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Review

The role of molecular markers of angiogenesis in disease prediction in breast cancer patients

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Summary. Breast cancer is the most common malignant tumor in women around the world. It is a disease of complex etiology, characteristics and response to therapy. Oncology therapy is the most expensive form of treatment and represents a significant fraction of the overall health budget of both developed and transition countries. Progress in molecular biology has had a major impact on the development of a more personalized approach. Our study clearly indicates that cancer causes changes in both cancerous and peritumoral tissue that are detectable on the molecular but not the pathohistological level. Therefore, analysis not only of cancer but also of the peritumoral tissue is very valuable, because often the changes that are very significant in the prognostic sense occur predominantly in the microenvironment of the carcinoma. Markers of angiogenesis in cancer and peritumor tissue such as MMP-9 concentration, expression of *VEGF-A, CXCL-12*, *HIF-1* and *iNOS* genes can serve as reliable predictors of disease outcome in patients with breast cancer, which can give useful suggestions for the choice of treatment. Using modern methods of molecular biology, a group of patients with an increased risk of metastases and recurrence can be identified, providing important information to guide decision making with respect to further treatments.

INTRODUCTION

Breast cancer is a global health problem, not only because of the fact that the disease has epidemic proportions, but also because its consequences affect practically all segments of society. The epidemic wave of this disease affects both developed and developing countries. Oncology therapy is the most expensive form of treatment, and represents the largest fraction of health budgets. By combining clinical, pathohistological, and molecular parameters of a cancer tissue, we can predict whether each individual patient will develop metastases. This information is significant for the selection of further oncological treatment, which represents a practical, clinical application of personalized, individualized therapy (Chan et al. 2017). The first examples of the development of individual therapy in oncology were related to the analysis of estrogen expression in breast cancer cells. The results of such a 'personal approach' identified groups of patients for whom ovariectomy was justified, or would benefit from the use of endocrine therapy (Engelsman et al. 1973; Jensen and Sombre 1973). After introduction of immunohistochemical methods, determination of the estrogen, progesterone and HER-2 status of breast cancers became a routine part of the diagnosis of breast cancer patients. This discovery opened an era of drugs that are specific for certain groups of patients. A major shift in diagnosis was the introduction of molecular techniques that were shown to have high sensitivity and specificity (Inic et al. 2014).

The hypothesis that cancer growth depends on angiogenesis was greatly supported by seminal papers from

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Folkman in the 1990s (Folkman and Klagsbrun 1987). Since then, the efforts of many scientists around the world have been invested in the recognition of angiogenesis mediators and antagonists; as well as their role in development of both primary breast cancer and metastases - secondary deposits. Thus, to date, more than 20 proangiogenic factors and approximately 30 anti-angiogenic agents have effects and therefore a possible role in the treatment of malignant diseases, as evidenced by the large number of studies in the world's scientific databases. It is known that each cell in the human body must be at a maximum of 200 μm distance from the nearest blood vessel (i.e. capillaries) in order to satisfy its needs for oxygen and nutrients (Cai et al. 2015). In low-grade cancers, the vascular network cannot reach the malignant tissue and cancer growth, which is too dense. Due to the rapid decrease in pO_2, a lack of nutrients and other factors in the tumor, an "angiogenic switch" is initiated, after which numerous factors that induce "germination" and chemotaxis, i.e. the movement of endothelial cells toward the cancer mass, are freed (Folkman 2007). Hypoxic conditions stabilize Hypoxia Inducible Factors - HIF-1, which further activate the expression of multiple genes that contribute to the process of angiogenesis (Smith et al. 2008). Hypoxic conditions due to rapid cancer tissue growth induce increased expression of HIF-1, which is the principal regulator of oxygen homeostasis in the cell (Smith et al. 2008). This heterodimeric molecule consists of two subunits HIF-1α and HIF-1β, wherein the first subunit has a dominant role. It affects the transcription of over 60 genes involved in many cancer development processes and largely determines its biological behavior, including angiogenesis, apoptosis, proliferation, resistance to chemotherapy and radiotherapy, and cancer cell glucose metabolism (Kuonen et al. 2012). Different analyses and tests of *HIF-1* gene expression in cancer tissue can be used to provide prognostic information and thus identify subgroups of patients requiring more intensive therapy. It has been suggested that modulating the molecular pathway of HIF-1 could be effective in treating pancreatic cancer. Many new therapeutic agents have been designed with the goal of blocking HIF signaling, and have antineoanigenic effects, including Trastuzumab (Harris 2002). The rate of angiogenesis is an important parameter of cancer aggressiveness (Stegmann et al. 2000).

It is relatively easy to distinguish new blood vessels from the normal vascular bed, as these blood vessels are highly disorganized with abnormal segmental enlargements, unusual branching, and unnatural shunting of lateral branches. Not only their appearance, but also the wall composition shows numerous abnormalities, with defects in the endothelium, discontinuity of the basal membrane, etc. This composition of the wall of new created blood vessels allows the entry and transport of malignant cells into the vascular bed and their further migration, which is the basis of the metastasis process (Bach-Gansmo and Tobin 2008).

The transcription activity of many genes, including one that determines the production of VEGF (Vascular Endothelial Growth Factor - VEGF), have been substantially altered in hypoxic conditions under the effect of HIF-1 α (van Hinsbergh and Koolwijk 2008; Ni et al. 2013). VEGF is one of the most significant angiogenetic factors related to breast cancer growth and its ability to develop secondary deposits. Considering that breast cancer is one of the leading causes of death, in both developed and developing countries, it is not surprising that scientists of various disciplines have focused their research on this carcinogenesis mediator, which may be one of the most important targets for future oncology therapies. VEGF can activate VEGFR-1 and VEGFR-2 tyrosine kinase receptors that are localized to the endothelium and their activation affects endothelial migration, proliferation, permeability, and survival. Many cells, mainly macrophages, stem cells, and many cancer cells have VEGFR-1 receptors on their surface (Srabovic et al. 2013). VEGF is a family of five isoform mediators (VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor, PGF) (Lee et al. 2015). Each of these factors can activate one or more VEGFR1, VEGFR2 and VEGFR3 receptors, which is followed by the activation of angiogenesis through stimulation of growth, migration, and invasion of endothelial cells. Breast cancer is characterized primarily by lymphatic metastases, which were previously assumed to develop through existing lymph vessels. However, it is known today that the process of lymphangiogenesis is also induced by the formation and development of cancer lymph vessels, analogously to angiogenesis, and that VEGF is responsible for both processes. VEGF-C is the major lymphangiogen and angiogenic factor expressed in carcinoma tissue (Rauniyar et al. 2018), which stimulates angiogenesis, lymphangiogenesis, and metastasis to lymph nodes by activating a specific VEGFR-3 receptor. Recent studies suggest that induced cancer lymphangiogenesis is directly proportional to metastatic potential. Termination of this signaling pathway in previous experiments has proven to be a promising strategy for cancer therapy.

In hypoxic conditions, the expression of numerous chemokines is specifically regulated, such as the stromal-cell-derived factor -1 α also called CXCL12 or SDF-1 α, which mobilizes proangiogenic cells from the bone marrow (Devignes et al. 2018). Factors released in the cancer microcirculation activate cancer-associated macrophages, which subsequently produce angiogenic factors such as VEGF and MMP and further support the process of angiogenesis (Guo et al. 2013). Stromal fibroblasts located in the cancer tissue, under the influence of VEGF, produce CXCL-12, which binds to CXCR-4 cancer cell receptors, initiating formation

of new blood vessels and the mobilization of proangiogenic cells from the bone marrow (Zheng et al. 2007; Chavakis et al. 2008). This creates conditions that are conducive for angiogenesis, invasion and dissemination of malignant cells to other organs, but also provides the nutrients and other necessary factors for the growth and development of malignant cells (Sakurai and Kudo 2011; Senger and Davis 2011). These mediators of vasculogenesis as well as numerous, relatively well-studied signaling pathways represent possible therapeutic targets (Jardim-Perassi et al. 2014). Therefore, therapy that has anti-invasive, anti-angiogenic and anti-metastatic effects would have enormous significance in the treatment of patients with cancer.

MOLECULAR MECHANISMS OF ANGIOGENESIS IN PATIENTS WITH BREAST CANCER

Using modern molecular biology methods, a group of patients with increased risk of metastasis and relapse can be identified, providing important information to guide further treatment. However, groups of patients that are over-treated should not be underestimated. Namely, studies indicate that although aggressive breast cancer therapy aims to prevent progression of the disease, for each patient whose life is saved by this treatment, it is estimated that 3 patients undergo unnecessary, and even harmfully excessive therapy. While it is logical to assume that aggressive treatment regimes should results in prevention of recurrence and distant metastases, numerous studies have shown that about 70% of patients who have undergone such aggressive treatment protocols would survive without them. The ability to predict the outcome of such treatments with high probability would allow physicians to make the best decision regarding the extent of surgical procedures, chemotherapy and radiotherapy in an adjuvant or neoadjuvant protocol. Molecular mechanisms of angiogenesis in breast cancer patients are presented in Fig. 1.

Individualization of therapy is the basic premise of modern oncology. Toward this aim, the molecular parameters of angiogenesis in cancer and peritumoral tissue were monitored in patients with breast cancer, a crucial mechanism in the spread and development of cancer. The study included 50 patients, whose clinical and pathological characteristics are described in Fig. 2. Patients were diagnosed with invasive ductal (86%) or invasive lobular carcinoma (14%). At the moment of diagnosis, the most common were carcinomas up to 5 cm (T2) with detected metastases in the lymph nodes (N2 41%) and distant organs (M1 52%). After histopathological verification of the preparations, poor carcinoma differentiation was detected in more than a half cases (G2 56%) and receptor status was determined (ER + 60%, PR-54% and Her- 52%) (Cvetković et al. 2019).

Concentration of MMP-9 as prediction marker in breast cancer

Metalloproteinases have numerous functions in normal physiological processes. MMP-9 is a proteolytic enzyme that degrades the basal membrane and extracellular matrix. It promotes the progression of cancer by increasing the proliferation of cancer cells, migration, invasion, metastasis and angiogenesis. These effects of MMP-9 are involved in remodeling and degradation of a diverse group of substrates which are structural components of the extracellular matrix. MMP-9 also regulates several signal pathways influencing growth factors, cytokine concentration, tyrosine kinase receptor expression, etc. (Bjorkklund and Koivunen 2005). Numerous papers suggest that MMP-9 can serve as a prognostic marker, although the role of MMPs as cancer modulators and their role in micro-circulation remains unclear, so further research is required in this field (Yousef et al. 2014). MMP-9 is found in the cytoplasm of cancer cells, but also in stromal fibroblast cells that are components of the cancer microenvironment (Pellikainen et al. 2004). Although cancer cells penetrate more tissue barriers for the purpose of prolifera-

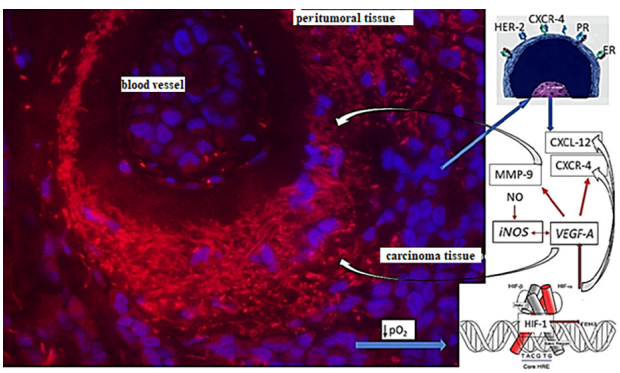

Fig. 1. Molecular mechanisms of angiogenesis in patients with breast cancer.

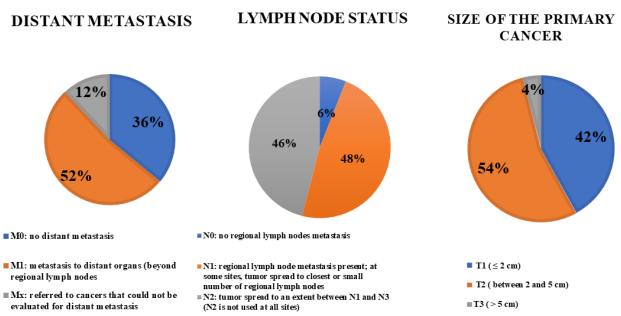

Fig. 2. Characteristics of breast cancer patients.

tion and expansion, there is evidence suggesting that cells in the cancer microenvironment, such as stromal cells, support such processes, and affect cancer progression (Charous et al. 1997; O-Charoenrat et al. 2001; Zhang et al. 2008).

We analyzed total metalloproteinase-9 concentrations in peritumoral (PT) and carcinoma tissue (CT) in patients with breast cancer (Fig. 3).

The microenvironment around the cancer tissue produces 1.42 fold higher concentrations of MMP-9 than in the carcinoma itself (Cvetković et al. 2019). This study confirms that breast cancer causes changes in the peritumor tissue, which can be detected on the molecular, but not the pathohistological level. The cancer environment in the advanced stage of disease takes on a more dominant role and becomes the main source of MMP-9. Peritumoral tissue at a distance of 3 cm from the cancer produces more MMP-9 than the cancer itself. An analysis of MMP-9 ratios between peritumoral and carcinoma tissue in breast cancer patients indicates a group of patients with high risk of metastases, proving that MMP-9 is a good prognostic indicator of disease progression in breast cancer.

Quantitative mRNA analysis of angiogenesis parameters in breast cancer

The relative expression of *VEGF-A, HIF-1, CXCL-12* and *iNOS* genes were calculated in relation to the expression of β-actin for every sample, using qPCR methods. Unlike MMP-9, whose protein expression was significantly higher in peritumoral tissue, gene expression of these parameters was statistically significantly higher in carcinoma tissue, when compared with microenvironment (Fig. 4). Based on these results, *VEGF-A* gene was found to be the most expressed in the cancer tissue, while the most expressed parameter in peritumoral tissue was *iNOS*. A large difference was observed in gene expression between PT and CT. In fact, *VEGF-A* gene expression was 4.57 folds higher in CT than in PT; similar to *CXCL-12*, whose expression was 4.13 fold higher in cancer tissue over peritumoral tissue.

The link between hypoxia and cancer progression has long been known to researchers. Hypoxia is a consequence of various factors, such as size, localization, type and circulation of the cancer. One of the most important parameters that occurs as a result of hypoxia is *HIF-1*. Intratumoral hypoxia is a parameter of poor prognosis in breast cancer patients. It has been documented that such cancers are biologically aggressive, often metastasize and, unfortunately, become resistant to most standard modalities of therapy, such as chemotherapy and radiotherapy (Cerci et al. 2016). Increased *HIF-1* gene expression is found in many cancers, and especially in breast cancer. Van den Bos and associates noted elevated *HIF-1* expression in cases of ductal breast cancer, but not in normal breast tissue, or in ductal hyperplasia (van den Bos et al. 2016). Figure 4 shows the relative expression of the *HIF-1* gene in patients with breast cancer. As can be seen, the cancer tissue has greater *HIF-1* gene expression than in the microenvironment of the cancer. Although it is known that normoxic conditions are standard in normal breast tissue, our results indicate that the peritumoral tissue becomes altered under the influence of cancer.

There are a number of papers in the literature dealing with the effects of angiogenesis, VEGF and VEGF-R in cancer tissue (Kerbel et al. 2008; Zhuang et al. 2013; Feng et al. 2017), but very few papers have examined the influence of these parameters in peritumoral tissue, which plays an important role in cancer progression. Our results show significantly higher expression of *VEGF* in cancer tissue than in the cancer environment (Fig. 4). In the literature, similar data has been reported for breast cancers in the initial stages of progression as well as for hepatocellular carcinoma (Lin et al. 2001). Macrophages in the peritumoral environment expressing VEGFR-1 receptors are particularly important

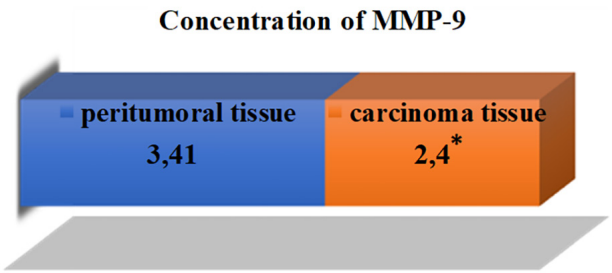

Fig. 3. Concentrations of matrix metalloproteinase-9 in peritumoral and cancer tissue in patients with breast cancer. The results are presented as the mean ± SE for the total number of samples (n = 50); *p < 0.05 statistically significant difference between PT and CT.

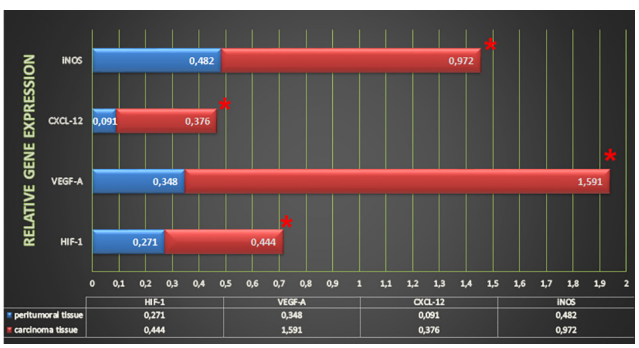

Fig. 4. Relative expression of HIF-1, *VEGF-A, CXCL-12 and iNOS* genes in peritumoral and carcinoma tissue in patients with breast cancer. Results are presented as the mean ± SE for the total number of samples (n = 50); *p < 0.05 statistically significant difference between PT and CT. β-actin is a reference gene for the normalization of gene expression.

and can play an important role in cancer recurrence. They can produce factors that promote cancer growth and metastasis, which again results in poor outcomes (Lin et al. 2001). Whether the expression of *VEGF* in peritumoral tissue is a consequence of cellular interactions (cancer tissue and environments), or is a consequence of relative hypoperfusion due to mechanical pressure of the cancer on the peritumoral tissue, is still not clear (Li et al. 2011).

Chemokine CXCL-12, also known as stromal cell-derived factor-1 (SDF-1) and VEGF, produced by hypoxic cancers, are potent angiogenesis stimulants. CXCL-12 is a new generation biomarker, and there is not yet enough published data for comparison with our results. According to our knowledge, studies involving measurements of *CXCL-12* expression in cancer tissue of breast cancer are rare, and this is one of the first studies analyzing its expression in peritumoral tissue (Fig. 4). 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 complementary effects on SDF-1 / CXCR-4 signaling pathways during the induction of angiogenesis (Carretero-Ortega et al. 2010). Migration of endothelial cells 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, and expression of proangiogenic factors, among which one of the more important is CXCR-4 (Kryczek et al. 2005). Stromal fibroblasts located in the cancer tissue produce CXCL-12, which binds to CXCR-4 receptors on cancer cells, initiating formation of new blood vessels and mobilization of proangiogenic cells from the bone marrow (Zheng et al. 2007; Chavakis et al. 2008). Muller et al. (2001) proved that CXCR-4 was not expressed in normal breast tissue, but was expressed in breast carcinoma and metastatic cells. Our results have shown that *CXCL-12* gene expression was 4 fold higher in carcinoma than in the cancer environment (Fig. 4). Also, these results suggest that the ligand also occurs in peritumoral tissue, which again confirms the fact that the peritumor tissue becomes altered under the influence of malignant cells. This evidence suggests disease progression in the examined patients with breast cancer.

Many angiogenic stimulants can induce the production of nitric oxide (NO), an important inter- and intracellular signal molecule synthesized from L-arginine in the presence of nitrogen monoxide synthetase (NOS). Among three NOS isoforms, the inducible one produces the largest amount of NO and is associated with numerous pathologies. Although their precise role is unclear, inducible and endothelial NOS are involved in processes such as cancer progression and angiogenesis, and their activity appears to depend on the distribution, concentration, and duration of exposure, as well as essential cell susceptibility to NO (Kostourou et al. 2011). In the present study, we examined the gene expression of *iNOS* and links with angiogenic potential in breast cancer patients (Fig. 4). Our results showed overexpression of *iNOS* gene in cancer tissue. It is known from the literature that high concentrations of iNOS are cytotoxic to cancer cells, stimulating the expression of proapoptotic genes. Also, the environment in which carcinoma cells are located can also be a source of NO (Vanini et al. 2015). Our results show that peritumoral tissue displays the highest level of *iNOS* expression comparing with all analyzed genes. It is known that lower NO concentrations, less than 100 nM, prevent certain types of carcinoma cells from entering apoptosis, thereby favoring angiogenesis and cancer progression (Oronsky et al. 2014). Therefore, iNOS expression analysis can serve as a reliable predictor of disease outcome in patients with breast cancer, which can give useful suggestions in the choice of treatment.

CONCLUSION

Markers of angiogenesis in cancer and peritumoral tissue such as MMP-9 concentration, expression of *VEGF-A, CXCL-12*, *HIF-1* and *iNOS* genes can serve as reliable predictors of disease outcome in patients with breast cancer, providing useful suggestions with respect to the choice of treatment. Using modern methods of molecular biology, a group of patients with an increased risk of metastases and recurrence can be identified, which is highly important information that can guide decisions on further treatment.

Conflict of interest

The authors declare that they have no conflict of interest.

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