS or RET/PTC mutations in patients with PTC measuring < 20 mm, mainly microPTC.

Methods: The prevalence of RET/PTC fusion genes was examined by quantitative RT-PCR. BRAF V600E and NRAS Q61 mutations were determined by PCR sequencing. To further elucidate why some small PTC are less responsive to radioactive iodine treatment therapy, we explored if these genetic alterations may modulate the expression of iodine metabolism genes (*NIS*, *TPO*, *TG*, *TSHR* and *PDS*) and correlated with clinico-pathological findings that are predictors of recurrence.

Results: This study shows that tumors measuring ≤ 20 mm exhibited higher prevalence of BRAF V600E mutation, which correlated with aggressive histopathological parameters, higher risk of recurrence, and lower expression of *NIS* and *TPO*. Although this correlation was not found when microPTC were evaluated, we show that tumors measuring 7–10 mm, which were positive for BRAF mutation, presented more aggressive features and lower expression of *NIS* and *TPO*.

Conclusion: We believe that our findings will help to decide the realistic usefulness of BRAF V600E mutation as a preoperative marker of poor prognosis in small PTC, primarily in microPTC.

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Introduction

Thyroid cancer is the fastest increasing cancer worldwide. The increase is predominantly due to an increased detection of small tumors, predominantly from papillary subtype (papillary thyroid carcinoma (PTC)). Eighty-seven percent of the cases consisted of PTC measuring 20 mm or less, and nearly 50% of the increase consisted of PTC measuring 10 mm or less in size (papillary thyroid microcarcinoma (microPTC)) (1, 2, 3).

Once a nodule has been identified, the first decision is whether fine-needle aspiration (FNA) should be performed to exclude malignancy. The Guidelines of the American Thyroid Association (4) recommend that only nodules > 10 mm should undergo FNA and that nodules measuring ≤ 10 mm should be biopsied on the basis of suspicious ultrasonographic findings or if there is family history of thyroid cancer or history of radiation exposure.

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However, there is still no consensus in the literature on whether a nodule of 10 mm or smaller should be selected for biopsy and if it should undergo follow-up. The controversy is mainly related to the fact that, despite the excellent clinical outcome of patients with microPTC, which are commonly seen as being low-risk tumors, a significant number of cases were associated with extracapsular invasion, lymph node metastases at presentation, tumor recurrence, distant metastases, advanced tumor stage and even disease-specific mortality (5, 6, 7). Therefore, it is of clinical importance to identify biomarkers that would help to risk-stratify and appropriately manage patients with small thyroid nodules.

PTCs commonly have genetic alterations that lead to the activation of the MAPK pathway. BRAF V600E mutation is the most common event found in PTC, followed by *RET/PTC* rearrangements. Although more than 13 *RET/PTC* rearrangements have been described, *RET/PTC1* and *RET/PTC3* are the most prevalent isoforms (8). Finally, activating mutation of *RAS* genes (*NRAS*, *KRAS* and *HRAS*) has also been described in a small subset of PTC. It has been observed that these driver alterations that affect the same pathway occur in a mutually exclusive pattern and are associated with distinct clinical, microscopic and biological features of PTC (9, 10, 11, 12). BRAF V600E mutation has been associated with more aggressive features such as extrathyroidal extension, presence of lymph node metastases, advanced tumor stages, higher risk of recurrence and disease-specific mortality (10, 11, 12, 13). *RET/PTC* and *RAS* were less frequently associated with an aggressive phenotype.

Within this perspective, several studies have assessed the clinical prognostic use of these genetic alterations in microPTC. Few studies correlated the presence of BRAF V600E mutation with a more aggressive phenotype (7, 11, 12, 14, 15, 16, 17, 18, 19, 20). Although the prevalence of *RET/PTC* rearrangement is higher in microPTC than in larger tumors, its role as a prognostic indicator is still unclear (21).

The primary goal of this study was to evaluate if the most frequent genetic alterations along MAPK pathway found in PTC may help to recognize those small PTC with a more aggressive phenotype, primarily microPTC. The current treatment for PTC includes thyroidectomy, TSH suppression and adjuvant therapy with radioactive iodine treatment (RAI), PTC recurrence and death essentially occurs in tumors that have lost radioiodine avidity. Loss of expression of thyroid-specific proteins involved in the iodine uptake, concentration and organification lead to the loss of radioiodine avidity and, consequently, failure of radioiodine therapy (22, 23, 24, 25).

Recently, in a landmark article by the Cancer Genome Atlas Research network (TCGA), the authors used comprehensive multiplatform analysis to characterize the genomic landscape of PTC. A molecular classification of PTCs, based on combined analysis of genomic variants, transcriptoma, proteome and methyloma with clinico-pathological information of 496 PTC, identified two major clusters defined as BRAF V600E-like and RAS-like. The BRAF V600E-like pattern comprise mainly less differentiated variants of PTC enriched for classical and tall variants, showed over-activation of the MAPK pathway and reduced expression of proteins involved in iodine uptake and metabolism. RAS-like patterns were enriched with more differentiated variants of PTC and lower activation of MAPK signaling than BRAF V600E-like PTCs (26).

As a percentage of microPTC do not respond to standard therapy, tumor size was not an independent variable of the TCGA dataset and also is not a feature available for comparisons (www.genome-cancer.ucsc.edu), it seems rational to investigate the expression of genes associated with iodine uptake and organification in these tumors and to explore whether the *BRAF* and *RAS* mutations and *RET/PTC* fusion genes downregulate the expression of genes involved in iodine metabolism, as it has been suggested for larger PTC. Therefore, we correlated the mutational status with the expression of sodium-iodine symporter (*NIS*), thyroglobulin (*TG*), thyroperoxidase (*TPO*), thyrotropin receptor (*TSHR*) and pendrin (*PDS*) and clinico-pathological features.

As far as we know, this is the first study that investigates the prognostic impact of *BRAF*, *RAS* and *RET/PTC* in the same series of microPTC and addresses the specific question of how these events affect the expression of key genes associated with iodine metabolism.

Methods

Tissue samples

The series consists of 118 PTCs and seven normal adjacent thyroid tissues obtained from patients who underwent thyroid surgery at Hospital São Paulo, UNIFESP and Hospital das Clínicas, Faculdade de Medicina da Universidade de São Paulo. The specimens were frozen in liquid nitrogen immediately after surgical resection and stored at -80°C until use. Final histological classification was obtained from paraffin-embedded sections. The clinical and pathological features such as the age at onset, gender, tumor size, histological variant,

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multifocality, extrathyroidal invasion and presence of lymph node metastasis were evaluated. Additionally, all patients were grouped into three risk categories at the time of initial treatment (27): i) Very low risk: unifocal T1 (≤ 10 mm) with no extension beyond the thyroid and no lymph node metastases (T1aN0M0). ii) Low risk: T1bN0M0 (tumor >10 mm and <20 mm), T2N0M0 or multifocal T1N0M0. iii) High risk: any T3 and T4 or any T, N1 or any M1. Follow-up protocol included neck ultrasound (US), serum TG (sTg), anti-TG antibodies (TgAb) and whole body scan. Patients were considered with 'no evidence of disease' when they presented negative neck US and undetectable sTg (below 1 ng/ml) and TgAb levels (below 40 IU/ml). Informed consent was obtained from all subjects involved in this study. The study was conducted under the approval of the Review Boards and Research Ethical Committees of the affiliated institutions.

NRAS Q61 and BRAF V600E mutation analysis

According to the catalogue of somatic mutations in cancer (<http://sanger.ac.uk/cosmic>), different types of cancer appear to be coupled to mutation of a particular *RAS* isoform (*NRAS*, *KRAS* and *HRAS*). *NRAS* is the most frequently mutated *RAS* isoform in PTC, with the highest rates of mutation found at Q61 (exon 2). *NRAS* was also reported as the second most common mutation found in PTC by the TCGA project (26). The Q61 mutation in the exon 2 of *NRAS* gene was investigated by PCR. PCR products were resolved by electrophoresis, purified and submitted to direct sequencing as described (13). The samples were sequenced at least twice and in both directions. BRAF V600E mutation was previously investigated in this series of PTC (28). The PCR primers and conditions are summarized in Supplementary Table 1, see section on supplementary data given at the end of this article.

Detection of *RET/PTC* fusion genes by RT-PCR

All tumors were screened for *RET/PTC1*, *RET/PTC2* and *RET/PTC3* rearrangements by RT-PCR, as previously described, with few modifications (29). Briefly, 1 μ l of cDNA synthesized as described (30) was used in 12 μ l reactions containing SYBR Green PCR Master Mix (PE Applied Biosystems) and predesigned primers that span the known breakpoints specific for each of the *RET/PTC* transcripts (29) or internal control (*RPS8*). PCR reaction was performed in triplicate and the threshold cycle (Ct)

was obtained using Applied Biosystem Software. Post-amplification fluorescent melting curve analysis was performed. The PCR products were analyzed by electrophoresis on a 2% agarose gel and visualized on a Bio-Rad Gel Doc EZ system (Bio-Rad). cDNA generated from PCCL3 cells transiently transfected with plasmids encoding *RET/PTC2* or *RET/PTC3* isoforms or TPC1 cell line (positive for *RET/PTC1*) were used as positive controls. The samples were considered positive when at least two replicates had amplification curves that crossed the threshold before cycle 35, the melt curve aligned with the positive control, and the gel analysis revealed a band of the expected product size. To confirm the identity of the amplified products, randomly selected positive samples were sequenced using the BigDye Terminator Cycle Sequencing kit (PE Applied Biosystems).

In addition, the presence of *RET/PTC* rearrangements was confirmed by expression analysis of both extracellular (EC) domain and tyrosine kinase (TK) domain of *RET*, as previously described (31, 32, 33, 34, 35, 36). Two pair of primers were designed to amplify the region encoding the EC domain (exons 6 and 7) and TK domain of *RET* (exons 16 and 18). An aliquot of cDNA was used in 12 μ l PCR reaction containing SYBR Green PCR Master Mix (PE Applied Biosystems) and 2.5 pmol of each primer. qPCR reactions were performed in triplicate and the Ct was averaged (s.d. <1.0). Samples with balanced ratio (i.e. the expression of the EC and the TK domain was $\sim 1:1$) were considered negative for *RET* fusion transcripts. Samples with unbalanced expression (i.e. the expression of TK domain was higher than the expression of the EC domain) were considered positive for *RET/PTC* fusion transcripts. The PCR primers and conditions are summarized in Supplementary Table 1.

Expression of iodine metabolism genes using quantitative RT-PCR

cDNA, synthesized as in the preceding, was used in a 12 μ l PCR reaction containing SYBR Green PCR Master Mix (PE Applied Biosystems) and 2.5 pmol of each primer for the target genes (*NIS*, *TG*, *TPO*, *TSHR*, *PDS*) or internal control (*RPS8*). The PCR reactions were performed in triplicate, and the Ct was obtained using Applied Biosystem software and averaged (s.d. <1.0). The relative expression (RE) was calculated according to the comparative $\Delta\Delta$ Ct method. Normal thyroid samples were used as the control group. The PCR primers and conditions are summarized in Supplementary Table 1.

Statistical analyses

Raw data from qRT-PCR was ln-transformed before submitted to statistics. The association between mutational status and clinico-pathological features was determined using Fisher's exact test. The association between tumor size and clinico-pathological features was determined using Student's *t*-test. The comparison between the expression of the EC and the TK domains of RET were performed using Student's *t*-test. The association between mutational status and gene expression was performed using the one-way ANOVA test followed by Bonferroni's *post-hoc* test. Statistical analyses were performed using STATA Software (StataCorp, College Station, TX, USA). The results with $P < 0.05$ were considered to be statistically significant.

Results

Mutational analysis of PTC

We initially investigated *RET/PTC* rearrangements by RT-PCR. For the direct detection of *RET/PTC1*, *RET/PTC2* and *RET/PTC3*, RT-PCR was performed with forward primer mapping on the respective fusion partners and reverse primer on the *RET* gene. Overall, *RET/PTC* fusion transcripts were identified in 27% (32 out of 118) of PTC. *RET/PTC1* was detected in 9% ($n=11$), *RET/PTC2* in 10% ($n=12$) and *RET/PTC3* in 5% ($n=6$). Multiple *RET/PTC* rearrangements were found in 3% ($n=3$), being two cases of *RET/PTC1* associated with *RET/PTC2* and one case of *RET/PTC1* associated with *RET/PTC3* (Supplementary Table 2, see section on supplementary data given at the end of this article).

Furthermore, to confirm the presence of *RET* fusion transcripts, the expression both EC and TK domains of RET were evaluated in samples positive for *RET/PTC1*, *RET/PTC2* and *RET/PTC3* isoforms ($n=25$) and samples negative for *RET/PTC* isoforms ($n=25$). Comparison of Ct value showed balanced expression of exons 6–7 (encoding the EC domain) and 16–18 (encoding the TK domain) of *RET* in all cases negative for *RET/PTC* fusion transcripts. The expression of the TK was significantly higher than expression of the EC domain in all cases considered positive in the direct detection of *RET/PTC1*, *RET/PTC2* or *RET/PTC3* assay ($P < 0.001$; Fig. 1). Representative Ct plots of a sample without *RET/PTC* fusion transcripts and a sample with multiple *RET/PTC* fusion transcripts are shown (Fig. 1). Random selected cases that were positive for *RET/PTC* rearrangements, according to the criteria

Figure 1

Analysis of *RET* unbalanced expression in PTC samples. The figure above shows the mean Ct value of exons 6–7 (encoding EC domain) and exons 16–18 (encoding TK domain) of *RET* in *RET/PTC*-negative ($n=25$) and *RET/PTC*-positive ($n=25$) samples according to qPCR analysis. *RET/PTC*-negative samples have a comparable Ct value for both domains (ratio 1:1; 37.3 ± 0.6 vs 37.1 ± 0.6) although *RET/PTC*-positive samples have a higher expression of TK domain compared to EC-domain of *RET* (36.4 ± 0.6 vs 33.3 ± 0.6). The panel below shows representative Ct plots from qPCR of exons 6–7 and exons 16–18 of a sample without *RET* fusion oncogene (left) and a sample with multiple *RET* fusions (right). *** $P < 0.001$.

described in Methods section, were also positive by sequencing data.

NRAS mutation was found in 9% ($n=11$) of PTCs. The Q61R substitution was found in 10/11 PTC, whereas the Q61K substitution was identified in 1/11 case. BRAF V600E mutations were found in 48% ($n=57$) of PTC, as previously described (13) (Supplementary Table 2).

Additionally, concomitant mutations were found in 17% ($n=20$) of PTC. Fifteen cases carried both *RET/PTC* and BRAF V600E mutation and five cases had both *RET/PTC* and *NRAS* Q61R (Fig. 2A). *RET/PTC2* was the most prevalent isoform found in association with BRAF or *NRAS* (Supplementary Table 2).

In summary, 32% of PTCs were negative for mutations. BRAF V600E only was detected in 36% of PTC cases, *RET/PTC* only in 10%, *NRAS* only in 5% and concomitant mutations in 17% (Fig. 2A). Importantly, although in the majority of studies, BRAF V600E, *RET/PTC*

Figure 2

Mutational status and clinico-pathological parameters of papillary thyroid carcinomas. (A) Prevalence of BRAF, RET/PTC and NRAS in PTC. (B) Samples were further classified into three-age groups. RET/PTC or NRAS alone are more prevalent in younger patients, whereas the prevalence of concomitant mutations increases with age (C) Tumors measuring ≤ 10 mm were further evaluated according to tumor size and clinico-pathological parameters associated with recurrence and death and presence of BRAF V600E. MicroPTC measuring ≥ 7 mm had more frequent extrathyroidal invasion ($P=0.023$), presence of lymph node metastasis ($P=0.028$), high risk ($P<0.001$) and BRAF V600E mutation ($P=0.049$), compared to microPTC measuring <7 mm. * $P<0.05$ and *** $P<0.001$.

and RAS exhibited a mutually exclusive pattern, concomitant mutations in these genes were previously reported in PTC (Table 1).

Association of mutational status with clinico-pathological features

In general, mutations along MAPK were associated with pathological features indicative of more aggressive behavior such as larger tumor size ($P=0.021$), presence of lymph node metastases at diagnosis ($P=0.048$) and

high risk ($P<0.001$), suggesting that mutations along MAPK pathway may have a direct effect on the biological behavior of PTC. However, when each mutation was considered as an independent variant, only BRAF V600E was significantly associated with classic variant of PTC ($P<0.001$), larger tumor size ($P=0.040$), presence of lymph node metastases at diagnosis ($P=0.002$) and high risk ($P=0.005$) (Table 2). These findings suggest that the association with clinical and pathological features representative of a more aggressive feature is limited to BRAF mutated cases.

We further explore the hypothesis that the frequency of mutations may differ according to age groups. Therefore, patients were subdivided into 20-year age groups. The overall prevalence of BRAF V600E and RET/PTC is similar among age groups (ranging from 49 to 53% and 27 to 31%, respectively). However, the prevalence of BRAF V600E, RET/PTC and RAS alone was higher among younger patients (20–39 years) and declined with age, especially when compared to older individuals (≥ 60 years). Instead, the prevalence of tumors with concomitant mutation increased with patient age (Fig. 2B). Interestingly, the TCGA researchers found that mutation densities correlated with age and were not associated with other variables like genotype or radiation exposure. Precisely, five BRAF V600E mutant tumors with aggressive behavior had higher mutation densities (26).

Association of mutational status with clinical course of disease

Follow-up (2–16 years; median 7.75 years) shows that most patients (74%; $n=87$) have no evidence of disease. Recurrence was observed in 15 out of 118 patients (13%). Nearly 53% (8/15) of recurrences were detected within the first 3 years of follow-up and 80% (12/15) were detected within 4 years. All patients who recurred were classified as high risk at initial diagnosis and had metastases to central or lateral compartment ($n=10$), lung ($n=4$) and bone ($n=1$). Sixty-seven percent (10/15) of patients who developed recurrent disease harbored BRAF V600E mutation alone or in association with RET/PTC2 (Table 3).

Mutational status helps to identify a more aggressive behavior in small PTC, primarily in microPTC

We initially investigated whether BRAF, RET/PTC or RAS could predict a more aggressive phenotype of small PTC (≤ 20 mm) and, therefore, could improve preoperative risk

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Table 1 Summary of studies with concomitant RET/PTC rearrangement and BRAF V600E mutation in PTCs.

References	n	Total	BRAF V600E			
			RET/PTC1	RET/PTC2	RET/PTC3	Multiples
(68)	137	3 (2%)	0	NE	3/3	0
(69)	11	1 (9%)	1/1	NE	0	0
(70)	60	2 (3%)	0	NE	2/2	0
(71) ^a	23	1 (4%)	0	NE	1/1	0
(72)	32	2 (6%)	1/2 (50%)	NE	1/2 (50%)	0
(56) ^a	65	5 (8%)	2/5 (40%)	NE	3/5 (60%)	0
(42)	78	4 (5%)	1/4 (25%)	NE	3/4 (75%)	0
(73)	125	5 (4%)	3/5 (60%)	NE	2/5 (40%)	0
(54)	54	5 (9%)	0	2/5 (40%)	3/5 (60%)	0
(74)	107	6 (6%)	6/6	0	0	0
(75)	266	1 (0.4%)	0	NE	1/1	0
(76)	70	4 (6%)	3/4 (75%)	NE	1/4 (25%)	0
(53)	72	14 (19%)	4/14 (29%)	NE	9/14 (64%)	1/14 (7%)
(55) ^a	88	7 (8%)	7/7	0	0	0
This study	118	15 (13%)	3/15 (20%)	9/15 (60%)	2/15 (13%)	1/15 (7%)
Total	1306	75 (6%)	31/75 (41%)	11/75 (15%)	31/75 (41%)	2/75 (3%)

NE, not evaluated.

^aCoexistence of RET/PTC rearrangement and RAS mutation were also described.

stratification and management of this set of thyroid tumors. When the presence of any mutation along MAPK pathway was investigated, we observed an association with presence of lymph node metastases ($P=0.020$) and high risk ($P=0.047$). However, when each mutation was considered independently, only BRAF V600E mutation was significantly associated with these clinico-pathological parameters (Table 4).

When considering tumors measuring ≤ 10 mm, no association was found (Table 4). Although not statistically significant, 64% (7/11) of microPTC with lymph node metastasis at diagnosis were positive for BRAF V600E alone or associated with RET/PTC, while only 35% (8/23) of non-metastatic microPTC were positive for BRAF V600E (Table 4). None of RAS or RET/PTC-positive microPTC had lymph node metastasis at diagnosis.

As it has been suggested that the selected cut-off size of 10 mm can be further refined, we investigated whether there is a relationship between tumor size and clinico-pathological parameters associated with recurrence and death or the presence of BRAF V600E mutation in this subgroup. We found that extrathyroidal invasion (8.7 ± 0.71 , $P=0.023$), presence of lymph node metastases (8.2 ± 0.48 , $P=0.028$), high risk (8.6 ± 0.39 , $P<0.001$) and BRAF V600E mutation (7.8 ± 0.38 , $P=0.049$) were significantly more frequent in tumors measuring 7–10 mm than smaller tumors (Fig. 2C). Although not significant, recurrence was more frequent in larger tumors (8.5 ± 0.64 vs 6.6 ± 0.41 ; Fig. 2C).

Loss of expression of *NIS* and *TPO* is associated with the presence of BRAF V600E mutation, independently of tumor size

We next investigated the effect of different mutations along MAPK in the expression of thyroid iodine-metabolizing genes. Overall, the expression of *NIS* and *TPO* was lower in BRAF V600E-positive tumor than in tumors negative for the five-mutation panel and normal thyroid (Fig. 3A). Although the expression of *PDS* and *TG* was lower in BRAF V600E-positive tumors than normal thyroid, the difference was not statistically significant and the expression of *TSHR* was similar in all subgroups (data not shown). In contrast, neither RET/PTC nor NRAS Q61 modulates the expression of *NIS* and *TPO* (Fig. 3A). The expression pattern observed in PTCs harboring coincident mutations was similar to that observed in samples harboring BRAF V600E alone (Fig. 3A).

When considering the expression according to tumor size, *NIS* level was significantly lower in PTCs measuring > 10 mm (i.e. 11–20 mm and > 20 mm). Regarding *TPO*, the expression levels were consistently lower in all PTCs, independently of tumor size (Fig. 3B).

As in microPTC, the expression of *NIS* was comparable to the expression observed in a normal thyroid, we assessed the expression of *NIS* and *TPO* in these tumors classified according to mutational status. *NIS* and *TPO* expression were consistently lower in microPTC that were BRAF V600E-positive or with concomitant mutations (Fig. 4A).

Table 2 Correlation of mutational status with clinico-pathological features of 118 PTC.

	Negative		Mutated PTC ^a		BRAF ^a only		NRAS ^a only		RET/PTC ^a only		RET/PTC1 ^a		RET/PTC2 ^a		RET/PTC3 ^a																				
	No.	n=38	No.	(%)	n=80	No.	(%)	P	n=42	No.	(%)	P	n=6	No.	(%)	P	n=12	No.	(%)	P	n=6	No.	(%)	P	n=1	No.	(%)	P	n=4	No.	(%)	P			
Gender																																			
Female	30		69	(70)	0.421	37	(55)	0.366	4	(12)	0.061	10	(25)	1.000	6	(17)	0.523	1	(3)	1.000	2	(6)	0.236	6	(17)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
Male	8		11	(58)		5	(38)		2	(20)		2	(20)		0	(0)		0	(0)		2	(20)		0	(0)		0	(0)		2	(20)		2	(20)	
Tumor size ^b																																			
≤10 mm	19		21	(53)	0.021	11	(37)	0.040	2	(10)	0.666	3	(14)	0.303	2	(10)	1.000	0	(0)	1.000	1	(5)	0.608	2	(10)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
>10 mm	19		57	(75)		30	(61)		4	(17)		8	(30)		4	(17)		8	(30)		1	(5)		3	(14)		1	(5)		3	(14)		3	(14)	
Variant																																			
CVPTC	16		56	(78)	0.005	35	(69)	0.000	2	(11)	1.000	6	(27)	0.743	3	(16)	1.000	0	(0)	1.000	3	(16)	0.313	3	(16)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
FVPTC	22		24	(52)		7	(24)		4	(15)		6	(21)		3	(21)		6	(21)		1	(4)		3	(21)		1	(4)		3	(21)		1	(4)	
Multifocality ^b																																			
No	22		37	(63)	0.235	17	(44)	0.173	3	(12)	0.682	4	(15)	0.183	2	(8)	0.380	0	(0)	0.421	2	(8)	1.000	2	(8)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
Yes	15		42	(74)		24	(62)		3	(17)		8	(35)		4	(21)		8	(35)		1	(6)		4	(21)		1	(6)		2	(8)		2	(8)	
Extrathyroidal invasion ^c																																			
No	28		45	(62)	0.097	21	(43)	0.058	6	(18)	0.315	6	(18)	0.258	3	(10)	0.593	1	(3)	1.000	1	(3)	0.068	3	(10)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
Yes	9		31	(78)		18	(67)		0	(0)		5	(36)		2	(8)		5	(36)		0	(0)		2	(8)		0	(0)		3	(25)		3	(25)	
Lymph node metastases																																			
No	27		41	(60)	0.048	15	(36)	0.002	6	(18)	0.313	7	(21)	0.486	4	(13)	1.000	1	(4)	1.000	1	(4)	0.100	4	(13)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
Yes	11		39	(78)		27	(71)		0	(0)		5	(31)		2	(15)		5	(31)		0	(0)		2	(15)		0	(0)		3	(21)		3	(21)	
Risk stratification ^{d,e}																																			
High	19		58	(75)	0.000	34	(64)	0.005	4	(17)	0.666	7	(27)	0.506	3	(14)	1.000	0	(0)	1.000	0	(0)	0.608	3	(14)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
Low	19		21	(53)		8	(30)		2	(10)		4	(17)		2	(10)		4	(17)		1	(5)		2	(10)		1	(5)		3	(14)		1	(5)	

CVPTC, Classical variant of papillary thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma.

^aFor statistical analysis, PTC samples positive for mutations were compared to samples that were negative for the five-panel of mutation.^bInformation not available for two of 118 cases.^cInformation not available for five of 118 cases.^dInformation not available for one of 118 cases.^eLow-risk cases include very low risk and low risk.

Table 3 Clinico-pathological parameters and mutational pattern of PTC cases with recurrence.

ID ^a	Gender	Age (years)	Size (mm)	Variant	Mutation	Risk	pT	pN	Treatment	Surgery	Time to recurrence (years)	Site of recurrence	End of follow-up findings	Time of final visit (years)
MicroPTC														
4	F	55	8	CVPTC	BRAF V600E	High	T3	N0	TT+RAI	2004	3.0	Central compartment	No evidence of disease	8.0
7	F	42	9	CVPTC	BRAF V600E	High	T1a	N1	TT+LCD+RAI	2005	2.0	Lateral compartment	LF	6.0
87	F	44	7	CVPTC	None	High	T1a	N1	TT+LCD	2003	1.3	Lateral compartment	No evidence of disease	9.0
104	F	76	10	FVPTC	None	High	T1a	N1	TT+LCD+CCD	2002	0.3	Lateral compartment	Persistent disease	4.0
Non-microPTC														
27	F	21	30	CVPTC	BRAF V600E	High	T3	N1	TT+LCD+CCD+RAI	2001	4.0	Lateral compartment	No evidence of disease	11.0
28	F	59	30	CVPTC	BRAF V600E	High	T3	N1	TT+LCD+CCD+RAI	2006	2.0	Lung	Persistent disease	6.0
30	F	29	35	CVPTC	BRAF V600E	High	T4	N1	TT+LCD+RAI	2003	6.0	Central compartment	No evidence of disease	10.0
34	F	58	90	CVPTC	BRAF V600E	High	T4	N1	TT+LCD+CCD	2005	4.0	Bone	Persistent disease	5.0
35	M	NA	NA	CVPTC	BRAF V600E	High	NA	N1	TT+LCD+RAI	1989	7.0	Central compartment	LF	9.0
42	F	38	50	FVPTC	BRAF V600E	High	T3	N1	TT+LCD+RAI	2006	0.2	Central compartment	No evidence of disease	6.0
65	F	46	15	CVPTC	BRAF V600E and RET/PTC2	High	T3	N1	TT+LCD+CCD+RAI	2002	4.0	Lung	Persistent disease	10.0
67	F	69	20	CVPTC	BRAF V600E and RET/PTC2	High	T4	N0	TT	2004	5.0	Central compartment	Persistent disease	8.0
60	F	27	50	FVPTC	RET/PTC1 and RET/PTC3	High	T3	N0	TT+RAI	2005	3.0	Lung	Persistent disease	7.0
108	M	23	25	FVPTC	None	High	T3	N1	TT+LCD	1997	2.0	Central compartment	LF	NA
117	F	68	80	FVPTC	None	High	T3	N0	TT+CCD+RAI	2005	4.0	Lung	Persistent disease	7.0

CVPTC, classical variant of papillary thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma; TT, total thyroidectomy; LCD, lateral compartment dissection; CCD, central compartment dissection; RAI, radioiodine; NA, not available; LF, lost to follow-up.

^aID according to Supplementary Table 2.

Table 4 Correlation of mutational status with clinico-pathological features of small PTCs divided into two subgroups according to tumor size.

	Negative		Mutated PTC ^a		BRAF ^b only		NRAS ^a only		RET/PTC ^c only		Concomitant mutations ^a		BRAF positive ^{a,b}	
	No.	(n=19)	No. (%)	P	No. (%)	P	No. (%)	P	No. (%)	P	No. (%)	P	No. (%)	P
PTC ≤ 10 mm (n=40)			(n=21)		(n=11)		(n=2)		(n=3)		(n=5)		(n=15)	
Extrathyroidal invasion ^c														
No	16	16 (50)	0.418		9 (36)	0.622	2 (11)	1.000	2 (11)	0.386	3 (16)	0.194	11 (41)	0.375
Yes	2	5 (71)			2 (50)		0 (0)		1 (22)		2 (50)		4 (67)	
Lymph node metastases														
No	15	14 (48)	0.488		6 (29)	0.225	2 (12)	1.000	3 (17)	1.000	3 (17)	0.568	8 (35)	0.151
Yes	4	7 (64)			5 (56)		0 (0)		0 (0)		2 (33)		7 (64)	
Risk stratification ^d														
High	6	10 (63)	0.349		6 (50)	0.266	0 (0)	1.000	1 (14)	1.000	3 (33)	0.326	9 (60)	0.165
Low	13	11 (46)			5 (28)		2 (13)		2 (13)		2 (7)		6 (32)	
PTC ≤ 20 mm (n=67)			(n=42)		(n=25)		(n=2)		(n=4)		(n=11)		(n=34)	
Extrathyroidal invasion ^c														
No	19	25 (57)	0.174		15 (44)	0.216	2 (10)	1.000	2 (10)	0.253	6 (24)	0.227	19 (50)	0.094
Yes	5	17 (77)			10 (67)		0 (0)		2 (29)		5 (50)		15 (75)	
Lymph node metastases														
No	20	21 (51)	0.020		10 (33)	0.009	2 (9)	1.000	3 (13)	1.000	6 (23)	0.224	14 (41)	0.004
Yes	5	21 (81)			15 (75)		0 (0)		1 (17)		5 (50)		20 (80)	
Risk stratification ^d														
High	11	28 (72)	0.047		19 (63)	0.013	0 (0)	0.512	2 (15)	1.000	7 (39)	0.288	26 (70)	0.008
Low	16	14 (47)			6 (27)		2 (11)		2 (11)		4 (20)		8 (33)	

^aFor statistical analysis, PTC samples positive for mutations were compared to samples that were negative for the five-panel of mutation.^bAll BRAF V600E positive cases (BRAF V600E only and BRAF V600E with RET/PTC).^cInformation not available for one case.^dLow-risk cases include very low risk and low risk.

Figure 3

Relative expression (RE) of *NIS* and *TPO* in PTC. (A) Expression of *NIS* and *TPO* classified according to the mutational status. Negative groups correspond to those samples negative for the five-mutation panel ($n=38$). (B) *NIS* expression is diminished in PTC measuring 11–20 mm and >20 mm. *TPO* expression is diminished in all PTC, independently of tumor size. Symbols represent means and bars represent the S.E.M. * $P<0.05$; ** $P<0.01$ and *** $P<0.001$.

Finally, as pathological features indicative of more aggressive behavior and BRAF V600E were found mainly in microPTC with ≥ 7 mm, we also investigated the expression level of *NIS* and *TPO* in these set of tumors. Interestingly, the expression of *NIS* and *TPO* was consistently lower in microPTC measuring ≥ 7 mm ($n=25$) than tumors with <7 mm ($n=15$) (Fig. 4B). These findings suggest that the diminished expression of *NIS* and *TPO* in microPTC is more likely to be associated with the presence of BRAF mutation and more aggressive phenotype.

Discussion

The increasing incidence of thyroid cancer is primarily due to increased detection of small PTC. Therefore, the management of these small tumors, mainly microPTC, turned out to be one of the most relevant clinical problems in endocrinology. Though most microPTC are classified as low-risk tumors and, therefore, less prone to recur and can be successfully treated with a more conservative approach, a substantial percentage of the cases have extrathyroidal extension and lymph node metastases at presentation; a small number of these tumors may have distant metastases (7, 37). These findings suggest that microPTC may include two subgroups with distinct biological behavior. As parameters of the tumor are still unknown,

currently it is not possible to distinguish those tumors that are within the good prognosis group from those that are within the poor prognosis group. In fact, there is no consensus in the literature to support either a more conservative approach or a more aggressive treatment. Hence, it is of clinical value to identify a molecular marker that can preoperatively improve risk stratification and hence tailor the management of patients with small PTC, mostly microPTC.

The knowledge that *BRAF* V600E, *RAS* and *RET/PTC* are the major driver mutations in PTC has had a profound impact on preoperative diagnosis of thyroid nodules. Although still remaining controversial (38), it has been proposed that *BRAFV600E* can potentially be used as prognostic factor in the risk stratification of microPTC (7, 11, 12, 14, 15, 16, 17, 18, 19, 37, 39). Regarding *RET/PTC*, only a limited number of studies have evaluated its prognostic value. Some studies were unable to find any significant association between the presence of *RET* rearrangements and several clinical pathological features of aggressiveness (21, 40, 41). Few studies suggested that *RET/PTC3* correlates with more advanced stage of disease (42, 43) and aggressive variants such as solid (33) or tall-cell (44). Additionally, *RET/PTC3* transgenic mice developed locally invasive solid PTC with lymph node metastases (45). Other studies reported that *RET/PTC*

Figure 4

Relative expression (RE) of *NIS* and *TPO* in microPTC. (A) The expressions of *NIS* and *TPO* are lower in BRAF V600E-positive PTC (BRAF V600E only and concomitant mutations), compared to normal thyroid tissues or PTC that are negative for the five-mutation panel. (B) *NIS* and *TPO* expression are lower in microPTC, measuring ≥ 7 mm than microPTC measuring <7 mm. Symbols represent means and bars represent the S.E.M. * $P<0.05$; ** $P<0.01$ and *** $P<0.001$.

rearrangements are significantly more common in smaller and less aggressive PTC, which points toward a good prognosis impact (8, 46).

Despite a number of descriptive reports on the prevalence of *BRAF* or *RET/PTC* mutations in microPTC and their correlation with clinico-pathological characteristics, there has been no analysis that evaluated the prognostic value of different mutations along with MAPK in the same series of microPTC. We considered this essential, as most studies correlate clinico-pathological parameters between *BRAF*-positive and *BRAF*-negative tumors. As *RAS* or *RET/PTC* are commonly found in PTC and were not investigated, the *BRAF*-negative group possibly includes cases that are indeed positive for these mutations.

In this study, we investigated the prevalence of *NRAS* Q61 mutations and *RET/PTC* rearrangements in a large series of PTC in which *BRAF* V600E mutational status was previously determined (13). The overall rate of *BRAF* V600E mutation is 48%, *RET/PTC* is 27% and *NRAS* is 9%. In few tumors, concomitant mutations along the MAPK pathway were identified.

It is worth mentioning that in most series mutations in genes within the MAPK pathway, they rarely co-occur in PTC (9, 47, 48, 49). Data from the Cancer Genome Atlas also showed that mutations in the MAPK-related genes were virtually mutually exclusive. *BRAF*, *NRAS*, *KRAS* and *HRAS* were present in the majority of tumor cells, i.e. they are largely clonal. The authors reported one PTC case with two driver mutations in the MAPK-related genes. In these tumors, *KRAS* mutation was clonal while *BRAF* was subclonal (26). Most likely, the mutually exclusive pattern occurs in most tumors, mainly with clonal origin. However, coincident mutations in genes that encode proteins along the MAPK pathway have been previously reported in thyroid tumors, as shown in Table 1. Furthermore, using high-throughput mutation profiling in 1000 human tumor samples, a high number of co-occurring mutations in the same pathway were detected in several tumor types. The authors found that *BRAF* mutations involving codon 600 and 601 were likely to co-occur with a *RAS* family mutation (50). Simultaneous *BRAF* and *RAS* mutations were also reported well as in the breast, gynecologic malignancies and colon (51, 52).

Comparable to our findings, other groups reported that *BRAF* V600E was associated with more aggressive tumor phenotype. *RET/PTC* alone or combined with *BRAF* V600E did not represent a risk factor for a more aggressive disease (53). However, few studies suggested that multiple genetic changes might be associated with tumor progression (52, 54, 55). Henderson *et al.* (54) reported that

prevalence of tumors with simultaneous *BRAF* and *RET/PTC* mutations in the recurrent population far exceeds the prevalence reported for patients with primary PTC. Zou *et al.* (55) found that concomitant mutations are a frequent event in advanced PTC and are associated with poor response to treatment and shorter disease-free survival. The authors examined different tumors sections from samples with *BRAF* V600E and *RAS* mutations. *BRAF* V600E mutation was found in all sections from the same tumor while *RAS* mutation was detected only in some sections. Based on these findings, the authors suggested that concomitant mutations may represent intratumoral heterogeneity and it is likely that *RAS* mutation occurred later than *BRAF*, and might play a role in disease progression (55). Evidence for intratumoral genetic heterogeneity of PTC involving both *RET/PTC* and *BRAF* has also been previously reported (56, 57).

Remarkably, our study found that the prevalence of *BRAF*, *RET/PTC* and *RAS* mutations alone decreases with age, whereas the incidence of concomitant mutations increases with age. Regarding the association between older age and increased prevalence of concomitant mutation, it was previously reported that most cases of PTC with concomitant mutations occurred in patients older than 45 years of age (54, 55).

Finally, the discrepancy may be a result of different sensitivities of the detection methods, mainly if *RET/PTC* is present in a small fraction of cells. Using FISH analysis and PCR-based assays that detect expression of *RET/PTC* transcripts, it was demonstrated that the presence of *RET/PTC* rearrangement within each tumor can vary from involving almost all tumors cells to only a small proportion of cells (56).

Our study used standard RT-PCR for detection of *RET/PTC* rearrangement, since the authors suggested this method is sufficient to detect *RET/PTC* in all tumors with at least 17% of cells harboring *RET/PTC* rearrangements. To confirm the expression of *RET* fusion transcripts, we additionally evaluated the expression of both the TK and EC domains of *RET* was performed, as described (31, 32, 33, 34, 35, 36).

All together, tumor heterogeneity, age of patients and methodology used may explain the divergence obtained in different series.

We next determined the prevalence of *BRAF* V600E, *NRAS* Q61 and *RET/PTC* in microPTC. While it has been suggested that *RET/PTC* rearrangements are particularly more prevalent in microPTCs than in clinically evident PTCs (41, 46), in our study no differences were found in the prevalence of *RET/PTC* and *RAS* in these subgroups.

Our study, however, confirmed the increased prevalence of BRAF V600E in tumors measuring >10 mm than in microPTC. Regarding prognosis implications, BRAF V600E was associated with larger tumor size, presence of lymph node metastases at diagnosis and higher risk of recurrence. *RET/PTC* and *RAS* were not correlated with clinical-pathological parameters indicative of a more aggressive phenotype.

Recently, a few groups have suggested that tumor size alone is not a significant predictor of tumor persistence or recurrence and, therefore, thyroidectomy and postoperative radioactive iodine ablation should be performed in patients whose one or more parameters such as multicentricity, positive lymph nodes, or capsular or vessel invasion are found (5, 58). Consequently, it has been suggested that the selected cut-off size of 10 mm may be too arbitrary and can be further refined (59). There is a trend to use cut-off value of 7 mm as a reasonable threshold for aggressiveness in microPTC (60), although other groups have suggested cut-off values from 5 mm to 9 mm.

In an attempt to better separate those microPTC that have more aggressive features from those tumors that have a limited disease, we correlated tumor size with BRAF V600E or clinical-pathological parameters of aggressiveness. The present study showed that extrathyroidal invasion, presence of metastases at diagnosis, high risk of recurrence and the presence of BRAF V600E mutation were significantly more prevalent in tumors measuring ≥ 7 mm.

Although current data suggest that RAI is of no benefit in preventing recurrence in microPTC (4), some cases show high metastatic potential and high persistence/recurrence rate justifying a more aggressive treatment. As BRAF V600E mutation is frequently found in tumors that are refractory to treatment with radioiodine (25) and small-molecule MAPK inhibitors restore the expression of thyroid-specific genes with consequent restoration of RAI uptake (61), it seems rational to explore whether BRAF V600E, NRAS Q61 or *RET/PTC* modulate the expression of proteins associated with iodine metabolism and may explain why some microPTC could be less responsive to RAI therapy and, therefore, RAI did not prevent the recurrence rate of some microPTC (59, 62).

This study showed that the expressions of *NIS* and *TPO* were significantly lower in PTC with *BRAF* mutations. No difference was observed in the expression of genes that code for proteins involved in iodine metabolism in PTC samples bearing *RAS* mutations or *RET/PTC* rearrangements.

The distinct expression profile of genes involved in iodine-uptake and metabolism between BRAF-V600E and

RAS-mutant tumors has been also confirmed in other studies (49, 63, 64, 65, 66, 67).

Recently, using exome and RNA sequencing data, the TCGA study group evaluated the spectrum of gene signature in BRAF V600E-mutated and RAS-mutated tumors. This signature showed strong separation between BRAF V600E and RAS-driven tumors. Therefore, they developed a score system, which quantify the extent to which the expression profile of a given tumor resembles either BRAF V600E or RAS-mutant profiles, the BRAF V600E and RAS Score (BRS). Next, they used BRS as a reference to interrogate the signaling consequences of less common mutations. All BRAF mutations other than BRAF V600E and BRAF fusions, PAX8/PPARG fusion and most EIF1AX mutations exhibited RAS-like behaviors. Nearly all of the *RET/PTC* were weakly BRAF-like and *NTRK3* and *ALK* fusion were largely neutral (26).

Next, they summarized the expression levels of thyroid metabolism and function genes to produce a score metric designated thyroid differentiation score (TDS). Even they are based on different signature, BRS and TDS measures were highly correlated across all tumors. The BRS and TDS score also correlated with different omics data and histology. In other words, BRAF V600E-Like comprise mainly less differentiated tall and classic variants of PTC, signaling preferentially through MAPK and downregulated expression of genes involved in iodine metabolism. Interestingly, BRAF V600E-like is less homogeneous than initially considered, containing distinct subgroups. The RAS-like PTCs result in highly differentiated tumors enriched for follicular variant, low risk of recurrence, occurs in young patients, less prominent levels of MAPK signaling and lower effect on the genes associated with iodine-uptake and metabolism (26).

All together, regarding the expression profile of genes involved in iodine-uptake and metabolism, our data is in agreement with TCGA study group. BRAF-mutant tumors showed lower expression of *TPO*, compared to *RET/PTC* and RAS-mutant tumors. Although very few samples with *RET/PTC1* and *RET/PTC3* exhibited BRAF V600E-like behavior, a large proportion has a RAS-Like behavior.

Remarkably, when we stratified microPTC according to mutational status, the expression of *NIS* and *TPO* were significantly diminished in BRAF V600E-positive microPTC. Finally, *NIS* and *TPO* expression were significantly lower in microPTC with ≥ 7 mm.

As nearly 87% of PTC detected consisted of tumors measuring 20 mm or less, we also explored an association between BRAF V600E mutations with different clinico-pathological features associated with poor prognosis in

PTC measuring ≤ 20 mm. We found an association between BRAF V600E and presence of lymph node metastases and higher risk of recurrence. Our findings corroborate with those previously reported, in which the authors suggested that BRAF V600E is useful to improve risk stratification even for tumors measuring ≤ 20 mm that are limited to the thyroid gland and, therefore, classified as low risk tumors (17). Nevertheless, when we considered only tumors measuring 10 mm or less, no significant association was found between mutational profiling and clinico-pathological parameters.

In summary, BRAF V600E mutation was associated with clinico-pathological parameters that are associated with more aggressive phenotype in small PTC. A strong association was also found in microPTC larger than 7 mm, suggesting that this subgroup of microPTC is more prone to recur. Moreover, BRAF-mutated cases showed lower expression of genes involved in iodine uptake, which points for an effect of BRAF on the expression of these genes. These findings are similar to the data found in larger aggressive PTC, suggesting that, although microPTC are considered clinically indolent, the subgroup of 7–10 mm might be treated as conventional PTC. We believe that our findings will help to decide the realistic usefulness of BRAF V600E mutation as preoperative marker of poor prognosis in small PTC, even in microPTC.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-15-0254>.

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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