



Evaluation of Programmed Death Ligand-1 Immunohistochemical Expression and Tumor-Infiltrating Lymphocytes in Different Types of Endometrial Carcinoma

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Abstract

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BACKGROUND: Endometrial cancer (EC) identified at an early stage is successfully treated in a majority of patients with surgery with or without radiotherapy or chemotherapy. For patients with advanced disease, however, the prognosis is poor; 5-year survival rates are less than 50% in patients with lymph node metastases and less than 20% with peritoneal or distant metastases. Previous studies proved that programmed death-1/programmed death ligand-1 (PD1-/PD-L1) blockers are currently effectively used as immunotherapies in a number of tumors such as melanoma and non-small cell lung cancer.

AIM: This study was conducted to determine the expression of PD L1 in endometrial carcinoma and to assess its potential role as a biomarker for different types that can be used to screen candidates fit for immunotherapy.

MATERIALS AND METHODS: This cross-sectional study was carried out on 32 cases of endometrial carcinoma cases that underwent endometrial biopsies, dilatation, and curettage or radical hysterectomies at Ain Shams University Hospitals Pathology Units from 2018 to 2020 with their clinical and radiological assessments. Correlation between hematoxylin and eosin-stained histopathological sections and PD-L1 immunohistochemical staining of the same sections, mainly emphasizing the tumor-infiltrating lymphocytes, was done.

RESULTS: PDL-1-positive expression of both tumor cells and TILs was significantly more frequent in type II endometrial carcinoma ($p = 0.04$ and 0.03 , respectively) using a cut-off value 10%, compared to type I. Moreover, Grade III tumors showed significantly more frequent PDL-1 expression in both tumor cells and TILs than Grade I and II tumors, using 5% and 10% cut-off values indicating that PDL-1 is overexpressed in aggressive tumors.

CONCLUSION: PD-L1 staining is significantly related to high-grade tumors and type II endometrial carcinomas, the aggressive types, which support their probable benefit from immunotherapy. Separate assessment of PD-L1-positive staining in both tumor cells or TILs with a cut-off value 10% can significantly reflect the aggressiveness of the tumor and its probable benefit from immunotherapy.

Introduction

Two types of endometrial carcinoma are distinguished with respect to behavior and clinical course. Type-I carcinoma is related to increased estrogen levels by association with endometrial hyperplasia, frequent expression of estrogen and progesterone receptors, and younger age, whereas type-II carcinoma is unrelated to estrogen, associated with atrophic endometrium, frequent lack of estrogen and progesterone receptors, and older age. Histologically, endometrioid and mucinous carcinomas are considered type I while serous and clear cell carcinomas are type II [1].

Molecular data from multiple studies support the hypothesis of different genetic pathways in the development of endometrioid and serous carcinoma. The most frequent genetic alteration in endometrioid carcinoma is PTEN inactivation by mutation, followed by microsatellite instability (MIN) and mutations of

K-ras and β -catenin. In serous carcinoma, p53 mutation is the most frequent genetic alteration, followed by inactivation of p16 and E-cadherin and amplification of her2/neu [2].

Programmed death-1 (PD-1, CD279) is an immunosuppressive molecule that is upregulated on activated T cells and other immune cells. PD-1 binding to its ligand programmed death-1 (PD-L1) (B7-H1, CD27) results in intracellular responses that reduce T-cell activation [3].

Upon recognition of tumor antigens, T effector cells or tumor-infiltrating lymphocytes (TILs) produce interferon-gamma ($\text{IFN-}\gamma$), which drives PD-L1 expression in the tumor cells. The consequences of the binding of PD-1 to PD-L1 are apoptosis and the exhaustion of activated immune cells [4], [5].

Aberrant PD-L1 expression observed on cancer cells led to the development of PD-1/PD-L1-directed cancer therapies, which have shown promising results in late-phase clinical trials. Blockade of the PD-1

and PD-L1 interaction led to good clinical responses in several, but not all cancer types, and the heterogeneous cellular expression of PD-1/PD-L1 may underlie these selective responses [6].

Immune modulatory antineoplastic agents demonstrated marked success in solid tumors including melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma, bladder cancer, and head-and-neck cancer [7], [8], [9].

Thus, those checkpoint inhibitors that target PD-1 and its ligand PD-L1 were approved by the Food and Drug administration for melanoma therapy in late 2014 and for NSCLC therapy March 2015 [10], [11].

Recent studies indicate that PD-L1 expression may identify patients who are more likely to benefit from immunotherapies. These agents and biomarkers could revolutionize the management of gynecological malignancies that have developed resistance to standard chemotherapies [12]. Pembrolizumab, an anti-PD-1 monoclonal antibody, was approved for the treatment of metastatic or unresectable metastatic endometrial cancers (ECs) [13].

Ongoing research is being conducted to identify which tumors may respond to PD-1/PD-L1 treatment. A research gap about the optimum method of assessing PD-L1 expression in various types and grades is questioned [14]. The issue is complicated by a lack of commonly accepted test methodologies for the assessment of PD-L1 status since a multitude of antibodies, staining protocols, readout methods, and cut-off definitions are being used in different studies [15]. Thereby, more research studies about Pd-L1 status in different patients should be done for better choice of selected candidates who can get benefit from immunotherapy [16].

Materials and Methods

This retrospective cross-sectional study comprised previously confirmed endometrial carcinoma patients who underwent either dilatation and curettage (D and C) or endometrial biopsy or hysterectomies by a convenience sample at Ain shams university hospitals pathology units including both El-Demerdash Pathology Unit and Early Cancer Detection Unit Since 2020.

- A convenience sample was chosen according to strict inclusion criteria conditioned mainly on patients who did not receive any type of immunotherapy with all their clinical data present. Cases with missing clinical data and cases that received neoadjuvant immunotherapy before hysterectomy were excluded from the study
- The study included 32 cases, the specimens of

which comprised (16) D and C, (4) endometrial biopsies, and (12) radical hysterectomies.

Statistical package was calculated using Gpower program, setting alpha error at 5% and power at 80%. Assuming an effect size of 0.9 (Cohen d) on the studied markers between the two types of ECs produced a sample size 16 cases per group (32 total).

- Data collection from patients' reports was performed, including age, type of tissue biopsy, gross findings and description, number of lymph nodes if present, the histopathological diagnosis and description and the subtype specification
- Extracting the H and E-stained slides of the chosen cases followed by selection of the appropriate formalin-fixed and paraffin-embedded (FFPE) blocks were done. Two sections were obtained from each block, one on a neutral slide for H and E staining and the other on a positively charged slide for PD-L1 IHC staining
- Examination of the newly stained hematoxylin and eosin slides and comparing the findings to the original reports and the already established previous histopathological diagnosis was done according to the WHO [10].

Ethical considerations

The study was conducted according to the stipulations of the Ain Shams University ethical and scientific committee. FWA 00017585.

Study tools

- Available clinicopathologic records of previously diagnosed endometrial carcinoma cases
- FFPE tissue blocks of endometrial carcinoma cases
- Hematoxylin and eosin slides of the selected cases
- Programmed death-ligand 1 receptor (PD-L1) immuno-histochemical marker.

Study procedures

- FFPE tumor tissue specimens of endometrial carcinoma in endometrial biopsies or hysterectomies received at Ain Shams University Pathology Lab and Early Cancer Detection Unit at Obstetric and Gynecology Ain shams University hospital were extracted and revised (according to the WHO)
- Revision of Hematoxylin and eosin slides of the selected cases
- Sections were obtained from those specimens and stained by PD-L1
- PD-L1 expression in endometrial carcinoma tumor cells and TILs was assessed separately.

This was done according to Zajac *et al.* using two cut-off values in assessing PD-L1 status [17].

- PDL1 expression in tumor cells and in TILs was correlated with all available clinicopathological data

Immunohistochemistry

For analysis of PD-L1 expression using IHC, sections were prepared from the paraffin blocks and treated them by PT link for antigen retrieval; then, monoclonal rabbit antibody was added against PD-L1 (clone MD21R) and HRP detection kit (Dako-Envision FLEX) using DAB as chromogen and hematoxylin as counter stain.

PD-L1s were assessed using semi-quantitative assessment of both positive tumor cells and positive TILs separately with two cut-off values 5% and 10% [17].

Placental tissue was used as positive control. IHC analysis was performed using the Discovery-Ultra immunostainer (Ventana Medical Systems, Tucson, AZ).

Finally, to detect the nuclei, the slides were counterstained with hematoxylin II reagent (Ventana Medical Systems) for 32 minutes, followed by a bluing reagent for 8 minutes.

The slides were then dehydrated, cleared, and mounted using routine processing.

Immunohistochemical analysis

The immunohistochemical preparations were assessed by three authors using a light microscope. Only cytoplasmic and membranous staining was considered as a positive reaction for PD L-1. Due to different staining properties, the assessment of the degree of immunohistochemical staining was made according to scoring scales based on the percentage of the stained tumor cells and TILs separately as described by Gozde *et al.* as follows: TPS (Tumor proportion score) = Number of positive PD-L1 TILs/ Number of viable tumor cells × 100 [18].

The quantitative evaluation of PDL-1 was categorized into positive and negative expression according to the follows: < or > 5% of both tumor cells and TILs and another evaluation with another cut off value < or >10% [17].

Statistical package

Statistical analysis using G power program with alpha error at 5% and power at 80% for calculation of sample size. Assuming an effect size of 0.9 (Cohen d) on the studied markers between the two types of ECs produced a sample size 16 cases per group (32 total).

Statistical analysis

Statistical analysis was done using SPSS program version 23. Appropriate descriptive and inferential statistical tests were used.

Quantitative data were presented as minimum, maximum, mean, and SD.

Qualitative data were presented as number and percentage.

Chi square test and Fisher exact test were used to compare qualitative data between different groups and $p \leq 0.05$ was considered statistically significant.

Results

This study incorporated 32 female patients in a cross-sectional study in 2 years in Ain shams university hospital laboratories with the diagnosis of endometrial carcinoma. The age range was 42 to 73 years with mean age of 60.91 years.

Half of the cases were in the form of D and C (50%), 31.3% had performed TAH and BSO (total abdominal hysterectomy and bilateral salpingo-oophorectomy), while only 6.3% had performed TAH alone, and 12.5% had performed diagnostic endometrial biopsy.

The gross features of the sampled endometrial masses included 83.9% that appeared as polypoid fragments, while 12.9% appeared as masses, and the rest showed rough irregular endometrium.

The signed out diagnoses among the sampled specimens were 64.5% diagnosed as endometrioid carcinomas, 25.8% serous carcinomas, 6.5% clear cell carcinoma, and 3.2% mucinous carcinoma.

Signed out grades of tumor among the sampled specimens included 31.3% grade III, 15.6% Grade II, and 53.1% Grade I.

Fifty percent of panhysterectomy cases showed myometrial invasion involving more than half the thickness of the endometrium, of which 18.7% showed positive serosal involvement, and 58.3% showed positive lymphovascular invasion, and 41.6% showed positive lymph nodes for metastases.

The distribution of PD- L1 expression in tumor cells with two cut off values (5%) and (10%) was as follows: 59.3% of cases showed positive expression in more than 5% of tumor cells, while only 53.1% crossed the 10% cut off value.

The distribution of PD- L1 expression in TILs with two cut-off values (5%) and (10%) was as follows: 37.5% of cases showed positive expression in more than 5% of TILs, while only 34.3% crossed the 10% cut-off value.

PDL-1-positive expression of both tumor cells and TILs was significantly more frequent in type II endometrial carcinoma ($p = 0.04$ and 0.03 , respectively) compared to Type I, using 10% cut-off value.

However, such relation was insignificant when using 5% cut-off value.

Grade III tumors showed significantly more frequent PDL-1 expression of tumor cells than grades I and II tumors, using 5% and 10% cut-off values. ($p = 0.03$ and 0.01 , respectively).

Moreover, positive PDL-1 expression of TILs significantly associated grade III tumors using 5% and 10% cut-off values ($p = 0.007$ and 0.02 , respectively).

The relation between PDL1 expression in tumor cells and TILs versus the extent of myometrial invasion, serosal involvement, lymphovascular invasion, and lymph node involvement showed a statistically insignificant difference when using both 5% and 10% cut-off value as shown in Table 1 and Figure 1.

Discussion

A series of studies have explored the prognostic value of programmed death-ligand 1 (PD-L1) in patients with endometrial carcinoma (EC) using different methods of assessment and different cut-off values, however, the results are controversial [19], [20], [21].

Of note, the wide range positivity across different studies can be attributed to the different study populations as well as the significant variability in PD-L1 assessment and sampling techniques, usage of polyclonal versus monoclonal antibodies, different antibody clones, and variable scoring systems with variable cut-off values [17], [22]. The use of a monoclonal antibody in our study aimed to achieve better reproducibility.

In this study, we semi-quantitatively assessed positivity of PD-L1 expression separately in tumor cells and TILs in different types of endometrial carcinoma using two cut off values 5% and 10% to highlight any significant difference in PDL-1 localization (in tumor cells and TILs) or between cut off values of 5% and 10%.

Wang *et al.* (2020) evaluated the expression of PD-L1 in carcinoma cells (Ca) and TILs across histopathologic and the Cancer Genome Atlas molecular subgroups of endometrial carcinoma [23]. Within various histotypes, non-endometrioid carcinomas displayed the highest PD-L1 expression in tumor cells and TILs. In concordance, our study demonstrated that PDL-1-positive expression of both tumor cells and TILs was significantly more frequent in Type II endometrial carcinoma compared to Type I, using 10% cut-off value. However, such relation was insignificant when using a 5% cut-off value, suggesting that a cut-off value of 10% might be more sensitive to segregate endometrial

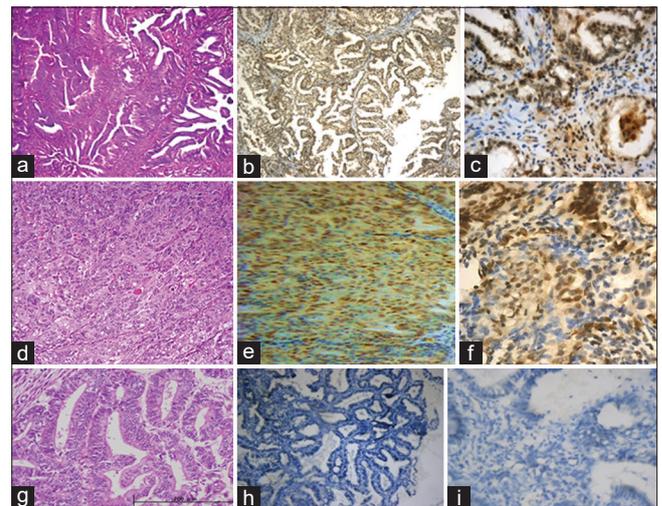


Figure 1: (a-c) Represent a case of serous carcinoma (H and E, $\times 100$) with positive PD-L1 staining in both tumor cells and TILs in PD-L1, $\times 100$ in b and (PD-L1, $\times 400$ in c). (d-f) Show high-grade endometrioid endometrial carcinoma (H and E, $\times 100$) with positive PD-L1 staining in both tumor cells and TILs in PD-L1, $\times 100$ in E and PD-L1, $\times 400$ in F. (g-i) Represent a low-grade endometrioid endometrial carcinoma (H and E, $\times 100$) with negative PD-L1 staining in both tumor cells and TILs in PD-L1, $\times 100$ in H and PD-L1, $\times 400$ in i

Table 1: Correlation of programmed death ligand 1 staining with clinical and pathological parameters

Correlated parameters	PD-L1 tumor cells with cut off value 5%		p	PD-L1 tumor cells with cut off value 10%		p	PD-L1 TILs cells with cut-off value 5%		p	PD-L1 TILs cells with cut off value 10%		p
	<5%	>5%		<10%	>10%		<5%	>5%		<10%	>10%	
Type												
Type 1	52.2	47.8	0.10 (NS)	60.9	39.1	0.04 (significant)	73.9	26.1	0.06 (NS)	78.3	21.7	0.03 (significant)
Type 2	12.5	87.5		12.5	87.5		37.5	62.5		37.5	62.5	
Grades												
Grade I and II	54.5	45.5	0.03 (significant)	63.6	36.4	0.01 (significant)	77.3	22.7	0.007 (significant)	72.7	27.3	0.02 (significant)
Grade III	0	100		10	90		20	80		20	80	
Extent of myometrial invasion (%)												
<50	50	50	0.2 (NS)	50	50	0.2 (NS)	75	25	1 (NS)	83.3	16.7	1 (NS)
>50	16.7	83.3		16.7	83.3		83.3	16.7		75	25	
Serosal involvement												
Positive	16.7	83.3	0.2 (NS)	16.7	83.3	0.2 (NS)	50	50	0.5 (NS)	50	50	0.5 (NS)
Negative	50	50		50	50		50	50		50	50	
Lymphovascular invasion												
Positive	28.6	71.4	0.6 (NS)	28.6	71.4	0.6 (NS)	57.1	42.9	0.6 (NS)	42.9	57.1	1 (NS)
Negative	40	60		40	60		60	40		60	40	
Lymph node involvement												
Positive	20	80	0.06 (NS)	20	80	0.07 (NS)	20	80	0.09 (NS)	40	60	0.6 (NS)
Negative	42.8	57.2		28.6	71.4		85.7	14.3		71.4	28.6	

PD-L1: Programmed death-ligand 1, NS: Not significant, TIL: Tumor-infiltrating lymphocytes.

carcinomas of aggressive subtypes, as type II, that could benefit from immunotherapy.

This is further supported by the frequent PD-L1 expression in serous carcinoma reported by Mo *et al.*, who claimed a potential role of the PD-1/PD-L1 pathway as a potential therapeutic target for these tumors [19]. Serous carcinomas frequently demonstrate P53 and E-cadherin mutation [2]. On the other hand, a significant correlation between P53 and PDL-1 was demonstrated in different tumors including lung carcinoma [24] and oral squamous cell carcinoma [25]. Moreover, Suda *et al.* reported that e-cadherin knockdown decreased PD-L1 expression in lung cancer cell lines. Taken all together, positive PDL-1 expression in type II carcinomas might be one of the mechanisms involved in the aggressive behavior of these endometrial carcinomas through a cross-talk between the above-mentioned genes and PDL-1 [26].

Bregar *et al.* reported significantly higher PD-L1 expression in high-grade tumors than low grade when utilizing two cut-off values $\geq 1\%$ and $\geq 5\%$ of total cells. Although we used higher cut-off values (5% and 10%), in agreement with their study, we still demonstrated that Grade III tumors showed significantly more frequent positive PDL-1 expression in both tumor cells and TILs than grades I and II tumors, using 5% and 10% cut-off values [21]. Moreover, increased expression was noted in the undifferentiated component of some tumors. Our results were also in line with Ling *et al.* and Khailfa *et al.* who found a significant association between PDL-1 and tumor grade [27], [28]. Moreover, Al-Hussaini *et al.* claimed that immunotherapy might be considered in the adjuvant setting of "undifferentiated endometrial carcinoma" which has poor response to traditional therapies [29]. Rationalizing this relation, according to a study performed by Sloan *et al.*, the majority of mismatch repair (MMR)-deficient endometrial carcinomas were PD-L1 positive (53%) while PD-L1 expression in TILs was seen in (100%) of MMR-deficient tumors [30]. On the other hand, MMR protein defects that lead to microsatellite instability (MSI) [31] were found to significantly associate high tumor grades [32], [33]. With impaired MMR and consequent increased mutation burden of tumor cells, a parallel augmentation of the number of neoantigens occurs. Thereby, such tumors become highly immunogenic and will probably retreat from immunotherapy [34]. Thus, MMR and MSI might be the linkers between PDL-1 and high tumor grade in endometrial carcinomas.

Nonetheless, immunotherapy (specifically PD-1 and/or CTLA-4 checkpoint blockade) has proved to be highly effective for the treatment of patients with advanced dMMR colorectal cancer [35]. Based on our data, regarding association of PDL-1 expression with aggressive endometrial carcinomas (serous carcinoma) and higher tumor grades, in addition to the above outlined previous studies, PDL-1 status combined with MMR and MSI status may be biomarkers predictive for response

to immunotherapy independent of tumor histology depending solely on genetic composition of tumors.

Regarding correlation with clinicopathological parameters, advanced cancers showed more frequent PD-L1 positivity compared with early disease [23]. In a meta-analysis performed to estimate the associations between PD-L1 expression and the prognosis as well as clinicopathological features of endometrial carcinoma although Ling *et al.* found that high expression of PD-L1 did not significantly correlate with overall survival, PD-L1 expression was significantly associated with advanced stage [27]. Nevertheless, Khailfa *et al.* demonstrated a significant association between positive PDL-1 expression and presence of lymph node metastasis and higher tumor stage [28]. However, in the present study, PDL-1 positivity in tumor cells and in TILs did not significantly correlate with myometrial invasion, serosal involvement, or lymph node involvement using both cut-off values 5% and 10%. Reasonable explanations for such contradiction may be tumor heterogeneity and complex interactions of tumor immune microenvironments [36]. These variables should be addressed using a larger number of cases to further elucidate such relation.

In the current study, since PDL-1 positivity in both tumor cells and TILs were significantly associated with the same variables, assessment of PDL-1 in either tumor cells or TILs may be used separately to reflect PDL-1 immunohistochemical status of endometrial carcinomas and in turn the biologic behavior as well as responsiveness of tumors to immune checkpoint inhibitors. TILs can boost PD-L1 expression in tumor cells in an IFN- γ -dependent manner whereas PD-L1 overexpression can, in turn, trigger immune tolerance of T-cells [37]. Similar results were proposed in colorectal adenocarcinomas where positive PD-L1 expression in tumor cells significantly associated CD8 or PD-1 overexpression of TILs in a subset of tumors suggesting that these tumors are appropriate targets for immunotherapy [36].

Conclusion

PD-L1 staining is significantly related to high-grade tumors and type II endometrial carcinomas, the aggressive types, which support their probable benefit from immunotherapy.

Both separate assessments of PD-L1-positive staining in tumor cells or TILs with a cut-off value 10% can significantly reflect the aggressiveness of the tumor and its probable benefit from immunotherapy.

More clinical trials are needed to observe the prognosis of patients receiving immunotherapy for PD-L1 positive cases

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