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PD-L1 immunohistochemical expression in ovarian serous carcinoma: Its relation to tumor grade, TILs, clinico-pathological prognostic parameters and patient's survival

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ABSTRACT

Background: The most common reason for cancer-related mortality in women is ovarian cancer. Among all histological types, the serous carcinoma is the commonest. Immunotherapy has developed to be used in treating different cancers; among which Immune Checkpoint Inhibitors (ICIs) demonstrated great role in such cases. One of the significant challenges in cancer treatment is the selection of studied cases who will benefit from ICIs. It is established that ICI sensitivity in malignancies can be predicted using Programmed Cell Death Ligand 1 (PD-L1) expression. Prior research has confirmed a correlation among PD-L1 expression in ovarian tumours and Tumour Infiltrating Lymphocytes (TILs). However, regrettably, the Food and Drug Administration (FDA) has not yet approved use of anti-programmed cell death ligand 1 (anti-PD-L1) in the treatment of ovarian cancer.

Aim and Scope: This study assesses an immunohistochemical expression of PD-L1 in ovarian serous carcinoma to testify its usage as a prognostic and predictive marker. We also investigate its association with tumor grade, TILs, clinico-pathological prognostic factors, and studied case's survival.

Materials and Method: This work is retrospective research performed upon 100 cases of surgically resected ovarian serous carcinomas. Immunohistochemistry (IHC) for PD-L1 was applied to such biopsies then assessed and scored. TILs were also assessed using Haematoxylin and Eosin-stained slides. Finally, the outcomes were tabulated for statistical analysis.

Results: In this research, elevated PD-L1 expression had been significantly related to aggressive features of tumor including older age group, larger tumor size, higher tumor grade, advanced International Federation of Obstetrics and Gynecology (FIGO) stage and shorter patients' mean survival time. On the other hand, tumors with positive TILs were significantly associated with younger age group, smaller tumor size, lower tumor grade, early FIGO stage and longer mean survival time. Consistently, PD-L1 overexpression show statistically significant association with tumors showing negative TILs ($p \leq 0.001$).

Conclusion: We observed PD-L1 overexpression in aggressive features of ovarian serous carcinomas suggesting its poor prognostic and predictive role in such cases that require further studies

Keywords: ovarian serous carcinoma, immunotherapy, Immune Checkpoint Inhibitors (ICIs), Programmed Cell Death Ligand 1 (PD-L1), Tumour Infiltrating Lymphocytes (TILs)

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INTRODUCTION

The eighth most frequent cancer in the world is Ovarian Cancer (OC) and among gynecologic malignancies, the most frequent reason for cancer-related mortality [1-3]. About 1 females in 70 females are affected, and only 45% of them survive for five years after being diagnosed. The Global Cancer Observatory 2020 expects 36% increase in OC incidence by 2040 [4, 5].

The Epithelial Ovarian Cancers (EOC) represent more than 90% of all OC and among these epithelial cancers, the serous carcinoma has been most common histological type; from which the advanced high-grade serous carcinoma has been most lethal disease [2, 3, 6-11] .

The EOCs are complex and therapeutically challenging malignancies that are usually diagnosed late with most patients have advanced stage associated with extensive peritoneal spread and relapse, thus showing poor prognosis [3, 12]. This delayed diagnosis is related to that most cases have vague non-specific symptoms or no symptoms at all in early stages in addition to lack of screening programs and specific diagnostic markers [2, 5, 13, 14].

Until now, the well-known standard therapy for EOC has been optimal surgical debulking followed by platinum-based chemotherapy and although the initial response to the already used chemotherapy, later, most patients develop chemotherapy toxicity, drug resistance (that may be due to cancer heterogeneity), tumor recurrence/relapse, extensive malignant ascites, cancer metastasis, high mortality and lowered five-year survival rate with worsening of the prognosis in such cases [1, 3-5, 8, 11, 12, 14]. Thus, we are in deep need to develop new therapeutic strategies for these patients [1, 12].

Immunotherapies, particularly immune check point inhibitors, are from these novel therapeutic applications that are thought to be useful in treatment of patients with OC as they aim to strengthen our adaptive immune system [3, 4]. They are used successfully in different malignancies containing malignant melanoma, non-small cell lung carcinoma, Hepatocellular Carcinoma (HCC) and Renal Cell Carcinoma (RCC) [1]. But against our expectations, their response rate in OC is only 4%-15% especially in high grade ovarian serous carcinoma and up till now, no current Food and Drug Administration (FDA) or Europian Medicines Agency approval was obtained [3, 4].

However, there is hope for increasing the response rate in OC

via adequate patient selection and the usage of combination chemotherapy and immunotherapy. Thus, recognition of some prognostic and predictive immunological biomarkers is mandatory to recognize such studied cases who will benefit from these new treatment modalities [8].

One of the new promising ICIs that has emerged recently is that targets programmed cell death protein1 or its ligand; programmed death ligand-1 whose higher expression in certain tumors has been associated with chemoresistance [15].

PD-1 has been an immune inhibitory receptor that belongs to the CD28 family and has been expressed mainly by activated T-lymphocytes. It has 2 ligands; PD-L1 and PD-L2 [7, 16].

PD-L1 has been a surface trans-membrane glycoprotein; encoded by CD 274 gene on chromosome 9 and belongs to β2/CD28 co-stimulatory factor family. It has been expressed by normal immune cells particularly macrophages, T cells, B cells, dendritic cells, and mast cells in addition to some hematopoietic cells [2, 3].

Also, PD-L1 has been expressed in various tumor cells as malignant melanoma, glioblastoma, Non-Small Cell Lung Cancer (NSCLC), RCC, head and neck cancers, esophageal, gastric, colonic, pancreatic, breast, cervical and ovarian carcinomas [2, 3, 17].

The physiological interaction between PD-1 and PD-L1 inactivates T cells causing inhibition of Tcells cytotoxic properties by inducing T cell anergy, thus, controlling the autoimmunity and preventing marked tissue injury but in tumor microenvironment, when PD-L1 has been engaged by tumor cells, binding of PD-1 to its ligand PD-L1 causing T cell function inhibition by inducing T cell apoptosis, enabling the tumor to escape the adaptive anti-tumoral immune response allowing tumor growth and progression. Thus, tumors that express PD-L1 has been related to poor prognosis [3, 5, 7, 13, 16-22].

Therefore, blocking PD-L1 and/or PD-1 may become an effective therapy in many malignancies, as the reverse of the above mechanism will occur via restoring the immunological reaction thus stopping cancer progression and furthermore, eliminating the cancer cells and may improve patient's outcome. So, patients with different malignancies treated by antibodies against PD-1/PD-L1 showed longterm survival [1-3, 8, 16, 19].

From this point of view, tumors that have higher PD-L1 expression give more response rate to such therapies. But this is not constant in all tumor types as some cases with little or no PD-L1 expression can respond to anti-PD-L1 antibodies [16, 19].

Ovarian carcinoma cells may activate PD-1/PD-L1 mechanism making the tumor more aggressive with therapy resistance, higher risk for mortality and worse prognosis [2, 7, 13]. Recently, it is found that high plasma levels of PD-L1 were demonstrated in studied cases with ovarian carcinoma compared to healthy females suggesting its important role as an immunotherapy biomarker [2]. So that it is recommended to identify PD-L1 expression in OC before the use of immunotherapy [23].

Only a minority of OC patients gets benefits from immunotherapy and is not long-lasting effect [16]. This unsatisfactory response rate can be because of low tumoral PD-L1 expression, low mutational burden; as OC cell harbor low neo-antigen load, and high Tumor Infiltrating Lymphocytes (TILs) of OC [4]. Thus, further studies to recognize the patients who can benefit from this treatment are recommended [4, 5, 11, 21].

In OCs, there are controversial findings regarding the association among the PD-L1 expression and different clinico-pathological characteristics and with patients' survival [5]. On the other hand, there is a thought that in ovarian serous carcinoma, detection of PD-1/PD-L1 expression is essential for their molecular classification and it has been known that the PD-L1 expression level has been related to favorable patients' survival and prognosis [3].

Ovarian carcinomas are usually associated with TILs; the degree of which is strongly related to improved survival as TILs act by decreasing tumor growth and depending on their density, OC were classified as hot or cold tumors [2, 4, 13].

PD-L1 is related to an immunological milieu that is high in T cells. "Hot" tumours have been reported to have this immunological milieu, in contrast to "cold" tumours that have no immune cells detected [20]. It is suggested that PD-L1 expression in ovarian carcinoma cells may be induced by cytokines released from T-lymphocytes in the surrounding stroma; thus, high PD-L1 expression on ovarian carcinoma cells could be related to TILs [13].

To develop effective therapies and to validate new prognostic and predictive biomarkers in OC, we should improve our understanding about the tumor microenvironment that may be attributable to treatment resistance [4, 11, 13, 16].

From the previous data and the confused information about the prognostic role of PD-L1, the aim of the study has been to evaluate the expression of PDL-1 in both low and high grades serous ovarian carcinoma; being the most common and the most fatal type of epithelial ovarian cancer, using Immunohistochemistry (IHC) technique to testify its possible use as a prognostic and predictive marker and investigates the association between this marker and the Tumor Infiltrating Lymphocytes (TILs), different clinico-pathological prognostic parameters in addition to its relation to patients' survival.

MATERIALS AND METHODS

Patients, clinical and histopathological classifications

Formalin-fixed paraffin-embedded tissue blocks from 100 studied cases' biopsied ovarian serous carcinomas were used in this retrospective investigation. Between January 2016 and August 2020, biopsies were referred to the Pathology Department Laboratory at the same institution from the Department of Surgery and Medical Oncology, Oncology Centre at the Faculty of Medicine, Mansoura University, Egypt (OCMU).

The patient's demographic and clinico-pathological data of the included cases had been obtained from our institute database for the studied cases' medical records retrospectively as regard patients' age, marital status, parity, presence or absence of ascites, serum Cancer Antigen (CA)-125 level, tumor site and size.

Hematoxylin Eosin (Hand-E)-stained histopathological sections had been prepared and reviewed to confirm the diagnosis of the tumors based on histopathological (Hand-E) and immunohistochemical examinations (antibodies for pan-CK,

CK7, CK20, vimentin, WT1, P53, CEA, P16 and Napsin) for all biopsies. Tumor histological subtyping and grading were also performed according to the 5th edition of World Health Organization (WHO) classification of female genital tumors [24]. Staging of all tumors had been evaluated according to the Tumor Node Metastasis (TNM) system regarding the AJCC (American Joint Committee on Cancer) 6th edition classification and the International Federation of Gynecology and Obstetrics classifications [25].

All studied cases had been followed up periodically after the surgery every 3 months for tumor recurrence and patient's survival considering the date of the surgery as the start point for follow up and the end point had been defined as three years and the survival time had been defined as 36 months for those who survived more than 3 years. Disease recurrence/relapse was confirmed by local reappearance of the tumor at the same site or at metastatic site either radiologically or histopathologically.

The overall survival, which was defined as the period from surgical resection to death or the end of follow-up, up to a maximum of 3 years, was the main outcome of this study. Recurrence/disease free survival, which is the interval between surgery and the occurrence of a local recurrence, metastatic disease, or death, was the secondary end point [22, 26]. Progression free survival had been calculated from the date of surgery for those cases underwent primary cytoreduction to the date of disease-free progression or death from any cause [27].

Platinum-based chemotherapies were administered to all cases after initial surgical resection. None of the patients received ICIs treatment. None of the included cases received pre-operative neo-adjuvant chemoradiotherapy.

Selection of the included cases had been based on the availability of the paraffin blocks, where there had been enough tissue in the paraffin blocks for further IHC research and availability of full clinical data.

Where as the exclusion criteria included cases with secondary, non-epithelial, border line, or non-serous ovarian tumors, inappropriate paraffin blocks, patients who died from events not related to ovarian serous carcinoma or lost follow-up, and patients received chemo or radiotherapy due to other diseases.

Immunohistochemistry (IHC)

IHC was performed for the 100 biopsies that were included in the study in accordance with the manufacturer's datasheet on paraffin sections that were about four μm thick on heat-fixed, positively charged slides. The next steps were carried out in a microwave: deparaffinization, rehydration, and epitope exposure using 0.01 M citrate buffer (pH 6.0) for ten minutes. An incubation of 3% hydrogen peroxide for ten minutes inhibited the activity of endogenous peroxidase. To prepare for immunohistochemical staining of formalin-fixed, paraffin-embedded tissues, all sections had been rinsed with phosphate buffer saline and incubated for 1 hour at room temperature with the primary antibodies directed against monoclonal rabbit antibody PD-L1(ZR3) (Cell Marque antibodies/Sigma-Aldrich, code: 438R-28, made in USA EMERGO EUROPE, the Netherlands). The standard avidin-biotin-peroxidase method was used, covering the results with coverslips after hematoxylin counterstaining for 30 seconds and Diaminobenzidine (DAB) for visualization after five minutes of incubation.

Appropriate negative controls were prepared consisting of histologic sections processed without the addition of primary antibody and we use the normal ovarian tissue as internal negative control [14]. In addition, an external positive control sections were prepared from tonsillar tissue (where strong membranous positivity had been observed in crypt epithelium and weak to moderate membranous staining in follicular macrophages). Also, the peritumoral inflammatory cells (including lymphocytes and macrophages) had been considered as internal positive control [22].

Evaluation of IHC reaction

IHC reaction was examined by the pathologist (who was blinded to the studied cases' clinical data) with a light microscope (Olympus, Tokyo, Japan). In all IHC analyses, areas of fibrosis, adipose tissue, necrotic areas, and edges of tissue sections had not been involved in the counting as to avoid possible false positivity. The PD-L1 expression in intra-luminal contents and the positive staining of immune cells for PD-L1 were excluded from the scoring [22].

PD-L1 has been expressed in the cytoplasm and cell membrane of tumor cells [7, 13, 14].

PD-L1 expression was defined as positive when equal or more than 1% of tumor cells within the whole tumor area stained positive for PD-L1 and those less than 1% were considered negative [5, 22].

A semi-quantitative evaluation of the average proportion of PD-L1 positively stained cells relative to the total number of tumour cells had been carried out using "hot-spot" analysis on a light microscope. The hot-spots were identified by first scanning the entire section at a low magnification (× 40), then using a light microscope set at × 200 magnification to analyze 3 chosen fields that had the highest index. The only staining that was deemed positive were cytoplasmic and membrane. If counterstaining is done appropriately, staining intensity is irrelevant. The PD-L1 score was divided into low (<10%) and high (≥ 10%) categories for statistical analysis (Table 1).

Tab. 1. PD-L1 immunoexpression had been assessed according to the % age of the positive cells and scored into four categories as follows [13, 14]

Score	Expression	Description
Score 0	Negative	No positive cells or with a single positive cell (<1%)
Score +1	Low	1% to 10% positive cells
Score +2	Moderate	10% to 50% positive cells
Score +3	Strong	More than 50% positive cells

Tumors with moderate (+2) and strong (+3) expressions had been considered as high PD-L1 expression [13, 14].

Evaluation of Tumor Infiltrating Lymphocytes (TILs)

Regarding the lymphocytic infiltrate had been analyzed at whole

slides. The presence of stromal lymphocytic infiltrate had been estimated on microscopic magnification × 100 on HandE-stained tissue samples and categorized into 2 groups [13]:

- Prominent lymphocytic infiltrate had been considered as TILs positive.
- Absent or rarely lymphocytic infiltrate which was considered as TILs negative.

All mononuclear inflammatory cells had been evaluated containing lymphocytes, macrophages and plasma cells. However, polymorph neutrophils leukocytes were not included. TILs outside the tumor borders, crushed artifacts and in areas of necrosis were excluded from scoring [21].

Ethical standards

Archive material from paraffin tissue blocks stored in the pathology laboratory provided the material for the investigation. The Institutional Research Board (IRB), code R.22.08.1780) at Mansoura University's Faculty of Medicine has approved the task proposal. Throughout the trial, patient confidentiality was protected by utilizing their code numbers rather than their names. Every study technique followed the most recent version of the Helsinki Declaration on the use of human beings in research.

Statistical analysis

IBM Co., Armonk, NY, USA used Statistical Package for the Social Sciences (SPSS) version 28 for statistical analysis. The Mann Whitney test had been used to assess the quantitative data, which were displayed as the median and interquartile range. The Chi-square test had been used to analyze the frequency and proportion of categorical data. To compare the survival distributions of the groups, the Kaplan-Meier curve and Log-rank test had been used to estimate the survival analysis. A P-value with two tails less than 0.05 had been deemed statistically significant.

RESULTS

Demographic and clinicopathological characteristics of all 100 studied cases

Papillary serous ovarian carcinoma biopsies of 100 patients had been contained in this research. The median age of the studied cases had been 65 years as 52 patients had been in the (≥ 65 years) age group, the vast majority (94 women) was married and 83 women were parous. More than two thirds of patients (67 patients) suffered from ascites. The median CA-125 level was 345 U/mL with IQR between 70 U/mL and 847 U/mL.

Concerning tumor characteristics, the median size was 12 cm (≥ 8 cm in 67 cases). As for tumor site, 35 cases, 32 cases and 33 cases were in the right, left side and bilaterally, respectively. Sixty cases were low grade and FIGO stage IV was the most frequently detected one among studied 42 cases. Regarding PD-L1 expression, 34 cases had negative expression, 16 cases had less than 10% positive cells indicating low (+1) expression, 11 had 10% to 50% positive cells indicating moderate (+2) expression while 39 showed more than 50% positive cells indicating strong (+3) expression (Figures 3-6). Regarding Tumor-Infiltrating Lymphocytes, more than half of the included 53 cases were positive (Table 2 and Figures 1-8).

Tab. 2. Demographic and clinicopathological characteristics of studied 100 cases

		Total patients (n=100)	
		N	%
Age (years)	<65	48	48
	≥ 65	52	52
	Median (IQR)	65 (50-70)	
Marital status	Single	5	5
	Married	94	94
	Widow	1	1
Parity		83	83
Ascites		67	67
CA-125 (U/mL)	Median (IQR)	345 (70-847)	
Size (cm)	<8	33	33
	≥ 8	67	67
	Median (IQR)	12 (5-19.23)	
Site	Right	35	35
	Left	32	32
	Bilateral	33	33
Grade	Low	60	60
	High	40	40
Stage	I	16	16
	II	18	18
	III	24	24
	IV	42	42

PD-L1 expression	**0**	34	34
	1	16	16
	2	11	11
	3	39	39
TILs	**Negative**	47	47
	Positive	53	53

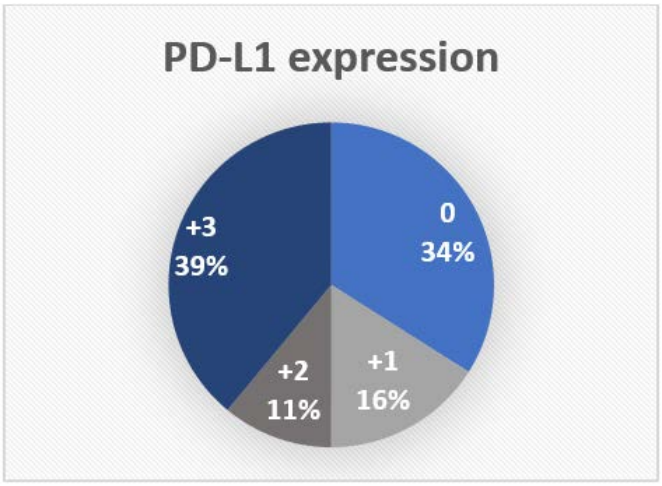

Fig. 1. Distribution of the studied cases according to PD-L1 expression

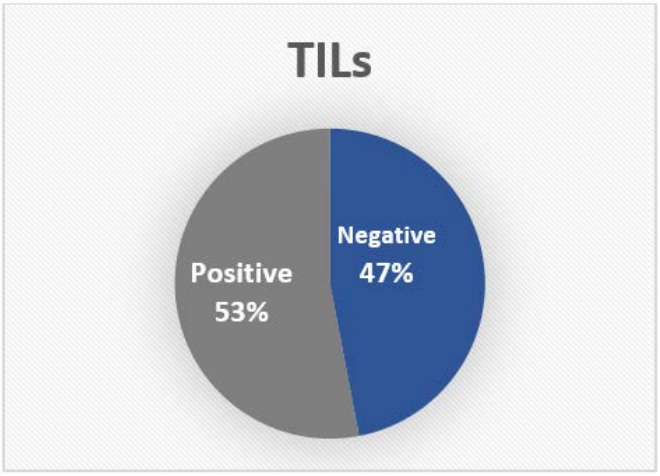

Fig. 2. Distribution of the studied cases according to the presence of TILs

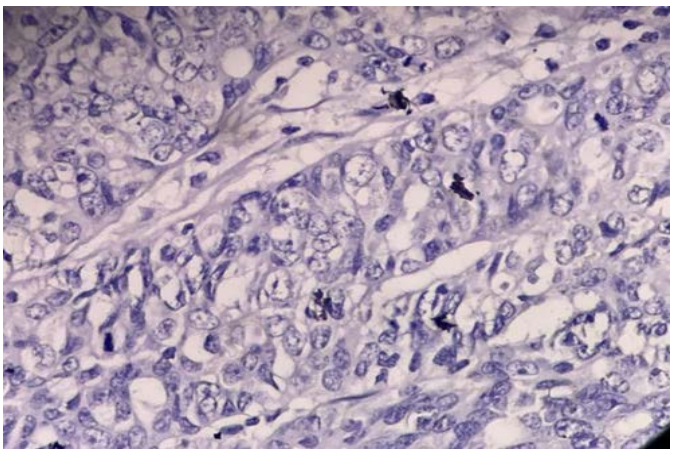

Fig. 3. IHC-stained sections revealed negative staining for PD-L1 (score 0; negative expression) (× 400)

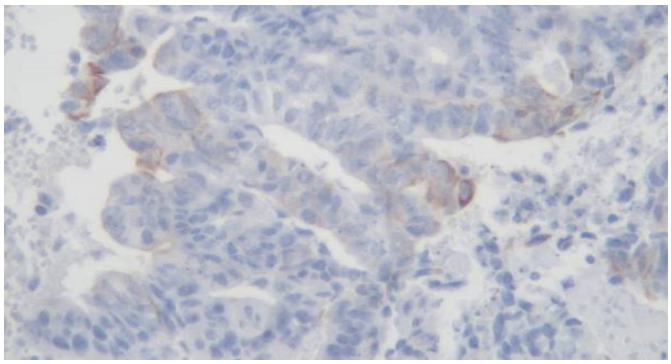

Fig. 4. IHC-stained sections revealed positive staining for PD-L1 in 1%-10% of tumor cells (score 1; low expression) (× 400)

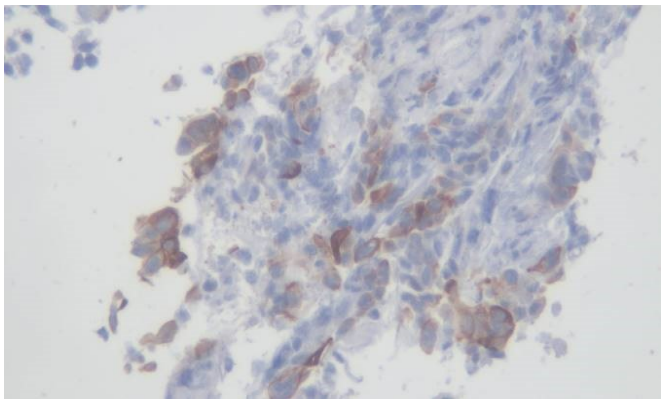

Fig. 5. IHC-stained sections revealed positive staining for PD-L1 in 10%-50% of tumor cells (score 2; moderate expression) (× 400)

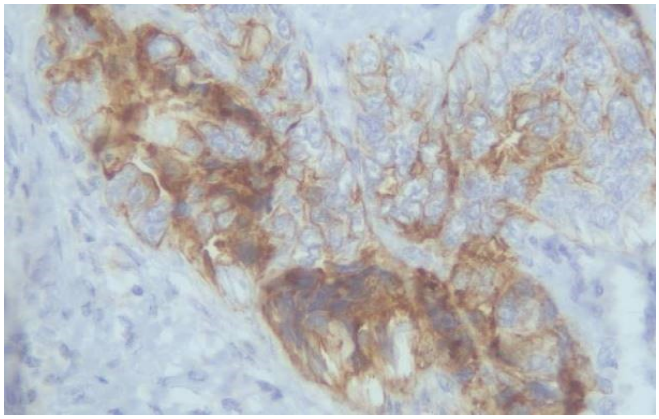

Fig. 6. IHC-stained sections revealed positive staining for PD-L1 in more than 50% of tumor cells (score 3; strong expression) (× 400)

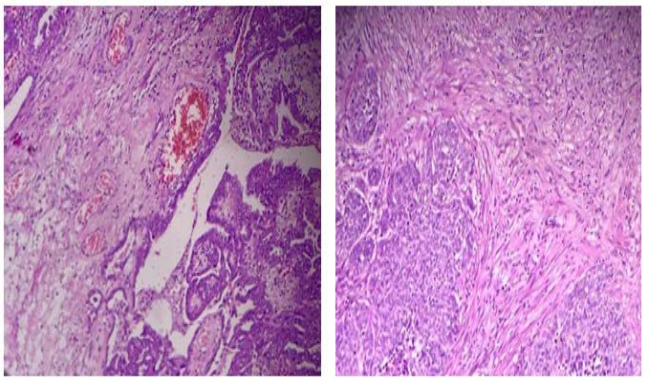

Fig. 7. Hand E-stained sections with negative TILs showing absent or rarely lymphocytic infiltrate in stroma between tumor cells (× 100)

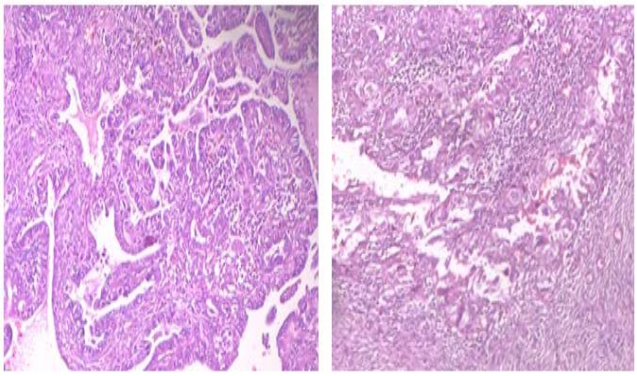

Fig. 8. Hand E-stained sections with positive TILs showing prominent lymphocytic infiltrate in stroma between tumor cells (× 100)

Relation among PD-L1 expression and demographic and clinicopathological characteristics of the studied cases

There had been a statistically significant association among PD-L1 expression and age of studied cases (p<0.001) as the level of PD-L1expression had been higher in older studied cases (≥ 65 years) than the younger ones. As for tumor characteristics, PD-L1 expression had been significantly higher in studied cases with ≥ 8 cm ovarian serous carcinomas than those with <8 cm (p<0.001). Also, PD-L1 expression had been significantly more intense in high grade serous carcinomas than the low-grade ones and in stage IV cases than stage I, II and III ones (p<0.001). Meanwhile, we found no statistically significant relation among PD-L1 expression level and marital status (p=0.537), parity (p=0.79), ascites (p=0.137), CA-125 level (p=0.07) and EOC site (p=0.137) (Table 3 and Figures 9-12).

Tab. 3. Relation between PD-L1 expression and demographic and clinicopathological characteristics of the studied patients

		PD-L1 expression		p value
		Absent/low (0/+1)	High (+2/+3)	
Age (years)	<65	33 (68.8%)	15 (31.3%)	<0.001*
	≥65	17 (32.7%)	35 (67.3%)	
	Median (IQR)	55 (42-67)	68 (60-74.5)	
Marital status	Single	3 (60%)	2 (40%)	0.537
	Married	46 (48.9%)	48 (51.1%)	
	Widow	1 (100%)	0 (0%)	
Parity		41 (49.4)	42 (50.6%)	0.79
Ascites		30 (44.8%)	37 (55.2%)	0.137
CA-125 (U/mL)	Median (IQR)	190 (38-593.5)	475 (106.75-1000)	0.07
Size (cm)	<8	28 (84.8%)	5 (15.2%)	<0.001*
	≥8	22 (32.8%)	45 (67.2%)	
	Median (IQR)	5.5 (3-15.08)	15.5 (11.75-24.2)	
Site	Right	21 (60%)	14 (40%)	0.137
	Left	17 (53.1%)	15 (46.9%)	
	Bilateral	12 (36.4%)	21 (63.6%)	
Grade	Low	49 (81.7%)	11 (18.3%)	<0.001*
	High	1 (2.5%)	39 (97.5%)	
Stage	I	14 (87.5%)	2 (12.5%)	<0.001*
	II	13 (72.2%)	5 (27.8%)	
	III	16 (66.7%)	8 (33.3%)	
	IV	7 (16.7%)	35 (83.3%)	

Data are presented as frequency (%) unless otherwise mentioned
*: Statistically significant as p value<0.05
CA-125: Cancer Antigen 125

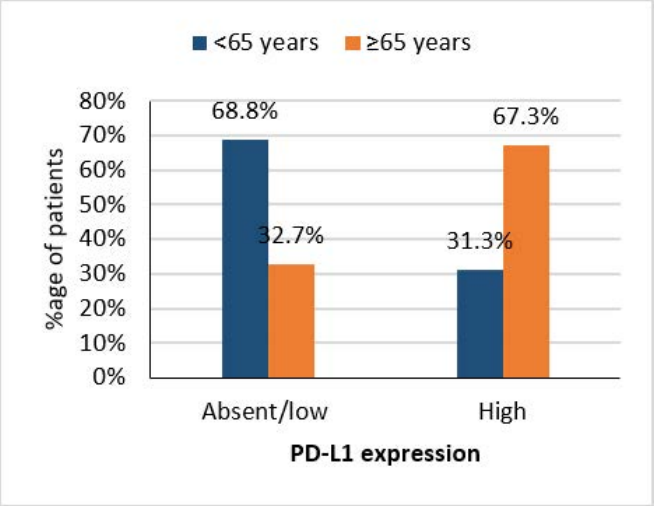

Fig. 9. PD-L1 expression in relation to age of the studied patients

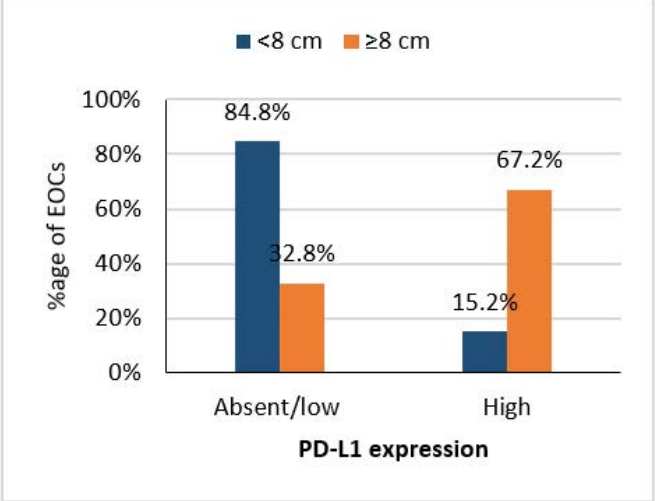

Fig. 10. PD-L1 expression in relation to tumor size

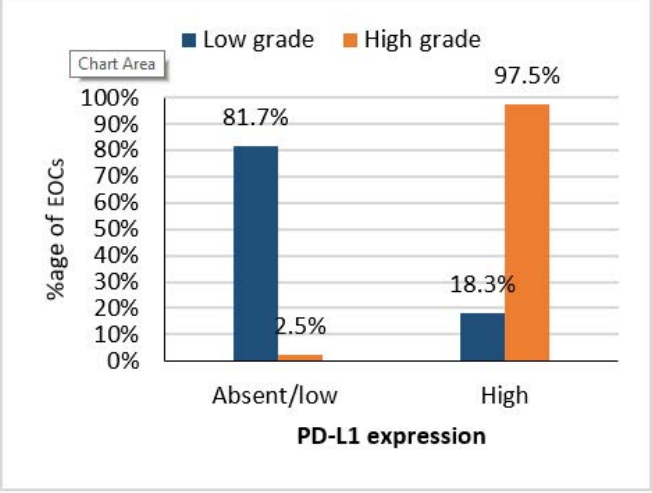

Fig. 11. PD-L1 expression in relation to tumor grade

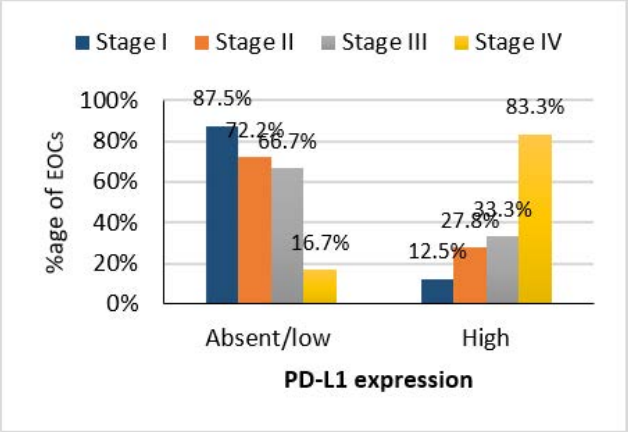

Fig. 12. PD-L1 expression in relation to tumor FIGO stage

The relation among PD-L1 expression and TILs of the studied cases

Table 4 shows a statistically significant relation among PD-L1 expression and TILs in studied cases with ovarian serous carcinoma as PD-L1 expression level had been significantly lower in TIL positive studied cases than TIL negative ones (p<0.001) (Table 4 and Figure 13).

Tab. 4. Relation between PD-L1 expression and TILs of the studied patients

		PD-L1 expression		p value
		Absent/low (0/+1)	High (+2/+3)	
TILs	Negative	3 (6.4%)	44 (93.6%)	<0.001*
	Positive	47 (88.7%)	6 (11.3%)	

Data are presented as frequency (%),
*: Statistically significant as p value<0.05
TILs: Tumor-Infiltrating Lymphocytes

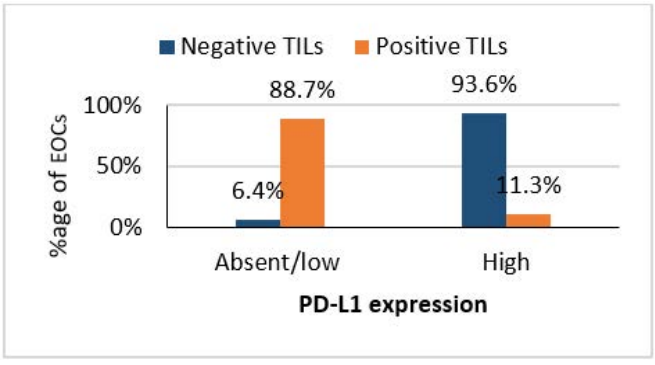

Fig. 13. PD-L1 expression in relation to TILs

The relation among PD-L1 expression and overall survival of the studied cases

There was a statistically significant impact of PD-L1 expression level on overall survival of EOC studied cases (p<0.001) as studied cases with high PD-L1 expression had significantly shorter mean survival time in comparison to those with absent/low expression (17.08 *vs.* 32.34 months, respectively) with higher HR (8.14, 95% CI: 4.49 to 14.77) (Table 5 and Figure 14).

Tab. 5. Relation between PD-L1 expression and overall survival of studied cases

		N. of events	Mean survival (months)	HR (95%CI)	Log-rank p value
PD-L1 expression	Absent/low (0/+1)	12 (24%)	32.34	Ref	<0.001*
	High (+2/+3)	42 (84%)	17.08	8.14 (4.49 to 14.77)	

HR: Hazard Ratio
*: Statistically significant as p value<0.05

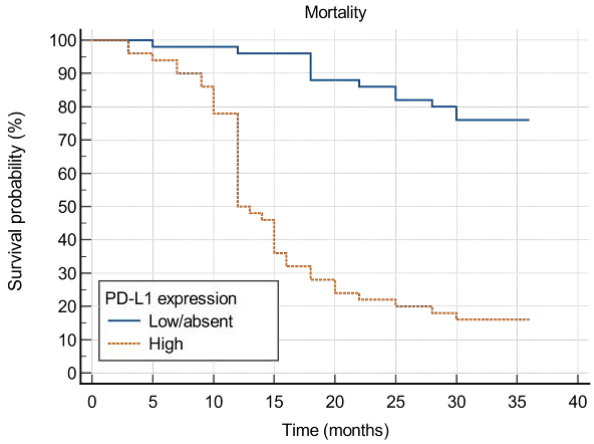

Fig. 14. Kaplan Meier curve for overall survival analysis of EOC patients according to PD-L1 expression level

The relation among PD-L1 expression and disease-free survival of the studied cases

There had been a statistically significant impact of PD-L1 expression level on disease free survival of EOC studied cases (p<0.001) as studied cases with high PD-L1 expression remained free from recurrence after 1ry cytoreduction for a shorter duration than those with absent/low expression (mean survival= 9.88 vs. 31.02 months, respectively) with higher HR (11.61, 95% CI: 6.17 to 21.87) (Table 6 and Figure 15).

Tab. 6. Relation between PD-L1 expression and disease free survival of studied cases

		N. of events	Mean survival (months)	HR (95% CI)	Log-rank p value
PD-L1 expression	Absent/low (0/+1)	8 (16%)	31.02	Ref	<0.001*
	High (+2/+3)	40 (80%)	9.88	11.61 (6.17- 21.87)	

HR: Hazard Ratio,
*: Statistically significant as p value<0.05

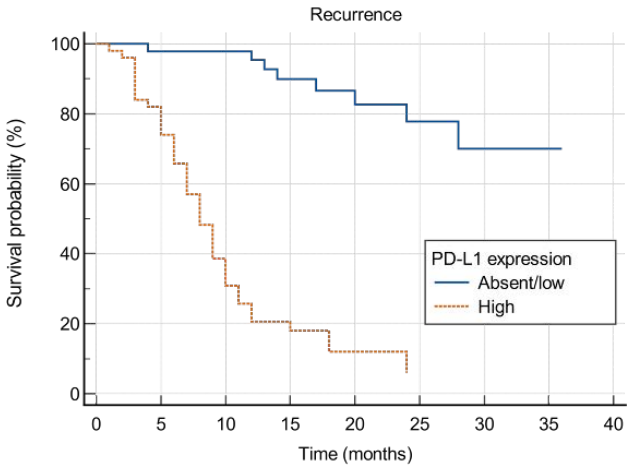

Fig. 15. Kaplan Meier curve for disease free survival analysis of EOC patients according to PD-L1 expression level

The relation among PD-L1 expression and disease progression of the studied cases

As shown in table 7, there was a statistically significant relation among progression free survival of EOC patients and PD-L1 expression (p<0.001) as studied cases with high PD-L1 expression remained free from metastasis for a shorter duration than those with absent/low expression (mean survival=11.59 months vs. 33.74 months, respectively) with higher HR (14.85, 95% CI: 8.22 to 26.82) (Table 7 and Figure 16).

Tab. 7. Relation between PD-L1 expression and progression free survival of studied cases

		N. of events	Mean survival (months)	HR (95% CI)	Log-rank p value
PD-L1 expression	Absent/low (0/+1)	4 (8%)	33.74	Ref	<0.001*
	High (+2/+3)	49 (98%)	11.59	14.85 (8.22-26.82)	

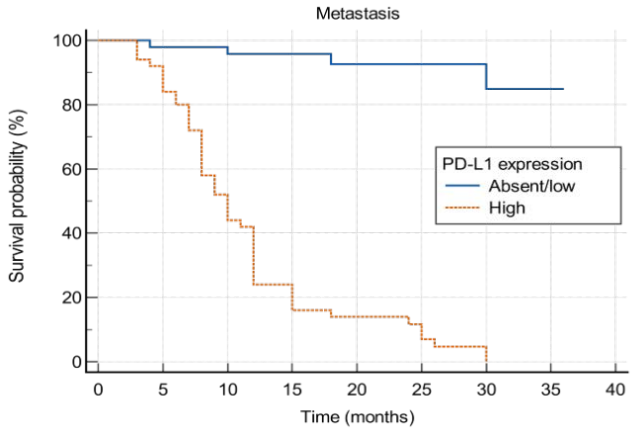

Fig. 16. Kaplan Meier curve for progression free survival analysis of EOC patients according to PD-L1 expression level

The relation between TILs and demographic and clinicopathological characteristics of the studied cases

There was a statistically significant association among TILs and age of patients as TILs were more frequently present in younger studied cases (<65 years) than the older ones (p=0.001). Regarding tumor characteristics, smaller EOCs (<8 cm) had more TILs than the larger ones (p<0.001). Positive TILs were significantly more predominant in low grade EOCs than the high-grade ones (p<0.001). Additionally, there had been a significant relation between TILs and tumor FIGO stage as stage IV EOCs elicited significantly less TILs than stages I, II and III (P<0.001). On the contrary, no statistically significant relation was detected between TILs and marital status, parity, ascites, CA-125 level and tumor site (Table 8 and Figures 17-20).

Tab. 8. Relation between TILs and demographic and clinicopathological characteristics of the studied cases

		TILs		p value
		Negative	Positive	
Age (years)	<65	14 (29.2%)	34 (70.8%)	0.001*
	≥ 65	33 (63.5%)	19 (36.5%)	
	Median (IQR)	69 (60-74)	56 (42.5-67)	<0.001*
Marital status	Single	2 (40%)	3 (60%)	0.602
	Married	45 (47.9%)	49 (52.1%)	
	Widow	0 (0%)	1 (100%)	
Parity		39 (47%)	44 (53%)	0.996
Ascites		33 (49.3%)	34 (50.7%)	0.52
CA-125 (U/mL)	Median (IQR)	453 (74.25-1091.75)	238 (67.05-593.5)	0.128
Size (mm)	<8	7 (21.2%)	26 (78.8%)	<0.001*
	≥ 8	40 (59.7%)	27 (40.3%)	
	Median (IQR)	15 (10.5-22)	8 (3-16.25)	
Site	Right	14 (40%)	21 (60%)	0.486
	Left	15 (46.9%)	17 (53.1%)	
	Bilateral	18 (54.5%)	15 (45.5%)	
Grade	Low	10 (16.7%)	50 (83.3%)	<0.001*
	High	37 (92.5%)	3 (7.5%)	
Stage	I	2 (12.5%)	14 (87.5%)	<0.001*
	II	7 (38.9%)	11 (61.1%)	
	III	7 (29.2%)	17 (70.8%)	
	IV	31 (73.8%)	11 (26.2%)	

Data are presented as frequency (%) unless otherwise mentioned
*: Statistically significant as p value<0.05
CA-125: Cancer Antigen 125
TILs: Tumor-Infiltrating Lymphocytes

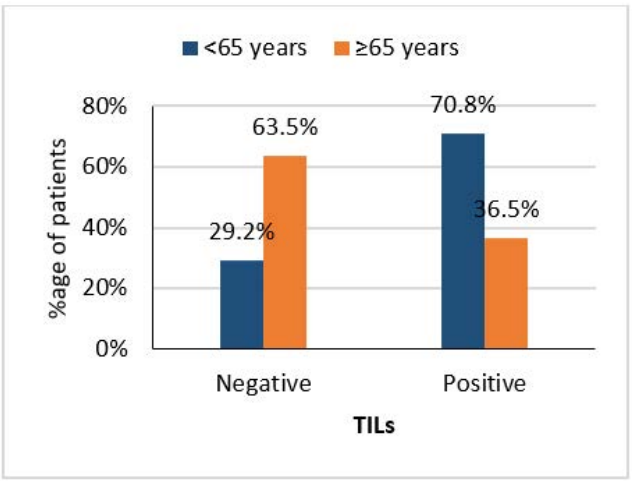

Fig. 17. KTILs presence in relation to age of the studied patients

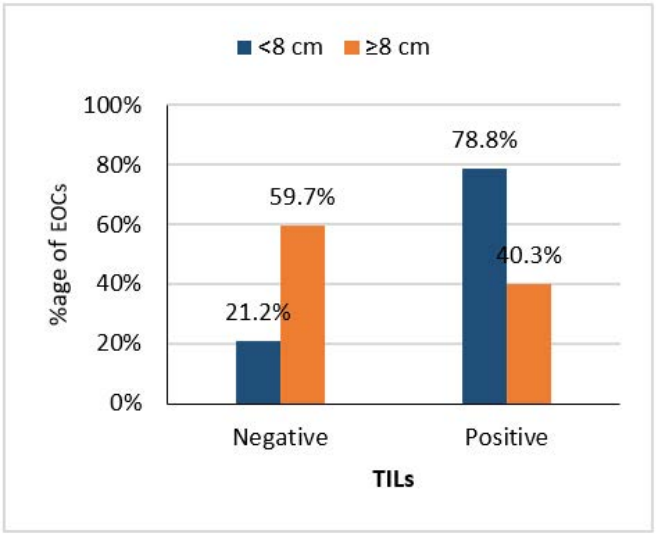

Fig. 18. TILs presence in relation to EOC size

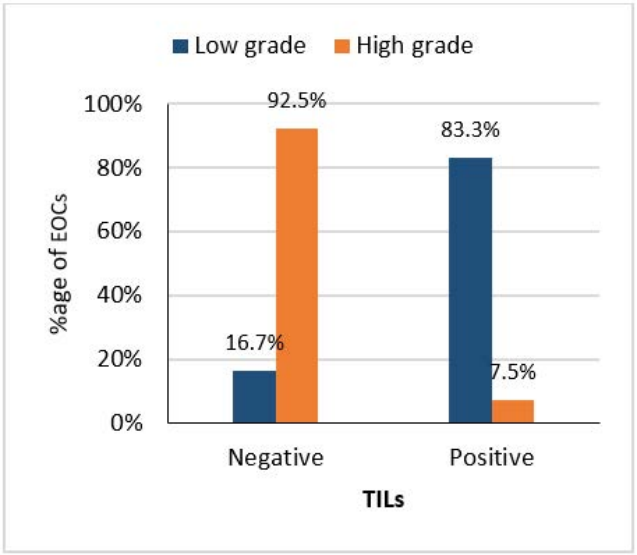

Fig. 19. TILs presence in relation to EOC grade

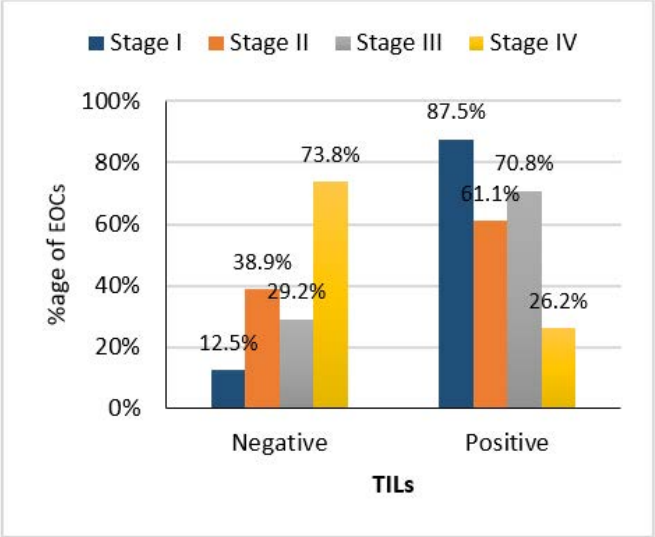

Fig. 20. TILs presence in relation to EOC FIGO stage

The relation between TILs and overall survival of the studied cases

There had been a statistically significant effect of TILs presence on overall survival of EOC studied cases (p<0.001) as TIL positive patients had significantly longer mean survival time in comparison to negative TIL ones (30.25 months *vs.* 18.47 months, respectively) with lower HR (0.2, 95% CI: 0.11 to 0.36) (Table 9 and Figure 21).

Tab. 9. Relation between TILs and overall survival of studied cases

		N. of events	Mean survival (months)	HR (95% CI)	Log-rank p value
TILs	Negative	37 (78.7%)	18.47	Ref	<0.001*
	Positive	17 (32.1%)	30.25	0.2 (0.11 to 0.36)	

HR: Hazard Ratio,
*: Statistically significant as p value<0.05

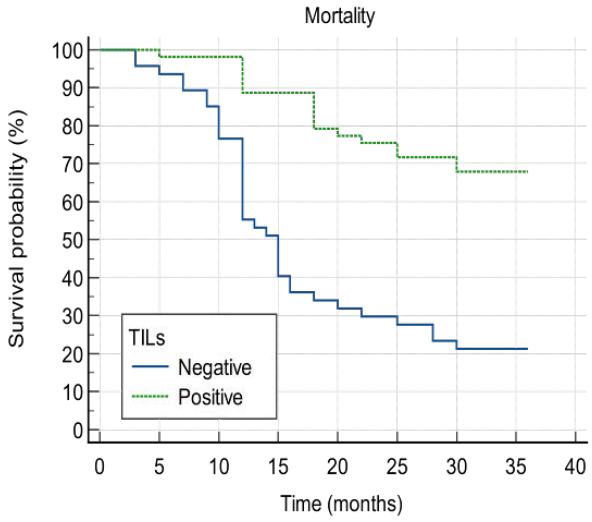

Fig. 21. Kaplan Meier curve for overall survival analysis of EOC patients according to TILs presence

The relation between TILs and disease-free survival of the studied cases

There was a statistically significant effect of TILs presence on disease free survival of EOC studied cases (p<0.001) as TIL positive patients remained free from recurrence for a longer duration than negative TIL ones (mean survival= 28.88 months *vs.* 11.69 months, respectively) with lower HR (0.14, 95% CI: 0.07 to 0.26) (Table 10 and Figure 22).

Tab. 10. Relation between TILs and disease free survival of studied cases

		N. of events	Mean survival (months)	HR (95% CI)	Log-rank p value
TILs	Negative	36 (76.6%)	11.69	Ref	<0.001*
	Positive	12 (22.6%)	28.88	0.14 (0.07-0.26)	

HR: Hazard Ratio,
*: Statistically significant as p value<0.05

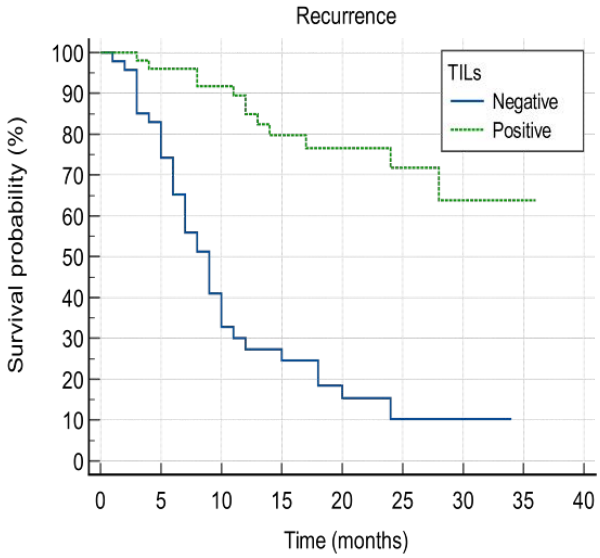

Fig. 22. Kaplan Meier curve for disease free survival analysis of EOC patients according to TILs presence

The relation between TILs and progression free survival of the studied cases

As shown in table 11, There was a statistically significant relation among progression free survival of EOC studied cases and the presence of TILs (p<0.001) as TIL positive patients remained free from metastasis for a longer duration than negative TIL ones (mean survival=30.61 months *vs.* 12.8 months, respectively) with lower HR (0.12, 95% CI: 0.07 to 0.21) (Table 11 and Figure 23).

Tab. 11. Relation between TILs and progression free survival of studied cases

		N. of events	Mean survival (months)	HR (95% CI)	Log-rank p value
TILs	Negative	43 (91.5%)	12.8	Ref	<0.001*
	Positive	10 (18.9%)	30.61	0.12 (0.07-0.21)	

HR: Hazard Ratio,
*: Statistically significant as p value<0.05

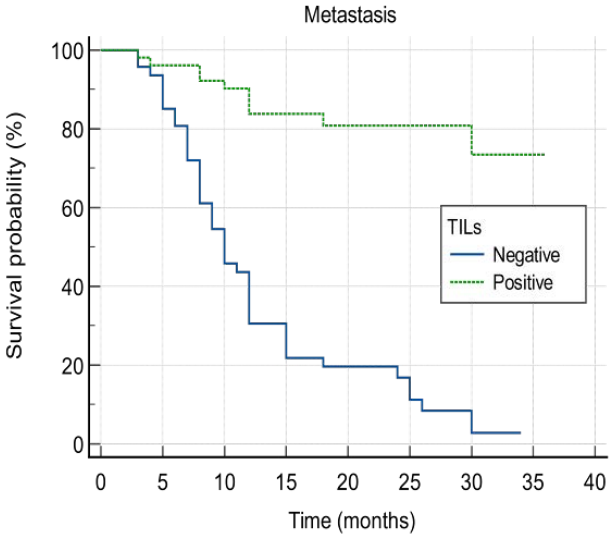

Fig. 23. Kaplan Meier curve for progression free survival analysis of EOC patients according to TILs presence

DISCUSSION

Ovarian carcinoma is one of the commonest tumors between females being associated with the worst prognosis even after optimal treatment [3, 5, 21, 22]. Most ovarian malignancies are of epithelial origin and the serous carcinoma has been the most common of them with poor prognosis and unsatisfactory survival rate [21]. This is because most patients develop recurrence and metastases with treatment resistance [5].

The ICIs including anti-PD-L1 immunotherapy show successful role in treating many solid tumors but their use in ovarian serous carcinoma is still limited [4]. Additionally, it has been essential to detect PD-L1 expression using IHC before the usage of anti-PD-L1 [13].

Also, the existence of TILs has a positive impact on prognosis, PFS and OS in addition to its relation to the PD-L1protein expression by tumor cells [21].

The tumor heterogeneity with subsequent PD-L1 different expression in different areas of the same tumor may represent a challenging problem for the use of anti-PD-L1 therapies [13]. Thus, it is suspected to improve the treatment efficacy by adequate patient selection based on combined PD-L1 and TILs analysis. Also, we should perfectly understand the immunotherapy markers and predictors of better response [11].

Surprisingly, in the included ovarian serous carcinoma cases, 34% of cases showed negative expression for PD-L1, 16% showed low expression and 50% indicating overexpression. This result has been consistent with that had been observed by Alwosaibai et al., (2023) who reported high PD-L1 expression in 47.8% of ovarian cancer samples where the highest levels of expression was detected in serous OCs [5].

In this research, we observed higher PD-L1 expression in older age group than younger patients with a statistically significant difference ($p<0.001$). But this disagrees with Riadi, et al. (2021) who observed statistically insignificant difference among the age and PD-L1 status among their studied cases [21].

Consistently, a larger tumor size (≥ 8 cm in its greatest dimension) had been seen to be accompanied by a higher PD-L1 expression with highly statistically significant relationship ($p<0.001$). This coincides with the findings of Nhokaew et al., 2019 but disagrees with Zhu et al., 2017 who observed no statistically significant relation with tumor size [14, 19].

In addition to the previously mentioned results, a statistically significant relation results were detected among PD-L1 expression and tumor grade ($p<0.001$), so that higher PD-L1expression had been more observed in the high-grade serous carcinomas. This agrees with Parvathareddy et al., who found that PD-L1 overexpression is related to high tumor grade [27]. Also, this runs parallel to the findings of revealed PD-L1 overexpression in higher tumor grades, but they didn't demonstrate any statistical relation among them. Also, demonstrated no significant statistical association among PD-L1expression and tumor grade. In addition to showed stronger PD-L1 expression in High-Grade Serous Carcinoma (HGSC) than Low-Grade Serous Carcinoma (LGSC) but with no statistical difference demonstrated no statistically significant relationship among PD-L1 and tumor grade [9, 21, 28].

Moreover, there had been a statistically significant relation among PD-L1 expression and advanced cancer stage ($p<0.001$). This is consistent with the outcomes of Alwosaibai Kh et al., (2023) who revealed PD-L1 overexpression in advanced cancer stages, but they didn't demonstrate any statistical relation among them. Whereas Nhokaew et al., 2019 showed that high PD-L1 expression had been related to advanced stage [5, 19].

Conversely, there had been a statistically insignificant relation among PD-L1 expression level and marital status ($p=0.537$). Also, there had been insignificant relation among PD-L1 expression and patients' parity status ($p=0.79$) which disagrees with Nhokaew et al., 2019 [19]. Additionally, statistically non-significant association among PD-L1 expression and serum CA-125 level ($p=0.07$) that agrees with Zhu et al., 2017, presence of ascites ($p=0.137$), and tumor site ($p=0.137$). The later agrees with Zhu et al., 2017 [13, 14].

The awesome finding of our study was the high statistically significant relationship among PD-L1 expression and the occurrence of tumor infiltrating lymphocytes as PD-L1 showed lower expression in TILs positive patients than those with negative TILs ($p<0.001$). This runs in parallel to the study done by Hamanishi et al. (2015) who demonstrated an inverse relationship among PD-L1 expression and TILs [17, 29]. Also, demonstrated that patients with PD-L1 overexpression had a significant inverse correlation with intraepithelial lymphocytic count. Thus, they suggested the possible prognostic role of the ovarian cancer cell PD-L1 expression. This also coincides with the observation of Riadi, et al. (2021) who noticed that by increasing PD-L1expression, the number of TILs decreases [21]. This can be explained by the inhibition of anti-tumor immune response by PD-L1 molecules on the cancer cells. In contrast, this disagrees with the research done by Alwosaibai et al., (2023) who documented that PD-L1 positive expression had been significantly related to positive TILs which is known to be related to favorable prognosis [5]. Riadi, et al. (2021) noticed a strong relationship among PD-L1 expression and TILs in serous carcinoma and thus suggested the possibility of using anti-PD-L1 in treatment of serous carcinoma. Our result is on the contrary to found more prominent TILs in HGSC with higher PD-L1 expression on tumor cells and explained this finding by the upregulation of PD-L1 receptors on tumor cells by activated T-lymphocytes [13].

Also, a statistically significant association had been detected among PD-L1 expression and the patients' outcome where studied cases with high PD-L1 expression had significantly shorter overall survival time ($p<0.001$), remained free from recurrence after primary cytoreduction for a shorter duration ($p<0.001$) and remained free from metastasis for a shorter duration ($p<0.001$). This agrees with Dergham et al. (2023) who stated that PD-L1 overexpression is related to lower OS and attributed this to the decreased lymphocytes in tumor microenvironment suggesting that PD-L1 expression may inhibit the tumoral lymphocytes infiltration [11]. Also, Hamanishi et al. (2007) suggested that high PD-L1 expression is related to worse OS and PFS [17]. This is also consistent with Jovanovic et al., 2021 who observed indirect correlation among PD-L1 expression on HGSC and studied case's survival [13]. Nhokaew et al., 2019 showed that high PD-L1 expression had been related to shorter median PFS, thus, it is associated with worse outcomes [19]. This disagrees with Alwosaibai et al., (2023) who found that studied cases with positive PD-L1 expression had better cancer-free survival rate compared to those with negative PD-L1 expression, however, no significant difference was detected among these 2 groups, thus suggested a minimal role of PD-L1 on prognosis [5]. Additionally, revealed no significant relationship among PD-L1 expression and OS in ovarian cancer [30]. Chang

et al., 2023 showed no significant association among PD-L1 expression and patients' OS [10]. Also, some older studies Chen et al., 2020 reported a positive correlation among PD-L1 expression on tumor cells and favorable prognosis in HGSC [31, 32]. Webb et al., 2016 revealed that PD-L1 positivity had been significantly related to disease-specific survival in HGSC but shows no prognostic significance in LGSC [18]. Cheng et al., 2018 demonstrated that positive PD-L1expression had been significantly related to prolonged OSin studied cases with OC but high PD-L1 expression had not been significantly related to longer PFS [23].

From these contradictory outcomes regarding the relation among PD-L1 expression and studied cases' outcomes, we thought that the effect of hormones especially estrogen on the ovarian carcinoma growth and cellular proliferation may be an attributable factor. Also, we attribute the controversial results among different studies to the use of different staining patterns and scoring system (that can be because of the use of different anti-PD-L1 antibodies), different detection methods, lack of standardized guidelines for PD-L1 IHC assays and in availability of validated cut-off values.

As regards TILs, 53% of the included cases were negative with absent or rare lymphocytes in the inter-tumoral stroma and 47% were positive where there was prominent lymphocytic infiltrate into the stroma. This disagrees with Alwosaibai et al., (2023) who found that most of his cases (about 81%) showed positive TILs [5].

There was statistically significant association among positive TILs and younger studied cases ($p=0.001$), smaller tumor size (<8 cm in its greatest dimension) ($p<0.001$). In addition, positive TILs were significantly more prominent in low grade tumors than the high-grade tumors ($p<0.001$). Also, a significant relation between TILs and tumor FIGO stage as advanced stages showed significantly lower TILs than early stages ($p<0.001$). Conversely, there had been a statistically insignificant relation among TILs and marital status ($p=0.602$), parity ($p=0.996$), ascites ($p=0.52$), CA-125 level (0.128) and tumor site ($p=0.486$). To the best of our knowledge, no available studies that correlate TILs with ovarian carcinomas' clinico-pathological features.

Our results showed that positive TILs showed statistically significant effect on the included ovarian serous carcinoma patients' overall survival ($p<0.001$). Additionally, TILs positive cases remained free from both recurrence and metastasis for longer duration than negative TILs cases ($p<0.001$ for each). This runs in parallel to the results of Zhang et al. (2003) who concluded a positive prognostic impact of TILs on OS in OC studied cases. This also agrees with Jovanovic et al., 2021 who observed that prominent TILs are associated with better outcomes [13, 33].

The discrepancy between the results of our work and the previous studies may be related to some limitations in this research. These include small sample size, the retrospective nature of the research, short follow-up period, in addition, we performed this study on ovarian serous carcinoma only and we didn't include the other histological types. Additionally, we assess PD-L1 expression only with IHC without other method to verify its actual expression status.

Despite of these limitations, our study highlights the prognostic value of PD-L1 expression in ovarian serous carcinoma and its relation to clinico-pathological parameters in those cases.

CONCLUSION

Our study suggests the possible prognostic and predictive role of PD-L1 overexpression in ovarian serous carcinoma where it indicates cases with aggressive features like high tumor grade, advanced cancer stage and poor survival. Thus, it is possible to use anti-PD-L1 in HGSC particularly if accompanied by prominent TILs and may improve the outcome in such patients.

We suggest that PD-L1 cannot be the only target for immunotherapy in OC. However, applying PD-L1 IHC in ovarian serous carcinoma could be a step in evaluating its predictive value as an immune biomarker for such cases.

RECOMMENDATIONS

Further prospective studies on larger sample size are one of our recommendations. Additional studies are required to unify PD-L1 expression scoring system. Integration of IHC with molecular and cytogenomic investigations in tumor cells and their microenvironment may be useful for prediction of treatment response.

DISCLOSURE

The authors report no conflicts of interest in this work.

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