Original Article

Combined expression of the *BRAF*^{V600E} mutation and PD-L1 in early papillary thyroid carcinoma and its relationship with clinicopathological features and recurrence—a retrospective cohort study

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Background: Identifying the high recurrence group of patients with early-stage papillary thyroid cancer (PTC) is the greatest challenge in the management of this disease. It has been noted that B-type Rafkinase (BRAF) V600E mutation and programmed death ligand 1 (PD-L1) are associated in PTC and highly expressed in PTC, correlating in PTC as potential prognostic biomarkers. However, whether they can be used to predict the aggressiveness and recurrence of early PTC remains unclear.

Methods: Clinicopathological data of 137 patients with early PTC [tumor-node-metastasis (TNM) stage I–II] who underwent surgery in Zhejiang Cancer Hospital between 2008 and 2010 were retrospectively analyzed. *BRAF*^{V600E} mutation and PD-L1 was detected by immunohistochemistry. The median follow-up time was 136 months (interquartile range 5.8). The presence of tumor confirmed by imaging or pathology or lymph node metastasis was considered as tumor recurrence. The association of both alone and in combination with clinicopathological features and recurrence was statistically analyzed respectively. The risk of recurrence was assessed using Cox regression models.

Results: Most of the 137 early PTC were female (78.1%). The mean age was 43.2±12.1 years. The median tumor size was 1.4 cm; 14 patients developed recurrence during follow-up period; 56 patients (40.9%) were detected positive for *BRAF*^{V600E} mutation; 76 patients (55.5%) were detected positive for PD-L1. Patients with both *BRAF*^{V600E} mutation and PD-L1 expression had larger tumors (P=0.038), were more likely to have extrathyroidal invasion (P=0.045), and had a lower rate of cervical lymph node metastasis (P=0.046). The recurrence rate was 17.5% (7/40) in patients with *BRAF*^{V600E} mutation and PD-L1 double expression compared to 8.9% (4/45) in patients with *BRAF*^{V600E} mutation and PD-L1 double negative [hazard ratio (HR) =1.267; 95% CI: 0.841–1.909; P=0.257]. Survival curves showed flatter recurrence-free survival (RFS) curves in positive *BRAF*^{V600E} mutation only and PD-L1 expression only, whereas decreased sharply in positive

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expression of both *BRAF*^{V600E} mutation and PD-L1; however, the differences were not significant (P>0.05).

Conclusions: The combination of *BRAF*^{V600E} mutation and PD-L1 to identify group at higher risk of recurrence in early PTC has insufficient clinical evidence and should be used with caution in the clinical management of PTC.

Keywords: *BRAF*^{V600E} mutation; programmed death-ligand 1 (PD-L1); early stage; papillary thyroid carcinoma prognosis

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Introduction

In the past 30 years, the incidence of thyroid cancer has been steadily increasing, and it is expected to continue to increase in the next decade (1). According to statistics, thyroid cancer accounted for 93.8% of the new cases of endocrine system malignant tumors in 2021 (2). Papillary thyroid carcinoma (PTC) is the most common histological subtype of thyroid cancer. Improvements in diagnostic techniques, an increase in monitoring methods, and the screening of thyroid diseases under large-scale physical examination have led to the increasing incidence of PTC. Most of the new cases are early stage [tumor-node-metastasis (TNM) stage I–II] indolent PTC, with a high survival rate and good prognosis. After surgical treatment and regular postoperative follow-up, the 10-year survival rate is 95% (3,4). However, some patients still show invasive clinical processes, especially recurrence (5). Therefore, early detection of PTC patients with high rates of aggression and recurrence is particularly important. Early identification of patients with high recurrence in early PTC is the current clinical management dilemma of PTC. It has been suggested that new molecular-based management will help address this challenge.

With the in-depth study of the pathogenesis of PTC, the significance of molecular biomarkers in the occurrence and development of PTC has become more apparent. Studies have found that molecular indicators play important roles in assessing and predicting the biological behavior and prognosis of tumor invasion, helping to guide clinical treatment and determine prognosis (6-8). The murine sarcoma filtering viral oncogene homolog B1, known as BRAF, is a serine/threonine kinase that is usually activated by binding to RAS to affect the MAPK signaling pathway and has the ability to inhibit tumor cell proliferation and induce cell apoptosis. BRAF can cause oncogenic mutations in a variety of malignant tumors, especially malignant melanoma and colorectal cancer. The mutation frequency of BRAF is as high as 40–80% in PTC, and BRAF mutations mostly occur in the early tumor stage and are initiators of tumorigenesis (9). The most common BRAF mutation site in PTC is the T1799A point mutation, expressly, the replacement of A by T at position 1799 in exon 15 (T1799A), resulting in the substitution of valine with glutamic acid at position 600 in the encoded product, in other words, the *BRAF*^{V600E} mutation. This mutation abnormally activates the RAF/MEK/MAPK signaling pathway, leading to the occurrence of PTC and affecting tumor progression (10-13). The *BRAF*^{V600E} mutation can be used to guide the diagnosis of PTC. Preoperative fine-needle aspiration biopsy (FNAB) with detection of the *BRAF*^{V600E} mutation

Highlight box

Key findings

- *BRAF*^{V600E} mutation and PD-L1 are significantly correlated in early PTC. The combined expression of *BRAF*^{V600E} mutation and PD-L1 suggests a higher risk of invasiveness and recurrence.

What is known and what is new?

- *BRAF*^{V600E} mutation in PTC is correlated with the PD-L1 protein. When considered separately, these two factors are often associated with higher aggressiveness and poor prognosis of PTC.
- The coexistence of the *BRAF*^{V600E} mutation and PD-L1 suggests a higher risk of invasiveness and recurrence.

What is the implication, and what should change now?

- Using these two factors can help to identify patients with relatively high risk and prone to recurrence in the early stage of PTC, improve the risk stratification of PTC, and be used to develop individualized treatment plans and optimize follow-up plans. it is recommended to detect *BRAF*^{V600E} mutation and PD-L1 in early PTC patients after surgery.

facilitates the determination of benign or malignant thyroid nodules that cannot be diagnosed by FNAB alone, effectively improving the accuracy of the clinical diagnosis of thyroid nodules (14). Many studies have found that PTC patients with the *BRAF*^{V600E} mutation often have high disease invasiveness and poor prognosis, thus making it a valuable prognostic marker of the disease (13,15-19). Given the high prevalence of *BRAF*^{V600E} mutation, a universal recommendation for aggressive treatment for all positive PTC is unrealistic. More precise risk stratification strategies need to be developed in conjunction with other molecular markers to guide treatment. Meanwhile, some researchers have noted that for nonhigh-risk early (TNM stage I–II) PTC patients, the *BRAF*^{V600E} mutation maybe cannot predict recurrence and tumor invasiveness after long-term follow-up. It has even been suggested that to avoid unwarranted anxiety, *BRAF*^{V600E} should not be tested to determine prognosis in such patients (20-22).

Programmed death-ligand 1 (PD-L1) negatively regulates the immune response process, can mediate the immune escape of tumors, promote tumor growth, is often overexpressed in PTC, is associated with increased tumor invasiveness, and is associated with metastasis and poor prognosis (23,24). The association between the *BRAF*^{V600E} mutation and PD-L1 expression has been reported in a variety of cancers. For example, the simultaneous expression of these two markers in colon cancer has been shown to have prognostic significance (25). Multiple current studies have shown that the *BRAF*^{V600E} mutation in PTC is correlated with the PD-L1 protein (26,27). Therefore, this study aimed to examine the expression and correlation between the *BRAF*^{V600E} mutation and PD-L1 in early PTC and to investigate their relationship, clinicopathological characteristics, and recurrence to further improve the risk stratification of early PTC, and more accurately identify high-risk patients who would benefit from aggressive treatment and surveillance. We present the following article in accordance with the REMARK reporting checklist (available at https://gs.amegroups.com/article/view/10.21037/gs-22-701/rc).

Methods

Patients and clinicopathological data

To explore the feasibility of combining *BRAF*^{V600E} mutation and PD-L1 to predict recurrence of early PTC, this study preliminary retrospectively enrolled a total of 200 patients with early PTC who underwent surgical treatment in the Department of Head and Neck Surgery of Zhejiang Cancer Hospital between December 2008 and March 2010 in succession. The inclusion criteria were as follows: (I) 18–80 years of age; (II) routine postoperative pathological examination suggesting PTC; (III) complete clinical and pathological data; and (IV) no serious organic lesions in important organs. The exclusion criteria were as follows: (I) a history of thyroid surgery or treatment; (II) a history of other malignant tumors; and (III) a history of radiotherapy or chemotherapy or exposure to radioactive materials.

The surgical plan was developed based on the American Thyroid Association (ATA) guidelines for the management of patients with thyroid nodules and differentiated thyroid carcinoma (28). On the basis of each individual's condition, total thyroidectomy or unilateral thyroid lobe combined with isthmus resection was performed. All patients underwent ipsilateral cervical lymph node dissection (CLND). Referencing guideline recommendations and considering individualized differences, the decision to perform lateral CLND and radioactive iodine treatment was determined based on each patient's condition. Pathological examination of the resected lymph nodes was performed routinely after surgery. General demographic data were collected, such as age at diagnosis, sex and so on. Pathological data such as tumor size, number of lesions, capsule invasion, extraglandular invasion, lymphatic, vascular, and nerve invasion, cervical lymph node metastasis (CLNM) in the central region, Hashimoto's thyroiditis (HT), and distant metastasis, were obtained through the routine pathological report after surgery. The recurrence of tumor was obtained through postoperative follow-up. Follow-up intervals were 3 months within 1 year, 6 months within 1 to 2 years, and annual follow-up thereafter. The patients in our hospital were mainly followed up through the outpatient department and electronic medical records, and the patients in other hospitals were mainly followed up by telephone. At each follow-up visit, routine physical examination, eight thyroid tests and neck ultrasound were performed, and if a suspected malignant thyroid mass or lymph nodes in the neck were present, fine needle aspiration pathology was performed. The starting point of follow-up is after the first thyroid cancer surgery, and the end point of follow-up is to detect tumor recurrence. Tumor recurrence refers to the presence of a PTC tumor or lymph node metastasis confirmed by cervical ultrasound, cervical computed tomography (CT), FNAB, or postoperative routine pathological examination. Recurrence-free survival

(RFS) refers to the time from the time of recurrence of the tumor after the first surgery. The follow-up time refers to the time from the first thyroidectomy to tumor recurrence, and for no tumor recurrence, the follow-up time refers to the time interval to the end of follow-up. The last follow-up time was November 2020. Disease staging was determined using the eighth edition of the American Joint Committee on Cancer (AJCC) TNM staging manual (29). The analysis of molecular markers in this study was performed after surgery and radioactive iodine treatment and did not affect the clinical treatment decisions for patients. None of the enrolled patients received anti-PD-L1 immunotherapy or BRAF inhibitor treatment during the follow-up period. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Review Committee of Zhejiang Cancer Hospital (No. IRB-2020-64) and informed consent was taken from all the patients.

Immunohistochemistry

Immunohistochemistry (IHC) was used to detect the expression of the *BRAF*^{V600E} mutation and PD-L1 protein in PTC tissue. All surgical specimens were fixed in 10% neutral formalin, embedded in paraffin, and prepared into 4 μm-thick serial tissue sections. Xylene (Chengdu Kelong Chemical Reagent Factory, Chengdu, China) was used to deparaffinize the sections (3 times, 5 minutes each), followed by dehydration in a gradient ethanol series (100%, 90%, 70%) (Shanghai Lingfeng Chemical Reagent Co., Ltd., Shanghai, China) (2 times, 5 minutes each) and a rinse with water (2 times, 5 minutes each). After soaking in 3% H_2O_2 solution for 5 minutes, phosphate buffer solution (PBS) composed of potassium chloride (GENERAL-REAGENT, Shanghai Titan Scientific Co., Ltd., Shanghai, China), sodium chloride (Guangdong Guanghua Sci Co., Ltd., Guangdong, China), disodium hydrogen phosphate, sodium orthovanadate dodecahydrate, and sodium dihydrogen phosphate (Aladdin Reagent Co., Ltd., Shanghai, China) was used to rinse the sections 3 times. Trisodium citrate (Shanghai Lingfeng Chemical Reagent Co., Ltd.) and citric acid monohydrate (Shanghai Lingfeng Chemical Reagent Co., Ltd.) were used to prepare antigen retrieval solution (10 mm citric acid with pH 6.0). After immersion in the solution, the sections were placed in a pressure cooker for 90 seconds and then allowed to cool to room temperature for approximately 30 minutes. The sections were blocked with blocking solution. The sections were incubated with either rabbit anti-human *BRAF*^{V600E} monoclonal antibody EP152Y (1:100; Abcam, Cambridge, MA, USA) or mouse anti-human PD-L1 monoclonal antibody 66248-1-Ig (1:5,000; Proteintech Group, Wuhan, China). The sections were incubated with the primary antibody for 1 hour at room temperature and then washed with PBS 3 times. Sections were incubated for 20 minutes at room temperature after the dropwise addition of reaction enhancement solution and then rinsed 3 times with PBS; 3,3'-diaminobenzidine (DAB) reagent was added dropwise for color development, and color development was terminated by rinsing the sections with tap water. Hematoxylin (Ningbo Tongsheng Biological Technology Co., Ltd., Ningbo, China) was used as a counterstain, and the sections were rinsed with PBS and mounted with neutral gum. As a negative control, PBS was used instead of the primary antibody. The DAB chromogenic reagent kit was purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China).

Assessment of the BRAF^{V600E} *mutation and PD-L1 expression*

The IHC-stained sections were observed under an optical microscope to assess the *BRAF*^{V600E} mutation and PD-L1 expression; 2 experienced pathologists performed independent readings and interpretations in the absence of knowledge of the molecular results. When there was a disagreement, a consensus was reached through consultation. At least 5 areas with the strongest staining intensity were randomly selected for each section under high magnification (×200 magnification) as the observation area, and the necrotic area and marginal area of the section were avoided as much as possible during the observation.

BRAF^{V600E} mutation: Mutated *BRAF*^{V600E} is mainly located in the cytoplasm of tumor cells. The semiquantitative integration method was used to determine the mutation status. The percentage of positive cells and the staining intensity of positive cells in each section were scored as follows: staining intensity: no staining (–), light yellow (+), brown (++), and brown (+++), was counted as 0, 1, 2, and 3 points, respectively; the number of positive cells was scored based on the percentage of staining: 0%, 0 points; ≤25%, 1 point; 26–50%, 2 points; 51–75%, 3 points; >75%, 4 points. The score for the number of positive cells was multiplied by the score for staining intensity; scores <2 points were considered negative for the

BRAF^{V600E} mutation, and scores ≥2 were considered positive for the *BRAF*^{V600E} mutation (+).

PD-L1 expression: PD-L1 protein expression in tumor cells is mainly located in the cell membrane and/or cytoplasm. When the cell membrane and/or cytoplasm was tan or brown, tumor cells were considered positive for PD-L1 expression; expression in the nucleus was considered meaningless. The number of positive cells in each field of view was manually counted, and the number of positive cells/total number of cells was used to calculate the average percentage of positive cells in each section. Judgment was based on the percentage of positive cells in tumor cells: if the percentage of positive cells was <1%, the cells were considered negative for PD-L1 expression (–), and if the percentage of positive cells was ≥1%, the cells were considered positive for PD-L1 expression (+).

After IHC and image analyses, 63 of the 200 PTC patients enrolled in this study were excluded due to IHC failure and the inability to read images. Therefore, a total of 137 PTC patients were included in this study.

Statistical analysis

Statistical analysis was performed using SPSS 26.0 software (IBM Corp., Armonk, NY, USA). Normally distributed measurement data are expressed as the mean ± standard deviation (mean ± SD), and the *t*-test was used for intergroup comparisons; skewed measurement data are expressed as the median (interquartile range) M (IQR), and the Wilcoxon Mann-Whitney test was used for intergroup comparisons. Count data and ranked data are expressed as frequency (percentage) [n (%)]. Intergroup comparisons were made using the χ^2 test or Fisher's exact probability method. The Kaplan-Meier method was used to plot recurrence-free survival curves and perform the log-rank test. Combining *BRAF*^{V600E} mutation and PD-L1, early PTC cases were divided into 4 groups. Every group was compared with each other and Cox regression models were used to assess the risk of recurrence. When *BRAF*^{V600E} mutation and PD-L1 were used alone to establish Cox regression model, factors such as the age, sex of the patients, multifocality, capsular invasion, extraglandular invasion, lymphatic vascular and nerve invasion, cervical lymph node metastasis, concomitant HT and ^{131}I therapy were adjusted to confirm the independent influence of molecular markers on recurrence. A two-sided P value <0.05 indicated that a difference was statistically significant. The test level was α =0.05.

Results

Patient characteristics

Referencing the 8th edition of the AJCC TNM staging manual, the 137 patients with early-stage PTC enrolled in this study were all stage I and stage II patients (133 patients with stage I disease and 4 patients with stage II disease). The clinical data of all patients are shown in *Tables 1*,*2*. Most patients were female (78.1%), and the male to female ratio was approximately 1:3. Age ranged from 16 to 82 years, with an average age of 43.2±12.1 years. The median tumor size was 14 mm, ranging from 5 to 45 mm. There were 53 patients with papillary thyroid microcarcinoma (PTMC; tumor size ≤10 mm). At the time of the initial surgery, more than half of the patients had central lymph node metastasis (80 patients, 58.4%), and no patients had distant metastasis. As of the last follow-up in November 2020, a total of 14 patients had recurrence. The median follow-up time of this study was 136 months (IQR, 5.8), and the longest follow-up time was 144 months.

The BRAF^{V600E} *mutation and PD-L1 expression in early-stage PTC*

IHC was used to detect the *BRAF*^{V600E} mutation and PD-L1 expression in PTC patients. As seen in *Table 3* and *Figure 1*, among the 137 patients with early PTC, 56 were positive for the *BRAF*^{V600E} mutation, 81 were negative, for a positive rate of 40.9%. A total of 76 patients were positive for PD-L1, and 61 were negative, for a positive rate of 55.5%. The positivity rates for the *BRAF*^{V600E} mutation and PD-L1 among the 53 PTMC patients were 34.0% (18/53) and 45.3% (24/53), respectively.

We further studied the correlation between *BRAF*^{V600E} mutation and PD-L1 expression. Among the 56 patients with *BRAF*^{V600E} mutation-positive PTC, 40 patients (71.4%) were positive for PD-L1 expression, and among the 81 PTC patients negative for the *BRAF*^{V600E} mutation, only 36 (44.4%) were positive for PD-L1 expression. Similarly, among the 76 PTC patients who were positive for PD-L1 expression, 40 (52.6%) were positive for the *BRAF*^{V600E} mutation; among the 61 PTC patients who were negative for PD-L1 expression, only 16 (26.2%) were positive for the *BRAF*^{V600E} mutation (*Figure 2*). There was a high correlation between the *BRAF*^{V600E} mutation and PD-L1 expression among PTC patients [odds ratio (OR): 3.125; 95% confidence interval (CI): 1.511–6.464; P=0.002]. Among all 137 PTC patients, 40 (29.2%) had both the *BRAF*^{V600E}

Table 1 Demographic characteristics of PTC patients with the *BRAF*^{V600E} mutation or PD-L1 expression

Parameters	Total (n=137)	*BRAF*^{V600E} mutation			PD-L1 expression		
		Positive (n=56)	Negative (n=81)	P value	Positive (n=76)	Negative (n=61)	P value
Age at diagnosis (years)	43.2±12.1	44.6±12.5	42.3±11.7	0.259	44.3±12.0	41.9±12.2	0.248
Sex							
Male	30 (21.9)	8 (14.3)	22 (27.2)	0.073	17 (22.4)	13 (21.3)	0.882
Female	107 (78.1)	48 (85.7)	59 (72.8)		59 (77.6)	48 (78.7)	
Tumor size							
≤1.0 cm	53 (38.7)	18 (32.1)	35 (43.2)	0.191	24 (31.6)	29 (47.5)	0.057
>1.0 cm	84 (61.3)	38 (67.9)	46 (56.8)		52 (68.4)	32 (52.5)	
Multifocality	45 (32.8)	16 (28.6)	29 (35.8)	0.376	28 (36.8)	17 (27.9)	0.266
Envelope invasion	77 (56.2)	36 (64.3)	41 (50.6)	0.113	45 (59.2)	32 (52.5)	0.429
Extraglandular invasion	40 (29.2)	19 (33.9)	21 (25.9)	0.311	28 (36.8)	12 (19.7)	0.028
Lymphatic, vascular and nerve invasion	12 (8.8)	6 (10.7)	6 (7.4)	0.715	8 (10.5)	4 (6.6)	0.414
Cervical lymph node metastasis	80 (58.4)	30 (53.6)	50 (61.7)	0.341	38 (50.0)	42 (68.9)	0.026
Concomitant HT	30 (21.9)	17 (30.4)	13 (16.0)	0.047	17 (22.4)	13 (21.3)	0.882
^{131}I dose (mCi)	0 [0]	0 [75]	0 [0]	0.667	0 [100]	0 [0]	0.213
Follow-up time (months)	136 [5.8]	136 [7.5]	136 [5.0]		136 [6.9]	135 [5.1]	

Normally distributed measurement data are expressed as the mean ± standard deviation, such as age at diagnosis. Skewed measurement data are expressed as the median [interquartile range], such as ^{131}I dose and follow-up time. Categorical data are expressed as frequency (percentage of all cases) [n (%)], including sex, tumor size, multifocality, envelope invasion, extraglandular invasion, lymphatic, vascular and nerve invasion, cervical lymph node metastasis, concomitant HT. PTC, papillary thyroid carcinoma; PD-L1, programmed death-ligand 1; HT, Hashimoto's thyroiditis.

mutation and PD-L1 expression, and 45 were negative for both the *BRAF*^{V600E} mutation and PD-L1 expression (32.8%), 16 (11.7%) carried the *BRAF*^{V600E} mutation alone; 36 (26.3%) had PD-L1 alone.

Association of the BRAF^{V600E} *mutation and PD-L1 expression with clinicopathological features of early-stage PTC*

The relationships of the *BRAF*^{V600E} mutation and PD-L1 expression with clinicopathological characteristics of PTC are shown in *Tables 1*,*2*. In the early PTC cohort (*Table 1*), the *BRAF*^{V600E} mutation was only associated with HT (P=0.047). Compared with *BRAF*^{V600E} mutation-negative patients, early PTC patients with the *BRAF*^{V600E} mutation more often had HT. There was no significant correlation between the *BRAF*^{V600E} mutation and factors such as sex, age at diagnosis, tumor size, multifocal tumor, capsular invasion, extraglandular invasion, lymphatic, vascular, and nerve invasion, and CLNM (P>0.05). PD-L1 expression was significantly correlated with external thyroid invasion (P=0.028) and CLNM (P=0.026). Compared with patients without PD-L1 expression, PTC patients with PD-L1 expression were more susceptible to extrathyroid invasion, and the rate of CLNM was lower. The clinicopathological characteristics of PTC patients, such as age, sex, tumor size, multifocal tumor, capsular invasion, lymphatic, vascular and nerve invasion, and concomitant HT (P>0.05) were not significantly correlated with PD-L1 expression. In the PTMC cohort (*Table 2*), only age at diagnosis was significantly correlated with the *BRAF*^{V600E} mutation (P=0.031), and the age of PTMC patients positive for the *BRAF*^{V600E} mutation was older. There was no significant correlation between other clinicopathological factors and the *BRAF*^{V600E} mutation and PD-L1 expression.

The association between the coexistence of the *BRAF*^{V600E} mutation and PD-L1 expression and the clinicopathological factors of PTC was further analyzed

Table 2 *BRAF*^{V600E} mutation or PD-L1 expression in PTMC patients

Parameters	Total (n=53)	*BRAF*^{V600E} mutation			PD-L1 expression		
		Positive (n=18)	Negative (n=35)	P value	Positive (n=24)	Negative (n=29)	P value
Age at diagnosis (years)	44.8±10.6	49.1±11.6	42.6±9.4	0.031	46.2±10.6	43.7±10.6	0.394
Sex							
Male	9 (17.0)	2 (11.1)	7 (20.0)	0.667	3 (12.5)	6 (20.7)	0.672
Female	44 (83.0)	16 (88.9)	28 (80.0)		21 (87.5)	23 (79.3)	
Multifocality	20 (37.7)	5 (27.8)	15 (42.9)	0.283	10 (41.7)	10 (34.5)	0.591
Envelope invasion	20 (37.7)	8 (44.4)	12 (34.3)	0.470	9 (37.5)	11 (37.9)	0.974
Extraglandular invasion	9 (17.0)	4 (22.2)	5 (14.3)	0.732	5 (20.8)	4 (13.8)	0.755
Lymphatic, vascular and nerve invasion	1 (1.9)	0 (0)	1 (2.9)	1.000	1 (4.2)	0 (0)	0.453
Cervical lymph node metastasis	26 (49.1)	10 (55.6)	16 (45.7)	0.497	9 (37.5)	17 (58.6)	0.126
Concomitant HT	7 (13.2)	3 (16.7)	4 (11.4)	0.916	4 (16.7)	3 (10.3)	0.788
^{131}I dose (mCi)	0 [0]	0 [0]	0 [0]		0 [0]	0 [0]	
Follow-up time (months)	135 [5.1]	134 [5.6]	135 [4.8]		133 [5.1]	135 [5.1]	

Normally distributed measurement data are expressed as the mean ± standard deviation, such as age at diagnosis. Skewed measurement data are expressed as the median [interquartile range], such as ^{131}I dose and follow-up time. Categorical data are expressed as frequency (percentage of all cases) [n (%)], including sex, multifocality, envelope invasion, extraglandular invasion, lymphatic, vascular and nerve invasion, cervical lymph node metastasis, concomitant HT. PD-L1, programmed death-ligand 1; PTMC, papillary thyroid microcarcinoma; HT, Hashimoto's thyroiditis.

Table 3 Relationship between the *BRAF*^{V600E} mutation and PD-L1 expression in 137 patients with early PTC

Expressing Status	PD-L1 expression			OR value	OR, 95% CI	P value
	+	–	Total			
BRAF^{V600E} mutation						
+	40	16	56	3.125	(1.511, 6.464)	0.002
–	36	45	81			
Total	76	61	137			

PD-L1, programmed death-ligand 1; PTC, papillary thyroid carcinoma; OR, odds ratio; CI, confidence interval.

(*Table 4*). When both molecular markers are considered simultaneously, in the early PTC cohort, compared with patients who were negative for the *BRAF*^{V600E} mutation and PD-L1 expression, patients who were positive for the *BRAF*^{V600E} mutation or PD-L1 expression alone failed to show significant risk effects. In contrast, patients with both the *BRAF*^{V600E} mutation and PD-L1 expression had larger tumors (P=0.038), were more prone to extrathyroid invasion (P=0.045), and had a lower CLNM rate (P=0.046). However, the *BRAF*^{V600E} mutation and PD-L1 expression were not correlated with age at diagnosis, sex, multifocality, capsular invasion, lymphatic, vascular and nerve invasion, or concomitant HT (P>0.05). These results indicated that the *BRAF*^{V600E} mutation and PD-L1 expression had a partial synergistic effect on tumor invasiveness. Analysis of the PTMC cohort showed that those with only the *BRAF*^{V600E} mutation were older; there were no significant differences in the coexistence of other clinicopathological characteristics with the *BRAF*^{V600E} mutation and PD-L1 expression.

Impact of the BRAF^{V600E} *mutation and PD-L1 expression on recurrence*

For early-stage PTC, the tumor recurrence rate for patients

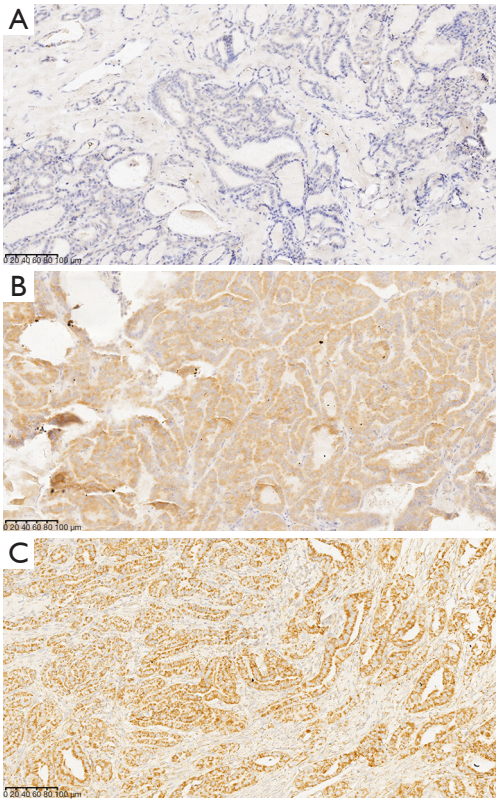

Figure 1 Detection of the *BRAF*^{V600E} mutation and PD-L1 protein expression in early PTC tumor tissue using IHC. (A) *BRAF*^{V600E} mutation and PD-L1 expression are negative; (B) *BRAF*^{V600E} mutation is positive, diffuse cytoplasmic staining can be seen in tumor cells; (C) PD-L1 expression is positive, tumor cell membrane and/or cytoplasm are brown (+++). IHC staining, ×200. PD-L1, programmed death-ligand 1; PTC, papillary thyroid carcinoma; IHC, immunohistochemistry.

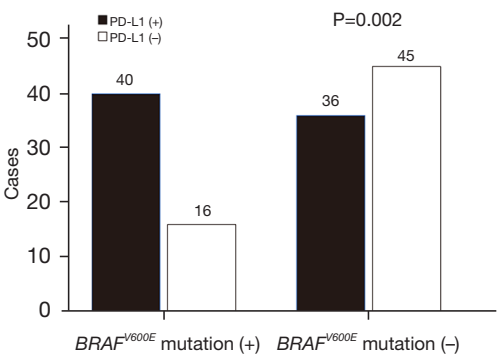

Figure 2 The *BRAF*^{V600E} mutation and PD-L1 expression in early PTC. PD-L1, programmed death-ligand 1; PTC, papillary thyroid carcinoma.

without the *BRAF*^{V600E} mutation was 7.4%, and the tumor recurrence rate for patients with the *BRAF*^{V600E} mutation was 14.3%. However, the correlation between the presence of the *BRAF*^{V600E} mutation and the risk of tumor recurrence was not significant (log rank P=0.197; *Figure 3A*). We further performed Cox regression analysis, and the results indicated that after adjusting for age at diagnosis and sex, the correlation between the presence of the *BRAF*^{V600E} mutation and the risk of tumor recurrence was still not significant; the hazard ratio (HR) was 2.552 (95% CI: 0.847–7.689; P=0.096). After further adjustment for tumor size, multifocality, capsular invasion, extrathyroidal invasion, CLNM, concomitant HT, and radioactive iodine 131 treatment, there was a significant correlation between the *BRAF*^{V600E} mutation and the risk of recurrence; the HR was 3.915 (95% CI: 1.087–14.106; P=0.037) (*Table 5*).

The tumor recurrence rate for patients who were negative for PD-L1 expression was 8.2%, and the tumor recurrence rate for patients who were positive for PD-L1 expression was 11.8%. Similarly, the correlation between the presence of PD-L1 and the risk of tumor recurrence was not significant (log rank P=0.222; *Figure 3B*). Further Cox regression analysis indicated that after adjusting for age at diagnosis and sex, the correlation between PD-L1 and the risk of tumor recurrence was still not statistically significant; the HR was 1.502 (95% CI: 0.503–4.489; P=0.467). After additional adjustments for tumor size, multifocality, capsular invasion, extrathyroid invasion, CLNM, concomitant HT, and radioactive iodine 131 treatment, there was still no significant correlation between PD-L1 expression and the risk of recurrence (HR =1.762; 95% CI: 0.537–5.778; P=0.350) (*Table 5*). The same results were also observed for patients with PTMC (*Figure 4A,4B*, *Table 5*).

Subsequently, we evaluated whether the coexistence of the *BRAF*^{V600E} mutation and PD-L1 affected the recurrence of early PTC and PTMC after long-term follow-up. We found that for both early PTC and PTMC, the *BRAF*^{V600E} mutation or PD-L1 expression alone did not significantly change the risk of recurrence compared with that for patients without the *BRAF*^{V600E} mutation and PD-L1 expression. Kaplan-Meier analysis showed that the RFS curves for double-negative and any single-positive PTC patients were flat and that the survival curves for PTC patients who were positive for the *BRAF*^{V600E} mutation and PD-L1 expression decreased sharply. However, the significance of this effect is not yet statistically meaningful (P>0.05, *Figure 5A,5B*). Combining the two molecular

Table 4 Effect of the *BRAF*^{V600E} mutation and PD-L1 expression alone or coexisting on the clinicopathological characteristics of PTC

Clinical pathological factors	BRAF-/PD-L1- (group 1)	BRAF+/PD-L1- (group 2)	P value (1 *vs*. 2)	BRAF-/PD-L1+ (group 3)	P value (1 *vs*. 3)	BRAF+/PD-L1+ (group 4)	P value (1 *vs*. 4)
Early PTC							
Number of cases	45	16		36		40	
Age at diagnosis (years)	40.9±11.9	44.8±13.0	0.277	44.0±11.5	0.239	44.6±12.5	0.164
Sex							
Male	12 (26.7)	1 (6.3)	0.175	10 (27.8)	0.911	7 (17.5)	0.311
Female	33 (73.3)	15 (93.8)		26 (72.2)		33 (82.5)	
Tumor size (cm)							
≤1.0	21 (46.7)	8 (50.0)	0.819	14 (38.9)	0.483	10 (25.0)	0.038
>1.0	24 (53.3)	8 (50.0)		22 (61.1)		30 (75.0)	
Multifocality	13 (28.9)	4 (25.0)	1.000	16 (44.4)	0.147	12 (30.0)	0.911
Envelope invasion	25 (55.6)	7 (43.8)	0.417	16 (44.4)	0.320	29 (72.5)	0.105
Extraglandular invasion	10 (22.2)	2 (12.5)	0.635	11 (30.6)	0.395	17 (42.5)	0.045
Lymphatic, vascular and nerve invasion	4 (8.9)	0 (0.0)	0.518	2 (5.6)	0.887	6 (15.0)	0.592
Cervical lymph node metastasis	32 (71.1)	10 (62.5)	0.746	18 (50.0)	0.052	20 (50.0)	0.046
With HT	7 (15.6)	6 (37.5)	0.137	6 (16.7)	0.892	11 (27.5)	0.179
^{131}I dose (mCi)	0 [0]	0 [0]		0 [0]		0 [100]	
Follow-up time (months)	135 [4.9]	135 [7.0]		137 [6.0]		136 [8.1]	
Tumor recurrence	4 (8.9)	1 (6.3)		2 (5.6)		7 (17.5)	
PTMC							
Number of cases	21	8		14		10	
Age at diagnosis (years)	41.1±9.2	50.4±11.5	0.032	44.8±9.5	0.260	48.1±12.2	0.086
Sex							
Male	5 (23.8)	1 (12.5)	0.874	2 (14.3)	0.796	1 (10.0)	0.672
Female	16 (76.2)	7 (87.5)		12 (85.7)		9 (90.0)	
Multifocal	8 (38.1)	2 (25.0)	0.821	7 (50.0)	0.486	3 (30.0)	0.969
Envelope invasion	9 (42.9)	2 (25.0)	0.647	3 (21.4)	0.345	3 (30.0)	0.583
Extraglandular invasion	3 (14.3)	1 (12.5)	1.000	2 (14.3)	1.000	6 (60.0)	0.611
Cervical lymph node metastasis	12 (57.1)	5 (62.5)	1.000	4 (28.6)	0.096	5 (50.0)	1.000
Concomitant HT	2 (9.5)	1 (12.5)	1.000	2 (14.3)	1.000	2 (20.0)	0.810
^{131}I dose (mCi)	0 [0]	0 [0]		0 [0]		0 [0]	
Follow-up time (months)	135 [5.3]	136 [3.7]		134 [4.7]		133 [61.5]	

Normally distributed measurement data are summarized as the mean ± standard deviation, such as age at diagnosis. Skewed measurement data are expressed as the median [interquartile range], such as ^{131}I dose and follow-up time. Categorical data are expressed as frequency (percentage of all cases) [n (%)], including sex, multifocality, envelope invasion, extraglandular invasion, lymphatic, vascular and nerve invasion, cervical lymph node metastasis, concomitant HT. The P values for each group were derived from comparisons with the *BRAF*^{V600E} mutation-/PD-L1-group (Group 1). PD-L1, programmed death-ligand 1; PTC, papillary thyroid carcinoma; HT, Hashimoto's thyroiditis; PTMC, papillary thyroid microcarcinoma.

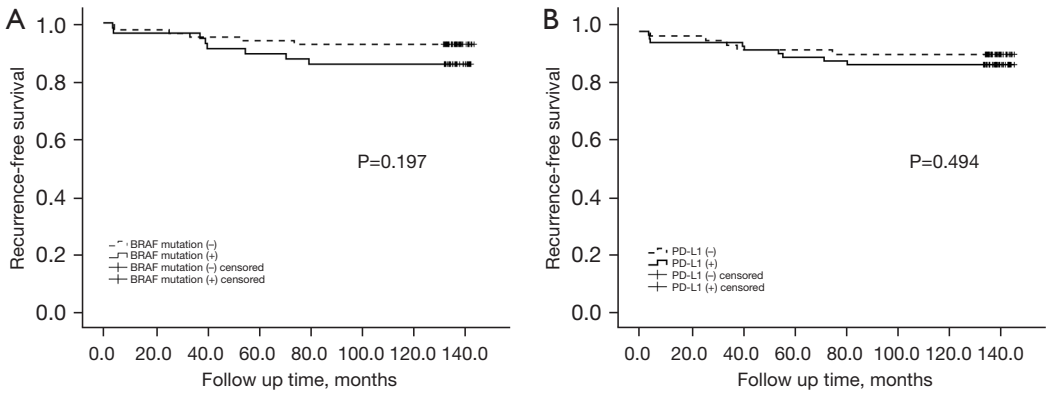

Figure 3 Survival curve for the *BRAF*^{V600E} mutation and the recurrence of early-stage PTC (log rank P=0.197) (A); survival curve for PD-L1 expression and the recurrence of early-stage PTC (log rank P=0.494) (B). PD-L1, programmed death-ligand 1; PTC, papillary thyroid carcinoma.

Table 5 Relationship of the *BRAF*^{V600E} mutation and PD-L1 expression with PTC recurrence

Grouping	Recurrence rate		Unadjusted		Adjusted *1		Adjusted *2	
	n	%	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
Early PTC	14/137	10.2						
BRAF^{V600E} mutation								
Negative	6/81	7.4	1.979 (0.687–5.705)	0.206	2.552 (0.847–7.689)	0.096	3.915 (1.087–14.106)	0.037
Positive	8/56	14.3						
PD-L1expression								
Negative	5/61	8.2	1.461 (0.490–4.360)	0.497	1.502 (0.503–4.489)	0.467	1.762 (0.537–5.778)	0.350
Positive	9/76	11.8						
PTMC	5/53	9.4						
BRAF^{V600E} mutation								
Positive	2/35	5.7	2.899 (0.484–17.357)	0.244	2.766 (0.406–18.867)	0.299	5.206 (0.647–41.872)	0.121
Negative	3/18	16.7						
PD-L1 expression								
Positive	2/29	6.9	1.770 (0.296–10.593)	0.532	1.548 (0.251–9.564)	0.638	1.310 (0.131–13.091)	0.818
Negative	3/24	12.5						

Adjusted *1: the age and sex of the patients were adjusted. Adjusted *2: the age at diagnosis, sex, tumor size, multifocality, capsular invasion, extraglandular invasion, lymphatic vascular and nerve invasion, cervical lymph node metastasis, concomitant HT, and ^{131}I therapy were adjusted. PD-L1, programmed death-ligand 1; PTC, papillary thyroid carcinoma; HR, hazard ratio; CI, confidence interval; PTMC, papillary thyroid microcarcinoma; HT, Hashimoto's thyroiditis.

markers for Cox regression, only 4 of 45 patients with *BRAF*^{V600E} mutation and PD-L1 double negative showed recurrence (8.9%), while 7 of 40 patients (17.5%) with *BRAF*^{V600E} mutation and PD-L1 double expression had the highest recurrence rate (HR =1.267; 95% CI: 0.841–1.909; P=0.257). Separate between-group comparisons revealed that the coexistence of the *BRAF*^{V600E} mutation and PD-L1 had a greater effect on PTC recurrence than either mutation alone; 1 of 16 early PTC cases carrying only the *BRAF*^{V600E} mutation developed recurrence (6.3%) (HR

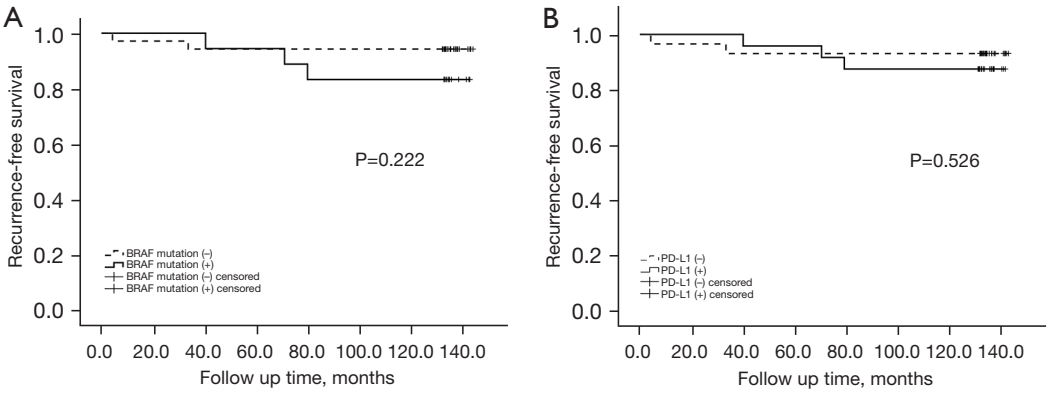

Figure 4 Survival curve for the *BRAF*^{V600E} mutation and PTMC recurrence (log rank P=0.222) (A); survival curve for PD-L1 expression and PTMC recurrence (log rank P=0.526) (B). PD-L1, programmed death-ligand 1; PTMC, papillary thyroid microcarcinoma.

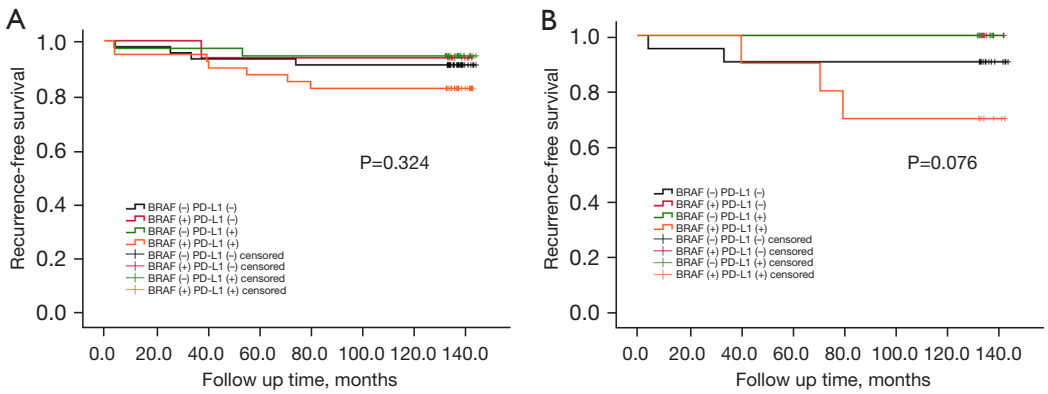

Figure 5 Survival curve for the *BRAF*^{V600E} mutation & PD-L1 and the recurrence of early-stage PTC (log rank P=0.324) (A); survival curve for the *BRAF*^{V600E} mutation & PD-L1 and PTMC recurrence (log rank P=0.076) (B). PD-L1, programmed death-ligand 1; PTMC, papillary thyroid microcarcinoma.

=1.704; 95% CI: 0.598–4.858; P=0.319) and 2 of 36 cases expressing PD-L1 only (5.6%) (HR =3.315; 95% CI: 0.689–15.958; P=0.135). However, the statistical significance of this effect was not significant (P>0.05) (*Table 6*).

Discussion

In recent decades, the incidence of thyroid cancer in China and globally has been increasing, of which the increase in new cases of PTC is the main reason. Improvements in diagnostic techniques, increased monitoring methods, and large-scale cancer screening have all led to an increase in the detection rate of small, low-risk, and indolent PTCs (3). Therefore, how to better deal with many early PTC and PTMC patients has become an urgent problem in China. For this part of the population, identifying patients with relatively high risk and excluding patients who do not need excessive active treatment is conducive to improving the current medical situation of the overtreatment of thyroid cancer in China.

The *BRAF*^{V600E} mutation is considered one of the most common specific diagnostic markers of thyroid cancer. It plays an important role in the occurrence and development of PTC, mainly through the abnormal activation of the MAPK signaling pathway (11). The mutation frequency of *BRAF*^{V600E} in PTC is generally higher in Asian countries than in Western countries; the mutation rate in China is 31–91%, with an average of 71.2% (30). The literature shows that (31) compared with methods such as gene sequencing and polymerase chain reaction (PCR), IHC using the VE1

Table 6 Comparison of PTC recurrence between the *BRAF*^{V600E} mutation and PD-L1 expression alone and combined

Group comparison	HR	95% CI	P value
Early PTC			
Group 1 *vs.* Group 2	0.683	0.076–6.115	0.733
Group 1 *vs.* Group 3	0.786	0.337–1.837	0.579
Group 1 *vs.* Group 4	1.267	0.841–1.909	0.257
Group 2 *vs.* Group 4	1.704	0.598–4.858	0.319
Group 3 *vs.* Group 4	3.315	0.689–15.958	0.135
Group 2 *vs.* Group 3	0.887	0.080–9.779	0.922
PTMC			
Group 1' *vs.* Group 2'	0.030	0–13655.040	0.597
Group 1' *vs.* Group 3'	0.146	0–49.834	0.518
Group 1' *vs.* Group 4'	1.464	0.806–2.659	0.211
Group 2' *vs.* Group 4'	7.674	0.073–807.719	0.391
Group 3' *vs.* Group 4'	112.910	0.007–NA	0.341
Group 2' *vs.* Group 3'	–	–	–

PTC, papillary thyroid carcinoma; PD-L1, programmed death-ligand 1; HR, hazard ratio; CI, confidence interval; PTMC, papillary thyroid microcarcinoma; NA, unable to calculate.

antibody has higher specificity, sensitivity, and positive and negative predictive values and is a reliable and highly sensitive method for the detection of the *BRAF*^{V600E} mutation in PTC. IHC has been widely applied in clinical practice. In this study, IHC was used to detect the *BRAF*^{V600E} mutation in 137 PTC patients, and the positive detection rate was 40.9%. The positive rate in the PTMC cohort was 33.9%, slightly lower than the average. The included cases were TNM stage I to stage II, which are relatively early stages. A recent meta-analysis of 9,908 patients with PTC (16) indicated that late TNM staging (stage III and IV) was an important risk factor for the *BRAF*^{V600E} mutation in PTC patients. Additionally, the results of this study also indicated that there was no association between or increased risk for tumor size >1 cm and the *BRAF*^{V600E} mutation in PTC patients. However, some studies (32,33) show that the *BRAF*^{V600E} mutation is associated with larger tumor size. This is consistent with the slightly lower *BRAF*^{V600E} mutation rate in the PTMC cohort in this study, which was at the lower limit for China. The detection of the *BRAF*^{V600E} mutation in PTMC indicates that this mutation may be an early event in the development of PTC. Fa *et al.* (30) conducted subgroup analyses and found that compared with studies that used formalin-fixed paraffin-embedded tissues, studies that used fresh frozen tissue and fine-needle aspirates showed a higher incidence of *BRAF*^{V600E} mutations. This may also be one of the reasons for the relatively low positive rate of the *BRAF*^{V600E} mutation in this study. In addition, the relationship between the *BRAF*^{V600E} mutation and clinicopathological parameters is still controversial. In this study, in the early stage of PTC, the *BRAF*^{V600E} mutation was only significantly correlated with HT but was not found to be significantly correlated with age at diagnosis, sex, tumor size, multifocality, capsular invasion, extrathyroidal invasion, lymphatic, vascular and nerve invasion, and CLNM. The *BRAF*^{V600E} mutation in PTMC was only associated with older age at diagnosis and was not significantly correlated with other clinicopathological features. This is consistent with the results reported in several studies (34,35).

The *BRAF*^{V600E} mutation is often regarded as a poor prognostic marker of PTC and is closely associated with PTC recurrence (18). Studies by Xing *et al.* (19,36) found that there was a significant association between the *BRAF*^{V600E} mutation and disease relapse in patients with low-risk stage I or II PTC and PTMC and various subtypes of PTC. Consistent with the above viewpoint, Kim *et al.* (37) proposed that the *BRAF*^{V600E} mutation can be used to predict clinical recurrence in patients with low-risk PTC. However, a single-center retrospective study (20) involving 461 patients with TNM stage I or II PTC reported that the *BRAF*^{V600E} mutation could not predict recurrence in patients with TNM stage I–II PTC after initial treatment with total thyroidectomy (TTE) and radioactive iodine therapy (RRA). Our findings are consistent with the results of this study. In our study, after 136 months of long-term follow-up of patients with stage I–II PTC and PTMC who underwent surgical treatment, the correlation between the *BRAF*^{V600E} mutation and PTC recurrence was not significant.

PD-L1 is expressed in a variety of tumor cells, such as malignant melanoma and non-small cell lung cancer. A series of studies (26,38) also proved that high PD-L1 expression in PTC patients is associated with tumor invasiveness and recurrence, different from the results for PD-L1 in this study. The reason may be that the included patients in our study were at an early stage; TNM stage has been shown to be one of the influencing factors of PD-L1 expression in PTC (39).

Several previous studies have reported an association between PD-L1 expression and the *BRAF*^{V600E} mutation in

PTC patients. However, the relevant results are controversial. Angell *et al.* (40) found that the positive status of PD-L1 in PTC patients was closely related to the *BRAF*^{V600E} mutation. Bastman *et al.* (41) did not observe a significant correlation between PD-L1 expression and *BRAF*^{V600E} mutation status in advanced PTC cases. Bai *et al.* (34) conducted a study of 110 cases of PTC with a diameter >1 cm and found that the correlation between PD-L1 and *BRAF*^{V600E} was not statistically significant. However, after excluding PTC patients with a background of chronic lymphocytic thyroiditis (CLT) or Hashimoto's disease, *BRAF*^{V600E} was positively correlated with PD-L1 expression statistically. The association between the *BRAF*^{V600E} mutation and PD-L1 has not been confirmed. A recent study (26) proposed that there is an association between PD-L1 overexpression and the *BRAF*^{V600E} mutation. Compared with patients with the BRAF mutation alone, the patients with both PD-L1 overexpression and the *BRAF*^{V600E} mutation had significantly less favorable disease-free survival (DFS). In addition, in vitro experiments confirmed that the overexpression of PD-L1 in PTC was significantly correlated with BRAF mutations. The results of this study showed that the *BRAF*^{V600E} mutation and PD-L1 expression were highly correlated in early PTC. These results further support that the coexistence of PD-L1 and *BRAF*^{V600E} mutations may be associated with the more invasive behavior of early PTC. This indicates that there may be a synergistic effect between the 2 in the development and progression of tumors.

Therefore, we further combined the *BRAF*^{V600E} mutation and PD-L1 to identify patients with strong invasiveness and a high risk of recurrence among early PTC patients. We observed that only the *BRAF*^{V600E} mutation or PD-L1 positivity alone had no significant effect on clinical pathological characteristics and the recurrence of PTC. However, when these 2 molecular markers coexist, it suggests that the tumor is larger and more prone to extraglandular invasion, and the RFS curve has a significant decreasing trend, indicating that the coexistence of the 2 may be related to more aggressive tumor behavior. Further Cox regression analysis also showed that patients with *BRAF*^{V600E} mutation and PD-L1 double positive had a higher recurrence rate than patients with double negative and single factor positive. A previous study (40) suggested that the *BRAF*^{V600E} mutation facilitates tumor immunosuppression through mechanisms such as PD-L1 and HLA-G expression and induces or recruits inhibitory immune cell populations to disrupt the immune surveillance and immune response of the host. In addition, in colon cancer, *BRAF*^{V600E} can transcriptionally upregulate the expression of PD-L1 and induce cell apoptosis (25). This further supports that the combined use of the *BRAF*^{V600E} mutation and PD-L1 has a predictive effect on the clinicopathological characteristics and prognosis of early PTC. However, the effect of the combination of the *BRAF*^{V600E} mutation and PD-L1 on clinicopathological characteristics and prognosis in PTMC has not been confirmed. This indicates that the combination of the *BRAF*^{V600E} mutation and PD-L1 serves as a poor indication for the prognosis of PTMC and should be interpreted with caution.

This study has some limitations. We used IHC to detect the *BRAF*^{V600E} mutation and PD-L1, but the time frame of the cases included in this study, the preservation conditions of the paraffin blocks, and the IHC operation procedures all can affect the final results, which can lead to the exclusion of some cases, resulting in a small number of cases included in this study. In addition, there was no negative case control in this study, potentially causing bias in the study results. Further studies are needed to increase the number of early- or low-risk PTC cases to confirm the effect of the combination of the *BRAF*^{V600E} mutation and PD-L1 on the clinicopathological characteristics and prognosis of early PTC. Therefore, the explanatory value of these data is limited, and the conclusion may lack credibility. Second, this study did not classify pathological subtypes because of the limitations of the conditions of the study. However, the median follow-up period of this study was 136 months, which to a certain extent effectively avoided the underestimation of the recurrence rate of PTC.

Conclusions

In summary, this study demonstrated a significant association between the *BRAF*^{V600E} mutation and PD-L1 in early PTC, and when only one of these indicators was expressed alone, the effect on the aggressiveness and prognosis of early PTC was small. The coexistence of the *BRAF*^{V600E} mutation and PD-L1 suggests a higher risk of invasiveness and recurrence but has insufficient clinical evidence. And there was no significant predictive effect for PTMC. To a certain extent, these results can assist identification of the relatively high-risk patients among the early PTC population in clinical practice, improve the risk stratification of PTC, and be used to develop individualized treatment plans and optimize follow-up plans. In the future, further prospective and larger-sample-size studies are

needed to provide more reliable evidence to support this point of view.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://gs.amegroups.com/article/view/10.21037/gs-22-701/rc

Data Sharing Statement: Available at https://gs.amegroups.com/article/view/10.21037/gs-22-701/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://gs.amegroups.com/article/view/10.21037/gs-22-701/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Review Committee of Zhejiang Cancer Hospital (approval ID: IRB-2020-64) and informed consent was taken from all the patients.

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