MORPHOLOGICAL FEATURES OF TUMOR ANGIOGENESIS IN INVASIVE DUCTAL BREAST CANCER

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Summary

Personalized treatment of patients with invasive ductal breast cancer is one of the most pressing issues in modern oncology. Today, information on the human genome and molecular markers is used to targeted therapy and optimize treatment strategies. One of the molecular markers is abnormal tumor angiogenesis. PECAM-1, or CD31, may be a marker of this process.

The aim of the study was to comprehensively assess the morphological features and clinical significance of different types of tumors microvessels in invasive ductal breast cancer.

CD31 expression was assessed by positive vascular endothelial staining. Using antibodies to CD31 (Ab-1, JC70A, Thermo scientific), we investigated 86 cases of invasive ductal breast cancer. In histological specimens stained with antibodies to CD31, the density of microvessels and areas with strong vascularization, the presence of dilated and atypical dilated capillaries in the intratumoral and peritumoral stroma, the presence of structures with local endothelial lining were assessed. The number of dilated and atypical dilated capillaries, as well as structures with partial endothelial lining was estimated by the semi-quantitative method. According to the results of our study, we showed some differences in the severity of vascular density of the tumor in patients with invasive ductal breast cancer. No differences in microvascular density were found depending on the age of the patients, T and N stages. At the same time, higher values of microvascular density in the intratumoral stroma were noted at G3 degree of differentiation, in triple-negative breast cancer and at HER2/neu positive. The most significant from the prognostic point of view were the number of atypical dilated vessels and structures with local endothelial lining, as well as the presence of characteristic porous structures in loose softly fibrous unformed connective tissue of the peritumoral stroma. Angiogenesis, morphology of tumor vessels is important for prognostic value and this marker can be used to predict the course of invasive ductal breast cancer.

Keywords: microvessels, breast carcinoma, immunohistochemistry, prognosis, angiogenesis.

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1. Introduction

Breast cancer is one of the most commonly diagnosed cancers and the main cause of cancer death among women in the Western world (WHO, 2021; Parkin DM et al., 2005]. In 2020, breast cancer was diagnosed in 2.3 million women, with 685,000 deaths from the disease reported worldwide, despite improved approaches to diagnosis and treatment. According to the WHO, at the end of 2020, 7.8 million women were diagnosed with breast cancer in the last five years, which means that this type of cancer is the most common cancer in the world. The number of lost years of healthy life of women with this diagnosis in the world exceeds that of any other type of cancer in the female population. Breast cancer occurs in all countries of the world in women of all ages after reaching puberty, but in older age the incidence rate increases (WHO) *(WHO, 2021)*. In the United States, the incidence of breast cancer is 300 thousand, in Europe – 458 thousand cases. In Ukraine, the incidence of breast cancer ranks 1st among all malignant tumors found in women. In 2018, there were 146,317 patients registered with an oncologist for breast cancer in Ukraine, the figure per 100,000 women is 762.1 *(Bulletin, 2020)*.

Systemic treatment improves the recurrence-free and overall survival of patients with breast cancer *(EBCTCG, 2005)*. Indications for systemic treatment are based on prognostic factors (Goldhirsch A et al., 2006], however, prognostic factors assess the risk of recurrence in a patient in the absence of systemic therapy and include age at diagnosis, histology and malignancy, tumor size, receptor status and lymph node status. Prognostic factors assess tumor susceptibility to specific treatments, such as estrogen receptor (ER) expression for endocrine therapy and human epidermal growth factor receptor 2 (HER2) overexpression / amplification for trastuzumab.

Prognostic factors are becoming increasingly important as the relative risk of dying from breast cancer is reduced through early diagnosis and improved treatment. In recent decades, a large number of studies have been conducted to study new prognostic factors based on the molecular characteristics of tumors in patients with breast cancer. Determination of genomic disorders that determine the development of the tumor, the degree of its malignancy, metastatic potential and rate of progression is a priority area of molecular genetic research in modern oncology. Therefore, molecular morphopathology, which takes into account the presence or absence of oncogenes and suppressors of tumor growth (molecular biological markers) in cells, is of paramount importance in the prediction of tumors. Differences in the expression of certain markers may explain why comparable in prevalence and histological structure of tumors differ in the aggressiveness of the disease. Determination of molecular biological markers in tumor tissue can provide additional information about the biological behavior of the tumor: its growth rate, ability to invade and metastasize, resistance to chemotherapy.

Angiogenesis is one of the key factors of tumor progression associated with the growth and metastasis of malignant neoplasms (Folkman J. 1976; Shen Y. et al., 2017; Liu H. et al. 2014). Currently, its assessment is considered an important marker of disease prognosis and susceptibility to anticancer therapy *(Şener E. et al., 2016; Kraby M.R. et al., 2017)*. It should be emphasized that technical and methodological approaches to the study of angiogenesis in malignant neoplasms differ greatly among different researchers. Some authors prefer to quantify the activity of angiogenesis. In breast cancer, it was noted that high microvascular density in the tumor and high expression of vascular endothelial growth factor expression are more common in advanced tumors, in the presence of metastases to regional lymph nodes and correlate with an unfavorable prognosis *(Han Z. et al., 2015; Shrivastav S. et al., 2016; Kraby M.R. et al., 2017; Zhang S. et al., 2017)*. However, it should be noted that not all researchers have found

correlations between angiogenesis activity and factors of tumor progression, including breast cancer *(Mohammed Z.M. et al., 2013; Chuangsuwanich T. et al., 2014)*. It is believed that this may be due to the fact that the vascular network in the tumor is heterogeneous in its origin and morphology *(Fukumura D. et al., 2010; Birau A. et al., 2012; Nagy J.A. et al., 2012)*. That is why other researchers to assess the role of angiogenesis in tumor progression prefer not only the number of vessels, but also take into account the method of their formation, the degree of maturity, as well as features of morphology *(Qian C.N. et al., 2016)*. We believe that this approach to studying the features of tumor angiogenesis has a number of advantages, because it allows to assess not only prognostic but also predictive significance of different types of vessels, which is especially important given the lack of effectiveness of angiogenesis inhibitors in clinical practice.

Thus, given the high morbidity and mortality, breast cancer is an urgent medical and social problem. Individualization of treatment of patients with breast cancer is directly related to the definition of prognostic factors, including a comprehensive study of morphological features of different types of microvessels in tumors and their correlations with clinical, morphological and molecular biological factors in the prognosis.

The aim of the study was to provide a comprehensive assessment of morphological features and clinical significance of different types of tumor microvessels in invasive ductal breast cancer.

2. Material and methods of research

The material of the current research was the analysis of case histories, outpatient cards of dispensary observation of 193 patients with invasive ductal breast cancer who had specialized antitumor treatment on the basis of the Lviv Regional Oncological Treatment and Diagnostic Center in 2017. Clinical and pathological characteristics included age of patients, tumor size, lymph node status, stage, ER, PR, HER2 / neu status, without neoadjuvant treatment and distant metastases. Given that the research material was collected in 2017, according to national and international recommendations, the TNM classification of the seventh edition was used. In all cases, the diagnosis of invasive ductal breast cancer was verified histologically. Histological type of cancer was determined in accordance with WHO recommendations *(WHO Classification, 2019)*. The degree of malignancy was determined by a modified scheme of P. Scarff, H. Bloom and W. Richardson *(Elston CW, Ellis IO., 1991)*.

The mean age at diagnosis was 54.7 years (28 to 85 years). The II and III stages of disease were dominated in patients, which amounted to 89 (46.11%) and 64 (33.16%) observations, respectively. The first stage of the disease was diagnosed in 40 (20.73%) patients. As for the size of the breast tumor, the distribution was as follows: stage pT1 was diagnosed in 61 (31.61%) patients, stage pT2 – in 96 (49.74%) patients, stage pT3 – in 19 (9.84%) patients and stage pT4 – respectively in 17 (8.81%) patients. More than 50.0% of patients were diagnosed with pN1-pN3 lymph node involvement at the time of diagnosis. Determination of the degree of malignancy (Grade) and the distribution was as follows: G1 was observed in 16 (8.29%) patients, G2 – in 129 (66.84%) patients, G3 – in 47 (24.35%) and G4 – in 1 (0.52%) of the patient. After studying the clinical and pathomorphological information and dividing the sample into molecular subtypes according to the consensus of St. Gallen 2015 *(Coates AS et al., 2015)* groups of observations were formed: luminal A subtype (79 cases); luminal B subtype (43 cases); Her2 / neu (39 cases); triple-negative (32 cases).

Histological examinations of the surgical material were performed using a universal light microscope Leica DM750 (Leica Microsystems GmbH). The research was carried out in compliance with the basic provisions of the "Statute of ethical principles of scientific medical research with human participation", approved by the Declaration of Helsinki (1964-2013), ICH GCP (1996), EEC Directive № 609 (dated 24.11.1986) , orders of the Ministry of Health of Ukraine № 690 dated 23.09.2009, № 944 dated 14.12.2009, № 616 dated 03.08.2012, approved by the Commission on Ethics of Scientific Research of the Danylo Halytsky Lviv National Medical University.

General histological processing of tissue samples of invasive ductal breast cancer was performed according to standard methods. Immunohistochemical studies (IHC) were performed in serial paraffin sections of tumor tissue using monoclonal antibodies. Incubation with primary antibodies was performed according to the instructions of the manufacturers, visualization of the IHC reaction was performed using the detection system DAKO EnVision + System with diaminobenzidine ("DAKO", USA). The sections were stained with Mayer's hematoxylin and encased in Canadian balsam. IHC study for CD31 (Ab-1, JC70A, Thermo scientific), estrogen receptor ER (monoclonal rabbit antibody, Clone SP1, Dako), progesterone receptor PR (monoclonal mouse antibody PgR 636, Dako Flex), c-erbB2 (monoclonal rabbit antibody) to Her2 / neu, Clone SP3, Thermo scientific) and Ki-67 protein (monoclonal mouse antibodies, clone MIB-1, Dako) was performed according to the manufacturer's protocol with the necessary controls. Immunohistochemical in situ fluorescence hybridization (FISH) was performed on samples with Her2 / neu 2+ status.

Evaluation of immunohistochemical staining. Positive ER and PR expression was found when ≥ 1% of neoplastic cells showed positive nuclear expression of any intensity *(Allison KH. et al., 2020)*. ER and PR status were assessed according to the recommendations of the American Society of Clinical Oncology / College of American Pathologists (ASCO / CAP) for ER and PR IHR testing. The threshold between low and high nuclear expression of Ki-67 was set at ≥ 20% of positive cells according to the Consensus of St. Gallen 2015. For Her2 /neu only membrane staining was considered, and more than 10% of strong membrane positivity was considered as positive (3+) Her2 / neu according to CAP recommendations.

CD31 expression was assessed by positive vascular endothelial staining. Using antibodies to CD31 (Ab-1, JC70A, Thermo scientific), we investigated 86 cases of invasive ductal breast cancer. Peri- and intratumoral areas were determined in the tumor stroma at low magnification (× 100). Vessels located in tumor tissue were regarded as intratumoral. Vessels located outside the tumor, but not more than 2 mm from the edge – as peritumoral.

In histological specimens stained with antibodies to CD31, the density of microvessels and areas with pronounced vascularization, the presence of dilated and atypical dilated capillaries in the intratumoral and peritumoral stroma, the presence of structures with partial endothelial lining were also assessed. The number of dilated and atypical dilated capillaries, as well as structures with partial endothelial lining was estimated by semi-quantitative counting at a magnification of × 200 (absent; single – no more than two in the field of view; multiple – more than two in the field of view) *(Bosari S et al., 1992)*.

Statistical processing of the obtained results was performed using a personal computer, the statistical package Statistica® for Windows was used for data analysis. For all types of analysis, differences were considered significant at $p < 0.05$.

3. Results of the research and their discussion

After studying the clinical and pathomorphological information and dividing the sample into molecular subtypes according to the consensus of St. Gallen 2015 *(Coates AS. et al., 2015)* groups of observations were formed: luminal A subtype; luminal B subtype; Her2 / neu; triple-negative.

The ratio of stroma and parenchyma is variable. For the most part, parenchyma was dominated in invasive ductal breast carcinoma. Various morphological structures were diagnosed in the infiltrative component, such as tubular, alveolar, trabecular, solid, single tumor cells and mixed structure. Tubular structures were formed by one or two rows of fairly monomorphic cells with normochromic, sometimes hyperchromic, rounded nuclei. The alveolar structures were accumulations of tumor cells of a rounded or slightly irregular, resembling a rounded shape. The morphology of the cells forming this type of structure varied from small cells with moderately pronounced cytoplasm and rounded nuclei to large cells with hyperchromic, irregularly shaped nuclei and moderate cytoplasm. Solid structures were characterized by fields different in size and shape, which consisted of small cells with moderately pronounced cytoplasm and monomorphic nuclei or large cells with abundant cytoplasm and polymorphic hyperchromic nuclei. The trabecular structures were short, formed by one row of small rather monomorphic cells, or long or wide, consisting of 2-3 rows of medium-sized cells with moderately pronounced cytoplasm, with rounded normochromic or hyperchromic nuclei. Separate groups of cells were clusters of two, sometimes 3-4 cells variable in their morphology with hyperchromic nuclei. But most often, especially in luminal cancer, the infiltrative component had a mixed structure.

Among the complexes of the tumor parenchyma are bundles of thin, curled, weakly eosinophilic or basophilic collagen fibers. Vessels are not numerous, mostly capillary type. However, in places between the layers of the tumor parenchyma are huge vessels of the sinusoidal type, resembling lacunae filled with erythrocyte masses or blood plasma. The wall of such vessels is thin, represented by one layer of endothelial or tumor cells. Thin layers of fibrous connective tissue with signs of edema, loosening and mucus are found around the vessels.

A detailed morphological study showed that in invasive ductal breast cancer, tumor vessels are heterogeneous in morphology. Depending on the morphological features, we have identified several types of tumor vessels and structures with endothelial lining. These were microvessels of ordinary structure. This type included capillaries and other microvessels with a diameter of 5-40 μm. The density of microvessels in the peritumoral stroma was 10.5 ± 2.5 per conventional unit area, in the intratumoral stroma – 11.2 ± 3.7 per conventional unit area. The vessels had a normal structure. The capillary wall is very thin, formed by endothelium, basement membrane and pericytes. The flattened endothelium lining such vessels had a flat hyperchromic nucleus. The cytoplasm of endothelial cells was uniformly and intensely stained with the CD31 marker and had clear, smooth contours. The described vessels did not have significant differences in peritumoral and intratumoral stroma and their density per unit area did not differ. Both erythrocytes and lymphocyte cells were found in the lumen of the microvessels (Fig. 1).

In a small number of cases, we found capillaries with weak expression of CD31, although the vessels had a normal structure, but along with this were found separately located endothelial cells in the intratumoral stroma (Fig. 2).

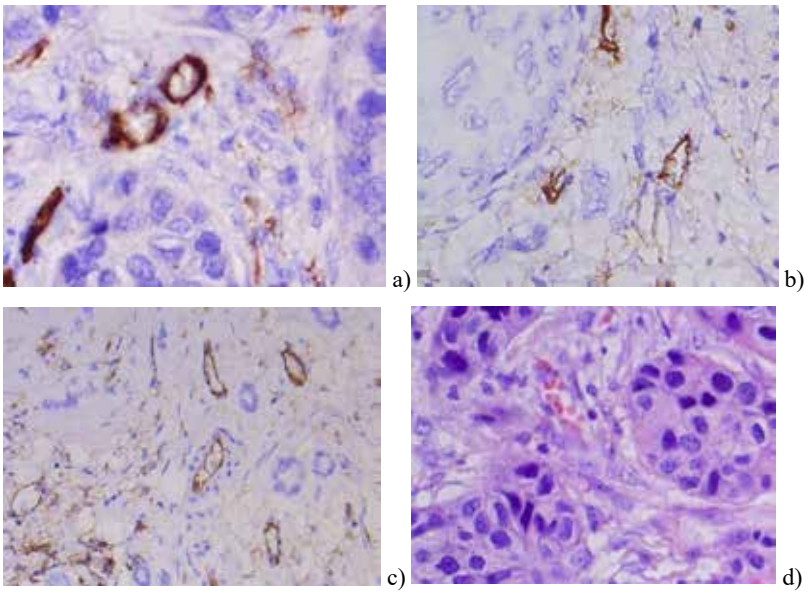


Fig. 1. Microvessels of normal structure in intratumoral (a, d) and peritumoral (b, c) stroma. The cytoplasm of endothelial cells is evenly and intensely stained with the marker CD31, has clear and smooth contours. IHC with CD31. a) × 600; b) and c) × 200; Staining with hematoxylin and eosin, d) × 600

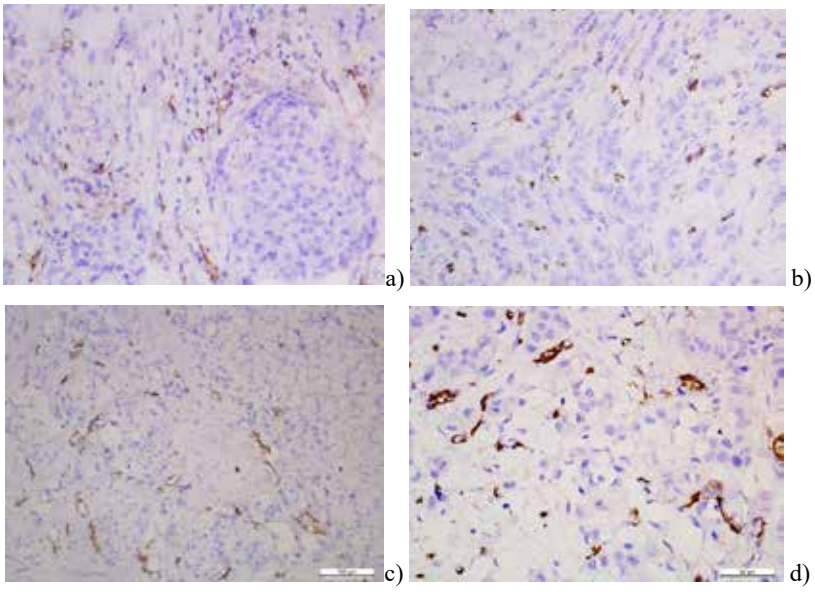


Fig. 2. Capillaries with weak expression of CD31 and single endothelial cells in the intratumoral stroma (a-b), increased expression of CD31 (d). IHC with CD31. a) b) and c) × 200; d) × 400

In the morphological study of invasive ductal cancer of the breast, the tumor complexes were surrounded by a sufficient number of stroma, represented by loose fibrous connective tissue, places with moderate edema and metachromasia. The number of vessels of the microcirculatory system is markedly increased compared to unaffected tissue, in some cases, vascularization even reached the degree of angiomatosis. In most cases, a significant portion of the capillaries were dilated with full-blooded with swollen endothelium. Some of the capillaries were filled with tumor cells and in a routine study with hematoxylin and eosin stain, mimicked small complexes of squamous cell carcinoma.

The dilated capillaries had some peculiarities. This type of vessel differed from ordinary capillaries in size. We have included vessels with a diameter of more than 40 μm to this type. We did not find the described vessels in 8.0% of cases, single and multiple dilated capillaries were equally common – in 46.0%. cases. Dilated capillaries often had a regular round or oval shape, but irregular angular capillaries were found. A characteristic feature of the described vessels was that in their endothelial lining cells with large light nuclei with a delicate reticulate structure of chromatin were often observed. The cross-sections of the nucleus were round or oval. The cytoplasm of endothelial cells was fairly evenly stained with a marker and had clear, relatively smooth contours. The most frequent dilated capillaries were observed in the peritumoral stroma, where their presence was associated with the characteristic structure of the connective tissue matrix, which was represented by immature connective tissue with fibroblasts and had a delicate thin-loop fibrous base (Fig. 3).

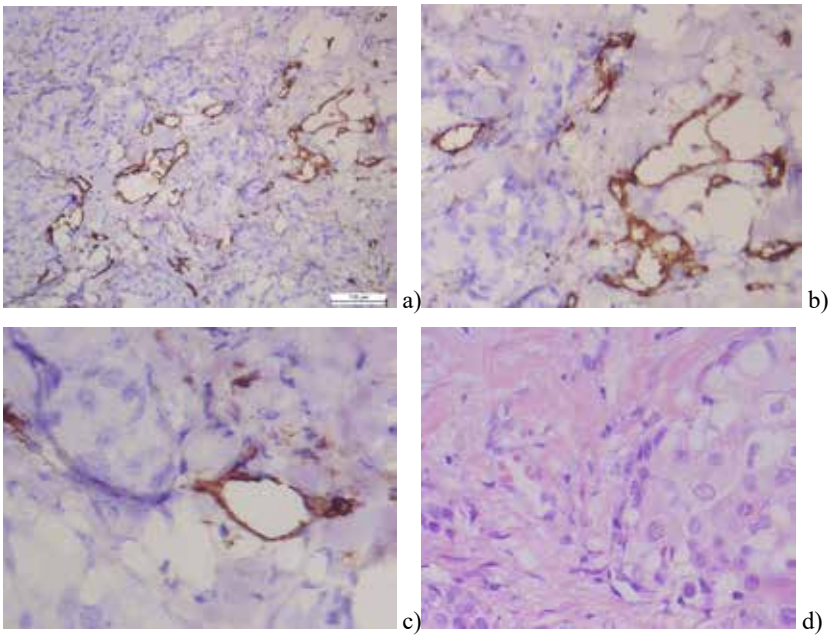


Fig. 3. Dilated capillaries of different shapes (a-d): round, oval and irregular angular shape. The cytoplasm of endothelial cells is evenly and intensely stained with a marker, has clear contours. IHC with CD31. a) × 200; b) × 400; c) × 600. The characteristic structure of the connective tissue matrix is represented by connective tissue with fibroblasts and has a delicate thin-lobed fibrous base. Hematoxylin and eosin staining. d) × 400

In some of our observations we also found dilated capillaries with weak expression of CD31, and the shape of the vessels is correct, the contours are smooth.

In addition, we found atypical dilated capillaries in the intratumoral stroma, which differed from the vessels near the invasive margin. They were represented by vessels of irregular shape, with a diameter of more than 40 μm. Atypical dilated capillaries were not detected in 20.8% of cases, were single – in 38.8% and multiple – in 40.4% of cases. The endothelial lining of such vessels was represented by chaotically arranged cells, which often layered on top of each other. The contours of the cells were blurred, uneven. CD31-positive cells not associated with endothelial lining were often detected in the lumen of such vessels. Such atypical sinusoids were located mainly in the intratumoral stroma. Erythrocytes were observed in the lumen of some vessels, in others only leukocytes (Fig. 4).

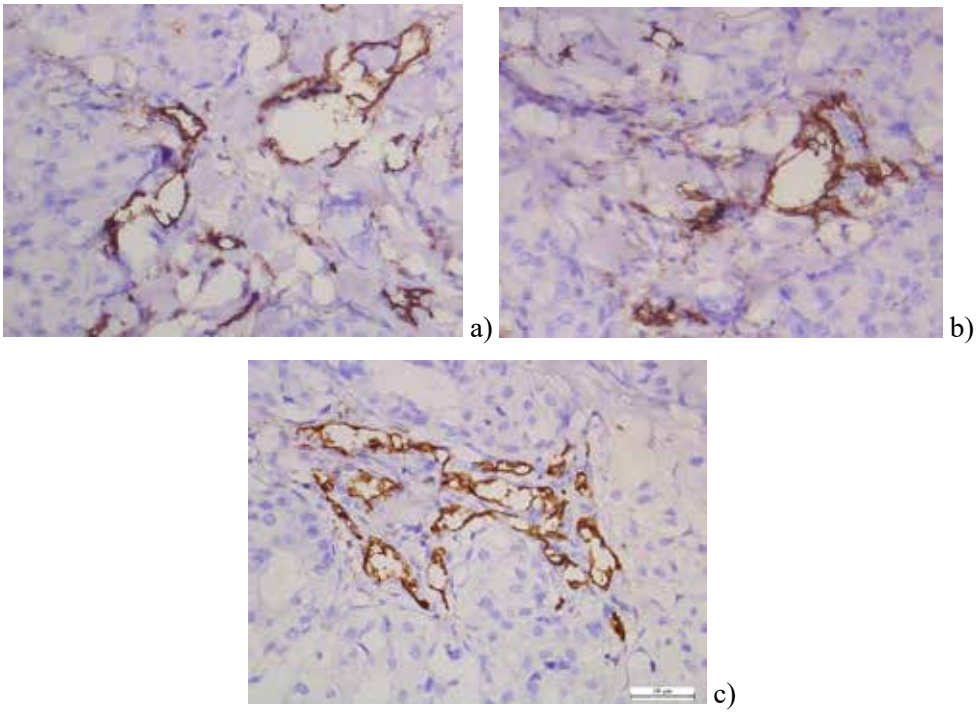
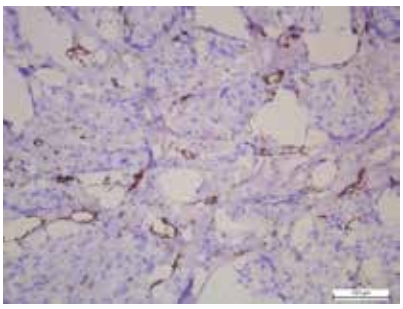


Fig. 4. Atypical dilated capillaries in the intratumoral stroma. The lining of the vessels is represented by chaotically arranged endothelial cells, which are layered on top of each other. IHC with CD31. × 400

Morphological examination, along with normal and dilated capillaries, revealed hollow round or oval structures with partial endothelial lining and characteristic cellular structures in loose softly fibrous unformed connective tissue. They were absent in 32.5% of cases, were single – in 38.9% and multiple – in 28.6%. It was characteristic that structures without endothelial lining and atypical sinusoids were also determined at the same time (Fig. 5).


a)

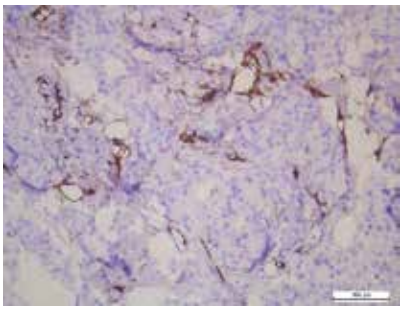

b)

Fig. 5. Structures of round or oval shape with partial endothelial lining in the intratumoral stroma, without endothelial lining next to normal and dilated capillaries. Endothelial cells in structures with partial endothelial lining are chaotic, the contours of the cells are not clear, the cytoplasm of the cells is unevenly stained with the marker CD31. IHC with CD31. × 200

The parameter for assessing the growth of new blood vessels, which is widely studied in various human diseases, especially in malignant tumors – is the density of microvascular density (microvascular density). Increased microvascular density is considered an unfavorable factor for many tumors, especially in prostate, breast and malignant diseases of the blood system *(Nico B. et al., 2008)*. Such a parameter as the density of microvessels is one of the important morphological criteria, which reflects not only the degree of vascularization of tumor tissue, but also the prognosis factor. According to the results of our study, we showed some differences in the severity of vascular density of the tumor in patients with invasive ductal breast cancer. There were no differences in microvascular density depending on the age of patients, T and N stage. At the same time, higher values of microvascular density in the intratumoral stroma were noted at G3 degree of differentiation, at triple-negative invasive ductal breast cancer and at HER2 / neu positive.

It is well known that angiogenesis is necessary for the growth of the primary tumor, its invasion and metastasis. It has been shown that a tumor cannot grow to more than 106 cells (or 1-2 mm^3) without adequate angiogenesis and intratumoral vascular network formation due to lack of oxygen and nutrients. Tumor angiogenesis consists of two phases separated by "angiogenic switching". The avascular phase is characteristic of tumors less than 1-2 mm in diameter. These tumors are "dormant" because the processes of proliferation and apoptosis are in balance. Some of these tumors enter the second, vascular phase, which is characterized by exponential tumor growth and imbalance of pro- and antiangiogenic factors *(Spirina L.V., et al., 2008)*.

Morphological studies have shown that the vessels in the tumor are unstable, immature, do not have a full basement membrane and pericytes, interendothelial pores are significantly enlarged. Unlike normal vessels, tumor vessels do not form venules, arterioles and capillaries, but form a chaotic network of vessels of all types simultaneously. The vascular network in tumors is "leaky" and often causes bleeding due to excessive production of VEGF *(Stepanova E.V. et al., 2006; Gershtejn E.S. etal., 2013)*. After reaching a certain critical size, the tumor cannot continue to grow without neovascularization, although non-angiogenic pathways have been shown breast cancer and non-small cell lung cancer due to the capture of pre-existing vessels or vascular mimicry. Vasculogenic mimicry is the process of forming channels from fluid-permeable tumor cells that have a high invasive potential. Characteristics of microvascular

mimicry are the lining of the canal by tumor rather than endothelial cells; ensuring blood flow to the tumor; positive reaction with Schiff's reagent (PAS reaction) and negative with CD31; intercellular matrix remodeling; expression of a multipotent phenotype similar to stem cells; association with poor prognosis and short 5-year survival *(Qiao L et al., 2015)*. There are two types of microvascular mimicry. The first type, tubular, morphologically similar to blood vessels lined with endothelial cells. The second type, matrix, does not resemble blood vessels morphologically or topologically. In such a matrix can be found laminin, heparan sulfate proteoglycan and collagen types IV and VI.

The study showed that multiple cavity structures with partial endothelial lining were more common in moderate and high tumor malignancy (Grade 2-3), with negative tumor status. Multiple structures with partial endothelial lining were observed in HER-2 / neu positive and triple-negative ductal breast cancer ($p < 0.05$). The presence of characteristic porous structures in the peritumoral stroma was the only factor associated with HER 2 / neu positive status. The described structures were significantly more common in the positive status of HER-2 / neu than in the negative ($p < 0.05$).

The most significant from the prognostic point of view were the number of atypical dilated vessels and structures with partial endothelial lining, as well as the presence of characteristic porous structures in loose softly fibrous unformed connective tissue of the peritumoral stroma.

4. Conclusions

Thus, the study revealed the following types of tumor microvessels and related structures: capillaries of normal structure, dilated capillaries, atypical dilated capillaries, structures with partial endothelial lining and characteristic porous structures in the loose soft fibrous stroma of the tumor. Dilated microvessels with a chaotic arrangement of endothelial cells and structures with partial endothelial lining were located mainly intratumorally and stained with antibodies to CD31.

An interesting component of the metastasis process may be the so-called vascular mimicry. In this case, cancer cells differentiate into endothelium-like structures, which is how malignant tumors may require vascularization. This ability is determined by the secreted proteins Serpine2 and Slpi, which are protease inhibitors and may perform anticoagulant function.

In general, the issue of angiogenesis in tumor tissue is undoubtedly of great interest, especially in connection with the development of methods of antiangiogenic therapy. At the same time, despite the large number of publications on this topic, the ways of new vessel formation and the influence of intra-tumor vessel density on the general and recurrence-free survival of invasive neoplasms remain unclear. There are very few works in which retraction slits in tumor tissue are studied. However, the obtained correlations indicate the possible involvement of cavities around tumor cells in the lymphangiogenic and, accordingly, the formation of metastases in the lymph nodes, which adversely affects the prognosis. Given this, the study of retraction slits, the mechanisms of their formation, the impact on the development of lymphatic vessels and possible targeted therapy are interesting and promising areas.

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