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Original Article

The prognostic significance of pdl1 and foxp3 expressions in tumor cells and the tumor microenvironment of ovarian epithelial tumors

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Abstract: Background: The aim of this study is to determine the prognostic values of PDL1 expression in ovarian epithelial tumors and to detect the presence of FOXP3-positive T reg cells in the tumor microenvironment. Methods: This study included patients with benign, borderline or malignant ovarian serous tumors (n=82), mucinous cancer (n=17) and endometrioid cancer (n=36). FOXP3 and PDL1 were immunohistochemically evaluated and compared with histopathological and clinical prognostic parameters. Results: There was no expression of PDL1 in any tumor cell. However, PDL1-positive inflammatory cells were seen in 10 cases (7.3%) with mucinous carcinoma (n=6), endometrioid carcinoma (n=2), borderline (n=1), and benign (n=1) serous tumors. It was also determined that there was a significant positive correlation between PD-L1 expression in tumor infiltrating cells and survival (P<0.01). In 47 (34.3%) cases, there were FOXP3-positive cells. The number of FOXP3-positive cells was significantly higher in ovarian cancer, especially in serous and endometrioid carcinomas, rather than benign and borderline tumors (P=0.007). But there was no statistically significant association between the survival times and the presence of T regs (P=0.241). Conclusions: This study demonstrated that the presence of FOXP3 and PDL1-positive regulatory T cells in TILs was associated with mainly malignant tumors. We also found that the presence of PD-L1-positive inflammatory cells has a positive effect on survival.

Keywords: PDL1, FOXP3, treg, ovarian epithelial tumors

Introduction

It is well known that the balanced immune response between activator and inhibitor pathways may be disturbed in several malignancies, where the inhibition of the immune system favors tumor progression [1]. Actually, cancer not only inhibits an immune response, but it also triggers the immune system. Within the immune system, cytotoxic CD8(+) and CD4(+) Th1 T cells, along with their characteristically produced cytokine IFN-γ, function as the major antitumor immune effector cells, whereas other tumor-associated cells (TAC) are generally recognized as dominant tumor-promoting forces thanks to their production of IL-6, TNF, IL-1β, and IL-23 [2]. It was determined that in the advanced stages of malignancies CD4+ helper and CD8+ cytotoxic T cells are found to be increased in the tumor microenvironment so as to assume an antitumoral role [1].

But not all T-lymphocytes are antitumor effector immune cells. A subpopulation of CD4+ T cells expressing CD25 and Foxp3, termed regulatory T cells (T regs), play a role in promoting tumor growth and progress by inhibiting the immune response against cancer [2]. FOXP3 is a transcription factor and probably the best marker currently available for identifying natural T regs in humans [3]. The presence of FoxP3-positive cells within tumors has been shown to predict a worse prognosis in some tumors, such as cervical cancer, T-cell lymphoma, bladder cancer, lung cancer, and breast cancer. The first studies about immune regulatory T-cell function were done on patients with multiple myeloma [1-3]. T regs, which are generally found to be increased

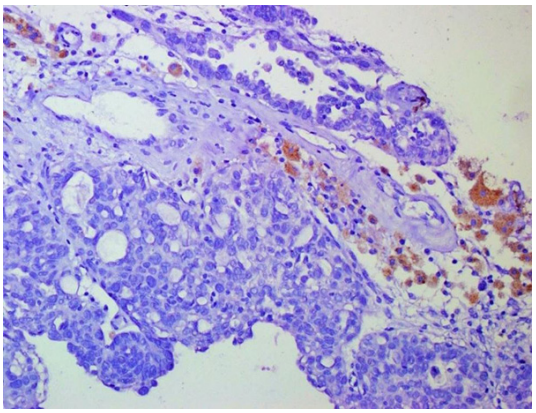

Figure 1. Note the presence of PD-L1-positive macrophages in a serous tumor (DAB ×200).

in cancers, play an important role in the maintenance of immune tolerance, but they also suppress immune responses to tumors, transplants, and infectious agents [2]. Therefore, effectiveness of immunotherapeutic approaches in cancer might be restricted by an increased number of T regs [1-4]. Programmed death-ligand 1 (PD-L1) is a member of the B7 superfamily, the most critical costimulatory molecules that regulate T-cell responses [5]. Programmed cell death 1 (PD1) is expressed at the surface of various immune cells, including T-lymphocytes. PD1 is activated by its ligands, PDL1 or PDL2, and expressed by antigen-presenting cells such as macrophages and B-lymphocytes [5-7]. The PDL1-PD1 interaction attenuates lymphocyte activation, promotes the development and function of T regs, and impairs the antitumor T-cell immune response. In summary, the PD1 pathway plays a major role in the negative regulation of cell-mediated immune responses. Recently, it has been determined that PDL1, PDL2, and PD1 are expressed on several tumor cells [8-11].

Ovarian cancer is the most lethal disease among the gynecological malignancies. Most cases present with massive peritoneal dissemination, and, although many of the cases respond to initial chemotherapy following tumor reductive surgery, most cases recur and eventually become resistant to chemotherapy [12]. Therefore, a new therapy for peritoneal dissemination other than conventional chemotherapy is required. Recently, FOXP3 and PDL1 expressions have been studied to predict their clinical responses in several cancers [13-18]. However, their expressions and impacts on the prognosis of patients with ovarian carcinoma have not been well studied and remain controversial. In this study, we aimed to explore the correlation between PD-L1 and $FOXP3^+$ T regs in ovarian epithelial tumors and further investigate their associations with clinic pathological features.

Materials and methods

The resection specimens of 137 patients who were diagnosed and treated in Izmir Tepecik Education and Research Hospital between 2002 and 2013 were included in this study. The study was approved by the Local Ethics Committee of the Hospital. The staging system developed by FIGO [Fédération Internationale de Gynécologie Obstétrique (International Federation of Gynecology and Obstetrics)] was used to describe the extent of the spread of these tumors. According to this system, stage I diseases are confined to one or both ovaries. In stage II, there is pelvic extension or implants, but the tumor is still limited to the pelvis. Stage III diseases demonstrate an extension to the peritoneum or the regional lymph nodes. There are distant metastases to the liver or outside the peritoneal cavity in stage IV disease.

Whole-tissue sections from 137 patients with ovarian epithelial tumors were examined for PD-L1 and FOXP3 expression by using immunohistochemical methods. Correlations between their expressions and their association with clinic pathological features, tumor subtypes and prognosis were studied.

Immunohistochemical staining of tumor tissues

For our immunohistochemical (IHC) studies, we used hematoxylin and eosin (HE) staining to select appropriate diagnostic paraffin blocks and to identify viable tumor areas. IHC tests were performed using the streptavidin-biotin peroxidase method (Invitrogen, Camarillo, 85-9043, USA). Serial 5-μm sections were obtained and placed on slides which were baked overnight at 60°C, dewaxed in xylene, and hydrated with distilled water through decreasing concentrations of alcohol. All slides were treated with the heat-induced epitope retrieval procedure in a microwave. In this procedure slides were left for 20 minutes in a 10 mM/L EDTA buffer at pH 8.0, cooled at room temperature for 20 min-

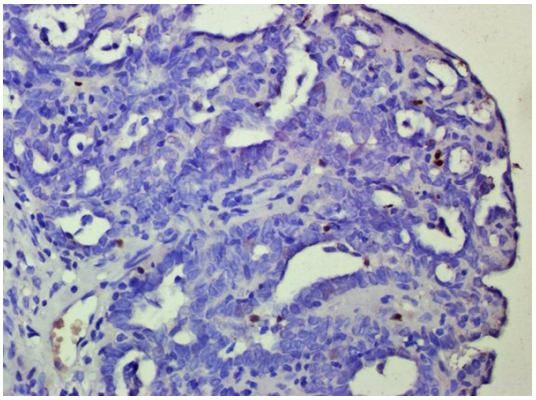

Figure 2. There were 1-9 FOXP3 positive cells in HPF of a serous carcinoma (DAB ×400).

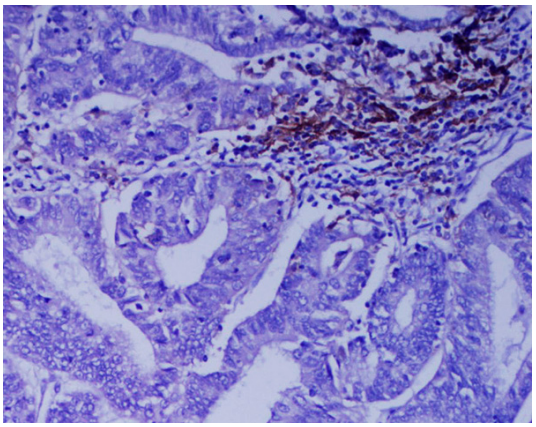

Figure 3. There were 10-49 FOXP3 positive cells in HPF of an endometrioid carcinoma (DAB ×400).

utes, and then blocked to retrieve endogenous peroxidase and biotin. Purified monoclonal mouse antibodies against PDL1 (abcam, ab-205921-pd-l1, RabMabAB, clone 28-8, 1:100 dilution) and FOXP3 (Anti-FOXP3 antibody [236A/E7] (ab20034, 1:300 dilution) were used. Two pathologists blinded to the clinical characteristics of the patients performed histopathological assessments. Immune reactivity for PD-L1, both the percentage and intensity of the complete membranous staining of tumor cells and the cytoplasmic staining of inflammatory cells were evaluated (**Figure 1**). The immune reactivity for FOXP3 was assessed using a scoring system. FOXP3 positive lymphocytes in the tumor microenvironment were counted, and the rates of positivity were categorized as negative (0), 1-9 cells/1 HPF (+/mild), 10-50 cells/HPF (++/moderate), and >50 cells/HPF (+++/high) (**Figures 2** and **3**).

Statistical analysis

Statistical analyses were performed with SPSS 18.0 software (SPSS, Chicago, IL). Spearman's correlation test was used to assess the association of PD-L1 expression with $FOXP3^+$ Treg infiltration and clinicopathological characteristics. *Chi*-square tests were used to compare PD-L1 expression and Treg infiltration among intrinsic subtypes. The cumulative survival (overall survival, OS; recurrence-free survival, RFS) times were calculated using the Kaplan-Meier method and analyzed with the log-rank test. *P* value of less than 0.05 considered statistically significant.

Results

In this series, there were 89 (64.9%) ovarian epithelial carcinomas, 12 (8.7%) borderline serous tumors, and 36 benign serous tumors (26.7%). Among the malignant tumors, 34 (38.2%) serous, 17 (19.1%) mucinous, and 38 (42.6%) endometrioid carcinomas were detected.

All twelve patients with borderline serous tumors had stage I disease (stages IA, n=8¸ 66.6%; IB, n=3; 25%; and IC, n=1, 8.3%). Most cases with serous carcinomas (n: 24/70.5%) have stage IIIC, while most cases with mucinous (n: 10/58.8%) and endometrioid carcinomas (n: 17/44.7%) have stage IC diseases. Patients with carcinomas have stage I (n=41; 46%), II (n=2; 2.2%), III (n=42; 47.1%), and IV (n=4; 4.4%) diseases.

The mean age of the patients was 46.2± 13.8 years (range, 17-78 years). The cases with serous carcinoma (52.4±9 years/30-70 years) were significantly older than the cases with both borderline (35.9±13.4 years/23-72 years) and benign serous tumors (38.5±14.8 years/17-62 years). Contrarily, there were no significant differences between the mean ages of the patients with carcinomas. The mean age of the patients with mucinous and endometrioid carcinomas were 46.1±15 years (18-78 years) and 51.1±10.5 years (34 to 77 years) respectively. All 48 cases with benign and borderline serous tumors were alive, while 17 cases with carcinoma died. The highest mortality rate was detected in the cases with serous carcinoma (32.4%, n=11). Five cases (13.2%) with endometrioid carcinoma and one case

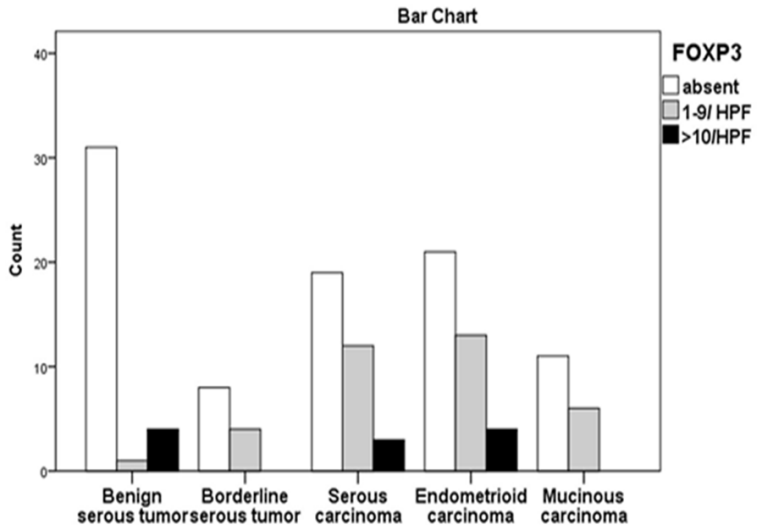

Figure 4. T regs decreased in benign and borderline serous tumors, while their levels increased in serous and endometrioid carcinomas (P=0.007).

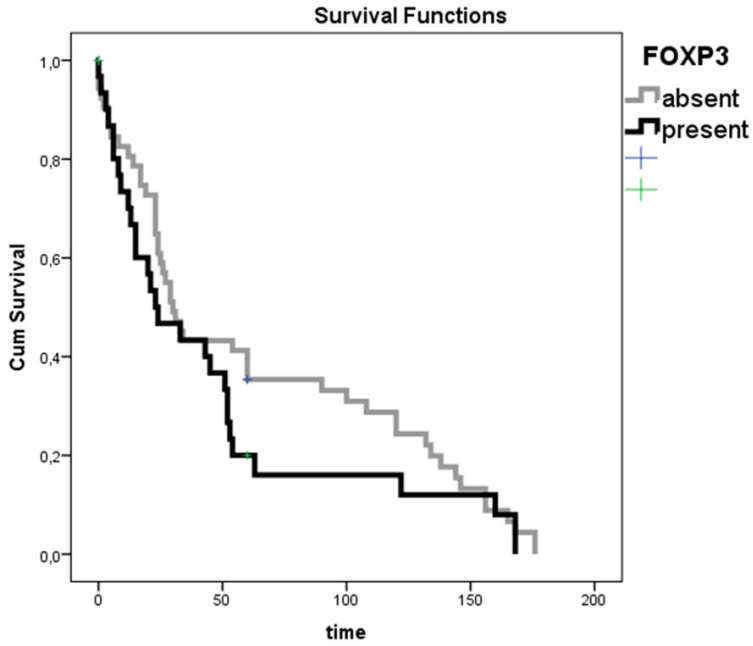

Figure 5. There was no significant association between overall survival of the patients with ovarian carcinomas and expressions of FOXP3 (P=0.241).

(5.9%) with mucinous carcinoma also died. In addition, three endometrioid and one mucinous carcinoma cases were lost to follow-up. The mean overall survival times for cases with serous, endometrioid, and mucinous carcinomas were 15.2±11.8 (0-45), 63.1±60.6 (3-185), and 96.1±66.5 (12-203) months, respectively.

There were FOXP3-expressing lymphocytes in peritumoral areas in 47 (34.3%) cases. FOXP3-positive T regs were detected in borderline serous tumors (33.3%), serous carcinomas (44.1%), endometrioid carcinomas (44.7%) mucinous carcinomas (35.3%), but only in 13.9% of benign serous tumors. The number of FOXP3 positive cells was significantly higher in ovarian cancer, especially in serous and endometrioid carcinomas rather than benign and borderline tumors (P=0.007) (**Figure 4**). But there was no statistically significant association between the survival times and the presence of T regs (P=0.241) (**Figure 5**).

None of the benign and malignant ovarian tumors in the series had PD-L1 expressions. However, PD-L1-positive inflammatory cells were seen in 10 cases (7.3%). These tumors consisted of mucinous carcinomas (n=6), endometrioid carcinomas (n=2), borderline (n=1) and benign (n=1) serous tumors. A statistically significant positive correlation was also determined between PD-L1 expression in tumor infiltrating cells and survival (P< 0.01) (**Figure 6**). Four of 6 mucinous tumors which were also positive for T regs, also included PDL1 positive cells in the tumor microenvironment. But there was no statistical significant association between PD-L1 and FOXP3 expressions (P= 0.085).

Discussion

Nowadays, huge developments have been achieved in our understanding of cancer immunology. The complex relationships between

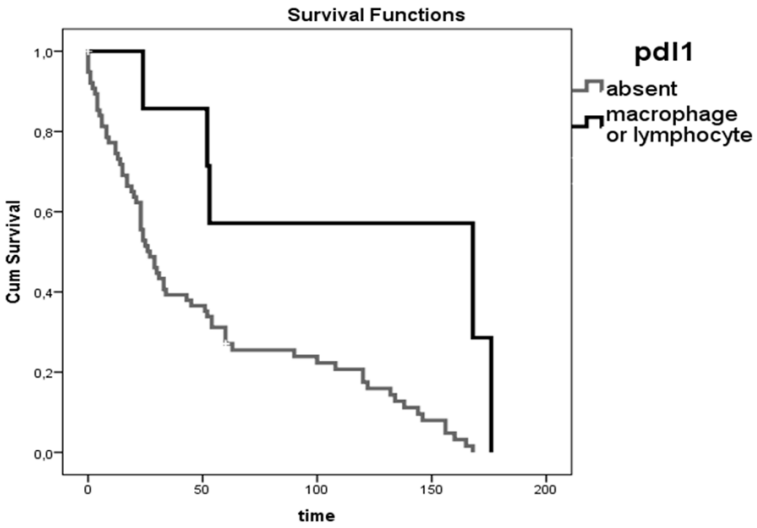

Figure 6. There was a significant association between overall survival of the patients with ovarian carcinomas and expressions of PDL1 (P=0.013).

tumor cells, the tumor microenvironment and immune system cells, especially the cytotoxic and helper T cells and the regulatory T cells, are beginning to be elucidated. Programmed cell death protein 1 (PD1), also known as CD279, functions as an immune checkpoint, and plays an important role in downregulating the immune system by preventing the activation of T-cells, which in turn weakens autoimmunity and promotes self-tolerance. Its inhibitory effect is accomplished through a dual mechanism which involves promoting apoptosis in antigen specific T-cells in lymph nodes and reducing apoptosis in regulatory T cells. Currently it has been determined that the immune checkpoint inhibitors targeting PD1 or PDL1 can reduce the tumoral growth and can be used in the treatment of several cancers. Recently, some tumors have been defined as immunogenic tumor types, and several breakthroughs in immunotherapies have led to a rapid expansion of the use of PD-L1 inhibitors such as nivolumab and pembrolizumab [8]. Only a few studies have recently been conducted on the prognosis of PDL1 expression in ovarian cancers. Although some notable findings were uncovered, there is no evidence to support the use of PD-L1 inhibitors in ovarian tumors yet [8, 18, 19]. In the present study, we also determined a statistically significant positive correlation between PD-L1 expression in tumor infiltrating cells and survival. However, in order to determine the prognostic and predictive role of PD-L1 in ovarian tumors, it is necessary to study PD-L1 in larger patient groups.

T regs are a subset of CD4+ helper T cells that possess a capacity to suppress the proliferation and cytokine secretion of effector T lymphocytes. FOXP3, *forkhead*/winged-helix transcription factor, appears to function as a master regulator in the development and control of T regs, and it has been regarded as the most specific and reliable surface marker of T regs [19]. Furthermore, T regs have been found in a number of human tumors and are considered as biomarkers and prognostic factors for human malignant tumors [20, 21]. An improved understanding of the molecular mechanisms that govern the host response to tumors has led to the identification of checkpoint signaling pathways that limit the anticancer immune response [22]. Understanding the mechanisms of T regs in cancer immunity in various cancers will provide valuable information on new tumor immunology-based tailored therapeutic approaches [1]. Still, new information about the role of T regs in cancer is needed to target them as poor prognostic markers in clinical oncology and to use them for the development of new therapeutic approaches such as the elimination or blocking of T regs within tumors or in the circulatory system [11, 23]. In this study, the number of FOXP3-positive cells was significantly higher in ovarian cancer when compared with benign and borderline tumors. Similarly, Que et al. [21] demonstrated that the number of T regs was higher in malignancies. They also reported that the presence of intratumoral FoxP3-positive cells are an independent poor prognostic factor and are associated with PDL1 positivity in soft tissue tumors [21]. But we didn't find any statistically significant correlations between FOXP3 expression and survival.

PD-L1 has been confirmed to play a critical role in the development and functional maintenance of T regs. The PDL1 can inhibit T cell responses by converting naïve CD4 T cells to iTreg cells, indicating that PDL1 has a pivotal role in regulating induced T reg (iTreg) cell development

and sustaining iTreg cell function [21, 22]. The infiltration of FOXP3+ T regs and PDL1 expression have been reported in patients with gastric cancer, colorectal carcinoma and breast cancer [13, 14]. In addition, varying proportions of PD-L1 expressions and their prognostic values in ovarian carcinomas have also been reported [23, 24]. PDL1 inhibitors have shown very promising results in clinical trials. notably in melanoma and renal, lung, prostate, and bladder carcinomas with durable tumor responses or stabilizations. In some cases, a relationship has been reported between therapeutic response and PDL1 expression on tumor and/or immune cells [13, 14]. PD1/PDL1 functions as a negative mediator of immune response through different pathways in antitumor immunity. Recent studies have reported that PDL1 plays a crucial role in the function and development of FOXP3-positive T regs. Although PDL1 is a potent negative regulator of antitumor immunity, little is known about the mechanism of cytoplasmic and membranous PDL1 regulation by the tumor microenvironment in ovarian cancer. We investigated the primary source of FOXP3 and PDL1 expression in epithelial ovarian cancer, its relationship to tumor-infiltrating lymphocytes (TIL) and associated gene products and their impact on survival in patients with such types of cancer. But there was no statistically significant association between PDL1 and FPXP3 expressions.

Currently, a blockade of the PD1/PDL1 signaling pathway has been proved to be one of the most promising immune therapeutic strategies in boosting the immune system to fight against cancer [18]. Blocking PD1 on tumor-infiltrating lymphocytes (TILs) or blocking PDL1 on tumor cells results in the restoration of the functions of tumor-specific T cells. The reactivated T cells can initiate the direct killing of tumor cells and the secretion of immune stimulatory cytokines such as interferon gamma (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor-alpha (TNF-α). Although the inhibition of these points is shown to produce beneficial results in different studies, this effect is not seen as homogeneous in the whole population. Phase 3 trials have shown that monoclonal antibodies targeting PDL1 or PD1 are useful in treating solid cancers such as malignant melanoma, lung carcinoma, non-Hodgkin lymphoma, renal cell carcinoma and triple negative breast cancer [1, 8, 11, 13, 14]. In clinical studies, PDL1 expression in cancer was mostly studied at the protein level using immunohistochemical tests (IHC). However, there are some limitations in PDL1 IHC standardization that may end up with discordant results. The previous studies revealed that patients with IHC-positive tumors may not respond to treatment [25]. As a result, discordant results have been reported throughout the studies, particularly in prognostic ones. The main reason for these differences is some limitations about the standardization of the IHC of PDL1. There are many PDL1 antibodies that lack specificity and reproducibility [25]. The optimal positivity cut-off value has not been defined yet, and the interpretation of the staining intensity is subjective. In previous studies, it was demonstrated that PDL1 is primarily expressed by macrophages in ovarian cancer and is strongly associated with subsets of both cytolysis and regulatory tumor-infiltrating lymphocytes (TILs), resulting in a positive association with survival. The PD1 expression rate was different in epithelial ovarian cancer. The difficulty in comparing results from methodologically different studies is illustrated by the fact that we observed PDL1 expression in cancer cells by the use of a particular, carefully validated antibody, while using another antibody which produced quite similar staining results for TILs, PDL1 cancer cell expression could not be elicited [25]. In this study we used a commercial anti-PDL1 antibody that was not indicated among the antibodies that the manufacturers of PDL1 inhibitors proposed to use.

Disclosure of conflict of interest

None.

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