Quantification of *BRAF* V600E alleles predicts papillary thyroid cancer progression

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Abstract

The *BRAF* V600E mutation is the most common genetic alteration in thyroid cancer. However, its clinicopathological significance and clonal mutation frequency remain unclear. To clarify the inconsistent results, we investigated the association between the allelic frequency of *BRAF* V600E and the clinicopathological features of classic papillary thyroid carcinoma (PTC). Tumour tissues from two independent sets of patients with classic PTC were manually microdissected and analysed for the presence or absence of the *BRAF* mutation and the mutant allelic frequency using quantitative pyrosequencing. For external validation, the Cancer Genome Atlas (TCGA) data were analysed. The *BRAF* V600E mutation was found in 264 (82.2%) out of 321 classic PTCs in the training set. The presence of *BRAF* V600E was only associated with extrathyroidal extension and the absence of thyroiditis. In *BRAF* V600E-positive tumours, the mutant allelic frequency varied from 8 to 41% of the total *BRAF* alleles (median, 20%) and directly correlated with tumour size and the number of metastatic lymph nodes. Lymph node metastases were more frequent in PTCs with a high ($\geq 20\%$) abundance of mutant alleles than in those with a low abundance of mutant alleles ($P=0.010$). These results were reinforced by validation dataset ($n=348$) analysis but were not reproduced in the TCGA dataset. In a population with prevalent *BRAF* mutations, quantitative analysis of the *BRAF* mutation could provide additional information regarding tumour behaviour, which is not reflected by qualitative analysis. Nonetheless, prospective studies are needed before the mutated allele percentage can be considered as a prognostic factor.

Key Words

- ▶ papillary thyroid carcinoma
- ▶ proto-oncogene
- ▶ proteins *BRAF*
- ▶ mutation

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Introduction

The V600E *BRAF* mutation is the most common genetic alteration in papillary thyroid carcinoma (PTC). The prevalence of the *BRAF* V600E mutation varies according to the histological subtype; the mutation is frequent in

the tall cell variant and classic PTC, but absent in the cribriform-morular variant (Trovisco *et al.* 2005). The clinical importance of the *BRAF* mutation derives from its role as a prognostic factor. Recently, mortality

has been related to *BRAF* mutation status, which had previously been considered supplemental information to conventional prognostic factors (Xing et al. 2013). Considering the availability of current targeted therapies that include a *BRAF* inhibitor, the identification of the *BRAF* mutation may also be a good candidate marker for the prediction of response to targeted therapies. Nevertheless, there remains controversy surrounding the prognostic role of the *BRAF* mutation, as multiple studies have failed to illustrate a prognostic role for the *BRAF* mutation (Kim et al. 2005, Cheng et al. 2011, Eloy et al. 2011, Nam et al. 2012, Paulson et al. 2012, Sassolas et al. 2012).

In the studies mentioned above, the *BRAF* mutation was evaluated in a qualitative manner. The presence of the *BRAF* mutation in tissues was considered important in the analysis. However, the subclonality of the *BRAF* mutation in PTCs has recently been suggested (Guerra et al. 2012a). In this context, the quantification of the mutated allele was emphasised in terms of predicting prognosis (Guerra et al. 2012b); however, several studies have shown results contradictory to those findings, as determined by the quantification of mutated alleles (Gandolfi et al. 2013) and immunohistochemical (IHC) staining (Ghossein et al. 2013). The proper value of the quantification of the mutated allele remains in debate and requires further evaluation.

In addition, there is a large variation in the prevalence of the *BRAF* mutation in PTCs between countries. Generally, the Asian population (especially in Koreans) exhibits up to an 86% incidence rate for the *BRAF* mutation compared with a rate that is typically below 60% in other populations (Chung et al. 2006, Lee et al. 2007, Jung et al. 2010, Xing et al. 2013). In this group, no quantification of mutated allele was evaluated.

Thus, in this study, the implication of quantifying the *BRAF* mutation will be evaluated in a population in which the mutation is highly prevalent.

Materials and methods

Patients and specimens

We retrospectively enrolled two independent sets comprising 670 PTC patients who underwent surgery at Seoul St Mary's Hospital between 2012 and 2014. Classic PTCs were only selected to minimise the bias due to the inclusion of different tumour types. Classic PTCs with tall cell features were excluded. The training set comprised 322 consecutive patients with classic PTC who underwent

surgery between February 2012 and January 2013. The independent validation dataset was obtained from a separate cohort of 348 consecutive classic PTC patients (who underwent surgery between February 2013 and December 2013) with the same enrolment criteria. We excluded classic PTCs with marked intratumoural lymphocytic infiltration or densely sclerotic stroma. One representative formalin-fixed and paraffin-embedded tissue block of classic PTCs was selected from each case by a pathologist after reviewing the haematoxylin and eosin-stained slides. In cases with multifocal PTC foci, the largest tumours were chosen for the study. All specimens were collected in accordance with the institutional protocols of our hospital.

Genomic DNA extraction from tumour tissue

Briefly, paraffin sections of 5-µm thickness were heated for 60 min at 57 °C, deparaffinised in xylene, rehydrated in decreasing concentrations of alcohols and air dried. Tumour areas were manually microdissected under a stereomicroscope to exclude possible contamination by the surrounding normal tissue. Genomic DNA was extracted from the microdissected tumour tissue using the QIAamp DNA FFPE Tissue kit (Qiagen).

PCR

A 98-bp fragment of exon 15 of the *BRAF* gene was amplified through PCR using the PyroMark PCR kit (Qiagen). The PCR mixtures included 12.5 µl of PyroMark PCR Master Mix (Qiagen), 2.5 µl of CoralLoad Concentrate (Qiagen), 5 µl of Q-solution, 0.5 µl of 10 µM forward primer (5'-biotin-TTCTTCATGAAGACCTCAGTAA-3'), 0.5 µl of 10 µM reverse primer (5'-CCAGACAACCTGTTCAAACCTGATG-3'), and 4 µl of 30 ng/ul genomic DNA in a final reaction volume of 25 µl. The PCR conditions were as follows: an initial activation at 95 °C for 15 min; 42 cycles of 30 s at 95 °C, 30 s at 53 °C and 30 s at 72 °C and a final extension at 72 °C for 10 min. The amplicons were evaluated through 2% agarose gel electrophoresis.

Pyrosequencing

After PCR, biotinylated PCR products were immobilised on Streptavidin Sepharose High Performance beads (GE Healthcare, Piscataway, NJ, USA). The reaction mixture for DNA immobilisation consisted of 2 µl of Streptavidin Sepharose High Performance beads, 40 µl of PyroMark

Binding Buffer (Qiagen), 28 µl of water (Qiagen), and 10 µl of PCR product in a final reaction volume of 80 µl. Single-stranded DNA for pyrosequencing was prepared on the PyroMark Q24 Vacuum Workstation (Qiagen) according to the manufacturer's instructions. Subsequently, the single-stranded DNA was annealed to the sequencing primer (5'-CCACTCCATCGAGATT-3') in a reaction mixture containing 0.8 µl of sequencing primer and 24.2 µl of PyroMark annealing buffer through a 2-min incubation at 80 °C. The sequencing primer was designed to include an analysis of codons 599–601 of *BRAF*. Pyrosequencing was performed using PyroMark Gold Q24 reagents (Qiagen) on a PyroMark Q24 system (Qiagen), and the data were analysed using the PyroMark Q24 software, version 2.0.6 (Qiagen). The cut-off value was calculated as the mean percentage plus 3s.d.s of the 60 normal controls, plus 3%. The *BRAF* mutation status was considered positive if the mutant allele percentage was 8% or more.

Statistical analysis

All statistical analyses were performed using the statistical package SPSS (SPSS, Inc.). Categorical variables are presented as the numbers and percentages in the table. Continuous variables are presented as their median and range. For the comparison of categorical parameters, the χ^2 -test was applied. For comparisons of continuous variables, the Mann–Whitney *U* tests and *t*-tests were applied for variables without a normal distribution and variables with a normal distribution respectively. The relationship between continuous variables was evaluated using a bivariate correlation. For the multivariate analysis, parameters that showed marginal significance (*P* values <0.25) were included in a binary logistic regression test.

Results

Baseline characteristics of the study population

The patient demographics and clinicopathological features are summarised in Table 1. In the training set, females comprised 76.1% of the study population, and the median age at diagnosis was 46 years. All *BRAF* mutations were the typical *BRAF* V600E. Based on the cut-off value of 8%, *BRAF* V600E was found in 265 (82.3%) out of 322 PTC samples and 188 (83.9%) out of 224 papillary thyroid microcarcinoma (PTMC) cases, which were defined by a size of 1 cm or less. There were no significant differences

The Cancer Genome Atlas dataset

For external validation, we investigated 500 thyroid cancer patients from The Cancer Genome Atlas (TCGA) dataset (<http://cancergenome.nih.gov/> and <http://www.cbioportal.org/>); next-generation sequenced DNA mutation data were available for analysis for 400 of these PTC patients. Pathological data were collected from CBioPortal for Cancer Genomics (<http://www.cbioportal.org/>).

Table 1 Baseline patient characteristics and clinicopathological features of *BRAF* mutation-positive and -negative classic papillary thyroid carcinoma in the training and validation sets

	Training set				Validation set			
	Patients	<i>BRAF</i> negative	<i>BRAF</i> positive	<i>P</i> value	Patients	<i>BRAF</i> negative	<i>BRAF</i> positive	<i>P</i> value
Total	322	57	265		348	63	285	
Females	245 (76.1%)	44 (77.2%)	201 (75.8%)	0.829	277 (79.6%)	51 (81%)	226 (79.3%)	0.768
Age (years) ^a	46 (13–77)	47 (23–77)	46 (13–76)	0.554	47 (17–83)	49 (17–75)	46 (18–83)	0.627
Tumour size (mm) ^a	7 (3–46)	8 (3–45)	7 (3–46)	0.884	6 (3–54)	6 (3–38)	6 (3–54)	0.583
PTMC	224 (69.6%)	36 (63.2%)	188 (70.9%)	0.247	275 (79%)	51 (81.0%)	224 (78.6%)	0.678
Multifocal	157 (48.8%)	28 (49.1%)	129 (48.7%)	0.952	136 (39.1%)	23 (36.5%)	113 (39.6%)	0.644
Extrathyroidal extension	139 (43.2%)	16 (28.1%)	123 (46.3%)	0.011	154 (44.1%)	20 (31.7%)	134 (47.0%)	0.027
pT3–4 stage	140 (43.5%)	16 (28.1%)	124 (46.8%)	0.010	150 (43.1%)	19 (30.2%)	131 (46.0%)	0.022
Lymphatic invasion	89 (27.6%)	17 (29.8%)	72 (27.2%)	0.684	95 (27.3%)	15 (23.8%)	80 (28.1%)	0.492
Vascular invasion	10 (3.1%)	3 (5.3%)	7 (2.7%)	0.303	5 (1.4%)	1 (1.6%)	4 (1.4%)	1.000
Thyroiditis	87 (27.0%)	24 (42.1%)	63 (23.8%)	0.005	105 (30.2%)	27 (42.9%)	78 (27.4%)	0.015
LN metastasis	161 (50.0%)	23 (41.8%)	138 (54.1%)	0.098	179 (51.4%)	26 (41.3%)	153 (53.7%)	0.074
Lateral LN metastasis	54 (16.8%)	12 (21.8%)	42 (16.5%)	0.343	39 (11.2%)	6 (9.5%)	33 (11.6%)	0.640

PTMC, papillary thyroid microcarcinoma (≤ 1 cm); LN, lymph node. Bold indicates statistical significance, $P < 0.05$.

^aThese parameters are presented as the median (range). A Mann–Whitney *U* test was used for comparison between two groups.

Table 2 Distribution of mutated *BRAF* allele percentages in the papillary thyroid carcinoma

Study	No. of cases	Histological subtypes	BRAF mutation	BRAF V600E allele percentage				Analysis method
				Min (%)	Max (%)	Mean (%)	Median (%)	
Guerra et al. (2012a,b)	168	Classic (n=147), follicular variant (n=21)	90 (53.6%)	5.1	44.7	21.9	20.6	Pyrosequencing
Gandolfi et al. (2013)	132	Classic (n=64), tall cell variant (n=38), follicular variant (n=21), diffuse sclerosing (n=2), solid (n=2), microcarcinoma (n=5)	58 (43.9%)	7.5	49.8	27.4	NA	Pyrosequencing
Nikiforova et al. (2013)	57	Classic (n=27), follicular variant (n=30)	21 (36%)	18.0	44.0	34.0	NA	NGS
de Biase et al. (2014)	155	85 BRAF V600E-positive tumours: classic (n=45), follicular variant (n=15), tall cell variant (n=25)	85 (54.8%)	2	53.6	33.8	32.5	Allele-specific locked nucleic acid PCR
TCGA	400	All subtypes ^a	247 (61.8%) ^b	4	57	33.6	35	NGS
		Classic (n=108)	83 (76.9%)	4	54	35.1	36	
		With thyroiditis (n=45)	32 (71%)	14	51	31.7	34	
Present study	322	Classic (n=322)	265 (82.3%)	8	41	20.3	20	Pyrosequencing
		With thyroiditis (n=87)	63 (72.4%)	8	36	16.2	15	
		Without thyroiditis (n=235)	202 (86.0%)	8	41	21.6	21	
Validation set	348	Classic (n=348)	285 (81.9%)	8	42	20.2	20	
		With thyroiditis (n=105)	78 (74.3%)	8	34	17.1	15	
		Without thyroiditis (n=243)	207 (85.2%)	8	42	21.4	22	

TCGA, The Cancer Genome Atlas (<http://cancergenome.nih.gov/>); NGS, next-generation sequencing; NA, not assessed.

^aClassic (n=108), classic with tall cell features (n=10), follicular (n=115), tall cell (n=25), oncocyctic (n=8), diffuse sclerosing (n=2), columnar cell (n=2), microcarcinoma (n=2), Warthin-like (n=2), Cribriform (n=2) variants, and NA (n=124).

^bOut of 247 papillary carcinomas with *BRAF* mutation, 245 (99.2%) have *BRAF* V600E mutation and two have *BRAF* fusion mutation.

in the baseline characteristics between the training and validation sets.

Approach to the status of *BRAF* mutation by conventional methods (whether positive or negative)

There was no association between *BRAF* mutational status and either age at diagnosis, tumour size or multifocality in the training and validation sets. The presence of extrathyroidal extension, a higher incidence of pT3–4 and the absence of chronic lymphocytic thyroiditis were significantly associated with *BRAF* in two independent sets (Table 1).

Distribution of mutated *BRAF* allele percentage

In only the *BRAF*-positive tumours, the median for the frequency of allele mutation was 20% (range 8–41%) and 20% (range 8–42%), respectively, in the training and validation sets. PTCs with thyroiditis had a lower percentage of the mutated allele than those without thyroiditis. We compared our results with previously published data and the results obtained from the

TCGA dataset. A detailed distribution of the allele mutation percentages is summarised in Table 2. Studies using next-generation sequencing (NGS) technology for *BRAF* mutation identification showed higher mutant allele percentages than those using pyrosequencing.

Impact of *BRAF* V600E allele percentage on clinicopathological features

When the study population was categorised into *BRAF* mutation negative (mutated allele frequency <8%) and low allele-mutation (allele frequency 8–19%) and high allele-mutation (allele frequency ≥20%) groups (based on the cut-off value derived from the median value of studied subjects), there was no significant difference in the clinicopathological features between the negative and low allele-mutation groups in two independent sets (Tables 3 and 4). However, the high allele-mutation group had a larger tumour size and a higher incidence of extrathyroidal extension, pT3–4 and lymph node metastasis than the negative and low allele-mutation groups (Table 3). These results were also consistent with those from the validation and total sets (Tables 4 and 5).

Table 3 Comparison of clinicopathological features according to the percentage of mutated *BRAF* alleles in the training set ($n=322$)

	Negative (A) ($n=57$)	Low (B) ($n=128$)	High (C) ($n=137$)	Comparison group	P value
Allele frequency ^a		15% (8–19)	25% (20–41)		
Females	44 (77.2%)	97 (76.4%)	104 (7.59%)	A vs B	0.835
				A vs C	0.849
				B vs C	0.980
Age (years) ^a	47 (23–77)	47 (21–75)	42 (13–76)	A vs B	0.963
				A vs C	0.270
				B vs C	0.173
Tumour size (mm) ^a	8 (3–45)	6 (3–40)	8 (3–46)	A vs B	0.057
				A vs C	0.126
				B vs C	<0.001
Multifocality	28 (49.1%)	67 (52.3%)	62 (45.3%)	A vs B	0.686
				A vs C	0.623
				B vs C	0.249
Extrathyroidal extension	16 (28.1%)	47 (36.7%)	76 (55.5%)	A vs B	0.252
				A vs C	<0.001
				B vs C	0.002
pT3–4 stage	16 (28.1%)	47 (36.7%)	77 (56.2%)	A vs B	0.252
				A vs C	<0.001
				B vs C	0.001
Lymphatic invasion	17 (29.8%)	28 (21.9%)	44 (32.1%)	A vs B	0.245
				A vs C	0.754
				B vs C	0.061
Vascular invasion	3 (0.5%)	1 (0.1%)	6 (4.4%)	A vs B	0.088
				A vs C	0.725
				B vs C	0.121
Thyroiditis	24 (42.1%)	47 (36.7%)	16 (11.7%)	A vs B	0.487
				A vs C	<0.001
				B vs C	<0.001
LN metastasis	23 (40.4%)	58 (45.3%)	80 (58.4%)	A vs B	0.530
				A vs C	0.022
				B vs C	0.010
Lateral LN metastasis	13 (22.8%)	15 (11.7%)	28 (20.4%)	A vs B	0.052
				A vs C	0.713
				B vs C	0.054
Number of metastatic LN ^a	0 (0–35)	0 (0–22)	1 (0–41)	A vs B	0.820
				A vs C	0.055
				B vs C	0.003

LN, lymph node; negative, <8%; low, 8–19%; high, 20–50%. Bold indicates statistical significance, $P<0.05$.^aThese parameters are presented as the median (range). A Mann–Whitney *U* test was used for comparison between two groups.

Correlation between mutated allele frequency and tumour progression

Among the tumours with the *BRAF* V600E mutation, the frequency of mutated alleles directly correlated with the tumour size ($P<0.001$, Fig. 1a and $P<0.001$, Fig. 1b respectively) and the number of metastatic lymph nodes ($P<0.001$, Fig. 2a and $P<0.001$, Fig. 2b respectively) in the training and validation sets. After the exclusion of PTMC, the association between the size of the tumours ($R^2=0.141$, $P=0.001$ and $R^2=0.125$, $P=0.005$ respectively) or the number of metastatic lymph nodes ($R^2=0.083$, $P=0.012$ and $R^2=0.020$, $P=0.272$ respectively) with the percentage of mutated alleles remained significant in the training and validation sets. Excluding

the population with concomitant chronic lymphocytic thyroiditis, the size of the tumour ($R^2=0.219$, $P<0.001$ and $R^2=0.163$, $P<0.001$ respectively) or the number of lymph node metastases ($R^2=0.070$, $P<0.001$ and $R^2=0.042$, $P=0.003$ respectively) was significantly related to the percentage of mutated alleles in the training and validation sets.

Qualitative and quantitative determination of *BRAF* mutational status in PTMC

There was a significant relationship between *BRAF* mutational status and extrathyroidal extension and the absence of chronic lymphocytic thyroiditis in PTMCs of

Table 4 Comparison of clinicopathological features among three study groups according to the percentage of mutated *BRAF* alleles in the validation set ($n=348$)

	Negative (A) ($n=63$)	Low (B) ($n=135$)	High (C) ($n=150$)	Comparison group	P value
Allele frequency ^a		13 (8–19)	26 (20–42)		
Females	51 (81.0%)	113 (83.7%)	113 (75.3%)	A vs B	0.633
				A vs C	0.374
				B vs C	0.082
Age (years) ^a	49 (17–75)	48 (18–75)	44 (18–83)	A vs B	0.688
				A vs C	0.221
				B vs C	0.036
Tumour size (mm) ^a	6 (3–38)	5 (3–40)	7 (3–54)	A vs B	0.119
				A vs C	0.017
				B vs C	<0.001
Multifocality	23 (36.5%)	46 (34.1%)	67 (44.7%)	A vs B	0.738
				A vs C	0.271
				B vs C	0.068
Extrathyroidal extension	20 (31.7%)	53 (39.3%)	81 (54.0%)	A vs B	0.307
				A vs C	0.003
				B vs C	0.013
pT3–4 stage	19 (30.2%)	51 (37.8%)	80 (53.3%)	A vs B	0.296
				A vs C	0.002
				B vs C	0.009
Lymphatic invasion	15 (23.8%)	32 (23.7%)	48 (32%)	A vs B	0.987
				A vs C	0.232
				B vs C	0.120
Vascular invasion	1 (1.6%)	0 (0%)	4 (2.7%)	A vs B	0.318
				A vs C	1.000
				B vs C	0.124
Thyroiditis	27 (42.9%)	49 (36.3%)	29 (19.3%)	A vs B	0.377
				A vs C	<0.001
				B vs C	0.001
LN metastasis	26 (41.3%)	64 (47.4%)	89 (59.3%)	A vs B	0.419
				A vs C	0.016
				B vs C	0.044
Lateral LN metastasis	6 (9.5%)	14 (10.4%)	19 (12.7%)	A vs B	0.854
				A vs C	0.515
				B vs C	0.545
Number of metastatic LN ^a	0 (0–21)	0 (0–29)	1 (0–23)	A vs B	0.685
				A vs C	0.025
				B vs C	0.009

LN, lymph node; negative, <8%; low, 8–19%; high, 20–50%. Bold indicates statistical significance, $P<0.05$.

^aThese parameters are presented as the median (range). A Mann–Whitney U test was used for comparison between two groups.

the training set (Supplementary Table 1, see section on supplementary data given at the end of this article). Among the PTMCs with *BRAF* mutation, the high allele-mutation group was significantly associated with a larger tumour size as well as the presence of extrathyroidal extension and the absence of thyroiditis (Supplementary Table 2). However, there was no significant relationship between the mutated allele percentages and lymph node metastasis (Supplementary Table 2).

Multivariate analysis of lymph node metastasis prediction

In the univariate analysis, lymph node metastasis was significantly associated with the size of the tumour, patient age, a high percentage of a mutated allele, tumour

multifocality, extrathyroidal extension and lymphatic invasion. However, in the multivariate analysis, the significance of multifocality and the percentage of mutated alleles were lost in the training and validation sets (Table 6).

TCGA validation set

Of the 400 PTC patients available for DNA sequencing data in TCGA, we selected only 108 patients with classic PTC to analyse the relationship between pathological features and *BRAF* mutation status (Table 7). *BRAF* positivity was correlated with the presence of extrathyroidal extension ($P=0.001$) and pT3–4 stage ($P=0.007$) but not with other factors. We further categorised 81 patients with *BRAF*

Table 5 Comparison of clinicopathological features among three study groups according to the percentage of mutated *BRAF* alleles in the total set ($n=670$)

	Negative (A) ($n=120$)	Low (B) ($n=263$)	High (C) ($n=287$)	Comparison group	P value
Allele frequency ^a		14 (8–19)	26 (20–42)		
Females	95 (79.2%)	210 (79.8%)	217 (75.6%)	A vs B	0.878
				A vs C	0.439
				B vs C	0.233
Age (years) ^a	47 (17–77)	48 (18–75)	43 (13–83)	A vs B	0.748
				A vs C	0.093
				B vs C	0.013
Tumour size (mm) ^a	7 (3–45)	6 (3–40)	8 (3–54)	A vs B	0.015
				A vs C	0.007
				B vs C	<0.001
Multifocality	51 (42.5%)	113 (43.0%)	129 (44.9%)	A vs B	0.932
				A vs C	0.650
				B vs C	0.640
Extrathyroidal extension	36 (30%)	100 (38.0%)	157 (54.7%)	A vs B	0.128
				A vs C	<0.001
				B vs C	<0.001
pT3–4 stage	35 (29.2%)	98 (37.3%)	157 (54.7%)	A vs B	0.123
				A vs C	<0.001
				B vs C	<0.001
Lymphatic invasion	32 (26.7%)	60 (22.8%)	92 (32.1%)	A vs B	0.413
				A vs C	0.281
				B vs C	0.015
Vascular invasion	4 (3.3%)	1 (0.4%)	10 (3.5%)	A vs B	0.035
				A vs C	1.000
				B vs C	0.009
Thyroiditis	51 (42.5%)	96 (36.5%)	45 (15.7%)	A vs B	0.263
				A vs C	<0.001
				B vs C	<0.001
LN metastasis	49 (40.8%)	122 (46.4%)	169 (58.9%)	A vs B	0.310
				A vs C	0.001
				B vs C	0.003
Lateral LN metastasis	18 (15%)	28 (10.6%)	47 (16.4%)	A vs B	0.188
				A vs C	0.892
				B vs C	0.069
Number of metastatic LN ^a	0 (0–35)	0 (0–29)	1 (0–41)	A vs B	0.876
				A vs C	0.003
				B vs C	<0.001

LN, lymph node; negative, <8%; low, 8–19%; high, 20–50%. Bold indicates statistical significance, $P<0.05$.

^aThese parameters are presented as the median (range). A Mann–Whitney *U* test was used for comparison between two groups.

V600E into low allele-mutation (allele frequency 4–35%) and high allele-mutation (allele frequency $\geq 36\%$) groups (based on the cut-off value derived from the median value of studied subjects). In the semi-quantitative analysis, the increase in mutated allele frequency was significantly associated with extrathyroidal extension and high pT stage. However, there was no correlation between the frequency of mutated alleles and tumour size or lymph node metastases.

Discussion

In this study, we evaluated the clinical implication of a quantitative approach of describing *BRAF* mutations (the percentage of mutated alleles) in a population with

a high *BRAF* mutation prevalence. We found that the quantitative analysis of *BRAF* mutation provided additional information regarding tumour behaviour that was not reflected by a qualitative analysis in both the training and validation sets. In addition, a substantial portion of PTC cases showed intratumoural heterogeneity in terms of the *BRAF* mutation.

In our training set, 82.3% of the population conventionally showed the *BRAF* mutation, which is consistent with previous Korean studies (Chung et al. 2006, Kim et al. 2009, Jung et al. 2010). The *BRAF* positivity rate appears to be higher in our study than in other studies performing a quantitative analysis of *BRAF* mutations (Guerra et al. 2012a,b, Gandolfi et al. 2013). As the incidence of *BRAF* mutation in PTCs varies

Figure 1

Association between the percentage of mutant alleles of *BRAF* V600E and tumour size in the training (a) and validation (b) sets.

depending on the histological subtype, such a difference in *BRAF* mutation positivity might derive from the histological subtypes included in the analysis. Previous studies have included follicular variants of PTC as well as classic variants, but we only selected classic PTCs and excluded any other histological subtype such as follicular variant, tall cell variant and classic PTCs with tall cell features. This may partly explain the lower incidence of *BRAF* mutations in western populations. In the TCGA dataset, the mutation rate was 76.9% for classic PTCs and 61.8% for all PTCs.

Using a conventional approach, there was no significant association of *BRAF* positivity with clinical/pathological parameters other than the presence of extrathyroidal extension and the absence of thyroiditis. The association of *BRAF* mutation with the presence of

extrathyroidal extension and the absence of thyroiditis (Kim et al. 2012, Nam et al. 2012) and the absence of thyroiditis (Sargent et al. 2006, Kim et al. 2009) was concordant with previous studies. By contrast, taking a quantitative approach to describe the *BRAF*-positive population revealed that groups with a high percentage of allelic mutation were closely related to poor prognostic parameters (such as tumour size, extrathyroidal extension, lymph node metastasis and the number of metastatic lymph nodes) compared with low mutation-percentage groups. A low percentage of allelic mutation was related to a high incidence of thyroiditis. PTCs with a low *BRAF* mutation-percentage had a clinical behaviour similar to that of *BRAF*-negative tumours. It is possible that there was a high prevalence of patients with a low percentage of mutant alleles in previous studies that described a lack of a relationship between *BRAF* positivity and unfavourable

Figure 2

Association between the percentage of mutant alleles of *BRAF* V600E and the number of metastatic lymph nodes in the training (a) and validation (b) sets.

Table 6 Multivariate logistic regression analysis for predicting lymph node metastasis in the training and validation sets of patients with classic papillary thyroid carcinoma

Parameters	Training set			Validation set		
	HR	95% CI	P-value	HR	95% CI	P-value
Tumour size	1.052	1.013–1.093	0.009	1.099	1.041–1.160	0.001
Age above 60	0.972	0.950–0.995	0.018	0.950	0.929–0.971	<0.001
High frequency of <i>BRAF</i> V600E allele	1.251	0.712–2.197	0.437	1.064	0.634–1.786	0.815
Multifocality	1.464	0.843–2.543	0.175	1.890	1.119–3.191	0.017
Extrathyroidal extension	2.770	1.541–4.978	0.001	2.244	1.312–3.839	0.003
Lymphatic invasion	8.647	3.933–19.008	<0.001	3.620	1.907–6.872	<0.001

HR, hazard ratio. Bold indicates statistical significance, $P < 0.05$.

parameters. Analysis of the percentage of mutated alleles in a population with prevalent *BRAF* mutations may provide clues to explain the discordant role of *BRAF* mutations in determining patient prognosis.

Intratumoural heterogeneity has been recognised since Nowell (1976) suggested that tumour heterogeneity could be acquired during progression. Several methods have been applied to detect intratumoural heterogeneity such as IHC staining, fluorescence *in situ* hybridisation, microarray analysis or DNA sequencing. In various cancers, intratumoural heterogeneity has already been demonstrated and has been related to patient prognosis or treatment response (Taniguchi et al. 2008, Gerlinger et al. 2012, Seol et al. 2012, Anaka et al. 2013). In our study, the *BRAF* mutation status was also found to be heterogeneous via quantitative pyrosequencing. Recently, the efficacy of several tyrosine kinase inhibitors, especially those targeting *BRAF* kinase activity, has been investigated in refractory thyroid carcinomas. The quantification of the

intratumoural heterogeneity of *BRAF* mutations would be useful to predict the response to such therapies.

The correlation between the percentage of allele mutation and the tumour size appears significant in terms of evaluating the role of the *BRAF* mutation in carcinogenesis. As suggested by the heterogeneity of RET/PTC rearrangements in post-Chernobyl PTCs (Unger et al. 2004), the heterogeneity of the *BRAF* mutation in PTCs could be caused by *BRAF* mutation development in tumour initiation with a later emergence of clones without mutation or by selection for cells harbouring the *BRAF* mutation during tumour progression. Considering the relationship between the percentage of mutated alleles and tumour size, the latter hypothesis based on the clonal evolution theory (Nowell 1976) appears plausible. This hypothesis is supported by several studies that analysed the status of the *BRAF* mutation in both primary lesions and metastases (Oler et al. 2005, Vasko et al. 2005). However, the issue of *BRAF* mutational heterogeneity and clonal evolution in thyroid cancer is

Table 7 Relationship between clinicopathological features and *BRAF* mutation status in 108 classic papillary thyroid carcinomas from TCGA dataset

	<i>BRAF</i> positivity				<i>BRAF</i> V600E allele frequency				
	Patients	<i>BRAF</i> negative	<i>BRAF</i> positive	P value	Patients	Negative (<4%)	Low (4–36%)	High (≥36%)	P value
Total	108	25 (23.1%)	83 (76.9%) ^a		106	25 (23.6%)	38 (35.8%)	43 (40.6%)	
Tumour size (mm) ^b	20 (7–82)	17 (7–55)	20 (10–82)	0.245	20 (7–82)	17 (7–55)	19 (11–50)	25 (10–82)	0.122
Multifocal	63/107 (58.9%)	14 (22%)	49 (78%)	0.738	62/105 (59.0%)	14 (23%)	20 (32%)	28 (45%)	0.417
Extrathyroidal extension	32/108 (29.6%)	1 (3%)	31 (97%)	0.001	31/106 (29.2%)	1 (3%)	13 (42%)	17 (55%)	0.006
pT3–4 stage	37/108	3 (8%)	34 (92%)	0.007	36/106 (34%)	3 (8%)	12 (33%)	21 (58%)	0.008
LN metastasis	57/92 (62%)	16 (28%)	41 (72%)	0.805	55/90 (61%)	16 (29%)	18 (33%)	21 (38%)	0.781
Lateral LN metastasis	26/92 (28%)	8 (31%)	18 (69%)	0.627	25/90 (28%)	8 (32%)	8 (32%)	9 (36%)	0.840
Thyroiditis	45/107 (42.1%)	13 (29%)	32 (71%)	0.250	45/105 (43%)	13 (29%)	20 (44%)	12 (27%)	0.054

LN, lymph node. Bold indicates statistical significance, $P < 0.05$.

^aOut of 83 classic papillary carcinomas with *BRAF* mutation, 81 (98%) have *BRAF* V600E mutation and two have *BRAF* fusion mutation.

^bThese parameters are presented as the median (range). A Mann–Whitney *U* test was used for comparison between two groups.

still controversial. Ricarte-Filho *et al.* (2009) found 100% concordance of *BRAF* mutational status between the primary thyroid cancers and the related metastatic sites.

In our study, the quantification of the mutated allele was evaluated using pyrosequencing. We observed that ~30% of PTCs with *BRAF* V600E had more than 25% of the mutated allele. These results are comparable to those obtained in the pyrosequencing study by Guerra *et al.* (2012a,b). However, these pyrosequencing data are in contrast to the results achieved using NGS and IHC staining. Nikiforova *et al.* (2013) evaluated *BRAF* mutated alleles using NGS and reported that 80% of PTCs with *BRAF* V600E had more than 25% of the mutated alleles. In the TCGA dataset, 86% of classic PTCs with *BRAF* V600E had more than 25% of the mutated alleles. In studies using a mutation-specific antibody against the *BRAF* V600E protein, the immunostaining pattern was homogenous in most PTCs, supporting the *BRAF* mutation as a clonal event (Bullock *et al.* 2012, Koperek *et al.* 2012, Ghossein *et al.* 2013). Recently, de Biase *et al.* (2014) have found the heterogeneous distribution of *BRAF* V600E within the PTC using both immunohistochemistry and quantitative DNA analytical methods. They also revealed the correspondence between mutated allele percentage and the extent and distribution of the *BRAF* V600E immunoreactivity in a subset of PTCs. However, there is still a puzzling discrepancy between the proportion of *BRAF* V600E-immunoreactive cells and mutant allele percentage based on DNA analysis in the study by de Biase *et al.* We are not sure as to why pyrosequencing underestimated the fraction of alleles carrying *BRAF* V600E compared with NGS. The clonal evolution theory is also contradictory to the previous belief that the *BRAF* mutation is an initial event in thyroid carcinoma carcinogenesis, which was supported by experimental methods (Knauf *et al.* 2005).

Considering that 69.6% (in training set) and 79.0% (in validation set) of the studied population were PTMC, it may be interesting to evaluate the possibility of the *BRAF* mutated allele percentage in PTMC as a biomarker for tumour aggressiveness. A high *BRAF* mutated allele percentage was related to tumour size, extrathyroidal extension and the absence of thyroiditis but not with lymph node metastasis. Therefore, the utility of *BRAF* mutation quantitative analysis in PTMC as a progression marker is not yet justified.

As there were many PTMCs in the current study, there may be some concern that contamination by normal tissue surrounding the tumour cells could be responsible for the results. In the presence of thyroiditis in particular, contamination of tumour cells by lymphoid cells should

be considered, as mentioned in a previous study (Marotta *et al.* 2013). To prevent the underestimation of mutated allele percentages by the contamination of non-tumour cells, careful microdissection was performed and tumours with marked lymphocytic infiltration were excluded in our study. The exclusion of PTMC or PTC with thyroiditis did not alter the association between the mutated allele percentages and tumour size. Although the association of the mutated allele percentage with the number of lymph nodes lost significance after the exclusion of PTMC, a significant association was still maintained after the exclusion of PTC with thyroiditis. Recently, a normalisation method has been introduced by de Biase *et al.* (2014) to overcome the problem of normal cell contamination. They normalised the frequency of mutated alleles to the real amount of tumour cells within each PTC sample. Most samples showed an increase in the percentage of mutated allele after normalisation. Thus, the inevitable contamination of non-tumoural cells still remained as fundamental weakness of the current study.

As this study did not have a follow-up period sufficient to estimate patient prognosis, the prognostic implications of a high percentage of *BRAF* mutation are limited. In addition, the same methodology should be evaluated in fine-needle aspiration samples, which could help with PTC patient management before surgery. If similar results are obtained, surgical decisions could be made based on the quantitative approach in cytology samples. However, as quantitative analysis of cytology samples may differ from that of tissue microdissection, this requires further evaluation in a future study. We used an arbitrary cut-off of 20% based on the median value for risk stratification. It may be arguable whether our results can be generalised. Although the mutated allele percentage was found to be a predictor of lymph node metastases in the univariate analysis, this relationship lost significance in the multivariate analysis. In the TCGA validation dataset, we did not find any relationship between the mutated allele percentage and lymph node metastasis or tumour size. Thus, prospective studies are needed before the mutated allele percentage can be considered to be a prognostic factor for predicting lymph node metastasis.

In conclusion, an allelic mutation rate high above the median value is associated with PTC aggressiveness in terms of tumour size and the number of lymph node metastases. Therefore, quantitative analysis of the *BRAF* mutation in mutation-prevalent populations may provide accurate characteristics of tumour behaviour among *BRAF*-positive PTCs.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-14-0147>.

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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