

Original Article

Association between BRAF V600E mutation and regional lymph node metastasis in papillary thyroid carcinoma

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Abstract: Background: *BRAF* V600E is the most frequent genetic alteration in papillary thyroid carcinoma (PTC); there are ongoing conflicts on its association with regional lymph node metastasis. And we aimed to test this association in a referred sample in a single institute in China. Methods: We analyzed *BRAF* V600E mutational status in the primary lesion of 150 PTC cases in Peking Union Medical College Hospital (PUMCH) and their corresponding lymph node metastasis (if present and available) using a validated Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR) method. Results: Among 150 PTC cases, 121 (80.6%) primary tumors harbored *BRAF* V600E mutation, 66.9% (81/121) and 79.3% (23/29) had regional lymph node metastasis (LNM) in cases detected with and without *BRAF* V600E mutation, respectively ($P = 0.195$). The *BRAF* V600E mutational status of most of the metastatic lesions was not different to that of their primary foci (73 out of 76 cases, 96.1%, Kappa value = 0.893). The 3 inconsistent cases were all mutation positive for primary tumors and mutation negative for LNM. Conclusion: No association was established between *BRAF* V600E mutation and regional lymph node metastasis in PTC in Chinese patients.

Keywords: BRAF, lymph node metastasis, intratumor heterogeneity

Introduction

PTC is an indolent malignancy. However, while most PTC patients have a favorable clinical outcome after surgery, quite a few of them suffer from compromised life quality imposed by the need for long term medical follow-ups, in the presence of the risk of metastasis and recurrence of the disease, the latter one of which greatly impairs prognosis for the patients [1].

To date, *BRAF* V600E analysis has been widely adopted in the management of PTC patients. *BRAF* V600E stands for a Valine to Glutamic acid substitution in codon 600 (V600E) that results in the activation of the Mitogen Activated Protein Kinase (MAPK) pathway, prompting tumor cells to grow, survive and invade [2]. Meanwhile, in *BRAF* V600E mutant PTC, the expression of iodine absorption-related genes was found to be impaired [3, 4]. In a recent large-cohort, multiple-center study by Xing et

al., *BRAF* V600E mutation was found to be associated with higher mortality rate in PTC patients [5]. However, the correlation between *BRAF* V600E mutation and parameters for aggressiveness such as tumor size, multifocality and metastasis remains controversial [6-10]. As most existing studies have been based on populations in western countries, data from China are limited.

In solid tumors, some cells may harbor certain genetic alterations that are absent in the others. Known as intratumor heterogeneity, it sometimes leads to distinct biological behaviors of cells in a single tumor. The study by Glinger et al. suggested that intratumor heterogeneity may foster tumor evolution and adaptation through Darwinian selection [11]. But heterogeneous *BRAF* was not recognized on PTC until one or two years ago [12]. And whether the mutation played a role in LNM of PTC remains unknown to the best of our knowledge.

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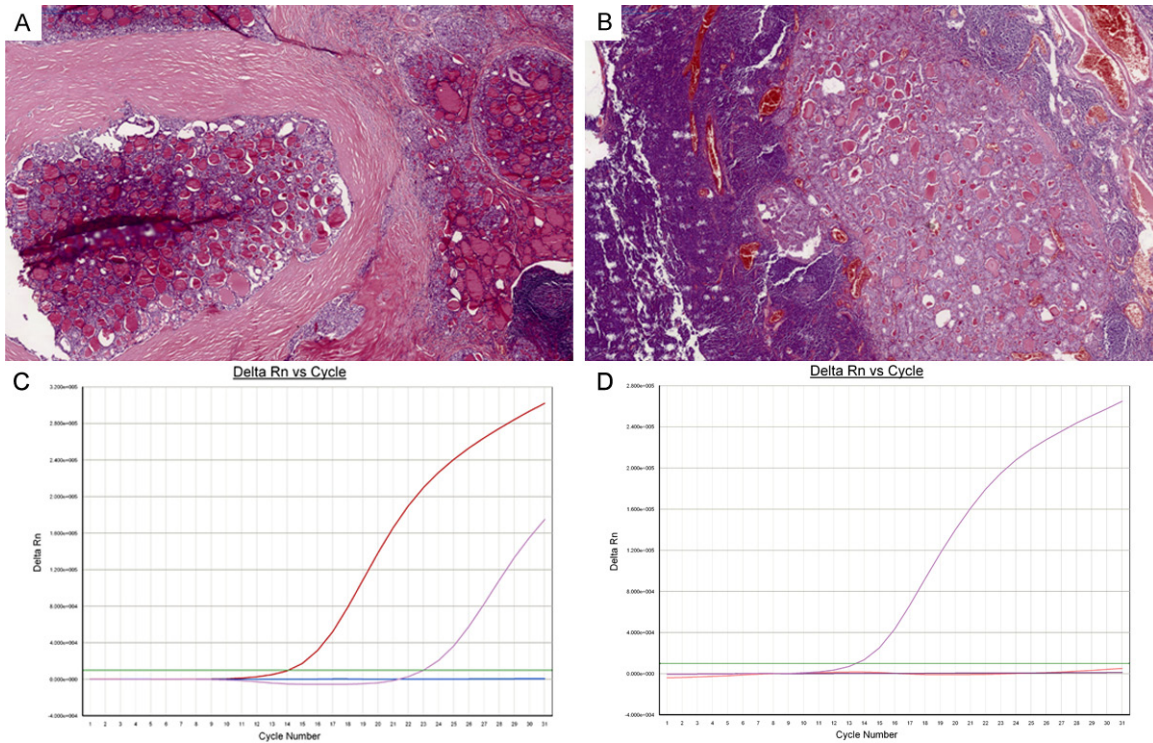


Figure 1. Exemplary figures of cases with distinct BRAF V600E status in primary tumor and LNM. A. Primary tumor. B. Corresponding lymph node metastasis. C. Amplification plot of primary tumor showing BRAF V600E mutation detected. D. Amplification plot of corresponding LNM showing BRAF V600E mutation undetected.

Therefore, in the present study, we sought to explore the association between *BRAF* V600E mutation and regional lymph node metastasis (LNM) in PTC, and determine whether the genetic mutation preferentially exerts PTC cells to invade regional lymph nodes by comparing *BRAF* V600E mutational status of primary lesions and corresponding metastatic lymph nodes.

Materials and methods

Materials

Formalin-fixed, paraffin-embedded (FFPE) tissue blocks from 150 patients who underwent thyroidectomy with histologically confirmed PTC from April 2013 to February 2014 in Peking Union Medical College Hospital (PUMCH) and referred to our laboratory for *BRAF* V600E mutation testing were retrieved from the tissue bank of pathology department. Detailed clinical pathological features of the enrolled patients were demonstrated in **Figure 1**. Written informed consent was acquired from each patient and the protocol was approved by the ethics committee of the hospital.

Among the overall 150 PTC cases, 104 had lymph node metastasis upon surgery. However, we were only able to collect sufficient tissue from the metastatic lesion in 76 cases due to either unavailable tissue blocks or insufficient cancer cells in the lymph nodes.

Sample preparation

Five μm -thick FFPE sections were mounted onto microscopic slides and deparaffinized using xylene and ethanol. For each tissue block one slide was preserved for Hematoxylin and Eosin (H&E) staining. Experienced pathologists reviewed the H&E slides and outlined cancerous regions. These very regions on corresponding, unstained slides were then macrodissected by dispensable syringe tips comparatively.

For small metastatic lesions in lymph nodes, in order to minimize contamination by lymphocytes, the unstained slides were placed under a microscope. After minimizing the aperture, the contour and pattern of the cancerous regions was identifiable when comparing to the H&E slide. Margin of the lesions was then outlined and trimmed off by a syringe tip.

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Table 1. Clinical pathological features of PTC cases

Characteristics	BRAF V600E status of primary tumor		P
	Mutation Detected	Mutation Not Detected	
Gender			
Male	39	3	0.021*
Female	82	26	
Age of onset			
< 45 years	75	21	0.119
≥ 45 years	46	8	
Tumor Size			
< 1 cm	71	12	0.172
≥ 1 cm	44	16	
Multifocality			
Yes	57	13	0.737
No	61	16	
LNM			
Yes	81	23	0.195
No	40	6	

Note: Data of tumor size and multifocality was unavailable in 7 and 3 cases, respectively.

All trimmed off tissues were collected to a 1.5 ml collection tube with 180 ul preloaded lysing buffer ATL (1063369, QIAGEN). In the case of multiple lymph node involvement, all metastatic lesions were collected to one collection tube when feasible.

DNA extraction, quantification and dilution

DNA of collected tissues was extracted using the QIAGEN QIAamp DNA FFPE Tissue Kit (56404, QIAGEN) following the protocol provided by manufacturer. Spectral absorbance of DNA was measured by a spectrophotometer (Merinton SMA4000), and DNA was diluted to approximately 2-3 ng/ul with elution buffer ATE (QIAGEN).

Mutation detection

A validated, China Food and Drug Administration (CFDA) - approved (State medical permitment number No. 2010-3401226) Human BRAF V600E ARMS-PCR Kit (Amoy Diagnostics, Xiamen, China) based on Amplification Refractory Mutation System (ARMS) was used for the detection of BRAF V600E mutation. For each sample, there was an external control assay and a mutation assay (in the same well). Each

run contained a negative control and a positive control. Thermo cyclings were as follows: Stage 1:5 minutes @ 95°C; Stage 2:25 seconds @ 95°C, 20 seconds @ 64°C, 20 seconds @ 72°C, repeated for 15 cycles; Stage 3:25 seconds @ 93°C, 35 seconds @ 60°C, 20 seconds @ 72°C, repeated for 31 cycles. Data was collected at 60°C stage 3 (Life Technologies Prism 7500 series real-time PCR instrument). Run files were analyzed and interpreted based on the manual of manufacturer.

Statistical analysis

Data collected was processed by IBM SPSS statistics 19 (IBM, USA). Consistency of BRAF V600E mutational status in primary and metastatic lesion was assessed by Kappa Test; correlation between BRAF V600E mutational status in primary lesion and LNM was estimated by Chi-Square Test; same method was adopted to estimate correlations between BRAF V600E mutation and age of onset and multifocality of primary lesions; Univariate analysis was also performed to evaluate the association between BRAF V600E mutation and primary tumor size. $P < 0.05$ is considered statistically significant.

Results

We included 150 cases in our study, with 42 males and 108 females, aged 13 to 73 years (median 43 y/o). Average primary tumor size was 1.00 ± 0.71 cm (**Table 1**).

Among the 150 cases enrolled, 121 (80.1%) were BRAF V600E mutation-detected judging from the primary lesions. 81 out of 121 (66.9%) mutation detected and 23 out of 29 (79.3%) mutation undetected cases had histologically confirmed regional lymph node metastasis, respectively. However, the association between regional lymph node metastasis and BRAF V600E mutation was statistically insignificant [$\chi^2 = 1.683$, $P = 0.195$; OR = 0.528, 95% Confidence interval = (0.199, 1.401)] (**Table 1**).

The mutational status of metastatic lymph nodes of 76 cases was studied. BRAF V600E mutation was detected in 56 (73.7%) cases. In 3 cases the primary lesions were mutation positive while their matched lymph node metastases were mutation negative (see **Figure 1**). In other 73 cases the BRAF V600E mutational status was highly concordant in the primary

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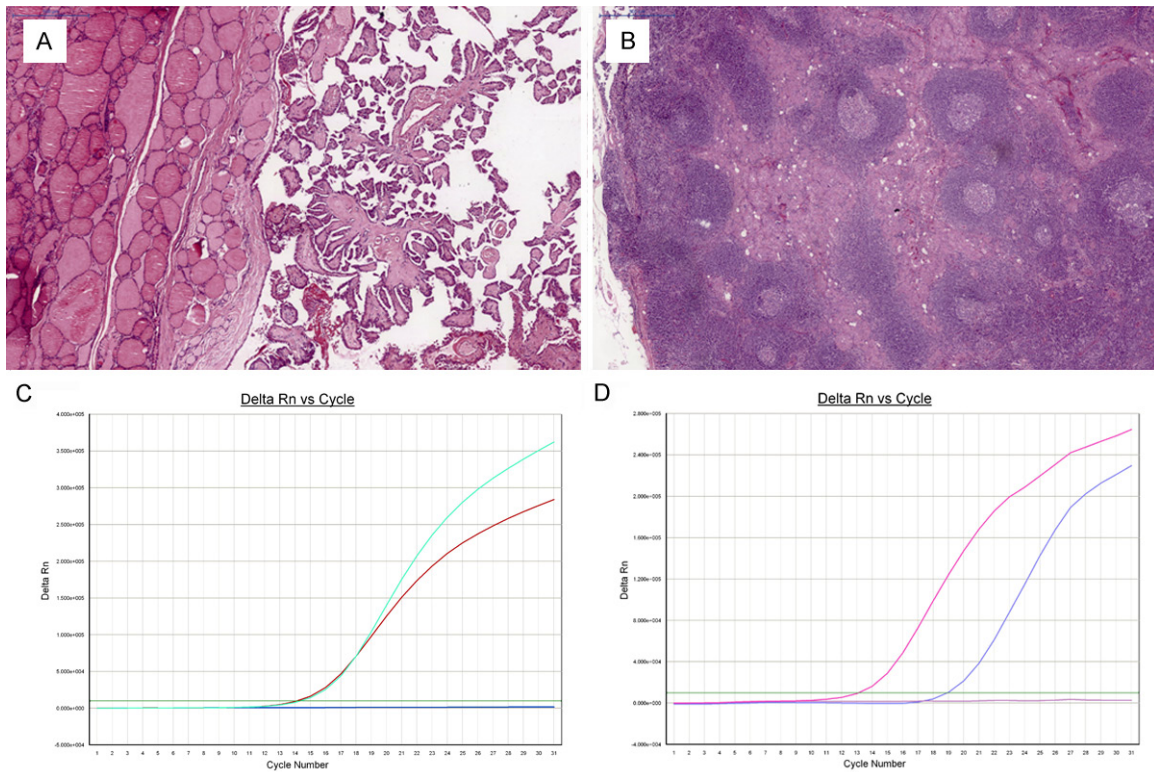


Figure 2. Exemplary figures of cases with identical BRAF V600E status in primary tumor and LNM. A. Primary tumor. B. Corresponding lymph node metastasis. C. Amplification plot of primary tumor showing BRAF V600E mutation detected. D. Amplification plot of corresponding LNM showing BRAF V600E mutation detected.

lesions and their matched lymph node metastases (96.1%, Kappa value = 0.893) (See **Figure 2**).

The associations between BRAF V600E mutation and other pathological features such as multifocality ($P = 0.737$), age of onset ($P = 0.119$) and primary tumor size ($P = 0.172$) were also studied and reported no statistical significance with one exception, which was the correlation with gender ($P = 0.021$).

Discussion

Growing number of evidence has shown that BRAF V600E mutational status is associated with a higher risk of recurrence and mortality, and can be a potential target for personalized treatment. But controversies persist in terms of its correlations with certain clinicopathological features such as LNM, extrathyroidal invasion, tumor size, multifocality and distant metastasis.

In the present study, we tested the association between BRAF V600E mutation and lymph

node metastasis in PTC in a Chinese population with a pre-validated, highly sensitive and specific method. Our study revealed a high prevalence of BRAF V600E mutation in the cases enrolled, and no association was established between BRAF V600E mutation and LNM of PTC. These two findings were in conflict with most studies that exist.

Tufano et al. reported 45% overall prevalence of BRAF mutation in their meta-analysis that included 2470 patients from 9 different countries in 2012 [13]; and research by Lim et al. reported 73.9% overall prevalence of the mutation in a Korean cohort of 3130 PTC cases in 2013 [8]. Both of them reported significant association between BRAF V600E mutation and LNM. However, neither study included patients from China, where iodine compound was a regular supplement in diet salt. As whether large iodine intake is a protective or a risk factor for PTC remains another controversy, whether excessive iodine constitutes de-novo carcinogenic pathway of PTC and thus results in different impact of BRAF V600E mutation in China awaits further exploration.

To further address this issue, we compared *BRAF* V600E mutational status in primary and matched metastatic lesions of our PTC cases. High concordance in *BRAF* status, which was consistent with an earlier study of similar design [14], suggested that codon 600 of *BRAF* gene, whether mutant or wild-type, was largely retained during the course of extra-thyroidal invasion. Furthermore, given that intratumor heterogeneity on codon 600 of *BRAF* was a frequent event in PTC [15], if *BRAF* V600E mutation promoted LNM, cells that harbor the mutation should out-compete their wild-type peers in the primary lesions and constitute the majority of metastatic lymph nodes. However three cases in our study lost mutant alleles during the course of regional lymph node invasion while not the contrary. Although central compartment lymph node invasion could happen early in PTC patients, it is undeniable that LNM happens at least one step later than the rise of primary lesion. Thus we tend to believe that *BRAF* V600E mutation is a rather early event. In the presence of intratumoral heterogeneity, whether it's *BRAF* mutant or not, certain cells were preferentially selected by factors other than *BRAF* V600E mutation during microevolution and invaded regional lymph nodes, grew faster or became multifocal. This hypothesis is agreed by the latest study of de Biase D et al. [16]. And we thus conclude that *BRAF* V600E mutation was not involved in the process of LNM.

Recent study by Xing M et al., which enrolled 1849 cases, claims that *BRAF* V600E mutation is associated with poorer outcome in PTC patients [5]. Another study by Yasuhiro et al., which enrolled 766 cases, drew similar conclusions [16]. As to how *BRAF* V600E increases mortality of PTC patients without prompting tumor growth or lymph node invasion, one explanation is that the mutation down-regulated the expression of genes associated sodium-iodine symporter, impairing the ability for PTC cells to absorb radioactive iodine, compromising the efficacy of radiotherapy and finally led to an increased risk of tumor recurrence [17]. However, in-depth study is required to provide further evidence for this hypothesis.

A decade ago Hingorani S.R. et al demonstrated that suppression of *BRAF* V600E led to inhibition of malignant transformation in melanoma, revealing its significance in the initiation of malignancy [18]. Later in 2008, Cheung et al.

suggested that *BRAF* V600E alone was not sufficient for nevi to transform into melanoma, as overly activated MAPK pathway inhibited tumor progression [19]. The feedback inhibition mechanism of MAPK pathway in melanoma was further recognized in the research of drug resistant mechanism later on [20]. If what happens to melanomas can be applied onto PTC, it will be reasonable to infer that *BRAF* V600E functions solely as an initiator in tumorigenesis of PTC, given that over 80% cases in our study harbored *BRAF* V600E mutation. It also explains why *BRAF* V600E mutant PTC cases failed to show more aggressive behavior than their wild-type peers to certain extent.

Major limitations of the present study include the followings: First of all, as most cases in our study harbored *BRAF* V600E mutation, the mutation-negative arm might be less well represented. Out of the interest of time and resources, we were unable to increase the sample size in the present study. But a larger sample is desired in the future.

Second, unlike Howell et al., who reported association between central compartment lymph node invasion and *BRAF* V600E mutation in PTC in their beautifully designed study, we did not stratified our patients with factors such as surgical regime and lateral/central compartment LNM due to unavailability of clinical information to control potential confounding factors.

While no association was established between *BRAF* V600E mutation and regional LNM or other parameters of tumor aggressiveness, testing for this mutation remains an important predictor for the sensitivity to radioactive iodine therapy and risk of recurrence in the clinical settings. The result of our study also indicates that regional lymph node metastasis is a feasible specimen source for the screening of *BRAF* V600E mutation when primary lesion is unavailable.

In conclusion, no association was established between *BRAF* V600E mutation and regional lymph node metastasis in PTC in Chinese patients.

Disclosure of conflict of interest

None.

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