ORIGINAL ARTICLE

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Immunostaining of stromal CD56 cells in ovarian malignancies

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SUMMARY

OBJECTIVES: The aim of this study was to evaluate CD56 immunostaining in the stroma of benign and malignant ovarian epithelial neoplasms and associate the CD56 immunostaining with prognostic factors and survival in ovarian cancer.

METHODS: Patients with ovarian epithelial neoplasia (n=77) were studied with a prospective cohort. The CD56 immunostaining was evaluated in the peritumoral stroma. Two groups were evaluated: benign ovarian neoplasms (n=40) and malignant ovarian neoplasms (n=37). Data were recorded for histological type and grade, International Federation of Gynecology and Obstetrics staging, molecular subtype, and lymph node metastases. Fisher's exact test and Kaplan-Meier survival curves were used, with a significance level of ≤0.05.

RESULTS: We found greater CD56 stromal immunostaining in malignant neoplasms when compared to the group of benign neoplasms (p=0.00001). There was no significant difference in relation to the prognostic factors and survival.

CONCLUSION: Malignant ovarian neoplasms showed higher stromal CD56 immunostaining. As the prognostic value of natural killer in ovarian cancer is controversial, knowing the specific function of each cell present both in the tumor tissue and systemically may help guide successful immunotherapies in the near future.

KEYWORDS: CD56 antigen. Killer cells, natural. Ovarian neoplasms. Prognosis.

INTRODUCTION

In 2020, 313,959 new cases of ovarian cancer were detected, and approximately 207,252 deaths from the disease occurred worldwide[1]. In Brazil, approximately 6,650 cases of ovarian cancer were registered each year of the 2020–2022 triennium, with 4,123 deaths. The estimate for new cases in 2023 is 7,310 cases, with about 3,921 deaths from the disease[2]. In the USA, there are an estimated 19,710 new cases with about 13,270 deaths in the year 2023[3].

Epithelial ovarian cancer (EOC) develops an inflammatory environment with the presence of immune cells that can promote its growth via the release of cellular signalers and cytokines. However, no consensus on this understanding of the disease has been reached[4-6]. The immune cells present in EOC comprise mainly tumor-infiltrating lymphocytes (TILs): T CD4+, T CD8+, natural killer (NK) cells (CD56), and CD3+ and CD20+ T lymphocytes[7,8]. Several theories have been proposed to describe the influence of the immune system on tumor cells and their signaling molecules. Interleukin (IL) levels in ovarian tissue and serum from patients with ovarian cancer have recently been linked to prognostic factors[6,9,10]. The immune system plays a multifaceted role, promoting and inhibiting tumor growth in different contexts. The role of the immune response against EOC has not been described clearly, given the different actions of immune cells, such as T lymphocytes, which can lead to proliferation or the inhibition of tumor growth[9].

The objectives of this study were to evaluate CD56 expression in the stroma of benign and malignant ovarian epithelial neoplasms and examine associations of CD56 immunostaining with prognostic factors and survival in patients with ovarian cancer.

METHODS

This study was conducted with a prospective cohort of 77 patients with ovarian epithelial neoplasms seen at the Pelvic Mass Outpatient Clinic of the Department of Gynecology and Obstetrics, Federal University of Triângulo Mineiro. CD56 immune expression in the peritumoral stroma of the ovarian epithelial neoplasms was evaluated. The patients were divided

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into two groups based on anatomopathological confirmation of diagnoses: those with benign (n=40) and primary malignant (n=37) epithelial ovarian neoplasms. Borderline cases were included in the malignancy group. The Research Ethics Committee of the Federal University of Triângulo Mineiro approved this study (protocol no. 34770014.4.0000.5154, October 30, 2014). Free and informed written consent was obtained from each patient or a family member.

The inclusion criterion was the postoperative diagnosis of primary ovarian epithelial neoplasia (benign or malignant) by the anatomopathological paraffin analysis. The exclusion criteria included secondary ovarian malignancy (metastasis) or primary nonepithelial ovarian tumor, torsion of the adnexal pedicle, receipt of treatment prior to surgery, neoplasm recurrence, and immunosuppressive disease or treatment with immunosuppressive drugs.

The data recorded were patient age, parity, ages at menarche and menopause, hormonal status, histological type and grade of EOC, International Federation of Gynaecology and Obstetrics (FIGO) stage, molecular subtype, lymph node metastasis, disease-free survival (DFS), and overall survival (OS).

DFS was calculated from the date of diagnosis to the date of the first recurrence. OS was calculated from the diagnosis to the date of death.

An experienced pathologist at the Surgical Pathology Service of the Federal University of Triângulo Mineiro performed an anatomopathological analysis of the cuts embedded in paraffin.

For the immunohistochemical analysis, specimens obtained by surgical resection were processed in paraffin and reviewed by an experienced pathologist. The selected cases were submitted to new cuts (4 μm) in silanized sheets (ATPS - Silane, Sigma® A3648). The technique was performed according to the manufacturer's instructions. The percentage of immunostained cells with these antibodies in 10 random stroma fields adjacent to the epithelium was determined by two observers (0, ≤25%; 1, 26–50%; 2, 51–75%; 3, ≥76%) at 200/400× magnification (Figure 1).

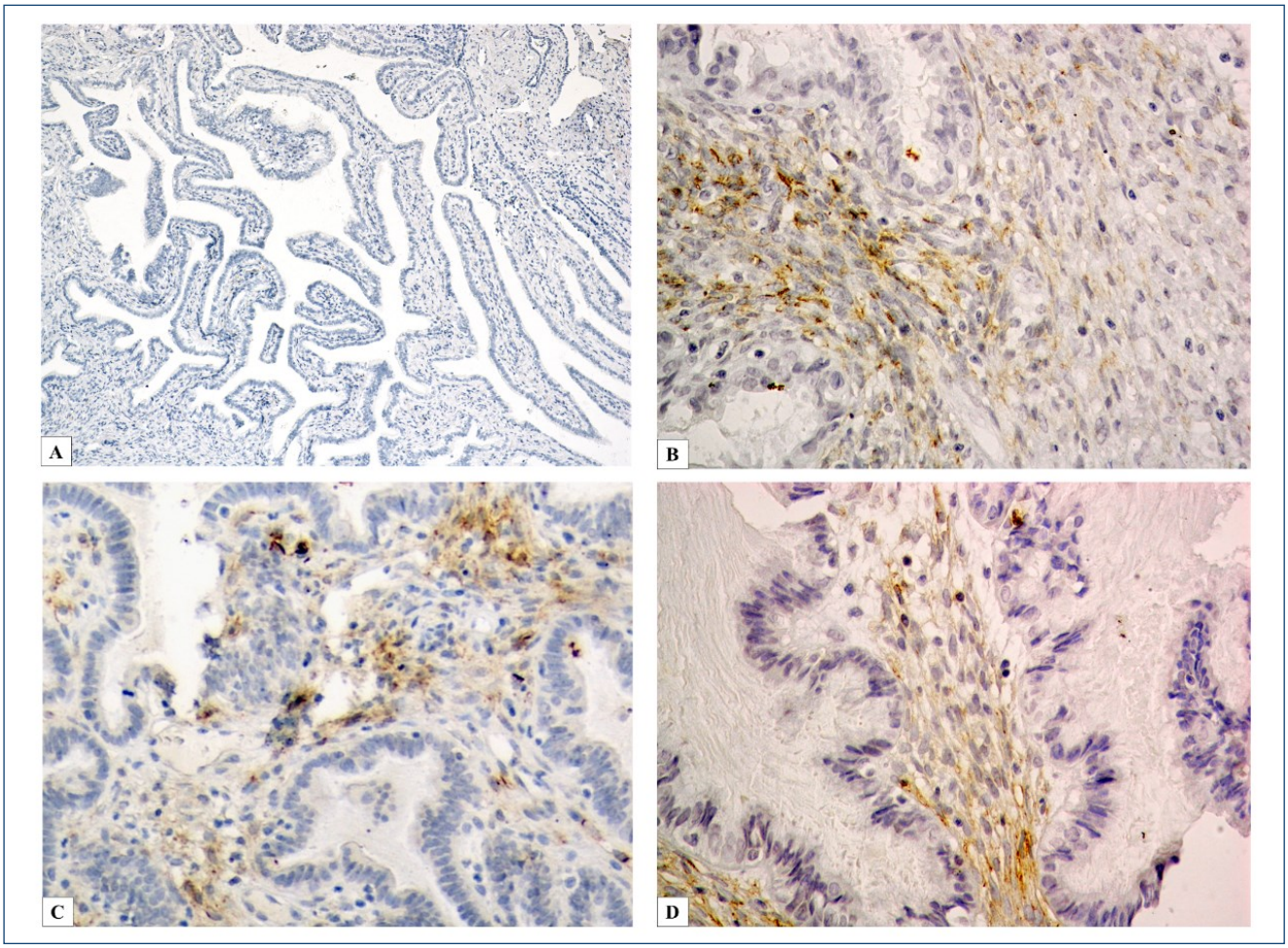

Figure 1. Immunohistochemical . of CD56. Histological sections of ovarian epithelial neoplasia. (A) Weak stromal CD56 immunostaining in serous cystadenoma (200×); (B) strong stromal CD56 immunostaining in serous borderline ovarian tumor (400×); (C) strong stromal CD56 immunostaining in serous borderline ovarian tumor (400×); (D): strong stromal CD56 immunostaining in serous borderline ovarian tumor (400×).

The GraphPad InStat and SPSS software were used for the statistical analysis. According to the data distribution (determined using the Kolmogorov-Smirnov test), the results are expressed as means and standard deviation or medians with percentiles, and Fisher's exact test was used. Kaplan-Meier curves were used to evaluate DFS and OS, in addition to the log-rank test. The significance level was 0.05. The agreement on immunohistochemical findings between the two observers was assessed using the kappa coefficient ($\kappa<0.4$, weak; $0.4\leq\kappa<0.8$, moderate; $0.8\leq\kappa<1.0$, strong; $\kappa=1.0$, perfect). Cases of disagreement were reviewed until consensus was reached.

RESULTS

The study sample comprised 77 patients, of whom 40 had benign and 37 had malignant neoplasms. For the malignant neoplasm group, the median age was 50 (range, 25–73) years, the median parity was 2 (range, 0–7) births, the median age at menarche was 13 (range, 9–16) years, and the median age at menopause was 50 (range, 38–57) years. In all, 22 (59.5%) of these patients were in menacme and 15 (40.5%) were menopausal, and 13 (35.1%) patients in this group died. For the benign neoplasm group, the median age was 48 (range, 18–69) years, the median parity was 2.5 (range, 0–9) births, the median age at menarche was 13 (range, 10–17) years, and the median age at menopause was 49 (range, 29–55) years. Of note, 23 (57.5%) patients were in menacme, and 17 (42.5%) were menopausal.

The benign ovarian neoplasm subtypes determined by the analysis of histological sections were 21 (52.5%) serous cystadenomas, 16 (40.0%) mucinous cystadenomas, 1 (2.5%) seromucinous cystadenoma, 1 (2.5%) Brenner tumor with mucinous cystadenoma, and 1 (2.5%) Brenner tumor with serous cystadenoma. The malignant neoplasm subtypes were 14 (37.9%) serous high-grade carcinoma, 12 (32.4%) mucinous borderline ovarian tumor, 3 (8.1%) serous low-grade carcinoma, 3 (8.1%) serous borderline ovarian tumor, 2 (5.4%) mucinous carcinoma, 1 (2.7%) endometrioid carcinoma, 1 (2.7%) clear cell carcinoma, and 1 (2.7%) endometrioid borderline ovarian tumor.

The FIGO stages of the malignant neoplasms were IA [n=14 (37.83%)], IB [n=2 (5.41%)], IC2 [n=2 (5.41%)], IIA [n=1 (2.70%)], IIB [n=2 (5.41%)], IIIA1 (ii) [n=1 (2.70%)], IIIA2 [n=1 (2.70%)], IIIB [n=1 (2.70%)], IIIC [n=10 (27.03%)], and IVB [n=3 (8.11%)]. The histological grades of these tumors were 1 [n=16 (43.2%)], 2 [n=14 (37.8%)], and 3 [n=7 (18.9%)]. Overall, 20 (54.05%) malignant neoplasms were of molecular subtype I and 17 (45.95%) were of molecular subtype II.

We observed greater CD56 stromal immunostaining in malignant than in benign neoplasms (p=0.00001; Table 1). No significant difference in immunostaining related to any prognostic factor was found (Table 2). The distribution of CD56

Table 1. Differences in stromal CD56 immunostaining between malignant and benign ovarian neoplasms.

		0	1/2/3	p-value
CD56	Benign neoplasms	39/40 (97.5%)	1/40 (2.5%)	0.00001*
	Malignant neoplasms	18/37 (48.6%)	19/37 (51.4%)	

*Fisher's exact test. 0: ≤25% of labeled cells; 1: 26–50% of labeled cells; 2: 51–75% of labeled cells; 3: ≥76% of labeled cells. Benign neoplasms: n=40; malignant neoplasms: n=37.

Table 2. Stromal CD56 immunostaining and association with histological grade, staging, molecular subtype, and lymph node metastasis in ovarian cancer.

		CD56 0	CD56 1,2,3	p-value*
Histological grade (n=37)	1	8/17 (47.1%)	9/17 (52.9%)	0.7463
	2/3	8/20 (40.0%)	12/20 (60.0%)	
Staging (FIGO) (n=37)	I/II	11/21 (52.4%)	10/21 (47.6%)	0.5085
	III/IV	6/16 (37.5%)	10/16 (62.5%)	
Molecular subtype (n=37)	I	11/20 (55.0%)	9/20 (45.0%)	0.3248
	II	6/17 (35.3%)	11/17 (64.7%)	
Lymph node metastasis (n=24)	Positive	2/5 (40.0%)	3/5 (60.0%)	1.0000
	Negative	7/19 (36.8%)	12/19 (63.2%)	

*Fisher's exact test. 0: ≤25% of labeled cells; 1: 26–50% of labeled cells; 2: 51–75% of labeled cells; 3: ≥76% of labeled cells. Malignant neoplasms: n=37.

Table 3. Distribution of CD56 stromal immunostaining readings according to histological subtypes of ovarian cancer.

Histological subtypes	IH 0	IH 1	IH 2	IH 3
Endometrioid adenocarcinoma	0	0	1	0
Clear cell carcinoma	1	0	0	0
Cystoadenocarcinoma	2	0	0	1
Mucinous cystadenocarcinoma	2	0	0	0
Serous papillary cystadenocarcinoma	4	4	4	2
Borderline atypical proliferative endometrioid tumor	1	0	0	0
Borderline mucinous tumor	7	1	2	2
Borderline serous tumor	0	0	0	3

IH: immunostaining.

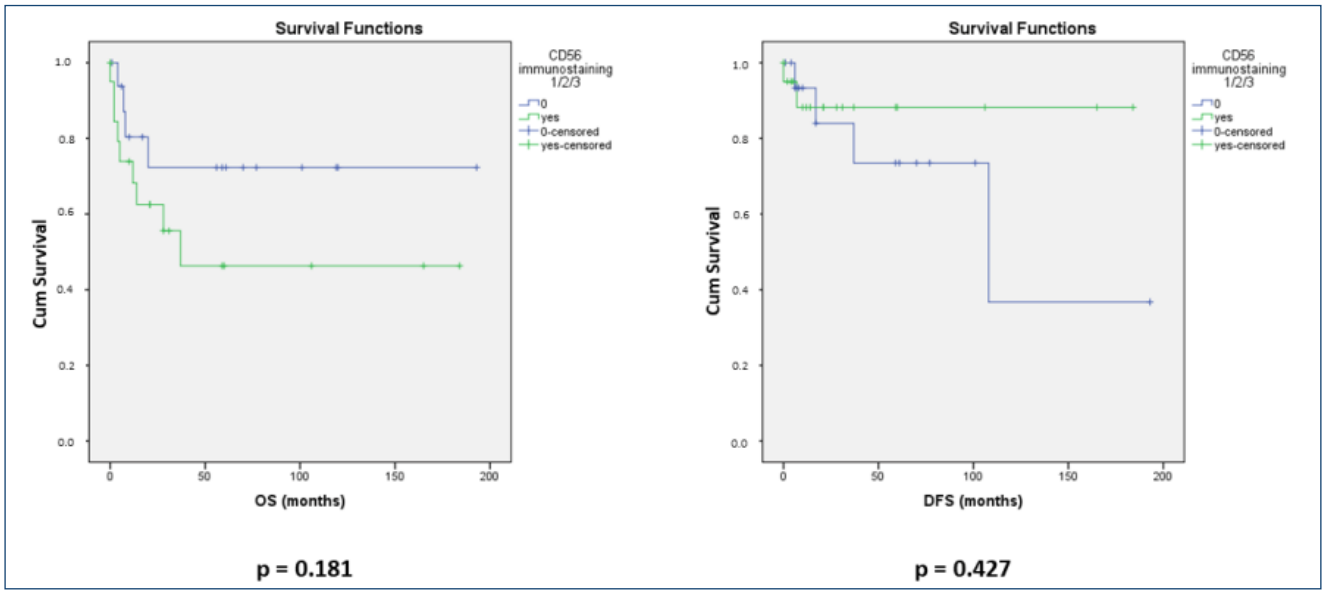

Figure 2. Evaluation of overall survival and disease-free survival in relation to stromal CD56 immunostaining.

stromal immunostaining readings according to the histological subtype of ovarian cancer is shown in Table 3. There was no statistical significance in the evaluation of OS or DFS (p=0.181 and p=427, respectively) (Figure 2).

DISCUSSION

Experimental data have shown that the inflammatory microenvironment of the EOC prevents the maturation of myeloid cells, favors the development of regulatory cells, and suppresses the cytotoxic activity of effector lymphocytes, allowing the tumor to escape the immune system and triggering the progression of cancer[11]. The immune system acts directly on tumor tissue with the expression of cytokines and their release into the serum, peritoneal fluid, and intracystic fluid of patients with cancer. This process makes evident the roles of defense cells in the tumor microenvironment, namely the promotion or inhibition of neoplastic cell growth[6,8-10,12,13].

The mechanisms by which TILs enter a tumor, crossing the vessel wall and migrating into the stroma, occur after the recruitment of T lymphocytes to the site. The complex stromal microenvironment is composed of noncancerous cells together with the extracellular matrix, and TIL migration results in interaction with tumor cells[14]. Activation of the stromal microenvironment has been identified as an important factor in the progression of cancer[15]. The rearrangement of the extracellular matrix and distinct cell clusters in the stroma create a specific microenvironment that promotes carcinogenesis, leading to

cancer cell proliferation, invasion, and survival. These events are based on activities orchestrated by cell-cell interaction[16].

We found greater stromal NK CD56 immunostaining in malignant than in benign neoplasms, but no association of this staining with any prognostic factor or survival was found. We attribute these findings to the heterogeneity of tumors evaluated. Despite their common cellular origin, ovarian neoplasms have heterogeneous, divergent, and difficult-to-understand genetic, biological, clinical, and immunological properties, especially in advanced stages[17,18].

NK are characterized phenotypically by the expression of a CD56 surface marker, although they lack CD3 expression, but they do not constitute a homogeneous population; they can be grouped into subpopulations based on maturity and functional characteristics. About 90% of NK CD56-expressing lymphocytes are cytotoxic, effectively inducing cell death. The remaining 10% have low degrees of cytotoxicity before activation, but they are the most efficient producers of cytokines with immunoregulatory properties, including interferon (IFN)-γ, tumor necrosis factor-α, granulocyte-macrophage colony stimulating factor, IL-10, and IL-13, thereby acting as regulatory T cells[5,19].

A study showed that most NK CD56 cells in patients with ovarian cancer have phenotypically regulatory and noncytotoxic characteristics, which prevent them from attacking tumor cells, thereby allowing tumors to proliferate. In the same study, however, the in vitro inhibition of certain receptors altered the activity of NK extracted from patients with ovarian tumors and healthy donors, activating them to eliminate tumor tissue[20]. Cells in the tumor microenvironment trigger the negative regulation of NK-activating receptors, thereby impairing their IFN-γ production and cytolytic functions. EOC cells' expression of the MUC-16 antigen protects them from recognition by NK, inhibiting the formation of intermembrane communication and leading to an increase in metastatic capacity[21].

On the contrary, another study revealed a larger proportion of cytotoxic NK than of those that behave as cytokine secretors in CD56+ clusters in the ascitic fluid of patients with EOC, comparing malignant and benign groups[22]. The cytotoxic functions of ascites-derived NK cells and those in peripheral blood from healthy donors are equivalent, although the former show less expression of activation markers than those in benign peritoneal fluid, which is associated with increased disease-free progression[23]. These data indicate that ascites-derived NK cells from patients with EOC and low survival have significantly less expression of activation receptors on their surfaces, an unknown variation that inhibits the cytotoxic function of these cells (in turn, this function can be activated with IL-15 stimulation)[22].

In a study by He et al., who studied the expression of CD56 in 16 normal ovaries, 17 ovarian fibromas, 11 ovarian cellular fibromas, 10 ovarian fibrothecomas, and 11 ovarian leiomyomas, the normal ovarian stromal cells were strongly positive for CD56 with the strongest immunostaining. CD56 is strongly expressed in ovarian stromal cells but not in endometrial stromal cells[24].

The frequency of these lymphocytes is much lower than that of T and B cells. The prognostic value of ovarian cancer NK is controversial, although greater NK activity in peripheral blood at the time of surgery is an indicator of better survival. Increased concentrations of NK in peritoneal and pleural exudates of metastatic tumors were associated with worse prognoses[25]. EOC-associated ascites showed a higher proportion of the subpopulation of NK $CD56^{bright}$ lymphocytes than in the blood, showing that the inflammatory profile can be different depending on the evaluated site[26]. Current experimental studies conducted with murine models have shown promise for the evaluation of the activation of NK cytolytic activity in breast and ovarian tumors. Changes in receptors obtained by the manipulation of oncological viruses associated with dendritic cell immunotherapy reduced the incidence of metastatic tumors and increased the survival of the study subjects[27].

This study has limitations. The use of immunohistochemical analysis in the present study may have limited the ability to evaluate NK activity because it does not allow the evaluation of phenotypic marking for cells that function as effectors against regulators in EOC. Another limitation of the study is the small sample of patients and the evaluation of different histological types. Future studies with a larger number of patients, enabling the stratification of histological types and NK subpopulations (CD56 dim and CD56 bright NK), are needed to clarify the role of stromal CD56 in the immune response and prognosis of ovarian cancer.

Therefore, the lymphocytes and NK cells in EOC have not been studied thoroughly, and their actions may differ at different points in the course of the disease. Knowledge of the specific functions of each cell in the tumor tissue can contribute systemically to the development of successful immunotherapies in the near future.

AUTHORS' CONTRIBUTIONS

CAL: Data curation, Methodology, Writing – original draft. **MPJ:** Investigation, Methodology, Writing – original draft. **RME:** Data curation, Methodology, Writing – original draft. **EFCM:** Conceptualization, Formal Analysis, Supervision, Validation, Writing – review & editing. **RSN:** Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Writing – review & editing.

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