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Original contribution

PD-L1 expression and association with malignant behavior in pheochromocytomas/paragangliomas☆,☆☆

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Summary The immunosuppressive effect of the programmed death (PD)–1/PD-L1 pathway plays an important role in the treatment of a variety of tumors, such as lung and breast cancer, but there is little literature about PD-1/PD-L1 in pheochromocytomas/paragangliomas (PCCs/PGLs). We explored the relationship of PD-L1 and malignant behavior in 77 cases of PCC/PGL using immunohistochemistry (IHC) to assess protein expression and RNAscope to detect mRNA expression in 20 cases. The IHC data showed that 59.74% of the PCCs/PGLs expressed PD-L1, and the extent of expression was highly correlated with Ki-67 ($P = .019$) and hypertension ($P = .013$) but not with age, sex, tumor size, capsular invasion, tumor necrosis, relapse/distant metastasis, secretion of noradrenaline/adrenaline/dopamine, or diabetes mellitus. In addition, we found an excellent correlation of PD-L1 mRNA and protein expression with a κ coefficient of 0.828, and further stratification of the IHC and RNAscope findings showed high consistency (Pearson coefficient 0.753). The correlation of PD-L1 and Ki-67 indicated that PD-L1 could be considered a malignant proliferation biomarker for PCCs/PGLs, which would be a putative biomarker for anti–PD-L1 therapies.

1. Introduction

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are highly vascular, catecholamine-secreting tumors that arise from sympathetic lineage–derived cells in the adrenal medulla (PCCs) and from extra-adrenal thoracic and abdominal paraganglia (PGLs) [1]. Most of these tumors do not spread and are curable with surgery. However, in a small number of patients (15%-17%), PCCs/PGLs exhibit malignant behavior characterized by metastases. These patients have lower overall survival rates; only 60% will be alive 5 years after diagnosis [2]. The treatments for metastatic PCCs/PGLs include surgery, localized radiotherapy, cytotoxic chemotherapy, and a few

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new therapies, namely, tyrosine kinase inhibitors, mammalian target of rapamycin inhibitors, and immunotherapy [2,3]. Unfortunately, only a fraction of patients respond to these measures, and responses usually last for only a short time or are only partial [2,4]. In recent years, immunotherapy has become a thriving field of basic research and clinical treatment. Compared with the traditional tumor therapies, immunotherapy could be effective against multiple cancers or distant metastases, functioning by inhibiting tumor cell growth or directly killing tumor cells with few adverse effects. Moreover, immunotherapy can promote repairs of the damaged tissues and organs caused by chemoradiotherapy [5-10].

The programmed death (PD)–1/PD-L1 interaction is involved in cell growth, proliferation, differentiation, signal transduction, and regulation of a variety of biological processes [11]. The immunosuppressive effect of the PD-1/PD-L1 pathway plays an important role in the development of a variety of immune-related diseases, including autoimmune diseases, infection, and tumor [12]. Because there is little literature discussing PD-1/PD-L1 and PCCs/PGLs, we explored the relation of PD-L1 expression and malignant behavior in PCCs/PGLs in the hope of providing a theoretical basis for targeted PD-L1 therapy.

2. Materials and methods

2.1. Patient information

Seventy-seven patients who underwent surgical treatment with a diagnosis of PCCs/PGLs at Peking Union Medical College Hospital (Beijing, China) between 2012 and 2016 were enrolled in this study. All final diagnoses were based on the morphology of tumor samples stained with hematoxylin and eosin (H&E), and all H&E slides for each tumor sample were reviewed by 2 experienced pathologists to confirm the final diagnosis. Clinical information, including patient age, sex, and smoking habits; hypertension; diabetes mellitus; tumor characteristics; capsular invasion; necrosis; metastasis to distant sites; secretion of norepinephrine, epinephrine, and dopamine; Ki-67 index; and vimentin and CD34 production, was collected from the clinical archives.

This study was approved by the Institutional Review Board of Peking Union Medical College Hospital. Informed consent was obtained from all patients.

2.2. Immunohistochemistry staining

Immunohistochemistry (IHC) staining was performed on 4-μm sections using the Dako Autostainer Link 48 (Dako, Glostrup, Denmark). The tissue epitopes were recovered with the automated water bath heating process in Dako PT Link; the sections were incubated in Tris-EDTA retrieval solution (10 mmol/L Tris buffer, 1 mmol/L ethylenediaminetetraacetic acid; pH 9.0) at 98°C for 20 minutes. The sections were subsequently incubated for 50 minutes at room temperature with the primary antibody anti–PD-L1 (clone E1L3N; Cell Signaling cat. no. 13684) diluted 1:200 in Dako Envision Flex antibody diluents (Dako, Glostrup, Denmark, cat. no.K800621) and followed by anti-rabbit immunoperoxidase polymer (Envision FLEX/HRP) for 20 minutes at room temperature (RT). Other antibodies, namely, CD34 (clone Qbend 10; Dako; dilution 1:25, 20 minutes at RT), vimentin (clone 3B4; Dako; dilution 1:150, 20 minutes at RT), and Ki-67 (MIB1; Dako; dilution 1:400, 20 minutes at RT followed by mouse linker 15 minutes at RT), were immunostained as indicated. The color reaction was developed with diaminobenzidine substrate–chromogen solution for 10 minutes. Finally, the sections were counterstained with hematoxylin. The PD-L1 positivity in the lung squamous cell carcinomas was used as a positive control, and the same tissue with omission of the primary antibody served as a negative control.

Expression of PD-L1 was scored independently by 2 experienced individuals using a semiquantitative immunohistoscore (IHS). Briefly, staining for PD-L1 on the membrane of the tumor cells was considered positive, and the proportion of positive cells was estimated on a scale of 0% to 100%. The average intensity of staining cells was given a score from 0 to 3 (0 = none; 1 = weak; 2 = intermediate; 3 = strong). The immunohistoscore (IHS) was calculated by multiplying the percentage and intensity scores, to yield a minimum value of 0 and a maximum value of 300 [13]. Cases with a focal pattern of PD-L1 expression of moderate intensity in more than 5% of the tumor cells were considered positive (immunohistoscore (IHS) > 10) [3,14].

2.3. In situ hybridization for PD-L1

In situ detection of PD-L1 mRNA was performed using an RNAscope Technology 2.5 HD Detection Kit-BROWN (Advanced Cell Diagnostics, Hayward, CA) for formalin-fixed, paraffin-embedded tissue according to the manufacturer's protocol. In brief, 3- to 5-μm formalin-fixed, paraffin-embedded tissue sections were baked, deparaffinized, hydrogen peroxidase blocked, antigen retrieved, protease plus treated, and hybridized with a probe specific for PD-L1 mRNA (No. 312351). After washing and sequential hybridization, slides were stained with diaminobenzidine (Dako, Glostrup, Denmark) and counterstained with hematoxylin. Peptidylprolyl isomerase B and the bacterial gene *dap*B were used as positive and negative controls, respectively.

Scoring of the RNAscope PD-L1 assay signals was performed according to the manufacturer using 20×-40× magnification with 0 or 1 dot per 10 cells = 0; 1-3 dots per cell visible = 1; 4-10 dots per cell (very few dot clusters visible) = 2; more than 10 dots per cell (less than 10% positive cells have dot clusters) = 3; and more than 10 dots per cell (more than 10% positive cells have dot clusters) = 4 [15]. A score of 1 or more was considered positive for PD-L1 expression [16,17].

2.4. Statistical analysis

Statistical analyses were carried out using SPSS17.0 (Chicago, IL) with nonparametric statistics for IHC of PD-

L1 and clinical characteristics and the McNemar-Bowker test for PD-L1 IHC and RNAscope. A κ value and Pearson coefficient were calculated to evaluate the consistency between PD-L1 mRNA expression and protein as measured by RNAscope and IHC. The significance of the κ value was assessed as follows: <0.40, poor to fair concordance; 0.41-0.60, moderate concordance; 0.61-0.80, substantial concordance; and 0.81-1.00, almost perfect concordance [18].

3. Results

3.1. Patient characteristics

The clinical characteristics of 77 patients, 75 (97.4%) with PCC and 2 (2.6%) with PGL, were collected (Table 1). Of the patients with PCC, 69 cases (89.6%) were unilateral. Among

Table 1 Relationship between PD-L1 protein expression and clinicopathologic characteristics of 77 cases of PCC/PGL

Characteristic	n (%)	PD-L1 IHC		
		+ (n = 46)	− (n = 31)	*P*
Sex				.165
Male	27 (35.1)	19	8	
Female	50 (64.9)	27	23	
Age (y)				.407
<40	24 (31.2)	16	8	
≥40	53 (68.8)	30	23	
Disease site				
PCC unilateral	69 (89.6)	41	28	
PCC bilateral	6 (7.8)	4	2	
PGL (extra-adrenal)	2 (2.6)	1	1	
Maximum tumor diameter (cm)				.177
<5	35 (45.5)	17	18	
≥5	42 (54.5)	29	13	
Ki-67				.019
<3	63 (81.8)	33	30	
≥3	14 (18.2)	13	1	
Vimentin positive				.088
Yes	9 (11.7)	3	6	
No	68 (88.3)	43	25	
CD34 positive				.15
Yes	3 (3.9)	3	0	
No	74 (96.1)	43	31	
Hypertension				.013
Yes	53 (68.8)	27	26	
No	24 (31.2)	19	5	
Diabetes				.412
Yes	16 (20.8)	11	5	
No	61 (79.2)	35	26	
Capsular invasion				.804
Yes	3 (3.9)	2	1	
No	74 (96.1)	44	30	
Necrosis				.884
Yes	7 (9.1)	4	3	
No	70 (90.9)	42	28	
Norepinephrine (μg/24 h)				.325
16.69-40.65	30 (39.0)	20	10	
>40.65	47 (61.0)	26	21	
Epinephrine (μg/24 h)				.243
1.74-6.42	68 (88.3)	39	29	
>6.42	9 (11.7)	7	2	
Dopamine (μg/24 h)				.447
120.93-330.59	64 (83.1)	37	27	
>330.59	13 (16.9)	9	4	

NOTE. Expression of PD-L1 correlated with the Ki-67 index (P = .019) and hypertension (P = .013).

the 6 cases of bilateral PCC, 3 (50%) were documented familial disease. The median age of the patients at diagnosis was 45 years (range 11-70 years). More than half of the patients were female (50 cases; 64.9%), and 68.8% of these had either resistant hypertension or labile blood pressure. The maximum tumor diameter was <5 cm for 35 patients (45.5%). The Ki-67 index, which is a cellular marker of proliferation, of 1 of every 5 patients was extremely strong (≥3) [19]. Capsular invasion and necrosis were observed in 3 (3.9%) and 7 (9.1%) cases, respectively. The secretion of catecholamines was higher than normal with 1 or more of norepinephrine, epinephrine, and dopamine. And among them, norepinephrine was higher in 47 cases (61.0%), epinephrine in 9 cases (11.7%), and dopamine in 13 cases (16.9%), respectively [2].

All patients had surgery as the principal treatment for their tumor. None of them received anti–PD-1 or PD-L1 targeted therapy. Patients were followed for an average of 45.5 months. Among the 45 patients with follow-up data, only 1 patient died of metastatic PGL after surgery.

3.2. PD-L1 expression in PCCs/PGLs via IHC

Tumor tissues of 77 patients with PCCs/PGLs who underwent surgery were stained with PD-L1 antibody to assess immunoreactivity. The subcellular locations of PD-L1 were observed. In total, 46 of 77 cases (59.7%) stained positively for PD-L1 according to the specified cutoff (HIS > 10), of which 24 cases (52.2%) stained weakly (IHS range 11-100), 10 (21.7%) showed moderate staining (IHS range 101-200), and 12 (26.1%) were strongly positive (IHS range 201-300). The prevalence of PD-L1 immunopositivity was 100% (1/1) and 59.2% (45/76) in metastatic and localized PCCs/PGLs, respectively (Fisher exact test $P = .597$). The PD-L1 positive samples showed appropriate cytoplasmic and membranous

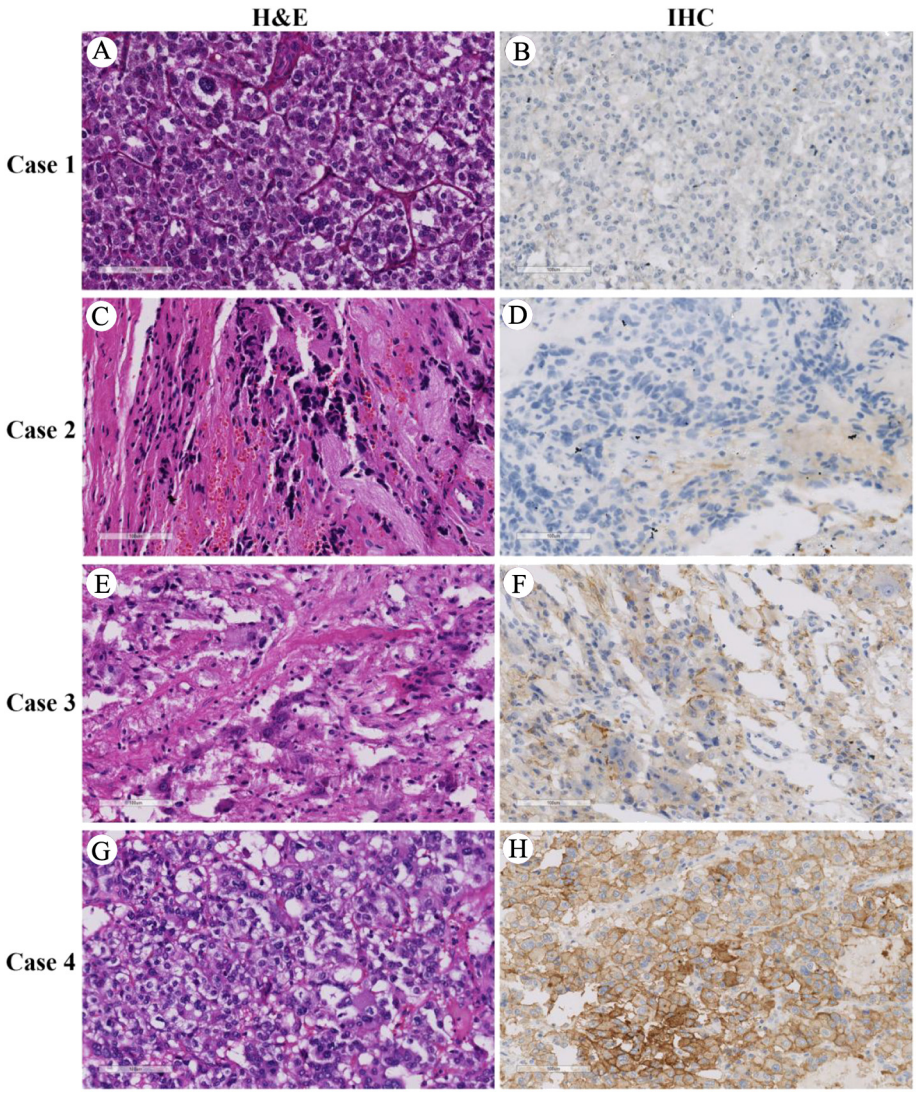

Fig. 1 H&E staining (A, C, E, and G) and immunohistochemical detection of PD-L1 expression (B, D, F and H) in PCCs/PGLs (original magnification of all images ×200). A and B, Negative. C and D, Weak staining. E and F, Intermediate staining. G and H, Strong staining.

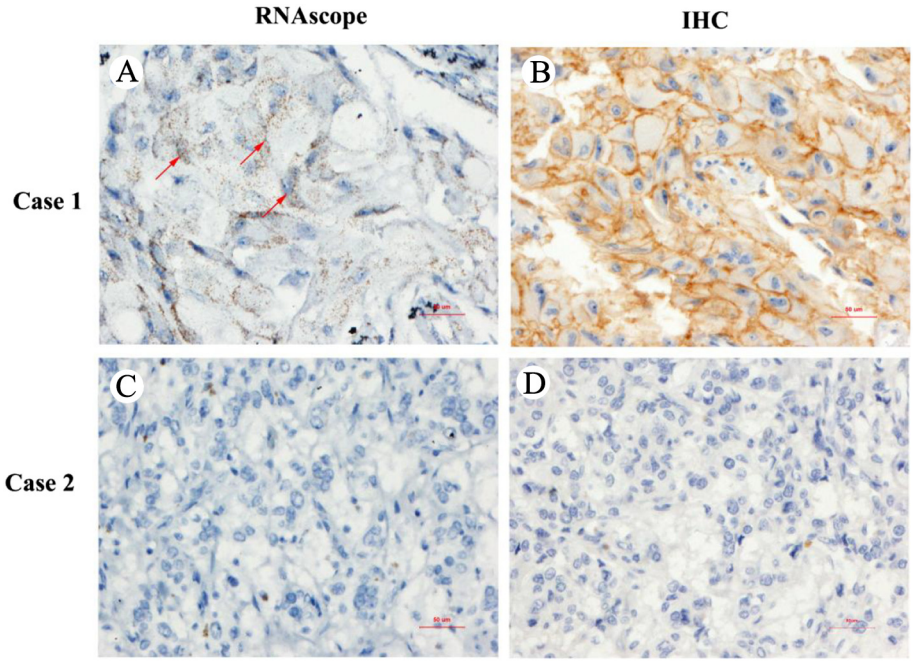

Fig. 2 RNAscope detection of PD-L1 mRNA expression in PCCs/PGLs (×200). Positive staining (red arrows) examined by RNAscope (A) and IHC (B). Negative staining by RNAscope (C) and IHC (D).

patterns in tumor cells and some stromal lymphocytes, indicating the specificity of our stain (Fig. 1).

There was no significant association between PD-L1 expression and most of the clinicopathologic characteristics (sex, age, disease site, tumor size, capsular invasion, vimentin expression, CD34 expression, diabetes, necrosis, catecholamine secretion). However, we found a higher rate of PD-L1 expression in cases displaying a high Ki-67 index (13/14; 92.9%; $P = .019$) or hypertension (19/24; 79.2%; $P = .013$).

3.3. PD-L1 expression in PCCs/PGLs via RNAscope

We also used the RNAscope to test for PD-L1 mRNA transcription. Of the 77 PCC/PGL tissues in this study, PD-L1 mRNA expression was detected in 20 cases, of which 3 (15%) were negative for PD-L1 (HIS ≤ 10) and 17 cases (85%) were positive with weak (3/17; 17.6%), moderate (7/17; 41.2%), or strong (7/17; 41.2%) staining. Thus, there was a high concordance between the IHC and RNAscope analyses. Of 17 PD-L1 samples positive by IHC, 94.1% were RNAscope positive. We did not detect any RNAscope-positive samples among the IHC-negative samples. Representative RNAscope results are shown in Fig. 2.

Furthermore, we found an excellent correlation between PD-L1 mRNA and protein expression as measured by RNAscope and IHC, respectively, with a κ coefficient of 0.828, and there was no significant difference between the results of the 2 methods according to the McNemar-Bowker test ($P = 1.000$; Table 2). Subsequently, we stratified the IHC results, with a score of 0 (IHS ≤ 10), 1 (IHS range 11-100), 2 (IHS range 101-200), or 3 (IHS range 201-300), and the RNAscope with scores of 0 to 3 as described above (Table 3). Of the discordant results, there were 7 samples varied in the scores of IHC and RNAscope (1 of IHC 1 versus RNAscope 2; 1 of IHC 2 versus RNAscope 0; 2 of IHC 2 versus RNAscope 1; 1 of IHC 3 versus RNAscope 1; and 2 of IHC 3 versus RNAscope 2); the other samples were within 1 point. The SPSS analysis showed that the stratified IHC and RNAscope results

Table 2 McNemar-Bowker test of IHC and RNAscope findings in 20 cases

PD-L1 RNAscope	PD-L1 IHC		κ	P
	+	−		
+	16	0	0.828	1.000
−	1	3		

NOTE. The test indicated excellent correlation of PD-L1 mRNA and protein expression by RNAscope and IHC, respectively.

Table 3 Stratified and correlated PD-L1 IHC and RNAscope scores

PD-L1 RNAscope	PD-L1 IHC				Pearson coefficient [a]
	0	1	2	3	
0	3	0	1	0	0.753
1	0	2	2	1	
2	0	1	4	2	
3	0	0	0	4	

[a] $P < .001$.

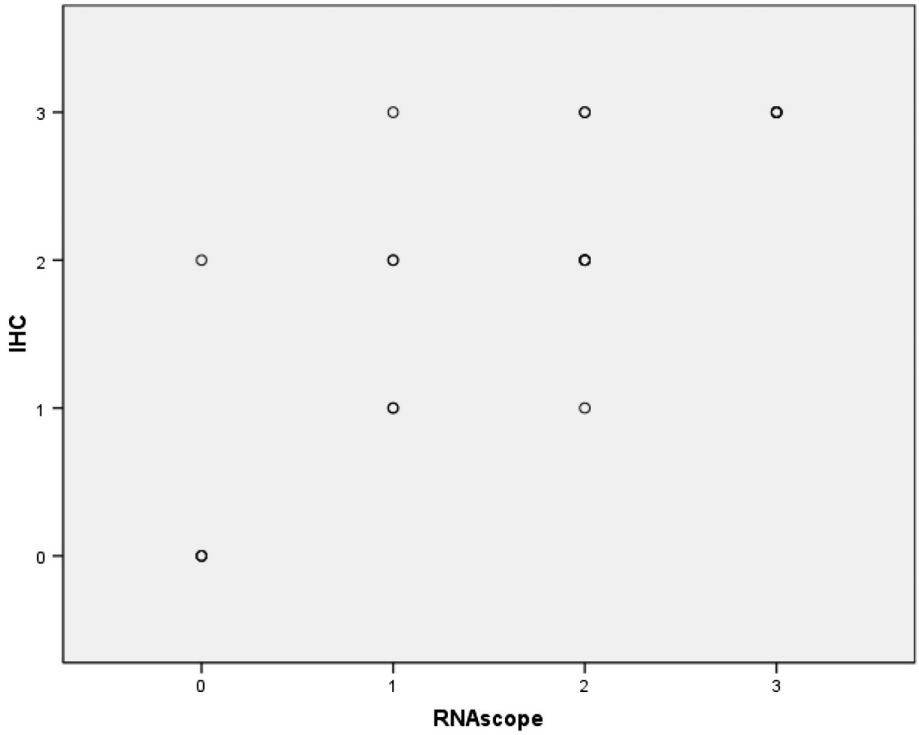

Fig. 3 Scatter plot of correlation of IHC and RNAscope findings for PD-L1.

were highly consistent (Pearson coefficient 0.753; $P < .001$) as shown in the scatter plot in Fig. 3.

4. Discussion

Currently, as summarized in the WHO 2017 classification of endocrine tumors [20], all PCCs/PGLs are believed to have malignant potential with an approximately 10% chance of metastasis, which contrasts with the almost 40% risk of metastasis from extra-adrenal sympathetic PGLs. However, the malignant behavior of PCCs/PGLs before distant metastasis is poorly understood. If the risk could be predicted before metastasis occurs, patients would get optimal therapeutic time. To this end, we studied PD-L1 expression in PCCs/PGLs and analyzed the relationship of PD-L1 expression and malignant behavior before distant metastases were established. The results showed that the expression of PD-L1 has no relation to age, sex, tumor size, capsular invasion, tumor necrosis, relapse/distant metastasis, secretion of norepinephrine/epinephrine/dopamine, or diabetes but correlated well with a Ki-67 value ≥3% ($P = .019$) and hypertension ($P = .013$), indicating that PD-L1 could be considered a malignant behavior biomarker for PCCs/PGLs. Among all 45 follow-up responses, only 1 patient died of PGL after surgery, and that patient's tumor tissue was PD-L1 positive.

As reported, there were several histomorphologic features of the Pheochromocytoma of Adrenal gland Scaled Score that may have a relationship to PCC/PGL's malignant behavior. Of these, necrosis, capsular invasion, vascular invasion, cellular monotony, high mitosis rate, atypical mitotic figures, and nuclear hyperchromasia were significant predictors of malignant behavior that appeared before distant metastasis [21,22]. In addition, the tumor size, spindle and small round cell morphology [19], a Ki-67 ≥3% [23,24], and S100 [21] are significantly associated with tumor behavior. Patients with primary PCCs/PGLs >5 cm have lower overall survival rates than those with smaller tumors because of a higher risk of distant spread [2,23].

However, there was no report of the correlation of PD-L1 and malignant behavior of PCCs/PGLs until the research of Pinato et al [3]. They showed that there was no significant association between PD-L1 positivity and any of the usually salient clinicopathologic features, including type of germline mutation, tumor size, tumor location, or survival, which is consistent with our results. Moreover, we analyzed the correlation between PD-L1 expression intensity and catecholamine secretion, hypertension, diabetes, capsular invasion, necrosis, and Ki-67 index and found that only a Ki-67 index ≥3% was positively correlated with PD-L1 expression. Ki-67 is a marker of cell proliferation usually used as an indicator of malignancy, especially in breast cancer. Its correlation with PD-L1 expression suggested that tumor growth would be associated with tumor immune escape for self-protection, and this finding is reasonable, as tumors with a self-protection apparatus could have a better chance to grow. However, the causal relation of tumor growth and immune escape in PCCs/PGLs is not clear, so whether suppression of the immune system leads to tumor growth or excessive cellular growth leads to PD-L1 expression

needs further investigation. Hypertension also correlated with the PD-L1 score, but no published data have addressed this correlation, so it requires further study.

The RNAscope is a novel RNA in situ hybridization method that allows observation of specific gene expression in individual cells through a novel probe design strategy. It has been used in several tumors, such as gastric cancer [25], triple-negative breast cancer [26], and bladder carcinoma [27]. The results of our RNAscope study agreed with classic IHC data, with a κ coefficient of 0.828 and a Pearson coefficient of 0.753, which indicated concordance between PD-L1 protein and mRNA expression. Moreover, the combination of mRNA analysis with in situ hybridization of protein through IHC could provide powerful methods to identify specific cell populations in situ at both the protein and mRNA levels.

In addition, approximately 40% of PCCs/PGLs carry a germline mutation in one of the following: *SDHA*, *SDHB*, *SDHC*, *SDHD*, *RET*, *VHL*, *NF1*, *TMEM127*, *MAX*, *SDHAF2*, and *FH*, although only *SDHB* is a strong indicator of malignancy [1]. Even the results of Pinato demonstrated a lack of PD-L1 overexpression in tumor samples harbouring mutations [3], but expression of PCC/PGL in tumors containing *SDHB* mutations should be investigated more closely.

For the treatment of metastatic and progressive PCCs/PGLs, cyclophosphamide, vincristine, and dacarbazine can be useful in controlling progression and improving survival [28], especially in *SDHB*-related metastatic PGL [29]. However, tumor shrinkage and decreased catecholamine secretion can be expected in only one-third of patients, so cyclophosphamide, vincristine, and dacarbazine are not currently recommended for all patients with PCCs/PGLs [28]. Immunotherapy targeting checkpoint inhibition to enhance or stimulate antitumor immune responses might have lower toxicity and fewer adverse effects and be powerful against recurrent and metastatic tumors [12]. This subject needs more attention [3].

Our results also showed lymphocyte aggregation in the PCCs/PGLs, which demonstrated the infiltration of immunocytes into the tumor microenvironment. Binding of PD-1 to PD-L1 inhibits $CD8^+$ T-cell signal transduction through PI3K/Akt and Ras-MEK-ERK, which could affect T-cell energy metabolism, inhibit proliferation, and eventually lead to apoptosis through release of cytokines and cytotoxic products, resulting in immune escape of tumor cells [30,31]. Thus, anti–PD-1/PD-L1 monoclonal antibody drugs are an attractive strategy. The US Food and Drug Administration has approved 2 monoclonal antibodies against PD-1 (nivolumab and pembrolizumab) and 3 monoclonal antibodies against PD-L1 (atezolizumab, avelumab, and durvalumab) for the treatment of melanoma, urothelial carcinoma, and metastatic Merkel cell carcinoma. These antibodies can significantly prolong the survival of these patients, with fewer adverse effects than are seen with chemotherapy [32]. An open-label phase II study of pembrolizumab in patients with metastatic PCCs/PGLs was recently initiated [2]. Whether anti–PD1/PD-L1 antibodies are effective to treat PCCs/PGLs needs further investigation.

Our findings provide a valuable clinical rationale for believing that PD-L1 should be considered as not only a malignant behavior biomarker for PCCs/PGLs but also a putative biomarker for anti–PD-L1 therapies as an immune checkpoint inhibitor.

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