

Association between BRAF^{V600E} mutation and the clinicopathological features of solitary papillary thyroid microcarcinoma

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Abstract. The B-Raf proto-oncogene serine/threonine kinase (BRAF)^{V600E} mutation is an important oncogene in the development of papillary thyroid carcinoma (PTC) and has been identified as a risk factor for poor prognosis in patients with PTC. However, whether the BRAF^{V600E} mutation is a prognostic marker in patients with solitary papillary thyroid microcarcinoma (sPTMC) has not yet been established. The present study aimed to identify the association between BRAF^{V600E} mutation and the clinicopathological features of patients with sPTMC. A total of 108 patients with sPTMC who underwent surgery at the Cancer Institute and Hospital of the Chinese Academy of Medical Sciences between December 2010 and December 2012 were analyzed retrospectively. Exon 15 of the BRAF gene was amplified using the polymerase chain reaction and direct sequencing was performed to detect the BRAF^{V600E} mutation. Statistical analysis was subsequently performed using SPSS software (version 16.0). The association between BRAF^{V600E} mutation and clinicopathological features of sPTMC was tested with the χ^2 test or Fisher's exact test, as appropriate. There were 27 males and 81 females in the cohort, who were aged between 22 and 66 years old, with an average age of 42 years. The BRAF^{V600E} mutation was found in 59 out of 108 (54.6%) patients with sPTMC. The presence of the BRAF^{V600E} mutation was demonstrated to be significantly associated with extrathyroidal extension (P=0.019), advanced Tumor-Node-Metastasis stage (P=0.007) and the presence of autoimmune thyroiditis (P=0.010). The BRAF^{V600E} mutation was not significantly associated with gender, anatomic

location or subtype of sPTMC (P>0.05). In addition, the BRAF^{V600E} mutation indicated poor prognosis in patients with sPTMC. These results suggest that the BRAF^{V600E} mutation is a risk factor for poor prognosis in patients with sPTMC. This knowledge will aid in the risk stratification and post-operative management of patients with sPTMC.

Introduction

Papillary thyroid carcinoma (PTC) is one of the most common types of endocrine malignancy and its prevalence is increasing. The incidence of PTC has continually increased between 1973-1977 and 1998-2002 (1). With increased awareness of thyroid nodular disease and advancements in ultrasonography, impalpable small papillary thyroid microcarcinomas (PTMCs) have been frequently detected. PTMC is defined as an incidental PTC with a maximum diameter ≤ 1 cm by the World Health Organization (WHO) (2). Within the past two decades, the incidence of PTMC among all thyroid cancers has nearly tripled in France (3). Patients with PTMC typically have a good prognosis, with a relatively low risk of developing distant metastases, resulting in reported mortality rates for PTMC as low as 0.5% (4). Although the prevalence of PTMC has increased in recent years, thyroid cancer-related mortality has not changed (5). The results from a meta-analysis of over 4,000 patients with PTMC demonstrated 28% lymph node metastasis, 0.6% distant metastasis, 3.3% disease recurrence and 0.3% tumor mortality incidences (6). These results suggest that the majority of PTMCs are indolent tumors that may not progress. Identifying the minority of patients with aggressive PTMCs is important in order to offer the most appropriate treatment.

Research into B-Raf proto-oncogene serine/threonine kinase (BRAF)^{V600E} mutations in PTC have increased in recent years. The activating somatic point mutation BRAF^{V600E} is the most frequent genetic alteration found in PTC and is a prognostic biomarker for the aggressive behavior of PTCs (7). BRAF^{V600E} mutation represents >90% of all the mutations found in the BRAF gene (8,9). BRAF^{V600E} mutation occurs in between 29 and 83% of PTC cases (average, 45%), depending on the population and geographical region being examined (8,10,11). The BRAF^{V600E} mutation occurs frequently in stage III and IV tumors (12).

Previous studies have reported an association between BRAF^{V600E} mutation and the severity of the clinicopathological

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features exhibited by patients with PTC, including those with multifocal disease and PTMC. However, to the best of our knowledge, an association between BRAF^{V600E} mutation and solitary PTMC (sPTMC) has not yet been reported. In the present study, the association between BRAF^{V600E} mutation and the clinicopathological features of patients with sPTMC was analyzed in order to elucidate the molecular mechanisms underlying the aggressive behavior of this tumor.

Materials and methods

Patients. The present study was retrospective and all patient data were kept anonymous. The data of 108 patients with sPTMC who had undergone thyroid surgery between December 2010 and December 2012 at the Cancer Institute and Hospital of the Chinese Academy of Medical Sciences (Beijing, China) was reviewed. The diagnosis of sPTMC was pathologically confirmed in all patients based on the specimen size (maximum diameter ≤ 10 mm) and the presence of a solitary PTMC tumor. The majority of patients underwent a lobectomy and isthmus gland resection under general anesthesia. Central compartment neck dissection was performed when an enlarged lymph node or invasion of the thyroid capsule was detected during surgery. All specimens were fixed in formalin and embedded in paraffin. In the present study, archived histological specimens from all patients were retrieved and analyzed. Specimen size and the presence of a solitary tumor was confirmed through hematoxylin and eosin staining. Other tumor features, such as histopathological subtype were classified based on the World Health Organization classification of tumors (2). Tumor staging was performed according to the seventh edition of the tumor-node-metastasis (TNM) classification by the American Joint Committee on Cancer (13).

Patient medical histories were analyzed, including their age at diagnosis, gender, lobe position of the tumor, primary tumor diameter, histological subtype of PTC, lymph node status and TNM stage. The tumors of all patients in the cohort were tested for the BRAF^{V600E} mutation.

DNA extraction and BRAF^{V600E} mutation analysis. Areas of the specimens that were enriched in tumor cell populations was marked through hematoxylin and eosin staining prior to analysis. Tissue was scraped from this preselected area and transferred to an Eppendorf tube for DNA isolation using the QIAamp[®] DNA Mini kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's protocol. The quality and concentration of DNA samples was examined using a Nano-Drop 1000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Exon 15 of the BRAF gene was amplified using PCR as previously described (14). Sequencing of the PCR products was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the same primers. Sequencing reaction products were put through capillary electrophoresis using a 3500xL Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Statistical analysis. Statistical analysis was performed using SPSS software (version 16.0; SPSS Inc., Chicago, IL, USA). Pearson's Chi-squared or Fisher's exact tests were used to compare the association between BRAF^{V600E} mutations and

Table I. Clinicopathological characteristics of patients with solitary papillary thyroid microcarcinomas.

Clinicopathological characteristic	No. of patients (%)
Age (years old)	
<45	65 (60.2)
≥ 45	43 (39.8)
Gender	
Male	27 (25)
Female	81 (75)
Anatomic location	
Left	59 (54.6)
Right	45 (41.7)
Isthmus	4 (3.7)
Tumor size (mm)	
≤ 5	15 (13.9)
> 5	93 (86.1)
Extrathyroid extension	
Absent	61 (56.5)
Present	47 (43.5)
Perineural invasion	
Absent	93 (86.1)
Present	15 (13.9)
Histological subtype	
Classic	97 (89.8)
Follicular	11 (10.2)
Tumor stage	
T1	65 (60.2)
T3	43 (39.8)
Node stage	
N _x	14 (13.0)
N0	52 (48.1)
N1a	32 (29.6)
N1b	10 (9.3)
TNM stage	
I	83 (76.9)
III	19 (17.6)
IVa	6 (5.5)
Thyroid background	
Nodular goiter	38 (35.2)
Hashimoto's thyroiditis	5 (4.6)
Lymphocytic thyroiditis	26 (24.1)
Granulomatous thyroiditis	1 (0.9)
Normal	38 (35.2)

clinicopathological features. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Clinicopathological characteristics of patients with sPTMC. Clinicopathological characteristics of the patients

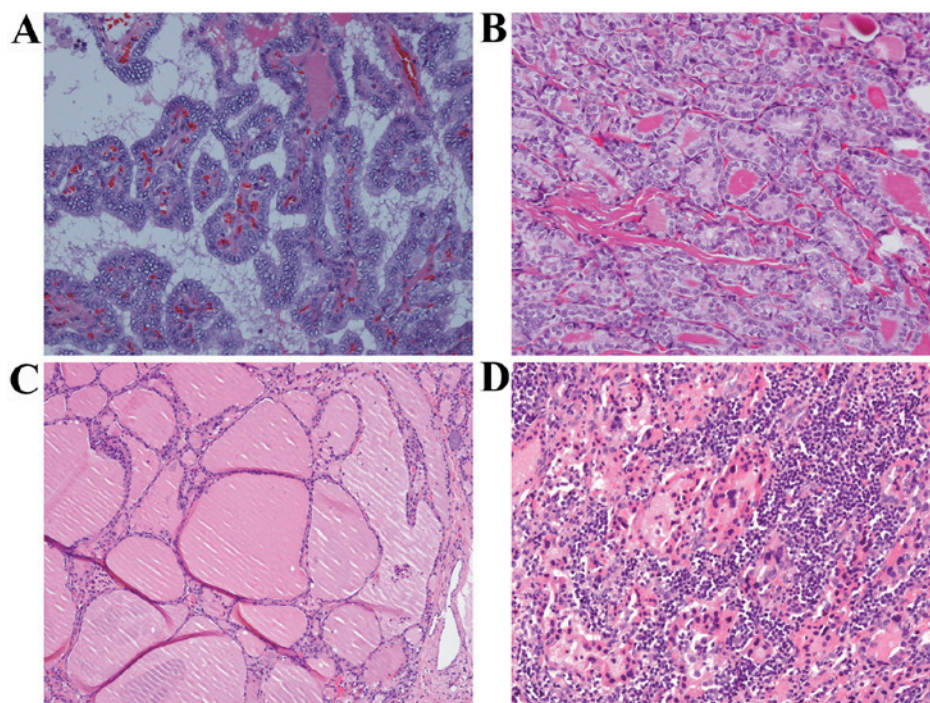


Figure 1. Hematoxylin and eosin staining of sPTMC samples for (A) the classical variant (magnification, x200), (B) the follicular variant (magnification, x200), (C) the nodular goiter (magnification, x100) and (D) Hashimoto's thyroiditis (magnification, x200).

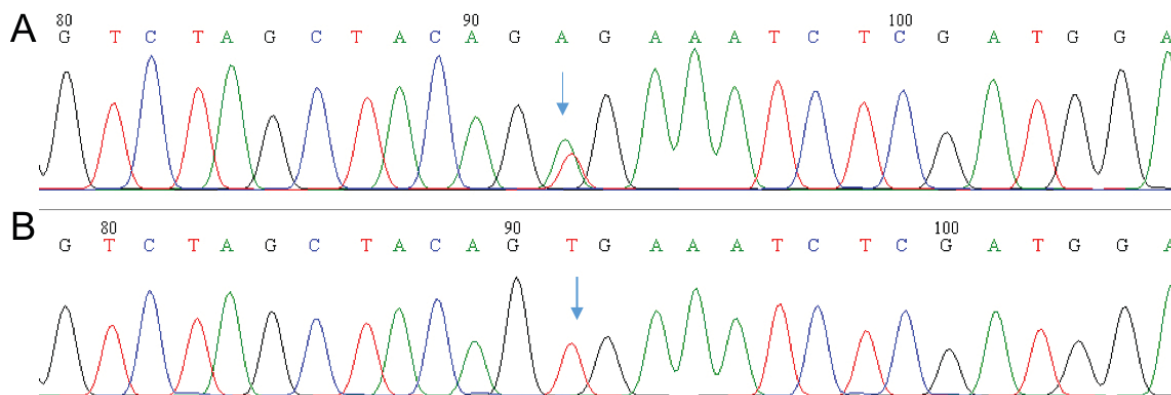


Figure 2. Representative sequencing of exon 15 of BRAF. (A) The V600E (GTG to GAG) mutation. (B) wild-type sequence. Blue arrow indicates mutated base in BRAF^{V600E} mutation. BRAF, B-Raf proto-oncogene serine/threonine kinase.

are illustrated in Table I. There were 27 (25%) male and 81 (75%) female patients in the present study. The mean age of the patients was 42 years old (range, 22-66 years). There were 65 patients <45 years old (60.2%) and 43 patients ≥45 years old (39.8%). The average tumor size was 7.2 mm (range, 2-10 mm). The number of patients with a tumor located in the left lobe, right lobe or isthmus was 59 (54.6%), 45 (41.7%) and 4 (3.7%), respectively. Extrathyroid extension was present in 47 patients (43.5%). In total, 97 (89.8%) and 11 (10.2%) patients had classic and follicular tumor subtypes, respectively (Fig. 1A and B). The lymph node status of 94 patients who underwent neck dissection was identified as the following: N0 (n=52; 48.1%); N1a (n=32; 29.6%); and N1b (n=10; 9.3%). Nx lymph node status was identified in the remaining 14 patients (13.0%). Patient thyroid backgrounds were nodular goiter (n=38; 35.2%), Hashimoto's thyroiditis (HT; n=5; 4.6%), lymphocytic thyroiditis (LT; n=26;

24.1%), granulomatous thyroiditis (n=1; 0.9%) and normal (n=38; 35.2%) (Fig. 1B and C). Sixty-five patients (60.2%) had T1 stage tumors, none had T2 and 43 (39.8%) had T3 stage tumors. Patients' TNM stage was separated into group I (n=83; 76.9%), group III (n=19; 17.6%) and group IVa (n=6; 5.5%).

Association between the clinicopathological characteristics of patients with sPTMC and BRAF^{V600E} mutation. The BRAF^{V600E} mutation was observed in 59 out of 108 patients (54.6%) through sequencing (Fig. 2). Table II demonstrates the association between clinicopathological characteristics and BRAF^{V600E} mutation in sPTMC. The following four clinicopathological characteristics were significantly associated with BRAF mutation: Extrathyroid extension (P=0.019, Pearson's Chi-squared test), tumor stage (P=0.032, Pearson's Chi-squared test), advanced TNM stage groups (III/IVa;

Table II. Association between clinicopathological characteristics and BRAF^{V600E} mutation in solitary papillary thyroid microcarcinoma.

Clinicopathological characteristic	BRAF gene status		P-value
	Wild-type [no. of patients/total (%)]	V600E mutation [no. of patients/total (%)]	
Age (years old)			0.081 ^a
<45	34/65 (52.3)	31/65 (47.7)	
≥45	15/43 (34.9)	28/43 (65.1)	
Gender			0.658 ^a
Male	11/27 (40.7)	16/27 (59.3)	
Female	38/81 (46.9)	43/81 (53.1)	
Anatomic location			0.543 ^b
Left	26/59 (44.1)	33/59 (55.9)	
Right	20/45 (44.4)	25/45 (55.6)	
Isthmus	3/4 (75)	1/4 (25)	
Tumor size (mm)			0.269 ^a
≤5	9/15 (60)	6/15 (40)	
>5	40/93 (43)	53/93 (57)	
Fibrous capsular invasion			0.159 ^a
Absent	13/22 (59.1)	9/22 (40.9)	
Present	36/86 (41.9)	50/86 (58.1)	
Extrathyroid extension			0.019 ^a
Absent	34/61 (55.7)	27/61 (44.3)	
Present	15/47 (31.9)	32/47 (68.1)	
Perineural invasion			0.582 ^a
Absent	41/93 (44.1)	52/93 (55.9)	
Present	8/15 (53.3)	7/15 (46.7)	
Histological subtype			0.541 ^a
Classic	43/97 (44.3)	54/97 (55.7)	
Follicular	6/11 (54.5)	5/11 (45.5)	
Tumor stage			0.032 ^a
T1a	35/65 (53.8)	30/65 (46.2)	
T3	14/43 (32.6)	29/43 (67.4)	
Node stage			0.495 ^b
N0	21/52 (40.4)	31/52 (59.6)	
N1a	17/32 (53.1)	15/32 (46.9)	
N1b	4/10 (40)	6/10 (60)	
TNM stage			0.007 ^b
I	44/83 (53)	39/83 (47)	
III	3/19 (15.8)	16/19 (84.2)	
IVa	2/6 (33.3)	4/6 (66.7)	
Thyroid background			0.010 ^b
Nodular goiter	19/38 (50)	19/38 (50)	
HT	4/5 (80)	1/5 (20)	
LT	16/26 (61.5)	10/26 (38.5)	
GT	0/1	1/1 (100)	
Normal	10/38 (26.3)	28/38 (73.7)	

^aPearson's Chi-squared test; ^bFisher's exact test. BRAF, B-Raf proto-oncogene serine/threonine kinase mutation; HT, Hashimoto's thyroiditis; LT, lymphocytic thyroiditis; GT, granulomatous thyroiditis.

P=0.007, Fisher's exact test) and thyroid background (P=0.010, Fisher's exact test) There were more cases of the BRAF^{V600E}

mutation in sPTMC where extrathyroid extension was present compared with sPTMC where it was absent [32/47, (68.1%)

vs. 27/61, (44.3%). Of the patients, there were 65 with T1a [BRAF^{V600E}, n=30 (46.2%)] and 43 with T3 [BRAF^{V600E}, n=29 (67.4%)]. In addition, out of the patients 83 had TNM stage I [BRAF^{V600E}, n=39 (47%)], 19 had TNM stage III [BRAF^{V600E}, n=16 (84.2%)] and 6 had TNM stage IVa [BRAF^{V600E}, n=4 (66.7%)] sPTMCs. BRAF^{V600E} mutation frequency was higher in advanced sPTMCs compared with early stage sPTMCs. The frequency of the BRAF^{V600E} mutation in patients with a normal thyroid background (n=28; 73.7%) was higher compared with those with a background of HT (n=1; 20%) or LT (n=10; 38.5%). Histological examination identified 97 classic and 11 follicular sPTMCs among the patients. The frequencies of the BRAF^{V600E} mutation were 55.7 and 45.5% (54/97 vs. 5/11) in patients with classic and follicular sPTMCs, respectively. The mutation was more frequently identified in classic subtype tumors compared with follicular variants. However, statistical analysis showed no significant difference in BRAF^{V600E} mutation frequency between these groups. The follicular sPTMC variant was uncommon in the study group (11/108; 10.2%). There were no other subtypes identified in the current study.

Discussion

In the present study, BRAF^{V600E} mutation was present in 54.6% (59/108) of patients with sPTMC. This frequency is higher when compared with another large Chinese study, which reported a frequency of 40.1% that included multifocal cases (15). Possible reasons for this difference might be the detection method for BRAF^{V600E} mutation or the cases chosen for the studies. Guerra *et al* (16) observed that the prevalence of BRAF^{V600E} mutation was higher when using pyrosequencing than compared with BigDye Terminator sequencing. Another reason for the differences in the results may be another effect of sPTMC that was present in the multifocal cases. These results indicate that BRAF^{V600E} mutation is associated with prognostic factors of sPTMC, including superficial (subscapular) tumor location and a more advanced tumor stage. Similarly, other reports identified the same association in PTC and PTMC (17-20). BRAF^{V600E} mutations have been identified in several histological variants of PTC. The prevalence of BRAF^{V600E} mutations was higher in conventional PTC (51-67.7%) compared with the follicular variant of PTC (17-24%) (16,21-23). In the current study, the prevalence of the BRAF^{V600E} mutation was not significantly associated with sPTMC subtype; however, the prevalence was higher in the classic compared with the follicular subtype (55.7 vs. 45.5%).

Several previous analyses have demonstrated that the BRAF^{V600E} mutation is a risk factor for disease persistence or recurrence in PTC (21,22,24). However, this could not be verified in the current study, as no follow-up data was included in the analysis due to the positive outcome of the patients.

The present study identified that the prevalence of BRAF^{V600E} mutation is lower in those with a background of LT or HT compared with other conditions, such as nodular goiter or a normal thyroid background. Lim *et al* (17) reported a similar result, observing that PTC with a background of LT was significantly lower in those with the BRAF^{V600E} mutation compared with those with wild-type BRAF (28.2 vs. 46.6%;

P<0.001). In addition, Lang *et al* (25) reported a negative association between BRAF^{V600E} mutation and HT. The BRAF^{V600E} mutation group in this study had significantly lower rates of co-occurring HT compared with the wild-type BRAF group (34.6 vs. 52.5%; P<0.001), which supports the results found in the current study. These results suggest that PTC with co-occurring HT or LT develop through a different biological mechanism compared with PTCs in patients with other thyroid backgrounds; however, this mechanism remains to be elucidated (26). In future, the respective roles and the association between BRAF^{V600E} mutation and thyroid background in the pathogenesis of PTC should be evaluated.

In general, patients with PTC have a good prognosis. Thus, the need for radioiodine ablation, the degree of thyroid stimulating hormone suppression and the intervals between follow-up visits are still in dispute, particularly in low-risk patients, such as those with sPTMC. The results of the current study indicate that the BRAF^{V600E} mutation is associated with factors that predict poor prognosis in patients with sPTMC. This suggests that BRAF^{V600E} mutation is a useful diagnostic and prognostic marker for PTC. However, further investigations into the underlying mechanisms and pathological diagnosis of PTMC are still warranted. In future, molecular subtyping will be useful in refining the risk stratification and personalizing the postoperative management of patients with PTC (27).

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