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Original Article

Relationship between BRAF V600E gene mutation and the clinical and pathologic characteristics of papillary thyroid microcarcinoma

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Abstract: Objective: This study aims to investigate the relationship between BRAF V600E gene mutation and the clinical and pathologic characteristics of patients with papillary thyroid microcarcinoma (PTMC). Methods: A retrospective analysis was conducted on the clinical and pathologic characteristics of 89 patients with PTMC, who had complete medical records. The BRAF V600E gene mutation was detected by real-time immunofluorescence quantitative PCR. Then, the BRAF V600E gene mutation and the clinical and pathologic data of these patients were statistically analyzed. Results: Among the 89 patients with PTMC, 67 patients had a BRAF V600E mutation with a mutation rate of 75.3%. 38 patients (42.6%) had cervical lymph node metastasis. 43 patients had capsular invasion (48.3%) and 40 patients (44.9%) had tumor multifocality confirmed by postoperative pathology. BRAF V600E mutation was not associated with gender, age, capsular invasion, tumor multifocality, cervical lymph node metastasis, or higher TNM stage. However, cervical lymph node metastasis was associated with age and tumor invasion of the capsule. Thus, cervical lymph node metastasis was most likely in patients < 45 years old and patients with capsular invasion. Conclusion: BRAF V600E mutation is common in PTMC, but is not associated with clinicopathologic factors, such as age, gender, tumor multifocality, cervical lymph node metastasis, capsular invasion, or tumor TNM stage. Cervical lymph node metastasis is more likely in patients < 45 years old and patients with capsular invasion.

Keywords: Thyroid carcinoma, papillary thyroid microcarcinoma, BRAF V600E gene, mutation

Introduction

Thyroid carcinoma is the most common endocrine malignancy, among which papillary thyroid carcinoma (PTC) is the most common pathologic type, accounting for approximately 90% [1]. The World Health Organization (WHO) defines thyroid papillary microcarcinoma (PTMC) as a PTC with a maximum diameter of ≤ 10 mm [2]. With improved health awareness and the development of cervical thyroid ultrasonography, thyroid nodules that cannot be detected by palpation can now be detected by high resolution ultrasonography, and the detection rate and incidence of thyroid microcarcinoma have greatly increased. According to the global cancer report published by the WHO in 2014, PTMC accounts for more than 50% of new-onset thyroid carcinoma [3]. At present, most PTMCs are considered inert, with slow progression and good prognosis, and the 10-year survival rate is 99.5% [2]. However, a certain proportion of PTMCs have been reported to be aggressive [4]. It has been reported [5] that 66.1% of PTMCs had extracapsular invasion and cervical lymph node metastasis, and one of the initial symptoms of PTMCs was distant organ metastasis. A large retrospective study conducted by Noguchi *et al.* [6] in 2008 revealed that the recurrence rate of PTMCs with a diameter of 1-5 mm was 3.3%, while the recurrence rate of PTMCs with a diameter of 6-10 mm was 14% during a 35-year follow-up period. A retrospective study [7] in the United States demonstrated that among 61,523 patients with thyroid carcinomas, PTMCs accounted for 5.1% of patients who died of thyroid disease. However, at present, commonly used im-

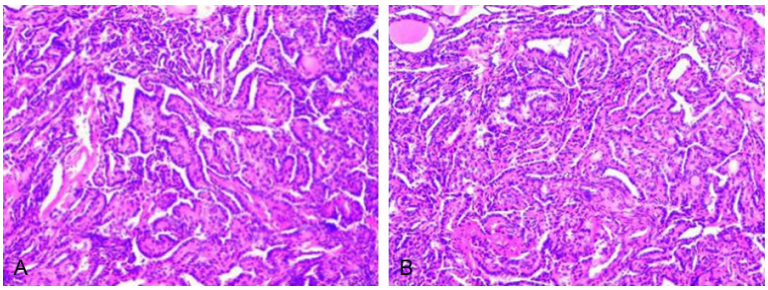

Figure 1. Hematoxylin and eosin staining of papillary thyroid carcinoma (PTC, with ×100 amplification).

aging examinations, such as ultrasonography, radionuclide imaging, and fine needle aspiration, are limited in assessing the biologic invasiveness of thyroid micronodules [8].

The V-raf murine sarcoma viral oncogene homolog B1 (BRAF) V600E gene mutation is a very common genetic event in PTC, with an incidence of 45.0%-73.4%. Mutations in this gene have been found to be associated with higher invasiveness, higher recurrence, higher risk of death, and worse prognosis [9, 10]. However, few studies have been conducted on the relationship between BRAF V600E gene mutation and the clinical and pathologic characteristics of PTMCs. As a retrospective study, the present research performed BRAF V600E gene detection through real-time immunofluorescence quantitative polymerase chain reaction in 89 patients with a clinical and pathologic diagnosis of PTMC. The relationship between BRAF V600E mutation and clinical and pathologic characteristics of PTMCs was preliminarily explored, in order to provide better treatment and prognosis tools for PTMCs.

Materials and methods

General materials

Data were obtained from 89 patients with PTMCs, who were hospitalized in the Department of Otolaryngology, Head and Neck Surgery of Beijing Shijitan Hospital from August 2012 to December 2015. All specimens were confirmed as PTMC by two pathologists during after surgery. All patients underwent thyroid color ultrasonography before the operation. According to the results of ultrasonography, thyroidectomy + isthmus section or total thyroidectomy were performed. Central lymph node dissection was performed. According to preoperative ultrasonography and intraoperative conditions, patients with cervical lymph node metastasis underwent cervical lymph node dissection. The clinicopathologic stage was based on the tumor-node-metastasis (TNM) staging standard issued by the American Thyroid Association in 2009 [11]. The inclusion criteria were as follows: (1) patients with PTMCs diagnosed by microscopic pathological examination, with a tumor diameter (measured by pathologists) of ≤ 1 cm; (2) patients with no preoperative topical radiotherapy or iodine ^{131} therapy, and thyroid hormone suppression therapy. The exclusion criteria were as follows: (1) patients with a post-operative pathologic diagnosis of follicular, medullary, undifferentiated or metastatic thyroid carcinoma; (2) patients diagnosed with multifocal PTMCs, with a focus > 1 cm in size; (3) patients with malignant tumor in any other part of the body, or with autoimmune disease.

Detection of BRAF mutation

BRAF mutation analysis was performed in the Department of Pathology in our hospital. Real-time immunofluorescence quantitative polymerase chain reaction (PCR) was used to detect the BRAF mutation. Before BRAF mutation analysis, the diagnosis of PTMC was confirmed by hematoxylin-eosin (H&E) staining under a microscope (**Figure 1**). Then, by comparing the H&E stains, the tumor-enriched areas were marked under the microscope, and the tumor-related regions from post-operative formalin-fixed paraffin-embedded (FFPE) tissues were selected. Five pieces of paraffin slices of 5 μm thick were cut and placed into five clean EP tubes. Then, the paraffined tissue was dewaxed. Afterwards, the DNA of these samples was extracted using a QIAamp DNA FFPE Tissue Kit, according to reference kit instructions (Beijing Kaijie Company). The mass and concentration of the DNA were measured using an ultraviolet spectrophotometer. The measured DNA concentration was diluted to 5-ng/ml for future assay. Using the ADx-ARMS-B-RAF detection kit (Xiamen Aide Company), the mutant gene of the exon 15 V600E of the BRAF gene was detected. The BRAF mutant and wild-type (**Figure 2**) were compared using the BRAF amplificat-

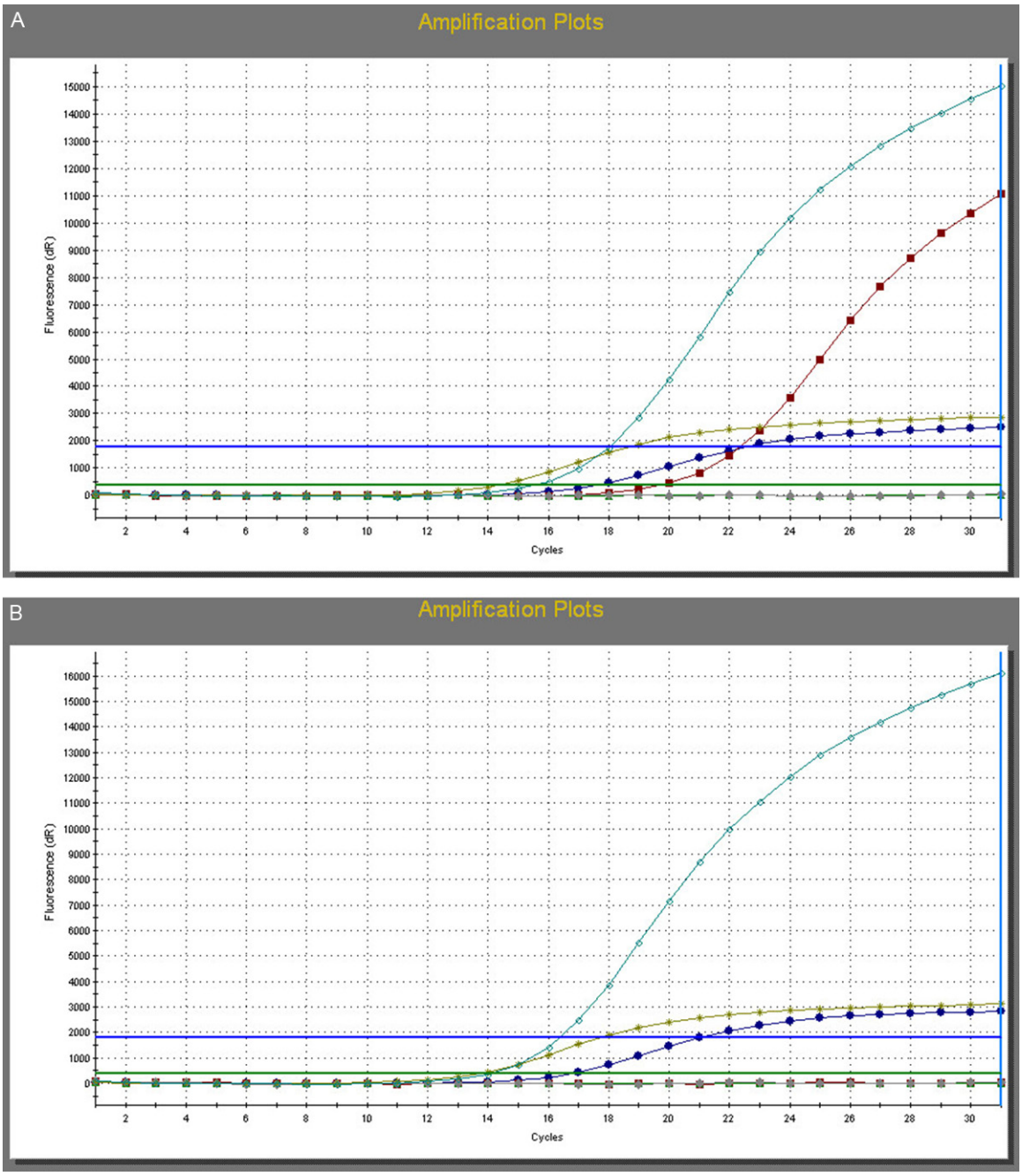

Figure 2. Amplification curve of BRAF: (A) BRAF mutation type demonstrated by the BRAF amplification curve; (B) BRAF wild-type demonstrated by the BRAF amplification curve.

ion curve to determine whether a BRAF mutation existed.

Statistical analysis

SPSS 17.0 software was used for the statistical analysis of data. Chi-square test was used in the univariate analysis. $P < 0.05$ was considered significant.

Results

Among the 89 patients with PTMCs, 21 patients were male, while 68 patients were female, and the male-to-female ratio was 1.00:3.24. The age of these patients ranged from 25-74 years, with an average age of 46.1 years and a median age of 45.5 years. Sixty-seven cases of BRAF V600E gene mutation were found in the

Table 1. Relationship between BRAF V600E gene mutation and the clinical and pathologic characteristics of papillary thyroid microcarcinoma

Variable		Cases	BRAF V600E gene				χ^2	P
			Mutation type		Wild-type			
			Cases	%	Cases	%		
Gender	M	21	17	25.4	4	18.2	0.1705	0.68
	F	68	50	74.6	18	81.8		
Age	< 45	36	28	41.8	8	36.4	0.0284	0.87
	≥ 45	53	39	58.2	14	63.6		
Capsular invasion	Yes	43	37	55.2	6	27.3	0.4800	0.49
	No	46	30	44.8	16	72.7		
Focus	Single focus	49	35	52.2	14	63.6	0.0351	0.85
	Multiple foci	40	32	47.8	8	36.4		
Lymph node metastasis	Yes	38	31	46.3	7	30.4	0.1307	0.72
	No	52	36	53.7	16	69.6		
Tumor stage	I	71	52	77.6	19	86.4	0.786	0.37
	II	0	0	0	0	0		
	III	9	7	10.4	2	9.1		
	IVa	9	8	11.9	1	4.5		

specimens, and the mutation rate was 75.3%. Age is regarded as a risk factor in the prognosis of patients with PTMC. According to the TNM staging criteria published by the American Thyroid Association in 2009, these patients were divided at 45 years of age. Among the 89 patients with PTMCs in the present study, 36 patients were under 45 years. Among these patients, 28 patients had the BRAF V600E gene mutation. Furthermore, 53 patients were over 45 years old, and among these patients, 28 patients had the BRAF V600E gene mutation. Among the 21 male patients, the BRAF V600E gene mutation occurred in 17 patients, while among the 68 female patients, 50 patients had the BRAF V600E gene mutation. Furthermore, 43 patients had capsular invasion. Among these patients, the surrounding strap muscles were invaded in two patients, while the BRAF V600E gene mutation occurred in 37 patients. Among the 46 patients without capsular invasion, 30 patients had the BRAF V600E gene mutation. Among the 49 patients with a singlefocus, the BRAF V600E gene mutation was detected in 35 patients. Among the 40 patients with multifocality in one lobe or in the bilateral lobes, 32 patients had the BRAF V600E gene mutation. Among the 37 patients with lymph node metastasis in the central part or the cervical region, the BRAF V600E gene mutation occurred in 31 patients. Among the 52 patients without lymph node metastasis, 36 patients had the BRAF V600E gene mutation. Tumor staging was performed in 89 patients, with seventy-one in stage I, 0 in stage II, nine in stage III, and nine in stage IV. Among the 71 patients in stage I + stage II, BRAF V600E gene mutations were detected in 52 patients, while among the 18 patients in stage III + stage IV, 15 patients had the BRAF V600E gene mutation. By statistical analysis, there was no statistical difference in terms of the association of BRAF V600E gene mutation with age, gender, capsular invasion, unifocal/multifocality, lymph node metastasis, and TNM stage (**Table 1**).

Among the 89 patients with PTMCs, 38 patients had lymph node metastasis confirmed by pathology after surgery, and the incidence of lymph node metastasis was 42.7%. Furthermore, 43 patients had capsular invasion, and the incidence was 48.3%. Among these patients, two of them whose thyroid's surrounding muscles were affected. In addition, 40 patients had multiple foci in one lobe or in the bilateral lobes, which accounted 44.9% of the study population. Lymph node metastasis was found in 11 of the 21 male patients, while 27 patients had lymph node metastasis among the 68 female patients. Lymph node metastasis occurred in 18 of 53 patients who were over 45 years old and in 20 of 36 patients who were less than 45 years old. Lymph node

Table 2. Relationship between lymph node metastasis, clinicopathologic features, and BRAF V600E gene mutation in thyroid microcarcinoma

Variable		Cases	Lymph node metastasis				χ^2	P
			Yes		No			
			Cases	%	Cases	%		
Gender	M	21	11	28.9	10	19.6	0.1705	0.68
	F	68	27	71.1	41	80.4		
Age	< 45	36	20	52.6	16	19.6	4.0855	0.043
	≥ 45	53	18	47.4	35	68.6		
Capsular invasion	Yes	43	26	68.4	17	33.3	4.103	0.045
	No	46	12	31.6	34	66.7		
Focus	Single focus	49	17	44.7	32	62.7	0.7602	0.38
	Multiple foci	40	21	55.3	19	37.3		
BRAF V600E mutation	Yes	67	31	81.6	36	70.6	0.2953	0.59
	No	22	7	18.4	15	29.4		

metastasis was found in 17 of 49 patients with a singlefocus and in 21 of 40 patients with multiple foci in one lobe or in the bilateral lobes. Lymph node metastasis occurred in 26 of the 43 patients with tumors invading the capsules and in 12 of the 46 patients without capsular invasion. Lymph node metastasis occurred in 31 of the 67 patients with BRAF V600E gene mutation and in seven of the 22 patients without BRAF V600E gene mutations. By statistical analysis, there was no difference in the association between lymph node metastasis and gender, multifocality, and BRAF V600E gene mutation (**Table 2**). However, there was a difference between lymph node metastasis and age and capsular invasion ($P < 0.05$); and cervical lymph node metastasis was more likely to occur in patients who were < 45 years old, and in patients with capsular invasion.

Discussion

In recent years, the incidence of thyroid carcinoma has greatly increased. Yang *et al.* [12] reported that the incidence of thyroid carcinoma in China increased by 137% in 2013, compared to that in 2010. According to the global cancer report published by the WHO in 2014, PTMC accounts for more than 50% of new onset thyroid carcinomas [3]. Studies have increasingly revealed that gene mutation is an important factor in tumorigenesis. The RAS/RAF/MEK/ERK/MAPK signaling pathway is an important transduction pathway that induces an intranuclear response [13]. In 2002, Davies *et al.* [14] first reported the BRAF V600E mutation in the MEK-ERK pathway in human malignant tumors. The BRAF V600E mutation is located downstream of RAS, which is considered as one of the key genes in PTC that initiate tumorigenesis [15]. Recent studies [16] have found that the BRAF V600E mutation is a common gene mutation in PTC. The frequency of mutations reported in the literature ranges within 25%-83%, but it does not appear in other pathologic types of thyroid malignant tumors and benign thyroid neoplasms. Since its discovery, the BRAF V600E mutation has been controversial as a diagnostic and prognostic marker of PTCs. In the present studies [17, 18] regarding the relationship between BRAF V600E gene mutation and the clinical and pathologic prognostic factors of PTMCs, it was found that there were conflicting results between the BRAF V600E gene mutation and bilaterality, multifocality, extracapsular invasion of the thyroid, and lymph node metastasis of PTMC. In order to clarify the relationship between the BRAF V600E gene mutation and the clinical and pathological prognostic factors of PTMCs, 89 patients with PTMC were grouped according to clinical and pathologic factors, such as age, gender, capsular invasion, multifocality, lymph node metastasis, and TNM stage [11], and their associations with the BRAF V600E mutation were investigated. Since the present studies have shown that cervical lymph node metastasis is the second independent risk factor that affects the survival of tumors, besides distant metastasis [19], the relationship between the clinical and pathologic factors of tumors and cervical lymph node metastasis were also analyzed.

According to these results, there were 21 male and 68 female patients in the 89 patients with PTMCs. 36 patients were < 45 years old and 53 patients ≥ 45 years old. Cervical lymph node metastasis was confirmed by pathology in 38 patients, and the rate of cervical lymph node metastasis was 42.7%. Multifocality was found in 40 patients, and capsular invasion was found in 43 patients under a microscope. Since cervical lymph node metastasis is a risk factor for local recurrence and increases the mortality of PTMCs [2, 7], these patients were stratified according to cervical lymph node metastasis, in order to investigate the association among age, gender, multifocality, capsular invasion, and BRAF V600E mutation. It was found that age and capsular invasion were risk factors for cervical lymph node metastasis ($P < 0.05$). However, gender, tumor multifocality, and BRAF V600E gene mutation was not associated with cervical lymph node metastasis. It was reported [20] that the lymph node metastatic rate ranged from 24.1%-64.1% in PTMCs. In our study, cervical lymph node metastasis was found in 38 patients (42.7%) among the 89 patients, which is similar to that reported in the literature. We found age was correlated with cervical lymph node metastasis. The patients' age was older than 45 years who had higher cervical lymph node metastasis. This is consistent with the results reported by Xu [21]. Age is a factor that affects lymph node metastasis in patients with PTMC, but it does not affect the prognosis, because as long as there is no distant metastasis, the TNM stage of tumors in patients younger than 45 years old remains stage I, regardless of cervical lymph node metastasis, and the mortality rate does not increase [11]. It has been reported [2, 18] that capsular invasion and the invasion of surrounding tissues is an independent risk factor for cervical lymph node metastasis in PTMC. In the present study, a significant correlation between tissue invasion and cervical lymph node metastasis in PTMCs was found, and cervical lymph node metastasis was more likely to occur in patients with thyroid capsular invasion. This suggests that central lymph node dissection should be performed when tumors invade the thyroid capsule during surgery in clinical practice. In the follow-up of patients after surgery, neck lymph nodes should be given attention in patients with capsular invasion, according to postoperative pathology reports. If there are suspected lymph nodes in the imaging report, a more positive approach, such as ultrasound-guided fine needle aspiration, should be adopted to determine whether the suspected lymph nodes are caused by tumor metastasis. According to the large amount of recent reports on the clinical and pathologic characteristics of patients with PTMCs in China [20, 21], cervical lymph node metastasis of PTMCs is significantly correlated with gender and the multifocality of tumors. These are high risk factors for patients with PTMCs. Males and patients with multiple foci are more likely to have cervical lymph node metastasis. However, no such association was found in our study, which may reflect a small number of cases included. Therefore, further studies are needed with an expanded number of cases.

Among the 89 patients with PTMC in the present study, 67 (75.3%) patients had the BRAF V600E gene mutation, suggesting that the BRAF V600E gene mutation is common in PTMCs. In our study, we found that the BRAF V600E gene mutation was more common in female patients, patients ≥ 45 years old, patients with capsular invasion, and patients with higher TNM stage of tumors, but there were no statistical significance. Furthermore, the BRAF V600E mutaion rate was lower in the patients' group with multifocal tumors and lymph node metastasis confirmed by pathology after surgery, but there was no statistical difference. This is different from the results of the meta-analysis of BRAF V600E mutations in PTMCs reported by Li *et al.* [18]. According to Li *et al.* [18], the mutation rate of BRAF V600E in PTMCs was 47.48%, and there was no correlation between BRAF V600E mutation and gender. In his study, there was also no difference in gene mutation rate between patients ≥ 45 years old and patients < 45 years old, when the patients were grouped by age. However, the BRAF V600E mutation was significantly associated with multifocality, extracapsular invasion of the thyroid gland, cervical lymph node metastasis, and higher TNM stage of tumors, suggesting that the BRAF V600E mutation in PTMCs might lead to more aggressive. But in the study conducted by Park *et al.* [17], real-time PCR was used in patients with PTMC, and the mutation rate of the BRAF V600E gene was 79.8%. Furthermore, the BRAF V600E gene mutation was correlated with age. Although the BRAF V600E mutation was more likely to

occur in older patients with PTMC, there was no correlation with gender, multifocality, extracapsular invasion of thyroid gland, or cervical lymph node metastasis. In our study, the BRAF V600E gene mutation rate was similar to the results reported by Park *et al.* [17]. The reason why our study's results were inconsistent with the report of Li *et al.* [18] might be that the BRAF V600E mutation was an early event in the onset of PTMCs [22]. Another possible reason might be the method we used to detect the BRAF V600E mutation was real-time PCR, which had a higher sensitivity and the rate was 75.3%, when compared to the mutation rate reported by Li *et al.* [18], which was 47.48%. However, the sample size of the present study was 89, which is relatively small. Therefore, it is necessary to further expand the sample size to probe the correlation between BRAF V600E gene mutation and the related clinical and pathologic factors in PTMC.

In conclusion, our study preliminarily explored the relationship between BRAF V600E gene mutation and the clinical and pathologic features in PTMCs. It was found that the BRAF V600E mutation is common in PTMC, but it is not associated with clinical and pathologic factors, such as age, gender, multifocality, cervical lymph node metastasis, capsular invasion, or TNM stage. However, we found that cervical lymph node metastasis was more prone to occur in patients < 45 years old and in patients with capsular invasion.

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Disclosure of conflict of interest

None.

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