

КЛІНІЧНА МЕДИЦИНА

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ANALYSIS OF ASSOCIATION BETWEEN THE DENSITY OF INFILTRATION IN PRIMARY CARCINOMA OF THE MAMMARY GLAND BY TUMOR-ASSOCIATED MACROPHAGES AND POSTOPERATIVE PROGNOSIS

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Пухлино-асоційовані макрофаги (ПАМ) M2-типу домінують у пухлинах і продукують сприятливі для їх росту молекули, стимулюючи ріст пухлини. Однак, зміна M2-типу на M1 може уповільнювати або припиняти цей ріст. Для реалізації напрямку модуляції M1/M2 при лікуванні карциноми/раку молочної залози (РМЗ) необхідна обґрунтована діагностика і підтвердження негативного прогнозу ПАМ. Метою роботи було оцінити відношення пухлино-асоційованих макрофагів до післяопераційного прогнозу/виживання пацієнток з карциномою/раком молочної залози (РМЗ). Матеріалом дослідження були інтраопераційні тканини пухлин та іпсилатеральних лімфовузлів при радикальному видаленні молочних залоз. Патоморфологічне дослідження лімфовузлів проводилося для уточнення діагностики стосовно NO/1. Щільність інфільтрації ПАМ визначали за допомогою імуногістохімічного забарвлення (ІГХ) CD68 та CD163 на 30 зразках п'яти молекулярно-біологічних типів РМЗ (по три клінічних випадки кожного). ІГХ дослідження по визначенню ПАМ і M2-подібних макрофагів проведено за допомогою стрептавідин-пероксидазного методу. Дослідження дозволило встановити, що кількісне представництво CD68+ та CD163+ Мф дуже різнилося від пацієнтки до пацієнтки, а також в межах зразку, що залежить зокрема від морфологічних особливостей РМЗ, охопленого біопсією. Щільність інфільтрації CD163+ макрофагами вогнища РМЗ негативно корелювала з післяопераційним виживанням, але не достовірно, проте це вкладається у загальну концепцію про негативний прогноз інфільтрації M2-подібними макрофагами. Потрібна більша кількість досліджень для підтвердження негативного значення щільності інфільтрації ПАМ первинного вогнища РМЗ для післяопераційного прогнозу. Не виключена захисна роль повноцінних M1-подібних ПАМ вогнища первинного ураження при РМЗ на рівні персоналізованого підходу. Перспективною є розробка диференційної діагностики і підходу до лікування РМЗ з урахуванням рівнів його інфільтрації субпопуляціями ПАМ.

Ключові слова: рак/карцинома молочної залози, пухлино-асоційовані макрофаги, молекулярно-біологічні типи РМЗ, післяопераційний прогноз виживання.

Tumor-associated macrophages (TAM) of the M2-type dominate in tumors and produce molecules, favorable for their growth, stimulating tumor growth. However, changing the M2-type for M1 can slow down or arrest this growth. For realization of the M1 / M2 modulation direction in the treatment of carcinoma / breast cancer (BC), a substantiated diagnosis and confirmation of the TAM negative prognosis is necessary. Therefore, the aim of the study was to evaluate the relation of tumor associated macrophages to the postoperative prognosis / survival of patients with 5 molecular-biological types of breast carcinoma. Materials of the study were intraoperative tissues of tumors and ipsilateral lymph nodes in radically removed mammary glands. Pathomorphological study of lymph nodes was conducted to clarify the diagnosis in relation to NO/1. The density of TAM infiltration was determined by immunohistochemical staining of CD68 and CD163 in 30 samples of five molecular biological types of breast cancer (three clinical cases of each type). Immunohistochemical (IHC) studies for the determination of TAM and M2-like macrophages were conducted using streptavidin-peroxidase method. The quantitative representation of CD68 + and CD163 + Mph is very different from patient to patient and also within one sample, which depends, in particular, on the morphological characteristics of breast cancer, studied by the biopsy. The density of infiltration by CD163 + macrophages of the BC focus negatively correlated with postoperative survival, which did not reach statistical significance, but is included in the general concept of a negative prognosis of infiltration by M2-like macrophages. Further research is needed to confirm the negative significance of the TAM infiltration density in the BC primary focus for postoperative prognosis. Promising is the development of differential diagnosis and approach to the treatment of breast cancer, taking into account the levels of its infiltration by sub-populations of TAM.

Key words: cancer / carcinoma of the mammary gland, tumor associated macrophages, molecular-biological types of breast cancer, postoperative survival prediction

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Introduction

Tumor-associated macrophages (TAM) of the M2-type dominate in tumors and produce molecules, favorable for their growth, stimulating tumor growth. However, changing the M2-type for M1 can slow down or arrest this growth. Such an effect is mediated by the direct activity of M1 and their ability to stimulate Th1-type cytotoxic T cells and other effector cells [1]. For realization of the M1 / M2 modulation direction in the treatment of carcinoma / breast cancer (BC), a substantiated diagnosis and confirmation of the TAM negative prognosis is necessary. Therefore, the aim of the study was to evaluate the relation of tumor associated macrophages to the postoperative prognosis / survival of patients with 5 molecular-biological types of breast carcinoma.

Materials and methods

Tissue samples. Biopsy samples and clinical data were obtained from patients undergoing treatment at Poltava Regional Clinical Dispensary. The study was approved by the Ethics Commission of UMSA. The average age of patients was 60 years, ranging from 30 to 79 years.

Materials of the study were intraoperative tissues of tumors and ipsilateral lymph nodes in radically removed mammary glands. Pathomorphological study of lymph nodes was conducted to clarify the diagnosis in relation to N0/1. Immunohistochemical (IHC) characteristics of the removed tumors (HER2, ER, PR, Ki67) were used to determine the molecular-biological subtype of BC in order to balance the study groups. IHC and pathologic findings were obtained at the diagnostic and advisory center CSD Health care, Kyiv).

Immunohistochemistry and antibodies. For IHC detection of TAM that infiltrated the primary focus of BC, we used the CD68 marker; to determine the M2-like TAM – the CD163 marker [2].

IHC studies for the determination of TAM and M2-like macrophages were conducted using streptavidin-peroxidase method. Paraffin sections, 2-3 μm thick, obtained by standard technique of the automated cycle of the pathologic-anatomical laboratory, were deparaffined, dehydrated, antigens were restored in citrate buffer (pH 6.0) in a microwave oven (at a power of ≈600 W, 3 cycles of 7 minutes with a break for 1 min), cooled for 20 min, washed in disodium and phosphate buffered saline (PBS, pH 7.2-7.4) for 2 min, blocked endogenous peroxidase with a reagent from the PolyVue HRP / DAB Detection System (For Mouse & Rabbit Primary Antibodies, Diagnostic BioSystems, USA), washed in PBS for 3 min. The slices were then incubated at 4°C overnight with mouse anti-CD68 monoclonal antibodies (clone PG-M1, REF PD M065-S, Diagnostic BioSystems, USA) and anti-CD163 (clone 10D6, REF Mob460-01, in dilution 1: 100 in Antibody Diluent Buffer for DTP, Antibody Diluent, Dako, USA). Further, the sections were treated in two steps with the Mouse / Rabbit PolyVue™ HRP / DAB Detection System (Diagnostic BioSystems, USA), a detector system for visualizing the chromosomal DAB response, the nuclei were bleached with hematoxylin Meyer and enclosed with a cedar balm under the cover glass. Antibody Diluent buffer was used instead of the primary antibodies as the negative control, the lymph nodes tissues – as the positive one.

Assessment of immunohistochemical staining. We conducted assessment by counting CD68⁺ TAM and CD163⁺ M2-like TAM under the light microscope (Biolam, LOMO, Russia: lens×40, eyepiece K7^x, magnification ×280, field diameter of the field of view 18 mm) in 7-10 consecutive fields of view for the IHC-reaction of each section, calculating the arithmetic mean, within the tumor nests and tumor stroma. The count included immunopositive cells with macrophage morphology. Microphotographs were obtained using a Microscope Leica DM500, Leica, Germany, lens × 40).

4-year follow-up. Patients were regularly observed during visits to the clinic or over the telephone for up to 4 years (or more precisely, up to 58 months maximum), or until the time of death. Overall survival was used for prognostic analysis.

Statistical analysis. All calculations were conducted using GraphPad Prism 5. The proportions were compared using the χ² test or Fischer's exact criterion. Overall survival was estimated using the Kaplan-Mayer method. The values of p≤0.05 were considered as statistically significant for all analyzes.

Results

The study included 6 patients with 5 immunohistochemical types of BC, equal in terms of metastases in the ipsilateral axillary lymph nodes, namely: 6 persons with non-luminal HER2⁺, 3 N0 and N1; 6 persons with luminal A, 3 N0 and N1, 6 persons with luminal B HER2⁻, 3 N0 and N1; 6 persons with luminal B HER2⁺, 3 N0 and N1; and 6 persons with triple negative BC, also 3 N0 and N1, 30 patients in total.

The primary results of the calculation of immunopositive Mph are given in Table 1.

*Table 1
Quantitative characteristics of tumor-associated macrophages, which infiltrate the primary focus of BC in each patient (average number in the field of view mg.×280)*

IHC BC type	N0		N1
	CD68 ⁺ TAM	CD163 ⁺ M2-like TAM	CD68 ⁺ TAM
Non-luminal HER2 ⁺	10.7	3.1	21.6
	3.6	1.9	32.1
	5.8	4.8	16.2
Luminal A	16.5	7.4	9.2
	8.8	4.3	23.1
	14.1	4.9	8
Luminal B HER ⁻	5.5	1	6.7
	16.6	1	4.3
	19.6	1	8
Luminal B HER ⁺	4.6	1	8.5
	6	1	2.5
	5.6	1	4.3
TNBC	12.5	5.1	23.5
	11.4	6.0	22.3
	12.5	1.0	26.6

In general, the quantitative characteristics of TAM showed that in all cases, the number of SD68⁺ Mph exceeded CD163⁺ Mph. All cells usually formed larger or smaller foci or clusters, which depended on the expressiveness of the stroma, the density of the tumor nests, the presence of necrosis centers and other morphological, very individual characteristics of the samples (Fig. 1, 2) [3].

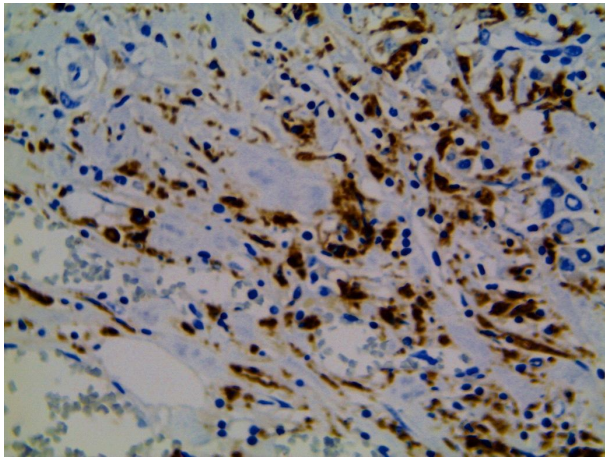


Fig. 1. Dense infiltration of the CD68 + TAM sample in triple negative BC N1.

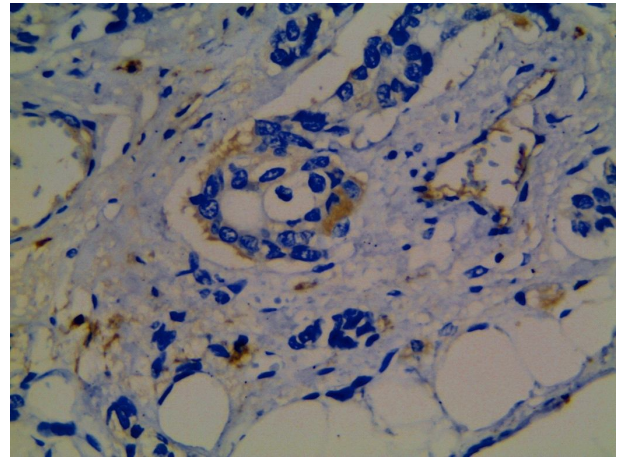


Fig. 2. Field of view with moderate density of infiltration of the CD163 + macrophages of luminal BC A N0.

For statistic analysis of levels of TAM infiltration and postoperative survival, the participants of the study were divided into 2 subgroups, by conditionally high (≥ 15 cells in the field of view mg. $\times 280$) and low (< 15) infiltration density of the primary focus of BC CD68⁺ TAM. In the subgroup with a high level of infiltration, one person died,

survival was 89%, in the subgroup with a low – two patients (83% survival), the difference was not reliable (Fig. 3, $p = 0.81$, log-rank test). With comparable proportions of 1/10 and 2/20, cumulative mortality in the subgroups is the same.

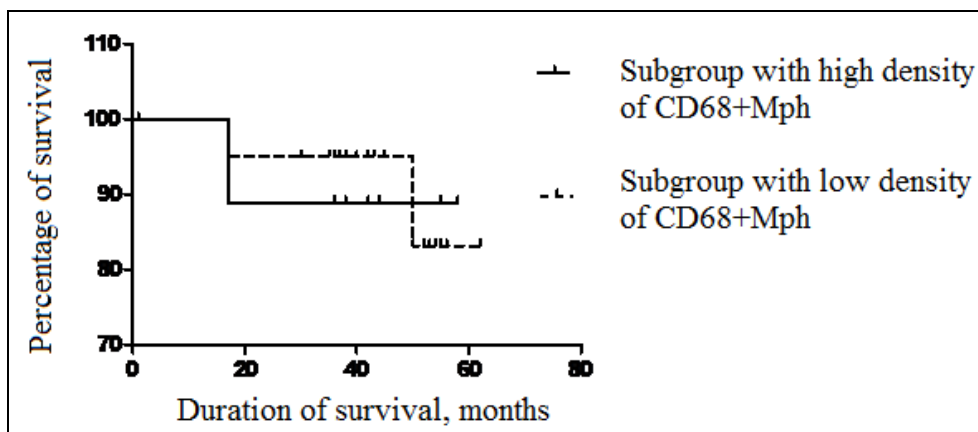


Fig. 3. Survival proportions and infiltration level of CD68 + TAM (Kaplan-Mayer Test).

To find out the relationship between the density of infiltration of BC CD163⁺ M2-like TAM and patients survival, the conditional division into two subgroups was conducted as follows: a moderate level of infiltration: > 3 cells in the field of view mg. $\times 280$, the low level is 0-3. In a subgroup with a moderate level of infiltration, two persons died (survival was 81%), in the subgroup with low –

one patient (survival - 91%), which did not reach statistical significance, when compared ($p = 0.94$, log-rank test, Fig. 4) and needs further observations for the conclusion, but in principle coincides with the concept that relatively higher infiltration of the primary focus with M2-like Mph is negative [1].

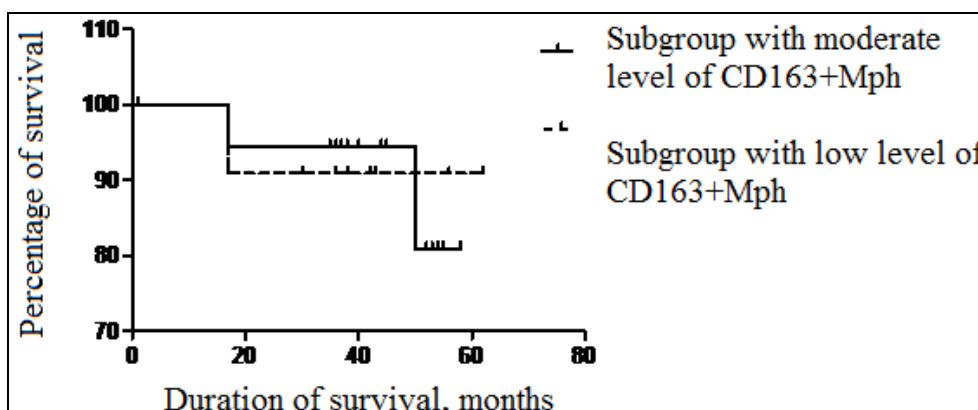


Fig. 4. Proportions of survival, depending on the level of infiltration of CD163 + M2 (Kaplan-Mayer test)

When comparing proportions of cumulative mortality: 2/18 (11%) (the subgroup with moderate infiltration density) versus 1/12 (8%) (the subgroup with low infiltration density), the differences also did not reach statistical significance, $p = 0.8$.

Discussion

The study presents the results of analysis of possible correlations between the infiltration density of separately M1-like TAM, which are conventionally considered CD68⁺ Mph, and M2-like - according to the widely used marker CD163, the primary focus of breast cancer: the relatively higher infiltration level by precisely CD163⁺ macrophages correlated with a decrease in survival (81%), which did not reach the reliability, however, is included in the general concept of the negative prognosis of tumor infiltration precisely with M2-like Mph [1].

The study is organized as a cross-sectional, balanced by immunohistochemical characteristics of tumors (HER2, ER, PR, Ki67) (or the surrogate classification for molecular-biological subtypes), pilot, and a small number of participants is a limitation of this study.

Previous studies have shown that increased macrophage density in biopsy samples of patients with breast cancer before treatment correlates with a decrease in non-recurrent and general survival [6]. The literature discusses the evidence that both M1 and M2 Mph have tumoral properties: in the early stages of transformation, M1-like TAM, due to the production of reactive oxygen and nitrogen forms, can potentially increase the rate of mutations in the epithelial cells and thus accelerate the tumor process; in the developed tumors, Mph demonstrate alternatively activated M2 functions, including the production of immunosuppressive factors (IL-10 and TGF- β) which can actively suppress the anti-tumoral immune response, produce growth factors and rebuild the matrix, supporting the growth of tumor cells and intensifying the invasion [4]. Nevertheless, in a study devoted to the research of macrophages localization in the human breast tumors, the high representation of CD68⁺ Mph in the gaps of the ductal tumors correlated with a decrease in metastases in the lymph nodes [5], reflecting some resistance to the tumor process.

Other studies, with a larger number of participants (100), found that high levels of CD68⁺ TAM infiltration of the tumor tissue were reliably associated with a worse prognosis compared with a relatively low level of infiltration [6], but these results were not correlated with molecular biological varieties of breast cancer

In its own study, we observed that both CD68⁺ and CD163⁺ Mph were grouped in different tumor sites and their localization was highly dependent on connective tissue structures of the tumor. By the way, almost all samples of breast cancer, in particular luminal, were characterized by a distinct desmoplastic response [3].

Probably, TAM is an important regulator of the development and remodeling of the intercellular matrix in the microenvironment of tumors, which has been studied less, but is a direct reflection of the standard functions of Mph [4,7]. This provision is indirectly confirmed by the data that the histological localization of TAM in different regions of breast cancer is correlated with the risks of metastasis and prognosis [8, 9].

According to earlier literature data, both M1 and M2 TAM in BC can suppress the proliferation of T cells, showing immunosuppression and anti-tumor efficacy [10]. Other data indicate that not all TAMs have the ability

to inhibit proliferation of CD8 T cells [11]. Consequently, some of the TAM functions are likely to be universal (recruitment, localization, matrix remodeling), whereas other properties (specific interactions with other infiltration cells) may depend on the tumor model under study [4]. These findings may explain the findings of negative effects of various, but not all, TAM subpopulations on the prognosis of breast cancer, and therefore leave an assumption about the protective role of some of them, in combination with individual tumor characteristics.

In our study, the average quantitative indices of CD68⁺ and CD163⁺ Mph were lower than in other researches, due to the count of immunopositive cells in successive tumor fields, and possibly due to the balanced representation of 5 molecular genetic types of carcinoma. The mean values of the amounts of CD68⁺ and CD163⁺ Mph were very different from patient to patient and also within one sample. In other studies, the authors showed a higher number of CD68⁺ TAM in breast cancer: an average of 61.14 ± 23.76 cells in the field of view of the total of $\times 400$, but the count of these cells was performed in "intensive reaction areas" and patients were not balanced by the molecular- biological types of breast cancer [6].

The prospect for research is the development of differential diagnosis and treatment of breast cancer, taking into account the levels of its infiltration by TAM subpopulations. Regarding the direction of "repolarization" of Mph within the microenvironment of the tumor to the M1 phenotype, it is necessary to take into account their potential tumoral properties [4].

Conclusions

1. The density of infiltration by CD163⁺ macrophages of the BC focus negatively correlated with postoperative survival, which did not reach statistical significance, but is included in the general concept of a negative prognosis of infiltration by M2-like macrophages. Further research is needed to confirm the negative significance of the TAM infiltration density in the BC primary focus for postoperative prognosis.

2. The protective role of full-rate M1-like TAM of the primary focus in breast cancer at the level of personalized approach is not excluded.

3. The quantitative representation of CD68⁺ and CD163⁺ Mph is very different from patient to patient and also within one sample, which depends, in particular, on the morphological characteristics of breast cancer, studied by the biopsy.

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