RESEARCH ARTICLE

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Immunohistochemical Expression of Programmed Death Ligand 1(PDL1) in Endometrial Carcinoma and Its Relation to CD4 and CD8 Positive Immune Cells

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Abstract

Objectives: Endometrial cancer (EC) is the most common cancer of the female genital tract. Egypt showed a significant increase in incidence lately of which 25% were premenopausal. Advanced or recurrent disease are mostly unresectable and the traditional adjuvant therapy give modest results with devastating side effects. Late discoveries of immune checkpoint inhibitors have produced promising results. Programmed cell death 1 (PD1) is an immune inhibiting receptor on surface of lymphocytes, which plays critical roles in maintaining immunological self-tolerance. There are two ligands for this receptor, PDL1 and PDL2. PD-L1 is expressed on tumor cells; attaches to PD1, allowing tumor cells to escape from the host immune response. Its prognostic significance in various tumors is controversial and its significance in ECs has just begun to be investigated. Therefore, we investigated the relationship between PDL1 expression and different clinicopathologic parameters in EC cases and its correlation with CD4 and CD8 immune cells, in order to identify the predictive biomarkers for the outcome by immune therapy. **Methods:** Hundred, paraffin tissue blocks of EC cases were collected and stained with antibodies against PDL1,CD4 and CD8. **Results:** PDL1 was positive in 67% of cases in tumor cells and in 61% of cases in immune cells. CD4 and CD8 were expressed in 79% of cases. Statistically significant correlations were observed between PDL1 expression and patients mean age, LVSI, TILS score and CD4+/CD8+ expression. **Conclusion:** Those variables can stratify candidates who can benefit most from immunotherapy, or can be chosen for further high cost molecular investigations application.

Keywords: PDL1- endometrial carcinoma- immune- CD4- CD8

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Introduction

Endometrial cancer (EC) is the most common cancer of the female genital tract (Sung et al., 2021), which as well shows racial disparities, with a higher prevalence among non-Hispanic black women compared to white women (Clarke et al., 2019).

Although Egypt has significantly lower incidence of EC than other Middle East countries, but it showed a significant increase in incidence over the past 12 years (Alshahrani et al., 2018) as it accounts for 31.4% of female genital tract malignancies according to Cancer Pathology Registry, Cairo university, National Cancer Institute (Mokhtar et al., 2016). Although EC mostly occurs in postmenopausal women, but, up to 25% of women at time of diagnosis were premenopausal (Santoro et al., 2021).

While early stage ECs are surgically curable, patients with advanced or recurrent cancer are mostly unresectable. Traditional chemotherapy and cytoreductive surgery only give modest results with devastating side effects. However, late discoveries of targetable pathways, molecular therapies like immune checkpoint inhibitors have produced promising results, which usually work better in combination, rather than as single therapies. Recently, immunotherapy proved to be the most effective therapy for advanced EC in the future, especially immune checkpoint inhibitors which showed propitious results in these cases (Toboni and Mutch, 2020).

Programmed cell death 1 (PD1) is an immune inhibiting receptor expressed on the surface of activated T and B cells, which plays crucial roles in maintaining immunological self-tolerance. There are two ligands for this receptor, programmed cell death ligands 1and 2 (PDL1 and PDL2). PD-L1 is expressed on both immune cells and tumor cells; which when attached to PD1, allows tumor cells to escape from the host antitumor immune response. In other words, PDL1 expressed on the surface of tumor cells bind to PD1 receptor on immune cells prompting adaptive immune resistance with resultant poor prognosis (Kooshkaki et al., 2020). Moreover, tumor microenvironment can be classified according to tumor infiltrating lymphocytes (TILs) and PDL1 expression into

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PD-L1+ TIL+ group which responds very well to immune checkpoint blockade (Zhan et al., 2020).

The prognostic significance of PDL1 in various tumors showed marked controversy. However lately, therapies targeting this immune checkpoint system showed excellent clinical responses in various kinds of tumors like non small cell lung carcinoma, melanoma, renal cell carcinoma and others (Liu et al., 2020).

But the significance of the PD1/PDL1 pathway has just begun to be explored in ECs together with few ongoing clinical trials and there are only few studies done on its prognostic importance in ECs up till now. Therefore, the purpose of this study is to investigate the relationship between PDL1 expression and different clinicopathologic factors in EC cases and its correlation with CD4 and CD8 immune cells, in order to spot the predictive biomarkers for the outcome using immune therapy, which sure provides significant implications on new therapeutic strategy for the tumor.

Materials and Methods

This study included 100 stored, formalin-fixed, paraffin-embedded tumor tissue blocks of EC cases (from total or subtotal hysterectomy, ± salpingo-oophorectomy specimens ± pelvic lymphadenectomy). The specimens were anonymous for confidentiality and replaced by numbers. The study was approved by the Medical Ethics Committee. The data collected from the pathology reports of the EC cases included age at time of diagnosis, extent of myometrial invasion, presence of tumor involvement of cervix, serosa, adnexa and parametrium, nodal metastasis (if available) and lymphovascular space involvement (LVSI). Cases who received neo-adjuvant therapy, or with missing essential clinical and pathological data or showing extensive necrosis were excluded from the study.

Staining procedures

Each paraffin block was re-cut by rotatory microtome at 4 μm thickness then mounted on a glass slide and stained by hematoxylin and eosin (HandE) for routine histopathological examination and on charged slides for immunohistochemistry (IHC). IHC was performed using an automated staining system (Dako autostainer link 48) with monoclonal rabbit antibodies against PD-L1 (clone RBT-PDL1, dilution 1:100; Bio SB, Santa Barbara, CA, USA), monoclonal mouse antibodies against CD4 (clone MT310, dilution 1:100; Bio SB, Santa Barbara, CA, USA), and monoclonal mouse antibodies against CD8 (clone 32-M4, dilution 1:100; Bio SB, Santa Barbara, CA, USA). Human tonsil was used as positive controls for all markers according to the manufacturer recommendations, with each run of IHC staining.

Histopathological examination

All EC cases were segregated based on

Histological classification according to the latest WHO recommendations (Kir et al., 2020). Histological grading according to FIGO grading system (Zhou et al., 2018).

Pathological staging according to FIGO staging system (FIGO Committee on Gynecologic Oncology, 2014) and the eighth edition (2017) of the American Joint Committee on Cancer's AJCC Cancer Staging Manual (Powéll et al., 2017).

Tumor infiltrating leucocytes (TILs)

All mononuclear cells were scored, excluding polymorphonuclear leukocytes, by determining the percent of combined stromal and tumoral TILs in area occupied by mononuclear inflammatory cells over total stromal or tumor area after counting 10 high power microscopic fields (HPFs). Tumors were then defined as High-TILs (≥30%) or Low-TILs (<30%) (Tomioka et al in 2018).

PDL1 IHC interpretation

PDL1 expression was defined as partial or complete membranous staining in viable tumor cells (TCs), and membranous and/or cytoplasmic staining in immune cells (ICs). We determined the percentage of positive TCs and ICs separately. Semiquantitative scoring was adopted as follows:

0: <1% of cells - 1: 1% to 4% - 2: 5% to 9% - 3: 10% to 49% - 4: ≥ 50%.

The cutoff for positive PDL1 staining was set at 1%. The cutoff for strong positivity was set at ≥ 50% for TCs and ≥ 10% for ICs. (Pasanen et al., 2020).

CD8 and CD4 IHC interpretation

The number of CD8+ and CD4+ TILs was counted in at least four different high-power fields for each specimen. Fields with the most abundant TILs in a specimen were selected for counting. The average number of positive cells of all specimens was used as the threshold level for determination of high or low CD4+ and CD8+ density for each specimen (Zhang et al., 2020).

Statistical analysis

The previously mentioned clinical, histopathological and immunohistochemical data were entered on Microsoft excel 2013 and then transferred to the Statistical Package of Social Science (SPSS) Software program, version 25 to be statistically analyzed. Numerical data were checked for normality and were statistically described in terms of mean (±standard deviation) or median (range) as appropriate. Categorical data were described as numbers and percentages. Comparison between 2 numerical variables was done using Student t-test if normally distributed and Mann-Whitney U test if not normally. Comparison between more than 2 variables was done using Analysis of variance (ANOVA) with Bonferroni adjustment if normally distributed and Kruskal Wallis test if not normally distributed. Spearman correlation coefficient was calculated for EH- PDL1 scoring and PDL1 score in TCs. When comparing categorical data, Chi square test or Fisher's exact test were performed as appropriate. Logistic regression analysis was used with Forward LR variable selection method and included all significant variables on the univariate analyses. Odds ratios with 95% confidence interval were calculated for the significant variables in the finalstep of the logistic regression. P-value is always 2 tailed and set significant at <0.05.

All slides were screened using a Leica DM500 microscope. Microscopic photos were captured using Leica EC4 camera using The Leica LAS EZ software.

Results

Demographic data

This preliminary study was done on 100 cases of EC. The age of the studied cases ranged from 34 up to 84 years with mean 60.9±9.1. According to the histological subtyping, endometrioid EC showed the major predominance (79%) while the rarest was the dedifferentiated type. As for histological grading, ECs grades I and II grouped as low grade constituted 79% of cases while the rest of cases were high grade. The majority of cases were FIGO stage I (69%) followed by stage III then II, only one endometrioid EC case was FIGO stage IV. Ninety five cases showed myometrial invasion with variable depths. Cervical involvement was seen in 19 cases. Lymph node metastasis could be assessed in only 33 out of the 100 studied cases of which 18 cases were positive for metastasis (5.9%), however 29% of studied cases showed lymphovascular invasion. Regarding TILS score, absent TILS was seen in 21 cases only while the rest of cases showed appreciable TILS with variable percentages. TILS score <30% was seen in 41 cases while TILS score ≥30% was seen in 38 cases.

Immunohistochemical expression

PDL1 expression was positively stained in tumor cells of 67% of cases mostly showing strong positivity (figure 1). Likewise PDL1 expression was positively stained in TIL cells of 61% of cases mostly showing strong positivity (Figure 2).

CD4+ as well as CD8+ immunoexpression were noticed in 79 cases (79%). CD4+ as well as CD8+ high density were seen in 80% of positive cases (figures 3 and 4 respectively).

Correlations

Cases showing positive PDL1 expression in either TC or IC showed a higher mean age compared to those showing negative expression. This correlation was statistically significant (p 0.006). The majority of cases showing positive PDL1 expression in either TC or IC also showed LVSI (79.3%), while the majority of cases lacking PDL1 expression also lacked LVSI. The correlation between PDL1 expression and LVSI was statistically significant (p 0.016).

Over 70% of cases showing combined TILS high and TILS low score were associated with positive PDL1 expression in both TC as well as IC, moreover, the majority of cases showing absent TILs also showed negative PDL1 expression in both TC as well as IC. The correlation between PDL1 expression and TILs score was

Table 1. relation between PDL1 Expression on One Hand and CD4+ as well as CD8+ Expression on the Other Hand

PDL1 expression	CD4+	CD8+		P value
	Negative	Low density	High density	<0.001
Negative	21 (100%)	0	0	
Positive	0	16 (20%)	63 (80%)	
Total	100			

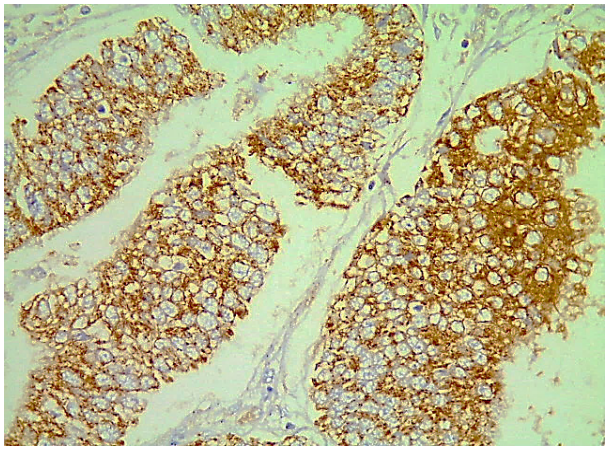

Figure 1. A Case of Endometrial Carcinoma Showing Strong Positive PDL-1 Immunohistochemical Staining in Tumor Cells (PDL-1 x 200 original power).

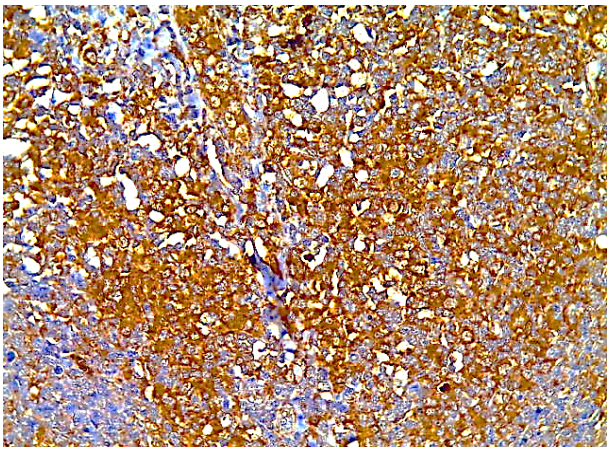

Figure 2. A Case of Endometrial Carcinoma Showing Strong Positive PDL-1 Immunohistochemical Staining in Tumor Infiltrating Immune Cells (PDL-1 x 200 original power).

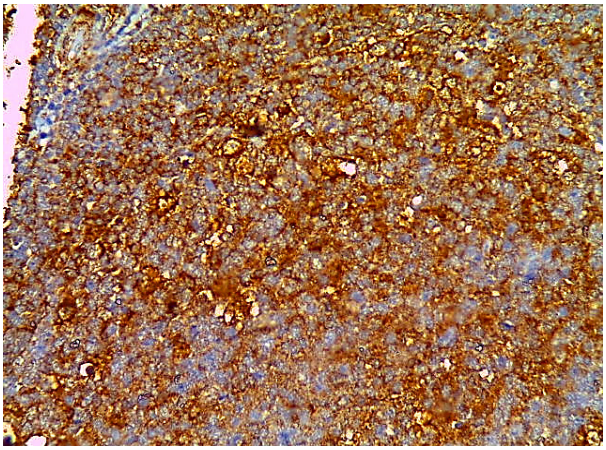

Figure 3. A Case of Endometrial Carcinoma Showing High Density Positive CD-4 Immunohistochemical Staining in Tumor Infiltrating Immune Cells (CD-4 x 200 original power).

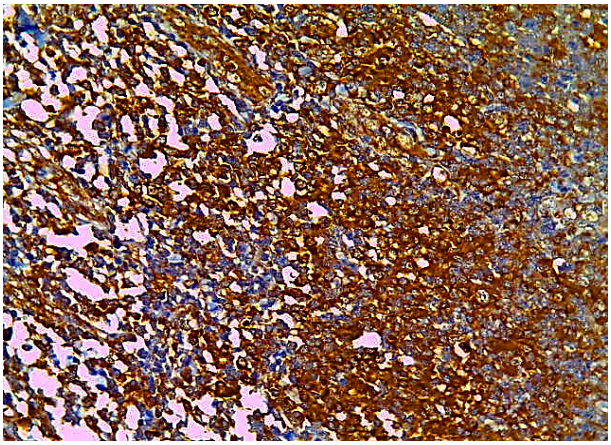

Figure 4. A Case of Endometrial Carcinoma Showing High Density Positive CD-8 Immunohistochemical Staining in Tumor Infiltrating Immune Cells (CD-8 x 200 original power).

statistically significant when studying TC-PDL1 (p 0.005) and highly significant when studying IC-PDL1 (p <0.001).

A highly significant correlation was seen between PDL1 expression on one hand and CD4+ as well as CD8+ on the other hand as shown in Table 1.

The rest of data showed insignificant correlations with PDL1 expression such as histologic subtypes, histologic grades, FIGO stage, myometrial invasion, cervical involvement as well as nodal metastasis.

Discussion

EC is a rising problem in Egypt, although Egypt still shows significantly lower incidence of EC than other Middle East countries, but the incidence has been increasing lately with a remarkable number of women diagnosed in the premenopausal age (Alshahrani et al., 2018). The integration of molecular study with usual histopathological study is highly demanded by the oncologist to amplify the diagnosis of EC and to achieve better targeted treatment and prognosis. But, unfortunately the health system in the developing countries doesn't support these high cost tests, making immunohistochemical studies the best ancillary techniques so far.

Cancer immunotherapy using PD-L1 monoclonal antibodies significantly improved survival of patients, and proved to be more advantageous than conventional therapies treating advanced and metastatic cancers especially those with high PD-L1 expression (Sun et al., 2020).

Our current study provides data about PD-L1 expression in ECs from Middle Eastern ethnicity (Egyptian patients) which is limited. It also highlights the most important and highly indicative statistically significant factors that correlate with PD-L1 expression in every case, which can suggest the case that could be a strong candidate for immunotherapy, meeting the cost saving strategy adopted by the developing countries governments.

Our study included 100 stored, formalin-fixed, paraffin-embedded tumor tissue blocks of EC cases. Positive TCs PD-L1 expression was reported in 67 cases (67%), with strong positivity in 46% of cases. Close to our results were those observed by Al-Hussaini et al in 2018 (66.7%) and Ono et al in 2019 (53%). On the contrary, the study done by Siraj et al in 2021 showed a marked lower results than ours, although it was done on Middle Eastern population like ours. This could be due to usage of tissue microarray. Other lower results were obtained by Pasanen et al., 2020 (8.6%), Kir et al., 2020 (10.2%), Zong et al., 2021 (14%), Siraj et al., 2021 (18.9%) and Bregar et al., 2017 (44%). This difference between various studies can be explained by the different clones of the antibodies applied, the use of full-face sections versus tissue micro-arrays (TMA) which may not represent the whole tumor spectrum, resulting in false negativity and the composition of the studied population itself in addition to the difference between the observers which may serve as a further factor. In the current study, the relatively high rate of TCs PD-L1 expression can be attributed to the use of full-face sections covering the heterogeneous nature of PDL1 expression, inclusion of a wide variety of EC subtypes in addition to the low cut-off of positivity (1%) applied.

Concerning the ICs PD-L1 expression, it was positive in 61 cases (61%) of our cases, with the majority of cases (49%) showing strong PD-L1 expression. Likewise, Kir et al., 2020 observed ICs PD-L1 expression in 67.8% of cases. Nevertheless, the studies done by Pasanen et al., 2020 and Zong et al., 2021 were significantly lower than ours (27.7% and 37.3% respectively).

The rate of ICs PD-L1 expression in our study (61%) was slightly lower than TCs PD-L1 expression (67%), in agreement with the studies done by kir et al., 2020 and Zong et al., 2021. However, Pasanen in 2020 found that PD-L1 expression was higher in immune cells than in tumor cells, in contrast to our results. This can be explained by the bigger study sample (842 patients) and the use of TMA.

In the 100 EC cases of our study, 19 cases showed nearby areas of EH. Positive PD-L1 expression was observed in 14 cases of them (73.7%), all showing atypia. While the remaining 5 (26.3%) that were PD-L1 negative showed no atypia. These results were statistically significant, (P<0.01). This was consistent with Antomarchi et al study in 2019 that found unchanged PD-L1 gene expression by (real-time PCR) in the hyperplasia without atypia group, while it was significantly increased in all tumor groups. Also, Chew et al in 2020 found all 32 non-neoplastic endometrial samples were PD-L1 negative, while the 59 ECs showed PD-L1 positivity in 72.7% of ICs and 28.8% of TCs, with statistically significant correlations. The positive PD-L1 expression in our EH cases was only linked to the presence of atypia which is the precursor for EC, emphasizing the role of this marker in tumor progression. In contrast to our study, Liu et al study in 2015 detected positive PD-L1 expression in 78% of normal endometrium samples, while it was upregulated in tumor samples showing positivity in 83% of 1ry EC, 68% in recurrent EC, and 100% in metastatic EC, with significant correlation (P<0.01).

In our study, cases showing positive TCs and ICs PD-L1 expressions showed a slightly higher mean of age (62.9) compared to those with negative PD-L1 expression (57.7), with statistically significant correlation (P= 0.006) supporting the fact that age is an important independent prognostic factor in ECs. In agreement with our study were those done by Sungu et al 2019 and Chew et al in 2020. Contradictory to our results were those observed by Mo et al in 2016 and Siraj et al study in 2021 who found insignificant association with age.

In our study, both TCs and ICs PD-L1 expressions were associated with LVSI achieving a statistically significant correlation (P = 0.016). Same observations were stated by Li et al in 2018 and Zong et al in 2021 and Crumley et al study in 2019. On the other hand, Mo et al in 2016 and Tawadros and Khalafalla in 2018 found no significant relation between LVSI and PD-L1 expressions.

Concerning TILs scores in our studied cases, 38% were TILs-high and 41% were TILs-low. We observed statistically significant correlation between PD-L1 expression and TILs score. Similar results were stated by Pasanen et al in 2020, who found that ECs with abundant T-cell density had TCs and ICs PD-L1 positivity more frequently than ECs with scarce T-cells, with statistically significant correlation.

CD+4 and CD+8 expression in our work showed a highly significant positive correlation with PDL1 expression. Going with our results, Zhang et al., 2020 concluded high PD-L1 expression in TICs associated with high density of CD8+ TICs, suggesting that PD-L1-induced adaptive immune resistance may involve killer T cells, as CD8 is marker for killer T cells. Likewise study done by Khalifa et al., 2021, observed significant correlation between PDL-1 expression and TILs CD4 and CD8 expression.

The previously discussed variables (age, LVSI and TILs score and CD+4 and CD+8 expression) were the only variables that showed statistically significant correlation with PD-L1 expression in both TC and IC in our research. The rest of variables revealed statistically insignificant correlations.

To sum up, both TCs and ICs PD-L1 expressions in our study achieved significant correlations with patient's age, LVSI, TILs score as well as CD8+4 and CD+8 immunoexpression. Those variables can stratify candidates who can benefit most from immunotherapy, or can be chosen for further high cost molecular investigations application. Unfortunately our study has limitations in the form of its modest size and lack of patient's follow up to detect the outcome, particularly occurrence of recurrence and survival, in addition to the lack of correlation with EC molecular subtyping due to fund limits.

Author Contribution Statement

The authors confirm contribution to the paper as follows: study conception and design: Salama M. and Khairy D., data collection: Salama M., analysis and interpretation of results: Salama M. and Khairy D., draft manuscript preparation: Salama M. All authors reviewed the results and approved the final version of the manuscript..

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