Expressions of PD-L1 and FOXP3 in uterine cervical neoplasms may indicate tumor invasion and squamous differentiation

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Objectives: Increasing evidence has demonstrated that upregulation of programmed death cell ligand-1 (PD-L1) and FOXP3-positive regulatory T cells in different malignancies plays a critical role in tumor progression. In the present study, the evaluation of PD-L1 and FOXP3 tissue expressions in a spectrum of cervical neoplasms were performed. Material and method: Immunohistochemical PD-L1 and FOXP3 expressions were evaluated in a total of 107 formalin-fixed, paraffin-embedded uterine cervical neoplasm specimens, and their association with some pathological parameters was tried to be elucidated. Results: Cases with low or high squamous intraepithelial lesion (n = 59), squamous cell carcinoma (n = 27), adenosquamous carcinoma (n = 15) and adenocarcinoma (n = 6) were included in this study. Tumoral PD-L1 was detected in most squamous cell carcinomas while it was found in almost none of the intraepithelial lesions (p < 0.001). Similarly, the number of both PD-L1, and FOXP3-positive inflammatory cells was statistically significantly higher in invasive tumors than in intraepithelial lesions (p < 0.001). Conclusion: Our findings demonstrated the association between the histological types of uterine neoplasms and PD-L1 or FOXP3 expressions, as well as the correlation between presence of invasion and tumoral PD-L1 expression. Therefore, it may be suggested that PD-L1 plays an important role in the pathogenesis of cervical neoplasms.

Keywords
Uterus, Cervical carcinomas, Squamous intraepithelial lesions, PD-L1, FOXP3

1. Introduction
The presence of a close association between the Human Papilloma Virus (HPV) and cervical cancers has been reported in early epidemiological studies concerning cervical neoplasia. Therefore, the conduction of screening studies especially among patients with cervical dysplasia associated with HPV carries an utmost importance [1–4]. Cervical cancer is one of the most common cancer types affecting women globally and HPV is present in approximately 90% of cases with cervical neoplasia [5]. But despite efficient and reliable screening tests, malignancies of the uterine cervix remain to be among the predominant causes of cancer mortality in women worldwide [3]. Their most frequently encountered histological type is squamous cell carcinoma (SCC) detected in 75–80% of the cases with invasive uterine cervical carcinomas [4]. Adenosquamous carcinoma (ASC) consists of malignant squamous and glandular cells, which stem from the same cell origin. As the second most frequently reported cervical cancer, the incidence of ASC ranges between 3.6% and 25% among all cervical cancers. The prevalence of ASC is higher in young women. ASC metastasizes to pelvic lymph nodes twice as often as SCC or adenocarcinoma (AC). ASCs and pure ACs of the uterine cervix have been mostly associated with a poorer prognosis [1]. Today, there has been a tendency to classify cervical neoplasms as HPV-related and unrelated rather than their histopathological types. So, the treatment and follow-up algorithm will be determined according to HPV status [5].

The balanced immune response between activator and inhibitor pathways may be disturbed in several malignancies, where the inhibition of the immune system favors tumor growth [6, 7]. It has been very well known that the CD8-positive cytotoxic T cells and the CD4-positive Th1 T cells, along with their typically 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 cells thanks to their expressions of IL-6, TNF, IL-1β and IL-23 [8]. It has also been determined that in advanced stages of malignancies, CD4-positive helper and CD8-positive cytotoxic T cells increase in tumor microenvironment suggesting their antitumoral roles [7]. But a subpopulation of CD4-positive T cells expressing CD25 and FOXP3, termed regulatory T cells (Tregs), play a role in promoting growth and proliferation of tumor cells by inhibiting the immune response against cancer [7]. FOXP3 is a transcription factor and probably the best marker currently available for identifying natural Tregs in humans [9]. Presence of FOXP3-positive cells in tumors has been shown to predict worse prognosis in some tumors such as cervical cancer, T-cell lymphoma, bladder cancer, lung cancer, and breast cancer [6, 7, 9, 10].
Programmed death-ligand-1 (PD-L1) is a member of the B7 superfamily that contains the most critical regulator on T-cell responses [9]. Programmed cell death-1 (PD-1) is expressed on the surface of various immune cells including T-lymphocytes. PD-1 is activated by its ligands, PD-L1 or PD-L2, and expressed by antigen-presenting cells such as macrophages and B-lymphocytes [10–12]. The interaction between PD-L1, and PD-1 attenuates lymphocyte activation, promotes development and function of Tregs, and impairs antitumor T-cell immune response. In summary, the PD-1 pathway plays a major role in the negative regulation of cell-mediated immune responses [9–13]. Recently, it has been determined that PD-L1 is expressed on the surface of some tumor cells and the expression of PD-L1 helps tumor progression by escaping from immune surveillance. Subsequently, special drugs that affect PD-L1 receptors have been developed for the treatment of PD-L1 expressed tumors. Therefore, evaluation of PD-L1 expression is very important for exploring new treatment strategies for tumors with poorer prognosis [14–16].

Until today, many markers have been evaluated as an indicator to reveal the proliferative activity and tumor cell dynamics of uterine cervical lesions. However, FOXP3 and PD-L1 have not yet been widely scrutinized. In this study, we aimed to explore the diagnostic significance of these two markers.

2. Material and method

One hundred and seven patients diagnosed as invasive or noninvasive uterine cervical tumors at the Tepecik Training and Research Hospital were identified using pathology databases. The study was approved by the local Ethics Committee of the hospital (2019/1-9/9 January 2019). The paraffin block most suitable for immunohistochemical (IHC) evaluation was selected via evaluating the archival slides stained with Hematoxylin Eosin. IHC tests were manually performed using the streptavidin-biotin peroxidase method (Invitrogen, Camarillo, CA, USA). Serial 5-µm sections were obtained on slides. These slides were incubated overnight at 60 °C and then deparaffinization procedures were applied. For heat-induced epitope retrieval procedure in a microwave, slides were left for 20 minutes in 10 mM/L EDTA buffer at pH 8.0, cooled at room temperature for 20 minutes, and then blocked against endogenous peroxidase and biotin. Purified monoclonal mouse antibodies against PD-L1 (Abcam, ab205921-PD-L1, RabMabAB, clone 28-8, 1 : 100 dilution) and FOXP3 (Anti-FOXP3 antibody [236A/E7] (ab20034, 1 : 300 dilution) were used. At least two pathologists, uninformed about the clinical characteristics of the patients, performed histopathological assessments. Immune reactivity for PD-L1 was evaluated in consideration of both percentage, and intensity of complete membranous staining of tumor, and cytoplasmic staining of inflammatory cells was also assessed (Figs. 1,2). Immune reactivity for FOXP3 was assessed using a scoring system. FOXP3-positive lymphocytes in tumor microenvironment were counted and categorized as negative (0), weakly (+: 1–9 cells/HPF), moderately (++: 10–50 cells/HPF and strongly (+++: >50 cells/HPF) positive (Fig. 3).

Statistical analysis was performed using the statistical package SPSS 25.0 (IBM, Armonk, NY, USA). In the statistical analysis, for the comparison of the quantitative data chi-square test and for the comparison of non-parametric data, Mann-Whitney U test were used. For the comparison of the measurements in more than 2 groups, a non-parametric Kruskal-Wallis test was utilized. p values of less than 0.05 were considered to be statistically significant.

3. Results

This series consisted of cases with low-grade (n = 30: 28%) and high-grade (n = 29: 27.1%) cervical squamous intraepithelial lesions (SILs), SCC (n = 27: 25.2%), ASC (n = 15: 14%) and AC (n = 6: 5.6%). Patients aged between 20 and 80 years
of age were included in the study. The mean age of all cases was 47.9 ± 12.8 years. The cases with noninvasive lesions (44.01 ± 11.1 years/20 to 66 years) were younger than the cases with invasive carcinomas (52.6 ± 13.2 years/30 to 80 years). However, the mean ages of all invasive tumor groups was fairly similar (SCC: 52.8 ± 14.8; ASC: 52.9 ± 12.1, and AC: 51.5 ± 9.2 years).

In 26 cases (24.3%), membranous expressions of PD-L1 were mainly detected in tumor cells (Fig. 1). PD-L1-positive inflammatory cells were seen in 23 (21.5%) tumor samples (Fig. 2). In 68 (63.6%) cases, FOXP3-positive lymphocytes were detected (Fig. 3). The mean number of FOXP3-positive cells was 18.4 ± 15.4 (range, 2–70) on each HPF. The median number of PD-L1-positive inflammatory cells was 6.8 (range: 1–25) on each HPF. On each HPF, the percentage of PD-L1-positive tumor cells ranged between 1 and 70 (mean: 18.9 ± 18.3). There was no statistically significant correlation between the age and presence of Tregs ($p = 0.162$). However, statistically significant correlations were determined between age and PD-L1 expression ($p = 0.042$); invasive potential and PD-L1 or FOXP3-positivity ($p < 0.001$), and the coexistence of PD-L1 and FOXP3 expressions ($p < 0.01$) (Fig. 4). Most importantly, it was found that expressions of PD-L1 and FOXP3 varied according to the tumor subtypes (Figs. 5,6). Statistically, the expressions of both PD-L1 and FOXP3 have been significantly upregulated in SCCs when compared with SILs, ASCs, or ACs ($p < 0.001$) (Fig. 7).

4. Discussion

PD-1/PD-L1 pathway and Treg cells are important in maintaining peripheral tolerance in tumors [16–18]. PD-1 and PD-L1 play a leading role in the escape of tumor cells from the host immune system [15, 18, 19]. PD-L1 is also effective in the formation, proliferation, and maintenance of FOXP3 expression in Treg cells. This way, PD-L1 indirectly suppresses antitumoral activity via induction of Treg cells. Treg cells mediate suppressing effector T cells and as well as, inhibit immune-mediated damage [20]. Some researchers have suggested that FOXP3-positive T cells in humans are heterogeneous and contain both suppressor Treg cells and non-suppressor simple T cells. Non-suppressor simple T cells are natural CD4 + T cells that show transient FOXP3 expression by stimulation of T cell receptors (TCRs). PD-L1 can inhibit T cell responses by converting naïve CD4 T cells to induced Treg (iTreg) cells, indicating that PD-L1 has a pivotal role in regulating iTreg cell development and sustaining iTreg cell function [10, 21, 22]. In the present study, we could not perform immunohistochemical staining with antibodies as CD4, CD8, CD3, CD20, to reveal the exact combination of immune cells infiltrating the tumor. Therefore, we do not know whether some of the FOXP3-negative cells are different from T cell subtypes that show transient expression. We think that this situation is the most important limitation of this study.

Recently, major developments have been performed in the understanding of cancer immunology. It has been determined that the immune checkpoint inhibitors targeting PD-1 or PD-L1 can suppress the tumoral progression and be used in the treatment of some cancers. It was previously reported that the response to anti-PD-L1 immunotherapy correlates with intense staining of tumor cells with immune markers as CD4, CD8, CD3, CD20, to reveal the exact combination of immune cells infiltrating the tumor. Therefore, the treatment of tumors with immune checkpoint inhibitors has only been approved if there is intense staining of tumor cells in most country. In clinical studies, PD-L1 expression in cancer was mostly studied at the protein level using immunohistochemical tests (IHC). However, there are discordant results in the rate and intensity of PD-L1 expressions in different studies. The main reason for these differences is some limitations about standardization of the IHC of PD-L1. There are many PD-L1 antibodies that lack specificity and reproducibility in use [25]. The optimal positivity cut-off value has not been defined yet, and the interpretation of staining intensity is influenced by subjectivity.

The previous studies also revealed that patients with IHC-positive tumors may not respond to treatment [25]. A dif-
different opinion related to the mechanism of this resistance is the proliferation of CD8+ T cells infiltrating the tumor has an impact on the treatment [8, 13, 24–26]. In a recent study, Koh et al. [19] reported that even though tumoral PD-L1 expression has a high rate in patients with non-small cell lung cancer, the presence of Tregs in tumor microenvironment leads to immunotherapy resistance. A significant positive correlation between the expressions of PD-L1 and FOXP3. The use of immune checkpoint inhibitors has not been widely approved for the treatment of cervix cancers and few experimental studies have yet been done [27–33]. Considering the findings of Koh et al. [19], the presence of FOXP3 positive lymphocytes can be regarded as an indicator of resistance to treatment. However, as the accumulation of knowledge about PD-L1 expression rate in uterine cervical neoplasms increases, new immunomodulatory therapy options will arise, especially for cases with advanced cervical cancers.

As it is known, the accepted positivity limits for some anti-PD-L1 primary antibodies such as 28-8, 22C3 coded antibodies of the DAKO/Agilent company; and SP142, SP263 coded antibodies of the Ventana Company are clear [22]. Even for some immunomodulatory drugs, the use of special antibodies is recommended in immunohistochemical tests in which PD-L1 positivity is investigated. These are 28–8 for Nivolumab, 22C3 for Pembrolizumab, SP142 for Atezolizumab, and SP263 for Durvalumab [22]. However, the antibody used in this study has not been validated in that way. Instead of determining the cut-off value like these antibodies, we directly used the positivity rate in both tumor and inflammatory cells in statistical analysis.

In our study, most invasive tumors expressed PD-L1 to varying degrees. Also, we found a higher mean PD-L1 expression in invasive tumor cells compared to SILs. As indicated in a study by Ring et al. [27] we have not found any statistically significant correlation between tumor cells and the surrounding peritumoral microenvironment regarding

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**Fig. 5. Immunohistochemical expression of PD-L1.** (A) strong membranous PD-L1 positivity of a SCC and (B) negativity of adenocarcinoma. Presence of only a few PD-L1-positive cells in stroma (DAB × 200).

**Fig. 6. FOXP3 expression in microenvironment of a SCC (A) and an AC (B) (DAB × 200).**

**Fig. 7. Percentages of both PD-L1 expressing tumor cells and inflammatory cells were higher in invasive neoplasms (p < 0.01).**
PD-L1 expression [27]. These findings suggest that invasive cervical cancers, especially SCCs may be appropriate candidate tumor types for the therapy with immune checkpoint inhibitors. Therefore, invasive cervical carcinomas must be evaluated for PD-L1 expression. While most cervical cancers, especially SCCs express membranous PD-L1, dichotomous classification as PD-L1 positive and negative cases may not be sufficient in the identification of patients that may benefit from these therapies. Because the percentage of tumor cells expressing PD-L1 in the tumor must be above a certain limit in order to benefit from immune checkpoint inhibitor therapy. Some authors have reported various PD-L1 staining intensities in tumor cells which were higher in SCCs than ACs [27, 28]. In the present study, we also found that PD-L1 expressions were predominantly confined to invasive tumors, especially SCCs.

FOXP3, forkhead/winged-helix transcription factor, appears to be the most specific and reliable surface marker of Tregs [10]. Furthermore, FOXP3-positive Tregs found in a number of human tumors are considered as biomarkers and prognostic factors for human malignant tumors [10, 31–33]. Improved understanding of the molecular mechanisms that regulate/modulate the host response to tumors has led to the identification of checkpoint signaling pathways that limit the antitumor immune response [7–10]. Understanding the mechanisms of Tregs in cancer immunity in various cancers will provide valuable information on new tumor immunology-based targeted therapies [31–33]. Still new information about the role of Tregs 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 elimination or blocking of Tregs in tumor or circulation [10]. In this study, the number of FOXP3-positive cells was significantly higher in invasive cervical tumors than SILs. We thought that as an indicator for invasive potential, FOXP3-positive cells in the tumor microenvironment are associated with PD-L1 positivity.

The most important limitation of this study is the lack of data concerning the follow-up and clinical characteristics of the patients. The follow-up data of the patient population was not available, and all data was collected retrospectively. This is basically a histopathological study. We determined that PD-L1 and FOXP3 expressions changed in two basic histopathological observable issues. One of them was the invasion and the other was squamous differentiation. It is very well known that there was extensive knowledge of cervical neoplasms. Malignant transformation rate in LSIL and HSIL patients, how cervical neoplasia progress according to their types and stages, the effect of HPV infection on uterine cervical carcinogenesis, and survival rates of all these situations have been settled at fixed rates worldwide. Therefore, even without follow-up, such a study in which the expression rates of FOXP3 and PD-L1 are revealed, especially in patients with cervical ACS and AC, which are rare, may contribute to the knowledge base on this subject.

5. Conclusions
In conclusion, the blockade of the PD-1/PD-L1 signaling pathway is one of the most promising immunotherapeutic strategies in boosting the immune system to fight against cancer [10]. Blocking PD-L1 on tumor-infiltrating lymphocytes (TILs) or tumor cells results in the recovery of the functions of tumor-specific T cells. The restoration of T cells can induce direct killing of tumor cells and secretion of immunostimulatory cytokines. Although inhibition of PD-L1 is shown to produce beneficial results in different studies, this effect is not seen homogeneously in the whole population. This undesirable situation may be due to the failure of the tumor to express PD-L1 at a sufficiently higher rate and concentration.

Future trials should include a more precise definition of PD-L1 positivity based on location, extent, and intensity of PD-L1 expression in different tumors. The findings of the present study point to a key role of PD-L1 in immune escape of cervical cancer and provide a rationale for therapeutic targeting of the PD-L1 pathway [10, 27–30].

Author contributions
SS, Protocol/project development, Data collection. GD, Project development, Manuscript writing, Data analysis. DA, Protocol/Data collection. DSK, Data Collection, Manuscript writing. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study was approved by the Local Ethics Committee of the Hospital (2019/1-9/9 January 2019). Our study is a retrospective study performed in archive paraffin block sections, patient identities are hidden and there is no patient monitoring. Therefore it is not necessary to obtain informed consent from patients specific for this study. Nevertheless general informed consent was obtained from patients before the surgical operation for use their specimens for scientific studies.

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Conflict of interest
The authors declare no conflict of interest.

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