

Original Article

Clinical significance of PD-L1 (CD274) enhanced expression in cervical squamous cell carcinoma

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Abstract: Programmed death ligand 1 (PD-L1) is a trans-membrane protein that can reduce the immune response in both infectious diseases and cancers and is commonly expressed in various solid tumors. Despite the success of immunotherapy directed at inhibiting of PD-L1/PD-1 signaling, it is not established that whether PD-L1 expression correlates with the clinical response and outcome in cervical squamous cell carcinoma. To investigate the clinical significance of PD-L1 expression in cervical cancer, we analyzed the expression of PD-L1 in 219 cervical squamous cell cancers and 30 healthy controls, characterized the expression level of PDL-1 in tumor-infiltrating lymphocytes (TILs), and assessed the relationship between them and prognosis of cervical cancers. The expression of PD-L1 was observed in 32.4% (71/219) cervical carcinomas and 10.0% (22/219) in partial TILs. However, there was no expression of PD-L1 in normal cervical epithelium. Statistical analysis showed that increased PD-L1 expression was significantly associated with high TNM stage, reduced number of TILs, and worse prognosis in cervical carcinomas, but there was no significant statistic difference in age, tumor size, HPV infection and other clinicopathology features. PD-L1 expression in TILs was found significantly associated with the TILs amount. Furthermore, the presence of prominent lymphocytic infiltrates was also significantly associated with a clear trend towards longer survival. In conclusion, these data suggested that PD-L1 could act as a significant biomarker in the worse prognosis and adverse clinicopathologic features of cervical cancer. Anti-PD-L1 therapy may have a role in the treatment of cervical squamous cell carcinoma.

Keywords: Programmed cell death ligand 1 (PD-L1), cervical cancer, human papillomavirus (HPV), tumor-infiltrating lymphocytes (TILs), prognosis

Introduction

Cervical cancer is one of the most common gynecological malignancies. In recent years, it has an incidence at younger age and approximately one-third of such cases lead to mortality [1]. High-risk human papillomavirus (HPV) types that are capable of affecting host anti-virus/tumor immune responses, predominantly HPV16 and HPV18, are associated with >90% of cervical cancer cases [2]. Currently, it is believed that immune evasion is one of the important mechanisms in the pathogenesis of many malignant tumors, as well as cervical cancer [3]. Inhibitors of immune check-point molecule, PD-1 (Programmed cell death-1) and its ligand PD-L1, have attracted much attention recently in cancer immunotherapy due to their

durable antitumor effects in various malignancies, especially the advanced ones [4]. The expression of PD-L1, which belongs to the B7/CD28 costimulatory factory superfamily of proteins, can be induced in certain types of solid and hematologic cancer and is exploited by tumors to evade immune responses [5-9]. Moreover, it has reported that increase expression in PD-L1 was correlated with poor clinical prognosis [8, 9]. Despite the success of immunotherapy directed at inhibiting of PD-L1/PD-1 signaling [1, 5, 6], whether PD-L1 expression correlates with the clinical response and outcome in cervical squamous cell carcinoma are still under debate. Up to now, only several studies have been published that assess expression levels of PD-L1 in cervical squamous cell carcinoma using IHC. To assess the clinical

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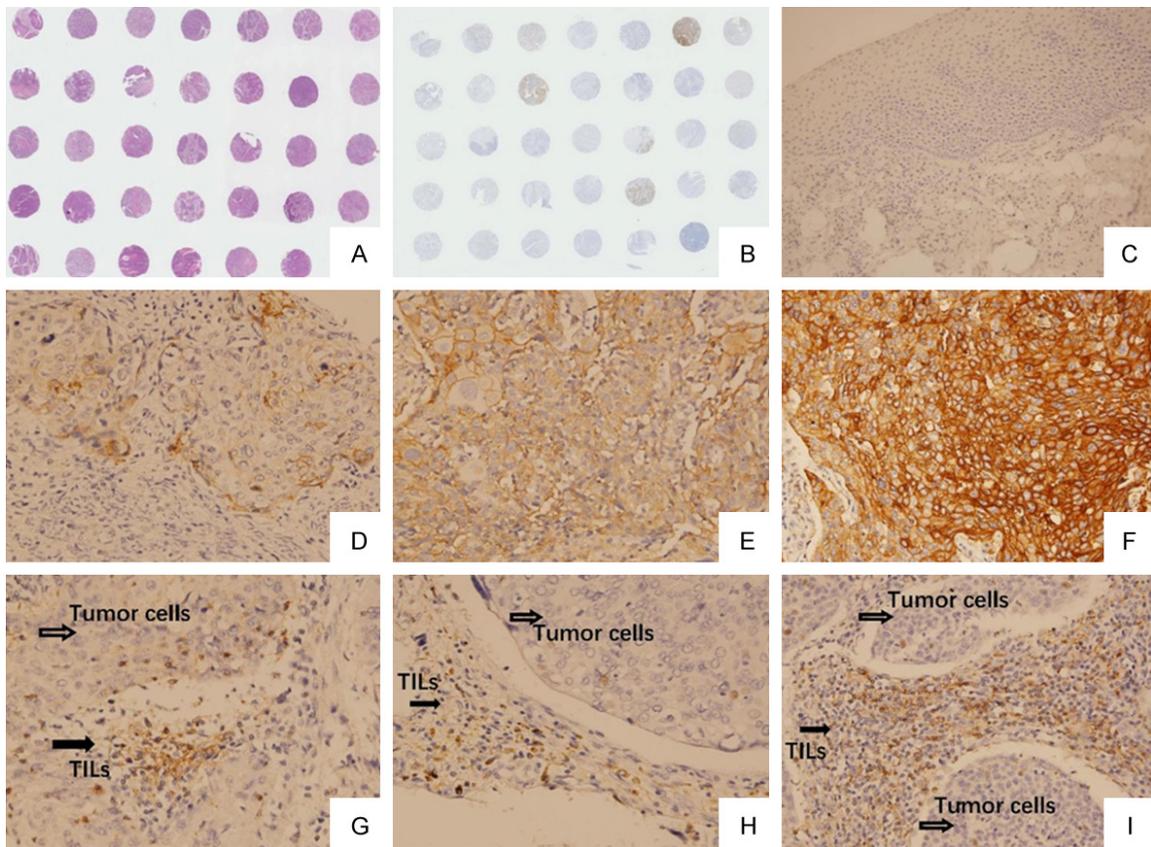


Figure 1. H&E staining and intensities of the PD-L1 reactions in cervical squamous cell carcinoma and tumor-infiltrating lymphocytes (TILs): (A) H&E staining of the tissue array; (B) Immunohistochemistry results of the tissue array; (C) Negative expression in normal cervical epithelium; (D) Weak intensity in tumor cells; (E) Moderate intensity in tumor cells; (F) Strong intensity in tumor cells; (G) Weak intensity in TILs; (H) Moderate intensity in TILs; (I) Strong intensity in TILs.

value of PD-L1 as a novel prognostic biomarker of cervical squamous cell carcinoma, which accounts for the majority of cervical cancers, we thus examined PD-L1 expression in 219 cervical cancers and 30 healthy controls, and evaluated the association between the clinicopathological parameters and PD-L1 expression.

Materials and methods

Participants

219 cases of cervical squamous cell cancer with no known history of cancer and 30 cases of healthy controls treated from March 2013 to July 2016 were included in this study. All cases were selected from department of pathology, West China Second University Hospital, Sichuan University. Medical charts and clinical data such as age, diagnoses, tumor size and TNM

stage were retrieved, and all cases were investigated by light microscopy and immunohistochemistry. HPV-DNA genotype detection and survival analysis were performed.

Tissue microarray construction

Formalin-fixed paraffin-embedded samples including 249 cores of both tumoral and normal cervical tissues were assembled into a tissue microarray (TMA) with a core size of 0.6 mm. Upon examination, the sections were 4-6 μ m in thickness.

Immunohistochemical staining and intensity

A TMA of a well-defined group of 219 patients and 30 healthy controls was stained with antibody against PD-L1. Hematoxylin and eosin staining was performed on one slide for histopathology evaluation (**Figure 1A**). To reduce

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Table 1. Clinicopathologic data in relation to PD-L1 immunohistochemical expression

Clinicopathologic measures	PD-L1 in tumor, n		P	PD-L1 in TILs, n		P
	Positive	Negative		Positive	Negative	
Overall						
Age at diagnosis						
≥45 y	40	85	0.942	18	107	0.619
<45 y	31	63		4	90	
Tumor size						
≥4 cm	24	39	0.604	4	59	0.926
<4 cm	47	109		18	138	
Tumor differentiation						
Moderate-high	22	28	0.357	6	44	0.380
Low	49	120		16	153	
Infiltration depth						
<10 mm	25	24	0.069	12	37	0.202
≥10 mm	46	124		10	160	
Lymph node metastasis						
Positive	15	36	0.494	7	44	0.513
Negative	56	112		15	153	
Vessel-invasion						
Positive	34	88	0.218	10	112	0.117
Negative	37	60		12	85	
TNM stage						
I+II	54	107	0.017	14	147	0.168
III+IV	17	41		8	50	
HPV testing						
Positive	65	122	0.563	18	169	0.082
Negative	6	26		4	28	
TILs amount						
High	46	82	0.021	19	109	0.033
Low	25	66		3	88	

TILs = tumor-infiltrating lymphocytes. P value has been calculated using Pearson's chi square.

nonspecific binding, sections were incubated overnight at 4°C with 10% rabbit serum. The PD-L1 antibody (rabbit anti-PD-L1 monoclonal antibody, ZA-0629; Zhongshan Jinqiao, Beijing) was used at 1:200, respectively. The tissue array was evaluated and scored by two experienced researchers independently. Immunohistochemistry results were semi-quantitatively assessed and graded on a four-tier scale based on the percentage of membranous positive cells: 0 = <5% positive cells, the staining intensity was assessed as negative. When ≥5% of the tumor cells were positive, the expression of PD-L1 was designated as positive: 1 = 5%-29% (weak); 2 = 30%-59% (moderate); 3 = >60% (strong) positive [7]. Moreover, immune

staining was qualitatively classified the same as in tumor-associated lymphocytes (Figure 1B).

Evaluation of tumor-infiltrating lymphocytes (TILs)

The scoring of TILs was performed in hematoxylin-eosin stained TMA preparations independently by two pathologists using a four-tiered scale based on the visual estimation of the amount of lymphocytes in each histospot, as described: a score of 0 indicated virtual absence of TILs; 1+, TILs <30%; 2+, TILs = 30%-60%; and 3+, marked increase in the lymphocytic infiltrate (> 60%) [7]. For statistical analyses, we regarded score 0 and 1+ as the low number group, 2+ and 3+ the high number group.

HPV-DNA testing

HPV-DNA was isolated from formalin-fixed, paraffin-embedded tissue of primary tumor samples using the Nucleic Acid genotyping kit for Human Papillomavirus (Flowcytometry Fluorescence Hybridization Method) according to the supplier's instructions, which was got from Shanghai Tellgen Cooperation.

Statistical analyses

Statistical analyses

Correlations was done by the χ^2 exact test between PD-L1 expression with clinicopathologic parameters of cervical squamous cell cancer, including age, tumor size, TNM stage, tumor differentiation, infiltration depth, lymph node metastasis, vessel-invasion, HPV-testing results, and the (high or low) number of tumor-infiltrating cells. $P < 0.05$ was considered significant. All P values are two-tailed. Survival analysis was performed using Kaplan-Meier with

Table 2. Immunohistochemical results of PD-L1 expression in tumor and TILs

	Negative, n (%)	Positive, n (%)		
		Weak	Moderate	Strong
PD-L1 in Tumor	148 (67.6)	12 (16.9)	32 (45.1)	27 (38.0)
PD-L1 in TILs	197 (90.0)	5 (22.7)	9 (40.9)	8 (36.4)

log-rank tests. Statistical analyses were carried out with the SPSS software package 22.

Results

Characteristics of cervical cancer patients and controls

The retrospective cohort consisted of 219 patients, who underwent surgical resection of squamous cell carcinoma of the cervix between 2014 and 2016 at West China Second University Hospital. Grading and staging was undertaken according to the current World Health Organization Classification of Tumors of the Breast and Female Genital Organs (2014) and the AJCC tumor, node, metastasis (TNM) classification. Patients' age ranged from 26 to 75 years (mean 46.7; median 49.0). 169 cases were poorly differentiated, 50 cases were moderately-well differentiated. 132 (60.3%), 29 (13.2%), 51 (23.3%), and 7 (3.2%) patients were diagnosed with TNM stages I-IV, respectively. Complete clinicopathologic data for all patients including age, tumor size, nodal metastasis and vessel-invasion were available (**Table 1**).

PD-L1 expression in benign tissue

Control cases were completely negative for PD-L1 in both the epithelium and in the stromal compartment aside from very occasional single positive lymphocytes (**Figure 1C**).

Tumoral PD-L1 expression and correlation with clinicopathologic features

PD-L1 expression was evaluated in tumor cells of primary cervical squamous cell carcinoma by immunohistochemistry (**Table 2**). PD-L1 expression positive was found in 71/219 (32.4%) of cervical squamous cell carcinoma. Strong, membranous staining (3+) was found in 27/71 (38.0%) cases, intermediate staining (2+) in 32/71 (45.1%) cases, whereas 12/71 (16.9%) cases demonstrated weak staining (1+) (**Figure**

1D-F). Overall, the expression level of PD-L1 was moderate to high. Among 32 HPV-negative cases, only 7/32 (21.9%) cases showed PD-L1 positive, while 64 (34.2%) cases showed PD-L1 positive in 187 HPV-positive cervical squamous cell carcinomas. Statistical analyses

showed that there was no significant statistic difference between PD-L1 expression and HPV-infection in cervical squamous cell carcinoma. Patients with PD-L1 immunopositive tumors had a significantly higher risk for high TNM stage ($P = 0.017$), but it was not relevantly correlated with age, tumor size, histologic grade, infiltration depth, lymph node metastasis, or vessel-invasion. Moreover, increased PD-L1 expression was significantly associated with reduced number of tumor infiltrating lymphocytes in cervical carcinomas and worse prognosis ($P = 0.021$, and 0.004). All features of clinicopathological data in relation to PD-L1 immunohistochemical expression are summarized in **Table 1**.

Characterization of tumor-infiltrating lymphocytes (TILs)

PD-L1 expression was also evaluated in tumor-infiltrating lymphocytes by immunohistochemistry (**Table 2**). PD-L1 positive TILs were seen in 10.0% (22/219) of cervical cancer in our study. Strong, membranous staining (3+) was found in 8/22 (36.4%) cases, intermediate staining (2+) in 9/22 (40.9%) cases, and 5/22 (22.7%) demonstrated weak staining (1+) (**Figure 1G-I**). Among the 22 cases, only 4 cases were HPV-negative, demonstrating that TILs with HPV-positive tumors were more likely to be PD-L1 positive relative to HPV-negative tumors. Representative pictures of cervical tumors cases showing different levels of TILs are depicted in **Figure 2**. The number of cases with more prominent lymphocytic infiltrates (scores 2+ and 3+) was 128 (58.4%), while 91 (41.6%) cases showed low TILs (scores 0 and 1+). Cases showed significantly higher PD-L1 levels with elevated TILs ($P = 0.033$), but not with age, tumor size, vessel invasion, lymph node positivity, TNM stage, and histologic grade. Furthermore, the presence of prominent lymphocytic infiltrates was also significantly associated with a clear trend towards longer survival ($P = 0.048$).

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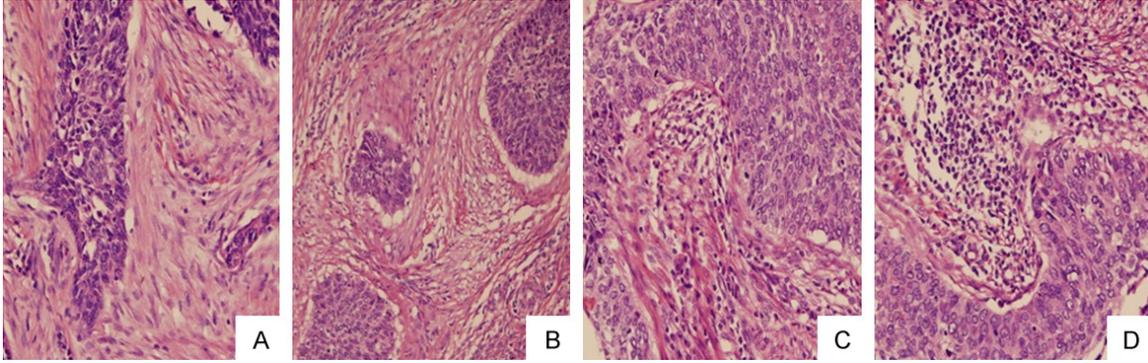


Figure 2. Different amounts of tumor-infiltrating lymphocytes: (A) Score 0, showing absence of TILs; (B) Score 1+, showing low amount of TILs (<30%); (C) Score 2+, showing moderate amount of TILs (30%-60%); (D) Score 3+, showing marked increase in TILs (>60%).

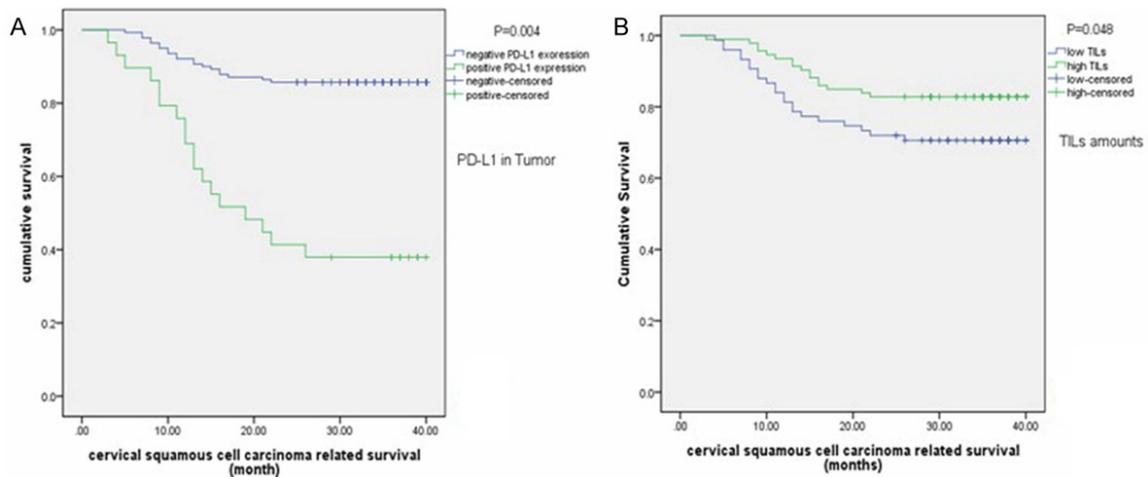


Figure 3. Survival analysis: (A) Survival analysis according to PD-L1 status in cervical squamous cell carcinoma; (B) Survival analysis according to TIL amount in cervical squamous cell carcinoma. *P*-value has been calculated using the log-rank test.

HPV analysis

Of all the 219 cervical cancer specimens, only 32 cases were HPV-negative by HPV-DNA testing, and among the 187 HPV-positive tumors, HPV16 is the most common subtype (162/187, 86.63%) in both single infection cases and combined infection cases.

Follow-up

All patients followed up to May 2017, 48 cases were lost and 171 (78.1%) cases were available. Mean follow-up was 33 months (range 6-40 months). At the end of the follow-up period, 19 patients had died of this disease, 14 patients had a recurrence, and 5 patients had a metastasis, the other 132 were alive. In the

cervical squamous cell carcinoma patient cohort with positive PD-L1 expression and reduced number of tumor-infiltrating lymphocytes, the risk of poor prognosis was significantly increased ($P = 0.004$, and 0.048) (Figure 3). However, the expression of PD-L1 of TILs was not significantly associated with the risk of recurrence or metastasis in multivariate analysis.

Discussion

The etiology of cervical cancer is a complex, multistep, and multifactorial process. A few risk-modulating variants for cervical cancer have been identified by a candidate gene association study, which are involved in DNA repair, cell cycle and apoptosis, cell proliferation and

differentiation, and various immune responses [10]. Aberrant PD-L1 is highly expressed on a variety of solid tumors by many different authors, and its increased expression in carcinomas can contribute to tumor evasion of the immune system, thereby engendering a poor clinical prognosis [1, 5, 6, 11, 12]. However, data on PD-L1 expression in cervical squamous cell carcinoma concerning prevalence, prognostic impact and variation of expression in disease course are limited to this day. Results from this study showed that PD-L1 expression pattern was a prognostic factor as a diffuse expression with high TNM stage and reduced number of tumor-infiltrating lymphocytes. At the same time, decreased amounts of tumor-infiltrating lymphocytes and increase in PD-L1 expression in tumor cells were found to be associated with poor prognosis. The results of the current study provide information that may be important in the investigation of immune checkpoint blockage in cervical squamous cell carcinoma.

PD-L1 is a surface glycoprotein known to be widely expressed on a majority of tumor cells and other immune cells including antigen presenting cells (APC), activated CD4+ and CD8+ T cells, and B cells, macrophages and thymic cortical epithelial cells. Its receptor PD-1 is an immune checkpoint protein, mainly expressed in activated T cells [13, 14]. Interactions between PD-L1 and PD-1 have been shown to inhibit a wide range of immune responses against pathogens, tumor, and self-antigens through attenuated T-lymphocyte activation and proliferation, suppressed cytokine secretion, induced T cell apoptosis, and maintained peripheral tolerance [5, 6, 15-17]. Relevant studies demonstrated that malignant tumors, including melanoma, gastric, colorectal, pancreatic, breast, renal, lung, bladder, brain and blood cancers, frequently show abnormal PD-L1 expression, and utilize the PD-L1 pathway to avoid an immune response, resulting in tumor progression and metastasis [5-9, 18-21]. In gynecologic malignancies, high rates of PD-L1 expression were identified in ovarian sex cord-stromal tumors (75.0%), uterine sarcoma (46.5%) and endometrial cancer (24.9%) [22], while lower rates were identified in HPV-associated cancers of the lower genital tract, such as vaginal and vulvar [23]. Several studies of abnormal expression of PD-L1 showed that

the rate of PD-L1 expression was in close proximity to 70% in cervical squamous cell carcinoma [24, 25]. Additionally, some other researchers observed PD-L1 in peripheral blood of cervical carcinoma patients was significantly increased compared with CIN or cervicitis [26]. However, not all investigators reached the similar results. In Herzog's study, only 16.7% were observed in cervical squamous cell cancer [23], and in Reddy's study, the rate was 34.4% [27]. The results of this present study showed that PD-L1 expressed in 32.4% cervical carcinomas. The variation in the level of PD-L1 expression might be due, at least in part, to the distinct properties, heterogeneity of subjects, a small sample size, and independent information provided by PD-L1 protein. Methodologic differences might also account for this apparent inconsistency. Thus, further studies are needed to better characterized PD-L1 expression status in addition to incorporation in clinical trials.

Currently, the level of PD-L1 expression in cervical squamous cell carcinoma and its association with patient clinical significance remains unclear. A retrospective clinicopathologic analysis showed PD-L1 positive cervical carcinoma had a significantly higher risk for nodal metastasis at the time of diagnosis and poor prognosis or with lower overall survival [11], while the data from Herzog's study suggested that the HPV-associated cancers of the lower gynecologic tract (e.g. carcinomas of the cervix, vagina and vulva) may derive less benefit based on absence of PD-L1 [23]. When the clinicopathologic features were considered in our limited data, the positive expression of PD-L1 was found to be significantly associated with high TNM stage, reduced amounts of TILs, and worse prognosis of cervical squamous cell carcinoma, but no significant relationship was detected between PD-L1 over-expression and other clinical characteristics. Our findings might strengthen the sensitivity and specificity of PD-L1 in predicting the clinical survival of cervical squamous cell carcinoma, which was partly similar to previous studies in other cancers of gynecologic tract [11, 12, 22, 27, 28]. It might be that high expression of PD-L1 indicates activation of the PD-L1/PD-1 pathway to promote tumor evasion of immune clearance, and enhance local and distant metastasis. Thus, a prognostic role of PD-L1 expression in cervical

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cancer is suggested and PD-L1 targeted immunotherapy may be useful in an advanced stage of cervical cancer.

HPV infection, predominantly HPV16 and HPV18, is a major risk factor for the development of cervical cancer [1]. Studies have found that HPVs perturb cell growth, apoptosis, differentiation, and additionally involved in affecting host anti-virus/tumor immune responses, which can influence the occurrence, development, metastasis and prognosis of cervical cancer [2, 10, 29, 30]. In our study, tumor cells with PD-L1+ were observed in 65 (34.8%) cases in HPV-positive group, but 6 (18.8%) cases in HPV-negative group. Although an obvious increase in the expression of PD-L1 in HPV-positive cervical cancer was observed when compared with HPV-negative cervical cancer, there was no statistically significant difference between these two groups. Some other studies have reported conflicting results; Liu's and Yang's studies demonstrated that expression of PD-L1 in cervical squamous cell carcinomas was of prognostic value and associated with human papillomavirus status [30, 31]. Furthermore, a study by Che et al. observed a reduction in PD-L1 protein expression by knock-down of the HPV16 E7 in HPV16-associated cervical cancer cells with a relevant siRNA, as well as a significant increase in peripheral blood mononuclear cell proliferation and cytotoxic T-cell activity [26]. These results suggest that increased PD-L1 expression on the cell membrane surface, induced by HPV16 E7, may be responsible for lymphocyte dysfunction during chronic HPV infection, and inhibiting this pathway may be beneficial to restore the function of tumor infiltrating lymphocytes [2]. However, the data and mechanisms in the over-expression of PD-L1 protein with HPV-associated and HPV-negative cervical cancer are still limited. Whether HPV infection affects PD-L1 expression in cervical squamous cell carcinoma remains to be determined.

Tumor-infiltrating lymphocytes (TILs) are an important component of the tumor immune microenvironment, and the quantity and function of these cells is reflective of the strength of the anti-tumor response [14-16]. Inflammatory tumor microenvironments contribute to the carcinogenesis and progression of many malignant cancers [6, 8, 18, 32]; however, few studies have investigated the association between

PD-L1 expression and TILs of the tumor immune microenvironment in cervical carcinoma. In the present study, PD-L1+ TILs were seen in 10.0% (22/219) of cervical cancer and they were associated with increased inflammatory infiltrates. Among the 22 cases, only 4 cases were HPV-negative, which demonstrated that TILs with HPV-positive tumors were more likely to be PD-L1 positive relative to HPV-negative tumors (81.8% vs. 18.2%). When analyzing the expression level, we found that a high level of PD-L1 protein expression in TILs was significantly associated with the presence of elevated TILs, but not with age, tumor size, lymph node positivity, or histologic grade. Additionally, we observed that tumors with higher accumulation of TILs are associated with improved survival in cervical cancer, which pointed to a critical role of local immunity in limiting tumor progression. It is possible that rather than an indication of total immune evasion, expression of PD-L1 by tumor cells might reflect the presence of antigenic induced anti-tumor-immune pressure mediated by TILs, which is thought to play a similar role in dampening the antitumor immune response and may contribute to tumor progression [7, 32]. The biologic determinants of the association between PD-L1 expression, increased TILs and better outcome are not well understood; clinical trials are necessary to investigate whether PD-L1 positivity within immune stroma is a sufficient predictor of therapeutic response.

In conclusion, the results of the present study demonstrated that PD-L1 is expressed in only a minority of cervical cancers and does influence the survival of patients with cervical cancer. Treatment with PD-L1 blocking antibodies may provide a viable option in cervical squamous cancer according to our limited study. However, the poor association between PD-L1 expression and other clinicopathologic features in tumor cells and TILs status highlights the complex interactions between the tumor and its microenvironment. Further investigations into cancer cell senescence and immune evasion in the microenvironment are required.

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Disclosure of conflict of interest

None.

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