POREĐENJE PD-L1 EKSPRESIJE U CITOLOŠKOM I HISTOLOŠKOM MATERIJALU SEROZNOG HIGH-GRADE KARCINOMA JAJNIKA

ORIGINALNI RAD

ORIGINAL ARTICLE

THE CORRELATION OF PD-L1 EXPRESSION IN CYTOLOGICAL AND HISTOLOGICAL MATERIAL OF SEROUS HIGH-GRADE OVARIAN CANCER

Ljubiša Jovanović[1], Anđa Ćirković[2], Ljubinka Nikolić[3], Milena Jović[4], Darko Mikić[4,5], Svetlana Milenković[1], Radmila Janković[6]

[1] Univerzitetski klinički centar Srbije, Klinika za ginekologiju i akušerstvo, Služba za patologiju i medicinsku citologiju, Beograd, Srbija
[2] Univerzitet u Beogradu, Medicinski fakultet, Institut za medicinsku statistiku i informatiku, Beograd, Srbija
[3] Univerzitetski klinički centar Srbije, Laboratorija za hematologiju i transfuziju, Beograd, Srbija
[4] Vojnomedicinska akademija, Institut za patologiju i sudsku medicinu, Beograd, Srbija
[5] Univerzitet odbrane, Medicinski fakultet Vojnomedicinske akademije, Beograd, Srbija
[6] Univerzitet u Beogradu, Medicinski fakultet, Institut za patologiju, Beograd, Srbija

[1] *University Clinical Center of Serbia, Clinic for Gynecology and Obstetrics, Department of Pathology and Medical Cytology, Belgrade, Serbia*
[2] *University of Belgrade, Faculty of Medicine, Institute for Medical Statistics and Informatics, Belgrade, Serbia*
[3] *University Clinical Center of Serbia, Laboratory for hematology and transfusion, Belgrade, Serbia*
[4] *Military Medical Academy, Institute of Pathology and Forensic Medicine, Belgrade, Serbia*
[5] *University of Defence, Military Medical Academy, Belgrade, Serbia*
[6] *University of Belgrade, Faculty of Medicine, Institute of Pathology, Belgrade, Serbia*

SAŽETAK

Uvod: Tumorske ćelije u peritonealnom lavatu eksprimiraju različite proteine sa značajnim prognostičkim i terapijskim potencijalom. Takva ekspresija se može razlikovati od ekspresije u primarnom tumoru ili u metastazama. U ovoj studiji upoređivali smo ekspresiju PD-L1 markera (programirani ligand ćelijske smrti-1) na karcinomskim ćelijama jajnika u citološkom materijalu, u peritonealnim metastazama i u primarnom tumoru.

Materijali i metode: Studija je obuhvatila 30 pacijentkinja koje su operisane zbog high-grade seroznog karcinoma jajnika (HGSC) u IIIC FIGO stadijumu tumorske bolesti, u jednogodišnjem periodu. Formirani su citoblokovi, citološki i tkivni mikronizovi koji su imunohistohemijski bojeni PD-L1 antitelom. Četiri kategorije PD-L1 ekspresije su: negativna, slaba, umerena i jaka ekspresija. Udružene umerena i jaka ekspresija smatrane su visokom PD-L1 ekspresijom.

Rezultati: Umerena ekspresija je najčešći obrazac PD-L1 ekspresije u primarnom HGSC (50%) kao i u peritonealnim metastazama (omentumu) (60%). Citološki uzorci su uglavnom pokazivali slabu PD-L1 ekspresiju (57%). Statistička analiza nije pokazala značajnu razliku među analiziranim grupama kada je reč o PD-L1 ekspresiji. Pokazana je pozitivna korelacija PD-L1 ekspresije između različitih, udruženih tumorskih uzoraka kod svakog pacijenta sa statisičkom značajnošću ($p < 0,05$) između svih analiziranih uzoraka.

Zaključak: PD-L1 ekspresija je dovoljno slična u svim analiziranim uzorcima tumora. To bi moglo ukazati na slične peritumorske regulatorne mehanizme HGSC primarnog tumora i tumorskog okruženja citološkog materijala. Imunohistohemijska analiza na formiranim citoblokovima pokazuje zadovoljavajuću pouzdanost određivanja ekspresije PD-L1 markera na karcinomskim ćelijama jajnika izolovanim iz citološkog materijala.

Ključne reči: karcinom jajnika, PD-L1, peritonealna lavaža, metastaze

ABSTRACT

Introduction: Neoplastic cells in peritoneal lavage express various proteins with significant prognostic and therapeutic potential. Such expression could differ from the expression in a primary tumor or in metastases. In this research, we compared PD-L1 (programmed cell death ligand-1) expression on ovarian cancer cells in cytological material with its expression on peritoneal metastases and a primary tumor.

Materials and methods: The study included 30 patients who had been operated on for high-grade serous ovarian cancer (HGSC) in FIGO IIIC, over the period of one year. Cytoblocks, cytological and tissue microarrays were assembled and immunostained with PD-L1 antibody. For each tumor compartment we determined four PD-L1 expression categories: negative, low, moderate, and strong expression, according to the percentage of membrane positive tumor cells. Moderate and strong positivity together were considered as high PD-L1 expression.

Results: Moderate PD-L1 expression was the most frequent pattern in primary HGSC (50%) and in peritoneal metastases (omentum) (60%). Cytological samples mostly showed low PD-L1 expression (57%). Statistical analysis did not show a significant difference in PD-L1 expression between the study groups. We found a positive correlation of PD-L1 expression between different, matched tumor samples in every patient, with statistical significance ($p < 0.05$) between all the analyzed samples.

Conclusion: PD-L1 expression was similar in all three tumor compartments. This could point to similar peritumor regulatory mechanisms of HGSC in primary tumor tissue and cytology tumor samples. Immunohistochemical analysis of the assembled cytoblocks is sufficiently reliable in the assessment of PD-L1 expression on cancer ovarian cells from cytological material.

Keywords: ovarian carcinoma, PD-L1, peritoneal lavage, metastases

Autor za korespondenciju:
Ljubiša Jovanović
Klinika za ginekologiju i akušerstvo, Univerzitetski klinički centar Srbije,
Dr Koste Todorovića 26, 11000 Beograd, Srbija
Elektronska adresa: jovanovicljubisa18@gmail.com

Corresponding author:
Ljubiša Jovanović
Clinic for Gynecology and Obstetrics, University Clinical Center of Serbia
26 Dr Koste Todorovica Street, 11000 Belgrade, Serbia
E-mail: jovanovicljubisa18@gmail.com

Primljeno • *Received*: August 22, 2023; **Revidirano • *Revised*:** September 5, 2023; **Prihvaćeno • *Accepted*:** September 8, 2023; ***Online first*:** September 25, 2023

DOI: 10.5937/smclk4-46109

UVOD

Karcinom jajnika nalazi se na osmom mestu po učestalosti među karcinomima ženskih polnih organa [1]. Maligni ascites kod žena je česta posledica karcinoma jajnika [2]. Određivanje stadijuma tumora obično predstavlja indikaciju za postupak peritonealne lavaže. Prema Međunarodnoj federaciji ginekologa i opstetičara (FIGO), u I i II stadijumu, učestalost prisustva malignih tumorskih ćelija u peritonealnom lavatu iznosi 7%, dok je u slučaju III i IV stadijuma to čitavih 89% [3,4]. High-grade serozni karcinom jajnika (HGSC) je najčešći maligni tumor ženskih polnih organa sa prisustvom malignih tumorskih ćelija u peritonealnom lavatu [3].

Ligand programirane ćelijske smrti1 (PD-L1) je molekul sa heterogenom ekspresijom koja nije karakteristična samo za tumorske ćelije [5,6]. U ovom istraživanju analizirali smo ekspresiju PD-L1 na ćelijama high-grade karcinoma jajnika, uzimajući u obzir različite tipove uzoraka. Jedna od najznačajnijih imunosupresivnih interakcija jeste interakcija proteina PD-L1 u tumorskim ćelijama sa PD-1 molekulom na površini T-limfocita. Ovaj odnos dovodi do supresije T ćelija i inaktivacije njihovih efektorskih funkcija [5-7].

Peritonealni izliv kod karcinoma jajnika ima imunosupresivni efekat. Ranije studije su pronašle razne imunosupresivne faktore u uzorcima malignog ascitesa [2,8]. Molekuli PD-1 i PD-L1 su svakako prisutni u malignom peritonealnom izlivu i mogu biti od značaja kao potencijalni prognostički i terapijski markeri [8].

Tumorsko tkivo u omentumu ukazuje na uznapredovali stadijum karcinoma jajnika i agresivnije biološko ponašanje. Omentum verovatno ima lošiji anti-tumorski imunski odgovor nego primarni tumor. Takve tumorske ćelije su često manje diferencirane u poređenju sa primarnim tumorom [9].

U ovoj studiji poredili smo ekspresiju PD-L1 na tumorskim ćelijama u citološkom materijalu peritonealnog lavata, peritonealnih metastaza, i primarnog HGSC.

MATERIJALI I METOD

Učesnici

Studija je zamišljena kao studija preseka koja obuhvata pacijentkinje prethodno operisane zbog high-grade karcinoma jajnika tokom perioda od jedne godine na Klinici za ginekologiju i akušerstvo Univrzitetskog kliničkog centra Srbije. Odabrali smo 30 pacijentkinja sa HGSC u IIIC FIGO stadijumu tumorske bolesti, sa peritonealnim metastazama i peritonealnim lavatom za imunohistohemijsko ispitivanje.

INTRODUCTION

Ovarian cancer is the eighth most common gynecologic cancer [1]. Malignant ascites in women is a common consequence of ovarian cancer [2]. Cancer staging is usually an indication for peritoneal lavage procedure. According to the International Federation of Gynecology and Obstetrics (FIGO), in stages I and II, the frequency of malignant tumor cells in peritoneal lavage is about 7%, which increases to 89% in stages III and IV [3,4]. High-grade serous ovarian cancer (HGSC) is the most common gynecologic malignant tumor with the presence of malignant tumor cells in the peritoneal lavage [3].

Programmed cell death ligand-1 (PD-L1) is a molecule with heterogenic expression which is not specific only for tumor cells [5,6]. In this research we analyzed PD-L1 expression on ovarian cancer cells in HGSC, in different sample types. One of the main immunosuppressive interactions is that of PD-L1 protein on tumor cells with programmed cell death-1 (PD-1) on T-lymphocytes. This relation leads to suppression of T-cells and inactivation of their effector functions [5-7].

Peritoneal effusion in ovarian cancer has an immunosuppressive effect. Previous studies reported various immunosuppressive factors in samples of malignant ascites [2,8]. PD-1 and PD-L1 molecules are certainly present in malignant peritoneal effusion. They could be significant as potential prognostic and therapeutic markers [8].

Tumor tissue in the omentum point to advanced stages of ovarian cancer and more aggressive biological behavior. The omentum probably has worse antitumor regulatory immune system than the primary site. Such tumor cells are often less differentiated compared to the primary tumor [9].

In this study we analyzed the differences between PD-L1 cancer cell expression in cytological material of peritoneal lavage, in peritoneal metastases, and in primary HGSC.

MATERIALS AND METHODS

Patient cohort

The study was designed as a cross-sectional study, including patients who had surgery due to high-grade ovarian cancer over the period of one year, at the Clinic for Gynecology and Obstetrics, University Clinical Centre of Serbia. We selected 30 patients with HGSC in FIGO IIIC, with peritoneal metastases and peritoneal lavage for immunohistochemical study.

Material collection and processing

All cytological samples (5 ml) were centrifugated for 10 minutes at 1800 rpm. Cytological paraffin blocks

Prikupljanje i obrada materijala

Svi citološki uzorci (5ml) su centrifugirani 10 minuta na 1800 obrtaja/min. Parafinski blokovi su dobijeni plazma-trombin metodom [10]. Centrifugiranim uzorcima najpre smo dodali 0,5 ml plazme. U narednom koraku, dodali smo 0,3 ml trombina i epruvete su ostavljene na temperaturi od 37°C. Odmah se stvorio ugrušak. Ugrušak je prebačen u vrećicu za tkivo, fiksiran u rastvoru formalina, i podvrgnut rutinskoj histološkoj proceduri kao i uzorci uzeti iz primarnog tumora i metastaza. Odabran je reprezentativan citoblok za sva tri slučaja radi dalje imunohistohemijske analize.

Tkivni i citološki mikronizovi

Prema pravilima koja se odnose na tkivne i citološke mikronizove, uzorci cilindara su uzeti iz svakog parafinskog bloka uz pomoć igle dijametra 3mm. Tkivni cilindar je deo uzorka sa najvećim udelom tkiva. Uzimali smo u obzir žarišta sa više od 50 tumorskih ćelija radi adekvatne procene. Cilindri tumorskog tkiva uzeti sa mesta primarnog tumora i metastaza sadržali su najhomogenije vitalne delove tumora, sa najmanjim stepenom nekroze. Cilindri su potom premešteni u recipijentne parafinske blokove i formiran je niz mikro uzoraka. Tkivo placente je korišćeno za unutrašnju kontrolu imunohistohemijske analize i orijentacije blokova [11,12].

Imunohistohemijska analiza

Imunohistohemijsko bojenje je obavljeno uz pomoć instrumenta Autostainer Link 48, Agilent (Danska), na Institutu za patologiju Medicinskog fakulteta u Belgradu. Za imunohistohemijsku analizu korišćeno je PD-L1 antitelo (klon 22C3) i sistem za vizualizaciju EnVision FLEX K8023. EnVision FLEX solution pH 6,1, K8005, Agilent, korišćen je za demaskiranje epitopa. Kao primarno antitelo, korišćeno je monoklonsko, antihumano M3653, Agilent, u razblaženju 1:30. Imajući u vidu imunoskor PD-L1, definisali smo četiri kategorije PD-L1 ekspresije na primarnom tumoru i metastatskom tkivu: negativnu (bez pozitivnih ćelija/sa jednom pozitivnom ćelijom (<1%)), slabu (sa manje od 10% pozitivnih ćelija), umerenu (sa 10-50% pozitivnih ćelija), i jaku (ekspresija iznad 50%). Udružene umerena i jaka ekspresija smatrane su visokom PD-L1 ekspresijom [13]. Ekspresija PD-L1 u tkivnim uzorcima procenjivana je na definisanim žarištima sa 50 tumorskih ćelija, gde su definisane sledeće kategorije: negativna ekspresija bez ijedne pozitivne ćelije, slaba ekspresija (<10 ćelija), umerena ekspresija (10-30 ćelija), i jaka ekspresija (>30 cells). Poredili smo ekspresiju PD-L1 u različitim uzorcima (metastaze, peritonealni lavat) svakog tumora. PD-L1 ekspresija u razli-

were made using the Plasma-Thrombin method [10]. To centrifugated samples we first added 0.5 ml of plasma. In the next step, we put 0.3 ml of thrombin and the tubes were left on 37°C. A clot was formed immediately. The clot was transferred to a tissue bag, fixed in formalin, and subjected to routine histological procedure, like the samples taken from a primary tumor and metastases. In each case, a representative tissue block was selected for further immunohistochemical analysis.

Tissue and cytological microarray

According to tissue and cytological microarray procedures, cylinders were sampled from each paraffin block using a 3 mm puncture needle. A cytological cylinder is the most cellular part of a sample. We counted hot spot areas with more than 50 tumor cells for appropriate evaluation. Tumor tissue cylinders taken from primary sites and metastases contained most homogeneous vital parts of the tumor, with the least necrosis. Cylinders were then moved to recipient paraffin blocks. A series of micro samples were formed. Placental tissue was used as an internal control of immunohistochemical analysis and block orientation [11,12].

Immunohistochemical analysis

Immunohistochemical staining was done on the Autostainer Link 48, Agilent, Denmark at the Institute of Pathology, Faculty of Medicine, Belgrade. PD-L1 antibody (clone 22C3) and EnVision FLEX visualization system K8023, Agilent were used for immunohistochemical analysis. EnVision FLEX solution pH 6.1, K8005, Agilent was used for epitope unmasking. When it comes to PD-L1 antibody, it was a primary, monoclonal, anti-human, M3653, Agilent, dilution ratio 1:30. Considering PD-L1 immunoscore, we defined four categories for evaluation of a primary tumor site and metastatic tissue PD-L1 expression levels: negative (with no positive cells/with a single positive cell (<1%)), low (with less than 10% of positive cells), moderate (with 10-50% of positive cells), and strong (expression above 50%). Moderate and strong positivity were regarded as high PD-L1 expression [13]. PD-L1 expression in cytological samples was evaluated on defined hot spots with 50 tumor cells, where the following categories were defined: negative expression with no positive cells, low expression (<10 cells), moderate expression (10-30 cells), and strong expression (>30 cells). We compared PD-L1 expressions in different samples (the primary site, a metastasis, peritoneal lavage) of each tumor. PD-L1 expression in different samples was correlated with available histopathology and clinical parameters [14].

čitim uzorcima bila je u korelaciji sa dostupnim histopatološkim i kliničkim parametrima [14].

STATISTIČKA ANALIZA

Statistička analiza je sprovedena primenom deskriptivnih statističkih metoda i odgovarajućih statističkih testova (Studentov *t*-test, Fišerov egzaktni test, Man-Vitnijev test, Kruskal-Valisov test, ANOVA) za nivo značajnosti 0,05 uz upotrebu SPSS21 za Windows. Poređenje ekspresije PD-L1 među različitim uzorcima tumora rađeno je uz pomoć Spirmanove korelacije (r_s).

REZULTATI

Ekspresija PD-L1 u primarnom high-grade seroznom karcinomu jajnika

Prosečna starost pacijentkinja u ovoj posmatranoj grupi bila je 57,5±10,14 godina. Umerena ekspresija bila je najzastupljenija kategorija ekspresije PD-L1 u primarnom HGSC (50%). Jedna trećina (33%) primarnih tumora imala je slabu ekspresiju. Jaka ekspresija PD-L1 i odsustvo ekspresije PD-L1 bili su manje uobičajeni, uz sličnu frekventnost (Slika 1).

Visoka PD-L1 ekspresija (udružene umerena i jaka ekspresija) bila je prisutna u 18 (60%) primarnih tumo-

STATISTICAL ANALYSIS

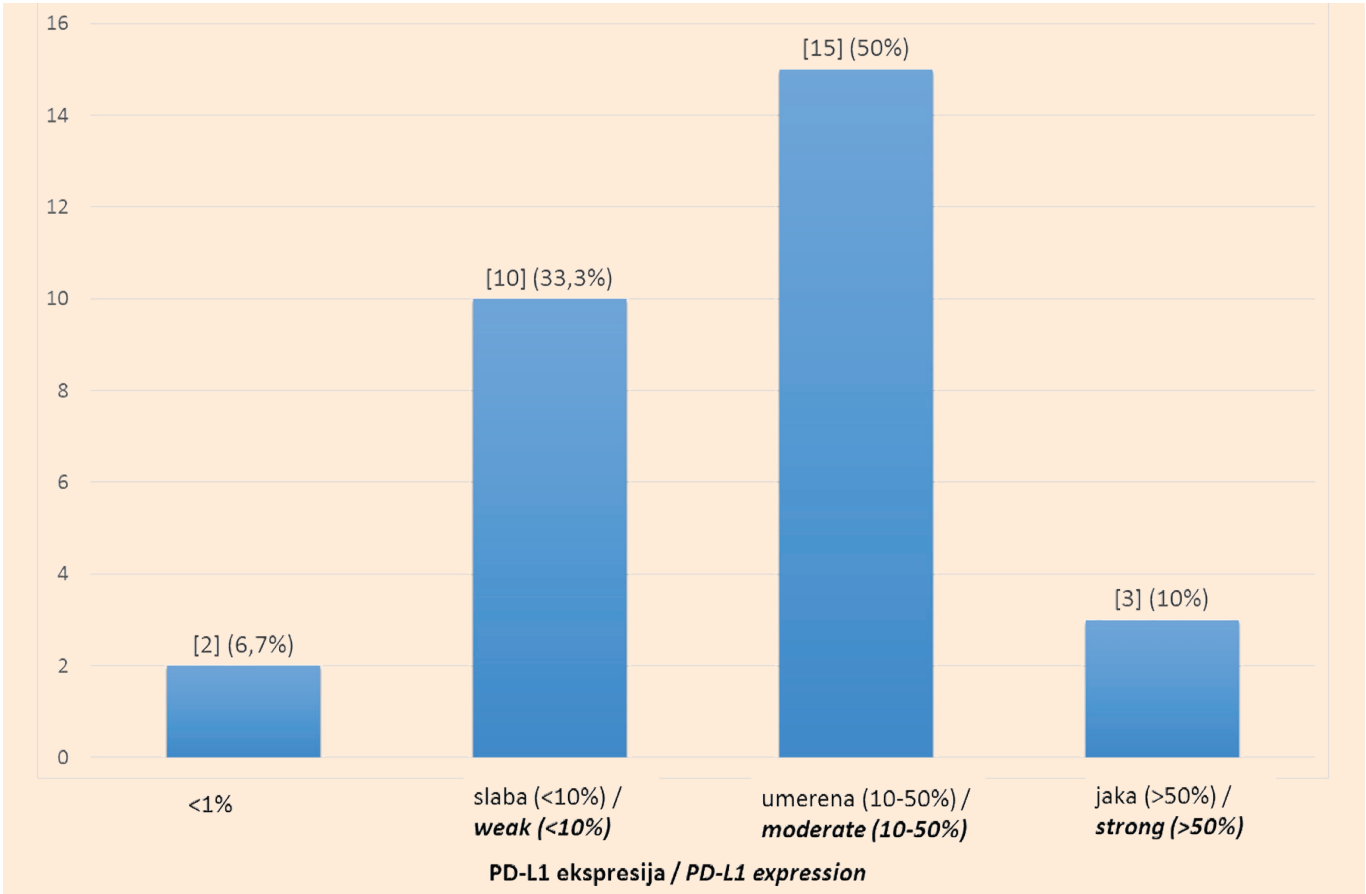
Statistical analysis was done using descriptive methods and adequate statistical tests (Student t-test, Fisher`s exact test, Mann–Whitney, Kruskal–Wallis, ANOVA) for the level of significance of 0.05, using SPSS21 for Windows. The comparison of PD-L1 expression between different tumor samples was performed using Spearman correlation analysis (r_s).

RESULTS

PD-L1 expression in primary high-grade serous ovarian cancer

The mean age of patients in this study group was 57.5±10.14 years. Moderate expression was the most frequent pattern of PD-L1 expression in primary HGSC (50%). One third (33%) of primary tumors had low expression. Strong PD-L1 expression and the absence of PD-L1 expression were less common, with similar frequency (Figure 1).

High PD-L1 expression (moderate and strong expression) in primary tumors was found in 18 (60%) cases. Clinical data and tumor size in high and low PD-L1 expression groups are shown in Table 1.



Slika 1. Učestalost kategorije PD-L1 ekspresije u primarnom high-grade seroznom karcinomu jajnika

Figure 1. Categories of PD-L1 expressions in primary high-grade serous ovarian cancer

Tabela 1. Categories od PD-L1 expression according to clinical parameters and tumor size

Table 1. Categories od PD-L1 expression according to clinical parameters and tumor size

Kategorije PD-L1 ekspresije / *PD-L1 expression categories*	Prosečna starost (godine) / *Mean age years (X±SD)*	*p*	Menopauza / *Menopausis*	*p*	Veličina tumora *(mm)* / *Tumor size (mm) Med (min-max)*	*p*
Visoka PD-L1 ekspresija / *High PD-L1 expression*	68.17±8.44	0.028	94.4%	0.548	65.0 (18.0-190.0)	0.484
Slaba PD-L1 ekspresija / *Low PD-L1 expression*	60.00±10.82		83.3%		75.0 (28.0-160.0)	

ra. Klinički podaci i veličina tumora u grupama sa visokom i slabom ekspresijom PD-L1 dati su u Tabeli 1.

Nije bilo statistički značajne razlike u PD-L1 ekspresiji u odnosu na menopauzalni status ($p = 0,741$ Fišerov test). Prosečna veličina tumora se nije značajno razlikovala u odnosu na PD-L1 ekspresiju u tumorskim ćelijama primarnog HGSC ($p = 0,864$ Kruskal-Valisov test).

Ekspresija PD-L1 u peritonealnim metastazama

PD-L1 ekspresija u peritonealnim metastazama (omentumu) najčešće (60%) je bila umerena (18 pacijenata). Osam uzoraka (27%) imalo je jaku ekspresiju, dok su preostale PD-L1 kategorije imale nisku frekventnost.

Prosečna starost pacijentkinja sa visokom PD-L1 ekspresijom u peritonealnim metastazama bila je 64,9±10,14 godina. Bilo je 23 pacijentkinje u menopauzi (88%). U grupi pacijentkinja sa visokom PD-L1 ekspresijom u peritonealnim metastazama, srednja veličina primarnog HGSC bila je 40 mm (18-190 mm).

Nije bilo statistički značajne razlike u prosečnoj starosti pacijentkinja u odnosu na kategorije PD-L1 ekspresije u metastazama HGSC ($p = 0,586$ ANOVA), kao ni statitistički značajne razlike PD-L1 ekspresije u PD-L1 ekspresiji u odnosu na menopauzalni status pacijentkinja ($p = 0,699$ Fišerov test). Veličina primarnog tumora nije se značajno razlikovala u odnosu na PD-L1 ekspresiju u peritonealnim metastazama ($p = 0,073$ Kruskal-Valisov test). Nađena je statistički značajna razlika u veličini primarnog tumora između slabe i umerene PD-L1 ekspresije ($p = 0,006$ Man-Vitnijev test).

Ekspresija PD-L1 u citološkom matrijalu iz peritonealnog lavata

Adekvatna celularnost za analizu bila je prisutna kod 28 pacijentkinja. Slaba ekspresija nađena je u 16 uzoraka (57%), dok je 9 uzoraka (32%) imalo umerenu ekspresiju. Nije bilo statistički značajne razlike u PD-L1 ekspresiji u odnosu na menopauzalni status ($p = 0.741$ Fisher's test). Prosečna veličina tumora se nije značajno razlikovala u odnosu na PD-L1 ekspresiju u tumorskim ćelijama primarnog HGSC ($p = 0.864$ Kruskal-Wallis).

There was no statistically significant difference in PD-L1 expression according to menopausal status ($p = 0.741$ Fisher's test). The mean tumor size was not significantly different according to PD-L1 expression in tumor cells of primary HGSC ($p = 0.864$ Kruskal-Wallis).

PD-L1 expression in tumor metastases

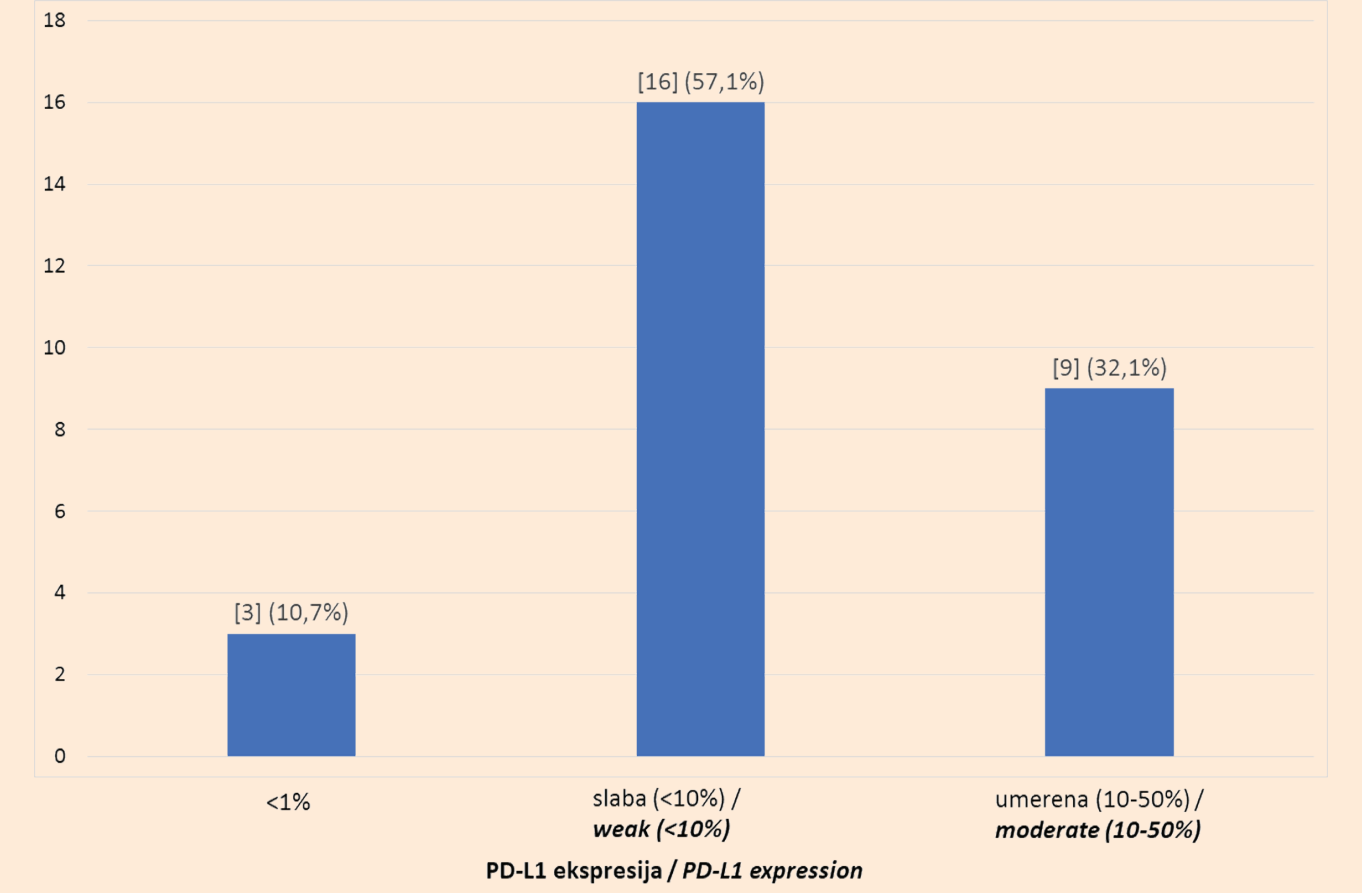
PD-L1 expression in peritoneal metastases (omentum) most frequently (60%) showed moderate expression (18 patients). Eight samples (27%) had strong positivity, while other PD-L1 categories showed lower frequencies.

The mean age of patients with high PD-L1 expression in peritoneal metastases was 64.9±10.14 years. There were 23 patients in menopause (88%). In the group of patients with high PD-L1 expression in peritoneal metastases, the mean size of primary HGSC was 40 mm (18-190 mm).

There was not a statistically significant difference in the mean age of patients according to PD-L1 expression categories in metastases of HGSC ($p = 0.586$ ANOVA). There was no statistically significant difference of PD-L1 expression considering the menopausal status of patients ($p = 0.699$ Fisher's test). The size of the primary tumor was not significantly different in relation to PD-L1 expression in peritoneal metastases ($p = 0.073$ Kruskal-Wallis). Statistically significant difference was found in the size of the primary tumor between low and moderate PD-L1 expression ($p = 0.006$ Mann Whitney test).

PD-L1 expression in cytological material from peritoneal lavage

Adequate cellularity for analysis was present in 28 patients. Low expression was found in 16 samples (57%), while 9 samples (32%) had moderate PD-L1 expression. There was no strong PD-L1 expression in cytological material (Figure 2).



Slika 2. Učestalost kategorija PD-L1 ekspresije u peritonealnom lavatu

Figure 2. Categories of PD-L1 expression in peritoneal lavage

spresiju PD-L1. Nije bilo jake ekspresije u citološkom materijalu (Slika 2).

Prosečna starost pacijentkinja sa visokom PD-L1 ekspresijom bila je 64,9±10,31 godina. Većina ih je bila u menopauzi (89%). Prosečna veličina primarnog HGSC u odnosu na visoku PD-L1 ekspresiju u citološkom materijalu bila je 72.5 mm (18-190 mm).

Nije bilo statistički značajnih razlika u high-grade PD-L1 ekspresiji u odnosu na godine starosti ($p = 0,938$ ANOVA), menopauzalni status pacijentkinja ($p = 0,699$ Fišerov test), i prosečne veličine tumora ($p = 0,132$ Kruskal-Valisov test).

Korelacija PD-L1 ekspresije u različitim uzorcima high-grade karcinoma jajnika

Poređene su kategorije visoke PD-L1 ekspresije u omentumu (87%) i visoke PD-L1 ekspresije u primarnom HGSC (60%). Nije bilo statistički značajnih razlika. Ćelije karcinoma jajnika u peritonealnom lavatu imale su slabiju PD-L1 ekspresiju u poređenju sa tumorskim ćelijama u peritonealnim metastazama. Pri poređenju učestalosti umerenog nivoa ekspresije između ova dva uzorka nisu nađene statistički značajne razlike (12 vs.18; $p = 0,273$).

Imunohistohemijska analiza pokazala je raznovrsnu učestalost pozitivnosti na PD-L1 u različitim ma-

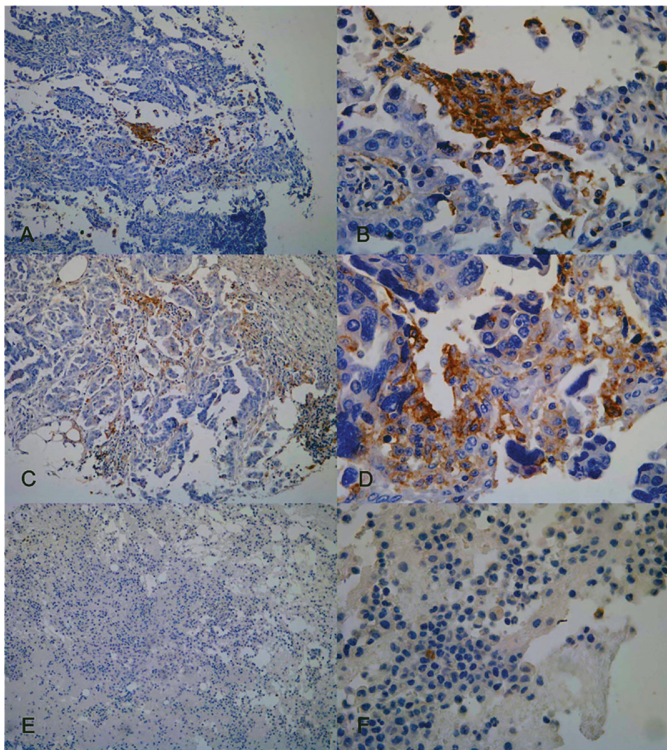
Patients with high PD-L1 expression in cytological material were 64.9±10.31 years. Most of them were menopausal (89%). The mean size of primary HGSC considering high PD-L1 expression in cytological material was 72.5 mm (18-190 mm).

There were no statistically significant differences in high-grade PD-L1 expressions considering age ($p = 0.938$ ANOVA), the menopausal status of patients ($p = 0.699$ Fischer test), and mean tumor size ($p = 0.132$ Kruskal-Wallis).

Correlation of PD-L1 expression in different samples of HGSC

Categories of high PD-L1 expression in the omentum (87%) and high PD-L1 expression in primary HGSC (60%) were compared. There were no statistically significant differences. Ovarian cancer cells in peritoneal lavage had lower PD-L1 expression compared to cancer cells in omental metastases. Comparing the frequency of moderate expression levels between these two samples, no significant difference was found (12 vs.18; $p = 0.273$).

Immunohistochemical analysis showed various frequencies of PD-L1 positivity in different materials. The most frequent PD-L1 expression in primary HGSC and metastases was moderate. In cytological material low PD-L1 expression was dominant (Figure 3).



Slika 3. Prikaz najčešćih kategorija PD-L1 ekspresije u primarnom HGSC (A) x100, (B) x400, u peritonealnim metastazama (omentumu) (C) x100, (D) x400, i u citološkom materijalu (E) x100, (F) x400

Figure 3. The most common categories of PD-L1 expression in primary HGSC (A) x100, (B) x400, in metastases (omentum) (C) x100, (D) x400, and in cytological material (E) x100, (F) x400 are presented

terijalima. Najučestalija kategorija ekspresije PD-L1 u primarnom HGSC i metastazama bila je umerena. U citološkom materijalu dominirala je slaba PD-L1 ekspresija (Slika 3).

U ovom istraživanju analizirali smo korelaciju PD-L1 ekspresije među različitim uzorcima tumora, koji su upareni prema pacijentkinjama, i pronašli smo statistički značajne rezultate među svim posmatranim grupama. Otkrili smo značajnu pozitivnu korelaciju u PD-L1 ekspresiji između primarnog HGSC i metastaza ($p < 0{,}001$; $r_s = 0{,}620$). PD-L1 ekspresija između primarnog HGSC i citoloških uzoraka takođe je pokazala postojanje pozitivne korelacije ($r_s = 0{,}599$), i to statistički značajne ($p < 0{,}001$). Kada je reč o metastazama, postoji pozitivna korelacija PD-L1 ekspresije u odnosu na citološke uzorke ($r_s = 0{,}817$), takođe statistički značajna ($p < 0{,}001$).

DISKUSIJA

Maligni ascites ima karakterističnо tumorsko mikrookruženje sa različitim antitumorskim regulatornim mehanizmima koji mogu stimulisati proliferaciju tumorskih ćelija i njihovu sposobnost da metastaziraju. Različiti citokini, proinflamatorni i imuni faktori u peritoneal effusion alter the host's immune system. Numerous molecules which are expressed in peritoneal lavage have prognostic and therapeutic significance. Their expression could be different from the one in a primary tumor site or in metastases [15].

In this research we analyzed the correlation of PD-L1 expression between different tumor samples, matched according to patients, and found statistically significant results between all study groups. We detected a significant positive correlation in PD-L1 expression between primary HGSC and metastases ($p < 0.001$; $r_s = 0.620$). PD-L1 expression between primary HGSC and cytology samples also showed a positive correlation ($r_s = 0.599$), that was statistically significant as well ($p < 0.001$). Metastases showed PD-L1 expression in positive correlation according to cytology samples ($r_s = 0.817$), also statistically significant ($p < 0.001$).

DISCUSSION

Malignant ascites has a characteristic tumor microenvironment with different antitumor regulatory mechanisms which could stimulate cancer cell proliferation, and the ability of these cells to metastasize. Various cytokines, proinflammatory and immune factors in the

There are not many studies on PD-L1 expression on cytological specimens and their correlation with PD-L1 expression in standard histopathological samples. Current reference associations (Papanicolaou Society of Cytopathology and Pulmonary Pathology Society) are very reticent about the idea of using cytological preparations to examine PD-L1 expression. Not enough papers and research on this topic, and undefined interpretation of results, make clinicians and researchers doubt reliability of this analysis [16]. Current studies compare the expression levels of PD-L1 markers in histological and cytological material on lung cancer tissue [17].

Our research is based on the analysis of differences in PD-L1 expression in ovarian cancer cells located in different tumor microenvironments. Since most primary tumor samples show high PD-L1 expression, the PD-L1 regulatory mechanism is thought to be significantly active in HGSC. We confirmed that high PD-L1 expression is associated with poorer differentiation of ovarian cancer (grade 3) and with an advanced cancer stage (FIGO IIIC).

In our study, PD-L1 expression on ovarian cancer cells from peritoneal lavage showed some frequency differences in expression compared to primary cancer tissue. There was a lower number of patients (32%) with high PD-L1 expression on tumor cells in peritoneal lavage than in a primary tumor (60%). Lower expression of PD-L1 markers on tumor cells in peritoneal lavage

ritonealnom izlivu menjaju imuni sistem domaćina. Brojni molekuli koji se eksprimiraju u peritonealnom lavatu imaju prognostički i terapijski značaj. Njihova ekspresija može se razlikovati od one u primarnom tumoru ili u metastazama [15].

Nema mnogo istraživanja koja se bave ekspresijom PD-L1 na citološkim uzorcima i njihovoj korelaciji sa ekspresijom PD-L1 u standardnim histopatološkim uzorcima. Aktuelna referentna udruženja (*Papanicolaou Society of Cytopathology* i *Pulmonary Pathology Society*) veoma su suzdržani po pitanju korišćenja citoloških preparata u svrhu istraživanja PD-L1 ekspresije. Zbog nedovoljnog broja radova i istraživanja u ovoj oblasti, kao i nedefinisanog načina interpretacije rezultata, klinički i istraživači sumnjaju u pouzdanost ove analize [16]. Aktuelne studije porede nivoe ekspresije PD-L1 markera u histološkom i citološkom materijalu na tkivu karcinoma pluća [17].

Naše istraživanje se zasniva na analizi razlika u PD-L1 ekspresiji u ćelijama karcinoma jajnika koje su smeštene u različitim tumorskim mikrookruženjima. Pošto većina uzoraka primarnog tumora pokazuje visoku ekspresiju PD-L1, smatra se da je PD-L1 regulatorni mehanizam u značajnoj meri aktivan u HGSC. Potvrdili smo da je visoka ekspresija PD-L1 povezana sa lošijom diferencijacijom ćelija karcinoma jajnika (trećeg stepena) i sa uznapredovalim stadijumom karcinoma (FIGO IIIC).

U našoj studiji, PD-L1 ekspresija na ćelije karcinoma jajnika iz peritonealnog lavata pokazala je neke razlike u frekventnosti ekspresije u poređenju sa primarnim tkivom kancera. Manji broj pacijentkinja (32%) imao je visoku PD-L1 ekspresiju na tumorske ćelije u peritonealnom lavatu nego što je to bio slučaj sa primarnim tumorom (60%). Slabija ekspresija PD-L1 markera na tumorske ćelije u peritonealnom lavatu može se objasniti dodatnim imunoregulatornim mehanizmima koji svakako utiču na tumorsko mikrookruženje. Pretpostavlja se da su ovi mehanizmi različito regulisani u peritonealnom lavatu u odnosu na primarno tumorsko tkivo. Signalni putevi kao što su LAG-3 (limfocitni aktivacioni gen 3) i TIM 3 (T ćelijski imunoglobulinski mucin 3) utiču jedan na drugi kao i na ekspresiju PD-L1 markera. U regulaciji ovih procesa važna je i aktivnost proinflamatornih citokina (TNF-α, IL-6), galektina-9, i sličnih faktora [18]. Detaljna analiza interakcije ovih i sličnih imunih kontrolnih puteva mogla bi dati sveobuhvatnija objašnjenja antitumorskog imuniteta.

Imunohistohemijski metod koji koristi citoblokove veoma je reprezentativan za adekvatno otkrivanje ćelija karcinoma jajnika u peritonealnom lavatu [19]. Ovo bi moglo biti od velikog značaja za pacijentkinje koje nije moguće operisati i kod kojih su dijagnostičke i terapijske opcije ograničene. Izolovanje ćelija karcinoma

could be explained by additional immunoregulatory mechanisms which certainly affect the tumor microenvironment. The assumption is that these mechanisms are differently regulated in peritoneal lavage according to primary cancer tissue. Signal pathways such as LAG-*3 (lymphocyte activation gene 3)* and TIM-3 *(T cell immunoglobulin and mucin domain-containing 3)* affect each other as well as the expression of PD-L1 markers. The activity of proinflammatory cytokines (TNF-α, IL-6), galectin-9, and similar factors is also important in the regulation of these processes [18]. A detailed analysis of the interaction of these and similar immune control pathways could lead to more comprehensive explanations of the antitumor immunity process.

The immunohistochemical method using cytoblocks is very representative for adequate detection of ovarian cancer cells from peritoneal lavage [19]. This could be very significant for inoperable patients where diagnostic and therapeutic options are limited. Isolating cancer cells that way could be less invasive and reliable enough to define further procedures. Cancer cells in cytoblocks show the identical type of membrane PD-L1 expression as in histological samples, which further confirms the validity of this method. Using cytoblocks, the material shows a significant correlation of immunohistochemical expression with standard histological preparations. Considering the applicability of cytological material for interpreting PD-L1 expression, there was acceptable reliability of results by cytoblock formation, in contrast to conventional cytological smears and the Liquid-Based method [20].

We found that frequencies of high PD-L1 expression are higher in omentum cancer cells (87%) than in primary cancer (60%) and cytological material (32%). In matched samples, we found a positive correlation between PD-L1 expression in different tumor samples. We got statistically significant results which showed PD-L1 expression on ovarian tumor cells from HGSC with enough reliability, regardless of whether a primary tumor, a metastasis, or a cytological sample were analyzed. These results demonstrate the possibility of applying PD-L1 immunohistochemical analysis to different types of materials with equal reliability.

A limitation of this research is certainly a small sample size. Considering the prospective study design and availability of adequate cytology samples we found this study significant enough to point to further similar and more comprehensive research projects in future. Tumor microenvironment is a great and unexplored field. Comparing its regulatory mechanisms and different molecular behavior could lead to a better understanding of antitumor interactions such as immunosuppression.

na taj način moglo bi biti manje invazivno i dovoljno pouzdano za utvrđivanje daljih procedura. Tumorske ćelije u citoblokovima imaju identičnu membransku ekspresiju PD-L1 kao u histološkim uzorcima, što još jednom potvrđuje validnost ovog metoda. Uz upotrebu citoblokova, materijal ispoljava značajnu korelaciju imunohistohemijske ekspresije sa standardnim histološkim preparatima. S obzirom na primenjivost citološkog materijala za interpretaciju ekspresije PD-L1, postojala je prihvatljiva pouzdanost rezultata formiranja citoblokova, za razliku od konvencionalnih citoloških razmaza i liquid-based citologije [20].

Zaključili smo da je učestalost visoke PD-L1 ekspresije veća u tumorskim ćelijama omentuma (87%) nego u primarnom tumoru (60%) i citološkom materijalu (32%). U podudarnim uzorcima, našli smo pozitivnu korelaciju između ekspresije PD-L1 u različitim uzorcima tumora. Dobili smo statistički značajne rezultate koji su sa dovoljno pouzdanosti pokazali PD-L1 ekspresiju na ćelijama karcinoma jajnika HGSC, bez obzira na to da li je analiziran primarni tumor, metastaza, ili citološki uzorak. Ovi rezultati pokazuju mogućnost primene PD-L1 imunohistohemijske analize na različite vrste materijala sa podjednakom pouzdanošću.

Ograničenje ovog istraživanja svakako predstavlja mala veličina uzorka. Imajući u vidu da je reč o prospektivnoj studiji i da su adekvatni citološki uzorci dostupni, smatramo da je ova studija dovoljno značajna da ukaže na dalje slične i još modernije istraživačke projekte u budućnosti. Tumorsko mikrookruženje predstavlja sjajno i neistraženo polje. Poređenje njegovih regulatornih mehanizama i raznovrsnog ponašanja molekula moglo bi da dovede do boljeg razumevanja antitumorskih interakcija kao što je imunosupresija.

ZAKLJUČAK

Ekspresija PD-L1 bila je slična u sve tri posmatrane grupe. Ovo bi moglo da ukaže na slične peritumorske regulatorne mehanizme koji postoje između primarnih tumorskih tkiva i citoloških tumorskih uzoraka. Imajući u vidu dobijene rezultate, mogla bi da bude korišćena manje invazivna procedura, kao što je peritonealno ispiranje, koja bi pouzdano predstavila odlike primarnog tumora. Za potvrdu ovih zaključaka, potrebno nam je u budućnosti još studija.

AUTORSKI DOPRINOS

Ljubiša Jovanović je osmislio naslov, obradio podatke, i napisao rukopis; Anđa Ćirković je uradila obradu statističkih podataka; Ljubinka Nikolić se bavila eksperimentalnim delom rada, Milena Jović, Darko Mikić i Svetlana Milenković pomagali su izvan baze podataka, čitali i odobravali rukopis. Radmila Janković bila je supervizor i mentor.

CONCLUSION

PD-L1 expression was similar in all three tumor compartments. This could point to similar peritumor regulatory mechanisms between primary tumor tissue and cytology tumor samples. Considering these results, a less invasive procedure such as peritoneal washing, could be used in PD-L1 immunoanalysis and reliably represent characteristics of a primary tumor site. For confirming these findings, we need more comprehensive studies in future.

AUTHOR CONTRIBUTION:

Ljubisa Jovanovic designed the topic, processed the database, and wrote the manuscript; Andja Cirkovic did statistical data processing; Ljubinka Nikolic was involved in the experimental part of the study; Milena Jovic, Darko Mikic and Svetlana Milenkovic helped update the database, and read and approved the manuscript; Radmila Jankovic was a supervisor and mentor.

Conflict of Interest:

The authors declared no potential conflicts of interest with respect to research, authorship, and/or publication of this article.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of University Clinical Center of Serbia (747/3; 19.07.2018) and Faculty of Medicine, University of Belgrade, Serbia (1550/XI-40; 28.11.2019.).

All subjects gave their informed consent for inclusion before they participated in the study.

Conflict of interest: None declared.

Sukob interesa

Autori su izjavili da ne postoji potencijalni sukob interesa kada je reč o istraživanju, autorstvu i/ili objavljivanju članka/knjige.

Istraživanje nije finansirano od bilo koje agencije iz javnog, privatnog ili neprofitnog sektora.

Studija je sprovedena u skladu sa Helsinškom deklaracijom, a protokol je odobrio Etički komitet Univerzitetskog kliničkog centra Srbije (747/3; 19.07.2018) i Medicinskog fakulteta Univerziteta u Beogradu (1550/XI-40; 28.11.2019.)

Svi autori dali su svoj informisani pristanak za učešće u studiji.

Sukob interesa: Nije prijavljen.

LITERATURA / *REFERENCES*

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA Cancer J Clin 2020;70(1):7-30. doi: 10.3322/caac.21590.
2. Landskron J, Helland Ø, Torgersen KM, Aandahl EM, Gjertsen BT, Bjørge L, et al. Activated regulatory and memory T-cells accumulate in malignant ascites from ovarian carcinoma patients. Cancer Immunol Immunother 2015;64(3):337-47. doi: 10.1007/s00262-014-1636-6.
3. Ford CE, Werner B, Hacker NF, Warton K. The untapped potential of ascites in ovarian cancer research and treatment. Br J Cancer 2020;123(1):9-16. doi: 10.1038/s41416-020-0875-x.
4. Tyagi R, Gupta N, Bhagat P, Gainder S, Rai B, Dhaliwal LK, et al. Impact of SurePath® liquid-based preparation in cytological analysis of peritoneal washing in practice of gynecologic oncology. J Cytol 2017;34(2):95-100. doi: 10.4103/JOC.JOC_193_14.
5. Pawłowska A, Kwiatkowska A, Suszczyk D, Chudzik A, Tarkowski R, Barczyński B, et al. Clinical and Prognostic Value of Antigen-Presenting Cells with PD-L1/PD-L2 Expression in Ovarian Cancer Patients. Int J Mol Sci 2021;22(21):11563. doi: 10.3390/ijms222111563.
6. Bansal A, Srinivasan R, Rohilla M, Rai B, Rajwanshi A, Suri V, et al. Immunotyping in tubo-ovarian high-grade serous carcinoma by PD-L1 and CD8+ T-lymphocytes predicts disease-free survival. APMIS 2021;129(5):254-64. doi: 10.1111/apm.13116.
7. Piao J, Lim HJ, Lee M. Prognostic value of programmed cell death ligand-1 expression in ovarian cancer: an updated meta-analysis. Obstet Gynecol Sci 2020;63(3):346-56. doi: 10.5468/ogs.2020.63.3.346.
8. Nikas IP, Lee C, Song MJ, Kim B, Ryu HS. Biomarkers expression among paired serous ovarian cancer primary lesions and their peritoneal cavity metastases in treatment-naïve patients: A single-center study. Cancer Med 2022;11(11):2193-203. doi: 10.1002/cam4.4600.
9. Meza-Perez S, Randall TD. Immunological Functions of the Omentum. Trends Immunol. 2017;38(7):526-36. doi: 10.1016/j.it.2017.03.002.
10. Sung S, Sireci AN, Remotti HE, Hodel V, Mansukani MM, Fernandes H, et al. Plasma-thrombin cell blocks: Potential source of DNA contamination. Cancer Cytopathol 2019;127(12):771-7. doi: 10.1002/cncy.22203.
11. Choi CH, Kim KH, Song JY, Choi SJ, Kim L, Park IS, et al. Construction of high-density tissue microarrays at low cost by using self-made manual microarray kits and recipient paraffin blocks. Korean J Pathol 2012;46(6):562-8. doi: 10.4132/KoreanJPathol.2012.46.6.562.
12. Scognamiglio G, De Chiara A, Di Bonito M, Tatangelo F, Losito NS, Anniciello A, et al. Variability in Immunohistochemical Detection of Programmed Death Ligand 1 (PD-L1) in Cancer Tissue Types. Int J Mol Sci 2016;17(5):790. doi: 10.3390/ijms17050790.
13. Jovanović L, Janković R, Ćirković A, Jović M, Janjić T, Djuričić S, et al. PD-L1 Expression in Different Segments and Histological Types of Ovarian Cancer According to Lymphocytic Infiltrate. Medicina (Kaunas) 2021;57(12):1309. doi: 10.3390/medicina57121309.
14. Tejerina E, Garca Tobar L, Echeveste JI, de Andrea CE, Vigliar E, Lozano MD. PD-L1 in Cytological Samples: A Review and a Practical Approach. Front Med (Lausanne) 2021;8:668612. doi: 10.3389/fmed.2021.668612.
15. Liu D, Kong D, Li J, Gao L, Wu D, Liu Y, et al. HE4 level in ascites may assess the ovarian cancer chemotherapeutic effect. J Ovarian Res 2018;11(1):47. doi: 10.1186/s13048-018-0402-3.
16. Layfield LJ, Roy-Chowdhuri S, Baloch Z, Ehya H, Geisinger K, Hsiao SJ, et al. Utilization of ancillary studies in the cytologic diagnosis of respiratory lesions: The papanicolaou society of cytopathology consensus recommendations for respiratory cytology. Diagn Cytopathol 2016;44(12):1000-9. doi: 10.1002/dc.23549.
17. Bozzetti C, Squadrilli A, Nizzoli R, Lagrasta C, Gasparro D, Majori M, et al. Optimizing PD-L1 evaluation on cytological samples from advanced non-small-cell lung cancer. Immunotherapy 2020;12(3):183-93. doi: 10.2217/imt-2019-0138.
18. Imai Y, Hasegawa K, Matsushita H, Fujieda N, Sato S, Miyagi E, et al. Expression of multiple immune checkpoint molecules on T cells in malignant ascites from epithelial ovarian carcinoma. Oncol Lett 2018;15(5):6457-68. doi: 10.3892/ol.2018.8101.
19. Toth L, Nagy B, Mehes G, Laszlo E, Molnar PP, Poka R, et al. Cell adhesion molecule profiles, proliferation activity and p53 expression in advanced epithelial ovarian cancer induced malignant ascites – Correlation of tissue microarray and cytology microarray. Pathol Res Pract 2018;214(7):978-85. doi: 10.1016/j.prp.2018.05.014.
20. Hernandez A, Brandler TC, Zhou F, Moreira AL, Schatz-Siemers N, Simsir A. Assessment of Programmed Death-Ligand 1 (PD-L1) Immunohistochemical Expression on Cytology Specimens in Non-Small Cell Lung Carcinoma. Am J Clin Pathol 2019;151(4):403-15. doi: 10.1093/ajcp/aqy164.