

Determination of VEGF and CXCR4 in Tumor and Peritumoral Tissue of Patients with Breast Cancer as a Predictive Factor

Danijela Cvetković^{1,*}, Aleksandar Cvetković², Danijela Nikodijević³, Jovana Jovankić³, Milena Milutinović³, Vladislava Stojić⁴, Nataša Zdravković⁵ and Slobodanka Mitrović⁶

¹University of Kragujevac, Institute for Information Technologies, Department of Science, Kragujevac, Serbia

²University of Kragujevac, Serbia, Faculty of Medical Sciences, Department of Surgery, Kragujevac, Serbia

³University of Kragujevac, Faculty of Sciences, Department of Biology and Ecology, Kragujevac, Serbia

⁴University of Kragujevac, Serbia, Faculty of Medical Sciences, Department of Medical Statistics and Informatics, Kragujevac, Serbia

⁵University of Kragujevac, Serbia, Faculty of Medical Sciences, Department of Internal medicine, Kragujevac, Serbia

⁶University of Kragujevac, Serbia, Faculty of Medical Sciences, Department of Pathology, Kragujevac, Serbia

Abstract

INTRODUCTION: Despite the obvious progress in the field of diagnosis and therapy, further measures are needed to increase the effectiveness of treatment and reduce morbidity and mortality from breast cancer.

OBJECTIVES: To study the influence of peritumoral tissue on the growth and development of the tumor itself.

METHODS: An immunofluorescence method was used to determine the protein expression of VEGF and CXCR-4 in tumor and peritumoral tissue.

RESULTS: Peritumoral tissue is not only a passive factor, but actively participates in the process of tumor growth and development, as well as in the processes of recurrence and metastasis.

CONCLUSION: Markers of neoangiogenesis in tumor and peritumoral tissue such as protein expression of VEGF and CXCR-4 receptors may serve as reliable predictors of disease outcome in breast cancer patients, which may provide useful suggestions in treatment choices.

Keywords: Breast cancer, Peritumoral tissue, VEGF, CXCR-4, Predictive factor.

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*Corresponding author: danijela.cvetkovic@uni.kg.ac.rs

1. Introduction

Breast cancer is the most common malignant tumor in women around the world. The mortality from breast cancer in the European Union is about 58,000 women a year and it is estimated that 135,000 new patients are diagnosed every year. In the female population in the world, breast cancer accounts for 22.9% [1]. Despite the obvious progress in the field of diagnosis and therapy,

further measures are needed to increase the effectiveness of treatment and reduce morbidity and mortality [1].

In recent years, much attention has been paid to the possibility that certain markers in peritumoral tissue may play an active role in the processes of invasion and metastasis, and therefore may serve as predictors of disease development and outcome and should be considered as possible targets for certain types of antitumor therapy. Two such factors are VEGF and CXCR-4. Vascular endothelial growth factor is a signaling protein of cells that stimulate vasculo- and angiogenesis. It is the basis of a system that regenerates

tissues in conditions of reduced oxygen concentration. Serum VEGF concentrations are high in a variety of pathological conditions. The normal function of VEGF is to create new blood vessels during embryonic development, after injury or after exercise. When overexpressed, VEGF can contribute to the development of pathological conditions. Solid tumors cannot grow without an adequate amount of blood, so cancers with high VEGF expression grow rapidly and metastasize. VEGF ligands and receptors are key regulators of vasculo-, angiogenesis, lymph angiogenesis, and vascular permeability of blood vessels [2].

By limiting the ability of new blood vessels to form in the tumor, it is possible to limit neoplastic angiogenesis in tumor tissue, so its growth can be stopped and limited. Therefore, numerous attempts have been made in the application of antiangiogenic therapy, which aimed to block its further development by reducing the blood flow, and thus the circulation in the tumor tissue. The first antiangiogenic therapy of tumors with the monoclonal antibody Bevacizumab, which neutralizes VEGF, was applied in 2004 on the basis of the approval of the World Food and Drug Administration (FDA) in the USA, for metastasis of colon cancer [3]. Meanwhile, the active ingredient (Bevacizumab) has been used in the treatment of breast cancer [4], lung cancer and kidney cancer [5].

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Chemokines play a role in cell migration and are secreted by numerous stromal and epithelial cells. Chemokine ligand-12 (Stromal Cell-Derived Factor-1-CXCL-12) and chemokine receptor-4 (Chemokine Receptor-4-CXCR-4) signaling pathway is an example that perfectly illustrates the process of metastasis. When talk about breast cancer, CXCL-12 as a product of the tumor cell itself, but also is a product of cells from the peritumor environment (fibroblasts) leading to the formation of secondary deposits at typical distant sites such as bone, liver or lung. CXCL-12 has two known receptors, CXCR-4 and CXCR-7 (Atypical chemokine receptor 3-ACKR-3). CXCL-12 bound to CXCR-4 initiates survival, growth, and induces signaling pathways associated with chemotaxis. CXCR-4 receptor expression is increased in both primary and metastatic tumors, confirming high tumor aggressiveness [6]. In pathological conditions such as cancer, they regulate invasion, angiogenesis, migration, and metastasis [7]. CXCR-4 and CXCL-12 together promote metastases, in some tumors mediate proliferation, tumor cell migration, and angiogenesis [8; 9; 10]. Numerous studies support the high expression of CXCR-4 in tumor cells. Mechanisms that regulate hypoxia in tumor cells have also been shown to induce increased expression of this chemokine. The promoter for the CXCR-4 gene is part of the hypoxic

response, so hypoxia-induced factor (HIF-1 α) can activate CXCR-4 transcription [11].

Our goal was to show that peritumoral tissue is an active factor in the process of tumorigenesis and metastasis.

2. Material and Methods

The study was conducted on 50 patients with breast cancer. We analyzed tumor and surrounding peritumoral tissue. All patients had a decision from the Oncology Council on appropriate surgical intervention. The examination was performed in accordance with good clinical practice with the approval of the Ethics Committee of the Clinical Center in Kragujevac (KC Kragujevac no. 01-4990) when perioperative tissue was sampled. Samples were then examined in the Department of Histopathology, when the following parameters were determined: histological type, tumor grade (Nottingham Histological Scores), age of patients, lymph node status, estrogen, progesterone and HER2 / neu status was evaluated according to the American Joint Committee on Cancer protocol - AJCC, 7th ed., 2010 [12, 13]. All patient data are described in detail in the manuscript of Cvetković et al 2019 [14].

The exclusive criteria were previous (neoadjuvant) therapy, previous history of breast cancer, presence of metastatic deposits originating from other organs.

2.1 Determination of protein expression of VEGF and CXCR-4 receptors on the membrane

Immunofluorescence is a technique used to mark individual cell parts with fluorescent dyes. The technique is based on the specific binding of an antibody to an antigen. The specificity of the antigen-antibody reaction enables this method to monitor the presence and localization accurately and precisely, as well as the distribution of a certain protein in a cell or tissue. The antibody as an indicator molecule is labeled with fluorescent dye with different excitation and emission wavelengths, and detection is performed with a fluorescent microscope [15].

2.2 Preparation of coloring preparations

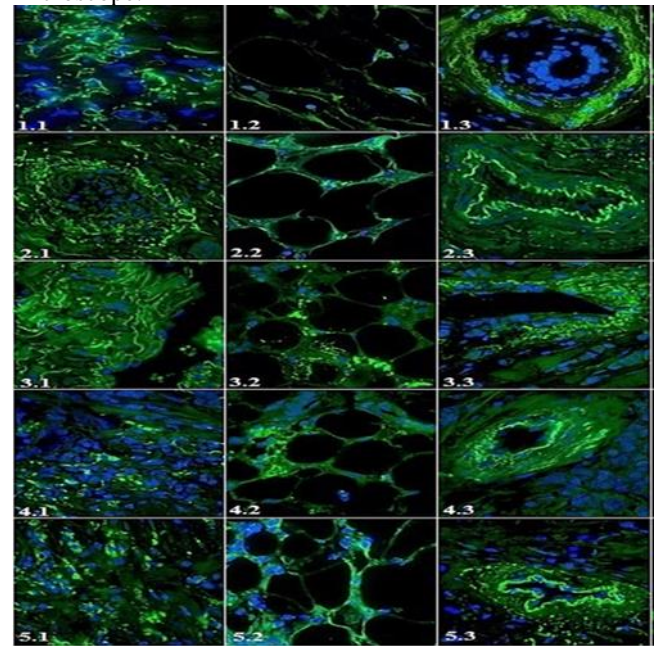
The samples used in this study to prove protein expression were processed by standard procedure at the Clinic for Pathological Anatomical Diagnostics at the Clinical Center Kragujevac. After surgery, the samples were fixed in 4% neutral, buffered formaldehyde for 24 hours and molded into paraffin. 4 μ m thick sections were mounted on special highly adherent SuperFrost® plates (Sigma-Aldrich, St. Louis, United States) and dried at 56°C for 1

hour. The sample sections obtained in this way were first subjected to deparaffinization, by passing through a series of alcohols of decreasing concentrations. The sample plates were first immersed in xylene for 2 x 5 minutes; then 2 x 5 minutes in absolute alcohol (Serva, Heidelberg, Germany), then 5 minutes in 96% alcohol, then 5 minutes in 70% alcohol, then 5 minutes in 50% alcohol, and at the very end the plates were washed in distilled water for 5 minutes. This was followed by "cooking" in a microwave oven at 560 W in citrate buffer. "Cooking" was performed in two intervals of 5 minutes and in one interval of 8 minutes, after which the plates with tissue samples were washed with running water for 5 minutes. This was followed by extensive rinsing of the preparation in distilled water and PBS (Thermo Fisher Scientific, Waltham, MA USA). The washed preparations were immersed in acetone for 5 minutes.

2.3 Immunofluorescence

100 μ L of Goat serum (Sigma-Aldrich, St. Louis, United States) was poured onto the wiped slides and allowed to incubate for 20 minutes at room temperature. After draining the excess serum, 100 μ L of primary antibody VEGF165b (RD System, Minesota, USA) (concentration 20 μ g / mL in PBS, R&D Systems) or primary antibody CXCR-4 (10 μ g / mL, (Thermo Fisher Scientific, Waltham, MA USA) was applied. An incubation of 60 minutes in a humid chamber followed. Excess antibody was washed three times with PBS. Secondary antibody was poured onto the washed and wiped slides and incubated for 45 minutes in a humid chamber in the dark. To demonstrate VEGF165b protein expression, a secondary Dnk pAb antibody (ABCAM, Waltham, MA USA) was poured into Ms IgG PE (ab7003), 1: 400 dilution in PBS with 1% BSA. Goat Anti-Rabbit IgG FITC secondary antibody (ABCAM abb717) dilution 1: 2000 in PBS with 1% BSA (ABCAM, Waltham, MA USA) was used to detect CXCR-4 protein expression. Excess secondary antibody was washed 3 times for 5 minutes in PBS. The slides were then wiped so that only the clippings remained wet. DAPI dye (4', 6-diamidino-2-phenylindole) (ABCAM, Waltham, MA USA) in a 1: 1000 dilution was used to visualize the blue-colored sails. The slides are covered with flakes (the ends of the flakes are glued). The preparations were allowed to dry overnight and then observed on a fluorescence microscope (Ti-Eclipse, Nikon), on filters of certain wavelengths and at a magnification of 600x. Protein expression of CXCR-4 and VEGF165b receptors, as predictors of tumor aggressiveness, was detected by fluorescence microscopy, using an inverted fluorescence

microscope.



Scale bare (— 100 μ m)

Figure 1. Protein expression of CXCR-4 receptors in peritumoral (column II) and cancer tissue (column I) in patients with breast cancer. Internal positive control is presented in column III.

Images were obtained on a fluorescent microscope, at a magnification of 600 \times . The nuclei were stained blue (DAPI color) and the CXCR-4 receptors green (secondary Goat Anti-Rabbit IgG FITC antibody).

3. Results and Discussion

3.1 CXCR-4 protein expression

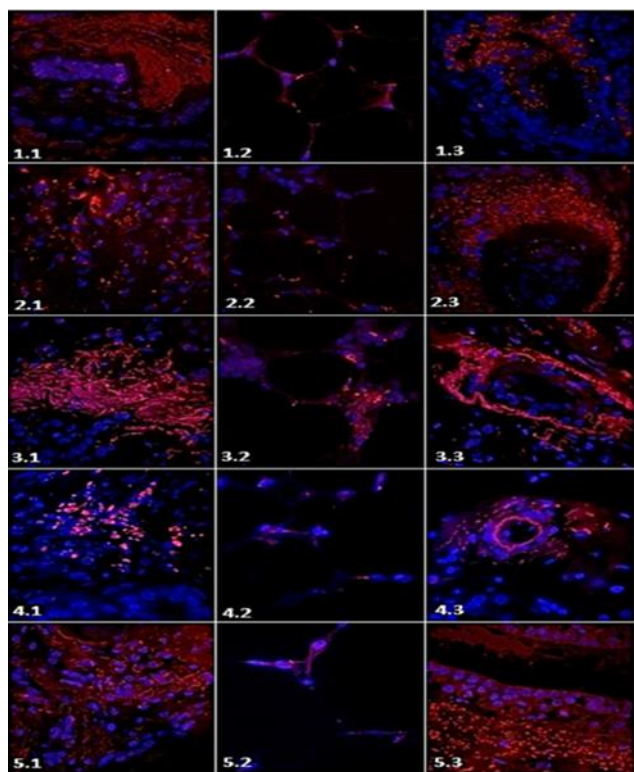
Figure 1 shows the protein expression of CXCR-4 receptors at cross-sections through carcinoma and peritumoral tissue in 5 cancer patients. Representative samples were selected, numbered 10, 12, 15, 16 and 23. Note the difference in protein expression of CXCR-4 receptors between cross sections through CT (cancer tissue) presented in the first column (I) compared to PT (peritumoral tissue) (column II). Protein expression of CXCR-4 receptors in cross-section through a blood vessel, represents an intrinsic positive control (column III).

Increased expression of CXCR-4 receptors (more intense green color of receptors) compared to PT (II) is observed in the cross section through CT (I). On micrographs (1.1, 2.1, 3.1, 4.1 and 5.1), tumor cells with an atypical structure, with a disturbed nucleus-cytoplasm ratio, can be observed. The nuclei are larger than the nuclei of healthy cells. Meganuclear structures are observed. Due to the high mitotic index, cancer cells often

number more nuclei, which is noticeable in Figure 20, because the cytoplasm does not manage to divide at the same rate.

The second column in Figure 20 presents cross-sections through the PT surrounding the cancer (1.2, 2.2, 3.2 4.2 and 5.2). The microenvironment around the tumor in breast cancer is most often adipose tissue. Micrographs 2.2, 3.2, and 4.2 show enhanced protein expression of CXCR-4 relative to other peritumoral tissue micrographs. Healthy breast tissue is known not to express the CXCR-4 receptor, so these results indicate altered function and morphology of peritumoral tissue.

The last column (III) in Figure 20 is the positive control. Increased expression of this receptor is observed, which is usually large in cross-sections of blood vessels. The picture shows sections of venous (2.3 and 5.3) and capillary networks (1.3, 3.3 and 4.3).



Scale bare (— 100 μm)

Figure 2. Protein expression of VEGF receptors in peritumor (column II) and cancer tissue (column I) in patients with breast cancer. Internal positive control is presented in column III.

Images were obtained on a fluorescent microscope, at a magnification of 600 \times . The nuclei were stained blue (DAPI color) and the VEGF receptors red (secondary antibody Dnk pAb in Ms IgG PE).

3.2 Protein expression of the VEGF165b receptor

Figure 2 shows the protein expression of the potent angiogenic and mitogenic factor VEGF165b receptor at cross-sections through carcinoma and peritumoral tissue in 5 breast cancer patients. Representative samples were selected, numbered 10, 12, 15, 16, and 23 in this study. of peritumoral tissue (column II). Protein expression of the VEGF165b receptor in cross-section through a blood vessel represents an intrinsic positive control (column III). Increased expression of VEGF165b protein (more intense red color of the receptor) was observed in the cross section through CT (I), compared to PT (II). On micrographs (1.1, 2.1, 3.1, 4.1 and 5.1), tumor cells with an atypical structure, with a disturbed nucleus-cytoplasm ratio, can be observed. The nuclei are larger than the nuclei of healthy cells. Due to the high mitotic index, cancer cells have more nuclei, which are blue-stained structures on micrographs.

The second column (II) in Figure 2 presents cross-sections through the PT surrounding the cancer (1.2, 2.2, 3.2 4.2 and 5.2). As already pointed out, significantly higher expression of VEGF165b protein in CT (I) is evident compared to PT (II). Micrographs 2.2 and 3.2 show increased expression of VEGF165b protein compared to other peritumor tissue micrographs.

The last column (III) in Figure 2 was taken as a positive control. Increased expression of this receptor is observed, which is usually large in cross-sections of blood vessels. Both venous and capillary network sections can be seen.

Figure 2. Protein expression of VEGF receptors in peritumor (column II) and cancer tissue (column I) in patients with breast cancer. Internal positive control is presented in column III. Neoangiogenesis, the formation of new blood vessels from pre-existing blood vessels, plays a key role in the formation and development of tumors. In low-differentiation tumors, the vascular network cannot follow the proliferation of malignant tissue and tumor growth that is too intense. Due to the rapid decrease in pO_2 (oxygen partial pressure), lack of nutrients and other factors in the tumor, an "angiogenic switch" is initiated, after which a number of factors that induce "germination" and chemotaxis are released, inducing the movement of endothelial cells towards tumor mass [16]. Under hypoxic conditions, HIF-1 α is stabilized, which further activates the expression of multiple genes that contribute to the process of angiogenesis. Increasing VEGF expression leads to increased vascular permeability, while fibroblast growth factor allows endothelial cell growth. HIF-1 α together with VEGF induces an increase in the expression of matrix metalloproteinases (especially MMP-9) which degrade the extracellular matrix and facilitate endothelial cell migration and release of growth factors [14]. Factors released in the tumor microenvironment activate tumor-associated macrophages, which later produce angiogenic factors, such as VEGF and MMP, and further support the

angiogenesis process [17]. Stromal fibroblasts located in tumor tissue, under the influence of VEGF produce CXCL-12, which binds to CXCR-4, receptors on tumor cells, which initiates the formation of new blood vessels and mobilization of proangiogenic cells from bone marrow [18 ;19]. This creates conditions for neoangiogenesis, invasion and dissemination of malignant cells into other organs, but also overcomes the barrier to nutrients and other necessary factors for the growth and development of malignant cells [20; 21].

High expression of VEGF leads to stimulation of endothelial cell proliferation, which further stimulates neoangiogenesis and lymphangiogenesis. The newly formed vascular and lymphatic network shows numerous dysfunctions, starting from irregular branching to increased permeability of the blood and lymph vessel wall. These newly formed abnormal vessels go hand in hand with tumor progression in two ways; first, they provide the tumor tissue with enough nutrients, and on the other hand, they enable easier penetration of malignant cells and their further hematogenous and lymphogenic dissemination, i.e., metastasis. It is already known from previous studies that there is a positive correlation between the levels of VEGF-C in the plasma of patients with breast cancer and the occurrence of metastases [22; 23].

Our results showed that there is an extremely high expression of VEGF in the peritumor and tumor tissue of patients with affected lymph nodes (N2 group). High VEGF expression leads to significant changes in the peritumor environment and stimulation of neoangiogenesis and lymphangiogenesis which can serve as a predictive parameter when it comes to metastases in "sentinel" lymph nodes [24].

Literature data show that VEGF stimulates the expression of CXCL-12 and CXCR-4 receptors. Cells previously treated with VEGF show an increased migratory and angiogenic response to CXCL-12, suggesting that VEGF stimulation has a complementary effect with the SDF-1 / CXCR-4 signaling pathway in inducing angiogenesis. Endothelial cell migration is a decisive step in the process of angiogenesis under the influence of VEGF and CXCL-12. VEGF participates in the processes of cell migration, proliferation, expression of proangiogenic factors, among which one of the most important is CXCR-4 [25]. Stromal fibroblasts located in tumor tissue produce CXCL-12, which binds to CXCR-4, receptors on tumor cells, initiating the formation of new blood vessels and the mobilization of proangiogenic cells from the bone marrow [19 ;20]. Muller et al. demonstrated that CXCR-4 is not expressed in normal breast tissue but is present in breast cancer tissue and metastatic cells [7].

The signaling pathway CXCL-12 / CXCR-4 is associated with the process of intravasation, which involves the entry of malignant cells into the blood and lymphatic network, which is a prerequisite for metastasis. Indeed, the papers describe many macrophages with increased expression of CXCR-4 receptors, which are

localized around blood vessels near the tumor. This allows the tumor to connect with the surrounding blood vessels [24]. Therefore, it is not surprising that there is a strong correlation between the mentioned axis on the one hand and lymph node and distant metastases on the other hand in patients with breast cancer. Our results support such literature findings.

Considering the protein expressions in the peritumoral and cancer tissues of patients, it can be concluded that the expression of VEGF165b and CXCR-4 receptors is higher in CT compared to PT. It should be taken into account that these receptors are not expressed in healthy breast tissue, which suggests that PT is by no means healthy tissue, because under the influence of the tumor it becomes an active factor in carcinogenesis, which is confirmed by the presented results. Also, high expression of these receptors in CT may indicate a poorer prognosis of the disease.

4. Conclusion

Carcinoma causes changes in tumor and peritumoral tissue detectable at the molecular level, which are not recorded on the pathohistological finding. Not only a detailed analysis of tumor, but also peritumoral tissue is sufficient, because often significant changes at the molecular level occur in the microenvironment of cancer. Peritumoral tissue is not only a passive factor, but actively participates in the process of tumor growth and development, as well as in the processes of recurrence and metastasis. Markers of neoangiogenesis in tumor and peritumoral tissue such as protein expression of VEGF and CXCR-4 receptors may serve as reliable predictors of disease outcome in breast cancer patients, which may provide useful suggestions in treatment choices.

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