Hormones and Cancer (2019) 10:97–106
https://doi.org/10.1007/s12672-019-0359-8

ORIGINAL PAPER

The Combination of RET, BRAF and Demographic Data Identifies Subsets of Patients with Aggressive Papillary Thyroid Cancer

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Received: 30 April 2018 / Accepted: 22 January 2019 / Published online: 22 March 2019

Abstract

The use of *BRAFV600E* and *RET/PTC1* as biomarkers to guide the extent of surgery in patients with papillary thyroid cancer (PTC) remains controversial. We assessed the combined use of demographic data (sex and age) with mRNA expression levels and/or mutational status (*BRAFV600E* and *RET/PTC1)* to identify potential subsets of patients with aggressive histopathological features (lymph node metastases and extrathyroidal extension). In a cohort of 126 consecutive patients, *BRAFV600E* and *RET/PTC1* mutations were found in 52 and 18%, respectively. By conditional bivariate analysis (CBVA), a 'high activity' profile of *BRAF* (*BRAFV600E* positive or high expression) and 'low activity' profile of *RET* (*RET/PTC1* negative or low expression) was associated with extrathyroidal extension (ETE) (OR 4.48). Alternatively, a 'high activity' profile of *RET* (*RET/PTC1* positive or high expression) and 'low activity' profile of *BRAF* (*BRAFV600E* negative or low expression) were associated with lymph node metastasis (LNM) (OR 12.80). Furthermore, in patients younger than 55 years, a low expression of *BRAF* was associated with LNM (OR 17.65) and the presence of *BRAFV600E* mutation was associated with ETE (OR 2.76). Our results suggest that the analysis of demographic and molecular variables by CBVA could contribute to identify subsets of patients with aggressive histopathologic features, providing a potential guide to personalised surgical management of PTC.

Keywords Papillary thyroid cancer · BRAF V600E · RET/PTC1 · Lymph node metastasis · Extrathyroidal extension

Introduction

Papillary thyroid cancer (PTC) is the most frequent endocrine malignancy [1]. In the last decades, an increased incidence of PTC has been reported, particularly among small tumours [2]. Despite that most of patients with PTC have an optimal response to surgical treatment, an uncertain subgroup will develop persistent or recurrent disease, increasing the risk of distant metastasis and mortality [3]. Currently, the identification of high-risk patients is performed by clinical stratification systems, mainly based on histopathological prognostic factors, including multifocality, tumour size, extrathyroidal extension (ETE) and lymph node metastases (LNM) [4]. However, since the histopathological report is only available after surgery, these systems cannot be used preoperatively to tailor the surgical planning to the individual patient risk [4]. For instance, in patients with clinically T1–T2 and N0 tumours, the treatment options could range from

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active surveillance to total thyroidectomy, depending on the presence of multifocality or ETE. In this context, the preoperative use of genetic biomarkers associated with certain histopathological features could potentially help clinicians to guide the most appropiate surgical approach.

A number of biomarkers have been proposed to identify histopathological prognostic factors for PTC, including *BRAFV600E*, *RAS*, and *TERT* mutations, as well as differential expression levels of several miRNAs (e.g. miR-212, miR-143, miR-9) [5–10]. Of these, one of the most promising but controversial biomarkers is the *BRAFV600E* mutation, which promotes a constitutive activation of the MEK/ERK pathway, inducing survival and cell proliferation [11]. Several studies have suggested that *BRAFV600E* is an independent risk factor for recurrence of PTC [7, 12–15]. However, these findings have not been reproduced by other authors [16–21]. Furthermore, there are no studies providing compelling data that tailoring the surgical approach based on *BRAFV600E* status improves the clinical outcomes. Additionally, the proto-oncogene *RET*, a proliferative tyrosine kinase receptor usually expressed in thyroid follicular cells and its rearrangement (*RET/PTC*), has been associated with PTC carcinogenesis through enhanced MEK/ERK signalling [22–24], being the most frequent *RET* rearrangement (60–70%) [25]. However, there are no robust evidence on the prognostic value of *RET/PTC1* rearrangement in PTC.

Recently, it has been proposed that the presence of concomitant PTC mutations could be useful to identify subsets of patients with a more aggressive disease [26]. For instance, the coexistence of *TERT* promoter mutations with *BRAF* or *RAS* mutations has been associated with a poorer prognosis in PTC patients [27–29]. Additionally, mRNA expression of *BRAF* and *RET* has been suggested as diagnostic biomarkers for PTC [30–34]. However, no studies have reported a combining the presence of *RET/PTC1* rearrangement, *BRAFV600E* mutation, and their mRNA expression levels, could be associated to histopathological prognostic factors of PTC.

In this study, we analysed the presence of both, *RET/PTC1* and *BRAFV600E* mutations, and their mRNA expression levels, to identify subgroups of PTC patients with high-risk histopathological factors. Further analysis was performed by stratifying the patients by demographic variables (i.e. sex and age). Here, we show that combined molecular-demographic analysis identifies subgroups of patients with aggresive histopathological features of PTC.

Subjects and Methods

Patients and Specimen Collection

A total of 126 consecutive fresh tissue samples were prospectively collected from patients with a preoperative diagnosis of PTC undergoing surgery. Patients were enrolled at the Clinical Hospital of the Pontificia Universidad Católica de Chile between 2013 and 2015. Inclusion criteria were patients > 18 years, with a cytological suspected diagnosis of PTC and confirmed surgical pathology diagnosis of PTC. Therapeutic lymph node dissection was performed in patients with ultrasound evidence of cervical adenopathy confirmed by fine needle aspiration or intra-operative pathological findings suggestive of lymph node metastases. In this study, patients did not undergo prophylactic lymph node dissection. The study was approved by the Ethics Committee of the Pontificia Universidad Católica de Chile and all patients included signed an informed consent. Tumour samples were obtained in the operating room and immediately placed in RNALater stabilisation solution (Ambion®, Carlsbad, CA). Samples were stored at 4 °C until RNA extraction which was usually performed 1 week after collection.

RNA Isolation and cDNA Synthesis

Total RNA was obtained with the RNeasy Plus-Mini Kit (QIAGEN®, Valencia, CA). RNA concentration was determined using the PicoCube spectrophotometer (Picodrop®, Cambridge, UK). Reverse transcription reaction from 150 ng of total RNA was performed in a final volume of 20 µl using the Improm II™ Reverse Transcription System (Promega®, Madison, WI) following the manufacturer instructions.

Real-Time Polymerase Chain Reaction and High-Resolution Melting qPCR

Briefly, 3.0 ng of total RNA from the RT reaction in a final volume of 20 µl was used in the real-time quantitative polymerase chain reaction (qPCR) reaction mixture containing 10 µl of 2× Brilliant II SYBR Green qPCR Master-Mix (Agilent®), 250 nM of each primer and nuclease-free water. Primer sequences were as follows: *GAPDH* forward 5'ATCATCAGCAATGCCTCCTGCA3' and *GAPDH* reverse 5'GTTTCCGGAGGGGCCAT3'; *RET* forward 5'AATTTGGAAAAGTGGTCAAGGC3' and *RET* reverse 5'CTGCAGGCCCCATACAAT3' and *RET/PTC1* forward 5'CGCGACCTGCGCAAA3' and *RET/PTC1* reverse 5'CAAGTTCTTCCGAGGGAATT CC3' with an annealing temperature of 60 °C. The qPCR reaction was performed in triplicate in the Rotor-Gene Q cycler (QIAGEN®, Valencia, CA). Thermocycling conditions were 10 min at 95 °C, followed by 40 cycles of 20 s at 95 °C, 20 s at 60 °C and 20 s at 72 °C. Amplicons were subjected to melting curve analysis by increasing the temperature from 72 to 95 °C with an increment of 1 °C per

second. All reactions with cycle threshold (CT) over 35 and deficient melting curves were not considered. Each qPCR run included cDNA of Nthy-Ori 3.1 and TPC-1 cell line as a negative and positive control for *RET/PTC1* arrangement, respectively.

High-resolution melting qPCR (HRM-qPCR) was set up using 3 ng of cDNA, 10 μl of 2× of Type-It HRM PCR kit (QIAGEN®, Valencia, CA) and 250 nM of each primer in a final reaction volume of 20 μl. Primer sequences for *BRAFV600E* were as follows: forward 5'TCATGAAGACCTCACAGTAAAAATAGG3' and reverse 5'TGGTGCCATCCACAAAATGG3' with an annealing temperature of 55 °C. The analysis was performed in triplicates using the Rotor-Gene Q cycler (QIAGEN®, Valencia, CA). Conditions for amplification were 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 55 °C. The HRM analysis was performed by increasing the temperature from 65 to 95 °C with an increment of 0.1 °C every 2 s. Each qPCR run included cDNA of Nthy-Ori 3.1 cell line as a *BRAF* wild-type control and cDNA of K1 PTC cell line as a mutant *BRAFV600E* positive control.

Statistical Analysis

Preoperative variables were separated into two subgroups: dichotomic variables, including sex (male or female), age (< 55 years old, and ≥ 55 years old) and mutational status (*BRAFV600E* and *RET/PTC1*), and continuous variables, including expression level for *BRAF*, *RET/PTC1* and *RET*. Histopathological prognostic factors for associative analysis were tumour size (< 2 cm and ≥ 2 cm), bilateral disease, multifocal disease, extrathyroidal extension (ETE), lymph vascular invasion (LVI) and lymph node metastasis (LNM). The odds ratio (OR) was calculated for three different statistical analysis: bivariate analysis (BVA), where each preoperative variable (demographic or genetic) was associated with each histopathological prognostic factor; multivariate analysis (MVA), where all the preoperative variables were integrated into a logistic regression model to predict the presence of a given outcome (LNM or ETE) and finally, conditional bivariate analysis (CBVA), where each dichotomic variable was used to define subsets of patients and then, for each subset, the remaining variables were associated with each outcome. The 95% of the confidence interval (95% CI) was also calculated for OR, resulting statistically significant when did not cross the unit value. Continuous variables (expression levels) were dichotomized by considering the best significant OR (i.e. 95% CI does not cross 1) by BVA at different cutoff values. Statistical analyses were performed by the SPSS 15.0 software.

Results

Cohort Description and Association with Histopathological Prognostic Factors

A total of 126 patients were included in the study. Overall, 108 (86%) patients were female, the mean age was 39 ± 13 years, and the mean tumour diameter was 1.5 ± 0.9 cm. (Table 1). One hundred and ten (87%) patients were younger than 55 years. Histopathologically, 56 patients (44%) had multifocal disease, and 41 patients (33%) had ETE; 31 patients (29%) were pN1a and 20 patients (16%) were pN1b according to the updated AJCC/TNM Staging System for Differentiated and Anaplastic Thyroid Cancer 8th edition (Table 1) [35]. By BVA, age < 55 years was associated with multifocal disease (OR 5.26, 95% CI 1.10–25.00) (Table 2). By MVA, male sex was independently associated to ETE (OR 2.97, 95% CI 1.00–8.85) (Table 3).

RET, BRAF and RET/PTC1 mRNA Expression Levels Are Associated with Histopathological Prognostic Factors

In our cohort, the prevalence of *BRAFV600E* and *RET/PTC1* was 52 and 18%, respectively. Coexistence of both mutations was detected in 10% of samples. In patients with *BRAFV600E*, ETE was found in 41%, compared to patients without *BRAFV600E*, wherein ETE was found in 23% of cases ($p = 0.039$, Table 1). No significant differences in *RET/PTC1* rearrangement prevalence were found by sex, age or histopathological prognostic factors (Table 1). Multivariate analysis only showed a significant independent association between *BRAFV600E* mutation and tumour size ≥ 2 cm. No significant association was identified for *RET/PTC1* rearrangement and the histopathological factors assessed (Table 3). Moreover, no associations were found for the transcript levels of *RET*, *RET/PTC1* nor *BRAFV600E* (Table 3).

To identify potential subgroups of patients in which genetic biomarkers could be significant, a CBVA was performed according to the status of BRAFV600E mutation and RET/PTC1 rearrangement. In *BRAFV600E*-positive patients, both, a low expression of *RET* and the absence of *RET/PTC1* rearrangement, were associated with ETE (OR 4.48, 95% CI 1.14–17.58 and OR 2.74, 95% CI 1.17–6.44, respectively) (Fig. 1). On the other hand, in *RET/PTC1*-positive patient, the absence of *BRAFV600E* mutation and a low expression of *BRAF* were associated with LNM (OR 12.80, 95% CI 1.21–135.58 and OR 7.88, 95% CI 1.10–56.12, respectively) (Fig. 1). In addition, in patients wild-type for *BRAF*, a high expression of *RET* was also associated with LNM (OR 3.95, 95% CI 1.34–11.64) (Fig. 1). No significant associations were found in

Table 1 Clinicopathological characterization of the PTC cohort considering $BRAF^{V600E}$ mutation and RET/PTC1 rearrangement

	Total cohort		$BRAF^{V600E}$ mutation				*p* value	RET/PTC1 rearrangement				*p* value
			Positive		Negative			Positive		Negative		
Sex												
Male	18	14%	10	15%	8	12%	0.804	3	14%	15	14%	1.000
Female	108	86%	56	85%	52	87%		19	86%	89	86%	
Age (years)												
< 55	110	87%	56	85%	54	90%	0.433	20	91%	90	87%	0.736
≥ 55	16	13%	10	15%	6	10%		2	9%	14	13%	
Size (cm)												
< 2	93	74%	48	73%	45	75%	0.432	16	73%	77	74%	0.443
2–4	29	24%	17	26%	12	21%		5	24%	24	24%	
> 4	4	3%	1	2%	3	5%		1	5%	3	3%	
Histology												
PTC - CV	89	71%	54	82%	35	58%	*0.022*	14	64%	75	72%	0.069
PTC - FV	23	18%	7	11%	16	27%		3	14%	20	19%	
PTC - OV	10	8%	4	6%	6	10%		2	9%	8	8%	
PTC - DSV	3	2%	0	0%	3	5%		3	14%	0	0%	
PTC - WLV	1	1%	1	2%	0	0%		0	0%	1	1%	
Multifocality												
No	70	56%	33	50%	37	62%	0.212	13	59%	57	55%	0.815
Yes	56	44%	33	50%	23	38%		9	41%	47	45%	
Extrathyroidal extension												
No	85	67%	39	59%	46	77%	*0.039*	18	82%	67	64%	0.138
Yes	41	33%	27	41%	14	23%		4	18%	37	36%	
Lymph vascular invasion												
No	110	87%	57	86%	53	88%	0.794	19	86%	91	88%	1.000
Yes	16	13%	9	14%	7	12%		3	14%	13	13%	
Lymph node metastasis												
N0	75	60%	41	62%	34	57%	0.651	13	59%	62	60%	0.922
N1a	31	29%	14	25%	17	33%		6	32%	25	29%	
N1b	20	16%	11	17%	9	15%		3	14%	17	16%	
Distant metastasis												
No	126	100%	66	100%	60	100%	1.000	22	100%	104	100%	1.000
Yes	0	0%	0	0%	0	0%		0	0%	0	0%	

PTC papillary thyroid carcinoma, *CV* conventional variant, *FV* follicular variant, *OV* oncocytic variant, *WLV* Warthin-like variant, *DSV* diffuse-sclerosant variant.

Italic $p < 0.05$

patients with concomitant mutations (*BRAFV600E* and *RET/PTC1*) or high expression of both, *BRAF* and *RET* transcripts. Finally, patients wild-type for *BRAF* and *RET/PTC1*, expressing *BRAF* and *RET* in low levels, showed a lower risk for ETE (OR 0.36, 95% CI 0.16–0.85) and LNM (OR 0.25, 95% CI 0.09–0.75) (Fig. 1).

In patients younger than 55 years, the presence of *BRAFV600E* mutation was associated with ETE (OR 2.76, 95% CI 1.21–6.27), while a low expression of *BRAF* was associated with LNM (OR 17.65, 95% CI 1.01–309.27) (Fig. 2). Finally, within males, the absence of *RET/PTC1* rearrangement was associated with ETE (OR 3.27, 95% CI 1.06–10.07). Decision trees with the most significant findings of this study are shown in Fig. 2.

Discussion

The preoperative identification of high-risk histopathological factors in patients with PTC may help to decide the extension of total thyroidectomy or neck dissection, offering a more personalised surgical management. In this

Table 2 Predictive statistical performance of demographic and molecular variables associated with histopathological prognostic factors by univariate analysis

	Size (≥ 2 cm)	Bilaterally	Multifocality	Extrathyroidal extension	Lymph vascular invasion	Lymph nodal metastasis (LNM)	
						pN0 v/s Central LNM	pN0 v/s Lateral LNM
Age (≥ 45)							
Odds ratio	0.75	1.16	1.02	0.96	0.74	0.43	0.98
95% CI	0.31–1.82	0.54–2.46	0.49–2.10	0.44–2.07	0.24–2.27	0.20–0.95	0.36–2.69
Sex (male)							
Odds ratio	2.64	1.67	1.30	3.10	2.29	1.84	1.77
95% CI	0.91–7.62	0.61–4.60	0.48–3.53	1.12–8.60	0.65–8.08	0.67–5.03	0.51–6.11
RET/PTC1 rearrangement							
Odds ratio	1.04	0.51	0.84	0.40	1.11	1.20	0.87
95% CI	0.34–3.11	0.17–1.50	0.33–2.14	0.13–1.28	0.29–4.26	0.47–3.08	0.23–3.28
RET/PTC1 expression level							
Odds ratio	n/d	n/d	n/d	n/d	0.03	n/d	n/d
95% CI					0.00–0.64		
$BRAF^{V600E}$ mutation							
Odds ratio	0.88	1.90	1.61	2.27	1.20	0.70	1.01
95% CI	0.38–2.05	0.90–4.05	0.79–3.27	1.05–4.93	0.42–3.44	0.34–1.45	0.38–2.69
BRAF expression level							
Odds ratio	n/d	n/d	n/d	n/d	15.57	n/d	n/d
95% CI					1.32–183.02		
cRET expression level							
Odds ratio	n/d	0.08	n/d	n/d	n/d	2.21	n/d
95% CI		0.01–0.62				1.06–4.60	

n/d not determined. Odds ratio analysis was not performed given that there were no cutoff points that showed significant differences

study, we propose a combination of demographic and molecular data, which could be assessed before surgery to identify subgroups of patients with high-risk histopathological factors.

In our cohort, we report an incidence rate of 52% for *BRAFV600E* mutation, which was significantly associated with ETE by BVA (OR 2.27; 95% CI 1.05–4.93). This result is consistent with previous studies that have reported an OR ranging from 1.98 to 2.23 [36–38]. Additionally, *RET/PTC1* was found in 18% of patients, and was not statiscally associated to any histopathological characteristic. This is consistent with prior evidence reported by Tallini et al. where the presence of *RET/PTC1* was not associated with advanced tumour stages nor progression to undifferentiated carcinomas [39]. Interestingly, the prevalence of *RET/PTC1* in our cohort seems to be higher than would be expected for a non-radiated cohort. However, the frequency of *RET* rearrangements (*RET/PTC*) in PTC varies significantly among patients from different regions, ranging from 2.5 to 85% [1]. Recently, Song and collaborators [2] have published the *RET/PTC* prevalence in Asian and Western countries which shows a significant variability in mutational frequency, even within the same geographical regions (0 to 54.5% in Asia, 2.4 to 72.0% in America and 8.1 to 42.9% in Europe). These discrepancies may be due to the genetic background and environment, different detection methods and additional factors like sex and age [3].

The significance of *BRAF* mRNA expression in thyroid tumours has been previously associated with adverse histopathological factors, disease recurrence and distant metastasis [34, 40]. In this study, we did not find any significant association for *BRAF* or *RET* mRNA expression. Additionally, we did not find differential mRNA expression between wild-type *BRAF* and *BRAFV600E*-positive tumours (data not shown), which is in contrast with a study based on RNA-Seq, where patients with *BRAFV600E* mutation presented an overexpression of *BRAF* and were associated with ETE and higher T stage [34]. This disagreement may be explained by the tumour cell heterogeneity in PTC [41], where *BRAF* mutated and non-mutated cells could coexist in the same tumour.

Table 3 Predictive statistical performance of demographic and molecular variables associated with histopathological prognostic factors by multivariate analysis

	Size (≥ 2 cm)	Bilaterally	Multifocality	Extrathyroidal extension	Lymph vascular invasion	Lymph nodal metastasis (LNM)	
						pN0 v/s Central LNM	pN0 v/s Lateral LNM
Age (≥ 45)							
Odds ratio	0.67	1.09	0.97	0.81	0.74	0.35	0.85
95% CI	0.28–1.61	0.49–2.42	0.43–2.17	0.33–1.99	0.20–2.75	0.14–0.89	0.28–2.55
Sex (male)							
Odds ratio	3.31	0.45	1.34	2.76	4.65	1.69	1.29
95% CI	0.81–13.50	0.14–1.42	0.44–4.05	0.88–8.62	1.08–19.61	0.52–5.55	0.32–5.27
RET/PTC1 rearrangement							
Odds ratio	0.39	1.39	1.06	0.58	1.31	1.45	0.74
95% CI	0.14–1.11	0.44–4.43	0.40–2.79	0.19–1.81	0.30–5.66	0.53–4.00	0.19–2.86
RET/PTC1 expression level							
Odds ratio	n/a	n/a	n/a	n/a	n/a	n/a	n/a
95% CI							
$BRAF^{V600E}$ mutation							
Odds ratio	3.98	0.52	2.09	1.68	1.27	0.66	0.89
95% CI	1.66–9.54	0.24–1.15	0.95–4.60	0.70–3.99	0.36–4.56	0.28–1.57	0.381–2.60
BRAF expression level							
Odds ratio	1.00	12.75	0.99	0.98	1.08	0.92	0.99
95% CI	0.99–1.01	0.13–1285.32	0.98–1.01	0.94–1.04	0.95–1.24	0.82–1.57	0.90–1.08
cRET expression level							
Odds ratio	1.00	0.01	1.00	1.00	0.94	1.01	0.99
95% CI	0.99–1.00	0.00–1.45	0.99–1.01	0.99–1.01	0.82–1.08	0.99–1.03	0.95–1.03

n/a not analysed. Odds ratio analysis was not performed given that there were not enough positive cases to determine a cutoff point

Considering that the presence of both *BRAFV600E* mutation and *RET/PTC1* was found in 10% of our cohort, it is important to highlight that, although evidence that dual mutations can rarely occur in well differentiated PTC [4], the description of concomitant events within the same tumour has been previously reported [5, 6]. Remarkably, while genetic alterations involving the MAPK pathway (e.g. *BRAFV600E* and *RET/PTC*) have been considered as mutually exclusive [7, 8], it has been proven that both mutations can be present at different cells within the same tumour, supporting the concept that, although monoclonal, tumours are heterogeneous [9].

The utility of *BRAFV600E* and *RET/PTC1* as biomarkers to identify patients with high risk of aggressive PTC remains controversial. While some studies have reported an association of *BRAFV600E* with ETE, LNM, a poor prognosis or an increased risk of recurrence [7, 12–15], this association has not been reproduced by others [16–20]. In recent years, it has been reported that in patients with the *BRAFV600E* mutation, the coexistence with the *TERT* promoter mutation is associated with a poorer prognosis of PTC when compared to patients presenting one or other mutation [10, 42, 43]. Thus, we explored if classic PTC molecular markers in combination with demographic variables could predict histopathological prognostic factors. In our study, we did not find associations between *BRAFV600E* and/or *RET/PTC1* and the histopathological prognostic factors by MVA. However, by CBVA, which is part of cluster analysis algorithms to group data by patterns [44], we were able to find and identify several subsets of patients with molecular profiles associated with relevant histopathological prognostic factors. The group with a 'high activity' profile of *BRAF* (presence of *BRAFV600E* mutation or high *BRAF* mRNA expression) and a 'low activity' profile of *RET* (absence of *RET/PTC1* or low *RET* expression) was associated with the presence of ETE (OR 2.74 and 4.48, $p < 0.05$) (Fig. 1). A second group with a 'low activity' profile of *BRAF* (absence of *BRAFV600E* or low *BRAF* expression) and a 'high activity' profile of *RET* (presence of *RET/PTC1* or high *RET* expression) was strongly associated to LNM (OR 3.95, 7.88 and 12.8, $p < 0.05$) (Fig. 1).

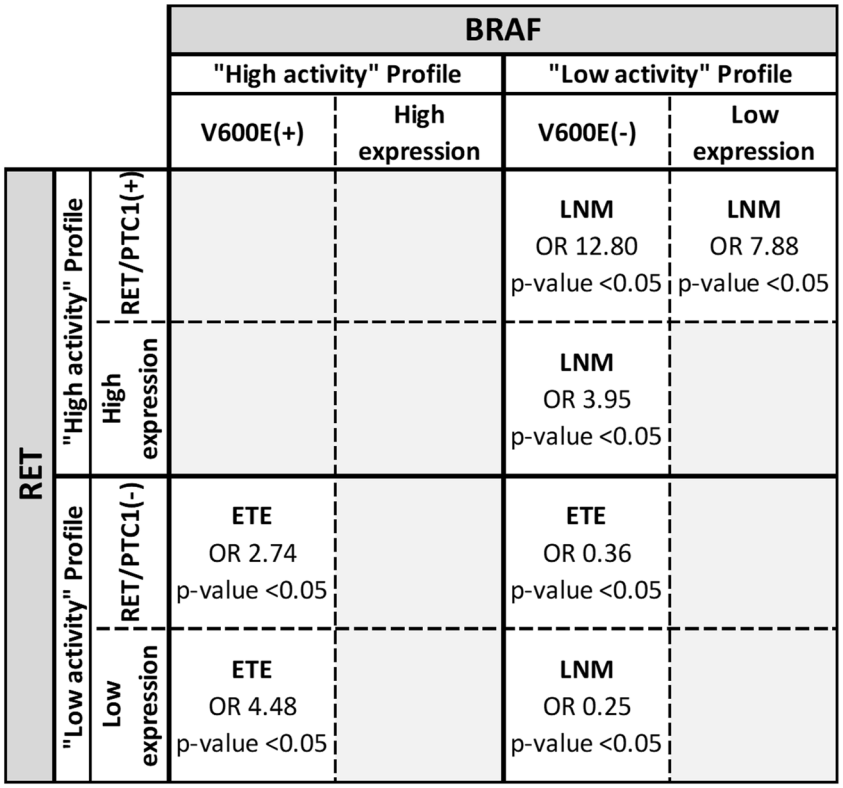

Fig. 1 Significative associations between histopathological prognostic factors and differential activity profiles of BRAF and RET. By bivariate analysis, several associations between activity profiles of BRAF and RET, and histopathological prognostic factors were found. A 'high activity' profile of BRAF and a 'low activity' profile of RET is associated with the presence of extrathyroidal extension (ETE). A 'low activity' profile of BRAF and a 'high activity' profile of RET is strongly associated with lymph node metastasis (LNM). A 'low activity' profile for both, BRAF and RET, is associated with the absence of ETE and LNM

Interestingly, when 'high activity' profile was detected for both (*BRAF* and *RET*), no significant associations were found, whereas a 'low activity' profile for both markers showed a significant association with the absence of LMN and ETE. This phenomenon may be due to the activation of different signalling pathways by RET and BRAF. Although RET and BRAF share the RAS/ERK/MAPK cascade, RET is also part of the Src signalling pathway [45]. In fact, while the RAS/ERK/MAPK signalling pathway is involved in cell differentiation and proliferation (processes associated with ETE) [46, 47], Src is associated with cellular migration and metastasis [48, 49].

The impact of age on the overall prognosis of PTC is well-known [35], and LNM is more frequent in patients less than 18 years old. However, the association of sex and age to specific histopathological prognostic factors in patients greater than 18 years remains controversial. By CBVA, our results showed that in patients under 55 years old, a low *BRAF* expression was strongly associated with LNM (OR 17.65, $p < 0.05$), while in this same group, the presence of the *BRAFV600E* mutation was associated with ETE (OR 2.76, $p < 0.05$). Previous studies have suggested that demographic data may help to stratify patients according to diverse tumour genetics. In fact, a recent study of Shen et al. reported that the age-associated mortality risk in PTC depends on *BRAF* status. In patients > 45 years old, the presence of *BRAFV600E* mutations was strongly associated with an increased risk of mortality, while not increased mortality was associated with wild-type *BRAF* patients [50]. Taken together, these data suggest that the combined use of demographic data and molecular markers, such as *BRAFV600E* and *RET/PTC1*, could be considered in assessing the risk stratification and management of patients with PTC.

Here, we report the identification of subsets of patients with aggressive histopathological prognostic factors of PTC by the combined use of demographic and molecular data. However, this study presented some limitations. First, since not all patients had lymph node dissections, there is a potential bias of underestimating lymph node metastasis. Another limitation is that we did not analyse the TERT promoter mutations given its low prevalence and limited prognostic utility in PTC [51, 52]. Nevertheless, we do not exclude performing a future study to further analyse the TERT mutation prognostic value in the identification of subsets of patients with aggressive PTC in combination with demographic or other mutations by CBVA. Finally, recurrence and mortality were not communicated given that the follow-up was incomplete in some patients. However, our cohort has an intrinsic therapeutic effect (since patients with cN0 did not undergo lymph node dissection and those with cN1 were all submitted to therapeutic dissection). Therefore, it would be necessary to evaluate two separate cohorts (i.e. patients with and without lymph

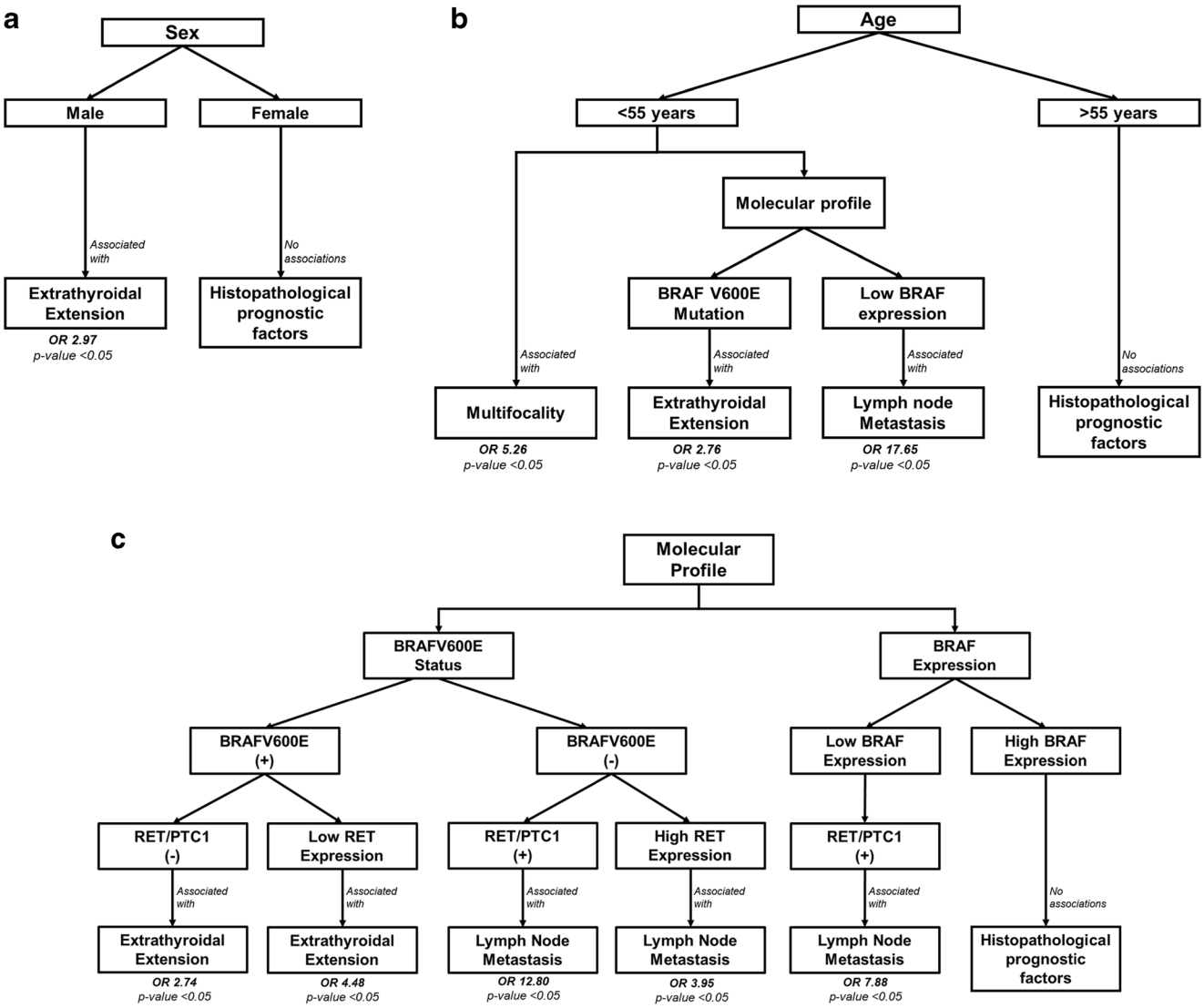

Fig. 2 Decision trees. Several decision trees were generated considering the most significant results obtained in this study. **a** Sex-based decision tree. **b** Age-based decision tree. c BRAF and RET-based decision trees

dissection) to properly assess the value of the associations reported in this study.

In summary, our results suggest that use of *BRAF*, *BRAFV600E*, *RET*, *RET/PTC1* and their combination with demographic variables may help identify patients with greater risk for aggressive histopathological characteristics in PTC such as extrathyroidal extension and lymph node metastases. Further validation studies are needed to determine if these combined demographic and molecular profiles are useful to help surgeons to preoperatively tailor surgical planning in patients with clinically apparent non-aggressive PTC (T1-T2 - N0).

Acknowledgments The authors acknowledge the grant from the Biomedical Research Consortium (BMRC), grant no. 13CTI-21526 P2.

Compliance with Ethical Standards

The study was approved by the Ethics Committee of the Pontificia Universidad Católica de Chile and all patients included signed an informed consent.

Conflict of Interest The authors declare that they have no conflict of interest.

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