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Original Article

Diagnostic significance of the *BRAF* V600E mutation in conventional papillary thyroid carcinomas

Bo Zhang[1], Chun-Wei Xu[1], Yong-Fang Wu[1], Qiu-Hong Man[1], Ye-Ying Song[1], Jing-Jing Wang[1], Huai-Tao Wang[1], Hai-Yan Wang[1], Xiao-Bing Li[1], Hao Zhang[2], Ting Ye[2], Zhe Zhang[2], Cong-Li Cai[2]

[1]*Department of Pathology, Affiliated Hospital of Academy of Military Medical Sciences, Beijing 100071, People's Republic of China;* [2]*Wuhan YZY Medical Science & Technology Co., Ltd, Wuhan 430075, Hubei, People's Republic of China*

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Abstract: Papillary thyroid carcinoma (PTC) is the most common histological thyroid cancer, accounting for approximately 80% of thyroid cancers. Although PTC is highly curable, some patients encounter high rates of morbidity and mortality. *BRAF* V600E mutations are the most common genetic mutation in PTC and the relationship between *BRAF* V600E mutations and PTC has recently been a focus of research. The aim of the present study was to determine the prevalence of *BRAF* V600E mutation in PTC tissue, pericarcinous tissue, and benign tumor samples, and explore the correlation between *BRAF* V600E mutations and different clinicopathological features. In the present study, 120 conventional PTC samples, 30 pericarcinous tissue, and 25 benign nodule samples were collected, and the *BRAF* V600E mutation was tested using the *BRAF* Gene V600E Mutation Detection Kit. Statistical analyses were performed for the data on *BRAF* V600E mutation in three kinds of tissue and in different clinicopathological categories of PTC. Finally, results revealed that the prevalence of *BRAF* V600E mutation in exact cancerous tissue of 120 PTC samples was 88.3%, and *BRAF* V600E mutations were absent in the pericarcinous tissue and benign nodule samples. There were no significant differences in the prevalence of *BRAF* V600E mutation depending on the age, gender, tumor size, lymph node metastasis, and lesion location ($P > 0.05$). Consequently, the *BRAF* V600E mutation was found to be highly prevalent in conventional PTC samples due to more accurate sample processing and the use of different detection methods, and absolutely negative in pericarcinous tissue and benign nodule samples. Therefore, *BRAF* V600E mutation could suggest a useful predictor of tumorigenesis in PTC, and such mutation may also affect local carcinoma development. There is no evidence that the *BRAF* V600E mutation significantly reflects aggressive characteristics and poor prognosis of patients with PTC.

Keywords: Papillary thyroid carcinoma, BRAF mutation, diagnostic significance, ARMS-qPCR

Introduction

Thyroid cancer is one of the most common endocrine malignancies with complicated processes, and 5-10% of thyroid nodules are thyroid carcinomas [1, 2]. Recently, the incidence of thyroid cancer as well as the tumor-targeted therapy has increased rapidly; in China, the incidence of thyroid cancer increased annually by 14.51%, whereas the mortality increased by 1.42% [3]. Thyroid cancer can be classified as papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), anaplastic thyroid cancer (ATC), and medullary carcinoma based on the histological characteristics [1, 4]. Among the four histological types, PTC is the most common thyroid cancer, accounting for more than 80% of all thyroid malignancies [1, 5]. PTC is highly curable through surgery and roentgenotherapy and has a good prognosis compared to the worst prognosis of ATC [6]. Some patients face the risk of death from the occurrence of PTC when it becomes surgically inoperable and radioiodine avidity is not available [7].

The pathogenesis of thyroid papillary carcinoma remains unclear; however, a variety of oncogenes and suppressor genes are involved in the occurrence and development of PTC [8, 9]. The RET/PTC-RAS-RAF-MEK-ERK signaling pathway (MAPK pathway) is a classical conserved intracellular signaling pathway, which plays a funda-

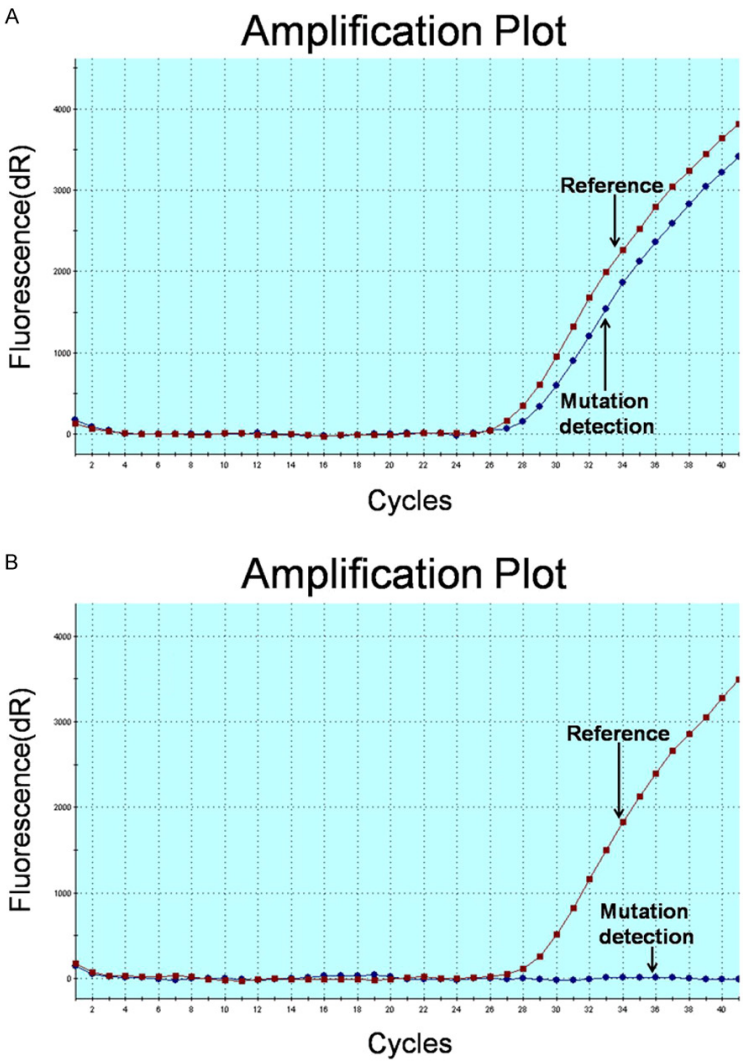

Figure 1. Amplification plot of *BRAF* V600E-positive (A) and -negative (B) results in Mx3000P. Results were analyzed using fluorescence quantitative PCR, The reference curve reflects the DNA quality in samples; the mutation-detection curve performs an obvious amplification curve and with the delta Ct ($Ct_{mutation}$-$Ct_{reference}$) < 9 in positive results, the mutation-detection curve showed a straight line when no mutation signal was collected.

mental role in cell proliferation, differentiation, apoptosis, and survival [8-10]. Recurrence of PTC involves a series of genetic events such as RAS/RAF gene mutation, RET/PTC rearrangement, and p53 inactivation, and these mutations are found in most PTC cases and rarely overlap in the same tumor [11-13].

BRAF kinase, encoded by the *BRAF* gene, also referred to as proto-oncogene B-Raf and v-Raf murine sarcoma viral oncogene homolog B, is a member of the RAF family of serine-threonine kinases. The *BRAF* gene is the strongest activator downstream of the MAPK pathway and tumorigenesis occurs when *BRAF* is aberrantly activated [14-16]. *BRAF* mutations exist in 66% of malignant melanomas and 15% of colorectal cancer [17]; however, the BRAF protein may be independent of the RAS protein in cancer occurrence. *BRAF* mutations showed a high prevalence in PTC, among which the T1799A point *BRAF* mutation is the most common, accounting for more than 90% of all *BRAF* mutations [18-20]. Based on the important role of *BRAF* mutations in papillary carcinomas, much research has focused on *BRAF* gene mutations and clinical pathological parameters of papillary carcinomas.

In the present study, we analyzed *BRAF* V600E mutations in 120 PTC tissues, 30 pericarcinous tissues, and 25 benign tumor (adenoma and hyperplasia) samples. This article will focus on the prevalence of *BRAF* mutations in 175 tissues and the discrepancy in the presence of *BRAF* mutations. These results may be inconsistent with other results because of different detection methods used. The aim of the present study will to elucidate the diagnostic value of the *BRAF* V600E mutation in PTC.

Materials and methods

Preparation of cancerous, pericarcinous, and benign tissue samples

All samples used for the present study were obtained from the Pathology Department at the Affiliated Hospital of the Academy of Military Medical Sciences. We selected 120 patients who were diagnosed with conventional papillary thyroid cancer from 2011 to 2014. All the tumors showed typical papillary carcinoma histology. Then, we analyzed the histology of the cancerous tissue microscopically using hema-

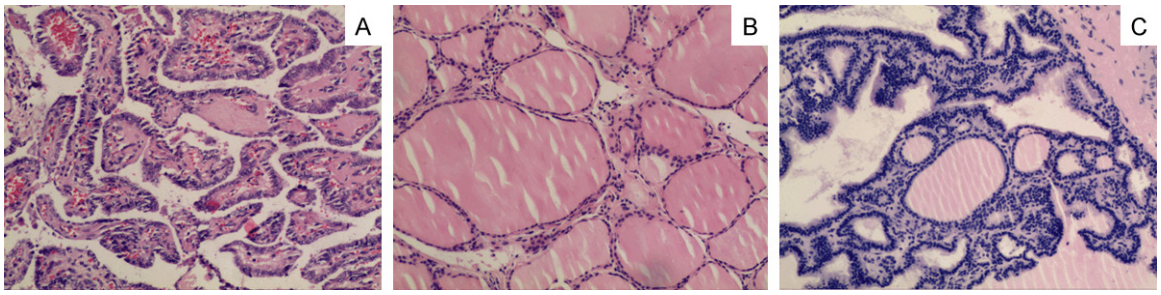

Figure 2. Representative HE staining results of conventional papillary thyroid carcinomas, pericarcinous tissue, and benign nodules. The cell nucleus was stained by hematoxylin with dark blue and acidophilic substances were stained by eosin with red or pink. A. Conventional papillary thyroid cancer cells are arranged in types of islands, rich in interstitial blood sinus and cancer cells, and form a relatively consistent size; B. Pericarcinous tissue shows natural tissue morphology with normal cells and uneven follicle sizes; C. Follicles covered with simple cuboidal epithelium cells, part of the follicular epithelial cells showed papillary hyperplasia histology.

toxylin and eosin (HE) staining, the exact cancerous part was marked in the slice. Corresponding formalin-fixed paraffin blocks were prepared and sliced into appropriate sections. Cancerous tissues were marked in paraffin sections and compared with the marked HE stained tissues. The cancerous tissue was scraped into 1.5 ml clean test tubes for DNA extraction. All cancerous samples used were large, excluding fine needle aspiration and needle biopsy tissues. The PTC patients included 94 females and 26 males ranging from 17 to 70 years old, with PTC tumor with sizes ranging from 0.3 to 4.5 cm. Pericarcinous samples were randomly collected from 30 patients with PTC. Pericarcinous tissues were also verified microscopically. We also collected 25 benign tumor samples including hyperplastic nodules associated with Hashimoto's thyroiditis.

Genomic DNA isolation

Genomic DNA was isolated using the QIAamp DNA FFPE Tissue Kit (Qiagen, Germany, Cat.56404). Samples embedded in paraffin were treated by dimethylbenzene dewaxing, and washed in ethanol. Dried samples were incubated in buffer ATL and proteinase K at 56°C overnight. DNA was purified and eluted, and the final concentration was measured using a Nanodrop 2000 spectrophotometer. DNA quality was evaluated by measuring the A_{260}/A_{280} ratio.

Detection of the BRAF V600E mutation

All DNA samples were diluted to 10 ng/μl, and detection of the *BRAF* V600E mutation was performed using the Human *BRAF* Gene V600E Mutation Detection Kit provided by the Wuhan YZY Medical Science & Technology Co., Ltd. The kit was developed using an Amplification Refractory Mutation System (ARMS)-qPCR detecting method. The reaction system was performed in two PCR tubes using 20 ng of DNA in a 25 μL reaction volume containing the reference and V600E mutation detection reagent (containing PCR buffer, dNTPs, specific primers, specific probes, and Taq polymerase). The amplification procedure was subjected to 40 cycles involving uracil-N-glycosylase treatment at 37°C for 10 min, initial denaturation at 95°C for 5 min, denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. The PCR was processed in the Agilent StrataGene Mx3000P QPCR System. The detection limit of this Kit was 1% *BRAF* V600E mutation in 20 ng genomic DNA; positive and negative results could be explicitly determined according to the user instructions.

Statistical analyses

BRAF V600E-positive and -negative papillary thyroid cancer numbers were compared for each category containing gender, age, tumor size, tumor location, and clinical stage. Comparison between groups was performed using SPSS 13.0 statistical software and a χ^2 test was used for the comparisons. $P < 0.05$ was considered to be statistically different.

Results

Basic clinicopathological features of patients

In all 120 cases of PTC patients, the female: male ratio was 3.6. For the TNM tumor stage, Stages I and II accounted for 86, and Stage III and IV for 34. About 30% of the individuals car-

Table 1. *BRAF* V600E mutation in three different tissues

Tissue type	Total (n)	*BRAF* V600E mutation (%)		*P* value*
		Positive	Negative	
Papillary thyroid carcinoma	120	106 (88.33)	14 (11.67)	< 0.0001
Pericarcinous tissue	30	0 (0.00)	30 (100.00)	
Benign nodules	25	0 (0.00)	25 (100.00)	

*Papillary thyroid carcinoma Vesus Pericarcinous tissue P < 0.001; Papillary thyroid carcinoma Vesus Benign nodules P < 0.001; Pericarcinous tissue Vesus benign nodules P = 1.000.

Table 2. Correlation of *BRAF* V600E mutation with different clinicopathological characteristics

Clinical features	Total (n)	*BRAF* V600E mutation (%)		*P* value
		Positive	Negative	
Gender				1.000
Male	26	23 (88.46)	3 (11.54)	
Female	94	83 (88.30)	11 (11.70)	
Age				0.755
≥ 50	31	27 (87.10)	4 (12.90)	
< 50	89	79 (88.76)	10 (11.24)	
Tumor size, cm				0.821
≥ 1.0	55	49 (89.09)	6 (10.91)	
< 1.0	65	57 (87.69)	8 (12.31)	
Lymph metastasis				0.352
Yes	36	30 (83.33)	6 (16.67)	
No	84	76 (90.48)	8 (9.52)	
Tumor stage				0.536
I and II	86	77 (89.53)	9 (10.47)	
III and IV	34	29 (85.29)	5 (14.71)	
Tumor lesion				0.399
Left	59	54 (91.53)	5 (8.47)	
Right	45	39 (86.67)	6 (13.33)	
Both	13	10 (76.92)	3 (23.08)	
Isthmus	3	3 (100.00)	0 (0.00)	

ried lymph node metastasis. Among 25 individuals with benign nodules, 4 were male and 21 were female with a female: male ratio of 5:3. The range of age was 33~62.

Pathological characteristics of three kinds of tissues

All samples diagnosed with PTC and benign nodules were traced using HE staining, and typical staining results of three tissue types are shown in **Figure 2**.

BRAF V600E mutations in three tissue types

Among all the procedures performed in Mx-3000P, the positive and negative controls in each procedure were effective and correct. One hundred and seventy-five cases of PTC, pericarcinous, and benign tumor tissues were analyzed for the *BRAF* V600E mutation (**Table 1**). The results indicated that in 120 cases of cancerous samples, *BRAF* V600E mutations were found in 106 (88.3%) conventional PTC tumors, and 14 cases were *BRAF* V600E-negative. No *BRAF* V600E mutations were detected in 30 cases of pericarcinous tissues and 25 cases of benign tumor tissues. Amplification plot of one *BRAF* V600E positive and negative result is shown in **Figure 1**.

The prevalence of *BRAF* V600E mutations in PTC is 88.3%, and 0% in pericarcinous tissue and benign nodules; therefore, the difference between PTC and other two groups is statistically significant (P < 0.05).

Analysis of BRAF V600E prevalence in different categories in PTC

As described above, different clinicopathological features involving age, tumor size, lymph node metastases, gender, tumor stage were classified and analyzed for the correlation with the status of the *BRAF* V600E mutation. No statistical significance were shown in the prevalence of *BRAF* V600E mutation depending on the age (P = 0.755), gender (P = 1.000), tumor size (P = 0.821), lymph node metastasis (P = 0.352), and lesion location (P = 0.399) (**Table 2**).

Discussion

The *BRAF* V600E mutation is the most common genetic mutation in PTC and generally occurs in approximately 29-83% of all cases [20]. In the present study, we analyzed the prevalence of the *BRAF* V600E mutation in different tissues, and especially in PTC, we reported an exceedingly high prevalence of the *BRAF* V600E mutation (88.3%). Moreover, no *BRAF* mutation was detected in benign nodules and pericarcinous tissue, which raised the possibil-

Table 3. Comparison of different detection methods used in somatic mutation

	Sanger sequencing	Pyrosequencing	HRM	PCR-SSCP	RFLP	ARMS-qPCR
Detection limit	20%	5%	5-10%	10-20%	5-10%	1%
Specificity	96-100%	90%	100%	70~80%	80-90%	100%
Operation time	6 hrs	4 hrs	2 hrs	6-8 hrs	6-8 hrs	2 hrs
Operational simplicity	Easy	Easy	Difficult	Medium	Medium	Easy
Cost	Low	Medium	Low	Medium	High	Low

ity that *BRAF* V600E acts as a determinant factor in PTC tumorigenesis. In fact, there are some differences in the prevalence of *BRAF* V600E mutations in different subtypes of PTC. Lee *et al*. [21] exhibited different distributions of *BRAF* V600E mutations in different histological subtypes. They found that *BRAF* V600E mutations are most frequent in tall cell variants of PTC (79.1%), and then in conventional PTC (59.1%). In the present study, we observed a higher prevalence of *BRAF* V600E mutations in PTC. Several reasons can be elucidated for this striking distribution: no fine-needle aspiration (FNA) biopsy tissue of papillary thyroid cancer was used, and inaccurate detection results could be obtained from minute tissue samples and operation instability, and in contrast, large mass tissues diagnosed pathologically were less susceptible; exact cancerous tissues were detected and scraped from the slice, contributing a significant role in reducing false-negative result from the pericarcinous portion. Interestingly, pericarcinous tissues in group B have been proven as negative.

Detection of somatic mutations in tumor tissues including direct sequencing, pyrosequencing, PCR-RFLP (polymerase chain reaction restriction fragment length polymorphism), SSCP (Single-Strand Conformation Polymorphism), was most used in current research [22-30]. Sensitivity, specificity, and other indexes of different detection methods were compared (**Table 3**), and direct sequencing was demonstrated to be a more reliable method with a sensitivity of 83% and specificity of 96% than PCR-RFLP with a sensitivity of 78.6% and specificity of 80% [30, 31]. The mutation detecting methods used in the present study, ARMS-qPCR is a conventional technology for measuring somatic mutation in tumors [32]. Kits can reach a detection limit at 0.5-1% mutation in 10 ng genomic DNA, which is exceedingly more sensitive than the methods mentioned above. According to the manufacturer's instructions, several samples harbored a *BRAF* V600E mutation less than 5% ($6 < Ct_{mutation}\text{-}Ct_{reference} < 9$), which could be assumed as negative by other detection methods. Consequently, we identified a higher prevalence of *BRAF* V600E mutations in the present study.

The prognostic value of *BRAF* V600E mutations in PTC has been controversial in different studies recently, Ito *et al*. [33] investigated *BRAF* V600E mutations in 631 patients with PTC having median follow-up periods of 83 months, and they finally indicated that *BRAF* V600E mutations did not significantly reflect the aggressive characteristics and poor prognosis of patients with PTC in Japan. Also, Kim *et al.* [34] studied 60 patients with conventional micro-PTC, and *BRAF* V600E mutations were detected in 31 of 60 PTC patients (52%), and the age distribution, tumor size, extrathyroid extension, and staging did not differ significantly between patients with and without the *BRAF* V600E mutation. On the contrary, many reports concluded that *BRAF* V600E mutations show poor prognostic relationships in positive PTC patients. In the present study, we discussed the mutation status in different groups classified by clinical features of PTC. It was observed that *BRAF* V600E mutations did not significantly correlate with age, gender, tumor size, lymph node metastasis, and different lesion locations ($P > 0.05$). Among 14 *BRAF* V600E-negative patients, we studied the correlation with clinical features, and determined that *BRAF* V600E-negative individuals did not show significant differences with age, gender, tumor size, lymph node metastasis, and lesion locations ($P > 0.05$).

In the present study, *BRAF* V600E mutation prevalence in PTC and benign tumors was significantly different. Samples harboring V600E mutations were all cancerous tissue and no *BRAF* mutations were found in benign tissue. *BRAF* V600E mutations showed extremely high

specificity in non-cancerous samples including pericarcinous tissues and benign tumor tissues. The detection of other genetic alterations in the MAPK pathway including *BRAF* V600K, *BRAF* V600D, *Kras,* and *Nras* mutation was also performed in 14 V600E-negative samples. Similarly to V600E mutations with a 1% detection limit, the mutations mentioned above were negative in the 14 samples (data not shown), which raised compelling evidence that the constitutive activation of the RAS/RAF gene in the MAPK pathway plays a predominant role in the pathogenesis of PTC. Therefore, individuals with conventional PTC tumorigenesis could harbor other mutations such as the RET/PTC rearrangement in the MAPK pathway or driver gene alterations in other signal pathways. These suggestions may assist pathologist to determine whether cancer occurred due to aberrantly activated BRAF proteins. Testing for the *BRAF* mutation may be a useful predictor of tumorigenesis in PTC.

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Disclosure of conflict of interest

None.

Authors' contribution

HZ performed the molecular genetic studies, nucleic acid extraction and detection, and drafted the manuscript. CWX, YFW, QHM, YYS, JJW, HTW, HYW, and XBL selected and prepared the samples used in this study, and analyzed their clinical features. HZ, TY, and ZZ designed the study and performed the statistical analyses. BZ and CLC conceived the study, and contributed to the manuscript design, coordination, and drafting. All authors read and approved the final manuscript.

Address correspondence to: Dr. Cong-Li Cai, Wuhan YZY Medical Science & Technology Co., Ltd, Wuhan 430075, Hubei, People's Republic of China. E-mail: caicongli@yzybio.com

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