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Tumor associated macrophages in breast cancer

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Abstract

Background: Cancer research was focused on the studying of proper tumor cells for a long time. Despite the huge progress, there are still a lot of questions, that's why new molecular markers must be identified. These could reveal new information about tumorigenesis.
Material and methods: 15 cases of ductal invasive breast carcinomas have been analyzed and researched on tumor associated macrophages via immunohistochemistry. CD68 was used as a macrophage marker and $CD68^+$ cells were evaluated in tumor nest and peritumoral area, as well as hormone receptors (ER, PR) and HER2 protein.
Results: Most of tumors (10 cases out of 15/ 66.7%) were moderately differentiated (G2). The mean and std. error of mean of intratumoral $CD68^+$ cells were 2.0±0.2, of peritumoral $CD68^+$ cells – 1.4±0.2. Intratumoral $CD68^+$ cells registered higher scores than those located in the peritumoral area.
Conclusions: $CD68^+$ cells are more likely to be present in the tumor nest rather than in the peritumoral area. This research did not establish any significant correlations between intratumoral and peritumoral $CD68^+$ cells and patients' age, tumor grade, expression of ER and PR. The content of peritumoral $CD68^+$ cells inversely correlated with the number of $HER2^+$ carcinoma cells.

Key words: breast cancer, tumor associated macrophages, CD68, ER, PR, HER2.

Introduction

According to Globocan 2018, there were reported 2 088 849 of new cases of breast cancer (11.2% incidence), being the most frequent neoplasia in women and the leading cause of female cancer related death [1].

Treatment strategy depends on tumor progression and its morphological type. Molecular classification, based on the evaluation of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), cytokeratin 5 (CK5) and epidermal growth factor receptor (EGFR), includes Luminal A, B, HER2 and triple-negative subtypes. This classification had a huge impact on individual prognosis, personalized treatment and response to the therapy. Despite the fact that many things about breast cancer have been discovered there are still a lot of questions. Molecular classification does not comprise and does not describe perfectly all of the entities, that's why new molecular markers must be identified. These could reveal new information about tumorigenesis [2, 3].

Cancer research was focused on the studying of proper tumor cells for a long time. But cancer cells do not act alone, tumor consists of both malignant and non-malignant elements like macrophages, lymphocytes, mast cells, fibroblasts, connective tissue fibers, nerve fibers. Among these, tumor associated macrophages represent the vast majority, sometimes more than 50%. Their importance should not be underestimated because they are able to control the immune response, cellular mobility and to stimulate/inhibit angiogenesis and lymphangiogenesis. Moreover, macrophages can modulate the drug resistance by various substances secreted into the microenvironment [4, 5].

Macrophages can be differentiated into 2 types depending on the chemical signals coming from the microenvironment: classically activated macrophages (M1) and alternatively activated macrophages (M2). M1 macrophages exhibit antitumoral activity because of their ability to activate type 1 helper T cells (Th1), recognize the cancer cells and phagocytose them. M2 macrophages on the other hand are involved in wound healing where they downregulate the inflammatory reactions, promote angiogenesis, recruit fibroblasts and regulate connective tissue remodeling. Thus, an increased number of M1 macrophages is associated with a lower tumor aggressiveness, whereas an increased number of M2 macrophages stimulates tumor growing and involves a poor prognosis [5, 6, 7].

The aim of this study was to determine the role of tumor associated macrophages in breast cancer pathology. We also researched on the localization of the macrophages (tumor nest/ peritumoral area) by studying the expression of CD68 marker and identified whether there does exist any correlation between $CD68^+$ cells and ER^+, PR^+, $HER2^+$ carcinoma cells. As a result, a single statistically significant negative correlation between $CD68^+$ cells and $HER2^+$ cells was established.

Material and methods

This research included 15 cases of ductal invasive breast carcinomas type NOS (not otherwise specified) collected

at Arad Clinical Hospital, Romania between 2013-2016. Patients ranged between 60 and 83 years old, mean of age being 69.6±2.1. All patients did not undergo chemo- or radiotherapy before surgery. The study was approved by the Ethics Committee of Nicolae Testemitsanu State University of Medicine and Pharmacy, Chisinau, Moldova (no. 33/ 37/ 12.02.2018).

Histological method. Specimens were obtained after surgery and fixed in 10% formalin. After the removal of fixative by washing with tap water, specimens were paraffin embedded (Paraplast High Melt, Leica Biosystems). Paraffin blocks were later used for creation of tissue microarrays by means of TMA Grand Master (3DHISTECH Ltd., Budapest, Hungary). Sections from these blocks were cut by using a Leica RM2245 microtome (Leica Biosystems, Newcastle UponTyne, UK) and mounted on glass slides (Surgipath X-tra Adhesive, Leica Biosystems, Newcastle UponTyne, UK).

Staining was accomplished by Leica Autostainer XL (Leica Biosystems, Newcastle UponTyne, UK). Mayer's hematoxylin (Merck, Germany) and aqueous eosin (Merck, Germany) were used. Slides were mounted automatically (Leica CV5030, Leica Biosystems, Newcastle UponTyne, UK). Tumor histology was reviewed by 3 pathologists and appropriate sections were selected for immunohistochemical stains.

Immunohistochemistry. Immunohistochemical staining was performed automatically by Leica Bond-Max (Leica Biosystems, Newcastle UponTyne, UK). Antigen retrieval was achieved by using of Bond Epitope Retrieval Solution 1 (pH 6) and 2 (pH 9) (Leica Biosystems, Newcastle UponTyne, UK). Primary antibody (ER, PR, HER2, CD68) was followed by 3% hydrogen peroxide (for endogenous peroxidase activity blocking). DAB (3, 3'- diaminobenzidine) was applied as a chromogen substrate for 10 minutes. Mayer's hematoxylin was the additional dye used for counterstaining (5 minutes). Then sections were placed in absolute alcohol for 5 minutes, dried and clarified in benzene for 5 minutes. Lastly, slides were mounted automatically (Leica CV5030, Leica Biosystems, Newcastle UponTyne, UK) using an ENTELLAN–like mounting medium (Leica CV Mount, Leica Biosystems, Newcastle UponTyne, UK) [8].

Methods of quantification. Hormone receptors (ER and PR) were evaluated according to Allred score. This score accounts the percentage of cells that test positive for hormone receptors, along with the intensity of staining [9]. HER2 protein was appreciated according to the recommendations of American Society of Clinical Oncology [10, 11].

CD68 is a glycoprotein found in lysosomes and to a lesser extent on the cell membrane. It is used for identification of macrophages, other members of the mononuclear phagocyte lineage and to describe the neoplasm of myeloid and macrophage/monocyte origin. Macrophages should show a moderate to strong cytoplasmic staining reaction [2, 12]. Quantification of brown stained macrophages was done by means of Axio Imager A2 microscope (Carl Zeiss, Germany). Sections were initially analyzed at a ×100 magnification in order to determine the most intensely stained regions. Then we analyzed intratumoral and peritumoral stroma, 2 microscopic fields for each one, at a ×200 magnification. The following score was applied: **"0"** – no staining observed; **"+1"** – up to 25% of $CD68^+$ cells; **"+2"** – 25-75% $CD68^+$ cells; **"+3"** – more than 75% $CD68^+$ cells. "+1", "+2" and "+3" were considered positive scores. The final value was the arithmetic mean of the values for the two fields.

Data analysis. A MS Excel 2010 database was used to store the data that were statistically analyzed by applying WinSTAT software. We considered a *p*-value of less than 0.05 as significant.

Results

Most of tumors (10 cases out of 15/66.7%) were moderately differentiated (G2). The other 5 cases (33.3%) were poorly differentiated (G3).

The mean and std. error of mean of ER^+ cells were 1.9±0.2; of PR^+ cells – 0.9±0.2; of $HER2^+$ cells – 0.5±0.2. Median's values were: "2" for ER, "1" for PR, "0" for HER2 (tab. 1).

Table 1

Patients' age, tumor grade, values of ER, PR, HER2, $CD68^+$ cells

	Age	Grade	ER	PR	HER2	CD68it	CD68pt
Valid cases	15	15	15	15	15	15	15
Mean	**69.6**	**2.3**	**1.9**	**0.9**	**0.5**	**2.0**	**1.4**
m	**2.1**	**0.1**	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**
Minimum	60	2	0	0	0	0	1
Maximum	83	3	3	2	2	3	3
Median	**67**	**2**	**2**	**1**	**0**	**2**	**1**

Note: CD68it – intratumoral $CD68^+$ cells; CD68pt – peritumoral $CD68^+$ cells; m – Std. error of mean.

Table 2

The comparative analysis of patients' age, tumor grade and molecular markers

	Age	Grade	ER	PR	HER2	CD68it	CD68pt
Age							
rs		0.31	0.26	0.09	-0.06	0.22	-0.02
P		0.13	0.18	0.38	0.42	0.22	0.47
Grade							
rs	0.31		0.19	-0.30	0.00	0.40	-0.19
P	0.13		0.25	0.14	0.50	0.07	0.25
ER							
rs	0.26	0.19		0.39	0.32	0.18	-0.30
P	0.,18	0.25		0.07	0.12	0.25	0.14
PR							
rs	0.,09	-0.30	0.39		0.42	0.23	-0.24
P	0.38	0.14	0.07		0.06	0.20	0.20

HER2							
rs	-0.06	0.00	0.32	0.42		-0.25	**-0.53**
P	0.42	0.50	0.12	0.06		0.19	**0.02**
CD68it							
rs	0.22	0.40	0.18	0.23	-0.25		0.33
P	0.,22	0.,07	0.25	0.20	0.19		0.11
CD68pt							
rs	-0.02	-0.19	-0.30	-0.24	**-0.53**	0.33	
P	0.47	0.25	0.14	0.20	**0.02**	0.11	

Note: Age – patients' age; Grade – tumor grade; ER – estrogen receptor; PR – progesterone receptor; HER2 – human epidermal growth factor receptor 2; CD68it – intratumoral $CD68^+$ cells; CD68pt – peritumoral $CD68^+$ cells; rs – Spearman rank correlation; p – statistical significance. Statistically significant cases were marked in **bold**.

The mean and std. error of mean of intratumoral $CD68^+$ cells were 2.0±0.2; median was "2". In case of peritumoral $CD68^+$ cells, the mean and std. error of mean were 1.4±0.2; median was "1". Intratumoral $CD68^+$ cells registered higher scores than those located in the peritumoral area.

The comparative analysis of intratumoral, peritumoral $CD68^+$ cells and molecular markers (ER, PR, HER2) revealed a single statistically significant negative correlation between the expression of HER2 protein and peritumoral $CD68^+$ cells (rs = -0.53, p<0.02), (tab. 2).

Discussion

Breast cancer is a heterogeneous disease in terms of histology, therapeutic response, dissemination patterns to distant sites, and patients' outcomes. A plausible explanation for this scenario is, in part, that we still lack a complete picture of the biologic heterogeneity of breast cancers with respect to molecular alterations, treatment sensitivity, and cellular composition. Importantly, this complexity is not entirely reflected by the main clinical parameters (age, node status, tumor size, histological grade) and pathological markers (ER, PR and HER2), all of which are routinely used in the clinic to stratify patients for prognostic predictions and to select treatments [13]. In this study we partially approached macrophages, just one actor of this complex scenario.

CD68 is a pan-macrophage marker used as a marker for tumor associated macrophages. However, CD68 recognizes both tumoricidal M1 (classically activated) and anti-inflammatory M2 (alternatively activated) macrophages [5]. The term of macrophage activation was introduced by Mackaness in the 1960s in an infection context to describe the antigen-dependent, but non-specific enhanced, microbicidal activity of macrophages toward *BCG* (bacillus Calmette-Guerin) and Listeria upon secondary exposure to the pathogens [14]. M1 macrophages, or classically activated macrophages, are aggressive and highly phagocytic, produce large amounts of reactive oxygen and nitrogen species, and promote a Th1 response [11]. This is a macrophage response usually seen during microbial infections. M1 macrophages secrete high levels of IL-12 and IL-23, two important inflammatory cytokines. IL-12 induces the activation and clonal expansion of Th17 cells, which secrete high amounts of IL-17, and thus contribute to inflammation. In the context of cancer, classically activated macrophages are thought to play an important role in the recognition and destruction of cancer cells, and their presence usually indicates good prognosis. For a long time, M1 macrophages were thought to be the only functional macrophages and that anti-inflammatory molecules were inhibitory to their function. Now we understand that anti-inflammatory molecules did not inhibit macrophage function but provided an alternative activation of macrophages. M2 macrophages, or alternatively activated macrophages, are anti-inflammatory and are not capable of efficient antigen presentation. Expression of IL-10 by M2 macrophages promotes a Th2 response, and Th2 cells, in turn, upregulate the production of IL-3 and IL-4. IL-4 is an important cytokine in the healing process because it contributes to the production of the extracellular matrix. The tumor microenvironment significantly affects macrophage polarization. The process of polarization can be diverse and complicated because of the complex environment of IL-10, glucocorticoid hormones, apoptotic cells, and immune complexes that can interfere with the function of innate immune cells [15].

According to Weagel et al., the tumor mass contains a great number of M2-like macrophages and these can be used as a target for cancer treatment. Reducing the number of M2s or polarizing them towards an M1 phenotype can help destroy cancer cells or impair tumor growth [15]. Unfortunately, our study did not reveal which macrophages have registered higher scores in the intratumoral area: M1s, being able to destroy the tumor or maybe M2 macrophages, promoting tumor growth and repair?

CD163 could be the answer to our questions. CD163 is a scavenger receptor upregulated by macrophages in an anti-inflammatory environment and regarded as a highly specific monocyte/macrophage marker for M2 macrophages. This research could be continued by studying the specific M1 and M2 macrophages' markers, the localization of cells rather than merely the presence of tumor associated macrophages [6]. Results must be confirmed in a higher number of cases.

Conclusions

$CD68^+$ cells are more likely to be present in the tumor nest rather than in the peritumoral area. This research did not establish any significant correlations between intratumoral and peritumoral $CD68^+$ cells and patients' age, tumor grade, expression of ER and PR. The content of peritumoral $CD68^+$ cells inversely correlate with the number of $HER2^+$ carcinoma cells.

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