THE RELATIONSHIP BETWEEN BRAFV600E MUTATION AND THE CLINICOPATHOLOGICAL FEATURESOFTHEPAPILLARY THYROID CARCINOMA

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ABSTRACT

There has been a dramatic increase in the morbidity of papillary thyroid carcinoma (PTC), which accounts for 85–90% of all thyroid malignancies. A somatic BRAF V600E oncogenic mutation has been recognized as the most frequent genetic alteration, occurring in approximately 32–83% of PTC cases and being associated with more aggressive clinical behaviors and worse prognoses. Still, the prognostic value of the mutation remains unvalidated, leading this study to investigate it by performing qPCR as well as immunohistochemistry (IHC) and analyzing the relationship between BRAF V600E mutation status and multiple clinicopathological features of 188 PTC patients. There was no significant difference in the prevalence of the mutation between patients of different gender (χ^2=1.252, P=0.263), ethnicity (χ^2=0.756, P=0.384) or age (younger than 40 years compared to 40 years or older; χ^2=0.002, P=0.957). Also, the study found no association between the frequency of the BRAF V600E mutation and various pathological characteristics of PTC; characteristics considered were calcification (χ^2=0.2186, P=0.640), hashimoto (χ^2=0.072, P=0.789), tumor size (χ^2=1.453, P=0.228), tumor multifocality (χ^2=0.183, P=0.668), thyroid capsular invasion (χ^2=0.138, P=0.710), vascular invasion (χ^2=1.132, P=0.860) and lymph node metastasis (χ^2=0.080, P=0.777). Considering that the mutation is widespread among PTC cases, it is speculated that the mutation is related to tumorigenesis rather than tumor progression. Moreover, the clinical value of an IHC approach to detecting the mutation was evaluated in comparison to the qPCR method; using IHC, the mutation was identified in 153 of 188 (81.38%) cases, whereas 160 (85.11%) patients were identified using the PCR assay. Thus, it can be concluded that the qPCR method features superior sensitivity to IHC.

Keywords: *Papillary thyroid carcinoma, BRAF V600E, tumorigenesis, qPCR and immunohistochemical methods.*

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Introduction

According to recent research, there has been a rapid and continuous increase in the worldwide incidence rate of thyroid cancer - the most common endocrine malignancy[1, 2] - with papillary thyroid cancer (PTC) accounting for the vast majority (85-90%) of all thyroid malignancies[3]. Despite the steadily rising morbidity, PTC-related mortality appears to be stable[1, 4]. Indeed, the intrinsically indolent behavior of the disease and the effective initial management mean that PTC prognoses generally promise with a 10-year overall survival rate for approximately 85% of patients[5]. However, relapses have been reported in about 5-25% of the patients[6, 7] and, where tumor metastasis occurs, that survival rate is reduced to 40%.

Interestingly, incidence rates for different thyroid cancer histotypes vary considerably. During the past few decades, the PTC rate has exclusively increased - especially the follicular variant[8] - while a considerably more modest increase has been observed for follicular thyroid cancers[9] and anaplastic thyroid cancer rate has remained stable or decreased[10]. This suggests that certain carcinogenetic factors promote PTC onset by deregulating specific molecular signaling. BRAF is a Ser/Thr specific protein kinase gene and a

member of the Raf kinase family of growth signal transduction protein kinases. This protein helps regulate the MAP kinase/ERKs signaling pathway, affecting cell division, differentiation, and secretion. The BRAF V600E mutation substitutes valine for glutamate in codon 600 of a serine- or threonine-specific protein kinase named BRAF, resulting in constitutive activation of the mitogen-activated protein kinase (MAPK) signaling pathway[11].

This occurs in approximately 36-83% of PTC cases[12] and critically maintains and progresses thyroid cancer[13]. Recent studies have reported an increasing trend for patients with PTC to carry the BRAF V600E oncogenic mutation[14, 15]. The BRAF V600E mutation is the most frequent genetic aberration correlating with more aggressive tumors based upon conventional staging; furthermore, patients harboring the mutation are more susceptible to increased risk of recurrence and lymph node metastasis compared to PTC patients without the mutation[16]. However, the value of the BRAFV600E mutation as a prognostic marker for PTC has been controversial and lacking clarity.

Thus, the present study aimed to define the correlation between the BRAF V600E mutation and PTC-related clinicopathological characteristics. Additionally, the study compared qPCR and immunohistochemical approaches to identifying the V600E mutation.

Materials and methods

Patients

A total of 188 patients with primary PTC diagnosed at the First Affiliated Hospital of Xinjiang Medical University (Urumqi, Xinjiang, China) from June 2015 to December 2015 were recruited for this study. Tumor tissues were obtained from surgical resections. We recorded and conducted statistical analyses on the gender, ethnicity, age, calcification, tumor diameter, hashimoto, thyroid focality, thyroid capsular invasion, vascular invasion, lymph node metastasis, BRAF immunity, and the BRAF V600E PCR results of each case. Written informed consent was provided by all participants and both the sample collection and the investigation were approved by the ethics committee at the First Affiliated Hospital of Xinjiang Medical University.

Quantitative PCR assay

All patients ate a low-residue semi-liquid diet 2 days Total DNA was extracted from paraffin-embedded tissues. The resuspended DNA samples were subjected to PCR analysis using a human BRAF V600E detection kit (ACCB Biotech Ltd., Beijing, China) according to the manufacturer's protocol. Amplification was performed using an ABI 7300 plus machine (Applied Biosystems, Foster City, CA, USA) (Figure 1). The amplification profile began with denaturation at 95°C for 10 min; this was followed by 40 15s cycles of denaturation at 95°C, annealing at 60°C for 30s, and extension at 72°C for 1 min.

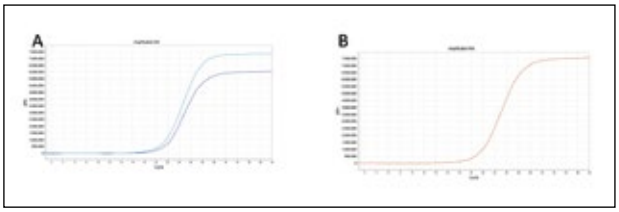

Figure 1: (A) BRAF gene mutant-type in the FAM channel. (B) BRAF gene Wild-type in the FAM channel.

Immunohistochemistry

Immunohistochemistry (IHC) was performed as has been described in the literature[17]. Briefly, formalin-fixed, paraffin-embedded tissue sections (4μm-thick) were deparaffinized and rehydrated before being incubated in a retrieval solution at 60°C overnight to retrieve antigens. Following inhibition of the preprimary peroxidase and blocking in 1% BSA in TBS for 2h at room temperature, slides were incubated with the anti-BRAF V600E (VE1) (1:50, Roche, Ventana Medical Systems, Tucson, AZ, USA) mouse monoclonal primary antibody at 37°C for 30 min. Next, following incubation with hematoxylin and a bluing reagent (for 4 min each), the primary antibodies were detected using an Opti View DAB IHC Detection kit (Ventana Medical Systems).

Statistical analyses were performed using SPSS software (version 11.0; SPSS Inc., Chicago, IL). A chi-square test was used to compare frequencies of BRAF V600E mutation between groups. Categorical variables were expressed as numbers and percentages. Statistical significance was assumed when the P-value was <0.05.

Results

Clinicopathological Features of Patients with PTC

The extensive application of ultrasound-guided fine-needle aspiration and imaging studies has produced increasing numbers of PTC cases in our hospital over the past few decades. During the study period, a total of 188 patients were diagnosed with

PTC; 160 of those patients carried the BRAF V600E mutation (85.1%), with 28 being mutation-negative (14.9%). The basic demographics of PTC patients were analyzed retrospectively and summarized in Table 1. Most patients were women (69.7%), of Han ethnicity (74.5%), and older than 40 (63.8%).

Calcification was detected in 60 cases (31.9%) and hashimoto was present in 63 cases (33.5%). Multifocal tumors were observed in 114 patients (60.6%). The frequencies of thyroid capsular invasion, vascular invasion and the lymph node metastasis were 36.2%, 4.8% and 30.9%, respectively. There were 62 patients (33.0%) with thyroid tumors of diameters greater than 1 cm.

Items	Cases	Positive rate (%)	χ^2	P value	Power
Gender			1.252	0.263	0.999
Male	57	87.02			
Female	131	80.70			
Ethnicity			0.756	0.384	0.677
Han	140	86.43			
non-Han	48	81.25			
Age			0.002	0.957	0.999
<40	68	85.29			
≥40	120	85			
Calcification			0.2186	0.640	0.952
None	128	85.94			
Yes	60	83.33			
Tumor diameter			1.453	0.228	0.999
≤1cm	126	87.30			
>1cm	62	80.64			
Hashimoto			0.072	0.789	0.973
None	125	84.12			
Yes	63	85.60			
Thyroid focality			0.183	0.668	0.999
Monofocal	74	86.49			
Multifocal	114	84.21			
Thyroid capsular invasion			0.138	0.710	0.993
None	120	85.83			
Yes	68	83.82			
Vascular invasion			1.132	0.860	0.047
None	179	85.47			
Yes	9	77.78			
Lymph node metastasis			0.080	0.777	0.903
None	130	84.62			
Yes	58	86.21			
BRAF immunity			78.054	1.00269E-18	0.378
Positive	153	96.08			
Negative	35	37.14			

Table 1: The association between PTC clinicopathological characteristics and patient BRAF V600E mutation status.

The relationship between PTC clinicopathological features of patients and BRAF V600E mutation status

Table 1 indicates that 46 of the 57 male patients (80.70%) carried the BRAF V600E mutation, compared to 114 (87.02%) of the 131 female patients, suggesting no significant correlation between gender and BRAF V600E mutation (χ^2=1.252, P=0.263).

To assess the correlation between BRAF V600E mutation and age, the 188 participants were divided into two groups: those younger than 40 and those aged 40 years and older. The BRAF V600E mutation was detected in 102 (85%) of the 120 older patients, compared to 58 of the 68 younger patients (85.29%). This suggests no direct association between BRAF V600E and patient age (χ^2=0.002, P=0.957).

The patients were also divided into two ethnic groups: Han and non-Han. There was no significant difference between the groups according to the incidence of the BRAF V600E mutation (86.43% vs. 81.25%; χ^2=0.756, P=0.384).

Next, we analyzed the association between the BRAF V600E mutation and the presence of either thyroid calcification or hashimoto. Among the 128 patients without thyroid calcification, 110 (85.94%) carried the mutation, compared to 50 of the 60 patients with calcification (83.33%). Likewise, 53 of 63 PTC patients with hashimoto (84.13%) carried the mutation, compared to 107 of the 125 patients without hashimoto (85.60%). This indicates no significant association between the BRAF V600E mutation and either thyroid calcification or hashimoto (χ^2=0.2186, P=0.640; χ^2=0.072, P=0.789, respectively).

The study also evaluated the association between BRAF V600E mutation and both focal number and tumor size. Multifocality was observed in 60.64% of all PTC patients. Among the 74 patients with monofocal PTC, 64 carried the mutation (86.49%), compared to 96 (84.21%) of the 114 patients with multifocal PTC, indicating that there was no significant difference between the two groups (χ^2=0.183, P=0.668).

Regarding tumor size, tumors with diameters bigger than 1 cm were observed in 62 (32.98 %) of patients; 67.02 % had tumors of a diameter smaller than 1 cm. Among those with larger tumors, 50 (80.65%) carried the BRAF V600E mutation, compared to 110 of the 126 patients with smaller tumors (87.30%).

Thus, there was no significant association defined for the relationship between the BRAF V600E mutation and tumor size (χ^2=1.453, P=0.228).

Aggressive characteristics of PTC were analyzed to define the factors related to the BRAF V600E mutation.

Thyroid capsular invasion, vascular invasion, and lymph node metastasis were respectively observed in 68 (36.17%), 9(4.79%), and 58 (30.85%) cases. Among the 120 patients without thyroid capsular invasion, 103 carried the BRAF V600E mutation (85.83%), compared to 57 of the 68 patients with thyroid capsular invasion (83.82%).

Thus, there was no direct correlation between the mutation's frequency and the incidence of thyroid capsular invasion (χ^2=0.138, P=0.710). Among the 9 patients with vascular invasion, the BRAF V600E mutation rate was 77.78%; among the non-invasive patients, 85.47% had the mutation. There was no statistical difference between the two groups (χ^2=1.132, P=0.860).

Of the 58 patients with lymph node metastasis, 50 carried the BRAF V600E mutation (86.21%), compared to 110 out of 130 patients without lymph node metastasis (84.62%). Thus, no significant difference in mutation frequency was observed between metastatic and non-metastatic forms of PTC (χ^2=0.080, P=0.777).

These univariate analyses of the relationship between the incidence of the BRAF V600E mutation and diverse clinicopathological factors (age, gender, ethnicity, tumor size, and the presence of calcification, hashimoto, multifocality, thyroid capsular invasion, vascular invasion, and lymph node metastasis) showed no apparent influence from these factors on the presence of the mutation.

Comparison of the BRAF V600E mutation using IHC and PCR

Mutation detection was also performed using IHC to determine whether the PCR method could be reliably used as a substitute for BRAF V600E detection. Figure 2 shows that, using IHC, the mutation was found in 153 cases.

The positive rate was 81.38%, relatively low compared to the rate found by qPCR (85.11%). Among the 153 mutation-positive cases detected by IHC, 147 (96.08%) could also have been identified by PCR; among the 35 mutation-negative cases, 13 were detected by PCR.

Given the superior performance of the qPCR approach–in terms of sensitivity–it can be concluded that PCR is preferable to IHC, likely due to its amplification effect on the mutant sequences in a small amount of tissue-based mutated tumor cells.

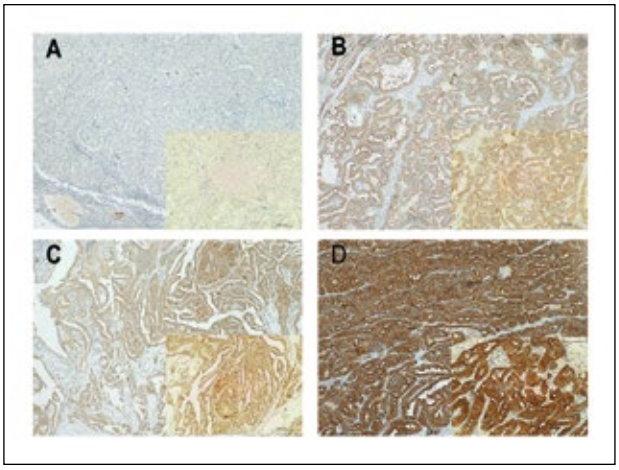

Figure 2: A-D show the expression of BRAF V600E in thyroid papillary carcinoma. A. Negative; B. Weakly positive; C. Medium positive; D. Strongly positive.

Discussion

The worldwide increase in PTC constitutes a significant public health concern, and clinical decision-making remains controversial in the absence of specific means of assessing malignancies[18]. Although the importance of the prognostic implications of other tumor-specific genetic alterations has become increasingly apparent[19], their clinical predictive power remains limited.

The RAS–ERK–MAPK signaling pathway is involved in cell responses to environmental stimuli and plays a crucial part in human carcinomas[18]. This pathway comprises RAS, MEK, ERK, and the protein kinases RAF, which include three protein kinases with nonredundant functions. Upon exciting cytokines or hormones, active RAS recruits RAF to the membrane; then, the RAF phosphorylate and activate the scaffold protein MEK, which, in turn, activates ERK. BRAF is a member of the RAF kinase family and an important transduction factor in the RAS–ERK–MAPK signaling pathway, helping to regulate a variety of biological events, including cell survival, division, differentiation, senescence, apoptosis, and normal cells secretion[19].

The BRAF V600E mutation is the most frequent genetic event observed in PTC, having been found in approximately 32-83% of cases; in contrast, it is rarely identified in the context of other thyroid tumors[12]. The mutation has been suggested as simulating constitutive phosphorylation on T599 and S602 residues, thereby abnormally activating the BRAF protein kinase[20]. In fact, it has been reported that BRAF V600E is activated 500-fold,

promoting the constitutively activated ERK–MAPK signaling and inducing the proliferation and survival of cancer cells. The BRAF V600E mutation has been observed extensively in cases of PTC, colorectal cancer, melanoma, and non-small-cell lung cancer[21-23]. The clinical researchers considering the BRAF inhibitors vemurafenib and dabrafenibim have demonstrated increased survival rates among patients with advanced melanoma[24] and objective response initiation in refractory hairy-cell leukemia[25]. Elsewhere, the BRAF-selective inhibitor vemurafenib restrained the growth of the BRAF-mutated anaplastic thyroid cancer in mice[26]. These results have validated BRAF V600E mutation as a therapeutic target in the context of many cancers; furthermore, BRAF-selective drugs have been used clinically and produced excellent effects in patients with BRAF-mutant melanomas[27].

Although results from certain individual studies have suggested that the BRAF V600E mutation was associated with multiple aggressive clinicopathological characteristics of PTC, including extra thyroid extension, multifocality, advanced tumor stage, and lymph node metastasis[28, 29], such results remain controversial and inconclusive. This additional study involving a larger number of patients with PTC was thus conducted to determine whether the frequency of the BRAF V600E mutation was correlated with those aggressive clinicopathological features or with other characteristics, namely tumor size, hashimoto presence, calcification, and patient age, gender, and ethnicity. A total of 188 PTC cases were studied retrospectively, with qPCR analyses used to identify whether the BRAF V600E mutation was present. A chi-square analysis demonstrated that there was no correlation between any of the variables and the BRAF V600E mutation; this indicated that none of the factors could function alone. Accordingly, we speculated that two or more factors might be collectively involved in the mutation. However, the number of mutation-negative cases was insufficient for an accurate evaluation of the phenotypic differences between mutation-positive and -negative tumors. In contrast, we have assumed that the mutation preferably contributes to tumorigenesis rather than the progression of PTC. Nonetheless, this study is potentially limited by not analyzing subtypes of PTC (i.e., the classical type, the follicular variant, the oncocytic variant, and the tall cell variant).

Recent studies have demonstrated that the BRAF V600E mutation has emerged as a useful diagnostic hallmark and a specific factor in poor clinical outcomes for PTC[12, 30, 31]. Therefore, an efficient and sensitive method with higher specificity needs to be developed to detect the presence of the BRAF V600E mutation. Of the 188 PTC patients in the present study, the mutation was identified using IHC in 153 cases and using PCR assay in 160 cases.

The detection rate for the mutation using IHC was 81.38%, slightly lower than the 85.11% achieved by the PCR method. Among the 153 mutation-positive cases detected by IHC, 147 (96.08%) could also be identified by PCR; among the 35 mutation-negative cases detected by IHC, 13 were detected by PCR. Given the superior performance of the PCR approach to detecting cells carrying the mutation, it can be concluded that PCR features superior sensitivity than IHC. Therefore, results obtained from PCR should be combined with those obtained from the IHC method for clinical evaluation of the BRAF V600E mutation.

The differential diagnostic value of the BRAF V600E mutation in the context of thyroid tumors has been widely recognized and applied. The results of this study indicate that the sensitivity of the ARMS PCR assay in the detection of the BRAF V600E mutation in the context of PTC is as high as 85%, comparing favorably with the average rate of 44% (36-83%)[33]. These results ar similar to those obtained by Wang and colleagues[34] (83.1%) and Wang and colleagues[35] (86.8%), indicating that the ARMS PCR method shows better clinical application prospects than the direct sequencing method[36].

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