Investigation of BRAF mutation in a series of papillary thyroid carcinoma and matched-lymph node metastasis with ARMS PCR

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ABSTRACT

Objectives: To figure out that if there is a consistency relationship of the $BRAF^{V600E}$ mutation in matched-lymph node metastasis and original papillary thyroid carcinoma (PTC) specimen for the same patient.
Methods: We collected the specimen of thyroids and matched-lymph node metastases of PTCs and tested the $BRAF^{V600E}$ mutation status with amplification refractory mutation system (ARMS) PCR.
Results: 20 patients with PTC and metastasis lymph node were hired. In this cohort, 16 (80%) patients had the same *BRAF* genetic mutation status in thyroid and metastasis, and the other 4 (20%) had an inconsistent situation.
Conclusions: Within our cohort, the data suggested that wild-type $BRAF^{V600E}$ oncogene in thyroid primary tumor does not rule out its mutation in lymph node metastasis, and vice versa.

1. Introduction

Papillary thyroid carcinoma (PTC) is the most common (accounts for approximately 80–90%) subtype of thyroid carcinoma [1,2], and its incidence is increasing all around the world [3]. Although PTC has an excellent prognosis with standard surgery, thyroid-stimulating hormone (TSH) suppressive therapy and ablation of the thyroid remnant with radioactive iodine (RAI), a significant recurrence rate (20% at 10 years and 30% at 30 years approximately) is observed, and many patients still succumb to it [4]. Besides, for recurrent patients who are probably resistant to radioiodine therapy and not eligible for surgery, the therapeutic option is very limited [5]. Therefore, stratification is important to identify patients with high risk of recurrence, then more aggressive therapy and monitoring can be implemented.

Lymph node metastasis ratio is very high in PTC patients [6,7]. In detail, they are found in 30%–65% of cases at the time of initial diagnosis, and 15% of tumors with lymph node metastasis also display very aggressive behavior, characterized by local invasion, distant metastasis, treatment resistance, and increased mortality [8,9]. As such, it is widely accepted currently that surgery for PTC patients should include thyroidectomy and also therapeutic central neck dissection (CND) in

patients who present with clinically evident positive lymph nodes [10]. While, for patients yet not shown evident positive lymph nodes, prophylactic lymphadenectomy or a more extensive primary resection is controversial [10].

BRAF is a serine-threonine kinase that strongly activate more distal pathways integral for cell proliferation, differentiation, division and growth [11]. The $BRAF^{V600E}$ mutation (T1799 A transversion in exon 15 of chromosome 7) is the most common and accounts for more than 90% of all the *BRAF* mutations [12]. The $BRAF^{V600E}$ mutation is one of the most prevalent somatic genetic events in human cancer, and constitutive activation of MAP kinase pathway with *BRAF* mutation leads to tumorigenesis [13,14]. Remarkably, this mutation has consistently been reported to be specific for PTC [15], it occurs exclusively in PTC and PTC-derived anaplastic thyroid cancers but not in benign thyroid neoplasms or follicular thyroid carcinoma (FTC) or medullary thyroid carcinoma (MTC) or normal thyroid tissue [16]. More interestingly, it has been reported that $BRAF^{V600E}$ mutation is potential to be an independent molecular marker with good sensitivity for the diagnosis of PTC patients. The presence of *BRAF* mutation in papillary thyroid carcinoma has been associated with the tumor recurrence, the absence of tumor capsule and tumor iodine avidity, and also treatment failure of

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recurrent disease, independently [16]. Sorafenib, the most commonly and widely used nonspecific small molecular *BRAF* inhibitor, has been reported to re-induce RAI uptake in patients with progressive metastatic or advanced RAI refractory PTC in several prospective phase II and III studies [17–21].

Although the previous findings support the correlation between the $BRAF^{V600E}$ mutation and more advanced clinical stage and aggressive tumor behavior [22], the association between this genetic event and the presence of lymph node metastasis is still controversial [23]. So, clinical decision making based on *BRAF* mutational status should be considered carefully, because it has been previously reported that the absence of the *BRAF* mutation in the primary tumors does not rule out its presence in metastatic lesions [24,25]. The objective of our research is to figure out that if there is a consistency relationship of the $BRAF^{V600E}$ mutation in lymph node metastasis and original thyroid specimen.

2. Methods and materials

2.1. Case selection

Initially, a representative series of 65 patients confirming with PTC and also lymph node metastasis who underwent thyroidectomy during the period of 2014–2015 was selected from Binzhou People's Hospital (Shandong province, China). And samples of 24 patients were collected for sequencing of $BRAF^{V600E}$ mutation with ARMS (Amplification Refractory Mutation System) PCR. Ultimately, 20 of them had successfully sequenced for both the thyroid and lymph node metastasis. The female to male ratio was 3:2, and the age at diagnosis ranged from 24 to 64 years (mean, 48.1 ± 11.7 years). All the patients were diagnosed with PTC through intraoperative frozen section examination and all samples were fixed in a timely manner within 30 min. The lymph node metastases were removed when radical surgery was operated and been fixed within in 30 min. All samples were fixed with 10% neutral buffered formalin, embedding with paraffin. The slides of haematoxylin-eosin (HE) staining were diagnosed by two high-age pathologists as PTC independently, all the diagnosis was made on the basis of the World Health Organization classification of tumors and the classification system of the American Joint Committee on Cancer. The study protocol was approved by the Hospital Human Ethics Review Committee.

2.2. DNA isolation

Briefly, 5 μm slides were cut from formalin-fixed paraffin-embedded (FFPE) samples. One slide was HE stained for pathologic review to make sure that the tumor part is more than 20% of the whole tissue. After the tumor area of interest was detected, other unstained slides were deparaffinized and the slides were manually scraped with a scalped blade. Using the FFPE DNA extraction kit (ADx-FF01, AmoyDx, Xiamen, Fujian, China) to isolate DNA from FFPE samples. The OD value of DNA samples were measured by NanoDrop 2000 spectrophotometer (Thermo fisher, Waltham, Massachusetts, USA).

2.3. Determination of $BRAF^{V600E}$ mutation with ARMS PCR

Using Human *BRAF* Gene^{V600E} Mutation Fluorescence Polymerase Chain Reaction (PCR) Diagnostic kit (Cat No. ADx-BR01, AmoyDx, Xiamen, Fujian, China) to detect the mutation status of *BRAF* oncogene. The sensitivity of this kit allows detection of 1% mutant DNA in a background of 99% normal DNA at 10 ng sample DNA amount. Amplification was performed in a reaction volume of 40 μl containing of Mix Buffer, *BRAF* Taq Polymerase, DNA sample or $BRAF^{V600E}$ mixed standard (STD) or ddH$_2$O (NTC). PCR conditions were as follows: (i) 95 ℃ for 5 min, (ii) 15 cycles of 95 ℃ for 25 s, 64 ℃ for 20 s, and 72 ℃ for 20 s, (iii) 31 cycles of 93 ℃ for 25 s, 60 ℃ for 35 s, and 72 ℃ for 20 s. Collect the fluorescence signal of FAM (external signal) and HEX/VIC (internal control) at the stage of 60 ℃ in (iii). The Ct value was calculated automatically after the reaction. ABI Prism model 7500 fluorescence quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA) was hired to sequence the BRAF gene mutant.

2.4. Statistical analysis

The $BRAF^{V600E}$ mutation in focal and corresponding lymph node metastasis were analyzed using chi-square test using Prism 6 (GraphPad, San Diego, CA, USA). P < 0.05 was considered as statistically significant.

3. Results

3.1. Case data and pathologic confirmation

Of the 65 PTC patients we initially screened diagnosed as PTC during 2014–2015, we collected the FFPE samples of both original thyroid and corresponding lymph node metastasis for 24 of them and sequenced the $BRAF^{V600E}$ mutation with ARMS PCR. Eventually, 20 PTCs were successfully sequenced in both sites in our research. This report represents those patients who were diagnosed with papillary thyroid cancer both in thyroid and lymph node metastasis (Fig. 1). This 20-case cohort contains of 12 females (60%) and 8 males (40%). The mean age is 48.1 ± 11.7 years (range, 24–64 years), with 7 (35%) being ≤45 years and 13 (65%) being > 45 years. Relationships between $BRAF^{V600E}$ and clinical pathological variables are shown in Tables 1 and 2. There was no significant relationship between BRAF mutation and gender, age (> 45 years), tumor size (> 1 cm), and number of primary PTC foci, at the time of diagnosis.

3.2. BRAF wild-type in the primary tumors does not rule out its mutation in metastatic lesions, vice versa

Both the FFPE samples of thyroid and corresponding lymph node metastasis were tested for $BRAF^{V600E}$ mutation status (Fig. 2). Sequencing test confirmed that PTC patients had a majority mutation of $BRAF^{V600E}$. In this cohort, 16 (80%) patients were mutated in thyroid and 14 (70%) were mutated in lymph node metastasis, separately. 13
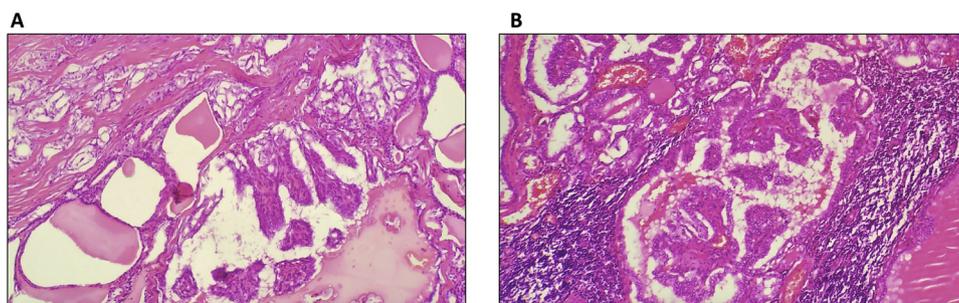
Fig. 1. Representative HE staining for PTC patient. (A) Original thyroid site, (B) Corresponding lymph node metastasis for the same patient.

Table 1
Relationships between the status of BRAF mutations and clinical pathological features of PTCs in thyroid focal.

	$BRAF^{V600E}$ positive (n = 16)	$BRAF^{V600E}$ negative (n = 4)	P
Sex			
Male	5	3	
Female	11	1	ns
Age			
≤ 45 years	5	2	
> 45 years	11	2	ns
Tumor size			
≤ 1 cm	3	0	
> 1 cm	13	4	ns
Multi-centric			
1	12	3	
≥ 2	4	1	ns

Table 2
Relationships between the status of BRAF mutations and clinical pathological features of PTCs in matched-lymph node metastasis.

	$BRAF^{V600E}$ positive (n = 14)	$BRAF^{V600E}$ negative (n = 6)	P
Sex			
Male	4	4	
Female	10	2	ns
Age			
≤ 45 years	5	2	
> 45 years	9	4	ns

(65%) were mutated both in thyroid and corresponding metastasis, only 3 (15%) were wild-type both in thyroid and corresponding metastasis, another 4 (20%) were mutated in one site. Of the 4 patients who had only one site mutation, 3 were mutated in thyroid focal, and 1 was mutated in lymph node metastasis. Taking together, 16 (80%) patients had the same *BRAF* genetic mutation status of this oncogene in thyroid and metastasis, and the other 4 (20%) had an inconsistent situation, but there was no statistical significance (p = 0.0609) (Fig. 3).

4. Discussion

In the present series, the overall prevalence of $BRAF^{V600E}$ mutation in original thyroid cancer is 80% (16/20), and 70% (14/20) in matched-lymph node metastasis. Our data are consistent with the observations of previous investigations [26,27]. Of all the patients, only 1 patient (5%) has wild type *BRAF* in original thyroid tissue and mutant

BRAF in corresponding lymph node metastasis, and 3 (15%) others have mutant BRAF in thyroid and wild type *BRAF* in metastasis, and 16 (80%) patients have the same *BRAF* status in both sites. So, there is a chance that the $BRAF^{V600E}$ wild-type status in thyroid does not rule out its mutation in lymph node metastasis, and vice versa. On the other side, there is a large opportunity that the metastasis *BRAF* gene status is in consistent with the thyroid focal, though no statistical significance is found based on our cohort.

In mainstream view, *BRAF* confers more aggressiveness to the behavior of PTC, correlated with more frequent early recurrences and predicting poorer outcomes. It is reported that about 40% of metastatic PTCs with $BRAF^{V600E}$ mutation show a progression from well-differentiated status at the time of diagnosis to a poorly differentiated status during the follow-up [28]. Those *BRAF* mutated patients progressively lose the functional features of thyroid follicular cells, and the ability to uptake and organify I^{131}, and induced a poor prognosis [29]. But, in our series, 3 of 20 cases with $BRAF^{V600E}$ positive tumor in primary not presenting the $BRAF^{V600E}$ in the respective lymph node metastases, and 1 of 20 cases did not harbor the $BRAF^{V600E}$ in the primary thyroid harboring the mutation in respective lymph nodes. It is possible because that the $BRAF^{V600E}$ is not absolutely the primary episode for PTC metastasis to lymph node, though this mutation is very important in the progression and seeding of PTC cells to lymph node metastasis.

Despite the generally good prognosis of PTCs, there are still a lot of patients who will develop metastatic diseases and fail to respond to radioactive iodine (RAI) treatment. Sorafenib, the molecular inhibitor targeted $BRAF^{V600E}$, has been approved for differentiated thyroid cancer refectory to RAI by Food and Drug Administration for metastatic RAI-refractory thyroid cancer [30,31]. When it is needed in clinical, we suggested both the samples of primary thyroid and lymph node metastasis should be used for $BRAF^{V600E}$ gene sequencing whenever it is possible.

To our knowledge, very few investigations have rigorously examined the $BRAF^{V600E}$ mutation status in primary and corresponding lymph node metastasis separately in the same patient with PTC for guiding the need for surgical intervention or therapy management. Though the number of cohort in this study is small, we systematically investigated *BRAF* mutation in primary and matched lymph node metastasis with ARMS PCR for the first time. Though direct sequencing is considered as the gold standard for genetic mutation detection, but it's easy to get false positive result because of low sensitivity, it need at least 20% mutant DNA in tumor tissue then can be detected [32]. ARMS PCR, based on the real-time PCR technology, is highly selective and sensitive (allowing detection of 1% mutant DNA in a background of 99% normal DNA at 10 ng sample DNA amount), with reasonable cost and simple operation, it is more suitable for clinical specimen for gene
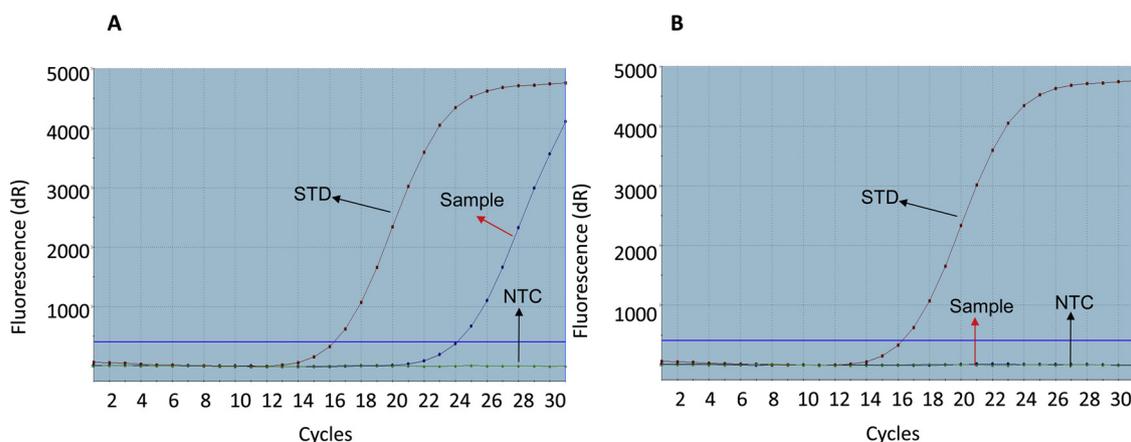

Fig. 2. BRAF V600E mutation detection with ARMS PCR. (A) Representative curve of sample with mutant BRAF. (B) Representative curve of sample with wild-type BRAF.
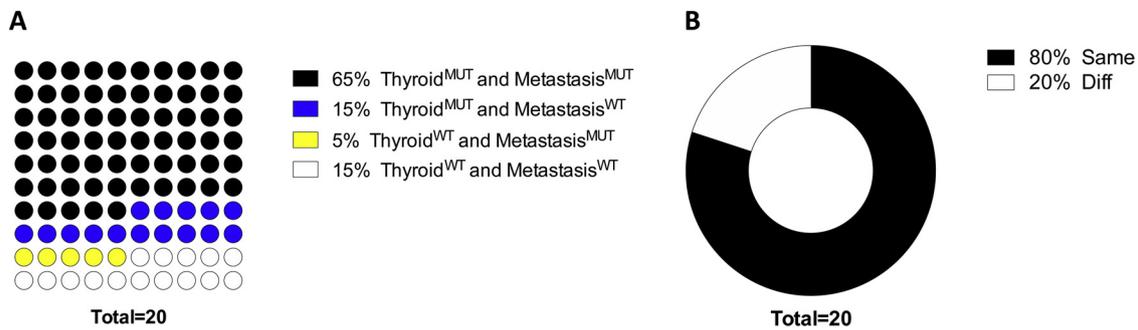
A

- ● 65% Thyroid^(MUT) and Metastasis^(MUT)
- ● 15% Thyroid^(MUT) and Metastasis^(WT)
- ● 5% Thyroid^(WT) and Metastasis^(MUT)
- ○ 15% Thyroid^(WT) and Metastasis^(WT)

Total=20

B

- ■ 80% Same
- □ 20% Diff

Total=20

Fig. 3. BRAF V600E mutation distribution status in thyroid focal and matched-lymph node metastasis with PTC patients. (A) Ratio of BRAF V600E mutation in thyroid and metastasis separately. (B) 80% of patients have the same BRAF V600E status in thyroid and metastasis though $p > 0.05$.

mutation detection.

We acknowledge the limitation of this study in the small cohort of patients. The study is insufficiently powered to clarify a statistically significant association of whether the *BRAF* mutation status between the thyroid and matched-lymph node metastasis is in consistent, if one truly exists. Besides, follow-up information of patients was not sufficient in this study. More samples and longer follow-ups would help to clarify the role of *BRAF* mutations in the metastatic process in the future investigation.

In conclusion, we hope that our data will contribute to an important question, that is, whether assessment of *BRAF* mutation status in original thyroid tumor can conclude the mutation status in matched-lymph node metastasis, vice versa. Further to improve the management of thyroid cancer, and help to decide whether kinase inhibitor (eg. Sorafenib, approved by US FDA for PTC) should be used as coadjutant of radioiodine therapy or not.

5. Conclusions

Within our cohort, the data suggested that wild-type *BRAF^(V600E)* oncogene in thyroid primary tumor does not rule out its mutation in lymph node metastasis, and vice versa. So, for iodine-refractory and progressive metastatic PTCs, who need more aggressive therapy and monitoring, we suggested both the samples of primary thyroid and metastasis should be supplied for BRAF gene sequencing to search for more chances of effective treatment.

Disclosure

The authors declare that there is no conflict of interest for this report.

Authors contributions

Jian Zhang and Chongyong Guo designed the experiments. Yaoyao Yang, Jing Zhao, Lifang Shi, Yanhua Xu performed the experiments. Yaoyao Yang and Kaikai Yu analyzed the data. Yaoyao Yang and Jian Zhang prepared the manuscript.

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