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Original contribution

Correlation between *BRAF* mutation and the clinicopathological parameters in papillary thyroid carcinoma with particular reference to follicular variant☆,☆☆

Robert Anthony Smith BSc (Hon), PhD, Ali Salajegheh MD, Stephen Weinstein MBBS, FRCPA, Mohammad Nassiri BSc, PhD, Alfred King-yin Lam MBBS, PhD, MD, FRCPA*

Department of Pathology, Griffith Medical School, Gold Coast, Queensland, Gold Coast QLD 4222, Australia

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Summary Mutation of the *BRAF* gene is common in thyroid cancer. Follicular variant of papillary thyroid carcinoma is a variant of papillary thyroid carcinoma that has created continuous diagnostic controversies among pathologists. The aims of this study are to (1) investigate whether follicular variant of papillary thyroid carcinoma has a different pattern of *BRAF* mutation than conventional papillary thyroid carcinoma in a large cohort of patients with typical features of follicular variant of papillary thyroid carcinoma and (2) to study the relationship of clinicopathological features of papillary thyroid carcinomas with *BRAF* mutation. Tissue blocks from 76 patients with diagnostic features of papillary thyroid carcinomas (40 with conventional type and 36 with follicular variant) were included in the study. From these, DNA was extracted and *BRAF* V600E mutations were detected by polymerase chain reaction followed by restriction enzyme digestion and sequencing of exon 15. Analysis of the data indicated that *BRAF* V600E mutation is significantly more common in conventional papillary thyroid carcinoma (58% versus 31%, $P = .022$). Furthermore, the mutation was often noted in female patients ($P = .017$), in high-stage cancers ($P = .034$), and in tumors with mild lymphocytic thyroiditis ($P = .006$). We concluded that follicular variant of papillary thyroid carcinoma differs from conventional papillary thyroid carcinoma in the rate of *BRAF* mutation. The results of this study add further information indicating that mutations in *BRAF* play a role in thyroid cancer development and progression.

1. Introduction

Papillary thyroid carcinoma (PTC) is the most common endocrine cancer [1]. Follicular variant of papillary thyroid carcinoma (FVPTC) is the third most common type of PTC, following conventional papillary thyroid carcinoma (CPTC) and papillary microcarcinoma [2]. Patients with FVPTC often present with larger tumor size and at a younger age than patients with CPTC [3]. FVPTC also shows less

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* Corresponding author.
E-mail address: a.lam@griffith.edu.au (A. K. Lam).

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calcification, psammoma bodies, and bone formation than CPTC. Furthermore, FVPTC has more favorable clinicopathological features and a better tumor risk group profile than CPTC. On the other hand, the long-term outcome is similar to that of CPTC patients [4].

FVPTC has created continuous diagnostic controversy among pathologists. FVPTC is the most difficult to differentiate from other benign thyroid and malignant thyroid lesions, both clinically and pathologically. The concordance rate for diagnosis of FVPTC between endocrine pathologists is less than 40% [5]. It is likely that the recent increase in incidence of thyroid cancer is related to the mislabeling of some of the benign mimics of FVPTC as FVPTC [6].

Molecular studies may provide more information about the pathogenesis and diagnosis of FVPTC. There is preliminary evidence that FVPTC has genotypic differences to CPTC that lead to the phenotypic difference between these 2 entities. For instance, data from our group and others have shown that FVPTC differs from CPTC by showing fewer *RET*, *p16* alterations, less COX-2 expression, and more *RAS* genetic alterations [7-11]. However, the number of cases analyzed in many studies was small. In addition, many studies did not apply strict criteria in the diagnosis of FVPTC. Some cases may be mislabeled as FVPTC, giving rise to the lower prevalence of genetic changes compared with CPTC.

BRAF is a serine/threonine kinase and a member of a family of *RAF* genes that are an integral part of one of the major pathways controlling cellular growth and differentiation [12-15]. *BRAF* has been one of the commonly studied genes in thyroid cancer in recent years. *BRAF* functions primarily as a signal transducer, carrying stimulation from other proteins to MAPK/ERK kinase (MEK), via phosphorylation of that gene. The pathway is summarized in Fig. 1. MEK then affects other downstream targets [13-15]. *BRAF* itself is an oncogene, with oncogenic mutations typically occurring in the kinase domain of the gene located on exons 11 to 15. The most common mutation identified is a point mutation causing a valine to glutamine transition at position 600 on the *BRAF* protein. This causes the kinase domain of *BRAF* to fold into a catalytically active state, no longer subject to normal repression. This in turn constitutively activates *BRAF*, resulting in constant phosphorylation of downstream targets and removing a layer of control in cellular reproduction [16].

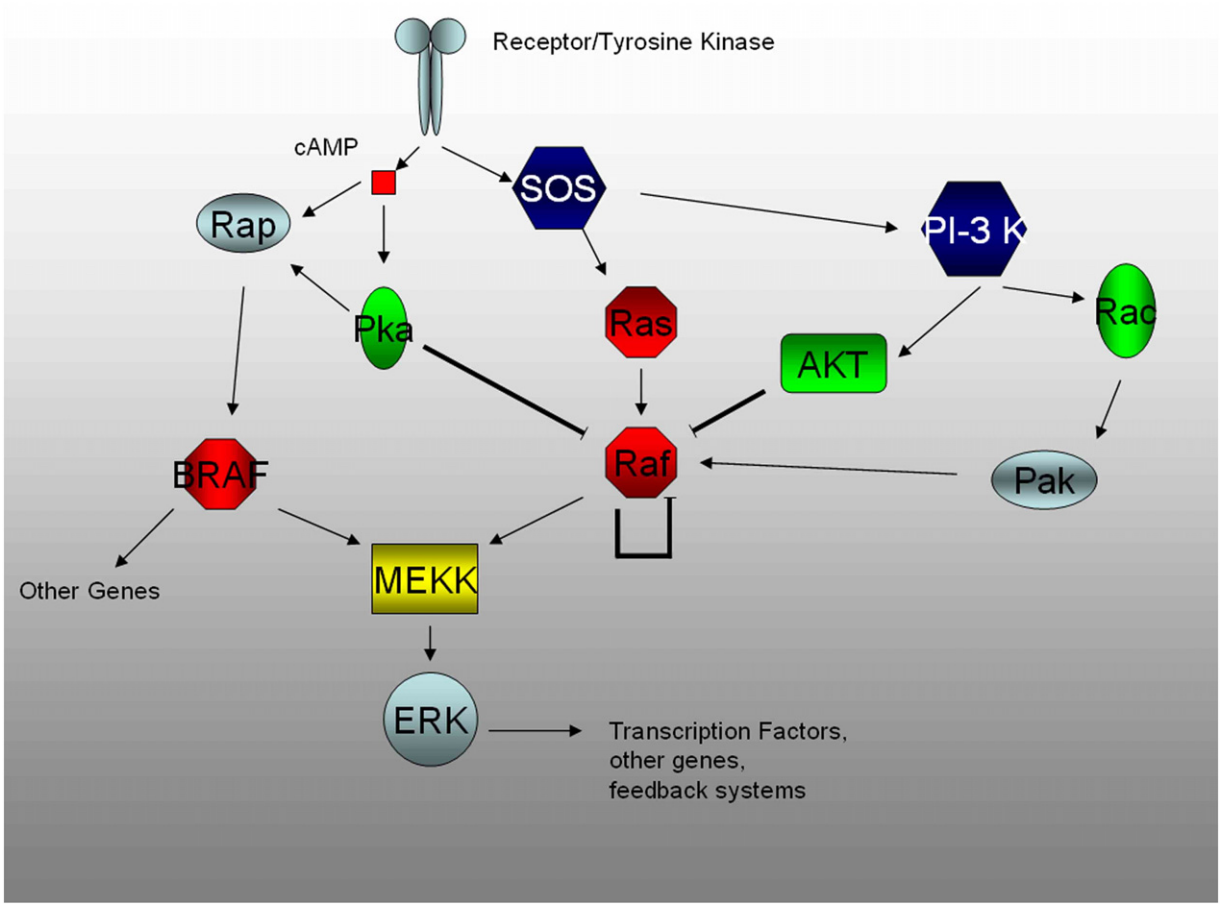

Fig. 1 The RAS/REF/MEK pathway. The complexity of even the simplified pathway can be seen as cell surface receptors stimulate *BRAF* to activate MEK and ERK, while the same receptors also stimulate other members of the pathway, some of which reduce stimulation on MEK and ERK.

BRAF V600E mutation has been associated with more aggressive phenotypes than other mutations, and it is possible that the mutation may be helpful in improving subtyping of thyroid cancers [17]. Several lines of evidence support this, first among which is that *BRAF* mutants show differential expression of genes in the *RAF/MEK/ERK* pathway compared with mutations in *RAS*, *RET*, or other tyrosine kinases [16]. In addition to this, *BRAF* mutants also show higher phosphorylation of downstream targets than *RAS* or *RET* mutants [16]. *BRAF* mutations have also been detected at high levels in thyroid lymph node metastases, indicating they may be related to the more invasive phenotypes of thyroid carcinoma [17]. Although not directly associated with thyroid cancer, a study in melanoma has identified a differential profile of copy number variations associated with *BRAF* mutants, which may indicate either that *BRAF* mutants are more likely to undergo certain mutations or that certain patterns of copy number variations predispose to *BRAF* transformation.

Although there is accumulating evidence of the role of *BRAF* in thyroid pathology, there is still a need to clarify the full effects of the mutations in PTC and in particular FVPTC. The aims of this study are to (1) investigate whether FVPTC has a different pattern of *BRAF* mutation from CPTC in a large cohort of patients with classic features of FVPTC and (2) to study the relationship of clinicopathological features of PTCs with *BRAF* mutation.

2. Materials and methods

2.1. Population

Thyroid cancer tissues were obtained from different hospitals in Australia after full ethical approval was obtained. Histologic slides of the thyroid cancers were retrieved and reviewed by the author (A. K. Y. L.). The malignant thyroid tumors were classified with reference to the criteria defined by the World Health Organization classification of malignant tumors [18].

Only thyroid cancers with typical morphologic features of CPTC and FVPTC were selected for the study. Multiple histologic sections were examined. Cases with mixed histologic patterns or equivocal diagnostic features were excluded for the study. For FVPTC, all the tumor cells must be arranged in a follicular pattern and have all the nuclear features including clear, grooved nuclei and intranuclear pseudoinclusions. If the diagnosis was in doubt or any of the above-mentioned features were absent, the case was not included in the study. A total of 76 patients with thyroid carcinomas (40 CPTCs and 36 FVPTCs) were included. A tissue block was chosen for each of the 76 carcinomas for mutation analysis. Of the 76 patients, 74% were female and 26% were male. The age of patients ranged between 18 and 72 years, with an average age of 43.13 years.

The clinicopathological features of patients with these cancers were analyzed. On pathological examination, the size and associated histologic features in the nontumor portion of the thyroid gland were noted. Lymph node metastases were recorded at the time of surgery. The thyroid cancers were staged according to the American Joint Committee on Cancer/International Union Against Cancer tumor-node-metastasis staging system for thyroid tumors [19].

2.2. Methods

From the chosen paraffin tissue block, 4-μm sections were stained with hematoxylin and eosin to locate the area of interest for DNA extraction (Fig. 2). The area with the tumor was marked on the slide and on the paraffin block (Fig. 2). Afterward, the selected area from the paraffin block was microdissected away from adjacent nontumor tissues, which were discarded to prevent the dilution effect of nontumor

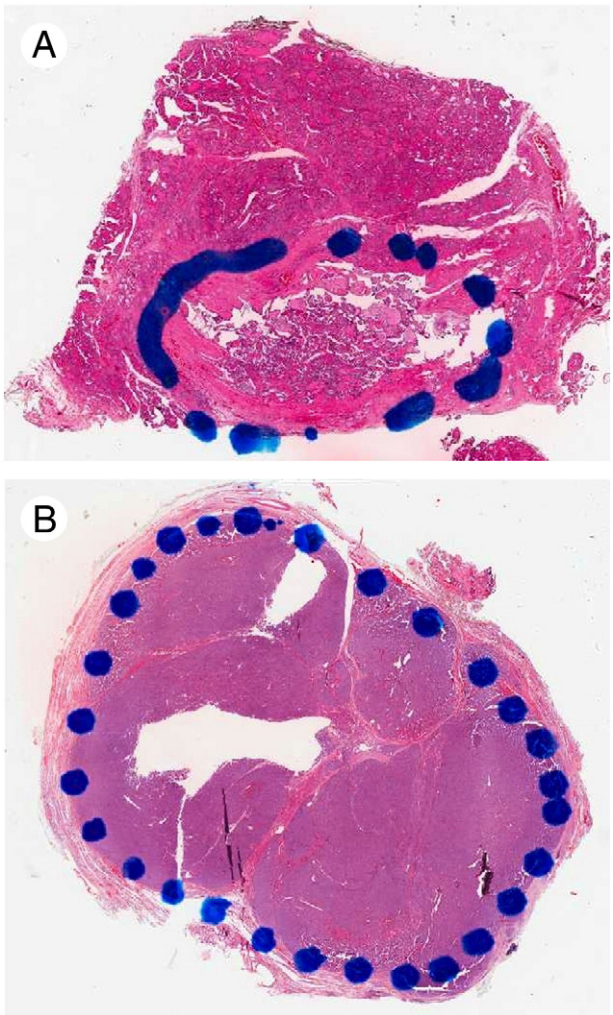

Fig. 2 The selection of PTC for the study. The nontumor portion was dissected from the tumor. Only the tumor portion was used for DNA extraction. A, CPTC. B, FVPTC. (Magnification 4×.)

thyroid tissue. For DNA extraction, five 10-μm unstained sections were cut.

DNA was extracted according to the manufacturer's protocol for the Qiagen FFPE Tissue DNA extraction kit (Qiagen, Hilden, NRW, Germany). DNA content was quantified by spectrophotometric absorption at 260 nm and evaluation of A 260/A 280 ratio (Nanodrop Spectrophotometer; BioLab, Scoresby, VIC, Australia). The DNA obtained was used to detect *BRAF* mutations in codons 600 and 601 by amplification of exon 15, using polymerase chain reaction (PCR) and DNA sequencing. The PCR was performed using an iCycler Thermal Cycler (Bio-Rad, Hercules, CA). For restriction enzyme digestion, a short amplicon was produced using the following primers: forward, 5′-ATG ACG GAA TAT AAG CTG GT-3′; reverse, 5′-CCT TAT AGT GGG GTC GTA TT-3′. For sequencing, the whole exon 15 of the *BRAF* gene was amplified using the primer pairs designed as follows: forward primer, 5′-TCA TAA TGC TTG CTC TGA TAG GA-3′; reverse primer, 5′-GGC CAA AAA TTT AAT CAG TGGA-3′. Amplifications were carried out using 60 ng of extracted genomic DNA in a 10-μL PCR mixture containing 5.5 μL MasterAmp 2× PCR premix A (Epicentre, Madison, WI), 0.4 μmol/L of each primer, and 4 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA). Initial denaturation at 95°C for 4 minutes was followed by 45 cycles comprising 30 seconds at 95°C, 45 seconds at 60°C, and 45 seconds at 72°C, and a final 7-minute extension at 72°C. A PCR without DNA template was used as a negative control in each run.

PCR samples were then subjected to restriction enzyme digestion to identify the presence of the *BRAF* V600E mutation. Samples underwent digestion using a restriction enzyme, *Mse*I (Genesearch Gold Coast, QLD, Australia), for 3 hours before being resolved on a 3% agarose gel. The *BRAF* amplicon was 136 base pairs long, and mutant alleles introduced a cutting site 21 base pairs into the fragment. After determination of mutation status by restriction enzyme digestion, genotypes for V600E were confirmed using DNA sequencing.

Fluorescent labeling of products was done with a BigDye terminator version 1.1 cycle sequencing kit (Applied Biosystems, Scoresby, VIC, Australia). For each sample, forward and reverse sequences were labeled in separate tubes. In each tube, 3 to 10 ng of the extracted amplicon was mixed with 3.2 pmol of primer (either forward or reverse), 2 μL of Big Dye terminator version 1.1, and 3 μL of 5× sequencing buffer provided by the manufacturer (Applied Biosystems). The master mix was put in the thermal cycler using 3 major steps. First, the samples were subjected to 96°C for 1 minute and then 30 repeated cycles of 96°C for 10 seconds, then 50°C for 5 seconds, and 60°C for 4 minutes. Finally, the samples were held at 4°C until the purification step. Purification was performed using a DyeEx Kit (Qiagen) according to the manufacturer's protocols, and purified products were analyzed by Applied Biosystems 3130 genetic analyzer using capillary electrophoresis.

All the data from the thyroid cancers were entered into a computer database. Statistical analysis was performed using analysis of variance for continuous variables and χ^2 test or CLUMP for categorical variables. CLUMP is a nonparametric, Monte-Carlo–style statistical test [20]. Significance was at $P < .05$. Statistical analysis was performed with the Statistical Package for Social Sciences for Windows (version 17.0; SPSS Inc, Chicago, IL).

3. Results

Restriction enzyme digestion (restriction fragment length polymorphism) and sequencing of the tumor population were completed with a 100% concordance rate. Although the concordance rate was high, restriction enzyme detection required stringent oversight to maintain an accurate result. Some cases of incomplete digestion resulted in possible false-negative interpretation using restriction fragment length polymorphism. Therefore, the findings from the more expensive option, gene sequencing, were presented.

Overall, *BRAF* mutation in codon 600 (*BRAF* V600E) was noted in 45% (n = 34 of 76) of PTCs. The mutation detected was the replacement of the nucleotide T to A (Fig. 3). No homozygote mutants were identified in the tissue population. No mutation was found in codon 601 (K601E).

Once the genotypes of each sample were known, analyses were undertaken to determine whether the presence of the *BRAF* V600E mutation was related to a number of pathological features of the tissue. Analyses were undertaken for age, sex, tumor subtype, tumor-node-metastasis staging, presence of nodular hyperplasia, and degree of lymphocytic thyroiditis (LT) in the adjacent thyroid. Initial results indicated that sex, tumor subtype, tumor staging, and degree of LT showed significant changes in wild-type versus mutants for *BRAF* V600E (Table 1).

The overall rate of *BRAF* mutation in women was 53% (30 of 56), whereas in men, the rate was 20% (4 of 20). To compensate for the low count in male mutant tumors violating the assumptions of the χ^2 analysis, a secondary analysis was undertaken using CLUMP. This analysis also proved that the sex differences were significant ($P = .016$). When the histologic subtypes were considered separately, the sex difference only remained significant in CPTC ($P = .042$) but not in FVPTC.

BRAF mutation was noted in 57% (23 of 40) of CPTCs. On the other hand, 31% (11 of 36) of FVPTCs showed *BRAF* mutation. This difference was found to be significant using χ^2 analysis ($P = .022$). It is worth noting that there is no significant difference in sex distribution, degree of LT, and pathological stage between the 2 variants of PTC ($P > .05$).

Stage I PTCs have a lower proportion of *BRAF* mutation than other tumors. However, when this was analyzed using χ^2 methods, the difference failed to reach significance, despite being close. The data were then transformed to

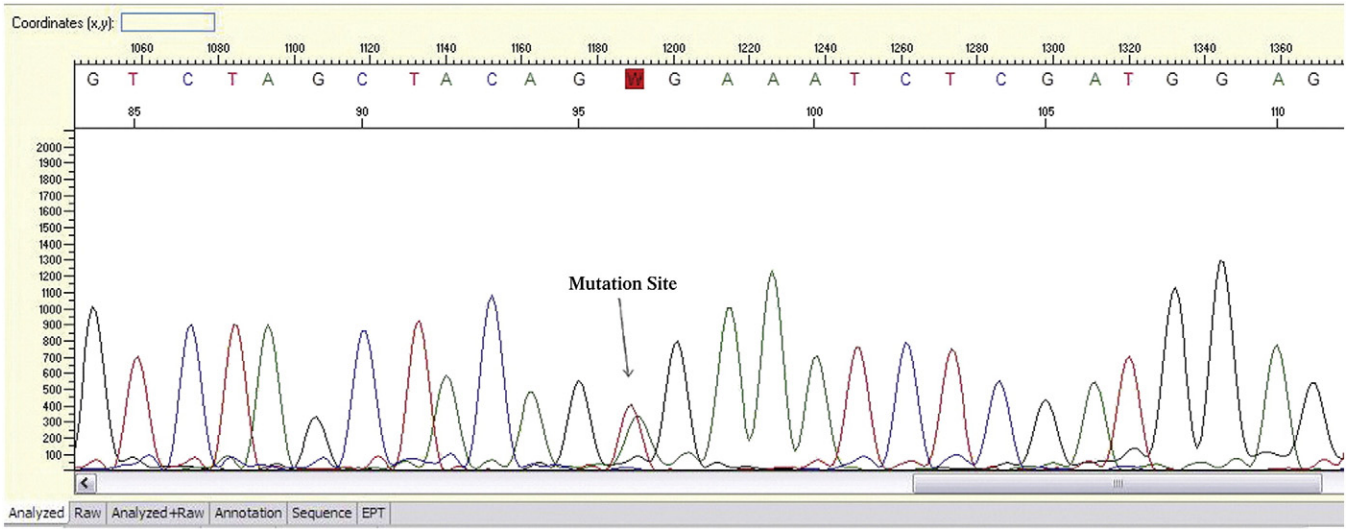

Fig. 3 The *BRAF* mutation detected in PTC. The codon (arrow) shows a mixture of T and A alleles, indicating a GTG to GAG mutation.

include all stage II and III tumors as a single category. It showed *BRAF* mutation in 37% (20 of 54) of stage 1 tumors. In contrast, *BRAF* mutation was noted in 64% (14 of 22) stage 2 and 3 tumors. The difference was statistically significant ($P = .034$)

The presence of LT showed a complex relationship with *BRAF* mutation. For tumors without LT, *BRAF* is mutated in 38% (18 of 48). On the other hand, *BRAF* mutation was noted in 85% (11 of 13) of tumors with mild LT (ie, without forming prominent germinal follicles) and 33% (5 of 15) of tumors with prominent LT (including Hashimoto thyroiditis). The difference was significant when the data were subjected to χ^2 and CLUMP analysis ($P = .006$ and .005, respectively).

Table 1 The relationship between *BRAF* mutation and clinicopathological features of patients with PTC

Characteristic		Mutants	Wild type	Total	*P* value
Age (y)	<45	18	28	46	.224
	≥45	16	14	30	
Sex	Male	4	16	20	.016
	Female	30	26	56	
Subtype	CPTC	23	17	40	.022
	FVPTC	11	25	36	
Staging	I	20	34	54	.034
	II	6	2	8	
	III	8	6	14	
Tumour size (mm)	<40	37	28	65	.479
	≥40	5	6	11	
Associated nodular hyperplasia	Present	13	14	27	.810
	Absent	21	28	49	
Associated LT	Negative	18	30	48	.006
	Mild	11	2	23	
	Prominent	5	10	15	

4. Discussion

A review of the literature has shown that the overall prevalence of *BRAF* mutations in PTC is approximately 45% [12]. In this study, the overall mutation rate of *BRAF* in 76 carefully selected PTCs (with typical pathological features) was also 45%. Thus, findings were in keeping with previous data. In addition, the results of this study indicated that a V600E mutation in the *BRAF* gene has a significant relationship with a number of features in PTC.

The first relationship detected in this study was a tendency for V600E mutations to be more common in female patients with CPTC. It is possible that the difference is due to a population effect and may not be replicable for a study with more male subjects. On the other hand, hormonally based signaling in women may favor using *BRAF* to drive cellular growth in the thyroid. Although this study has no further data regarding this possibility, studies into gene regulation in thyroid cancer may shed further light on this.

More interestingly, this study also revealed a significant difference between the prevalence of *BRAF* mutations in CPTC and the FVPTC. CPTC tended to have more mutations than FVPTC. These results replicate the findings of smaller and comparable studies, although it is worth noting that the proportion of *BRAF* mutations detected in this study is on the higher end of the previous data [21-25]. In addition, our study used a strict clinical definition of FVPTC, including only FVPTC with classic features and eliminating the possibility of the "dilution effect" of benign mimics of FVPTC. Also, the nontumor tissue was microdissected from the tumor. Furthermore, we sequenced all the cases to confirm the presence and type of mutation. Thus, this is the largest and most well-designed study to prove that the difference between FVTC and CPTC is a real phenomenon. *BRAF* mutations not only may play a role in

transformation of thyroid cells but also may influence the subtype fate of the tumor.

This hypothesis is supported by a study by Pratilas et al [16] who found that *BRAF* mutants displayed differential regulation of several genes compared with *RAS* mutants, including in a number of genes not previously associated with ERK pathway activation. Despite this, it is clear that *BRAF* mutation alone is not sufficient to induce either CPTC or FVPTC phenotypes, as both populations harbor wild types and mutants. It seems likely that other molecular events contribute to the range of cellular phenotypes in a carcinoma subtype and that other genes may also substitute for the effects of the V600E *BRAF* mutation.

BRAF has several naturally occurring splice variants in mice and humans that may influence cellular proliferation and resistance to regulation [26,27]. These splice variants may also play a role in early carcinogenesis, with shifts in splicing leading to cells undergoing more rapid proliferation without a mutation. Several aberrantly spliced *BRAF* messenger RNAs have recently been detected in thyroid carcinomas that lack sections of the *BRAF* regulatory regions, and are comparably oncogenic to the V600E mutation [28]. These splicing variants were present in a cross section of tumors and thyroid cell lines. They may provide some explanation as to how thyroid carcinomas with no major oncogenic mutations undergo transformation. In addition, *BRAF* splice variants may be a factor in the process that results in carcinoma subtypes such as FVPTC. Thus, investigation of these *BRAF* splice variants alongside classic *BRAF* mutation (*BRAF* V600E) may form an essential part of future screening processes.

In the literature, most studies showed that PTCs with *BRAF* mutations more often presented at an advanced stage [29,30]. In this study, we also demonstrated in a large cohort of patients that PTC with *BRAF* mutation presented at a more advanced stage. It is possible that this link to aggression may simply be a side effect of the increased growth rates resulting from *BRAF* V600E mutations. There is, however, some information indicating that *BRAF* expression up-regulates several genes involved in extracellular matrix remodeling, especially matrix metalloproteinases [31]. Because all tumors share a general increase in growth rates, it is likely that the link to aggressive status is itself a result of the effects of *BRAF* mutation on other gene pathways such as matrix metalloproteinases and those differentially expressed genes identified by Pratilas et al [16].

The data produced within this study also indicated that the *BRAF* V600E mutation may play a role in the presence of LT adjacent to thyroid carcinoma. The prevalence of *BRAF* mutations in carcinomas with mild LT was higher than that in carcinomas without any LT. This observation is unique in thyroid cancer, as far as the authors can discern; but similar relationships have been reported in colorectal cancer and melanoma [32,33]. Work in melanoma has established that *BRAF* V600E proteins provoke an immune response, which may attract immune cells to tumors [34]. Furthermore, Sumimoto et al [35] indicated that MAPK signaling on the *BRAF* axis is required for melanoma cells to evade immunogenic attack. Thus, *BRAF* V600E might both stimulate immune cells to invade the surrounding tissue and induce tolerance to the cancer. Such invasion may be a mechanism for *BRAF* to increase rates of tumor invasiveness, as migrating lymphocytic cells degrade the extracellular matrix, reducing the levels of remodeling required for tumor escape.

In contrast to the findings of high prevalence of *BRAF* mutation in PTC with mild adjacent LT, PTC with prominent LT (including Hashimoto thyroiditis) showed a slightly lower rate of *BRAF* mutation than PTC without LT. A recent study by Kim et al [36] also reported a similar finding. This implies that lymphocytic infiltration in Hashimoto thyroiditis, an autoimmune disease, is of a different nature than that of mild LT adjacent to PTC. The presence of prominent lymphocytic infiltration in Hashimoto thyroiditis adjacent to the PTC was unlikely to be related to *BRAF* mutation.

BRAF K601E mutation has been detected in PTC by only one group, who found that 4 FVPTCs with *BRAF* mutation were K601E [21]. That type of *BRAF* mutation has been described in follicular adenoma, colorectal carcinoma, and melanoma. However, in analyzing 36 FVPTCs with typical histologic features, we could not detect this mutation in *BRAF*. The total lack of this mutation in the target population reinforces the rare nature of this mutation and the difficulty in obtaining quality data for it and its effects in PTC.

This study confirmed the relatively low *BRAF* mutation rate in a large cohort of patients with typical features of FVPTC. We also identified a number of relationships between the *BRAF* V600E mutation and pathological features in PTC. Together, these results strengthen the evidence that *BRAF* plays a role in the biology of thyroid cancers. By continuing to refine our understanding of how the gene functions, we may increase our understanding of how to treat, diagnose, and manipulate thyroid and other cancers.

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