



High expression of CD8 in the tumor microenvironment is associated with PD-1 expression and patient survival in high-grade serous ovarian cancer

Tümör mikroçevresinde CD8'in yüksek ekspresyonu, yüksek dereceli seröz over kanserinde PD-1 ekspresyonu ve hasta sağkalımı ile ilişkilidir

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¹University of Health Sciences Turkey, Kanuni Sultan Süleyman Training and Research Hospital, Clinic of Obstetrics and Gynecology, İstanbul, Turkey

²University of Health Sciences Turkey, Gazi Yaşargil Training and Research Hospital, Clinic of Obstetrics and Gynecology, Diyarbakır, Turkey

³İstanbul Medipol University Faculty of Medicine, Department of Medical Oncology, İstanbul, Turkey

⁴University of Health Sciences Turkey, Prof. Dr. Cemil Taşcıoğlu City Hospital, Clinic of Gynecologic Oncology, İstanbul, Turkey

⁵İnönü University Faculty of Medicine, Department of Obstetrics and Gynecology, Malatya, Turkey

⁶University of Health Sciences Turkey, Kanuni Sultan Süleyman Training and Research Hospital, Clinic of Gynecologic Oncology, İstanbul, Turkey

⁷Kocaeli University Faculty of Medicine, Department of Gynecologic Oncology, Kocaeli, Turkey

⁸Aydın Adnan Menderes University Faculty of Medicine, Department of Gynecologic Oncology, Aydın, Turkey

Abstract

Objective: The current study assesses programmed death-1 (PD-1) receptor expression and CD3, CD4, and CD8 tumor-infiltrating lymphocytes (TILs) in high-grade serous ovarian cancer (HGSOC) and associates our results with neoadjuvant chemotherapy history and disease prognosis.

Materials and Methods: We included cases diagnosed with primary HGSOC with biopsy or surgical resection materials in this study. The immunoreactivity of CD3, CD4, CD8, and PD1 was assessed immunohistochemically in tumor tissue. We analyzed TILs in two predetermined groups of high and low TIL. The relationships between clinical characteristics, PD-1, and TIL were assessed by the χ^2 test or Fisher's Exact test. We used Kaplan-Meier survival analysis and Cox proportional hazards regression model to the connection between survival and the amounts of TIL, and PD1.

Results: Univariate analysis demonstrated that optimal debulking ($p<0.001$), early International Federation of Gynecology and Obstetrics stage ($p=0.046$), and higher scores of stromal CD8+ TIL expression ($p=0.028$) in tumor cells were all substantially correlated with longer disease-free survival (DFS), whereas the remaining variables analyzed, including PD-1 positivity, stromal CD3+, and CD4+ TILs, and intraepithelial CD3+, CD4+, and CD8+ TILs, were not correlated with DFS. Also, univariate analysis revealed that optimal debulking ($p=0.010$), and higher scores of stromal CD8+ TIL expression ($p=0.021$) in tumor cells were all substantially correlated with longer overall survival (OS).

Conclusion: Higher scores of stromal CD8+ TILs are substantially correlated with DFS and OS in univariate analyses, whereas scores of stromal CD3+ and CD4+ TILs, and intraepithelial CD3+, CD4+, and CD8+ TILs are not correlated with DFS and OS in both univariate and multivariate analyses. Also, we found a significant association between PD-1 positivity and the scores of stromal CD3+ TILs and intraepithelial CD8+ TILs. However, no remarkable relationship was revealed between PD-1 positivity and the survival of HGSOC cases.

Keywords: High-grade serous ovarian cancer, programmed death-1 receptor, tumor-infiltrating lymphocytes

PRECIS: High expression of CD8+ TILs in the tumor microenvironment is connected with PD-1 expression and longer patient survival in high-grade serous ovarian cancer.

Address for Correspondence/Yazışma Adresi: Süleyman Cemil Oğlak MD,

University of Health Sciences Turkey, Gazi Yaşargil Training and Research Hospital, Clinic of Obstetrics and Gynecology, Diyarbakır, Turkey

Phone: +90 506 402 11 57 **E-mail:** sampson_21@hotmail.com **ORCID ID:** orcid.org/0000-0001-7634-3008

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Öz

Amaç: Çalışmamızın amacı, yüksek dereceli seröz over kanserinde (HGSOC) programlanmış ölüm-1 (PD-1) reseptör ekspresyonunu ve CD3, CD4 ve CD8 tümör infiltrate edici lenfositleri (TIL) değerlendirmek ve bulgularımızın neoadjuvan kemoterapi öyküsü ve hastalık prognozu ile ilişkisini incelemektir.

Gereç ve Yöntemler: Biyopsi veya cerrahi rezeksiyon materyalleri ile primer HGSOC tanısı alan olgular çalışmaya dahil edildi. CD3, CD4, CD8 ve PD1'in immünoreaktivitesi, tümör dokusunda immünohistokimyasal olarak değerlendirildi. TIL, önceden tanımlanmış iki grup olan düşük ve yüksek TIL grubunda analiz edildi. Klinik özellikler, PD-1 ve TIL arasındaki ilişkiler χ^2 testi veya Fisher's Exact test ile değerlendirildi. TIL, PD1 ve hayatta kalma arasındaki ilişki için Kaplan-Meier hayatta kalma analizi ve Cox oransal hazard regresyon modeli kullanıldı.

Bulgular: Tek değişkenli analiz, tümör hücrelerinde optimal debulking ($p<0,001$), erken Uluslararası Jinekoloji ve Obstetrik Federasyonu evresi ($p=0,046$) ve daha yüksek stromal CD8+ TIL ekspresyonu skorlarının ($p=0,028$) tümünün daha uzun hastalısız sağkalım (DFS) ile önemli ölçüde ilişkili olduğunu gösterdi; oysa ki kalan değişkenler, PD-1 pozitifliği, stromal CD3+ ve CD4+ TIL'ler ve intraepitelyal CD3+, CD4+ ve CD8+ TIL'ler dahil olmak üzere, analiz edildiğinde DFS ile korele değildi. Ayrıca, tek değişkenli analiz, tümör hücrelerinde optimal debulking ($p=0,010$) ve daha yüksek stromal CD8+ TIL ekspresyonu skorlarının ($p=0,021$) tümünün daha uzun genel sağkalım (OS) ile önemli ölçüde ilişkili olduğunu ortaya koydu.

Sonuç: Daha yüksek stromal CD8+ TIL skorları, tek değişkenli analizde DFS ve OS ile anlamlı şekilde ilişkiliyken, stromal CD3+ ve CD4+ TIL'lerin ve intraepitelyal CD3+, CD4+ ve CD8+ TIL'lerin skorları, hem tek değişkenli hem de çok değişkenli analizlerde DFS ve OS ile ilişkili değildi. Ayrıca, PD-1 pozitifliği ile stromal CD3+ TIL'lerin ve intraepitelyal CD8+ TIL'lerin skorları arasında anlamlı bir ilişki bulundu. Ancak, PD-1 pozitifliği ile HGSOC hastalarının sağkalımı arasında anlamlı bir ilişki gözlenmedi.

Anahtar Kelimeler: Yüksek dereceli seröz over kanseri, programlanmış ölüm-1 reseptörü, tümör infiltrate edici lenfositler

Introduction

Globally, epithelial ovarian cancer is the third most frequent gynecologic cancer but has the highest death rate among gynecologic malignancies. Annually 313,000 women (3.4% of all cancer patients) are diagnosed with ovarian cancer worldwide and it is estimated to cause more than 200,000 deaths (4.7% of all cancer patients) occurred every year⁽¹⁾. Among these cancers, high-grade serous ovarian cancer (HGSOC) constitutes 80-85% of these cancers and represents the highest mortality rate⁽²⁾. Due to the absence of effective screening methods and its non-specific early symptoms, most of the patients are diagnosed at advanced stages, with a five-year survival rate below 45%⁽³⁻⁵⁾. Various variables have been elucidated for the prognosis of HGSOC patients such as the stage at diagnosis, the extent of debulking surgery, and chemotherapy response⁽⁶⁾. Despite the considerable advances in complete debulking surgery, chemotherapeutic drugs, and targeted agents, survival ratios for ovarian cancer have unsatisfactorily improved over the past few decades and most patients have experienced drug resistance and cancer progression⁽⁷⁾. Thus far, no useful biochemical markers have been detected that might accurately predict the treatment response and survival of HGSOC. Therefore, there is an imperative requirement for superior plans for early recognition, prediction of prognosis, and efficient treatments to improve clinical consequences.

Adaptive and innate immune system cells perform a crucial role in eliminating cancer cells and have a significant impact on clinical outcomes in cancer patients. Cancer cells induce alterations in the immune context of the patient to regulate a supportive but definitely immunosuppressive tumor microenvironment (TME), producing neoantigens that interest various cells of the immune system⁽⁸⁾. Emerging studies indicate that ovarian cancer is frequently accompanied by a greatly systemic immunosuppressive TME^(9,10). This is the crucial element compromising the success rate of anticancer

immunotherapy⁽¹⁰⁾. Patients who display a powerful immune response demonstrate a superior chemotherapy response and improved survival rates⁽⁹⁾. Tumor-infiltrating lymphocytes (TILs) are an essential constituent of the cellular immune system and crucial for cell-mediated anti-tumor immune responses⁽¹¹⁾. There are various types of TILs with different functions. TILs consist of all lymphocytic cell populations that have left the vasculature and migrated to tumors. These cells are localized in the peritumoral space and the tumor islet (intraepithelial), and display an endogenous anti-cancer immune response⁽¹²⁾. TILs are identified by the cell surface expression of diverse molecular biomarkers such as CD3, CD4, and CD8, and demonstrate significantly different antitumor activities and spatial distribution among tumor areas⁽¹³⁾. After the recognition of specific antigens in MHC-I, cytotoxic CD8 T lymphocytes (immune system's classic killers) kill target cells by expressing cytokines such as interferon- γ (IFN- γ) and tumor necrosis factor (TNF) and enzymes like perforin and granzyme-B. Conversely, CD4 T-cells are infrequently cytotoxic and instead promote other cells' recruitment and activation, including macrophages, B-cells, dendritic cells, and other T-cells⁽¹⁴⁾. TILs have been described in many solid tumors, and are considered to display a crucial role in mediating the chemotherapy response and improving survival in virtually entire solid tumor types, including ovarian cancer⁽¹²⁾. Ovarian carcinoma is an immunogenic disorder that is recognized and attacked by the immune system, and TILs are considered to recognize cancer cells to cause an immune response⁽¹⁵⁾. Numerous studies have concluded that the existence of TILs is significantly correlated with favorable prognoses in HGSOC cases^(8,13). However, there have also been some studies with conflicting results⁽⁹⁾. TILs might inhibit the immune response effectiveness due to adaptive immune resistance⁽¹⁶⁾. The inconsistency in results proposes that the prognostic value of TILs in patients with HGSOC remains controversial.

Measuring the levels of TILs extensively used the procedure to obtain detailed information on the interplay between the TME and the immune system. The current study assesses programmed death-1 (PD-1) expression and CD3+, CD4+, and CD8+ TIL in HGSOE and to associate our results with neoadjuvant chemotherapy (NACT) history and disease prognosis.

Materials and Methods

Patients

A total of 268 HGSOE patients were assessed and treated at the Kanuni Sultan Süleyman Training and Research Hospital, Department of Gynecologic Oncology, and partly Medipol University Department of Medical Oncology between February 2001 and April 2020, and 127 patients had adequate tumor samples and clinical data for analysis. We classified tumors histologically based on the criteria of the World Health Organization and staged based on the International Federation of Gynecology and Obstetrics (FIGO) system.

All patients with suspected advanced stage HGSOE were evaluated by a gynecologic oncologist before the beginning of treatment whether these cases were suitable for primary complete debulking surgery. Patients who had a low probability of achieving cytoreduction to <1 cm or had a high perioperative risk profile received NACT⁽¹⁷⁾. Maximal debulking surgery was defined as no visible disease remaining at the completion of the surgery. Optimal debulking surgery was defined as one or more tumor nodules <1 cm in maximal dimension remaining at the end of the surgical procedure. Suboptimal debulking surgery was described as any residual tumor nodule >10 mm in maximal dimension remaining at the end of the surgery⁽¹⁸⁾. All patients who experienced primary surgery and neoadjuvant and/or adjuvant platinum-based first-line chemotherapy were applied according to the stage. We obtained all the primary tumor tissue samples at the primary surgery time. The study was designed retrospectively and detailed clinical data of the cases were recorded from the patient's medical charts. The study was approved by the Medipol University Ethics Committee Resolution 10840098-604.01.01-E.17851, dated July 26, 2020.

Immunohistochemical Analysis of TIL and Scoring

Immunohistochemistry (IHC) was performed using primary antibodies against CD3+, CD4+, CD8+ TILs, and PD-1. Formalin-fixed, paraffin-embedded tissue sections (3 µm thick) were taken on 3-aminopropyltriethoxysilane coated glass slides. The sections were deparaffinized in xylene followed by hydration in graded ethanol. Histologic sections from tumors were stained with hematoxylin and eosin (H&E). For IHC representatives, prestained sections were selected, and 3-µm sections were obtained from paraffin-embedded tumors. CD3 rabbit monoclonal antibody (1:50 dilution, clone EP41, Biocare, Pacheco, USA), CD4 mouse monoclonal antibody (1:100 dilution, clone 4B12, Biocare, Pacheco, USA), CD8

mouse monoclonal antibody (1:100 dilution, clone C8/144B, Biocare, Pacheco, USA) and PD1 mouse monoclonal antibody (1:100 dilution, clone NAT105, Sigma-Aldrich, Germany) staining were performed according to the manufacturer's protocol (Figures 1-3).

The IHC slides were examined by two experienced pathologists under microscopy (Olympus) without any clinical data of the cases. Tumor cells, TILs, and PD-1 positivity were evaluated in H&E-stained sections.

The CD3, CD4, and CD8 protein staining was assessed based on the International TIL Working Group 2014 recommendations. Briefly, the assessment of TILs was carried out by manual visual evaluation of the percentage area covered by lymphocytes, after a standardized procedure⁽¹⁹⁾. Accordingly, the area within the tumor border is selected, the stromal and intra-tumoral compartments are defined, only mononuclear lymphocytic infiltrate areas are included, and the percentage area is evaluated at low (4x) and high (10x) magnification. Patients were identified to have a high expression of CD3, CD4, or CD8 TILs if these cases had ≥10% positive staining cells/HPF whereas low expression of TILs if these cases had <10% positive staining cells/HPF. We scored the stromal and intratumoral compartments separately. The stromal and intratumoral CD3, CD4, or CD8 TILs were classified into two groups for the analysis of low TIL (<10%) and high TIL (≥10%).

Tumor samples stained with anti- PD-1 were scored according to the intensity of cytoplasmic and/or membranous positivity as follows: 0 (no staining), 1 + (weak or equivocal staining), 2 +

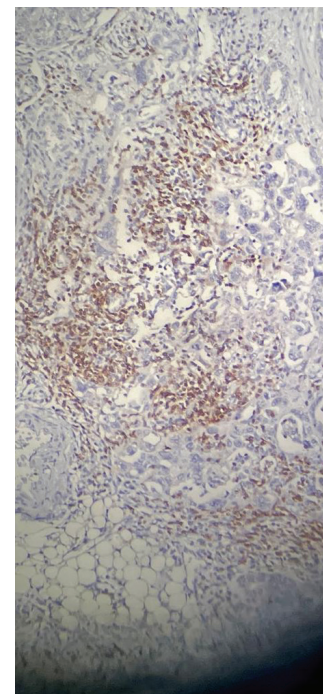


Figure 1. Stromal and intraepithelial CD3+ tumor-infiltrating lymphocytes (IHC, x200)

IHC: Immunohistochemistry

(moderate staining), or 3 + (strong staining). Tumor cells and micro-environment were considered as positive for staining if they had more than 5% staining⁽²⁰⁾.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY). We

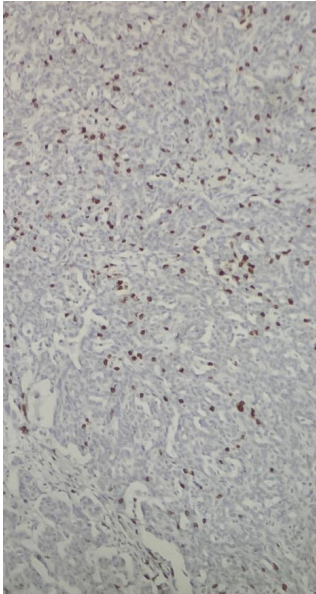


Figure 2. Intraepithelial CD4+ tumor-infiltrating lymphocytes (IHC, x200)

IHC: Immunohistochemistry

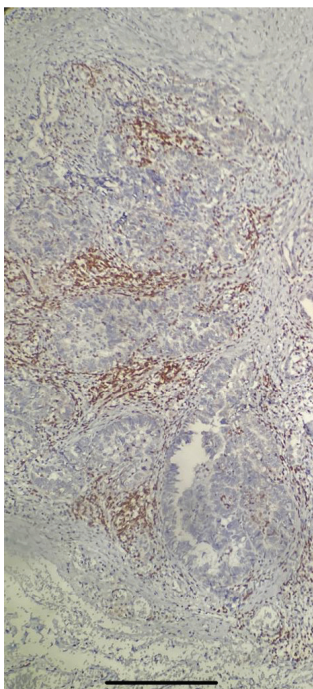


Figure 3. Stromal CD8+ tumor-infiltrating lymphocytes (IHC, x200)

IHC: Immunohistochemistry

used descriptive statistics to summarize baseline features. We used Fisher's Exact test and Pearson chi-square (χ^2) test to determine the relationship between clinicopathological factors and TIL status in patients with serous ovarian cancer. We made survival analysis via Kaplan-Meier analysis and comparisons via the Log-Rank test. We defined disease-free survival (DFS) as the date from diagnosis or curative surgery to recurrence or disease progression or the time of death or loss in the follow-up. We described overall survival (OS) as the date from diagnosis to the time of the patient's death or loss in the follow-up. Univariate analysis was performed to evaluate the significance of clinicopathological characteristics as prognostic factors. Subsequently, we performed a multivariate analysis with the Cox proportional hazards model to detect the independent prognostic factors for both DFS and OS. We used multivariate p-values to describe the independence of these factors. Data were presented as mean \pm standard deviation, median (minimum-maximum), 95% confidence interval, and percentage (%) where appropriate. P-values <0.05 were considered statistically significant.

Results

The demographic and basic data of the participants are listed in Table 1. Of the study cohort of 127 cases, the median age at diagnosis was 55 years with a range approximately 26-83 years. Most cases (n=89, 71.2%) presented FIGO stage III or IV. Within the 127 cases, 77.4% (n=96) of them received NACT. Of these, 89 cases presented with advanced stage of disease, and 7 patients had a high perioperative risk profile. Maximal debulking was achieved in 75.6% (n=93), and optimal debulking was achieved in 16.3% (n=20) patients. The median OS was 37.5 months (ranging between 3.7 and 94.9 months) for the entire study population.

The relationship between clinicopathological factors and PD-1 expression in patients with serous ovarian cancer is presented in Table 2. PD-1 was detected in intraepithelial and stromal TILs in 66.9% (n=85) of cases. PD-1 positive and PD-1 negative groups were similar regarding age, receiving NAC, surgery type, tumor grade, and FIGO stage at diagnosis. The PD-1-positive group had significantly higher numbers of stromal CD3+ TILs and intraepithelial CD8+ TILs than the PD-1-negative group (p=0.043, p=0.003, respectively). However, we found no association between PD-1 positivity and the scores of stromal CD4+ (p=0.073) and CD8+ TILs, and intraepithelial CD3+ and CD4+ TILs.

We summarize univariate and multivariate analyses of risk factors for DFS in cases of serous ovarian cancer in Table 3. Univariate analysis demonstrated that optimal debulking (p<0.001), early FIGO stage (p=0.046), and higher scores of stromal CD8+ TIL expression (p=0.028) in tumor cells were all substantially correlated with longer DFS, whereas the remaining variables analyzed, including PD-1 positivity, stromal CD3+, and CD4+ TILs, and intraepithelial CD3+, CD4+, and CD8+

TILs, were not correlated with DFS. Multivariate analysis showed that optimal debulking ($p < 0.001$) was an independent prognostic variable for DFS, whereas FIGO stage and higher scores of stromal CD8+ TILs ($p = 0.055$) were unsubstantial predictive factors for DFS in multivariate analysis. Although slightly far from statistical significance at a 0.05 threshold, a comparable tendency was detected in the DFS analysis; cases with CD8+ tumors seemed to have better DFS than those with CD8-negative tumors ($p = 0.055$).

Univariate and multivariate analyses of risk factors for OS in patients with serous ovarian cancer are shown in Table 4. Univariate analysis revealed that optimal debulking ($p = 0.010$), and higher scores of stromal CD8+ TIL expression ($p = 0.021$) in tumor cells were all substantially correlated with longer OS, whereas the remaining variables analyzed, including FIGO stage, PD-1 positivity, stromal CD3+, and CD4+ TILs, and intraepithelial CD3+, CD4+, and CD8+ TILs, were not associated with OS. Multivariate analysis showed that only optimal debulking ($p = 0.026$) was an independent prognostic variable for OS, whereas a higher score of stromal CD8+ TIL expression was not a significant predictive factor for OS in multivariate analysis.

Table 1. Demographic and basic patient information

| Characteristics (units) | Average (range) n=127 |
|---|--------------------------|
| Age (years) | 55 (26-83) |
| Preop plasma CA125 (IU/L) | 210 (4-9777) |
| FIGO | n (%) |
| Stage I/II | 36 (28.8) |
| Stage III/IV | 89 (71.2) |
| Surgery type | n (%) |
| Maximal debulking | 93 (75.6) |
| Optimal debulking | 20 (16.3) |
| Suboptimal debulking | 8 (6.5) |
| Inoperable | 2 (1.6) |
| Neoadjuvant therapy | n (%) |
| Absent | 28 (22.6) |
| Present | 96 (77.4) |
| Tumor grade | n (%) |
| 1 | 10 (8) |
| 2 | 29 (23.2) |
| 3 | 86 (68.8) |
| PD-1 expression | n (%) |
| Negative | 42 (33.1) |
| Positive | 85 (66.9) |
| Median OS (months) | 37.5 (3.73-94.93) |
| OS: Overall survival, FIGO: International Federation of Gynecology and Obstetrics | |

Figures 4 and 5 demonstrate the Kaplan-Meier curves of median DFS and median OS regarding the scores of stromal CD8+ TILs, respectively. Cases in the stromal CD8-high TIL group had substantially longer DFS (28.3 months) and OS (83.8 months) than those in the CD8-low TIL group (15.3 months and 65.7 months, $p = 0.028$ and $p = 0.021$, respectively).

Discussion

In this study, we investigated the effects of CD3, CD4, and CD8 T-cell status on survival in patients with HGSOc. Also, we clarified PD-L1 expression and TIL infiltration in HGSOc cases. As evidenced by our study, stromal CD8+ TILs alone were indicative of improved survival in patients with HGSOc. However, the presence of intraepithelial CD3+, CD4+, and CD8+ TILs, and stromal CD3+ and CD4+ TILs were not associated with the prognosis of ovarian cancer.

The heterogeneity of stromal TILs with cancer cell proliferation, invasion, and matrix remodeling that drive carcinogenesis ending in diverse survival periods generates a specific TME. Cancer cells interact with their TME both to induce tumorigenic inflammation and suppress T-cell activation⁽²¹⁾. Transformed tumor cells, as a source of neoantigens or tumor-related antigens, might stimulate the immune response, eventually ending in tumor cell elimination by TILs⁽²²⁾. The density, subtype, and location of TILs are determining factors of the prognostic importance of TILs in ovarian malignancy⁽²³⁾. Considering the beneficial treatments, particularly immunotherapy modalities to TILs, it must assess the presence and density of TILs and realize tumor-immune system interactions in ovarian malignancy⁽²⁴⁾. Various studies have reported explored the prognostic significance of TILs in ovarian cancer. Despite these studies using biomarkers to clarify the diverse subtypes of TILs that impact prognosis and survival, the outcomes were indicated have still been inconsistent⁽²⁵⁾. The possible reasons for inconsistent findings might involve sample size, tumor heterogeneity, tumor type, clinical stage, variations in the regions of the study cohort, and the technique for specimen processing⁽¹⁵⁾.

CD3 antigen, known as pan T-cell marker, is a receptor glycoprotein that exists in mature lymphocytes. Zhang et al.⁽²⁶⁾ reported that the increased expression of VEGF was related to the lack of CD3+ TILs, and thus early recurrence and short survival. They found that the CD3+ TILs associated with improved OS and PFS in ovarian cancer patients⁽²⁶⁾. However, in our study, similar to the study of Sato et al.⁽²⁷⁾ and Lo et al.⁽²⁸⁾, neither stromal nor intraepithelial CD3+ TILs alone were connected with the survival of patients with ovarian cancer.

CD4+ TILs exhibit a different variety of antitumor immune responses. CD4+ T-helper 1 (Th1) cells secreting cytokines, including TNF and IFN- γ might efficiently suppress angiogenesis and promote the proliferation and activation of CD8+ TILs. In contrast, CD4+ T-regulatory (Treg) cells display tumor-promoting activity by inhibiting Th1 cell function and inhibiting autoimmunity development⁽⁸⁾. Pinto et al.⁽⁹⁾ found

Table 2. Relationship between clinicopathological factors and PD-1 in patients with serous ovarian cancer

| Factors | n (%) | PD-1 positive n (%) | PD-1 negative n (%) | p-value |
|----------------------------|------------|---------------------|---------------------|--------------|
| All patient | 127 (100) | 85 (66.9) | 42 (33.1) | |
| Age (year) | | | | |
| <50 | 38 (29.9) | 29 (76.3) | 9 (23.7) | 0.156 |
| >50 | 89 (70.1) | 56 (62.9) | 33 (37.1) | |
| NAC | | | | |
| Absence | 96 (77.4) | 64 (66.7) | 32 (33.3) | 1.0 |
| Presence | 28 (22.6) | 19 (67.9) | 9 (32.1) | |
| Surgery type | | | | |
| Maximal debulking | 93 (75.6) | 65 (69.9) | 28 (30.1) | 0.287 |
| Optimal debulking | 20 (16.3) | 13 (65) | 7 (35) | |
| Suboptimal debulking | 8 (6.5) | 3 (37.5) | 5 (62.5) | |
| Inoperable | 2 (1.6) | 1 (50) | 1 (50) | |
| Tumor grade | | | | |
| 1 | 10 (8) | 8 (80) | 2 (20) | 0.621 |
| 2 | 29 (23.2) | 20 (69) | 9 (31) | |
| 3 | 86 (68.8) | 56 (65.1) | 30 (34.9) | |
| FIGO stage | | | | |
| I-II | 36 (28.8) | 26 (72.2) | 10 (27.8) | 0.531 |
| III-IV | 89 (71.2) | 58 (65.2) | 31 (34.8) | |
| Stromal CD3 | | | | |
| Low TILs | 39 (30.7) | 21 (53.8) | 18 (46.2) | 0.043 |
| High TILs | 88 (69.3) | 64 (72.7) | 24 (27.3) | |
| Stromal CD4 | | | | |
| Low TILs | 107 (84.3) | 68 (63.6) | 39 (36.4) | 0.073 |
| High TILs | 20 (15.7) | 17 (85) | 3 (15) | |
| Stromal CD8 | | | | |
| Low TILs | 80 (63) | 57 (71.3) | 23 (28.8) | 0.241 |
| High TILs | 47 (37) | 28 (59.6) | 19 (40.4) | |
| Intraepithelial CD3 | | | | |
| Low TILs | 45 (35.4) | 27 (60) | 18 (40) | 0.241 |
| High TILs | 82 (64.6) | 58 (70.7) | 24 (29.3) | |
| Intraepithelial CD4 | | | | |
| Low TILs | 104 (81.9) | 69 (66.3) | 35 (33.7) | 1.000 |
| High TILs | 23 (18.1) | 16 (69.6) | 7 (30.4) | |
| Intraepithelial CD8 | | | | |
| Low TILs | 35 (27.6) | 16 (45.7) | 19 (54.3) | 0.003 |
| High TILs | 92 (72.4) | 69 (75) | 23 (25) | |

PD-1: Programmed death-1, NAC: Neoadjuvant chemotherapy, FIGO: International Federation of Gynecology and Obstetrics, TILs: Tumor-infiltrating lymphocytes

Table 3. Univariate and multivariate analysis of risk factors for DFS in patients with serous ovarian cancer

| Factors | Median DFS time (months) | Univariate p-value | Multivariate p-value | HR (95% CI) |
|----------------------------|--------------------------|--------------------|----------------------|------------------|
| Age (year) | | | | |
| <50 | 23.0 | 0.423 | | |
| >50 | 17.0 | | | |
| NAC | | | | |
| Absence | 16.0 | 0.08 | | |
| Presence | 21.0 | | | |
| Surgery type | | | | |
| Maximal debulking | 28.3 | <0.001 | <0.001 | 1.69 (1.23-2.33) |
| Optimal debulking | 15.7 | | | |
| Suboptimal debulking | 11.4 | | | |
| Inoperable | 6.9 | | | |
| Tumor grade | | | | |
| 1 | NR | 0.055 | | |
| 2 | 25.6 | | | |
| 3 | 16.9 | | | |
| FIGO stage | | | | |
| I-II | 52.4 | 0.046 | 0.160 | 0.60 (0.29-1.22) |
| III-IV | 16.9 | | | |
| PD-1 expression | | | | |
| Negative | 23.5 | 0.64 | | |
| Positive | 18.4 | | | |
| Stromal CD3 | | | | |
| Low TILs | 16.0 | 0.621 | 0.136 | 0.52 (0.22-1.22) |
| High TILs | 18.8 | | | |
| Stromal CD4 | | | | |
| Low TILs | 20.3 | 0.763 | 0.316 | 1.61 (0.63-4.09) |
| High TILs | 15.7 | | | |
| Stromal CD8 | | | | |
| Low TILs | 15.3 | 0.028 | 0.055 | 2.23 (0.98-5.08) |
| High TILs | 28.3 | | | |
| Intraepithelial CD3 | | | | |
| Low TILs | 23.5 | 0.705 | 0.747 | 1.14 (0.50-2.56) |
| High TILs | 16.0 | | | |
| Intraepithelial CD4 | | | | |
| Low TILs | 16.9 | 0.216 | 0.175 | 0.50 (0.19-1.35) |
| High TILs | 32.8 | | | |
| Intraepithelial CD8 | | | | |
| Low TILs | 23.5 | 0.665 | 0.867 | 0.93 (0.39-2.18) |
| High TILs | 16.0 | | | |

PD-1: Programmed death-1, NAC: Neoadjuvant chemotherapy, FIGO: International Federation of Gynecology and Obstetrics, TILs: Tumor-infiltrating lymphocytes, CI: Confidence interval, DFS: Disease-free survival, HR: Hazard ratio

Table 4. Univariate and multivariate analysis of risk factors for OS in patients with serous ovarian cancer

| Factors | Median OS time (months) | Univariate p-value | Multivariate p-value | HR (95% CI) |
|----------------------------|-------------------------|--------------------|----------------------|------------------|
| Age (year) | | | | |
| <50 | 44.9 | 0.799 | | |
| >50 | 47.7 | | | |
| NAC | | | | |
| Absence | 50.5 | 0.111 | | |
| Presence | 37.0 | | | |
| Surgery type | | | | |
| Maximal debulking | NR | 0.010 | 0.026 | 1.56 (1.05-2.33) |
| Optimal debulking | 48.9 | | | |
| Suboptimal debulking | 35.5 | | | |
| Inoperable | 36.9 | | | |
| Tumor grade | | | | |
| 1 | 78.7 | 0.076 | | |
| 2 | 50.5 | | | |
| 3 | 39.9 | | | |
| FIGO stage | | | | |
| I-II | 50.5 | 0.642 | 0.517 | 0.75 (0.27-1.91) |
| III-IV | 45.0 | | | |
| PD-1 expression | | | | |
| Negative | 45.6 | 0.870 | | |
| Positive | 47.7 | | | |
| Stromal CD3 | | | | |
| Low TILs | NR | 0.385 | 0.817 | 0.87 (0.29-2.61) |
| High TILs | 60.8 | | | |
| Stromal CD4 | | | | |
| Low TILs | 72.6 | 0.776 | 0.724 | 0.75 (0.16-3.53) |
| High TILs | NR | | | |
| Stromal CD8 | | | | |
| Low TILs | 65.7 | 0.021 | 0.211 | 1.96 (0.68-5.66) |
| High TILs | 83.8 | | | |
| Intraepithelial CD3 | | | | |
| Low TILs | 72.66 | 0.309 | 0.719 | 1.23 (0.39-3.84) |
| High TILs | 60.8 | | | |
| Intraepithelial CD4 | | | | |
| Low TILs | 72.6 | 0.594 | 0.742 | 0.81 (0.24-2.71) |
| High TILs | 64.4 | | | |
| Intraepithelial CD8 | | | | |
| Low TILs | 72.6 | 0.365 | 0.794 | 1.17 (0.35-3.85) |
| High TILs | 60.8 | | | |

PD-1: Programmed death-1, NAC: Neoadjuvant chemotherapy, FIGO: International Federation of Gynecology and Obstetrics, TILs: Tumor-infiltrating lymphocytes, CI: Confidence interval, DFS: Disease-free survival, HR: Hazard ratio, OS: Overall survival

that CD4+ TILs are the early predictor factors of OS and PFS in patients with HGSOC. However, a recent study conducted in Turkey concluded that the existence of CD4+ TILs was more frequent in advanced stages ovarian cancer patients, and no significant relationship was found between this subtype of TILs and survival⁽²¹⁾. Likewise, we observed no significant association between higher levels of stromal and intraepithelial CD4+ TILs and a favorable prognosis. To demonstrate CD4+ TIL biology in connection with tumor progression in ovarian cancer patients, additional analysis on the functional subtypes of CD4+ TILs, including Th1 and Treg, is required in forthcoming research⁽⁸⁾.

CD8+ TILs perform a central role in immunity to cancer through their capacity to directly kill tumor cells after recognition of specific antigens in MHC-I molecules⁽¹⁴⁾. We demonstrated

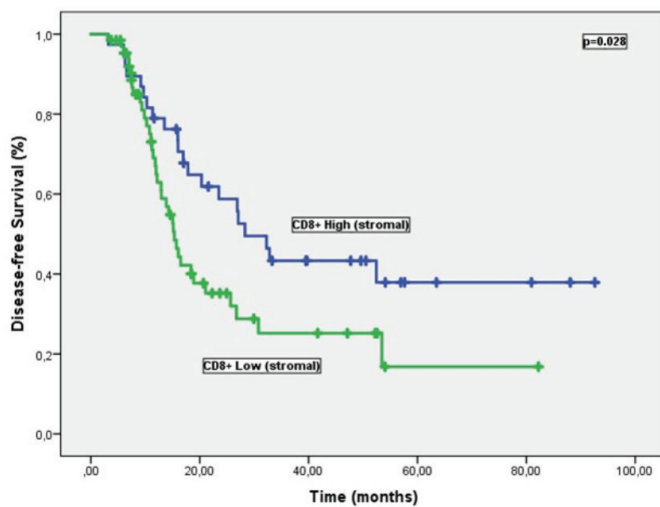


Figure 4. Analysis of disease-free survival regarding stromal CD8 positivity status

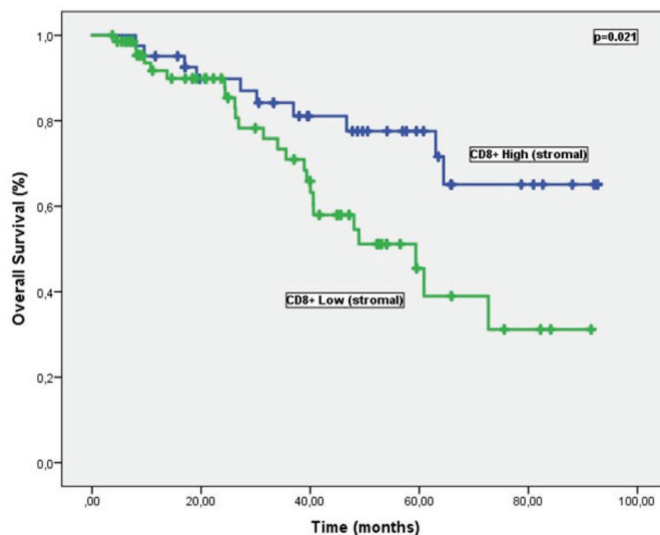


Figure 5. Analysis of overall survival regarding stromal CD8 positivity status

that the stromal CD8+ TIL infiltration was correlated with better DFS and OS in HGSOC patients in univariate analysis. Nonetheless, following adjusting the confounding variables in the multivariate analysis, we did not verify its significance as an outcome predictor of DFS and OS. Even though slightly far from statistical significance at a 0.05 threshold, HGSOC patients with CD8+ TILs seemed to have better DFS than those with CD8-negative cases. Also, we found that intraepithelial CD8+ TILs did not correlate with improved DFS and OS in our cohort of HGSOC patients. In a meta-analysis, Li et al.⁽¹⁵⁾ investigated whether the specific location of CD8+ TILs within the tumor mass is crucial for the prognostic impact on ovarian cancer. They indicated that intraepithelial TILs are related to a favorable prognosis in ovarian cancer, underlining the significance of assessing the localization of TILs within the TME⁽¹⁵⁾. However, Hao et al.⁽⁸⁾ confirmed that both intraepithelial and stromal CD8+ TILs are positively correlated with OS and progression-free survival (PFS) cases with HGSOC. A recently published study on the Turkish population indicated that CD8+ TIL infiltration was connected with advanced stage and worse prognosis in ovarian cancer⁽²¹⁾. Several experiments have been conducted to clarify the mechanisms that provide the location and infiltration of TILs into tumor islets in ovarian cancer⁽²³⁾. However, to date, due to the immune system plasticity and the tumor genome complexity, the precise mechanism remains unclear. Schietinger et al.⁽²⁹⁾ reported that CD8+ TILs might be involved in the destruction of stromal components such as endothelial cells, causing tumor necrosis. Also, this might end in impacts against antigen-negative cancer cells in tumors. Moreover, stromal cells might perform a function in antigen presentation to improve T-cell activity against cancer cells⁽²⁹⁾. Therefore, the stromal accumulation of CD8+ TILs without direct interplay with tumor cells is critical for cancer removal⁽²⁵⁾. Comprehension of the interplay between ovarian cancer and the immune system might be a significant stage toward detecting prognostic gene markers, overcoming drug resistance and providing longer life expectancy of patients with ovarian cancer⁽²⁴⁾.

The PD-1 receptor is expressed by activated T-cells and has two recognized ligands, of which PD-L1 can be expressed by tumor cells and adjacent immune cells in various solid tumors. The binding of PD-L1 to PD-1 inhibits T-cell receptor signaling, resulting in decreased T-cell proliferation and enhanced vulnerability to apoptosis⁽³⁰⁾. The PD-1/PD-L1 pathway is supposed to be the main regulator of tumor-induced immune suppression⁽¹¹⁾. Anti-PD-1 immunotherapy promotes persisted T-cell activity to prevent apoptosis of these cells and is effective in a broad variety of malignancies⁽³¹⁾. However, Hao et al.⁽⁸⁾ and Lo et al.⁽²⁸⁾ indicated that overexpression of PD-1+ TILs was not related to the survival benefit of HGSOC cases. Hao et al.⁽⁸⁾ also stated that the association between survival outcomes and PD-1+ TILs still warrants additional research because of the comprehensively increasing immunotherapy

implications⁽²⁸⁾. Because few studies have been published regarding the association between TILs and PD-1 positivity in ovarian cancer^(30,32-34), we assessed the correlations between PD-1 positivity and the scores of TILs. PD-1-positive cases include substantially greater numbers of stromal CD3+ TILs and intraepithelial CD8+ TILs than in the PD-1-negative cases, whereas no significant associations were observed between PD1 positivity and scores of intraepithelial CD3+ and CD8+ TILs, and stromal CD4+ and CD8+ TILs. We also showed no significant relationship between PD-1 expression and survival outcomes in TILs. Thus, the efficiency of PD-1/PD-L1 blockade in ovarian cancer is comparatively lower than that in melanoma, gastric cancer, and cervical cancer. A possible reason for the comparatively low efficiency of PD-1/PD-L1 blockade in ovarian cancer might be that the cases included in these studies lack existing CD8+ TILs and PD-L1 expression in tumors. Assessment of CD8+ TIL count and PD-L1 expression might be beneficial in the stratification of HGSOC cases for PD-1/PD-L1 blockade therapy.

Study Limitations

The main limitations of this study are the retrospective nature, comparatively small number of cases, and a comparatively short period of follow-up. The main study strength is that few studies in the literature investigate the clinicopathologic and molecular features of TILs in tumor cells in HGSOC in the Turkish population.

Conclusion

This study revealed that higher scores of stromal CD8+ TILs are substantially correlated with DFS and OS in univariate analyzes, whereas scores of intraepithelial CD3+, CD4+, and CD8+ TILs, and stromal CD3+ and CD4+ TILs are not correlated with DFS and OS in both univariate and multivariate analyses. Also, we found a significant association between PD-1 positivity and the scores of stromal CD3+ TILs and intraepithelial CD8+ TILs. However, no significant association was detected between PD-1 positivity and the survival of HGSOC patients.

Ethics

Ethics Committee Approval: The study was approved by the Medipol University ethics committee resolution 10840098-604.01.01-E.17851, dated July 26, 2020.

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: F.Ö., Ö.F.Ö., Ö.A., S.A., Concept: F.Ö., S.C.O., Ö.F.Ö., Ö.A., E.Y., S.A., M.K., N.A.S., A.K.K., Design: F.Ö., S.C.O., Ö.F.Ö., Ö.A., E.Y., S.A., M.K., N.A.S., A.K.K., Data Collection or Processing: F.Ö., Ö.F.Ö., Ö.A., S.A., Analysis or Interpretation: F.Ö., S.C.O., Ö.F.Ö., E.Y., Literature Search: F.Ö., S.C.O., Ö.F.Ö., E.Y., Writing: F.Ö., S.C.O., Ö.F.Ö., Critical Review: S.C.O., Ö.F.Ö.

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