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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Pulvinarly-asociytovani macrofagi (PAM) M2-tipy dominoychy u pulvinax i produkuyen spratilivyy dla iy rostru molekuly, stymyuyushchi rist pulvin. Odnak, zmena M2-tipy na M1 moze upovlyivaty abo prylinuty cey rist. Dl y realizatsii na-
pramy modulyacii M1/M2 pri likuvanniu karinomy/razku molnoy zaloz (RMZ) neobkhodnaya obruntovana diagnostika i pi-
detverdjeniya negativnogo pradguzu PAM. Metoq robity bu y otsnit vioischeniya pulvinly-asociytovannyh macrofagov do pi-
slavoperapidynogo pradguz/vyjivayenii yatsiht za karinomou/razkom molnemy zaloz (RMZ). Materialom dosledkii
乒iihtraoperalytynih tkanyux pulvin t i sipiliteralytyih lymfovuzyliv pri radykalnymy vidyali molnoy zaloz.
Pathomorfologichie dosledkii lymfovuzyliv provodilosya dlya utychniya dianstiki stoysto NO/1. Shlyynst
infiltrapii PAM yiznachali za dopolyamym ijuniqytsikhmyno zbaravleniya (IPX) CD68 t CD163 na 30 zryaka piy-
leukyyro-biologichynih typy RMZ (po tri klinichnyih vyadych kojnoy). IPX dosledkii po viznachhno PAM i M2-
podibnyih macrofagov pravdeno za dopolyamym streptavidin-perokсидnogo metodu. Dosledkii yizvolility vstanov-
ti, yh klykysne predstavitsytstvo CD68+ t CD163+ Mf duje riznylo vyi patsiynty do patsiynty, a taksak v menykh
razryku, yh zalenych zorkyma vyd morfologichynih osoblyvostiy RMZ, oholoynogo bioloyo. Shlyynst infiltrapii CD163-
macrofagami yoginza RMZ negativnoy koruylvova z pslavoperapidnymy vyjivayeniyam, ale ne dostoyvryu, pryste yu-
kladaetsya u zagalnu konceptii po negativnogo pradguz infiltrapii M2-podibnymi macrofagam. Potribna biyla kly-
kys dosledkii dlya pidvetverdjeniya negativnogo znacheniya shlyynst infiltrapii PAM pervynnogo yoginza RMZ
p pslavoperapidnymy pradguzu. Ne yizvolility zahkhisa roly povyonychnih M1-podibnyih PAM yoginza pervynnogo uyrakh-
nya pri RMZ na riveny peroksidnogo podkhu. Perspektivno y rozrobka diferenttsiynoi diagnostiki i podruxu
d do likuvannya riveny ikh infiltrapiy sypobolypami PAM.

Key words: primary carcinoma of the mammary gland, tumor associated macrophages, molecular-biological types of
RMZ, pslavoperapidny pradguz vyjivayenya.

**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 PolyVueTM 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+, 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 ps0.05 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+ [2], 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**

<table>
<thead>
<tr>
<th>IHC BC type</th>
<th>N0</th>
<th>N1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-luminal HER2+</td>
<td>CD68+ TAM</td>
<td>CD163+ M2-like TAM</td>
</tr>
<tr>
<td>CD163+ M2-like TAM</td>
<td>16.5</td>
<td>4.3</td>
</tr>
<tr>
<td>Luminal A</td>
<td>8.8</td>
<td>4.3</td>
</tr>
<tr>
<td>Luminal B HER2+</td>
<td>5.5</td>
<td>1</td>
</tr>
<tr>
<td>Luminal B HER2+</td>
<td>16.6</td>
<td>1</td>
</tr>
<tr>
<td>TNBC</td>
<td>12.5</td>
<td>5.1</td>
</tr>
<tr>
<td>N0</td>
<td>11.4</td>
<td>6.0</td>
</tr>
<tr>
<td>N1</td>
<td>12.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

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].
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. × 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.

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. × 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].
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 tumor 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 carcinomas. 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 × 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.

References


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