Comprehensive screening for PD-L1 expression in thyroid cancer

Soomin Ahn[1,*], Tae Hyuk Kim[2,*], Sun Wook Kim[2], Chang Seok Ki[3], Hye Won Jang[4], Jee Soo Kim[5], Jung Han Kim[5], Jun-Ho Choe[5], Jung Hee Shin[6], Soo Yeon Hahn[6], Young Lyun Oh[7] and Jae Hoon Chung[2]

[1]Department of Pathology, Ewha Womans University School of Medicine, Seoul, Korea
[2]Division of Endocrinology and Metabolism, Department of Medicine, Thyroid Center, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea
[3]Department of Laboratory Medicine and Genetics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea
[4]Department of Medical Education, Sungkyunkwan University School of Medicine, Seoul, Korea
[5]Division of Breast and Endocrine Surgery, Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea
[6]Department of Radiology and Center for Imaging Science, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea
[7]Department of Pathology and Translational genomics, Sungkyunkwan University School of Medicine, Seoul, Korea
(*S Ahn and T H Kim contributed equally to this work)

Correspondence should be addressed to Y L Oh or J H Chung
Email
bijou@skku.edu or thyroid@skku.edu

Abstract

PD-L1 expression is being considered a potential biomarker for response of anti-PD-1 or anti-PD-L1 agents in various tumors. The reported frequency of PD-L1 positivity varies in thyroid carcinomas, and multiple factors may contribute to the variability in PD-L1 positivity. We evaluated the PD-L1 expression in various thyroid cancers on a large scale. A total of 407 primary thyroid cancers with a median 13.7-year of follow-up were included. We evaluated the frequency of PD-L1 expression using a rabbit monoclonal antibody (clone SP142). In addition, we analyzed the relationships between PD-L1 expression and clinicopathologic factors, including *TERT* promoter, *BRAF* status and disease progression. Tumoral PD-L1 was expressed in 6.1% of papillary thyroid carcinomas, 7.6% of follicular thyroid carcinomas and 22.2% of anaplastic thyroid carcinomas. The distribution of PD-L1 positivity was different according to cancer histology types ($P < 0.001$). All PD-L1-positive cases of follicular thyroid carcinoma and anaplastic thyroid carcinoma showed strong intensity. The proportions of positivity in PD-L1 positive anaplastic thyroid carcinomas were more than 80%. PD-L1 in immune cells was positive in 28.5% of papillary thyroid carcinoma, 9.1% of follicular thyroid carcinomas and 11.1% of anaplastic thyroid carcinomas. There was no significant association between clinicopathologic variables, disease progression, oncogenic mutation and PD-L1 expression. PD-L1 was highly expressed in a subset of patients with advanced thyroid cancer, such as follicular and anaplastic thyroid carcinoma. Identification of PD-L1 expression may have direct therapeutic relevance to patients with refractory thyroid cancer.

Key Words
- thyroid
- PD1
- PDL1
- *TERT*
- anaplastic carcinoma

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Introduction

Programmed cell death 1 (PD-1) and the PD-1 ligand (PD-L1) show great promise for the treatment of various tumors, including melanoma and non-small-cell lung cancer (Ohaegbulam *et al.* 2015). PD-1 (B7-1) is a cell-surface glycoprotein normally expressed by macrophage lineage cells and T cells. The binding of PD-1 to one of its ligands, PD-L1 or PD-L2, can inhibit a cytotoxic T-cell immune response, leading to immune tolerance of cells expressing PD-L1 or PD-L2 (Dong *et al.* 2002). PD-L1 is constitutively expressed on tumor cells as a result of oncogenic signaling or dynamic IFNγ expression in tumor microenvironments (Taube *et al.* 2012). Based on the premise that anti-PD-1 therapy functions by blocking interactions between PD-1 and PD-L1, PD-L1 expression is being considered a potential biomarker for response of anti-PD-1 or PD-L1 agents (Page *et al.* 2014). Patients with higher levels of PD-L1 expression by immunohistochemistry have improved response rates during anti-PD-L1 therapy for lung cancer and melanoma (Patel & Kurzrock 2015). Currently, PD-L1 immunohistochemistry using a rabbit monoclonal antibody (clone 22C3) has been approved by the US Food and Drug Administration as a companion diagnostic test for patient selection of pembrolizumab (PD-1-blocking monoclonal antibody) treatment in metastatic non-small-cell lung cancer (Sul *et al.* 2016). Recently, another PD-1-blocking monoclonal antibody, atezolizumab, was approved by the US Food and Drug Administration for bladder cancer treatment (Rosenberg *et al.* 2016), and the US Food and Drug Administration also approved the diagnostic test, Ventana PD-L1 (SP142) immunohistochemistry assay, to detect PD-L1 expression of tumor-infiltrating immune cells.

There have been several studies investigating PD-L1 expression in thyroid cancer (Cunha *et al.* 2013, Angell *et al.* 2014, Wu *et al.* 2015, Bastman *et al.* 2016, Chowdhury *et al.* 2016). The frequency of PD-L1 positivity ranges from 23% to 87.5% of thyroid carcinoma, which varies according to studies and is higher than other cancer types (Cunha *et al.* 2013, Angell *et al.* 2014, Patel & Kurzrock 2015, Wu *et al.* 2015, Bastman *et al.* 2016 Chowdhury *et al.* 2016). Multiple factors may contribute to the wide range of PD-L1 positivity reported across studies, including different antibodies, assay methods, applied thresholds and interpretation (Phillips *et al.* 2015). Although only membranous staining of PD-L1 is considered positive and the cut-off values of 1%, 5% or 10% are frequently used to define the positive rate of PD-L1 staining for patient selection in clinical trials, the optimal cut-off value for PD-L1 staining has not been definitively validated.

Clinically, a previous study reported that PD-L1 expression correlates with a higher risk of recurrence and shortened disease-free survival in thyroid cancer patients (Chowdhury *et al.* 2016). In addition, the *BRAF* V600E mutation was once reported to be associated with increased PD-L1 expression in papillary thyroid carcinoma (Angell *et al.* 2014). Although most papillary thyroid carcinomas have indolent clinical behavior, follicular thyroid carcinomas show occasional metastasis and anaplastic thyroid carcinomas are highly aggressive with a mortality of almost 100% (Xu & Ghossein 2016). Accurate detection of PD-L1-positive tumors in aggressive thyroid cancers can be important to identify patients who may potentially benefit from anti-PD-L1 therapy.

Herein, we evaluated PD-L1 expression in various thyroid cancers on a large scale with more than ten years of follow-up. We used a Ventana PD-L1 (SP142) immunohistochemistry assay and interpreted the immunohistochemistry results semiquantitatively. The frequency of PD-L1 expression in various thyroid cancers and the relationship between PD-L1 expression and clinicopathologic factors including *BRAF*, *TERT* promoter status and disease progression were evaluated.

Materials and methods

Case selection

A total of 407 primary thyroid cancers were included (papillary thyroid carcinoma $n=326$, follicular thyroid carcinoma $n=66$, poorly differentiated thyroid carcinoma $n=6$ and anaplastic thyroid carcinoma $n=9$). Of six poorly differentiated thyroid carcinomas, one had co-existing papillary thyroid carcinoma and another had co-existing follicular carcinoma. The samples were obtained from patients who underwent surgical resection for primary thyroid cancer at the Samsung Medical Center, Seoul, Korea, between 1994 and 2004. Clinicopathological information, including age, sex, the presence of Hashimoto disease, histologic type, tumor size, multiplicity, margin status, pT, pN stage, disease relapse and survival data, was evaluated by reviewing the medical records. Patients lost during follow-up or who died of causes other than thyroid cancer were not included in the survival analysis. The study was approved by the institutional review board of Samsung Medical Center (IRB File No.: 2015-04-007).

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PD-L1 immunohistochemistry

In all cases, we performed PD-L1 immunohistochemistry using tissue microarrays. Tissue microarrays consisted of four 2.0 mm cores from each tumor. A pathologist (SA) reviewed the slide of thyroidectomy and marked areas for tissue microarray. Four areas including center and periphery of tumor were chosen for tissue microarray. Formalin-fixed, paraffin-embedded tissues were sliced to a 4-μm thickness and dried at 60°C for 30 min. The anti-PD-L1 immunohistochemistry (anti-human PD-L1 rabbit monoclonal, 1:25, clone SP142, Spring Bioscience, Pleasanton, CA, USA) staining was performed on a BenchMark automated immunostainer (Ventana, Tucson, AZ, USA). Antigen retrieval was performed for 92 min with CC1 buffer, and the antibody was incubated for 120 min at 37°C using the Ventana BenchMark XT platform. Signal visualization was achieved with the OptiView DAB immunohistochemistry detection kit (Ventana, Catalog number 760-700) and OptiView Amplification kit (Ventana, Catalog number 860-099). Tonsil squamous epithelium was used as a PD-L1 immunohistochemistry-positive control (Fig. 1A) (Phillips *et al.* 2015).

PD-L1 immunohistochemistry microscopy results were interpreted by two experienced pathologists (SA and YO). PD-L1 expression was evaluated in tumor cells and tumor-infiltrating lymphocytes. For tumor cells, positive PD-L1 staining was defined as complete and/or partial circumferential linear plasma membrane staining at any intensity that can be differentiated from background and diffuse cytoplasmic staining (Phillips *et al.* 2015). According to the *de-facto* consensus, membranous positivity of tumor cells was considered positive, whereas only cytoplasmic staining was disregarded (Scheel *et al.* 2016). The staining intensity was determined for each cell. The percentage of cells at each intensity was measured. Finally, the proportion and intensity of positive cells for each case were recorded. The positivity of PD-L1 status was determined based on 1% and 5% thresholds. For tumor-infiltrating lymphocytes, membranous and cytoplasmic staining cannot be reliably distinguished due to the small cell size, and PD-L1 staining of any intensity, either membranous or cytoplasmic, was considered positive (Scheel *et al.* 2016).

BRAF and *TERT* promoter mutation test

Genomic DNA was extracted using a Qiagen DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instruction. Semi-nested polymerase chain reaction (PCR) was carried out to identify *TERT* promoter mutations. First-round PCR was performed using primers *TERT*-F and *TERT*-236-R, as previously described (Sohn *et al.* 2016). The 235 bp-sized PCR amplicon was subjected to second-round PCR using primers *TERT*-F and *TERT*-163-R. PCR reactions were performed using a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems). Cycle sequencing was performed using Big Dye Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems) on an ABI 3730xl Genetic Analyzer (Applied Biosystems).

For detection of the *BRAF* V600E mutation, the mutant enrichment with 3′-modified oligonucleotides-PCR

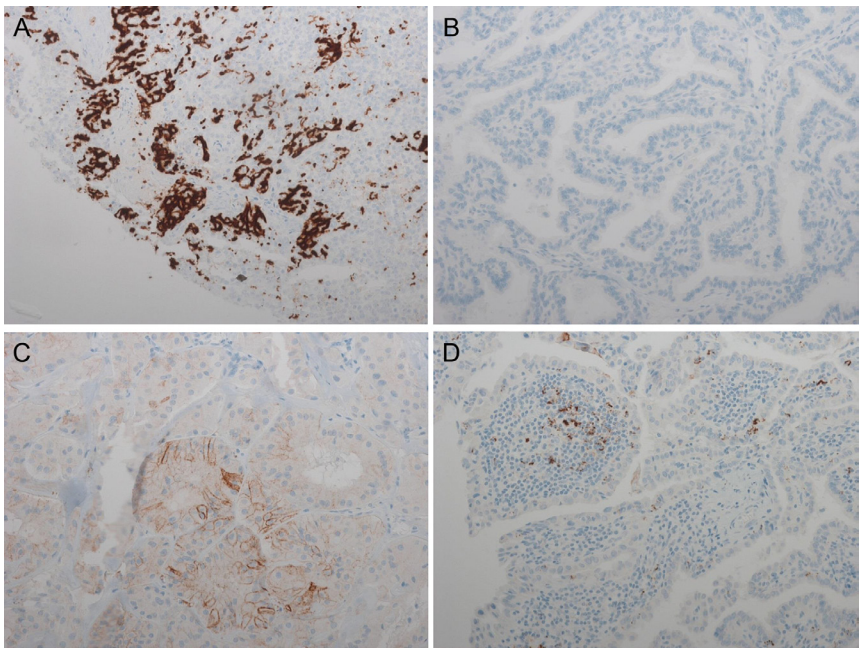

Figure 1
(A) Tonsillar squamous epithelium used as the positive control for PD-L1 staining. (B–D) PD-L1 staining in papillary thyroid cancers. (B) PD-L1-negative staining. (C) PD-L1-positive staining in tumor cells with intensity 2+ and 1+ and (D) PD-L1-positive staining in intratumoral lymphocytes. A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-16-0421.

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(MEMO-PCR) and direct sequencing was performed as described previously (Lee *et al.* 2012). The obtained sequences were analyzed using the Sequencher program (Gene Codes Corp., Ann Arbor, MI, USA) and were compared to a reference sequence (GenBank accession number NM_004333.4). *BRAF* mutation was tested in 322 available cases.

Statistical analysis

Statistical analysis was performed using SPSS, version 18.0. Contingency tables and χ^2 tests were used to correlate PD-L1 immunohistochemistry results with tumor type, age, sex, pT stage, pN stage, Hashimoto disease, multiplicity, *BRAF* status, *TERT* promoter status, disease recurrence and cancer-specific death. Structural recurrence, as opposed to biochemical recurrence, was defined as recurrent or persistent disease determined pathologically or cytologically to be malignant tissue and/or highly suspicious metastatic lesions. Disease-free interval curves were drawn using Kaplan–Meier estimates and were compared using log-rank tests. A P value <0.05 was considered to be significant.

Results

Patient demographics and clinicopathologic variables

Clinicopathologic characteristics of 407 patients according to PD-L1 status are presented in Table 1. The age of patients at the time of diagnosis ranged from 15.8 to 81.4 years (median 43.8 years). The median (IQR) follow-up was 13.7 (11.8–16.4) years. Among patients, 83% were females and 17% were males. The proportions of papillary thyroid carcinoma, follicular thyroid carcinoma, poorly differentiated thyroid carcinoma and anaplastic thyroid carcinoma were 80.1%, 16.2%, 1.5% and 2.2%, respectively. According to the AJCC classification, stages 1, 2, 3 and 4 consisted of 54.1%, 6.6%, 25.3% and 14.0%, respectively. Hashimoto disease was present in 49 of 407 patients.

Among 380 patients without systemic metastasis at the initial presentation, 99 (26.1%) developed structural recurrence. Of the 407 patients, 43 (10.6%) deceased during follow-up and 38 (9.3%) died of thyroid cancer. According to cancer types, 17 (5.2%) of 326 patients with papillary thyroid carcinoma, ten (15.2%) of 66 with follicular thyroid carcinoma, two (33.3%) of six with poorly differentiated thyroid carcinoma and all nine patients with anaplastic thyroid carcinoma died of thyroid

Table 1 Clinicopathologic characteristic of 407 thyroid tumors according to PD-L1 status.

Variables	PD-L1 immunohistochemisty in tumor		Total	*P*-value
	Negative	Positive		
Age				
<45	212 (55.8%)	13 (48.1%)	225	0.44
≥45	168 (44.2%)	14 (51.9%)	182	
Sex				
Female	318 (83.7%)	20 (74.1%)	338	0.192
Male	62 (16.3%)	7 (25.9%)	69	
Tumor size				
<2 cm	42 (11.1%)	2 (7.4%)	44	0.797
2–4 cm	282 (74.2%)	20 (74.1%)	302	
>4 cm	56 (14.7%)	5 (18.5%)	61	
Multiplicity				
No	280 (73.7%)	19 (70.4%)	299	0.706
Yes	100 (26.3%)	8 (29.6%)	108	
Extension				
No	102 (26.8%)	8 (29.6%)	110	0.957
Yes	231 (60.8%)	16 (59.3%)	247	
NA	47 (12.4%)	3 (11.1%)	50	
Margin				
Negative	291 (76.6%)	22 (81.5%)	313	0.648
Positive	68 (17.9%)	3 (11.1%)	71	
NA	21 (5.5%)	2 (7.4%)	23	
pT stage				
T1	62 (16.3%)	2 (7.4%)	64	0.062
T2	50 (13.2%)	6 (22.2%)	56	
T3	248 (65.3%)	16 (59.3%)	264	
T4a	19 (5%)	2 (7.4%)	21	
T4b	1 (0.3%)	0 (0%)	1	
Tx	0 (0%)	1 (3.7%)	1	
pN stage				
N0	191 (50.3%)	14 (51.9%)	205	0.22
N1a	113 (29.7%)	8 (29.6%)	121	
N1b	75 (19.7%)	4 (14.8%)	79	
Nx	1 (0.3%)	1 (3.7%)	2	
Initial metastasis				
No	354 (93.2%)	26 (96.3%)	380	0.45
Yes	26 (6.8%)	1 (3.7%)	27	
TNM stage				
1	207 (54.5%)	13 (48.1%)	220	0.11
2	22 (5.8%)	5 (18.5%)	27	
3	98 (25.8%)	5 (18.5%)	103	
4	53 (13.9%)	4 (14.8%)	57	
Recurrence				
No	264 (74.6%)	17 (65.4%)	281	0.303
Yes	90 (25.4%)	9 (34.6%)	99	
Death				
Alive	345 (90.8%)	24 (88.9%)	369	0.73
Dead	35 (9.2%)	3 (11.1%)	38	
TERT promoter				
Wild	333 (87.6%)	25 (92.6%)	358	0.758
Mutated	47 (12.4%)	2 (7.4%)	49	
BRAF				
Wild	112 (37.5%)	9 (39.1%)	121	0.873
Mutated	187 (62.5%)	14 (60.9%)	201	

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cancer. The *BRAF* V600E mutation was detected in 201 (62.4%) of 322 tested cases. According to histologic type, it was detected in 75.3% (198 of 263) of papillary thyroid carcinoma, 25% (1 of 4) of poorly differentiated thyroid carcinomas and 66.7 % (2 of 3) of anaplastic thyroid carcinoma cases. *BRAF* mutation was detected in none of the 52 follicular thyroid carcinomas. *TERT* promoter mutations were detected in 49 (12.0%) of 407 tested cases: 11.1% ($n=45$) *TERT* C228T and 1.0% ($n=4$) C250T mutations. *TERT* promoter mutations were detected in 9.8% (32 of 326) of papillary thyroid carcinomas, 16.7% (11 of 66) of follicular thyroid carcinomas, 33.3% (2 of 6) of poorly differentiated thyroid carcinomas and 44.4 % (4 of 9) of anaplastic thyroid carcinoma cases.

PD-L1 immunohistochemistry results

Overall, tumoral PD-L1 was expressed in 27 (6.6%) of 407 cases at a 1% threshold. Regarding cancer types, PD-L1-positive staining was found in 6.1% of papillary thyroid carcinomas (20 of 326), 7.6% of follicular thyroid carcinomas (5 of 66), 22.2% of anaplastic thyroid carcinomas (2 of 9) and none of the poorly differentiated thyroid carcinomas. The distribution of PD-L1 positivity was significantly different according to cancer histology types ($P<0.001$) (Table 2). Most PD-L1-positive papillary thyroid carcinoma showed weak (1+) intensity with <5% proportion of positivity (Fig. 1). When a 5% threshold was applied, the positivity of PD-L1 in papillary thyroid carcinoma decreased to 0.9% (3 of 326). Follicular and anaplastic thyroid carcinoma showed no change in PD-L1 positivity when a 5% threshold was applied. All PD-L1-positive follicular and anaplastic thyroid carcinomas showed strong intensity (2+ and 3+) (Fig. 2). The proportions of positive staining in two PD-L1-positive anaplastic thyroid carcinomas were 80% and 90%. The details of immunohistochemistry results and clinicopathologic information of PD-L1-positive tumors are summarized in Table 3.

Regarding tumor-infiltrating lymphocytes, PD-L1 was expressed in 93 (28.5%) of 326 papillary thyroid carcinomas, 6 (9.1%) of 66 follicular thyroid carcinomas, 1 (11.1%) of 9 anaplastic thyroid carcinomas and none of poorly differentiated thyroid carcinomas. Although there was no association between accompanied Hashimoto disease and tumoral PD-L1 expression ($P=0.551$), the frequency of PD-L1 expression in tumor-infiltrating lymphocytes was significantly higher in cases with Hashimoto disease (67.3%) than cases without Hashimoto disease (18.7%) ($P<0.001$) (Supplementary Table 1, see section on supplementary data given at the end of this article). There was no significant association between PD-L1 expression in tumor cells and those in tumor-infiltrating lymphocytes ($P=0.866$).

Association of PD-L1 status with clinicopathologic variables

The clinicopathologic characteristics of 407 patients according to tumoral PD-L1 status are presented in Table 1. There was no significant association between PD-L1 status and age, sex, tumor size, stage, initial metastasis, recurrence, death, *BRAF* and *TERT* promoter results.

As papillary thyroid carcinoma, follicular thyroid carcinoma, poorly differentiated thyroid and anaplastic thyroid carcinoma show distinctly different clinical behaviors, associations between clinicopathologic variables and PD-L1 results were separately analyzed. In thyroid carcinoma of papillary, follicular and anaplastic subtypes, there was no significant association between PD-L1 status clinicopathologic variables. Among 32 *TERT* promoter-mutated papillary thyroid carcinomas, two (6.3%) cases revealed PD-L1 positivity, despite no statistical significance.

In addition, there was no association between clinicopathologic factors and PD-L1 expression in tumor-infiltrating lymphocytes for any type of thyroid carcinomas.

Table 2 Frequency of PD-L1 positivity.

	PD-L1 immunohistochemistry in tumor			
		Positive		
	Negative	(1% ≤ Proportion <5%)	(Proportion ≥5%)	Total
Papillary carcinoma	306 (93.9%)	17 (5.2%)	3 (0.9%)	326
Follicular carcinoma	61 (92.4%)	0 (0%)	5 (7.6%)	66
Poorly differentiated carcinoma	6 (100%)	0 (0%)	0 (0%)	6
Anaplastic carcinoma	7 (77.8%)	0 (0%)	2 (22.2%)	9
Total	380 (93.4%)	18 (4.4%)	9 (2.2%)	407

$P<0.001$.

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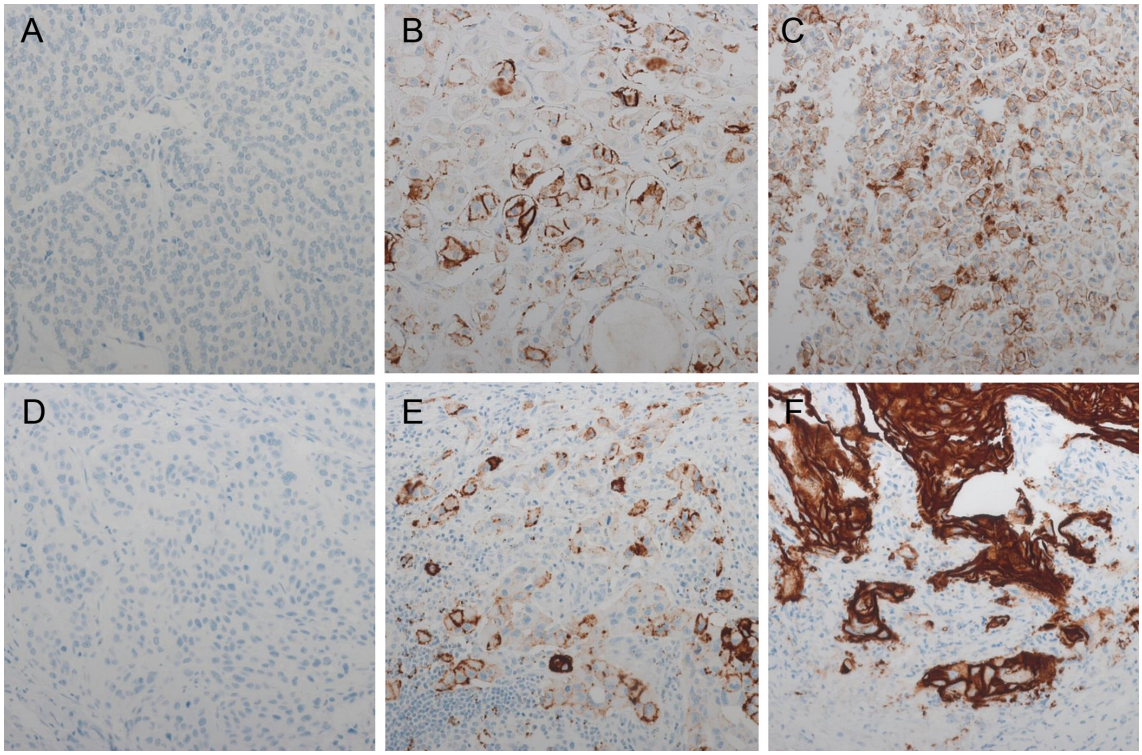

Figure 2
(A–C) PD-L1 staining in follicular thyroid carcinomas: (A) PD-L1-negative staining, (B) PD-L1-positive staining in tumor cells with intensity 3+ and 2+ and (C) PD-L1-positive staining in tumor cells with intensity 3+ and 2+. (D–F) PD-L1 staining in anaplastic thyroid carcinomas: (D) PD-L1 negative staining, (E) PD-L1-positive staining in tumor cells with intensity 3+ and 2+, and (F) PD-L1-positive staining in tumor cells with intensity 3+. A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-16-0421.

Association of PD-L1 status with clinical outcome

We did not find significant associations between tumoral PD-L1 expression and disease-free survival in papillary thyroid carcinoma ($P=0.123$), follicular thyroid carcinoma ($P=0.398$) and anaplastic thyroid carcinoma ($P=0.953$). In addition, there was no statistically significant association between PD-L1 status and cancer-specific survival in papillary thyroid carcinoma ($P=0.955$), follicular thyroid carcinoma ($P=0.345$) and anaplastic thyroid carcinoma ($P=0.667$).

PD-L1 negativity in tumor-infiltrating lymphocytes was associated with shorter cancer-specific survival in papillary thyroid carcinomas ($P=0.041$). There was no association between PD-L1 expression in tumor-infiltrating lymphocytes and cancer-specific survival in follicular thyroid carcinomas and anaplastic carcinomas.

Discussion

We evaluated the PD-L1 expression in a large cohort of thyroid carcinoma patients with a median 13.7 years of follow-up period. We identified tumoral PD-L1 expression in 6.6% of our thyroid cancer cohort. Remarkably, PD-L1 expression was more frequent and staining intensity was stronger in anaplastic thyroid carcinomas and follicular thyroid carcinomas. All PD-L1-positive anaplastic thyroid carcinomas showed strong PD-L1 staining intensity and were positive in ≥80% of tumor cells. Anaplastic thyroid cancer is known to have a poor prognosis due to its aggressive and rapid metastasis with a median survival of less than six months (Lim *et al.* 2012). The first treatment option for anaplastic thyroid carcinoma is palliative surgery, followed by radiotherapy, chemotherapy or both (Pierie *et al.* 2002, Lim *et al.* 2012). However, many patients present with inoperable status and complete resection is possible for only up to one-third of patients at presentation (McIver *et al.* 2001, Lim *et al.* 2012). Therefore, identification of novel therapeutic targets is warranted for treatment of this aggressive thyroid cancer. Although infrequent, identification of PD-L1 expression in anaplastic cancer might have direct therapeutic relevance to patients. Two previous studies reported PD-L1 expression in anaplastic thyroid carcinomas, and the frequency of PD-L1 positivity was 75% and 23%, respectively (Table 4) (Wu *et al.* 2015, Bastman *et al.* 2016).

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Table 3 Clinicopathologic information of PD-L1-positive thyroid cancers.

Case no	Histology type	PD-L1 in tumor (intensity, proportion)	Age	Sex	Size of the largest tumor	Multiplicity	pT stage	pN stage	TNM stage	*TERT* promoter	*BRAF*	Recurrence	Death
1	Papillary	1+, 1%	54	Female	2.5	No	T3	N1a	3	Wild	V600E	No recurrence	Alive
2	Papillary	1+, 1%	42	Male	1.8	No	T3	N1a	1	Wild	V600E	No recurrence	Alive
3	Papillary	1+, 2%	27	Female	5	Yes	T3	N1b	1	Wild	V600E	Recurrence	Alive
4	Papillary	2+, 1%	36	Female	2.5	No	T2	N1a	1	Wild	V600E	Recurrence	Alive
5	Papillary	2+, 1%	45	Female	2	Yes	T3	N1b	1	Wild	V600E	No recurrence	Alive
6	Papillary	1+, 2%	42	Female	2.9	Yes	T3	N1a	1	Wild	V600E	No recurrence	Alive
7	Papillary	1+, 2%	40	Female	3.6	Yes	T3	N1b	1	Wild	Wild	Recurrence	Alive
8	Papillary	1+, 2%	34	Female	2.7	No	T3	N0	1	Wild	V600E	No recurrence	Alive
9	Papillary	1+, 2%	47	Female	2.3	Yes	T3	N1a	3	Wild	V600E	No recurrence	Alive
10	Papillary	1+, 2%	39	Female	0.7	Yes	T3	N1a	1	Wild	NA	Recurrence	Alive
11	Papillary	1+, 2%	61	Male	10.5	No	T4a	N1b	4	C228T	V600E	Recurrence	Dead
12	Papillary	1+, 2%	53	Female	2.3	No	T3	N0	3	Wild	NA	No recurrence	Alive
13	Papillary	1+, 3%	45	Male	4	No	T3	N0	1	Wild	V600E	No recurrence	Alive
14	Papillary	2+, 2%	30	Female	2.8	No	T3	N0	1	Wild	Wild	Recurrence	Alive
15	Papillary	2+,2%; 1+,1%	50	Male	2	No	T1	N0	1	Wild	V600E	No recurrence	Alive
16	Papillary	2+, 3%	44	Female	2.5	No	T3	N0	1	Wild	V600E	Recurrence	Alive
17	Papillary	2+, 3%	54	Male	2.5	No	T3	N1a	3	Wild	V600E	No recurrence	Alive
18	Papillary	2+, 5%	57	Female	3.5	Yes	T2	N0	2	Wild	Wild	No recurrence	Alive
19	Papillary	2+, 5%; 1+, 30%	47	Female	2.5	No	T2	N0	2	Wild	NA	No recurrence	Alive
20	Papillary	3+, 5%; 2+, 10%; 1+, 40%	75	Female	5	No	T3	N0	3	C228T	Wild	Recurrence	Alive
21	Follicular	2+, 3%; 1+, 15%	44	Female	3	No	T1	N0	1	Wild	Wild	No recurrence	Alive
22	Follicular	3+, 10%; 2+, 5%	57	Female	5	No	T4a	N0	4	Wild	Wild	No recurrence	Alive
23	Follicular	2+, 20%; 1+, 20%	40	Female	2.5	No	T2	N0	2	Wild	Wild	No recurrence	Alive
24	Follicular	3+, 10%; 2+, 30%	46	Male	3	No	T2	N0	2	Wild	Wild	No recurrence	Alive
25	Follicular	3+, 30%; 2+, 40%	55	Female	4	No	T2	N0	2	Wild	Wild	No recurrence	Alive
26	Anaplastic	3+, 80%	64	Male	5.1	No	Tx	Nx	4	Wild	V600E	NA	Dead
27	Anaplastic	3+, 60%; 2+, 30%	72	Female	3	Yes	T3	N1a	4	Wild	NA	Recurrence	Dead

NA, not available.

It is highly important to accurately select patients who may benefit from anti-PD-L1 therapy. Although some studies have shown minimal predictive value for PD-L1 expression, others have shown significantly increased response rates in expressers over non-expressers (McLaughlin *et al.* 2016). Therefore, various studies by pharmaceutical companies have used their own companion diagnostic methods and various PD-L1 antibodies. However, PD-L1 assays for predicting response to monoclonal antibodies targeting PD-1 and PD-L1 are not standardized, with diverse commercially available assays yielding discordant results regarding PD-L1 expression and correlation to overall survival (McLaughlin *et al.* 2016). Several recent comparative studies of PD-L1 expression using different PD-L1 antibodies showed significant discordant results (McLaughlin *et al.* 2016, Scheel *et al.* 2016, Sun *et al.* 2016). Compared to other studies in thyroid cancer, PD-L1 positivity in papillary thyroid carcinoma patients was lower in our study (Table 4). In previous studies, PD-L1 positivity in papillary thyroid carcinoma was reported in up to 66.5% and 82.5% of cases (Cunha *et al.* 2013, Chowdhury *et al.* 2016). However, both used different antibodies (clone E1L3N and ab82059) from ours (SP142). As mentioned in a previous study, different antibodies were one contributing factor to different immunohistochemistry results. Three recent comparative studies including SP142 showed that SP142 stained less carcinoma cells compared to other immunohistochemistry

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Table 4 Prevalence of PD-L1 expression in thyroid cancers in reported studies.

	References	Tumor histology	Frequency of PD-L1 positivity	Localization of PD-L1 protein (interpretation)	PD-L1 detection antibody
1	Chowdhury *et al.* (2016)	Papillary carcinoma	123/185 (66.5%)	Cytoplasmic	E1L3N, cell signaling
			74/185 (40%)	Membranous	E1L3N, cell signaling
2	Angell *et al.* (2014)	Papillary carcinoma	10/33 (30.3%)	Not mentioned	#4059
3	Cunha *et al.* (2013)	Papillary carcinoma	209/254 (82.3%)	Cytoplasmic	ab82059
		Follicular carcinoma	35/40 (87.5%)	Cytoplasmic	ab82059
4	Bastman *et al.* (2016)	Differentiated carcinoma	7/12 (58.3%)	Membranous	SP142
		Anaplastic cancer	6/8 (75%)	Membranous	SP142
5	Wu *et al.* (2015)	Anaplastic cancer	3/13 (23.1%)	Not mentioned	5H1

clones (McLaughlin *et al.* 2016, Scheel *et al.* 2016, Sun *et al.* 2016). In addition, we found that interpretation methods utilized were also different (Table 4). We defined positive PD-L1 staining as membranous staining of tumor cells and/or cytoplasmic staining. On the other hand, some previous studies that reported higher frequencies also counted cytoplasmic positivity of tumor cells as PD-L1 expression (Cunha *et al.* 2013, Chowdhury *et al.* 2016). These data should be interpreted with caution. Several ongoing clinical trials adopt varying detection antibodies and immunohistochemistry cutoffs, but all counted only membranous positivity that are directly relevant to interaction with PD-1 receptors on immune cells (Garon *et al.* 2015, Patel & Kurzrock 2015). It is known that only membranous PD-L1 is functionally relevant, by contacting PD1+ T cells (Topalian *et al.* 2016). Accordingly, we interpreted cytoplasmic positivity without membranous positivity as a negative result. In our study, PD-L1 in tumor-infiltrating lymphocytes was positive in 28.5% of papillary thyroid carcinomas, 9.1% of follicular thyroid carcinomas and 11.1% of anaplastic thyroid carcinomas. PD-L1 expression in tumor-infiltrating lymphocytes has been reported in several cancer types (Powles *et al.* 2014, Sun *et al.* 2016). In bladder cancer, tumors expressing PD-L1-positive tumor-infiltrating lymphocytes had particularly high response rates for atezolizumab (Powles *et al.* 2014), and the Ventana SP142 test is the first US Food and Drug Administration approved test to detect PD-L1 positivity in tumor-infiltrating lymphocytes. However, the meaning and clinical implication of PD-L1 in tumor-infiltrating lymphocytes are yet to be established in other cancer types, and further studies are expected to elucidate these factors.

The limitation of our study is that PD-L1 immunohistochemistry was performed on a tissue microarray. As PD-L1 expression can show heterogeneity, evaluation on tissue microarray may miss some positive cases. We evaluated four cores with a relatively large size (2 mm) for each case and tried to overcome the limitation of tissue microarray usage. To address intra-tumoral heterogeneity issue, we randomly chose 18 PD-L1-negative cases and 15 positive cases in tissue microarray screening and performed PD-L1 in all cases with entire block. In 33 tested cases, there was no discordance between tissue microarray and whole slides. However, there is still a limitation of evaluating PD-L1 expression in tissue microarray. Another limitation is that we only tested one PD-L1 antibody. The SP142 antibody is a well-validated antibody used in several studies (Herbst *et al.* 2014, Powles *et al.* 2014), and has been recently approved as a companion diagnostic by the US Food and Drug Administration. Considering that different assays yield variable results, future studies are needed to compare PD-L1 positivity in thyroid cancer using variable assays with validation and propose a harmonized PD-L1 evaluation procedure.

Clinically, there was no significant association of PD-L1 expression with other clinicopathologic variables in this large-scale study. We did not demonstrate a significant prognostic value of tumoral PD-L1 expression in predicting poorer cancer-specific survival or disease-free survival, which was different from another study (Chowdhury *et al.* 2016). In addition, there was no association between the *BRAF* mutation and PD-L1 expression in our cohort, which was inconsistent with a previous report (Angell *et al.* 2014). The *BRAF* status was not available in subset of our cohort. Although the *BRAF* mutation rate (75.2%) in papillary carcinoma was within the reported range from the population, there remains a possibility that untested cases for *BRAF* mutation might alter the insignificant relationship between PD-L1 positivity and the mutational status. *TERT* promoter mutations are associated with an aggressive behavior of thyroid cancer including papillary thyroid carcinoma (Liu & Xing 2016). Among 32 *TERT* promoter-mutated papillary thyroid carcinomas, two (6.3%) cases revealed PD-L1 positivity. Of these two patients, one showed disease recurrence and the other

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patient died of thyroid cancer who might have benefited from anti-PD-1 immunotherapy. Lastly, the case number of anaplastic, poorly differentiated and follicular thyroid carcinomas was small in our study. Further large-scale study is expected to elucidate prevalence and prognostic significance of PD-L1 in aggressive thyroid cancers.

So far, few targeted therapies have been proven effective for anaplastic thyroid carcinomas. For patients with advanced differentiated (papillary and follicular) thyroid cancers, sorafenib, selumetinib, pazopanib and sunitinib have been investigated with promising results (Nixon *et al.* 2013). Anaplastic lymphoma kinase (ALK) rearrangement was reported in a subset of aggressive thyroid cancers (Kelly *et al.* 2014), and the availability of a small-molecule ALK inhibitor, crizotinib, has shown clinical promise in *ALK*-rearranged aggressive thyroid cancer (Godbert *et al.* 2015). As crizotinib was approved by the US Food and Drug Administration with a companion diagnostic test called the Vysis *ALK* Break Apart FISH Probe Kit, PD-L1 immunopositivity would be of clinical importance when considering anti-PD-L1 therapy in selected thyroid cancer patients. Currently, a trial with Atezolizumab for advanced solid tumors is recruiting patients with biomarker analysis (NCT02458638), which might address further questions.

In conclusion, we investigated PD-L1 expression using the SP142 antibody and found higher expression in a subset of advanced thyroid cancers such as anaplastic thyroid carcinoma and follicular thyroid carcinoma. Identification of PD-L1 expression may have direct therapeutic relevance to patients with refractory thyroid cancer.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-16-0421.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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