Expression of PD-1, PD-L1 and PD-L2 is associated with differentiation status and histological type of endometrial cancer

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Abstract. Endometrial cancer (EC) is the most frequent gynecological malignancy and a major cause of morbidity and mortality for women worldwide. Programmed cell death protein 1 (PD-1) and its ligands programmed death ligand 1 (PD-L1) and programmed death ligand 2 (PD-L2) have been well studied in lung cancer, melanoma and renal-cell cancer. However, few studies have been performed in EC. The purpose of the present study was to assess the expression of PD-1, PD-L1 and PD-L2 in 35 human normal endometrial tissue samples and 75 human EC tissue samples using immunohistochemical staining. It was found that 61.3% of ECs were positive for PD-1 staining, which was almost exclusively found in the tumor-infiltrating immune cells. By contrast, PD-1 was not expressed in the tumor cells or normal endometrial tissues. It was also found that 14.3% of normal endometria and 17.3% of EC tissues were positive for PD-L1 expression, while 20.0% of normal endometrium and 37.3% of EC tissues were positive for PD-L2 expression; however, there was no statistically significant difference between the normal endometrium and EC tissues. PD-1 expression in the tumor-infiltrating immune cells was more frequently found in the moderately and poorly-differentiated ECs and non-endometrioid (type II) ECs than in the well-differentiated ECs and endometrioid (type I) ECs. Similarly, PD-L1 and PD-L2 expression in the tumor-infiltrating immune cells was more frequently found in the moderately and poorly-differentiated ECs and type II ECs than in the type I ECs. The present findings indicate a possible better outcome for future treatment with anti-PD-1 or anti-PD-L1 antibody-based therapies against these subgroups of endometrial cancers with frequent expression of the PD-1/PD-L1/PD-L2 axis.

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Introduction

Endometrial cancer (EC) is the most common gynecological malignancy, with almost 200,000 cases diagnosed every year, and is a major cause of morbidity and mortality for women worldwide (1,2). EC has been broadly classified into two types, termed type I and type II, which possess different etiologies and patient survival rates (3). Type I EC includes endometrioid adenocarcinomas that represent 80-90% of EC arising from atypical endometrial hyperplasia with unopposed estrogen exposure (4,5). The remaining 10-20% of EC are classified as type II, including papillary serous EC, clear cell EC and other histological variants. Type II ECs are mostly poorly-differentiated, estrogen-independent and aggressive, with a ~50% recurrence rate and mortality rate of 50-60% of patients with stage I-II disease (6). The majority of EC patients are diagnosed at an early stage and are treated by surgery with favorable outcomes. However, certain women are diagnosed at a late stage or with type II EC, and often suffer from worse outcomes with limited adjuvant treatment options and low survival rates, for example, patients with low-grade (well-differentiated) type I ECs have a 5-year survival rate of ~90%, whereas patients with high-grade (poorly-differentiated) type I ECs have a 5-year survival rate of 45-77% (6-10). By contrast, patients with type II ECs, including papillary serous and clear cell ECs, have a 5-year survival rate of 35-53% (4,6).

Previous studies have identified certain risk factors for EC, such as nulliparity, early age at menarche, late age at menopause,

unopposed estrogen treatment, hereditary non-polyposis colorectal cancer and polycystic ovarian syndrome (11,12). However, EC pathogenesis remains poorly understood. Additional attention has been paid to the tumor microenvironment and mechanisms of immune evasion. For example, programmed cell death protein 1 (PD-1) has emerged as a key player in tumor immune evasion. PD-1 is a member of the B7/cluster of differentiation (CD)28 family, which is an immune check-point receptor expressed on T cells, natural killer cells, monocytes and B cells (13-15). The ligands for PD-1, namely, programmed death ligand 1 (PD-L1) and programmed death ligand 2 (PD-L2), interact with PD-1 to suppress T cell functions and induce tumor immune evasion (16). PD-L1 is expressed by tumor cells and tumor-infiltrating immune cells, including macrophages, dendritic cells and T cells (17). PD-L1 and PD-L2 mRNAs are found in human heart, placenta, spleen, lymph node and thymus tissues. Additionally, PD-L2 mRNA, but not PD-L1 mRNA, is found in human lung, liver, smooth muscle and pancreas tissues (18). In the tumor microenvironment, the PD-1/PD-L1/PD-L2 immune inhibitory pathway plays a pivotal role in the ability of tumor cells to evade the host's immune system by inhibiting cytotoxic T lymphocyte proliferation, inducing apoptosis of infiltrating T cells, and increasing the amount of regulatory T cells (19,20). Based on the understanding of the immunosuppressive function of the PD-1/PD-L1/PD-L2 axis, multiple clinical trials have demonstrated that antibodies against PD-1 or PD-L1 are effective therapeutics to block immune evasion and induce tumor regression in patients with melanoma, non-small cell lung cancer, and renal-cell cancer (21-27). Therefore, the US Food and Drug Administration (FDA) has approved the anti-PD-1 antibodies pembrolizumab (Keytruda®; Merck & Co., Inc., Kenilworth, NJ, USA) (28) and nivolumab (Opdivo®; Bristol-Myers Squibb, Princeton, NJ, USA) (29) for the treatment of patients with unresectable or metastatic melanoma. Nivolumab has also been approved for the treatment of patients with metastatic squamous non-small cell lung cancer, with progression on or after platinum-based chemotherapy (30). The FDA has assigned a priority review designation to pembrolizumab as a treatment for patients with advanced non-small cell lung cancer (31). Since the anti-PD-L1 antibody MPDL3280A (Genentech, Inc., South San Francisco, CA, USA) showed responsive rates of 13-26% in solid tumors, including non-small cell lung cancer (17), the FDA has assigned MPDL3280A a breakthrough therapy designation for the treatment of PD-L1-positive non-small cell lung cancer that has progressed during or subsequent to platinum-based chemotherapy, as well as a targeted therapy for patients with epidermal growth factor receptor-positive or anaplastic lymphoma kinase-positive tumors, pending the outcomes of ongoing phase II and III trials (32).

There have been an extremely limited number of studies on PD-1 and EC (33,34), and neither of the previous two studies has addressed the association between PD-1 expression and clinicopathological characteristics of EC patients. Therefore, the aim of the present study was to assess expression of PD-1, PD-L1 and PD-L2 in EC and to compare the expression of these proteins with clinicopathological characteristics. Immunohistochemical (IHC) staining was performed in 35 normal endometrium tissues and 75 EC tissues. It was found that PD-1 expression was significantly increased in EC than in normal endometrium and that expression of PD-1, PD-L1 and PD-L2 in the tumor-infiltrating immune cells was associated with differentiation status and histological type of EC.

Materials and methods

Human endometrial tissue samples. In total, 35 samples of normal endometrium and 75 samples of ECs that were archived from surgeries performed between January 2012 and December 2014, in the Department of Obstetrics and Gynecology at Shijiazhuang Maternal and Child Health Care Hospital and the Department of Obstetrics and Gynecology at Shijiazhuang First Hospital (Shijiazhuang, Hebei, China) were retrospectively collected. The samples were formalin-fixed and paraffin-embedded tissue blocks and the pathological diagnoses were re-confirmed by a pathologist. The present study was approved by the Institutional Review Boards of Shijiazhuang Maternal and Child Health Care Hospital and Shijiazhuang First Hospital. The procedures to obtain human endometrial tissues were in accordance with the Ethical Principles for Medical Research Involving Human Subjects, as formulated in the World Medical Association Declaration of Helsinki (2008 revision). The clinicopathological characteristics of the patients were summarized in Table I.

Immunohistochemistry. Tissue sections (4-μm thick) were baked at 60˚C for 60 min, deparaffinized in xylene and rehydrated through graded ethanol solutions to water. The antigens were retrieved by heating the tissue sections in 0.01 M ethylenediaminetetraacetic acid buffer at 95˚C for 5 min and then cooling down to room temperature for 20 min. Endogenous peroxidase activity was blocked by 0.3% H_2O_2 for 5 min. Non-specific binding was blocked with 1.5% normal goat or horse serum (VECTASTAIN Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). The tissue sections were incubated with primary antibodies in a humid chamber at 4˚C overnight. Rabbit anti-human PD-L1 polyclonal antibodies (dilution, 1:400; catalog no., ab58810; Abcam, Cambridge, MA, USA), rabbit anti-human PD-L2 polyclonal antibodies (dilution, 1:800; catalog no., SAB3500395-100UG; Sigma-Aldrich, St. Louis, MO, USA), and rabbit anti-human CD279 (PD-1) affinity-purified and validated polyclonal antibodies (dilution, 1:600; catalog no., PIPA520351; Fisher Scientific; Thermo Fisher Scientific, Inc., Waltham, MA, USA) were used as the primary antibodies. Subsequent to being washed 3 times in phosphate-buffered saline, the tissue sections were incubated with goat anti-rabbit polyclonal secondary antibodies (dilution, 1:200; catalog no., PK-6101; VECTASTAIN Elite ABC kit; Vector Laboratories) for 2 h at room temperature. The color was developed using 3,3'-diaminobenzidine substrate kit (Vector Laboratories) following the manufacturer's protocol. The tissue sections were then counterstained with hematoxylin. The tissue sections that had previously stained positively for PD-1, PD-L1 and PD-L2 in a pilot study were used as positive controls and the tissue sections stained with non-immune serum (Vector Laboratories) acted as negative controls. Positive staining for PD-1, PD-L1 and PD-L2 appeared as brown particles at the cytoplasmic membrane or in the cytoplasm. Under microscopy, 5 representative high-power fields (x400 magnification) per tissue section were randomly selected and evaluated by two investigators, who were blinded

Table I. Clinicopathological characteristics of patients.

Characteristics	Number
Normal endometrium	35
Age, mean ± SD (years)	45.3±5.6
Endometrial cancer	75
Age, mean ± SD (years)	57.3±10.1
<60 years	45
≥60 years	30
Differentiation	
Well	37
Moderate	23
Poor	15
Stage	
I	62
II	4
III	9
Histological type	
Endometrioid	63
Papillary serous	11
Clear cell	1
Vascular invasion	
Yes	7
No	68

SD, standard deviation.

to the clinicopathological data. An average of the scores obtained by the two examiners was used to represent each case. A two-score system based on a proportion score and an intensity score was used, as previously described by Allred *et al* (35). The proportion scores indicated the proportion of positive staining: 0, None; 1, less than one-hundredth; 2, one-hundredth to one-tenth; 3, one-tenth to one-third; 4, one-third to two-thirds; and 5, greater than two-thirds. The intensity scores represented the estimated average staining intensity of positive staining: 0, None; 1, weak; 2, intermediate; and 3, strong. The overall scores (Allred scores) were the sum of the proportion score and intensity score of each case (range, 0-8).

Statistical analysis. Statistical analysis was performed using SPSS version 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). Patient age was expressed as mean ± standard deviation. The comparison of clinicopathological characteristics between different groups was performed using the χ^2 test. Spearman's correlation coefficient was calculated to reveal the correlation between PD-1 scores and PD-L1 or PD-L2 scores. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PD-1, PD-L1 and PD-L2 are expressed in endometrial cancer. IHC staining for PD-1, PD-L1 and PD-L2 was performed using 35 normal endometrium tissues and 75 EC tissues. Representative photomicrographs of the stained samples are shown in Fig. 1. Any sample was defined as having positive staining if the Allred score was ≥1 and any sample was defined as having negative staining if the Allred score was 0. It was found that all normal endometrial samples were negative for PD-1 expression, whereas 61.3% of ECs were positive for PD-1 staining (Table II; $P<0.001$). In total, 14.3% of normal endometrial samples were positive for PD-L1 staining, while 17.3% of ECs were positive for PD-L1 staining (Table II; $P=0.687$). In addition, 20.0% of normal endometrial samples were positive for PD-L2 staining, while 37.3% of ECs were positive for PD-L2 staining (Table II; $P=0.069$). It was found that PD-1 was only expressed in the tumor-infiltrating immune cells, but not in the tumor cells (Fig. 1). By contrast, PD-L1 and PD-L2 were expressed in the tumor cells and infiltrating immune cells (Fig. 1).

PD-1 expression is associated with differentiation status and histological type of EC. As shown in Table III, the rate of positive PD-1 staining was 73.7% in the poorly and moderately-differentiated ECs, which was significantly increased compared with the well-differentiated ECs (48.6%; $P=0.026$). The rate of positive PD-1 staining was 100% in the non-endometrioid ECs, including 11 papillary serous ECs and 1 clear cell EC, which was significantly increased compared with the endometrioid ECs (54.0%; $P=0.006$; Table III). However, PD-1 expression was not different among patients with different ages, clinical stages or statuses of vascular invasion in the tumors (Table III).

PD-L1 expression in the tumor-infiltrating immune cells is associated with the differentiation status and histological type of EC. As shown in Table IV, the rate of positive PD-L1 staining in the tumor-infiltrating immune cells was ~73.7% in the poorly and moderately-differentiated ECs, which was significantly increased compared with the well-differentiated ECs (45.9%; $P=0.014$). The rate of positive PD-L1 staining in the tumor-infiltrating immune cells was 100% in the non-endometrioid ECs, which was significantly increased compared with in the endometrioid ECs (52.4%; $P=0.006$; Table IV). However, PD-L1 expression in the tumor-infiltrating immune cells was not different among patients with different ages, clinical stages or statuses of vascular invasion in the tumors (Table IV). In addition, PD-L1 expression in the tumor cells was not significantly different among patients with different ages, differentiation statuses, clinical stages, histological types or statuses of vascular invasion in the tumors (Table IV).

PD-L2 expression in the tumor-infiltrating immune cells is associated with the differentiation status and histological type of EC. As shown in Table V, the rate of positive PD-L2 staining in the tumor-infiltrating immune cells was 73.7% in the poorly and moderately-differentiated ECs, which was significantly higher than in the well-differentiated ECs (51.4%; $P=0.046$). The rate of positive PD-L2 staining in the tumor-infiltrating immune cells was 100% in the non-endometrioid ECs, which was significantly higher than in the endometrioid ECs (55.6%; $P=0.003$; Table V). However, PD-L2 expression in the tumor-infiltrating immune cells was not significantly different among patients with different ages, clinical stages or statuses of vascular invasion in the tumors (Table V). Additionally, PD-L2 expression in the tumor cells was not significantly different among patients with different

Table II. Expression of PD-1, PD-L1 and PD-L2 in normal endometrium and EC.

		PD-1		PD-L1		PD-L2	
Group	n	Positive, n (%)	P-value	Positive, n (%)	P-value	Positive, n (%)	P-value
Normal	35	0 (0.0)	<0.001	5 (14.3)	0.687	7 (20.0)	0.069
EC	75	46 (61.3)		13 (17.3)		28 (37.3)	

EC, endometrial cancer; PD-1, programmed cell death protein 1; PD-L1, programmed death ligand 1; PD-L2, programmed death ligand 2.

Table III. PD-1 expression and clinicopathological characteristics of patients with endometrial cancer.

Characteristics	n	Positive, n (%)	P-value
Patients	75	46 (61.3)	-
Age			
<60 years	45	26 (57.8)	0.439
≥60 years	30	20 (66.7)	
Differentiation			
Well	37	18 (48.6)	0.026
Poor/moderate	38	28 (73.7)	
Stage			
I	62	36 (58.1)	0.204
II/III	13	10 (76.9)	
Histological type			
Endometrioid	63	34 (54.0)	0.006
Non-endometrioid	12	12 (100.0)	
Vascular invasion			
Yes	7	4 (57.1)	1.000
No	68	42 (61.8)	

ages, differentiation statuses, clinical stages, histological types or statuses of vascular invasion in the tumors (Table V).

PD-1 expression is associated with PD-L1 and PD-L2 expression in the tumor cells and infiltrating immune cells. It was found that the PD-L1 and PD-L2 Allred staining scores were higher in the tumor-infiltrating immune cells than the tumor cells alone (Fig. 2). Since PD-1 was only expressed in the tumor-infiltrating immune cells, Spearman's correlation analysis was performed between PD-1 scores and PD-L1 or PD-L2 scores. It was found that the PD-1 score was positively associated with the PD-L1 or PD-L2 score in the tumor cells and infiltrating immune cells (Fig. 2).

Discussion

The present study found that 61.3% of human ECs stained positive for PD-1, which was almost exclusively found in the tumor-infiltrating immune cells. By contrast, PD-1 was not expressed in the tumor cells or normal endometrial tissues. It was also found that 14.3% of normal endometria and 17.3% of ECs were positive for PD-L1 expression, while 20.0% of normal endometria and 37.3% of ECs were positive for PD-L2 expression, though there was no statistically significant difference between normal endometrium and EC. Vanderstraeten *et al* (34) reported that PD-1 expression was present in all 15 cases of normal endometria, PD-L1 expression was present in 81% of 16 cases of normal endometria, and PD-L2 expression was present in 47% of 15 cases of normal endometria. In primary ECs, Vanderstraeten *et al* found that PD-1 expression was present in 100% of 30 cases of ECs, PD-L1 expression was present in 83% of 29 cases of ECs, and PD-L2 expression was present in 40% of 30 cases of ECs. The discrepancy between the present study and the study by Vanderstraeten *et al* (34) may be due to different antibodies and IHC protocols used. It may also be due to the different EC patients studied. For example, the study by Vanderstraeten *et al* included 28 cases of papillary serous and clear cell ECs who were compared with 16 cases of endometrioid ECs (34). By contrast, the present study compared 12 cases of papillary serous and clear cell ECs with 63 cases of endometrioid ECs. Therefore, it is expected that Vanderstraeten *et al* should find more PD-1, PD-L1 and PD-L2-positive cases than the present study, since the present study showed that non-endometrioid ECs were 100% positive for PD-1, PD-L1 and PD-L2 expression, if we did not distinguish between the tumor cells and infiltrating immune cells. In addition, Howitt *et al* (33) reported that PD-1 was overexpressed in tumor-infiltrating lymphocytes of 81% of polymerase ε-mutated ECs and 28% of microsatellite-instable ECs. In peritumoral lymphocytes, PD-1 was overexpressed in 90% of polymerase ε-mutated ECs and 28% of microsatellite-instable ECs. PD-L1 expression was infrequently noted in the tumor cells, but was common in intraepithelial immune cells and more frequent in polymerase ε-mutated ECs (39%) than in microsatellite-instable ECs (13%; P=0.02) (33). The present study also showed that PD-L1 and PD-L2 expression was less frequently found in the tumor cells than in the tumor-infiltrating immune cells, which is consistent with the findings of Howitt *et al* (33).

The present study went beyond the previous two studies (33,34) to show the correlation between the expression of PD-1, PD-L1 and PD-L2 and clinicopathological characteristics of ECs. It was shown that PD-1 expression in the tumor-infiltrating immune cells was more frequently found in the moderately and poorly-differentiated ECs and non-endometrioid (type II) ECs than in the well-differentiated ECs and endometrioid (type I) ECs. Similarly, it was shown that PD-L1 and PD-L2 expression in the tumor-infiltrating immune cells was more frequently found in the moderately and poorly-differentiated ECs and non-endometrioid (type II) ECs than in the well-differentiated ECs and endometrioid (type I)

Table IV. Association between programmed death ligand 1 expression and clinicopathological characteristics of endometrial cancer patients.

		Tumor cells		Immune cells	
Characteristics	n	Positive, n (%)	P-value	Positive, n (%)	P-value
Patients	75	13 (17.3)	-	45 (60.0)	-
Age			0.681		0.149
<60 years	45	7 (15.6)		24 (53.3)	
≥60 years	30	6 (20.0)		21 (70.0)	
Differentiation			0.141		0.014
Well	37	4 (10.8)		17 (45.9)	
Poor/moderate	38	9 (23.7)		28 (73.7)	
Stage			0.315		0.171
I	62	9 (14.5)		35 (56.6)	
II/III	13	4 (30.8)		10 (77.0)	
Histological type			0.237		0.006
Endometrioid	63	9 (14.3)		33 (52.4)	
Non-endometrioid	12	4 (33.3)		12 (100.0)	
Vascular invasion			0.764		0.427
Yes	7	2 (28.6)		3 (42.9)	
No	68	11 (16.2)		42 (61.8)	

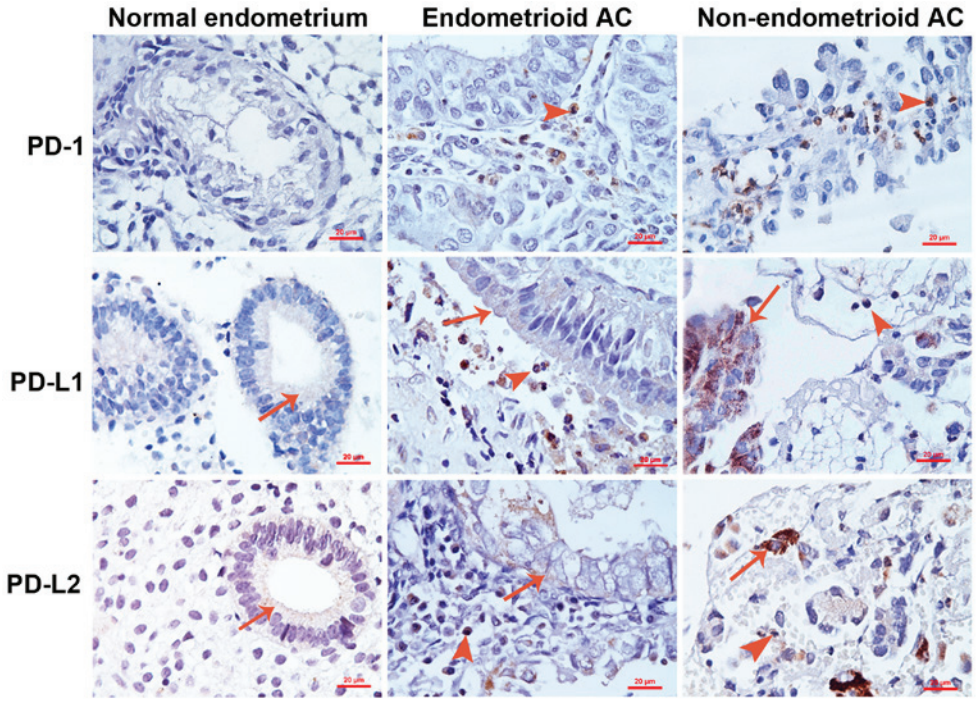

Figure 1. Representative photomicrographs of immunohistochemical staining. Arrows indicate the positively stained normal epithelial or tumor epithelial cells. Arrowheads indicate the positively stained tumor-infiltrating immune cells. Original magnification, x400. Scale bar, 20 μm. AC, adenocarcinoma; PD-1, programmed cell death protein 1; PD-L1, programmed death ligand 1; PD-L2, programmed death ligand 2.

ECs. It is known that moderately and poorly-differentiated ECs and type II ECs have a lower 5-year survival rate than the well-differentiated ECs and type I ECs (6-10). Therefore, the present findings suggest that more frequent expression of PD-1, PD-L1 and PD-L2 in the moderately and poorly-differentiated ECs and type II ECs may cause immunosuppression to favor tumor growth, thus negatively affecting the patient's survival. Future studies may address whether expression of PD-1, PD-L1 and PD-L2 may be used as an independent predictor of patient survival, once the follow-up data for the present

Table V. Association between programmed death ligand 2 expression and clinicopathological characteristics of endometrial cancer patients.

		Tumor cells		Immune cells	
Characteristics	n	Positive, n (%)	P-value	Positive, n (%)	P-value
Patients	75	28 (37.3)	-	47 (62.7)	-
Age			0.380		0.119
<60 years	45	15 (33.3)		25 (55.6)	
≥60 years	30	13 (43.3)		22 (73.3)	
Differentiation			0.698		0.046
Well	37	13 (35.1)		19 (51.4)	
Poor/moderate	38	15 (39.5)		28 (73.7)	
Stage			1.000		0.393
I	62	23 (37.1)		37 (59.7)	
II/III	13	5 (38.5)		10 (76.9)	
Histological type			0.188		0.003
Endometrioid	63	21 (33.3)		35 (55.6)	
Non-endometrioid	12	7 (58.3)		12 (100.0)	
Vascular invasion			0.095		0.413
Yes	7	5 (71.4)		3 (60.0)	
No	68	23 (33.8)		44 (64.7)	

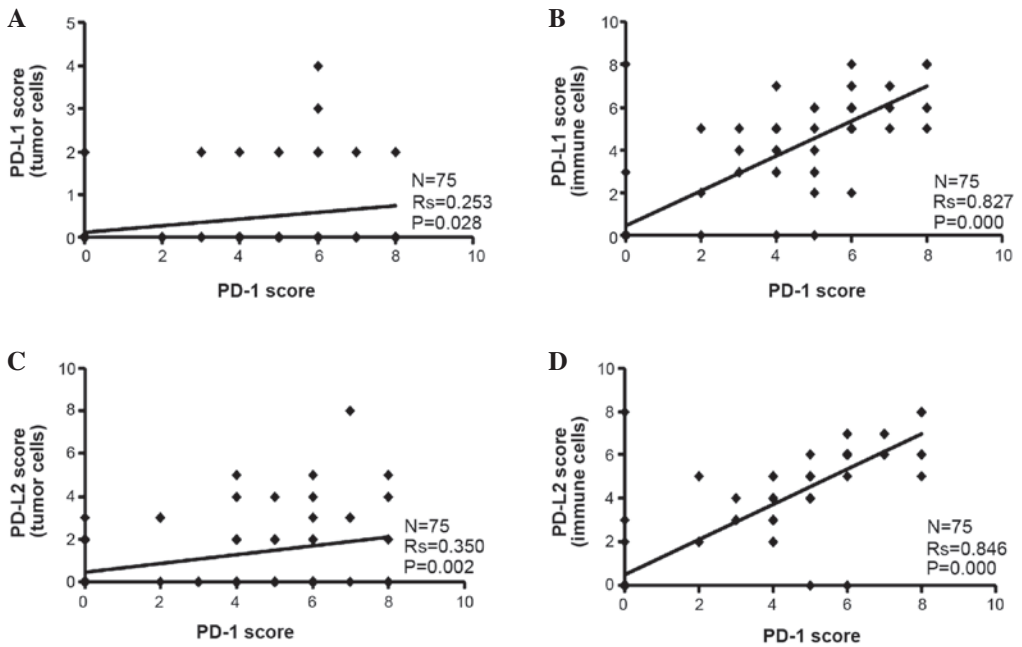

Figure 2. Correlation analysis of PD-1 and PD-L1 or PD-L2 expression. Correlation between PD-1 and PD-L1 scores in the (A) tumor cells and (B) immune cells. Correlation between PD-1 and PD-L2 scores in the (C) tumor cells and (D) immune cells. The expression levels are indicated by Allred scores and assessed by Spearman's correlation coefficient between two proteins. PD-1 expression was only found in the tumor-infiltrating immune cells. PD-L1 and PD-L2 expression was found in the tumor cells and infiltrating immune cells, which was assessed separately. A number of data points are identical, and therefore overlap in each panel. PD-1, programmed cell death protein 1; PD-L1, programmed death ligand 1; PD-L2, programmed death ligand 2.

patients are available. The follow-up data are not available at this time due to the short period subsequent to inclusion of the cases. By contrast, it is reasonable to speculate that the moderately and poorly-differentiated ECs and type II ECs may be more sensitive to anti-PD-1 or anti-PD-L1 antibodies-based therapies, since it has been demonstrated in clinical trials that PD-L1-positive tumors tend to be more responsive to anti-PD-1 or anti-PD-L1 therapies (17,26).

In summary, the present study demonstrates that the expression of PD-1, PD-L1 and PD-L2 is associated with moderately and poorly-differentiated endometrial cancer and type II endometrial cancer. Frequent expression of the

PD-1/PD-L1/PD-L2 axis in these subgroups of endometrial cancers may be potentially correlated with their aggressive progression and poor patient survival. The present findings indicate a possible improved outcome for future treatment with Keytruda and Opdivo in these subgroups of endometrial cancers with frequent expression of the PD-1/PD-L1/PD-L2 axis.

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