



In papillary thyroid carcinoma, expression by immunohistochemistry of BRAF V600E, PD-L1, and PD-1 is closely related

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Abstract

Immune checkpoint inhibitor therapies targeting PD-L1/PD-1 have been shown to be effective in treating several types of human cancer. In papillary thyroid carcinoma (PTC), little is known about the expression of PD-L1/PD-1 in the tumor microenvironment or its potential correlation with BRAF V600E mutation status. In this study, we examined the expression of PD-L1, PD-1, and BRAF V600E in PTC by immunohistochemistry and investigated the clinical significance of expression status. We studied the expression of PD-L1, PD-1, and BRAF V600E by immunohistochemical staining in 110 cases of PTC with a diameter > 1 cm. Cases with a background of chronic lymphocytic thyroiditis (CLT) were excluded, as differentiating lymphocytes in the context of CLT from tumor-infiltrating lymphocytes (TILs) is difficult. We classified PD-L1+/PD-1+ expression as type 1 (41%), PD-L1-/PD-1- as type 2 (17%), PD-L1+/PD-1- as type 3 (5%), and PD-L1-/PD-1+ as type 4 (37%). Significant correlations were found between expression of BRAF V600E and that of PD-L1 and PD-1. The positive correlation observed between expression of BRAF V600E and PD-L1/PD-1 suggests that immunotherapies targeting PD-L1/PD-1 might be effective for PTC patients with the BRAF V600E mutation, which are refractory to radioiodine therapy.

Keywords BRAF V600E · PD-L1 · PD-1 · Papillary thyroid carcinoma · Immunohistochemistry

Introduction

Thyroid cancer is the most common endocrine malignancy, and it accounts for the majority of endocrine cancer deaths. Papillary thyroid carcinoma (PTC) accounts for approximately

90% of thyroid cancers, and more than 10% of patients with PTC develop cancer recurrence or distant metastasis, and cancer-related death may occur [1, 2]. In the past decade, significant progress has been made in identifying the molecular changes responsible for PTC development and invasiveness. The RET-RAS-RAF-MEK-ERK (mitogen-activated protein kinase) pathway is the most widely accepted signaling pathway responsible for thyroid tumorigenesis and invasiveness, in which the BRAF V600E mutation plays a prominent role, resulting in overactivity of downstream signaling [3–5].

Human cancers harbor numerous genetic and epigenetic alterations, generating neoantigens that are potentially recognizable by the immune system [6]. Programmed death 1 (PD-1) is a key immune checkpoint receptor, mainly expressed by activated T cells, and it mediates immunosuppression [7]. PD-L1 (B7-H1) is the ligand of PD-1, of which the expression is greatly increased on some tumor cells. Inhibition of the interaction between PD-1 and PD-L1 can enhance T cell responses in vitro and mediate preclinical antitumor activity [7–10]. Clinical trials using anti-PD-1 or anti-PD-L1 antibodies to treat patients with cancers, such as melanoma, nonsmall cell lung carcinoma, renal cell carcinoma, prostatic carcinoma,

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and colorectal carcinoma, have provided encouraging results [10–15]. Furthermore, patients with PD-L1-positive tumors had an objectively better response to anti-PD-1 treatment than patients with PD-L1-negative tumors [14]. A positive response to immunotherapy usually relies on dynamic interactions between tumor cells and immunomodulators inside the tumor microenvironment, which plays an important role in dampening or enhancing immune responses [16, 17]. Teng et al. classified tumors into four groups on the basis of their PD-L1 status and presence or absence of the tumor-infiltrating lymphocytes. Different types of immunotherapeutic approaches might have to be applied to different groups of tumors [16].

Surgery and postoperative radioiodine therapy are the basic treatments for most patients with differentiated thyroid carcinoma. However, a subset of PTC patients does not respond to radioiodine therapy. The incidence of radioiodine-refractory PTCs is higher in tumors with the BRAF V600E mutation [18, 19]. For PTC patients who failed to respond to radioiodine therapy, it is important to develop novel treatment modalities. Little is known regarding PD-L1/PD-1 expression status in PTC cases, especially taking into account the BRAF mutation status [20–24]. Therefore, examining the relationship between BRAF V600E status and PD-L1/PD-1 expression is of great significance, as this might be used to select candidates for immunotherapy. Furthermore, it has been reported that combining BRAF inhibitors and anti-PD-L1 antibody therapies improves tumor regression and antitumor immunity in melanoma cell lines as well as in an immunocompetent murine model of anaplastic thyroid carcinoma [25, 26]. This indicates that combination approaches may be potential avenues to increase antitumor efficacy. Some PTC cases harbor a background of chronic lymphocytic thyroiditis (CLT). In these cases, differentiating CLT from tumor-infiltrating lymphocytes (TILs) is difficult, as conceivably changes in immunomodulatory receptor expression may overlap and thus complicate the results. The purpose of the present study is to clarify in PTC lacking a CLT background the expression status of BRAF V600E, PD-L1, and PD-1 and the clinical significance of BRAF V600E, PD-L1, and PD-1 expression and classify microenvironment in PTC and its correlation with BRAF V600E status.

Materials and methods

Patients and specimens

We reviewed the records of patients with a diagnosis of PTC who underwent surgical treatment at Peking University Cancer Hospital, Beijing, China. Patients with a background of CLT or Hashimoto's disease were excluded from this study. Tumor-bearing thyroid tissues with only a small or focal

lymphocytic reaction were not regarded as CLT specimens in the present study (Fig. 1a, b), since they represented types of nonspecific thyroiditis but not autoimmune thyroiditis. Patients with microcarcinoma were excluded from this study because they have a much better clinical outcome than patients with other types of PTC, as noted in our earlier study [27], and may not need additional treatment. In total, 110 patients were enrolled, consisting of 79 females and 31 males. All patients had no evidence of distant metastasis at surgery.

This study was approved by the ethics committee of Peking University Cancer Hospital & Institute.

Histological examination

Ninety cases of conventional-type PTC, 18 cases of follicular variant of PTC, one case of a solid variant, and one case of a tall cell variant were selected ($n = 110$). All sections were stained with hematoxylin and eosin and independently reviewed by two pathologists, YB and DN, to examine the presence of psammoma bodies, stromal calcification, bone formation within the tumor mass, tumor extrathyroidal invasion, and tumor multifocality. The definition of psammoma bodies, stromal calcification, and bone formation has been described in detail in our earlier study [28]. Psammoma bodies, stromal calcification, and bone formation were encountered in 49, 52, and 3% of the samples, respectively.

The pT, pN, and stage grouping were classified according to the cancer staging guidelines in the seventh edition of the American Joint Committee on Cancer.

Immunohistochemical analysis

We stained formalin-fixed, paraffin-embedded (FFPE) whole-tissue 4- μ m sections from 110 cases of PTC, including tumor center and tumor invasion front, for BRAF V600E, PD-L1, and PD-1. Two pathologists (YB and DN) independently evaluated all stained sections.

Immunohistochemical staining using a BRAF V600E mutation-specific antibody (VE1) identifies the BRAF V600E gene mutation specifically and sensitively according to previous studies [29–31]. Mouse monoclonal anti-VE1 primary antibody (Ventana Medical Systems, Oro Valley, AZ, USA) was used in a working dilution on the Benchmark XT platform (Ventana Medical Systems). Staining was visualized using the ultraView Universal DAB Detection Kit (Ventana Medical Systems). Prevalidated VE1-positive and VE1-negative PTC specimens were used as positive and negative controls, respectively. The immunoreactivity of VE1 was evaluated as positive if unequivocal diffuse cytoplasmic staining of tumor cells was identified [29–32]. Focal staining, any type of isolated nuclear staining, and weak staining of single interspersed cells were evaluated as negative.

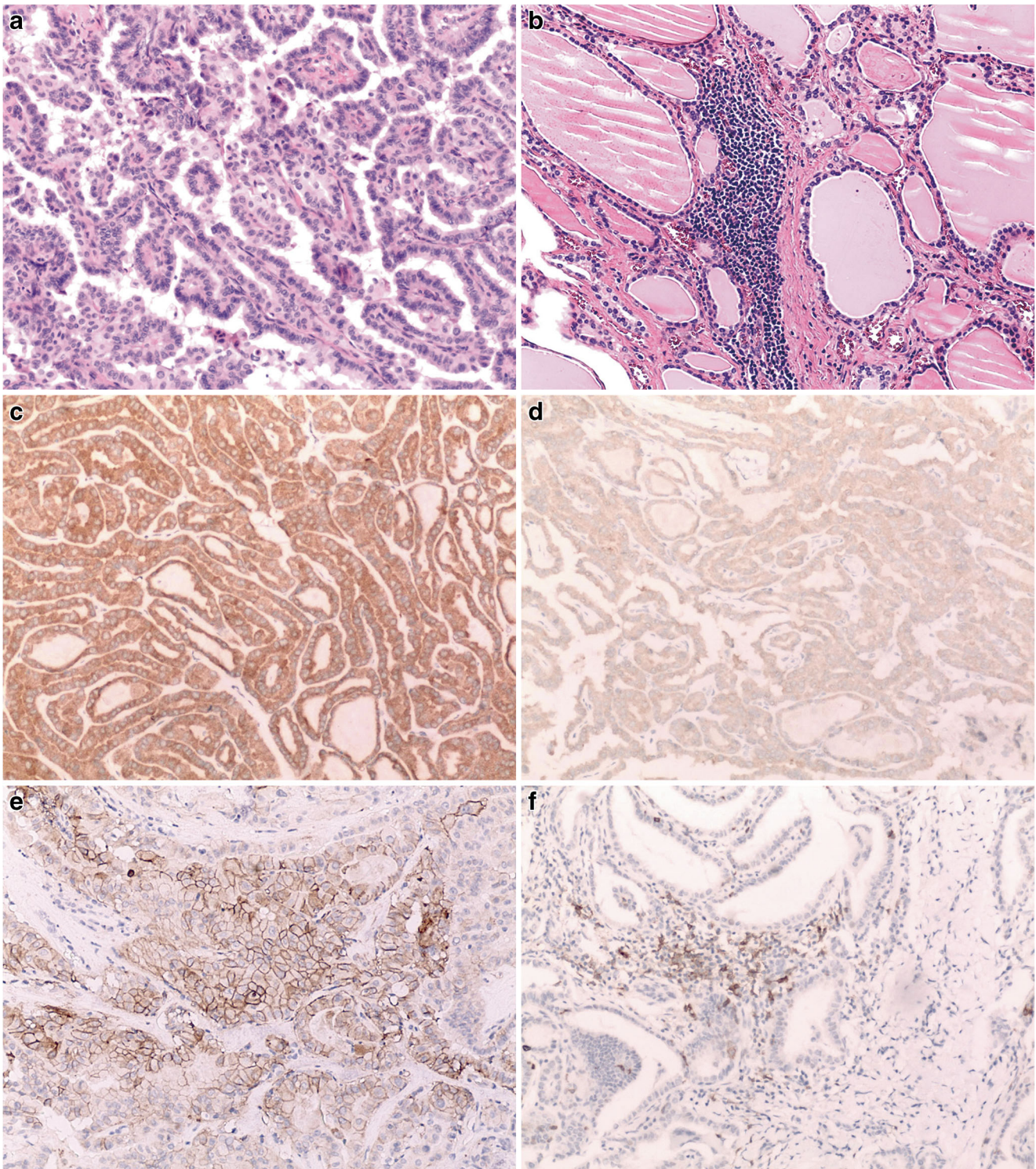


Fig. 1 **a** Papillary thyroid carcinoma (PTC) with papillary structures and typical nuclear features of PTC ($\times 100$). **b** Focal lymphocytic infiltration was noted in the background of PTC, which should not be regarded as chronic lymphocytic thyroiditis ($\times 100$). **c** BRAF V600E was strongly and diffusely expressed on the tumor cell cytoplasm ($\times 100$). **d** Mild but

diffuse expression of BRAF V600E was observed on the PTC ($\times 100$). **e** PD-L1 was unequivocally expressed on the tumor cell membrane and in part of the cytoplasm ($\times 100$). **f** PD-1 expression on tumor-infiltrating lymphocytes was identified in the tumor stroma ($\times 100$)

PD-L1 staining was performed with an anti-human PD-L1 rabbit monoclonal antibody (SP142; Zhongshan Golden

Bridge Biotechnology, Beijing, China) in a working dilution on an automated staining platform (BOND-III, Leica

Biosystems Ltd., Newcastle, UK) using a Bond Polymer Refine Detection kit (Leica Biosystems Ltd., Newcastle, UK) according to the manufacturer's instructions. Primary antibody was replaced with phosphate-buffered saline in the control sections to verify immunostaining specificity. Staining of 5% or more tumor cells was considered as positive and of less than 5% cells as negative [33].

For PD-1, antigen retrieval was carried out in a PTLink machine (Dako) after deparaffinization and rehydration of the FFPE sections. Subsequently, the sections were incubated in 3% H₂O₂ solution at room temperature for 10 min to block endogenous peroxidase activity. Sections were then incubated with mouse anti-PD-1 monoclonal antibody in a working dilution (UMAB199; Zhongshan Golden Bridge Biotechnology, Beijing, China) at 37 °C for 15 min, followed by incubation with anti-mouse IgG-HRP conjugate (EnVision FLEX/HRP; Dako) at room temperature for 25 min. Antibody binding was visualized using an EnVision FLEX DAB CHROMOGEN kit (Dako) according to the manufacturer's instructions. As negative control, primary antibodies were replaced with phosphate-buffered saline. Immunostaining of PD-1 was scored as positive when in a hot spot inside the tumor, or in the tumor invasive front, 3 or more TIL/high power field (HPF) were identified, using an Olympus BX43 microscope.

Statistical analysis

Data analysis was performed using SPSS 17.0 statistical software. Time-independent and categorical data were evaluated using the chi-squared test, continuity correction, or Fisher's exact probability test as appropriate. Statistical significance was defined as $P < 0.05$.

Results

Immunohistochemical expression of BRAF V600E, PD-L1, and PD-1 in papillary thyroid carcinoma without a background of CLT

VE1 staining was considered positive and was diffuse with either strong, moderate, or weak intensity (Fig. 1c, d) in 76% of PTC cases. Even tumors with weak diffuse staining have been shown to harbor the BRAF V600E gene mutation [29].

PD-L1 expression was located on the plasma membrane of tumor cells, with or without cytoplasmic staining. The staining pattern was either diffuse or focal (Fig. 1e). Strong staining on histiocytes inside the glandular lumina and in the fibrous stroma of PTC was not considered as positive. In six out of 18 follicular variant cases, the staining was focal. In the solid-type variant, the staining was diffuse, and the tall cell variant

was negative. PD-L1 expression was considered positive in 46% of all PTC cases.

Expression of PD-1 on TILs was found both in the tumor center and in the invasive front of the tumor in 78% of PTC cases (Fig. 1f).

Clinicopathological correlation of BRAF V600E, PD-L1, and PD-1 expression

BRAF V600E expression was not significantly correlated with the clinicopathological parameters patient age, gender, tumor size, extrathyroidal invasion, pT, pN, stage grouping, the presence of psammoma bodies, stromal calcification, bone formation, and tumor multifocality (data not shown).

PD-L1 expression significantly correlated with the absence of psammoma bodies ($P = 0.007$), but not with the other clinicopathological parameters (Table 1). Expression of PD-1 significantly correlated with small tumor size and absence of stromal calcification ($P = 0.022$ and 0.010 , respectively), but not with the other clinicopathological parameters (Table 2).

The correlation between BRAF V600E, PD-L1, and PD-1 expression

BRAF V600E expression was correlated with both PD-L1 and PD-1 expression ($P = 0.004$ and 0.001 , respectively) (Tables 1 and 2, Fig. 2a, b). PD-L1 expression correlated significantly with that of PD-1 ($P = 0.005$) (Fig. 2c).

Classification of tumor microenvironments based on PD-L1 and PD-1 expression

PD-L1 and PD-1 expression in the tumor microenvironment was classified as type 1 (PD-L1+/PD-1+, 41%), type 2 (PD-L1-/PD-1-, 17%), type 3 (PD-L1+/PD-1-, 5%), and type 4 (PD-L1-/PD-1+, 37%). BRAF V600E expression, the presence of psammoma bodies, and stromal calcification were significantly different between the four types. The BRAF V600E was positive in 89% of type 1, 37% of type 2, 80% of type 3, and 78% of type 4, respectively ($P = 0.001$). According to type, psammoma bodies were found in 33, 47, 40, and 68% of types 1, 2, 3, and 4, respectively ($P = 0.013$), while for stromal calcification, this was 47, 79, 60, and 44%, respectively ($P = 0.065$). In 8% of the 83 BRAF V600E-positive cases, PD-L1-/PD-1- (type 2) expression was found.

Discussion

We studied immunohistochemical expression status of both PD-L1 and PD-1 in a large number of human PTC cases. Expression of PD-L1 and PD-1 and BRAF status have been studied in follicular cell-derived thyroid carcinomas, as listed

Table 1 The correlation between the PD-L1 expression and other clinicopathological parameters in papillary thyroid carcinoma without background lymphocytic thyroiditis

	PD-L1		P value
	+ 51 (46%)	– 59 (54%)	
Age			
< 45 years	25 (49%)	33 (56%)	0.469
≥ 45 years	26 (51%)	26 (44%)	
Gender			
Female	41 (80%)	38 (64%)	0.063
Male	10 (20%)	21 (36%)	
Tumor size			
≤ 2 cm	36 (%)	36 (61%)	0.199
> 2 and ≤ 4 cm	11 (%)	21 (36%)	
> 4 cm	4 (%)	2 (3%)	
Extrathyroidal invasion			
–	6 (%)	14 (24%)	0.105
+	45 (%)	45 (76%)	
pT			
1b	5 (%)	12 (20%)	0.345
2	2 (%)	2 (3%)	
3	44 (%)	44 (75%)	
4a	0	1 (2%)	
pN			
0	18 (35%)	21 (36%)	0.536
1a	20 (39%)	18 (31%)	
1b	13 (26%)	20 (34%)	
Stage grouping			
I	3 (6%)	10 (17%)	0.271
II	1 (2%)	1 (2%)	
III	35 (69%)	32 (54%)	
IVA	12 (24%)	16 (27%)	
Psammoma body			
–	33 (65%)	23 (39%)	0.007
+	18 (35%)	36 (61%)	
Stromal calcification			
–	26 (51%)	27 (46%)	0.585
+	25 (49%)	32 (54%)	
Bone formation			
–	50 (98%)	57 (97%)	0.646
+	1 (2%)	2 (3%)	
Multifocality			
Unifocal	35 (69%)	40 (68%)	0.926
Multifocal	16 (32%)	19 (32%)	
BRAF V600E			
–	6 (12%)	21 (36%)	0.004
+	45 (88%)	38 (64%)	

in Table 3. Bastman et al. found in 92 cases of differentiated thyroid carcinoma expression of PD-L1/PD-1 mRNA; PD-L1

expression was higher in patients with nodal metastases [24]. This is different from our findings, most likely due to methodological differences. They also found no association of the BRAF V600E mutation with immunohistochemical expression of PD-L1, but on a small cohort of 22 advanced pT4 cases. Ahn et al. found PD-L1 expression in approximately 6% of PTCs, without correlation with PD-L1 status or oncogen mutation profile [21]. We found much more frequent expression of PD-L1 (46%) in PTCs, positively correlated with the expression of BRAF V600E and PD-1. The data of Ahn et al. should be interpreted with caution, since they used tissue microarrays which may not be representative of the whole tumor.

In recent years, a novel antibody specifically recognizing the BRAF V600E protein (clone VE1) has become available. Its specificity and sensitivity toward the BRAF V600E protein has been verified in many series of tumors, including in melanoma and PTC [29–31], and immunohistochemical staining with this antibody can be used as a surrogate for detection of the BRAF V600E mutation. We used this antibody to detect the BRAF V600E protein in PTC by immunohistochemistry, and our results were in accordance with those of Angell et al. [36] in that expression of BRAF V600E was associated with increased PD-L1 expression. We also found expression of BRAF V600E and PD-1 to be positively correlated. This provides a molecular basis for immunotherapy, targeting PD-L1/PD-1, for the subset of PTC patients who are refractory to radioiodine therapy. It should be noted that our cases were mostly conventional PTCs, and the correlation between BRAF V600E and PD-L1/PD-1 in specific PTC variants should be investigated in larger case series. We found expression of PD-L1 to be correlated with the absence of psammoma bodies, and of PD-1 with small tumor size and absence of stromal calcification. Stromal calcification arises by deposition of calcium phosphate in the fibrous stroma. The formation of both psammoma bodies and stromal calcification requires a certain amount of time. That PD-L1 is more often expressed in the absence of psammoma bodies, and PD-1 more often in the absence of stromal calcification, suggests that expression of PD-L1 and PD-1 is associated with a short clinical course and both may be involved in tumor growth, as has been shown in ovarian cancer [37].

A previous study has classified the tumor microenvironment into four groups based on PD-L1 expression and the presence of TILs [16]. Type 1 is PD-L1-positive in the presence of TILs which drives adaptive immune resistance, type 2 is PD-L1-negative in the absence of TIL which indicates immune ignorance, type 3 is PD-L1-positive in the absence of TILs indicating intrinsic induction, and type 4 is PD-L1-negative in the presence of TILs indicating the role of other suppressors in promoting immune tolerance. Patients with a type 1 tumor are most likely to benefit from single agent anti-PD-1/L1 blockage. In patients with a type 2 tumor, single

Table 2 The correlation between the PD-1 expression and other clinicopathological parameters in papillary thyroid carcinoma without background chronic lymphocytic thyroiditis

	PD-1		P value
	+ 86 (78%)	– 24 (22%)	
Age			
< 45 years	44 (51%)	14 (58%)	0.534
≥ 45 years	42 (49%)	10 (42%)	
Gender			
Female	61 (71%)	18 (75%)	0.695
Male	25 (29%)	6 (25%)	
Tumor size			
≤ 2 cm	59 (69%)	13 (54%)	0.022
> 2 and ≤ 4 cm	25 (29%)	7 (29%)	
> 4 cm	2 (23%)	4 (17%)	
Extrathyroid invasion			
–	16 (19%)	4 (17%)	0.828
+	70 (81%)	20 (83%)	
pT			
1b	13 (15%)	4 (17%)	0.193
2	4 (5%)	0	
3	69 (80%)	19 (79%)	
4a	0	1 (4%)	
pN			
0	31 (36%)	8 (33%)	0.647
1a	31 (36%)	7 (29%)	
1b	24 (28%)	9 (38%)	
Stage grouping			
I	9 (11%)	4 (17%)	0.668
II	2 (2%)	0	
III	54 (63%)	13 (54%)	
IVA	21 (24%)	7 (29%)	
Psmmoma body			
–	43 (50%)	13 (54%)	0.718
+	43 (50%)	11 (48%)	
Stromal calcification			
–	47 (55%)	6 (25%)	0.010
+	39 (45%)	18 (75%)	
Bone formation			
–	84 (98%)	23 (96%)	0.624
+	2 (2%)	1 (4%)	
Multifocality			
Unifocal	59 (69%)	16 (67%)	0.857
Multifocal	27 (31%)	8 (33%)	
BRAF V600E			
–	14 (16%)	13 (54%)	0.001
+	72 (84%)	11 (46%)	

agent checkpoint blockage would most likely not be successful, given the absence of pre-existing T cells, and combination

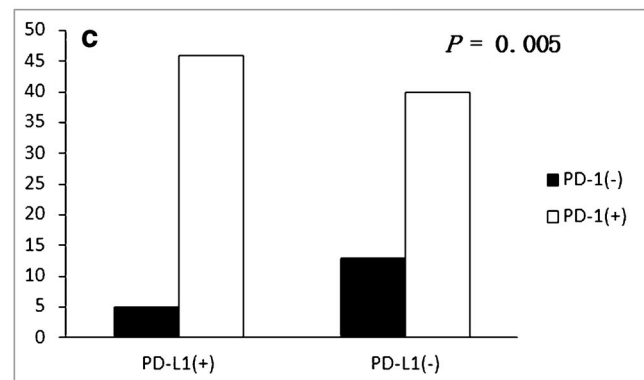
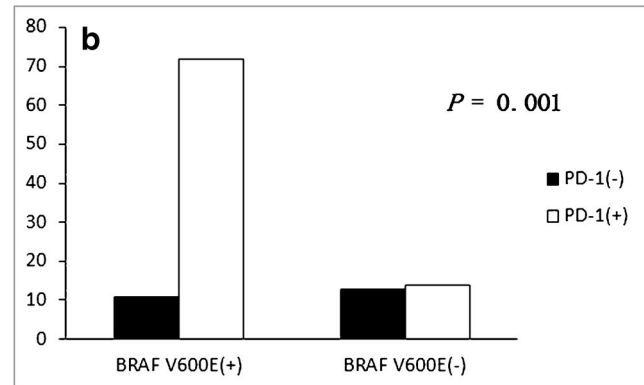
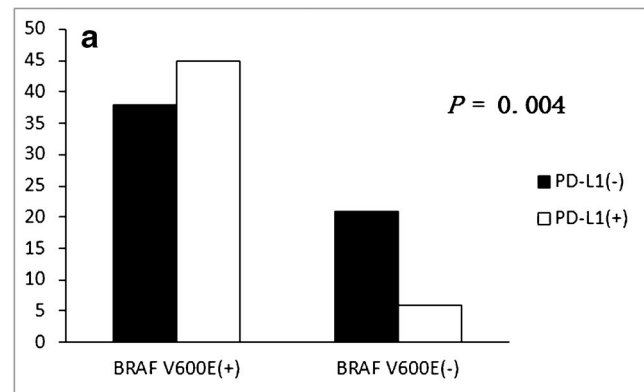


Fig. 2 **a** The correlation between BRAF V600E and PD-L1 expression. **b** The correlation between BRAF V600E and PD-1 expression. **c** The correlation between PD-L1 and PD-1 expression

therapies for this group should therefore be designed to bring T cells into tumors, such as a combination of anti-CTLA4 and anti-PD-1. For type 3 patients, a similar approach to that used for type 2 patients might be employed to recruit lymphocytes into tumors. For patients with a type 4 tumor, other immunosuppressor pathways such as metabolites and non-T cell effector strategies might be considered. The expression of PD-1 in TILs provides a more direct indicator of the tumor immunosuppression status. Therefore, we classified the tumor microenvironment into four groups, according to the expression of PD-L1 in tumor cells and the expression of PD-1 in the

Table 3 Summary of PD-L1 and PD-1 profiling in previous series of follicular cell-derived thyroid carcinomas

Authors, year	Journal	Sample	Tissue tested; methodology	Findings	Reference
Zwenepeol et al., 2017	<i>Histopathology</i>	49 cases of ATC	FFPE samples; immunohistochemistry (PD-L1, PD-1); RT-PCR (BRAF V600E mutation)	PD-L1 was found in 28.6% of the ATC cases. PD-1 was not expressed on the tumor cells. No association was observed between PD-L1 expression and BRAF mutation.	[34]
Chintakundlawar et al., 2017	<i>J Clin Endocrinol Metab</i>	16 cases of ATC	FFPE samples; immunohistochemistry (PD-L1, PD-1)	High PD-1 expression (> 40% staining) in inflammatory cells was associated with worse OS and trended toward worse PFS, whereas high PD-L1 expression in tumor cells (> 33% staining) trended toward worse PFS and OS.	[35]
Ahn et al., 2017	<i>Endocr Relat Cancer</i>	407 cases of primary thyroid cancers	Tissue microarray; immunohistochemistry (PD-L1, PD-1)	Tumoral PD-L1 was expressed in 6.1% of PTC, 7.6% of FTC, and 22.2% of ATC. There was no significant association between clinicopathologic variables, disease progression, oncogenic mutation, and PD-L1 expression.	[21]
Chowdhury et al., 2016	<i>Oncotarget</i>	66 benign thyroid nodules and 185 PTCs	FFPE samples; immunohistochemistry	Patients with increased PD-L1 membrane or cytoplasmic positivity had significantly shorter median DFS (36 and 49 months, respectively) as compared to those with PD-L1 negative tumors (DFS, both 186 months).	[20]
Bastman et al., 2016	<i>J Clin Endocrinol Metab</i>	Primary DTCs	Frozen tumor (DTC, n = 92); qRT-PCR (PD-L1, PD-1) FFPE samples (DTC, n = 12; PDTC, n = 2; ATC, n = 8); immunohistochemistry (PD-L1, PD-1)	At the RNA level, PD-L1 was higher in patients with nodal metastases. PD-L1 was expressed by tumor and associated leukocytes in 13 of 22 cases, and expression was more diffuse in ATCs. BRAF V600E mutation was associated with higher frequencies of tumor-associated lymphocytes, but not PD-L1 expression.	[24]
Angell et al., 2014	<i>Thyroid</i>	33 PTCs	FFPE samples; immunohistochemistry (PD-L1); direct DNA sequencing (BRAF V600E)	Mutated BRAF V600E tumors more often express high levels of PD-L1 (53 vs. 12.5%) compared to BRAF wild-type tumors.	[36]

ATC, anaplastic thyroid carcinoma; DTC, differentiated thyroid carcinoma; PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma; FFPE, formalin-fixed, paraffin-embedded; RT-PCR, reverse transcriptional-polymerase chain reaction; qRT-PCR, quantitative reverse transcriptional-polymerase chain reaction; OS, overall survival; PFS, progression-free survival

TILs, which might direct immunotherapy approaches more effectively. We found low incidence of BRAF V600E expression in PD-L1⁻/PD-1⁻ presumably immune ignorant tumors, and lack of psammoma bodies in PD-L1⁺/PD-1⁺ presumably immune-resistant tumors, which may help to improve the prediction of the efficacy of anti PD-L1/PD-1 immunotherapy.

In conclusion, we found that immunohistochemical expression of BRAF V600E was correlated with that of both PD-L1 and PD-1 in this series of PTC (of > 1 cm and without a background of lymphocytic thyroiditis). Some distinctive pathological features in PTC, including psammoma bodies and stromal calcification, were predictive of PD-L1 or PD-1 expression. Furthermore, patterns of PD-L1 and PD-1 expression might be helpful to predict antitumor efficacy of immunotherapy targeting PD-L1/PD-1 in a subset of PTC patients.

Author contributions BY, GT, KK, and LZ conceived and designed the experiments. HX and WQ performed the experiments. BY and ND reviewed the slides; JX and FQ analyzed the data. BY and GT wrote the manuscript.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

References

- Bai Y, Kakudo K, Li Y, Liu Z, Ozaki T, Ito Y, Kihara M, Miyauchi A (2008) Subclassification of non-solid-type papillary thyroid carcinoma identification of high-risk group in common type. *Cancer Sci* 99:1908–1915
- Carcangiu ML, Zampi G, Pupi A, Castagnoli A, Rosai J (1985) Papillary carcinoma of the thyroid. A clinicopathologic study of 241 cases treated at the University of Florence, Italy. *Cancer* 55:805–828
- Xing M (2005) BRAF mutation in thyroid cancer. *Endocr Relat Cancer* 12:245–262
- Baquero P, Sanchez-Hernandez I, Jimenez-Mora E, Orgaz JL, Jimenez B, Chiloeches A (2013) (V600E) BRAF promotes invasiveness of thyroid cancer cells by decreasing E-cadherin expression through a Snail-dependent mechanism. *Cancer Lett* 335:232–241
- Kimura ET, Nikiforova MN, Zhu Z, Knauf JA, Nikiforov YE, Fagin JA (2003) High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. *Cancer Res* 63:1454–1457
- Sjöblom T, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, Mandelker D, Leary RJ, Ptak J, Silliman N, Szabo S, Buckhaults P, Farrell C, Meeh P, Markowitz SD, Willis J, Dawson D, Willson JK, Gazdar AF, Hartigan J, Wu L, Liu C, Parmigiani G, Park BH, Bachman KE, Papadopoulos N, Vogelstein B, Kinzler KW, Velculescu VE (2006) The consensus coding sequences of human breast and colorectal cancers. *Science* 314:268–274
- Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N (2002) Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A* 99:12293–12297
- Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, Lennon VA, Celis E, Chen L (2002) Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 8:793–800
- Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, Chen S, Klein AP, Pardoll DM, Topalian SL, Chen L (2012) Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Sci Transl Med* 4:127ra37
- Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM, Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A, Wigginton JM (2012) Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* 366:2455–2465
- Ilie M, Hofman V, Dietel M, Soria JC, Hofman P (2016) Assessment of the PD-L1 status by immunohistochemistry: challenges and perspectives for therapeutic strategies in lung cancer patients. *Virchows Arch* 468:511–525
- Massari F, Santoni M, Ciccarese C, Santini D, Alfieri S, Martignoni G, Brunelli M, Piva F, Berardi R, Montironi R, Porta C, Cascinu S, Tortora G (2015) PD-1 blockade therapy in renal cell carcinoma: current studies and future promises. *Cancer Treat Rev* 41:114–121
- Sznol M, Chen L (2013) Antagonist antibodies to PD-1 and B7-H1 (PD-L1) in the treatment of advanced human cancer. *Clin Cancer Res* 19:1021–1034
- Zhang T, Xie J, Arai S, Wang L, Shi X, Shi N, Ma F, Chen S, Huang L, Yang L, Ma W, Zhang B, Han W, Xia J, Chen H, Zhang Y (2016) The efficacy and safety of anti-PD-1/PD-L1 antibodies for treatment of advanced or refractory cancers: a meta-analysis. *Oncotarget* 7:73068–73079
- Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M (2012) Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 366:2443–2454
- Teng MW, Ngiow SF, Ribas A, Smyth MJ (2015) Classifying cancers based on T-cell infiltration and PD-L1. *Cancer Res* 75:2139–2145
- Tang H, Qiao J, Fu YX (2016) Immunotherapy and tumor microenvironment. *Cancer Lett* 370:85–90
- Ricarte-Filho JC, Ryder M, Chitale DA, Rivera M, Heguy A, Ladanyi M, Janakiraman M, Solit D, Knauf JA, Tuttle RM, Ghossein RA, Fagin JA (2009) Mutational profile of advanced primary and metastatic radioactive iodine-refractory thyroid cancers reveals distinct pathogenetic roles for BRAF, PIK3CA, and AKT1. *Cancer Res* 69:4885–4893
- Xing M (2013) Molecular pathogenesis and mechanisms of thyroid cancer. *Nat Rev Cancer* 13:184–199
- Chowdhury S, Veyhl J, Jessa F, Polyakova O, Alenzi A, MacMillan C, Ralhan R, Walfish PG (2016) Programmed death-ligand 1 overexpression is a prognostic marker for aggressive papillary thyroid cancer and its variants. *Oncotarget* 31:32318–32328
- Ahn S, Kim TH, Kim SW, Ki CS, Jang HW, Kim JS, Kim JH, Choe JH, Shin JH, Hahn SY, Oh YL, Chung JH (2017) Comprehensive screening for PD-L1 expression in thyroid cancer. *Endocr Relat Cancer* 24:97–106

22. Cunha LL, Marcello MA, Morari EC, Nonogaki S, Conte FF, Gerhard R, Soares FA, Vassallo J, Ward LS (2013) Differentiated thyroid carcinomas may elude the immune system by B7H1 upregulation. *Endocr Relat Cancer* 20:103–110
23. Wu H, Sun Y, Ye H, Yang S, Lee SL, de las Morenas A (2015) Anaplastic thyroid cancer: outcome and the mutation/expression profiles of potential targets. *Pathol Oncol Res* 21:695–701
24. Bastman JJ, Serracino HS, Zhu Y, Koenig MR, Mateescu V, Sams SB, Davies KD, Raeburn CD, McIntyre RC Jr, Haugen BR, French JD (2016) Tumor-infiltrating T cells and the PD-1 checkpoint pathway in advanced differentiated and anaplastic thyroid cancer. *J Clin Endocrinol Metab* 101:2863–2873
25. Atefi M, Avramis E, Lassen A, Wong DJ, Robert L, Foulad D, Cerniglia M, Titz B, Chodon T, Graeber TG, Comin-Anduix B, Ribas A (2014) Effects of MAPK and PI3K pathways on PD-L1 expression in melanoma. *Clin Cancer Res* 20:3446–3457
26. Brauner E, Gunda V, Vanden Borre P, Zurakowski D, Kim YS, Dennett KV, Amin S, Freeman GJ, Parangi S (2016) Combining BRAF inhibitor and anti PD-L1 antibody dramatically improves tumor regression and anti tumor immunity in an immunocompetent murine model of anaplastic thyroid cancer. *Oncotarget* 7:17194–17211
27. Zuo H, Tang W, Yasuoka H, Nakamura Y, Ito Y, Miyauchi A, Kakudo K (2007) A review of 227 cases of small papillary thyroid carcinoma. *Eur J Surg Oncol* 33:370–375
28. Bai Y, Zhou G, Nakamura M, Ozaki T, Mori I, Taniguchi E, Miyauchi A, Ito Y, Kakudo K (2009) Survival impact of psammoma body, stromal calcification, and bone formation in papillary thyroid carcinoma. *Mod Pathol* 22:887–894
29. Sun J, Zhang J, Lu J, Gao J, Lu T, Ren X, Duan H, Liang Z (2015) Immunohistochemistry is highly sensitive and specific for detecting the BRAF V600E mutation in papillary thyroid carcinoma. *Int J Clin Exp Pathol* 8:15072–15078
30. Pyo JS, Sohn JH, Kang G (2015) BRAF immunohistochemistry using clone VE1 is strongly concordant with BRAF (V600E) mutation test in papillary thyroid carcinoma. *Endocr Pathol* 26:211–217
31. Liu H, Li Z, Wang Y, Feng Q, Si L, Cui C, Guo J, Xue W (2014) Immunohistochemical detection of the BRAF V600E mutation in melanoma patients with monoclonal antibody VE1. *Pathol Int* 64:601–606
32. Koperek O, Kornauth C, Capper D, Berghoff AS, Asari R, Niederle B, von Deimling A, Birner P, Preusser M (2012) Immunohistochemical detection of the BRAF V600E-mutated protein in papillary thyroid carcinoma. *Am J Surg Pathol* 36:844–850
33. Li Z, Lai Y, Sun L, Zhang X, Liu R, Feng G, Zhou L, Jia L, Huang X, Kang Q, Lin D, Gao J, Shen L (2016) PD-L1 expression is associated with massive lymphocyte infiltration and histology in gastric cancer. *Hum Pathol* 55:182–189
34. Zwaenepoel K, Jacobs J, De Meulenaere A, Silence K, Smits E, Siozopoulou V, Hauben E, Rolfó C, Rottey S, Pauwels P (2017) CD70 and PD-L1 in anaplastic thyroid cancer-promising targets for immunotherapy. *Histopathology* 71:357–365
35. Chintakuntlawar AV, Rumilla KM, Smith CY, Jenkins SM, Foote RL, Kasperbauer JL, Morris JC, Ryder M, Alsidawi S, Hilger C, Bible KC (2017) Expression of PD-1 and PD-L1 in anaplastic thyroid cancer patients treated with multimodal therapy: results from a retrospective study. *J Clin Endocrinol Metab* 102:1943–1950
36. Angell TE, Lechner MG, Jang JK, Correa AJ, LoPresti JS, Epstein AL (2014) BRAF V600E in papillary thyroid carcinoma is associated with increased programmed death ligand 1 expression and suppressive immune cell infiltration. *Thyroid* 24:1385–1393
37. Clark CA, Gupta HB, Sareddy G, Pandeswara S, Lao S, Yuan B, Drerup JM, Padron A, Conejo-Garcia J, Murthy K, Liu Y, Turk MJ, Thedieck K, Hurez V, Li R, Vadlamudi R, Curiel TJ (2016) Tumor-intrinsic PD-L1 signals regulate cell growth, pathogenesis, and autophagy in ovarian cancer and melanoma. *Cancer Res* 76:6964–6974