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ABSTRACT

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IMMUNOHISTOCHEMICAL STUDY OF M1 AND M2 MACROPHAGES IN BREAST CANCER WITH MICROCALCIFICATIONS

Introduction. Breast cancer (BC) is a significant medical and social problem, as it is the leading cause of cancer-related mortality in women worldwide. Microcalcifications in the breast tissue are essential in developing the pathological process and affect the prognosis and metastasis. The tumor microenvironment consists of cancer cells and stromal cells such as fibroblasts, endothelial cells, pericytes, and immune cells, including M1 and M2 macrophages.

The work *aims* to study the influence of microcalcifications on the polarization of macrophages in the tumor microenvironment of BC.

Materials and methods. The study was conducted on 60 samples of BC, divided into 30 samples of BC with microcalcifications (group I) and a control group of 30 samples of BC without calcifications (group II). All microcalcifications met the criterion of size up to ≤1 mm. To study the pathohistological changes, BC's tissue was analyzed using macroscopic description, histology, and immunohistochemical study with antibodies against CD68 and CD163.

Results. According to the results of an immunohistochemical study, it was found that the expression of CD68-positive macrophages of the M1 type is significantly higher in the tissue of samples of BC with microcalcifications, compared to samples of the control group (60.85 ± 2.71 cells in the field of view vs. 51.14 ± 2.89, $p < 0.05$). On the other hand, it was established that the average value of CD163 expression in group I was 53.21 ± 3.05 against 65.57 ± 3.75 ($p < 0.05$) cells in the group of BC without calcification. Nevertheless, the localization of M1 and M2 macrophages in the tumor had standard features and did not differ.

A possible mechanism of the effect of microcalcifications on the polarization of macrophages is their support of chronic inflammation in cancer tissues and, thus, the development of pro-inflammatory M1 phenotype in macrophages. Further studies are needed to obtain more unambiguous conclusions.

Keywords: breast cancer, microcalcifications, tumor microenvironment.

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ІМУНОГІСТОХІМІЧНЕ ДОСЛІДЖЕННЯ МАКРОФАГІВ М1 ТА М2 ПРИ РАКУ ГРУДНОЇ ЗАЛОЗИ З МІКРОКАЛЬЦИФІКАТАМИ

Вступ. Рак грудної залози (РГЗ) є важливою медичної і соціальною проблемою, оскільки являється основною причиною смертності жінок від раку у всьому світі. Мікрокальцифікати у тканині грудної залози (ГЗ) відіграють важливу роль у розвитку патологічного процесу та впливають на прогноз та метастазування. Пухлинне мікрооточення складається з ракових клітин і стромальних клітин, таких як фібробласти, ендотеліальні клітини, перицити та імунні клітини, в тому числі макрофаги типу М1 та М2.

Метою роботи є дослідження впливу присутності мікрокальцифікатів на поляризацію макрофагів пухлинного мікрооточення РГЗ.

Матеріали та методи. Дослідження було проведене на 60 зразках РГЗ, які поділялися на групу 30 зразків РГЗ з мікрокальцифікатами (група I) та контрольну групу 30 зразків РГЗ без кальцифікатів (група II). Всі мікрокальцифікати відповідали критерію розмір до ≤ 1 мм. Для дослідження патогістологічних змін тканина РГЗ вивчалася за допомогою методів макроскопічного описання, гістології, імуногістохімічного дослідження з антитілами проти CD68 та CD163.

Результати. За результатами імуногістохімічного дослідження було виявлено, що експресія CD68-позитивних макрофагів типу М1 достовірно вища у тканині зразків РГЗ з мікрокальцифікатами, у порівнянні зі зразками групи контролю (60,85 ± 2,71 клітин у полі зору проти 51,14 ± 2,89, $p < 0,05$). З іншого боку, було встановлено, що середнє значення експресії CD163 у групі I складало 53,21 ± 3,05 проти 65,57 ± 3,75 ($p < 0,05$) клітин у групі РГЗ без кальцифікації. Тим не менше, локалізація макрофагів типів М1 та М2 у пухлині мала спільні риси і фактично не відрізнялася.

Можливим механізмом впливу мікрокальцифікатів на поляризацію макрофагів є підтримка ними хронічного запалення у ракових тканинах і сприяння розвитку таким чином у макрофагах прозапального М1 фенотипу. Для отримання більш однозначних висновків необхідні подальші дослідження.

Ключові слова: рак грудної залози**,** мікрокальцифікати, пухлинне мікрооточення.

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INTRODUCTION / ВСТУП

Breast cancer (BC) is a significant medical and social problem, as it is the leading cause of cancer-related mortality in women worldwide. According to the WHO, in 2018, 2.1 million new cases of BC and 627,000 deaths were recorded worldwide [1, 2].

Microcalcifications in the breast tissue play an essential role in developing the pathological process and affect the prognosis and metastasis. Their presence in the tissue of BC is a criterion for determining the stage of the disease and early and differential diagnosis of BC [3, 4, 5].

The tumor microenvironment (TME) is an essential part of research in oncology, as its role in the development of the pathological process, metastasis, worse prognosis, and recurrence of tumors has been studied for many years and is of urgent importance in today's conditions. It consists of cancer cells and stromal cells, such as fibroblasts, endothelial cells, pericytes, and immune cells [6, 7, 8]. The primary role in developing neoplastic cell proliferation, angiogenesis, and invasion into surrounding tissues is played by M1 and M2 macrophages. Macrophages type M1 (CD68) has anti-inflammatory and antitumor effects, and M2 (CD163) is immunosuppressive and exhibits pro-tumor activity [9, 10, 11].

Objective: Our work aims to investigate the influence of microcalcifications on the polarization of macrophages in the tumor microenvironment of breast cancer.

Materials and methods

The study was conducted on material obtained during surgical operations at the Sumy Regional Clinical Oncology Center (RCOC). After a histological examination, 30 samples of BC tissue (group I) with microcalcifications were selected. As a control, 30 samples of BC tissue without signs of biomineralization were used (group II).

The Ethics Committee

The study was approved by the ethics committee of the Medical Institute of Sumy State University (proceedings 3/03, 09.02.21).

Histology. Breast tissue samples were fixed in a 10% neutral formalin solution for 24 hours for histological examination. The preparation of the material and the production of paraffin blocks were carried out according to the generally accepted method. Histological examination of breast cancer samples was the main criterion for forming research groups I and II. All detected microcalcifications in group I did not exceed the size of 1 mm (1000 μm).

Immunohistochemistry of breast cancer tissue

In general, the procedure of immunohistochemical staining of the tissue was carried out according to the method previously described in our works [5]. We used primary antibodies against CD163 (clone EP 324, Master Diagnostica, ready to use), and CD68 (clone Ab-3, Thermo Fisher Scientific, at a dilution of 1:200). At least six different fields of view (FOV) with a diameter of 1 mm were analyzed for each sample. The IHC results were presented as a mean number of OPN-positive cells per FOV.

Statistical analysis

The normality of all data sets was assessed by the Shapiro-Wilk test. Student's t-test was applied for data analysis with a normal distribution. Mann-Whitney's U-test was applied for nonparametric data sets. The results were considered statistically significant, with a probability of more than 95 % ($p < 0.05$). Statistical analysis was performed in Microsoft Office Excel 2016 with the addon AtteStat (version 12.0.5). All graphs were made with GraphPad Prism 9.0.

Results

Macroscopic description

Macroscopically, the tissue of BC of both groups was presented in the form of a well-defined nodule, dense in consistency and tightly connected to the surrounding tissue, and had jagged edges. The size of the tumor varied from 0.5 to 5.0 cm in diameter. On the section, the node was gray with yellowish necrosis and isolated hemorrhages. In some cases, rigid inclusions were found in the samples of the 1st group, which, when cut, showed a typical crunch.

Histology

The samples of both groups were represented by polymorphic glands formed by atypical cells and surrounded by a connective tissue stromal component. Cells formed nests, trabeculae, and clusters. Tumor cells contained polymorphic and hyperchromic rounded nuclei with chromatic and mitotic figures, nucleoli, and eosinophilic cytoplasm (Fig. 1 A, B). The cells had increased mitotic activity and pathological mitoses. The microscopic picture of most samples was consistent with invasive ductal breast cancer. In some samples, intraductal inclusions in the form of small polymorphic cells, which formed papillary, cribriform structures, and phenomena of inflammatory infiltration, were noted.

In samples of the I group, biomineral deposits were present in all tumor tissue components: in the parenchyma, stroma, and inside the ducts (Fig. 1 C, D).

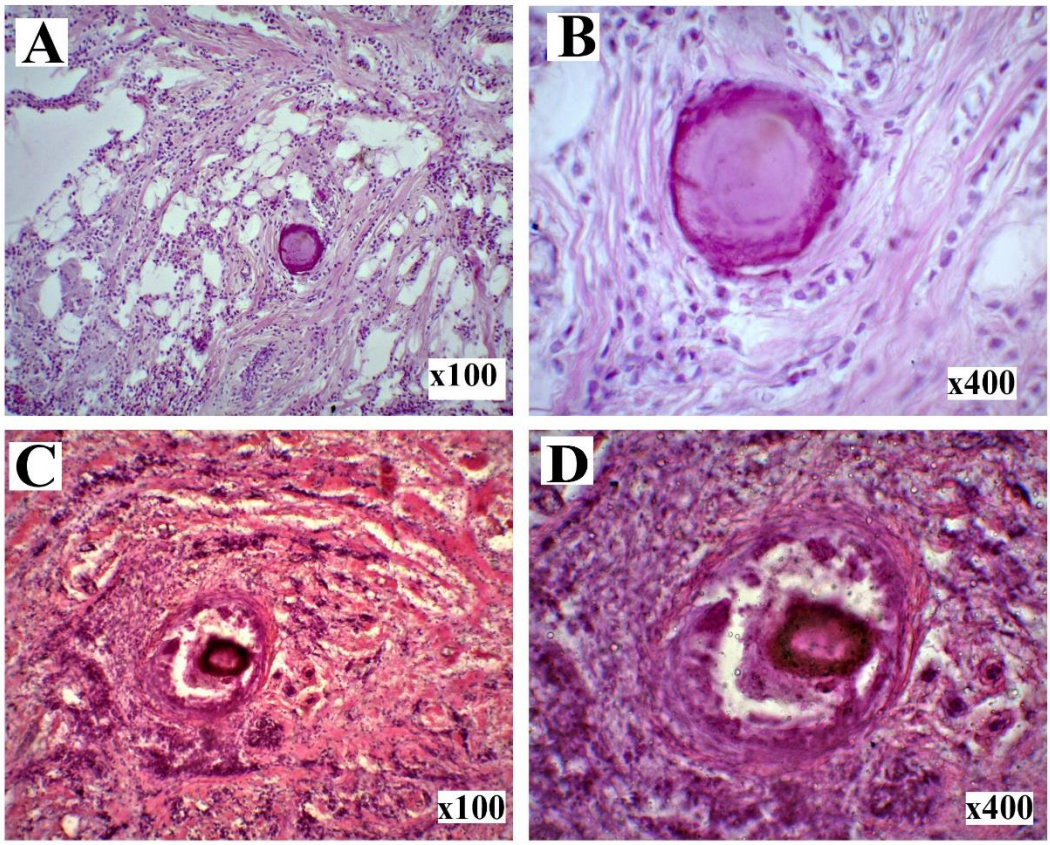

Figure 1 – Breast cancer tissue with microcalcifications. Staining with hematoxylin and eosin. Magnification is indicated in the lower right corner of each image

Immunohistochemistry

Immunohistochemical examination of ductal invasive breast cancer tissue with antibodies against CD68 showed an intense positive cytoplasmic reaction in macrophages of the tumor microenvironment. Macrophages were mainly localized in the stroma around the tumor tissue. Part of the cells penetrated between the solid layers of the tumor epithelium. In addition to macrophages, a positive reaction was observed in some endotheliocytes and fibroblasts (Fig. 2 A, B).

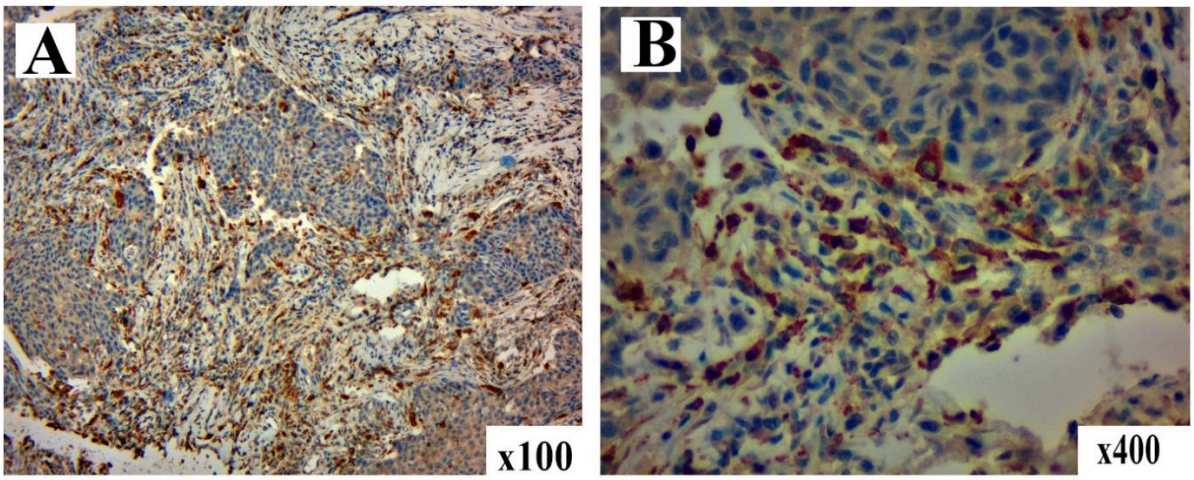

Figure 2 – Immunohistochemical study of the tissue of BC (group I) with antibodies against CD68. Staining of nuclei with Mayer's hematoxylin. Magnification is indicated in the lower right corner of each image

Immunohistochemical study of samples of group II (control group) shows the coincidence of localization of CD68-positive cells in tissue compartments. Yes, we note the presence of stained cells, mainly in stromal connective tissue and necrotized tumor tissue. A small number of CD68-positive cells is located among the tumor layers (Fig. 3 A, B). In the control group, the false staining of some other tumor microenvironment cells is also noted: endotheliocytes, fibroblasts, and pericytes.

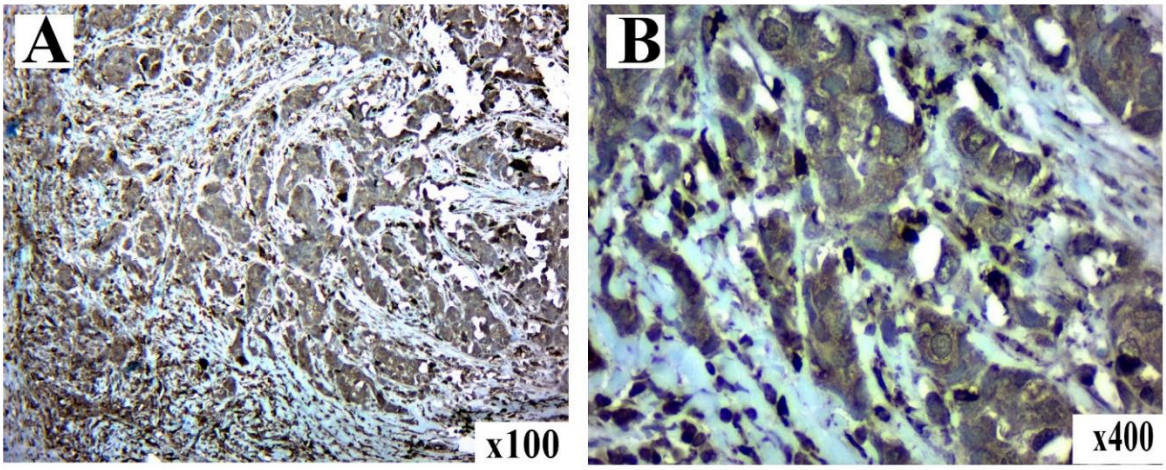

Figure 3 – Immunohistochemical study of the tissue of BC (group II) with antibodies against CD68. Staining of nuclei with Mayer's hematoxylin. Magnification is indicated in the lower right corner of each image

We performed an immunohistochemical study with anti-CD163 antibodies to establish the presence of M2-phenotype macrophages in breast cancer tissue with microcalcifications. We found many CD163-positive cells in the tumor microenvironment (Fig. 3 A, B). The cytoplasm and partially the membrane were intensely stained in positively stained cells. The vast majority of cells corresponded to the phenotype of macrophages. But, simultaneously, a noticeable number of spindle-shaped cells, similar to fibroblasts, was detected, and a positive cytoplasmic reaction was noted in parts of the endothelium.

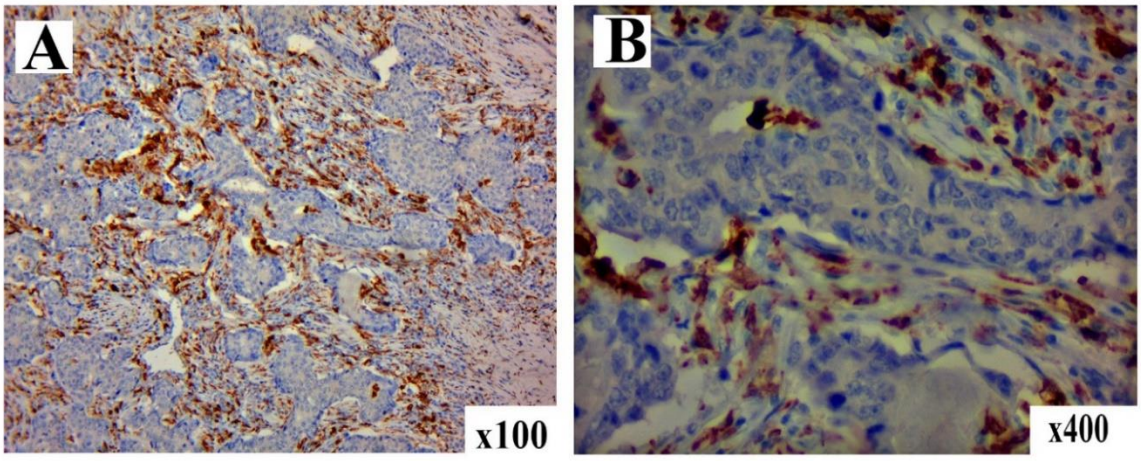

Figure 4 – Immunohistochemical study of BC tissue (group I) with antibodies against CD163. Staining of nuclei with Mayer's hematoxylin. Magnification is indicated in the lower right corner of each image

Immunohistological study of the polarization of tissue macrophages of samples of group II with antibodies against CD163 showed the similarity of the compartmentalization of positively stained cells to the results of group I. We found CD163-positive cells in the stroma and part of them in the thickness of tumor tissues (Fig. 5 A, B). Nevertheless, M2 macrophages were much less frequently found in necrotic tissues and inflammatory infiltrates. We also noted the overlapping of endothelium and fibroblasts during immunohistochemical staining of the tissue of group II samples with antibodies against CD163.

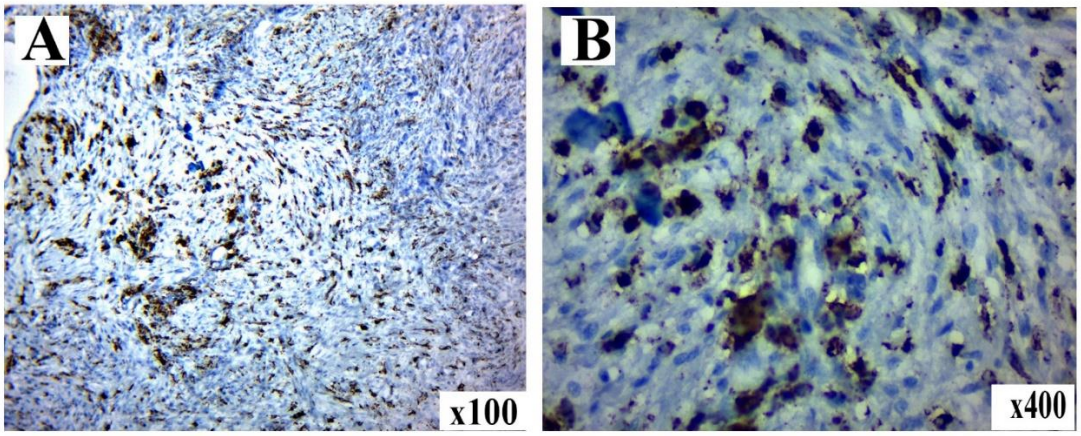

Figure 5 – Immunohistochemical study of BC tissue (group II) with antibodies against CD163. Staining of nuclei with Mayer's hematoxylin. Magnification is indicated in the lower right corner of each image

Statistical analysis

When examining samples of the 1st group of RGZ tissue with calcifications, it was established that the average value of CD68 expression was 60.85 ± 2.71. In the control group of RGZ tissue without calcification, the expression of CD68 was 51.14 ± 2.89 ($p < 0.05$).

When examining a group of breast cancer tissue samples with calcifications, it was found that the average value of CD163 expression was 53.21 ± 3.05. In the control group of RGZ tissue without calcification, CD163 expression was 65.57 ± 3.75 ($p < 0.05$).

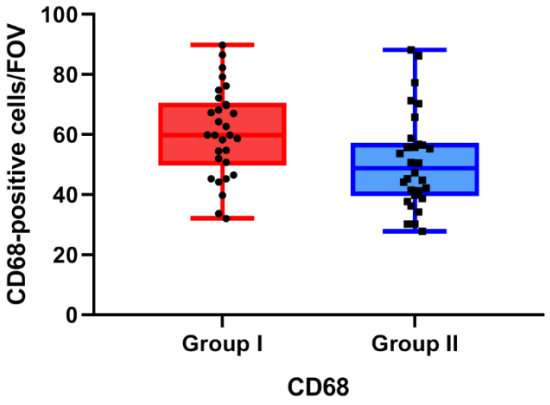

Figure 6 – IHC study of the expression of the CD68 marker in the breast cancer tissue of the studied groups. The column represents the mean value. The bar represents the 95% confidence interval, p < 0.05

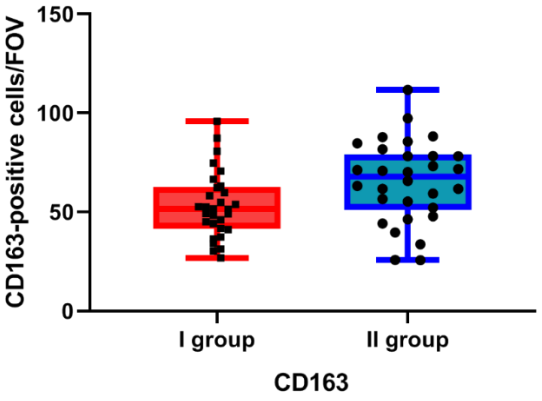

Figure 7 – IHC study of the expression of the CD163 marker in the breast cancer tissue of the studied groups. The column represents the mean value. The bar represents the 95% confidence interval, p < 0.05

Discussion

The tumor microenvironment occupies a leading place in the oncological process. It affects the proliferation of neoplastic cells, cancer progression, and invasion of adjacent tissues. The tumor microenvironment includes tumor and stromal cells, such as fibroblasts, endothelial cells, pericytes, and immune cells [12, 13]. Important importance is attached to the study of the role of macrophages in the development of the tumor microenvironment and the progression of the tumor process. Macrophages that are associated with the tumor are distinguished among immune cells. According to the mechanism of action, they are divided into two types: macrophages M1 and M2. M1 (CD68) has anti-inflammatory and antitumor effects, and M2 (CD163) are immunosuppressive cells and affect the spread of the tumor process [14, 15].

Studies by Zhang et al. indicate that tumor-associated macrophages are essential in the

prognosis of tumors. Their study showed that CD68 expression was associated with higher expression of vimentin and lower expression of E-cadherin, which are important markers of epithelial-to-mesenchymal transition. It may indicate that macrophages affect inducing epithelial-mesenchymal transition, rapid tumor process spread, and a worse prognosis [16]. In the experiments of Gwak et al., it was shown that it is essential to evaluate the polarization of macrophages and the areas of the tumor where they are located. According to their study, CD68 infiltration of macrophages in the intratumoral compartment or CD163 macrophages in the tumor stroma was associated with poor prognoses in BC samples. This review also showed that high expression of CD68 and CD163 macrophages in the epithelial part or increased density of CD68 or CD163 macrophages in the tumor stroma was also associated with a worse prognosis [17, 18].

Our study showed that the expression of CD68 level is higher in the tissue of BC with calcifications compared to the control group. Type 1 macrophages were found mainly in the stroma around the tumor, and some of these were found in cancer between layers of atypical cells.

According to the results of the Werhrhan study, it was found that suppressing the number of M2 macrophages in the tissue of triple-negative BC leads to a decrease in infiltration by macrophages in the tumor and a decrease in metastasis [19]. An immunohistochemical study of CD163 expression revealed that a significantly greater number of type 2 macrophages is found in the tissues of BC samples without microcalcifications ($p < 0.05$). The localization of most CD163-positive cells corresponded to the stroma of the tumor, and some of them were located between the tumor cells.

Based on our results, we can assume the predominance of M1 macrophages in the group of BC samples with microcalcifications. On the other hand, BC tissue without calcifications has more M2-type macrophages. In our opinion, this may indicate a specific effect of microcalcifications. Pathological biomineralization processes take place in the presence of an inflammatory process, which is supported, among other things, by M1 macrophages. The obtained data can be used to revise the assessment of the role of microcalcifications in the course of BC.

In general, these results contradict some of the studies that show the negative role of microcalcification in the prognosis of BC [3, 4, 19]. In other words, microcalcifications can indirectly reduce the negative impact of tumor-associated macrophages of type M2 due to a relative decrease in their number. The mechanism of this phenomenon remains unclear.

The immunohistochemical study of CD68- and CD163-positive cell expression has limitations and caveats. The main problem is the positive overlapping by these markers of some cell populations, such as fibroblasts, endotheliocytes, and pericytes, which are also residents of the tumor microenvironment. Further studies are needed to obtain more unambiguous conclusions.

CONCLUSIONS / ВИСНОВКИ

According to the results of the immuno-histochemical study, it was found that the expression of CD68-positive macrophages of the M1 type is significantly higher in the tissue of the samples of RGZ with microcalcifications, compared to the samples of the control group ($p < 0.05$). On the other hand, it was established that the number of CD163-positive cells is significantly higher in the tissue of RGZ without calcifications. Nevertheless, the tumor's M1 and M2 macrophage localization had standard features and did not differ.

A possible mechanism of the effect of microcalcifications on the polarization of macrophages is their support of chronic inflammation in tissues, thus promoting the development of the pro-inflammatory M1 phenotype in macrophages. Further studies are needed to obtain more unambiguous conclusions.

PROSPECTS FOR FUTURE RESEARCH / ПЕРСПЕКТИВИ ПОДАЛЬШИХ ДОСЛІДЖЕНЬ

We plan to investigate the immunohistochemical expression of osteoblastic markers in breast cancer tissue with microcalcifications and compare the results with a control group of breast cancer samples without pathological biomineralization.

CONFLICT OF INTEREST / КОНФЛІКТ ІНТЕРЕСІВ

The authors declare no conflict of interest.

FUNDING / ДЖЕРЕЛА ФІНАНСУВАННЯ

The work is a fragment and was carried out with the support of the research topic "The state of mineralized tissues when using new composites with Ag+ Cu2+ nanoparticles" (state registration number No. 0121U100471).

AUTHOR CONTRIBUTIONS / ВКЛАД АВТОРІВ

All authors substantively contributed to the drafting of the initial and revised versions of this paper. They take full responsibility for the integrity of all aspects of the work.

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