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

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Running title: *BRAF* in thyroid carcinoma

Abstract:

Mutation of the *BRAF* gene is a common event 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 from conventional papillary thyroid carcinoma in a large cohort of patients with typical features of follicular variant of papillary thyroid carcinoma and (2) the relationship of clinicopathological features of papillary thyroid carcinomas with *BRAF* mutation. Tissue blocks 76 patients with diagnostic features of papillary thyroid carcinomas (40 with conventional type and 36 with follicular variant) were recruited. From these, DNA was extracted and *BRAF* V600E mutations were detected by PCR 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%, $p=0.022$). Also, the mutation was often noted in female patients ($p=0.017$), high stage cancers ($p=0.034$) and in tumours with mild lymphocytic thyroiditis ($p=0.006$). It is concluded that follicular variant of papillary thyroid carcinoma differ from conventional papillary thyroid carcinoma in 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.

Keywords: Follicular variant; papillary thyroid carcinoma; *BRAF* V600E; lymphocytic thyroiditis; Hashimoto' thyroiditis.

Introduction:

Papillary thyroid carcinoma (PTC) is the most common endocrine cancer [1]. Follicular variant of papillary thyroid carcinoma (FVPTC) is third commonest type of PTC, following conventional papillary thyroid carcinoma (CPTC) and papillary microcarcinoma [2]. Patients with FVPTC often presented with larger tumour size and younger age groups than patients with CPTC [3]. It also showed less calcification, psammoma bodies and bone formation in comparison with conventional PTC. Also, FVPTC has a more favourable clinicopathological features and a better tumour risk group profile than CPTC. On the other hand, the long-term outcome was similar to conventional PTC patients [4].

FVPTC is a type of thyroid cancer that has created continuous diagnostic controversies among pathologists. FVPTC is the thyroid cancer that is most difficult to be differentiated from other benign thyroid and malignant thyroid lesions both clinically and pathologically. The concordance rate for diagnosis of FVPTC between endocrine pathologists was less than 40% [5]. It is likely that the recent increase of incidence of thyroid cancer is related to the mislabelling 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 some preliminary evidence that FVPTC has some genotypic differences compared with conventional PTC, which lead to the phenotypic difference between these two entities. For instance, data from our group and others have shown that FVPTC differs from CPTC by showing less *RET*, *p16* alterations, less COX-2 expression and more *RAS* genetic alterations [7-11]. However, the number of cases analysed in many studies was small. In addition, many studies did not apply strict

criteria in the diagnosis of FVPTC. There may be some cases mislabelled as FVPTC giving rise to the lower prevalence of genetic changes as 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]. It is one of the common studied genes in thyroid cancer in the recent years. *BRAF* functions primarily as a signal transducer, carrying stimulation from other proteins to the MAPK/ERK kinase (MEK), via phosphorylation of that gene. The pathway was summarized in Figure 1. MEK then goes on to affect other downstream targets [13-15]. *BRAF* itself is an oncogene, with oncogenic mutations typically occurring in the kinase domain of the gene located in exons 11-15. 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].

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 sub-typing of thyroid cancers [17]. There are several lines of evidence supporting this, first among which is that *BRAF* mutants show differential expression of genes in the *RAF/MEK/ERK* pathway compared to 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]. While 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 that *BRAF* mutants are either more likely to undergo certain mutations, or that certain patterns of copy number variations predispose to *BRAF* transformation.

While 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 papillary thyroid carcinoma 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 classical features of FVPTC and (2) the relationship of clinicopathological features of papillary thyroid carcinomas with *BRAF* mutation.

Materials and methods:

Population

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

Only thyroid cancers with typical morphological features of conventional papillary thyroid carcinoma (CPTC) and follicular variant of papillary thyroid carcinoma (FVPTC) were selected for the study. Multiple histological sections were examined. Cases with mixed histological patterns or equivocal diagnostic features were excluded for the study. For FVPTC, all the tumour cells must be arranged in follicular pattern and had all the nuclear features including clear, grooved nuclei and intra-nuclear pseudo-inclusions. If the diagnosis was in doubt or any of the above-mentioned features was absent, the case was not included in the study. A total of 76 patients with thyroid carcinomas (40 CPTC and 36 FVPTC) 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 analysed. On pathological examination, the size, and associated histological features in the non-tumour portion of the thyroid gland were noted. Lymph nodes metastases were recorded at the time of surgery. The thyroid cancers were staged according to the American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC) tumour-node-metastasis (TNM) staging system on thyroid tumours [19].

Methods

From the chosen paraffin tissue block, 4µm sections were stained with hematoxylin and eosin to locate the area of interest for DNA extraction (Figure 2). The area with the tumour was marked on the slide and on the paraffin block (Figure 2). Then, the selected area from the paraffin block was micro-dissected away from adjacent non-tumour tissues, which were discarded in order to prevent the dilution effect of non-tumour thyroid tissue. For DNA extraction, five 10µm unstained sections were cut.

DNA was extracted according to the manufacturer's protocol of 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 reaction was performed using an iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). For restriction digest, 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 60ng of extracted genomic DNA in a 10 µl PCR reaction mixture containing 5.5 µL MasterAmp 2X PCR premix A (Epicentre, Madison, WI, USA), 0.4 µM of each primer, and with 4 units of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). Initial denaturation at 95° C for 4 minutes was followed by 45 cycles comprising 30 seconds at 95° C, 45 seconds at 60° C,

45 seconds at 72°C, and a final 7 minutes extension at 72°C. A PCR reaction without DNA template was used as negative control in each run.

PCR samples were then subject to restriction digest to identify the presence of the *BRAF* V600E mutation. Samples underwent digestion using a restriction enzyme, *MseI* (Genesearch Gold Coast, QLD, Australia) for three 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 digest, genotypes for V600E were confirmed using DNA sequencing.

Fluorescent labeling of products by BigDye® terminator version 1.1 cycle sequencing kit (Applied Biosystems, Scoresby, VIC, Australia) was then performed. For each sample, forward and reverse sequence was labelled in separate tubes. In each tube, 3 to 10 nanograms of the extracted amplicon was mixed with 3.2 pmol of primer (either forward or reverse), 2ul of big dye terminator version 1.1 and 3 ul of 5X sequencing buffer provided by the manufacturer (Applied Biosystems). The master mix was put in the thermal cycler using three major steps. Firstly, they were subjected to 96°C for 1 minute and then 30 repeated cycles of 96°C for 10 seconds, 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 DyeEx Kit (Qiagen), according to manufacturer's protocols and purified products 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 ANOVA for continuous variables and chi-square test or CLUMP for categorical variables. CLUMP is a non-parametric, Monte-Carlo style statistical test [20]. Significance level was taken at $p < 0.05$. Statistical

analysis was performed with the Statistical Package for Social Sciences for Windows
(version 17.0, SPSS Inc., Chicago, IL, USA).

Results:

Restriction digestion (RFLP) and sequencing of the tumour population was completed with a 100% concordance rate. Although the concordance rate was high, restriction enzyme detection required stringent oversight in order to maintain an accurate result. There were some cases of incomplete digestion resulting in possible false negative interpretation using the RFLP. Therefore, the findings of the more expensive option, gene sequencing, was presented.

Overall, *BRAF* mutation in codon 600 (*BRAF* V600E) was noted in 45 % (n=34 of 76) of papillary thyroid carcinomas. The mutation detected was the replacement of the nucleotide T to A (Figure 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, gender, tumour subtype, TNM staging, presence of nodular hyperplasia and degree of lymphocytic thyroiditis in the adjacent thyroid. Initial observation of results indicated that a number of parameters showed significant changes in wild type versus mutants for *BRAF* V600E (Table 1). These parameters were gender, tumour subtype, tumour staging, and degree of lymphocytic thyroiditis.

The overall rate of *BRAF* mutation in females was 53% (30 of 56), whereas in males, the rate was 20% (4 of 20). In order to compensate for the low count in male mutant tumours violating the assumptions of the chi-square analysis, a secondary analysis was undertaken using CLUMP. This analysis also proved the gender differences was significant (p=0.016). When the histological subtypes were considered

separately, the gender difference only remained significant in the conventional papillary thyroid carcinoma ($p=0.042$) but not in FVPTC.

BRAF mutation was noted in 57% (23 of 40) of conventional papillary thyroid carcinoma. On the other hand, 31% (11 of 36) of FVPTC showed *BRAF* mutation. This difference was found to be significant using chi-square analysis ($p=0.022$). It is worth noting that there is no significant difference in gender distribution, degree of lymphocytic thyroiditis and pathological stage between the 2 variants of papillary thyroid carcinoma ($p > 0.05$).

Stage I papillary thyroid carcinomas have lower proportion of *BRAF* mutation than other tumours. However, when this was analysed using chi-square methods, the difference failed to reach significance, despite being close. The data was then transformed to include all stages II and III tumours as a single category. It showed *BRAF* mutation in 37% (20 of 54) of stage 1 tumour. In contrast, *BRAF* mutation was noted in 64% (14 of 22) stage 2 and 3 tumours. The difference was statistically significant ($p = 0.034$).

The presence of lymphocytic thyroiditis showed a complex relationship with *BRAF* mutation. For tumours without lymphocytic thyroiditis, *BRAF* is mutated in 38% (18 of 48). On the other hand, *BRAF* mutation was noted in 85% (11 of 13) of tumours with mild lymphocytic thyroiditis (i.e., without forming prominent germinal follicles) and 33% (5 of 15) of tumours with prominent lymphocytic thyroiditis (including Hashimoto's thyroiditis). The difference was significant when the data was subjected to chi-square and CLUMP analysis ($p=0.006$ and 0.005 respectively).

Discussion:

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

The first relationship detected in this study was a tendency for V600E mutations to be more common in female patients with conventional papillary thyroid carcinoma.. It is possible that the difference is due to population effects and may not be replicable for a study with more males. On the other hand, hormonally based signalling in women may favour using *BRAF* to drive cellular growth in thyroid. Though this study has no further data regarding this possibility, studies into gene regulation in thyroid cancer may shed further light onto it.

More interestingly, this study also revealed a significant difference between the prevalence of *BRAF* mutations in conventional papillary thyroid carcinoma and the follicular variant of papillary thyroid carcinoma. Conventional PTC tended to have more mutations than FVPTC. The result replicated the findings of smaller and comparable studies, though 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 utilised a strict clinical definition of FVPTC, including only FVPTC with classical features and eliminated the possibility of the “dilution effect” of benign mimics of FVPTC. Also, the non-tumour tissue was micro-dissected from the tumour. 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 the difference between

FVTC and CPTC is a real phenomenon. *BRAF* mutations may play a role not only in transformation of thyroid cells, but may also influence the sub-type fate of the tumour.

This hypothesis is supported by a study by Pratilas *et al* who found that *BRAF* mutants displayed differential regulation of several genes compared to *RAS* mutants, including in a number of genes not previously associated with ERK pathway activation [16]. Despite this, it is clear that *BRAF* mutation alone is not sufficient to induce either CPTC or FVPTC phenotypes, as both populations harbour wild types and mutants. It seems likely that other molecular events contribute to the range of cellular phenotypes in a carcinoma sub-type 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 which 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* mRNAs have recently been detected in thyroid carcinomas which 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 tumours and thyroid cell lines. They may provide some explanation as to how thyroid carcinomas with no major oncogenic mutations undergo transformation. Additionally, *BRAF* splice variants may be factors in the process that results in carcinoma subtypes such as FVPTC. Thus, investigation of these *BRAF* splice variants alongside classical *BRAF* mutation (*BRAF* V600E) may form an essential part of future screening processes.

In the literature, most of the studies showed that PTCs with *BRAF* mutations presented more often 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 remodelling, especially matrix metalloproteinases (*MMPs*) [31]. Since all tumours share a general increase in growth rates, it is likely that the link to aggressive status is itself a result of effects of *BRAF* mutation on other gene pathways, such as *MMPs* 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 lymphocytic thyroiditis (LT) adjacent to the thyroid carcinoma. The prevalence of *BRAF* mutations in carcinomas with mild LT was higher than those carcinomas without any lymphocytic thyroiditis. This observation is unique in thyroid cancer, so far as the authors can discern, but similar relationships have been reported before 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 tumours [34]. Also, Sumimoto *et al.* indicated that MAPK signalling on the *BRAF* axis was required for melanoma cells to evade immunogenic attack [35]. 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 tumour invasiveness, as migrating lymphocytic cells degrade the extracellular matrix, reducing the levels of remodelling required for tumour escape.

In contrast to the findings of high prevalence of *BRAF* mutation in PTC with mild adjacent lymphocytic thyroiditis, PTC with prominent lymphocytic thyroiditis (including Hashimoto's thyroiditis) showed a slightly lower rate of *BRAF* mutation than those PTC without lymphocytic thyroiditis. A recent study by Kim *et al* also reported a similar finding [36]. This implies that lymphocytic infiltration in Hashimoto's

thyroiditis, an autoimmune disease, is of different nature than that of mild LT adjacent to PTC. The presence of prominent lymphocytic infiltration in Hashimoto's 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. They found that four FVPTC with *BRAF* mutation were K601E [21]. The type of *BRAF* mutation has been described in follicular adenoma, colorectal carcinoma and melanoma. However, in analysing 36 FVPTC with typical histological 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 involved in obtaining quality data for it and its effects in papillary thyroid carcinoma.

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 papillary thyroid carcinoma. Together, these results strengthen the evidence that *BRAF* plays a role in the biology of thyroid cancers, and that 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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Figure Legends:

Figure 1: The RAS/RAF/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.

Figure 2. The selection of papillary thyroid carcinoma for the study – the non-tumour portion was dissected from the tumour. Only the tumour portion was used for DNA extraction. 2A – conventional papillary thyroid carcinoma (CPTC); 2B- follicular variant of papillary thyroid carcinoma (FVPTC).

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

Table 1. The relationship between *BRAF* mutation and clinicopathological features of patients with papillary thyroid carcinoma

Characteristic		Mutants	Wild Type	Total	p-value
Age	< 45	18	28	46	0.224
	= > 45	16	14	30	
Gender	Male	4	16	20	0.016
	Female	30	26	56	
Subtype	CPTC	23	17	40	0.022
	FVPTC	11	25	36	
Staging	I	20	34	54	0.034
	II	6	2	8	
	III	8	6	14	
Tumour Size	< 40mm	37	28	65	0.479
	=> 40mm	5	6	11	
Associated nodular hyperplasia	Present	13	14	27	0.810
	Absent	21	28	49	
Associated lymphocytic thyroiditis	Negative	18	30	48	0.006
	Mild	11	2	23	
	Prominent	5	10	15	

CPTC: conventional papillary thyroid carcinoma; FVPTC: follicular variant of papillary thyroid carcinoma