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Original Research Article

Distribution of tumor-associated macrophages and M1/M2 polarization in different types and grades of ovarian tumors

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ABSTRACT

Ovarian cancer represents one of the major causes of gynaecologic cancer mortality worldwide. Tumor-associated macrophages (TAM) are associated with the development and progression of ovarian cancer. TAMs are mainly represented by two types M1 and M2. We investigated the distribution of total, M1, M2 macrophages, tumor cell/macrophage ratio and M1/M2 ratio in mucinous and serous ovarian tumors. The study results showed that total TAM count is significantly higher in serous ovarian tumors, compared to mucinous tumors and the highest infiltration rate is detected in high-grade serous ovarian carcinomas. In addition, the number of M2 macrophages is significantly increased in higher-grade serous ovarian carcinomas. The evaluation of tumor/cell macrophage ratio could be used as an objective measure of macrophage infiltration in ovarian tumors.

1. Introduction

Ovarian cancer represents one of the major causes of gynaecologic cancer mortality worldwide.[1] In Georgia, ovarian cancer is the fourth most common cancer in females and third most common cause of cancer related mortality.[2] Due to lack of early screening methods and indolent clinical course, the majority of ovarian cancers are diagnosed at advanced stages when the disease prognosis is already poor.[3] Based on histological origin ovarian tumors are divided into epithelial, sex chord and stromal tumors. World health organisation (WHO) classifies ovarian carcinomas as serous, mucinous, endometroid, clear cell, transitional cell, mixed epithelial and undifferentiated and unclassified tumors,[4] from which ovarian serous carcinomas are most common.[5,6] Based on FIGO classification ovarian serous carcinomas are classified as low and high grade,[7] with prognostic importance.[8] In addition, subgroup of serous ovarian tumors are classified as borderline, which are characterised with histological features of both benign serous cystadenomas and malignant serous ovarian carcinomas. They are also characterised with borderline prognosis.[9]

The Immune microenvironment of ovarian carcinomas is mostly represented by macrophages, dendritic cells, neutrophils and lymphocytes.[10] Macrophages are associated with the development and progression of ovarian cancer.[11] They are known as Tumor-Associated Macrophages (TAMs).[11] TAMs are mainly represented by two functional and phenotypic subtypes - M1 and M2, which are considered immune activating and immune-suppressive respectively.[12] M1 and M2 macrophages are not distinguishable in standard, haematoxylin and eosin stained diagnostic specimens by light microscopy. However due to their phenotypic difference and the expression of

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different cell surface markers, M1 and M2 macrophages can be distinguished by immunohistochemical examination, using the most common and widely applicaple markers such as CD68 for both types of macrophages and CD163 for M2 macrophages.[13] It has been shown that M2 macrophages are most frequent in ovarian carcinomas. Some studies also report the association of M2 macrophages with tumor invasion, angiogenesis, metastasis and early relapse.[14] However, the detailed distribution of M2 macrophages in different grades of ovarian serous carcinomas, as well as in serous cystadenomas and borderline serous tumors is not well studied. Our study aims to characterise the distribution of M2 macrophages in ovarian serous cystadenomas, borderline serous tumors and low and high-grade serous carcinomas.

2. Material and Methods

2.1. Study samples

Formalin-fixed and paraffin-embedded (FFPE) tissue material was retrieved from the Research, Diagnostic and Teaching Laboratory of Tbilisi State Medical University, Georgia. Patients were diagnosed between 1^{st} of February 2021 and 28^{th} of February 2022 in the same department. Informed consent for the use of FFPE material and associated data have been retrieved from each patient and approved by Ethics Committee of Tbilisi State Medical University (N2-2022/95). Ovarian cancer cases have been randomly selected for each diagnosis of interest with equal numbers, in order to maximize the comparability between different diagnostic entities. Altogether 75 cases were included in the study which were distributed into the following groups: group I – ovarian serous borderline tumors (n=15), group II – ovarian mucinous borderline tumors (n=15), group III – ovarian mucinous carcinoma (n=15), group IV – low grade serous ovarian carcinoma (n=15), group V – high grade serous ovarian carcinoma (n=15).

2.2. Immunohistochemistry

FFPE tissue sections were deparaffinized in xylene, rehydrated by using serial dilutions of ethanol (96%, 80%, 70%) and heat mediated antigen retrieval has been performed. Ready to use antibodies against the following antigens were used: CD68 (514H12) and CD163 (10D6) (Novocastra). Staining and visualisation has been performed using Bond polymer refine detection system. Histopathological and IHC staining images are given in Figure 1.

2.3. Digital pathology analysis

IHC stained samples were digitally analysed using freely available digital pathology software QuPath (V0.1.2). First, 20 regions of interest (ROIs) were captured from each slide at 400x, using a Leica MC170 HD camera. Images were included in the software and IHC staining vectors were adjusted. Watershed nucleus detection has been used for the detection of total cell count. Positive cell detection was used for counting CD68 and CD163 positive macrophages. Automatic detections were revised and corrected by the pathologist.

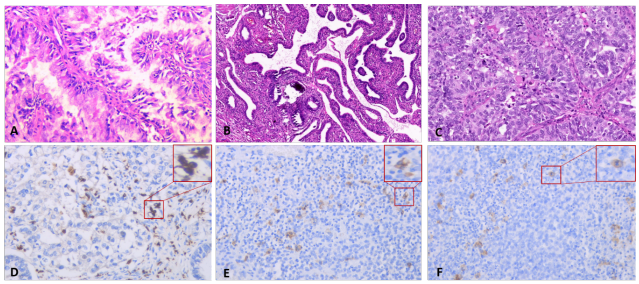

Fig. 1: The representative microphotograph of: **A:** Ovarian mucinous carcinoma, H&E, x400, **B:** Low grade serous ovarian carcinoma, H&E, x200, **C:** High grade serous ovarian carcinoma, H&E, x400, **D:** CD68+ macrophages in mucinous carcinoma, IHC, x400; **E:** CD163+ macrophages in low grade serous carcinoma, IHC, x400, **F:** CD163+ macrophages in high grade serous carcinoma, IHC, x400.

2.4. Statistical analysis of data

Total tumor cell and positive cell (macrophage) counts were recorded and the Tumor/Macrophage ratio has been calculated by dividing a total number of tumor cells by the macrophage number. M1/M2 ratio has been calculated as follows: the number of CD68 positive cells (total macrophage count) minus the number of CD163 positive cells (M2 macrophage count) divided by the CD163 positive cell count. Comparisons between groups were made using the Kruskal-Wallis test and correlations were assessed using Spearman's rank correlation. P values <0.05 were considered significant. All statistical tests were performed using IBM SPSS software V19.00.

3. Results and their Discussion

The counted average tumor cell number was 1321±269 in serous borderline tumors, 1445±321 in mucinous borderline tumors, 1310±227 in mucinous carcinomas, 1422±423 in low-grade serous carcinomas and 1347±231 in high-grade serous carcinomas. The total macrophage count labelled as CD68 positive cells were 44.13±12.1 in serous borderline tumors, 44.17±14.3 in mucinous borderline tumors, 58.3±17.2 in mucinous carcinoma, 78.9±22.1 in low-grade serous carcinoma and 102.3±32.6 in high-grade serous carcinomas. The average total tumor cell/total macrophage ratio was 30.5±5.8 in serous borderline

tumors, 33.9±3.7 in mucinous borderline tumors, 22.8±2.7 in mucinous carcinomas, 18±2.4 in low-grade serous carcinomas and 13.9±1.7 in high-grade serous carcinomas. The average M1/M2 ratio was 4.06±0.9 in serous borderline tumors, 5.4±1.1 in mucinous borderline tumors, 2.9±0.4 in mucinous carcinomas, 1.6±0.3 in low-grade serous carcinomas and 1.3±0.1 in high-grade serous carcinomas.

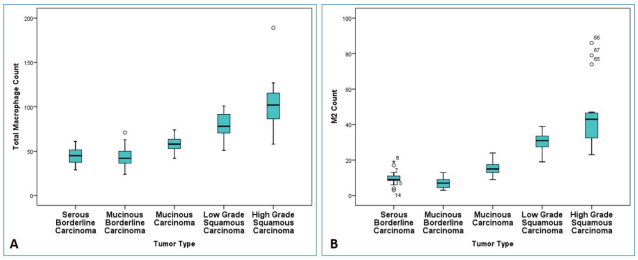

Fig. 2: Box and whisker plots showing: **A:** The distribution of total macrophage count in ovarian tumors, **B:** The distribution of M2 macrophage count in ovarian tumors

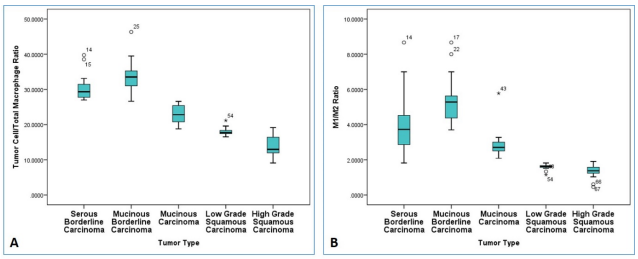

Fig. 3: Box and whisker plots showing: **A**. The distribution of tumor cell/total macrophage ratio in ovarian tumors, **B**. The distribution of M1/M2 macrophage ratio in ovarian tumors

Table 1: The distribution of tumor/total macrophage ratio and M1/M2 ratio

Tumor Type	Tumor/Total Macrophage Ratio	M1/M2 Ratio
Serous Borderline Carcinoma	30.5±5.8	4.06±0.9
Mucinous Borderline Carcinoma	33.9±3.7	5.4±1.1
Mucinous Carcinoma	22.8±2.7	2.9±0.4
Low Grade Serous Carcinoma	18±2.4	1.6±0.3
High Grade Serous Carcinoma	13.9±1.7	1.3±0.1

The results of our study indicate that highest tumor cell/total macrophage ratio is seen is mucinous borderline tumors, meaning the lowest number of macrophage infiltration. The tumor cell/total macrophage count was slightly lower in serous borderline tumors, compared to mucinous borderline tumors, which indicates that serous borderline tumors have more macrophage infiltration. With regard to ovarian carcinomas, the highest tumor cell/total macrophage ratio has been seen in mucinous carcinomas, followed by low grade serous carcinomas. The lowest tumor cell/total macrophage ratio has been seen in high grade serous carcinomas. In particular, it was 1.6 times lower compared to mucinous carcinomas and 1.3 times lower compared to low grade serous carcinomas. Overall, the results indicate that serous tumors have higher macrophage infiltration and it is further increased with the increase of malignancy grade. Highest M1/M2 ratio has been also demonstrated in mucinous borderline tumors, followed by serous borderline tumors. In carcinomas, the highest M1/M2 ratio has been seen in mucinous carcinomas, followed by low grade serous carcinomas and high grade serous carcinomas. In high grade serous carcinomas M1/M2 ratio was 2.2 times lower compared to mucinous carcinomas and 1.3 times lower compared to low grade serous carcinomas. These results indicate the significant increase to M2 macrophages in high grade serous carcinomas, compared to M1 macrophage count, known as a macrophage repolarization.

Recent decades witnessed significant advances in tumor immune microenvironment research, which became more important along with the development of novel immunotherapeutic drugs in various cancer patients.[11] Tumor associated macrophages (TAMs) are crucial component of tumor immune microenvironment, represented with different functions and phenotypes.[15] Most commonly investigated TAM subtypes are so called type M1 and M2 macrophages. The distinction is based on the cytokine secretion profile. M1s are classically activated macrophages whilst M2s represent alternatively activated macrophages. M2 macrophage derived cytokines play an important role in angiogenesis and tissue remodelling and bear pro-tumorigenic properties. TAMs are dynamic population of cells, which could change phenotype. Different studies indicate that M1/M2 repolarization is the frequent event in malignant tumors, including ovarian cancer.[16] In ovarian cancer the investigation of M1/M2 macrophages, showed that higher M1/M2 ratio is associated with better overall survival.[16] There are several markers for TAM characterisation and subtyping. However, most commonly used markers in clinicopathological studies are CD68 total macrophage marker and CD163 as M2 macrophage marker.[17] Xia et al., performed a meta-analysis including 9 studies and 794 patients. Study results showed that worse progression free survival was associated with higher density of CD163+ M2 macrophages and lower CD68+/CD163+ TAM ratio.[18] Similar to our study results, Kawamura et al., also demonstrated that TAM infiltration is more prominent in serous ovarian tumors compared to mucinous tumors, as well as in higher grade serous

carcinomas.[19] In addition to other studies, we have first investigated the tumor cell/macrophage ratio in ovarian cancer patients. We believe that such an assessment might be implemented in future studies for more accurate evaluation of the TAMs.

4. Conclusions

Total TAM count is significantly higher in serous ovarian tumors, compared to mucinous tumors and the highest infiltration rate is detected in high grade serous ovarian carcinomas. In addition, the number of M2 macrophages are significantly increased in higher grade serous ovarian carcinomas. The evaluation of tumor/cell macrophage ratio could be used as an objective measure of macrophage infiltration in ovarian tumors.

5. Source of Funding

None.

6. Conflict of Interest

None.

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