Update regarding the role of PD-L1 in oncocytic thyroid lesions on cytological samples

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Received 9 February 2022
Accepted 24 May 2022
Published Online First 14 June 2022

To cite: Dell'Aquila M, Tralongo P, Granitto A, *et al*. *J Clin Pathol* 2023;**76**:671–677.

ABSTRACT

Aims Several papers have shown that programmed death-ligand 1 (PD-L1) expression is a relevant predictive biomarker in anti-PD-L1 cancer immunotherapy. While its role in several human cancers is correlated with poor prognosis and resistance to anticancer therapies, in thyroid cancers the role of PD-L1 remains questionable. Few articles have studied PD-L1 in thyroid fine-needle aspiration cytology (FNAC), demonstrating a possible correlation with papillary thyroid carcinoma. However, its role in oncocytic thyroid lesions remains controversial. We accordingly examine the performance of PD-L1 immunostaining in liquid based cytology (LBC) from oncocytic lesions.

Methods From January 2019 to March 2021, 114 thyroid lesions diagnosed by FNAC from lesions with a predominant oncocytic component, were enrolled for evaluation by PD-L1 immunostaining on both LBC and corresponding histology samples.

Results The FNAC cohort included 51 benign (B, negative controls), 4 atypia of undetermined significance/follicular lesions of undetermined significance (AUS/FLUS), 57 follicular lesions (follicular neoplasm/suspicious for FN, FN/SFN) and 2 suspicious for malignancy (SFM) cases. Fifty-four cases (11B, 2 AUS/FLUS, 39 FN/SFN and 2 SFM) had histological follow-up including: 1B case resulted as a hyperplastic oxyphilic nodule in Hashimoto thyroiditis (HT), 10B as goitre, 2 AUS/FLUS cases as oncocytic adenomas (OAs); 39 FN/SFN included 27 OAs, 4 FA and 8 oncocytic follicular carcinoma (OFC). The two SFM cases were diagnosed on histopathology as OAs. Increased plasma membrane and cytoplasmic PD-L1 expression were found in 47 cases of the LBC cases (41.2%). Among the histological series, 67.3% of OAs and 75% of OFC had PD-L1 expression, while negative PD-L1 was found in hyperplastic oncocytic cells in HT. A positivity in more than 30% of the neoplastic cells was found in 72.9% of the cases including six OFC.

Conclusions These data suggest that PD-L1 expression is expressed in oncocytic thyroid lesions. While weak PD-L1 expression failed to discriminate benign from malignant lesions, OFC demonstrated more intense cytoplasmic and membranous expression.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Several papers have shown that programmed death-ligand 1 (PD-L1) expression is a relevant predictive biomarker in anti-PD-L1 cancer immunotherapy. While its role in several human cancers is correlated with poor prognosis and resistance to anticancer therapies, in thyroid cancers the role of PD-L1 remains questionable. Few papers have been published on the role of PD-L1 in thyroid lesions and its role in oncocytic thyroid lesions remains controversial. We accordingly examine the performance of PD-L1 immunostaining in liquid based cytology from oncocytic lesions.

WHAT THIS STUDY ADDS

⇒ We demonstrated an increased plasma membrane and cytoplasmic PD-L1 expression in 47 cases of the cytological cases (41.2%). Among the histological series, 67.3% of oncocytic adenomas and 75% of oncocytic follicular carcinoma (OFC) had PD-L1 expression, while negative PD-L1 was found in hyperplastic oncocytic cells in Hashimoto thyroiditis. A positivity in more than 30% of the neoplastic cells was found in 72.9% of the cases including six OFC.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE AND/OR POLICY

⇒ The results from our study, even if need to be supported by also larger series, suggest that PD-L1 expression is expressed in oncocytic thyroid lesions. While weak PD-L1 expression failed to discriminate benign from malignant lesions, OFC demonstrated more intense cytoplasmic and membranous expression. Its expression can be combined with morphology in suggesting a malignant nature of oncocytic lesions.

INTRODUCTION

It is well known that programmed death-ligand 1 (PD-L1) is expressed in a variety of tumour cells, working through its interaction with programmed cell death (PD-1) receptor in order to dampen the immune system response. Different research projects have confirmed the role of immune checkpoint inhibitors, blocking the PD-L1/PD-1 interaction, as a therapeutic option for patients with certain cancers.[1–3] Currently, evaluation of the therapeutic efficacy of these inhibitors relies on immunohistochemical (IHC) PD-L1 staining of various cancer specimens, mostly performed on histological samples as approved by the Food and Drug administration (FDA).[4] Specifically, the

FDA-approved-specific PD-L1 IHC tests as either companion or complementary diagnostic tests using different antibody clones and defined conditions.[5–7] Most literature dealing with the role of PD-L1/PD-1 has focused on advanced stage lung cancer, mostly non-small cell lung cancer (NSCLC), as well as head and neck carcinoma, melanoma and urogenital cancers.[8–15]

IHC evaluation of PD-L1 expression in cancer has been validated on different types of histological samples including formalin fixed, paraffin embedded (FFPE) tissue.[16–20] Issues have been encountered for IHC results obtained on cytological samples that are not fixed in formalin and when not employing FDA-approved PD-L1 antibodies. Apart from using FFPE cell blocks (CB) for cytological material, very few studies have examined the feasibility of evaluating PD-L1 expression in conventional cytology smears or with liquid based cytology (LBC) slides.[6 16–21] The major limitations of PD-L1 IHC ascribed to cytological material are attributed to non-specific nuclear and cytoplasmic positivity, high background staining, difficulty evaluating three-dimensional (3D) cellular clusters, and the high number of false negative interpretations. Nonetheless, some authors reported great success when staining cytology samples, demonstrating high concordance for PD-L1 staining in both CB and conventional smears compared with surgical samples in lung cancer.[6 8]

On the other hand, the role of PD-L1 expression in thyroid pathology has been documented in few papers.[22–30] To date, these limited studies indicate that PD-L1 expression in thyroid tumour cells of papillary thyroid carcinoma (PTC) correlates with poor prognosis in thyroid cancer, greater risk of recurrence and shortened disease-free survival.[28] This suggests there may be potential use for anti-PD-L1 immunotherapy in patients that are unresponsive to radioiodine or other chemotherapeutical measures. Indeed, a few trials, mostly including advanced differentiated thyroid cancers and anaplastic thyroid cancers, proposed using pembrolizumab to treat a minority of patients.[30] Some authors found PD-L1 expression only in invasive encapsulated follicular variant of PTC leading to the conclusion that PD-L1 might serve as a promising biomarker in predicting invasiveness.[25] Recently we found that there is weak PD-L1 cytoplasmic positivity in some oncocytic thyroid neoplasms.[9] Since then, however, PD-L1 expression in oncocytic cells, to the best of our knowledge, has not been described in the literature.

Therefore, the aim of this study was to analyse PD-L1 expression in cytology specimens from a large series of oncocytic thyroid lesions, and determine whether PD-L1 could be used as a marker to discriminate between benign and malignant oncocytic neoplasms of the thyroid gland.

MATERIALS AND METHODS

A prospective evaluation of PD-L1 IHC was performed of all consecutive thyroid cytological cases diagnosed as oncocytic follicular neoplasms (OFN), including those with subsequent surgical pathology follow-up diagnosed and recorded in the archives of the Division of Anatomic Pathology and Histology of the 'Fondazione Policlinico Universitario Agostino Gemelli'—IRCCS during the period between January 2021 and March 2021. A subset of oncocytic lesions were also discussed in a previous publication and included in this manuscript.[9] The institution's electronic medical record system (Armonia-Metafora, Italy) documented also the cases with surgical procedures (thyroidectomy specimens). The patient's age, gender, diagnosis, previous fine-needle aspiration cytology (FNAC) diagnosis and follow-up information were tabulated. All cytological material and thyroidectomy slides were reviewed.

The thyroid ultrasound evaluation was performed in the 'Centre for Thyroid Diseases' at our hospital. All aspirations (usually two passes performed for each lesion) were performed with 25–27 G needles. No rapid on-site assessment for adequacy of material was done because our cytology samples were processed using ThinPrep (Hologic, Marlborough, Massachusetts, USA). Then the prepared slides were fixed in 95% methanol and stained with the Papanicolaou stain. Any remaining material was stored in Preservcyt solution for possible preparation of additional slides and ancillary investigations (eg, immnostains and molecular analysis) if needed.and including the performance of PD-L1 . All patients consented to their procedure.

For adequacy we followed the lower limit for cytological adequacy of each sample established according to the Bethesda and British RCPath classification schemes; specifically, the minimum number of adequate cells in each sample was six groups of thyroid follicular epithelial cells per submitted slide where each of these groups contained at least 10 well-visualised epithelial cells.[31 32] The cytological diagnoses were classified according to the New Italian Working Group SIAPEC-IAP classification.[33 34] Specifically, these categories are defined as follows: TIR1: inadequate, TIR1C: cystic-haemorrhagic lesions, TIR2: benign nodules, TIR3A, FN (low-risk indeterminate lesions), TIR3B: FN (high-risk indeterminate lesions), TIR4: suspicious for malignancy (SFM) and TIR5: positive for malignant neoplasm. Nevertheless, all of cases were re-evaluated and then re-classified according to The Bethesda System for Reporting Thyroid Cytology (TBSRTC).[35] The definition of oncocytic cells and OFN followed the criteria defined by both the Italian and TBSRTC,[33–35] which are identical. For the purpose of this study, analyses were conducted using TBSRTC terminology. The entire FNAC series from the reference period included the following distribution of cases: 0% non-diagnostic including cystic cases, 21.1% benign; 17.7% atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS); 13.9% FN/suspicious for FN (FN/SFN); 22.4% SFM and 24.5% as malignant (M). All cytology and histology cases were reviewed by two cytopathologists. Furthermore, controversial cases were reviewed by additional pathologists in order to achieve a final consensus agreement.

Imunocytochemistry analysis

In our previous study, we performed PD-L1 staining on both LBC and corresponding resected thyroid tissue specimens. In this follow-up study, we adopted the same staining protocol as the previous study.[36–39] PD-L1 immunostaining was performed using the FDA-approved Dako PD-L1 pharmDx (clone 22C3; Dako/Agilent Technologies, Carpinteria, California) on a Dako Autostainer Link 48 autostainer (Dako/Agilent Technologies). Some of the authors were trained to interpret PD-L1 staining patterns by the company that produces the antibody. Antigen retrieval was performed using PT Link with Target Retrieval Solution (low pH; Dako/Agilent Technologies) as specified by the manufacturer without any modification to adjust for differences between cytology and histology. According to our long-standing performance of HBME-1 and Galectin-3 immunocytochemistry (ICC) previous described by our group in thyroid lesions, we adopted the same standard method.[36 37 40] We reviewed the FNA specimens to identify cases with adequate material for the study. Specifically, the minimal percentage of adequate lesional cells for the performance of PD-L1 evaluation was defined at 100

tumour cells in LBC samples. PD-L1 expression was evaluated with the Tumour Proportion Score system (TPS), defined as the percentage of viable tumour cells showing either partial/complete membranous and cytoplasmic positivity. Data from the literature documented thatPD-L1 in PTC has shown increased expression (using the E1L3N antibody) with both plasma membrane and cytoplasm localisation.[25–28] In the previous series, also in the current paper, ICC was carried out using the DAKO 22C3 clone, which also resulted in both cytoplasmic and membranous PD-L1 staining in tumour cells. To note, l even if a cytoplasmic expression of PD-L1 staining is considered unusual and non-specific in lung cancer,[22–29 41] it was a common finding in different thyroid lesions in both our LBC series and literature.[9 25–28]

According to the literature, we set the initial cut-off for the expression of PD-L1 at 1% staining of lesional cells; however, in our specific patient study group, the analysis of data, showed that two-thirds of all the positive cases had PD-L1 expression that was greater than 30% of lesional cells. Hence, because this was our second study of PD-L1 immunoexpression in thyroid lesions, and we did not have published reference cut-off values to follow as a benchmark, we arbitrarily used the 30% value as the minimal number of lesional cells required for PD-L1 staining to be interpreted as positive. However, we also compared the distribution of positive staining with a different value (ie, 50%). The staining intensity was defined as: 0=negative expression; 1=weak expression when visible with a 40x microscope objective lens; 2=moderate when visible with a 10x and/or 20x microscope objective lens and 3=strong when visible with a 4x microscope objective lens. For the evaluation of histological samples, the same staining cut-off values were used as for cytological samples; however, we did not report zonal PD-L1 positivity and noticed that the cases with PD-L1 expression were uniformly positive in up to 50% of the cells. PD-L1 controls were run concurrently; they included the Dako provided positive and negative cell-line control as well as an in-house tonsil control and PTC case that served as positive controls. Our LBC smears also showed positivity in macrophages. Instead of performing ICC on cellblocks obtained from LBC stored material, we decided to apply ICC to LBC for three reasons: (1) our cytology team has had long-standing success with validated ICC protocols on LBC, (2) our previous personal experience with ICC (Galectin-3 and HBME-1) resulted in contradictory results when LBC results were compared with FFPE CBs and (3) to demonstrate the feasibility of LBC for PD-L1 analysis. PD-L1 expression was evaluated independently by two different cytopathologists who scored these cytology slides, being blinded to the scoring results of concomitant histology specimens. Each case was evaluated for inter-observer agreement; any case with more than 10% discordance was reviewed together for a final consensus opinion. The assessment of PD-L1 was prospective so that the pathologists were blinded to PD-L1 staining when assessing diagnoses. The patients were not triaged to surgery based on the presence/absence of PD-L1 staining, but on the diagnosis of OFN on cytological samples.

Histology

All surgical specimens were fixed in 10% buffered formaldehyde, embedded in paraffin and then subsequent 5 μm thick sections were stained with H&E. The perithyroid adipose tissue, if present, was submitted and examined for lymph nodes. The diagnosis of oncocytic adenoma (OA) was based on finding well-circumscribed and capsulated lesions, composed of entirely oncocytic cells. The diagnosis of oncocytic carcinoma was based on the histological evidence of tumour infiltration defined by blood vessel invasion, located within or outside the fibrous capsule, with tumour emboli covered by endothelium. Extrathyroidal extension was defined as tumour invasion beyond the thyroid capsule into fat or skeletal muscle. All the cases were classified according to the eighth edition of the tumour-node-metastasis-based staging system recommended by the American Joint Commission on Cancer.[42] We also considered the revision for the encapsulated, non-invasive follicular variant of PTC by Nikiforov *et al* among the histological parameters for the series.[43] The follow-up period for thyroidectomy ranged between 2 and 15 months.

Tumour infiltrating lymphocytes (TILs) were defined as intratumoural infiltrating lymphocytes. Non-neoplastic thyroid that showed a diffuse dense lymphocytic infiltrate forming secondary lymphoid follicles with germinal centres was defined as concurrent lymphocytic thyroiditis (LT). Cases where non-neoplastic thyroid tissue showed only scattered lymphocytes with no lymphoid follicles were not considered to represent LT.

Statistical analysis

Statistical analysis was performed using GraphPad Prism V.6 software (Graph Pad Software, San Diego, California, USA) and MedCalc V.10.2.0.0 (MedCalc Software, Mariakerke, Belgium). Comparison of categorical variables was performed using the χ^2 test and the Fisher's exact test, with a 95% CI. P values that were lesser than 0.05 were considered as statistically significant.

RESULTS

During the 14-month study time period, we diagnosed 1120 cytological thyroid samples. Among them, our study included all lesions showing an oncocytic component in the cytological diagnosis. Patient demographics and clinicalpathological features of this oncocytic cohort are described in table 1. Furthermore, we also included a subset of 24 oncocytic lesions that were discussed in a previous publication.[9]

Table 1 Summary of clinicalpathological data

Clinical-pathological feature	Proportion (n=114 cases)
Age	
Mean	55.4 years
Median	55.5 years
Range	16–82 years
Gender	
Male	41 (35.9%)
Female	73 (64.03%)
Cytology diagnosis	
Benign	51° (44.7%)
AUS/FLUS	4 (3.5%)
FN/SFN	57 (50%)
SFM	2 (1,7%)
Malignant	0 (0%)
Histopathology*	
Benign†	46 (85,1%)
Malignant	8 (14.1%)

Additional 50° benign goitre cases are considered as negative control with 10 histological goitre.
*Histology available in 54 cases.
†Includes follicular adenomas.
AUS/FLUS, atypia of undetermined significance/follicular lesion of undetermined significance; FN/SFN, follicular neoplasm/suspicious for follicular neoplasm; SFM, suspicious for malignancy.

Table 2 Cytohistological correlation (54 cases) according to TBSRTC

Diagnosis	Goitre	FA	OA	OFC
Benign (11 case)	10*	1	/	/
AUS/FLUS (2 cases)	/	/	2	/
FN/SFN (39 cases)	/	4	27	8
SFM (2 cases)	/	/	2	/

*Ten benign cases out of 50 had histological follow-up and they are included in the tables (negative controls).
AUS/FLUS, atypia of undetermined significance/follicular lesions of undetermined significance; FA, follicular adenoma; FN/SFN, follicular neoplasm/suspicious for follicular neoplasm; OA, oncocytic adenoma; OFC, oncocytic follicular carcinoma; SFM, suspicious for malignancy; TBSRTC, The Bethesda System for Reporting Thyroid Cytology.

We included all cytological samples diagnosed as hyperplastic oncocytic lesions in Hashimoto thyroiditis (HT) and oncocytic indeterminate thyroid lesions (ie, AUS/FLUS and FN/SFN), including those with histological follow-up. For negative control cases, we added an additional 50 benign cases (from the same study time period). We excluded 10 cases due to the fact that these cases did not have adequate tumour cells to assess PD-L1, as well as another 15 samples that resulted in fewer than 100 tumour cells after having performed the PD-L1 stain. The series included 41 (35.9%) male and 73 (64.03%) female patients with a median age of 55.4 years (range 16–82 years and mean 55.5 years). Their thyroid neoplasms ranged in size from 0.5 to 6.9 cm (table 1). While for our prior study we excluded HT, due to possible bias of PD-L1 over-expression related to the presence of tumour-related immune cells and lymphocytes (TILs), in this study, we decided to include cases with these cyto-morphological features, exclusively focusing on PD-L1 expression in the oncocytic component.

For the study period, our cytological series (114 cases) included the following distribution of thyroid diagnoses: 50 goitre cases diagnosed as benign (ie, negative control cases), one (1.7%) hyperplastic oncocytic nodule in HT, four (6.8%) AUS/FLUS, 57 (89%) FN/SFN and two (3.1%) SFM. (table 1). All subcentimetre lesions were discovered incidentally during radiologic screening for causes unrelated to the thyroid gland. There was no significant difference in the size of lesions among the aforementioned diagnostic entities. No statistical correlation was found with clinicalpathological data.

Histological diagnoses were rendered in 54 cases as benign in 85.2% and malignant in 14,8 % cases. The surgical pathology follow-up of the different cytological categories is described in table 2. The 50 negative control cases included 10 benign cases with histological follow-up diagnosed as goitres nad they were included in the cytohistological serie As reported in table 2, for cytohistological correlation purposes the reference study series includes one hyperplastic oncocytic nodule in HT that turned out to be a follicular adenoma (FA), 2 AUS/FLUS cases diagnosed as 2 OAs, 39 FN/SFN cases confirmed to be either OA (27 cases), FA (4 cases) and eight oncocytic carcinoma (OFC). The two SFM cases included two OAs.

For the analysis of the expression of PD-L1 in our series of cases (table 3), among cases with an adequate TPS (114 cases), none of the benign lesions had PD-L1 expression (figure 1). Our series included 47 (41.2%) cases with PD-L1 expression on cytology. Neverthless 10 PD-L1 positive cases belonging to the FN/SFN did not have a surgical procedure so that we discussed only the

Table 3 Cytohistological (54 CASES) correlation related to PD-L1 expression

Cytology diagnosis	Histology diagnosis	PD-L1 negative	PD-L1 positive
Benign* (11 case)	FA	11 (20,3%)	0
AUS/FLUS (2 cases)	OA	0	2 (3,7%)
FN/SFN (39 cases)	FA OA OFC	0 2 (3,7%) 2 (3,7%)	4 (7,4%) 25 (46,2%) 6 (11.1%)
SFM (2 cases)	OA	2 (3.7%)	0/
Malignant (0 cases)	/	/	/

*Additional benign lesions (diagnosis of goitre on FNAC), all negative for PD-L1 expression and not included in the tables (negative controls).
AUS/FLUS, atypia of undetermined significance/follicular lesions of undetermined significance; FA, follicular adenoma; FNAC, fine needle aspiration cytology; FN/SFN, follicular neoplasm/suspicious for follicular neoplasm; OA, oncocytic adenoma; OFC, oncocytic follicular carcinoma; PD-L1, programmed death-ligand 1; SFM, suspicious for malignancy.

PD-L1 expression in the cytohistological series (54 cases) distributed according to the following categories of TBSRTC: 2 AUS/FLUS cases (3.7%) and 35 FN/SFN cases (64.8%). According to the cytohistological series, PD-L1 was expressed in OA and malignant (OFC) lesions. All of these positive cases were characterised by both positive cytoplasmic and membranous expression, defined as complete or partial membranous type staining (figures 1 and 2). As previously noted, we evaluated PD-L1 expression using the cut-off values of 30% and 50% (table 4). When analysing staining intensity, the majority of these cases (27 cases) had PD-L1 expression higher than 30%, including 15 cases with weak expression in more than 30%, but less than 50% of lesional cells; further, 12 cases demonstrated strong cytoplasmic expression of PD-L1 in more than 50% of the cells. These latter 12 cases also had strong membranous positivity. To confirm our cytology results, we evaluated PD-L1 staining in corresponding histological samples.

PD-L1 staining in LBC samples showed high concordance with matched histological specimens. All of the positive cases had diffuse and homogeneous positivity for PD-L1. All of the negatively stained samples showed 100% concordance with histology. Only a few discrepancies were identified, related mostly to differences in the intensity of staining between corresponding cytological and histological specimens, but there was no statistical significant difference and no clinical implications.

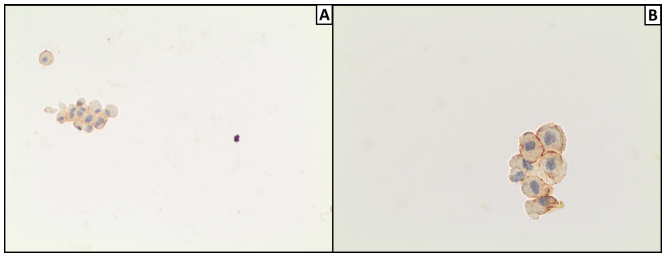

Figure 1 (A, B) Different magnification of the pattern of PD-L1 expression in oxyphilic cells in a case diagnosed as oxyphilic neoplasm and resulted as oncocytic adenoma on histology (×200 and ×400, LBC, ThinPrep, immunocytochemistry). LBC, liquid based cytology; PD-L1, programmed death-ligand 1.

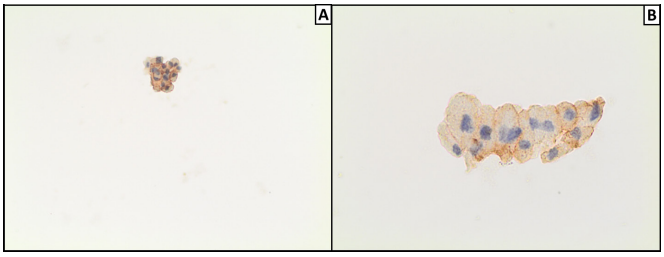

Figure 2 (A, B) Show different magnification of PD-L1 positivity in another case of oncocytic neoplasm resulted as oncocytic carcinoma on histology (capsular invasions) (×200and ×400 LBC, ThinPrep, immunocytochemistry). LBC, liquid based cytology; PD-L1, programmed death-ligand 1.

We attributed those discrepancies to the different technical procedures and material preparations (cytology vs histology) and the amount of oncocytic cells in our slides. Nevertheless, perhaps due to the limited number of oncocytic carcinoma cases included in this study, we found that the Fisher's Exact test used to compare PD-L1 positivity with benign and malignant histopathological diagnosis was not statistically significant. Furthermore, evaluation with the chi-squared test performed in order to compare PD-L1 positivity on cytological smears with subsequent histopathological diagnoses, was similarly found not to be statistically significant.

The current study confirms that oncocytic neoplasms show weak or moderate/strong cytoplasmic positivity for PD-L1, with mostly focal membranous positivity (figure 1A,B). Furthermore, stronger positivity was found in 6 out of 8 OFC cases (75%), also associated with stronger membranous expression (figure 2A,B). We acknowledge the limited number of oncocytic malignancy cases included in this stud. Further conclusions will need to be confirmed in larger studies. PD-L1 weak and moderate positivity is likely attributed to the mitochondrial rich cytoplasm of oncocytic cells, as found in OAs or carcinomas, but not in hyperplastic benign oncocytic lesions. It is plausible that the membranous expression seen in the six OFC cases serves to differentiate between adenoma and carcinoma.

Of note, only a few cases resulted in differences of PD-L1 intensity in cytological slides and the overestimation of PD-L1 expression in cytology cases. In particular, while weak and moderate expression maintained the same staining intensity on corresponding cytology and histology cases, only three cases with strong PD-L1 cytological expression exhibited moderate PD-L1 staining in matching histology specimens. The comparative analysis of lesions and PD-L1 staining did not show any significant correlation, confirming the lack of a relationship between PD-L1 expression and thyroid lesion aggressiveness. In fact, our results showed that the majority of cases, regardless of PD-L1 expression, were OAs.

Table 4 PD-L1 expression and localisation in different oncocytic thyroid lesions (37 cases)

Diagnosis	TPS <30%	TPS >30%<50%	TPS >50%
Benign (1 case)	/	/	/
AUS/FLUS (2 cases)	/	2	/
FN/SFN (35 cases)	10	13	12
SFM (0 cases)	/	/	/
Malignant (0 cases)	/	/	/

AUS/FLUS, atypia of undetermined significance/follicular lesions of undetermined significance; FN/SFN, follicular neoplasm/suspicious for follicular neoplasm; PD-L1, programmed death-ligand 1; SFM, suspicious for malignancy; TPS, Tumour Proportion Score.

DISCUSSION

The role of PD-L1 and its receptor has been widely studied in various cancers[1–5] emphasising the known evidence that PD-L1 and PD-1 regulate the activity of other cells, including T-cell, in the tumour microenvironment.[1 8–11] Some papers demonstrated that the upregulated expression of PD-L1 on the surface of cancer cells is likely to work as an immune-resistant mechanism against T-cells. The approval of immunotherapeutics for certain cancers has changed their treatment landscape, and among them PD-L1 has accordingly been investigated as a marker of tumour aggressiveness.[1–6 11–15] The vast majority of papers to date have confirmed the expression of PD-L1 in histological samples, with lack of data about cytological specimens. In fact, none of the FDA approved PD-L1 antibodies, have been validated on cytological samples despite the fact that PD-L1 testing by ICC is reasonable using different cytological preparations.[6 16–21]

The vast majority of papers have focused on lung cancers and excellent results, using cytological samples, have been mostly studied in publications dealing with lung cytology samples, demonstrating both successful PD-L1 staining and high concordance with histological samples.[16–21 44] For example, Kulac *et al* found that CBs obtained from cytological material are a good alternative.[8] Furthermore, Noll *et al* showed that NSCLC cytology samples evaluated for PD-L1 exhibit high concordance (78.6%) with paired core needle biopsy samples.[6–8] The feasable and reliable use of cytological samples is also supported by the fact that, no significant difference has been reported in the staining intensity when formalin was compared with alcohol and methanol fixation methods.

Right now, a great interest deals with the role and importance of interpreting PD-L1 expression for the clinical management of NSCLC and/or melanoma, as well as a few other types of malignancies. By comparison, relatively little is known about the role of PD-L1 in thyroid cancer.[13 22–29 41 45–55]

Well-differentiated thyroid cancers account for the majority of endocrine malignancies, of which PTC represents the leading thyroid cancer.[22 41 56] The indolent behaviour of well-differentiated thyroid cancers has always raised doubts about the role of immune cells and PD-L1. According to Fadia *et al*, the search for published data between 1946 and 2017 confirmed that only six relevant studies included the evaluation of PD-L1 in PTC.[57] They revealed that there was marked variability with PD-L1 results ranging from 6.1% to 82.5% due to varying factors such as: different PD-L1 IHC assays, quantitative reverse transcription polymerase chain reaction (qRT-PCR), the site of PD-L1 expression (membranous vs cytoplasmic), the evaluation on tumour cells alone versus tumour +lymphocytes, and the threshold for positive staining including intensity and extent.[57] Apart from PD-L1 expression in well-differentiated PTCs that are non-responsive to radioiodine therapy,[49–55] some studies demonstrated diffuse and strong PD-L1 expression in anaplastic thyroid carcinoma suggesting that anti-PD-L1 immunotherapy can help manage unresponsive patients with aggressive thyroid cancer,[26 47 50 58–63] those with high stage disease and distant metastases, or death.[22–28 41] In a paper by Chowdhury *et al*, evidence

is provided indicating that PD-L1 expression is correlated with a more aggressive behaviour, including invasiveness of PTC and poor prognosis, especially in stages III and IVa.[28] Furthermore, they found a correlation between the pattern of staining and localisation in different stages of PTC, as for instance, the evidence of less cytoplasmic staining in stage I cancer, increased cytoplasmic and membranous expression in stage II cancer, and significantly increased PD-L1 expression in stages III and IV. These same authors formulated the conclusion that PD-L1 expression could be a useful marker in predicting invasiveness of E-FVPTC and that PD-L1 negativity in NIFTP confirms the non-malignant nature of this new entity.[25]

The expression of PD-L1 has been well established on the plasma membrane of neoplastic cells, even though Fu *et al* mostly using the E1L3NL antibody reported both plasma membrane and cytoplasm localisation in their thyroid series.[25] In our both series, we performed ICC with the the DAKO 22C3 clone, finding both cytoplasmic PD-L1 expression and weak or strong membranous PD-L1 localisation. The non-thyroid published series assessed that the detection of cytoplasmic PD-L1 staining is unusual and has been considered to be non-specific in lung and other cancers, while it was a common finding in different thyroid lesions in our LBC and histological series[24 25 28 29 41]

To the best of our knowledge, this is the second paper dealing with the performance of PD-L1 testing in thyroid cytology samples including LBC. As previously noted, there are several variables that may impact the interpretation of PD-L1 staining in histological samples such as preanalytical factors, different antibody clones, different cut-off values, and TIL and intratumoural heterogeneity.[6 16–21] For cytological samples, there are even more variables that need to be taken into consideration when evaluating PD-L1 such as the presence of contaminating blood and 3D cell clusters. In our previous paper, we suggest that to the evaluation of PD-L1 can be feasibly and reliably performed on LBC specimens.[9] To date, no publications have specifically dealt with PD-L1 expression in oncocytic thyroid lesions and whether expression can play a role in discriminating between benign and malignant oncocytic neoplasms. To note, the majority of oncocytic lesions had a benign histology, with also eight out of 54 cases with a malignant histology (14.8%).

This study denotes that oncocytic lesions of the thyroid exhibit weak PD-L1 expression.[9] We evaluated PD-L1 staining in LBC thyroid samples at cut-off values of 1%, 30% and above 50%. We confirmed that the majority of positive PD-L1 cases had greater than 30% staining, but less than 50% positive tumour cells defined by moderate cytoplasmic and also membranous staining. In the current series, we show that up to 56% of both benign and malignant oncocytic lesions yield PD-L1 expression. Such PD-L1 positivity might be ascribed to the mitochondrial rich cytoplasm of oncyctic cells. The current study further documented that six out of the eight OFC had very strong cytoplasmic and membranous expression of PD-L1, although this was not statistically significant due to the limited number of cases. Furthermore, our OA cases were mostly defined by moderate to strong cytoplasmic positivity and only focal membranous expression. None of the benign lesions, including those cases of hyperplastic oncocytic lesions in HT, were PD-L1 positive. It is plausible that PD-L1 may serve as a possible marker of oncocytic neoplasms. However, this warrants further study. Moreover, because it is not possible to distinguish OA from carcinoma based on cytomorphology alone, perhaps the utility of PD-L1 could be explored for this purpose in larger series.

Handling editor Runjan Chetty.

Contributors EDR, LP and LML planned the study. PT, VF, AG, MM, SC and MC worked on the technical performance. MR, CPL, GF and AP revised the manuscript for contents. EDR is responsible for the overall content as the guarantor.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval We received institutional (Catholic University of the Sacred Heart) ethics approval for this study.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article.

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