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CORRELATIONS BETWEEN VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION, MICROVASCULAR DENSITY IN TUMOR TISSUES AND TNM STAGING IN BREAST CANCER

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Abstract - Breast cancer is the most frequent malignancy in women worldwide. In spite of its questionable reliability, the TNM (Tumor, Node, Metastasis) staging system is widely used for the prognosis of this disease. On the other hand, angiogenesis is considered to have an essential role in the evolution of breast cancer and the Vascular Endothelial Growth Factor (VEGF) has proven to be the key regulator of this process. Quantitation of VEGF and the tumor vasculature might play an important role in predicting tumor behavior and patient management. The aim of our study was to evaluate the potential correlation between the VEGF expression, microvessel density (MVD) in the tumor tissues and TNM staging in breast cancer. We included 34 patients with breast cancer who had undergone surgical treatment and evaluated each case clinically, histopathologically and by immunohistochemistry for VEGF and CD31 expression in the tumor tissues. VEGF expression was evaluated by calculating the average percentage of cytoplasmic positive cells from 3 high power fields. MVD was expressed as the average number of the CD31+ microvessels from 3 high power fields. Expression of VEGF was significantly associated with the number of lymph nodes with metastasis, the number of cells in mitosis, the presence of necrosis and the T-stage (inverse correlation). MVD correlated very well with the histological grading, the number of cells in mitosis, the presence of inflammation and the presence of necrosis. No correlation could be established between VEGF expression and MVD. Although their relationship with TNM staging remains unclear, VEGF expression and MVD proved to be important indicators of the malignant status in breast cancer, confirming the major involvement of angiogenesis in this type of cancer. Both of them are valuable prognostic factors in breast cancer, but the pattern of their relationship needs further analysis of the VEGF receptors in order to be described.

Key words: Angiogenesis, breast cancer, microvessel density**,** TNM staging, VEGF

INTRODUCTION

Breast cancer is the most frequent malignancy in women all over the world and therefore finding valuable means for its evaluation has always been a challenge for medical science. More than 50 years ago, Pierre Denoix, from the Institut Gustav-Roussy, France, devised the TNM staging system, and its application to breast cancer was published 24 years later in 1968 (Benson, 2003). At the introduction of this system, the International Union Against Cancer Committee defined the aims of cancer staging as: (i) to provide some indication of prognosis; (ii) to aid the clinician in planning cancer treatment; (iii) to assist in evaluating the results of treatment; (iv) to facilitate the exchange of information between treatment centers; and (v) to contribute to continuing investigation of human malignancies (Bonnefoi, 2007). The aims of the TNM classification defined 50 years ago are still valid, but need to be updated or rephrased in order to adapt to medical development. Another problem for discussion is that the TNM staging sys-

tem is based on an anatomic model, while nowadays more and more scientists talk about the "biological determinism" of this disease. Therefore, the question we need to answer would be: "Is the TNM system reliable?". While some researchers consider that the TNM staging system should be abandoned and prognosis should be based on the biology of the disease, others consider that other prognostic markers should be used in addition, but not instead of TNM (Mason, 2006). In spite of its debatable reliability, the TNM staging system is still widely used for the prognosis of this disease.

On the other hand, tumor-induced angiogenesis is considered to have an essential role in the evolution of breast cancer. The central importance of tumor neovascularization has been emphasized by clinical trials using antiangiogenic therapy in breast cancer (Fox et al., 2007). Angiogenesis, the generation of new blood vessels from the existing vasculature, consists of multiple coordinated, sequential and interdependent steps (Fox et al., 2007), controlled by various positive and negative regulatory signals (Wülfing et al., 2005). The process of neovascularization is driven by growth factors released into the stroma by tumor cells and immune cells (Pavlakis et al., 2008).

Most human tumors arise in the absence of angiogenic activity and exist in a dormant state for months to years without neovascularization (Naumov et al., 2006). In this "prevascular" phase, tumors are usually thin and the cell population is limited (Folkman, 1990). They are microscopic and asymptomatic cancerous lesions which remain occult for prolonged periods of time (Almog et al., 2009). The absence of angiogenesis in the preneovascular phase precludes expansion of the tumor population regardless of the proliferative capacity of the tumor cells (Folkman, 1990).

Angiogenesis is known to be a prerequisite for tumor growth beyond a few (1–3) mm^3 in size (Wülfing et al., 2005), the nonangiogenic tumors being invisible until the angiogenic switch (Naumov et al., 2006). In order to initiate neovascularization, a tumor must switch to an angiogenic phenotype (Fox et al., 2007).

This switch from the prevascular to vascular phase is accompanied by exponential growth of the tumor (Mousa, 2000). As the tumor continues to progress, so does the degree of neovascularization. The new vessels not only help to meet the growing metabolic demands of the tumor by supplying additional nutrients, but also provide potential routes for tumor dissemination and metastasis (Boudreau et al., 2003).

It has been proven that VEGF and its receptors are essential for neovascularization in cancer (Bando et al., 2005) and VEGF plays a relevant biological role in the progression of breast cancer (Gasparini, 2000). VEGF is a potent and selective endothelial mitogen that can induce a rapid and complete angiogenic response (Boudreau et al., 2003) and is likely an important tumor angiogenic factor (Weidner, 1995). The expression of angiogenic factors, such as VEGF, have been associated with a worse prognosis in patients with invasive breast disease (Relf et al., 1997).

Studies have revealed significantly higher VEGF concentrations in the plasma of women with breast cancer relative to levels of the same cytokine in a control group (Thielemann et al., 2008).

The high VEGF expression was significantly associated with the metastases in the regional lymph nodes, which indicates that VEGF expression is associated with tumor progression, spread and poor prognosis, probably by stimulating angiogenesis, macrophage infiltration and remodeling of tumor tissue (Valković et al., 2002). In addition, VEGF levels have been found to be of prognostic value in heterogeneous patient populations as well as in node-positive and node-negative subgroups, regardless of the type of adjuvant therapy administered (Gasparini, 2000).

VEGF can also sustain breast carcinoma survival independently of angiogenesis by stimulating autocrine survival-signaling in these cells (Bachelder et al., 2002). It has been proven that it stimulates the

growth of breast cancer, through angiogenesis rather than through a direct contribution to tumor cell survival (Lee et al., 2007).

Along with the size of the tumor, increased plasma VEGF levels are important for women with breast cancer (Thielemann et al., 2008). In spite of all the evidence of the relationship between the TNM system and VEGF expression, some authors maintain the idea that VEGF is not correlated with the conventional prognostic factors (Gasparini, 2000).

Microvessel density assessment is the most commonly used technique to quantify intratumoral angiogenesis in breast cancer (Uzzan et al., 2004). Evaluation of cancerous tissue in women suffering from breast cancer revealed an increased proportion of MVD values when compared to the proportion of vessels in control tissues (Thielemann et al., 2008), MVD being a powerful and often independent prognostic indicator in breast cancer (Sainson, 2008).

An increase in MVD in breast carcinoma has been shown to correlate with malignant and metastatic potential and hence with a poor prognosis (Mousa, 2000). An overwhelming majority of published reports have shown a significant correlation between the density of intratumoral microvessels in invasive breast carcinoma and the incidence of metastases and/or patient survival (Mousa, 2000).

It has been shown that women with metastases to the axillary lymph nodes had increased numbers of blood vessels compared with MVD levels in health breast tissue. However, there was no statistical relationship between the tumor vessel densities of women with metastases to the lymph nodes and those without (Thielemann et al., 2008). Another opinion is that MVD, as a marker of angiogenesis, does indeed predict poor survival in women with invasive breast cancer, especially in node-negative patients (Uzzan et al., 2004).

PECAM-1 (platelet endothelial cell adhesion molecule-1 or CD31), a member of the cell adhesion molecule family of surface immunoglobulin glycoproteins, is involved in cell–cell interactions during growth and is thought to play an important role in embryogenesis and development. Recent studies have concluded that PECAM-1 is involved during tumor angiogenesis and that vascular endothelial PECAM-1 may be a useful marker in assessing angiogenesis (Cao et al., 2002).

There is still much to learn, with the full complexity of the mechanisms of tumor neovascularization and their regulators still to be defined, not only in individual tumor types but also in individual patients. Thus, more information in terms of biomarkers that are predictive of response is required so that tailored treatment can be offered (Fox et al., 2007).

Quantitation of VEGF and the tumor vasculature in breast cancer seem to play an important role in predicting tumor behavior and patient management. The aim of our study was to evaluate the potential correlation between the VEGF expression, MVD in the tumor tissues and the TNM staging in breast cancer.

MATERIALS AND METHODS

Clinicopathologic data

The study includes 34 patients suffering from breast carcinoma, 32 female and 2 male with an average age of 56.32 years, who underwent surgical treatment as the first therapeutical option, between May 2005 and April 2006. Pathologic staging depending on tumor size and lymph node status was performed according to the American Joint Cancer Committee (AJCC) TNM Staging 2002.

The histopathological interpretation supposed the fixation of pieces in 4% formalin solution, inclusion on paraffin, sectioning at microtome at 3-5 μm thickness and a staining with hematoxylin-eosin.

Histologically, tumors were graded as I–III (Scarff-Bloom-Richardson grading) based on tubule formation, nuclear grade and mitotic count. The

presence of inflammation, necrosis, peritumoral, perineural or fat tissue invasion, were recorded.

Immunohistochemical analysis

Additional slides from the primary tumors were processed for immunohistochemical identification of VEGF expression with anti-VEGF antibody (VG-1 clone, ready-to-use, 30 min incubation time at room temperature, Labvision / Neomarkers, Fremont, CA, USA) and microvascular endothelial cells with anti-CD31 (clone JC/70A, prediluted, Dako, Denmark).

Before staining, tissue sections were dewaxed in xylem and rehydrated in a graded ethanol series. PT link modules (DakoCytomation, Denmark) were used for heat-induced epitope retrieval in citrate buffer pH 9 for 15 min (for VEGF) and citrate buffer pH 6 for 30 min (for CD31). The immunohistochemical technique used was based on the avidin-biotin method using LSAB+ working system, which followed incubation with the primary antibodies (for VEGF and for CD31). The chromogen was 3,3 diaminobenzidine. Staining of nuclei was performed with modified Lille's hematoxylin. The entire immunohistochemical procedure was performed with a DakoCytomation Autostainer. Evaluation of VEGF expression and MVD was performed using Eclipse 80i Nikon microscope and the images were captured and processed with Lucia G software system.

The local research ethics committee approved the protocol of the study, and informed consent was obtained from all subjects according to the World Medical Association Declaration of Helsinki.

VEGF immunoreactivity was obtained by calculating the average percent of cytoplasmic positive cells in 3 high power fields, and the grading consisted of 4 levels: (-): less than 10%; (1+): 11%–20%; (2+): 21–50%; (3+): more than 50%.

The areas of the highest neovascularization (hot spots) were found in each tumor by scanning the section at low power, and then individual microvessels were counted on high power fields. MVD was obtained as the average number of vessels in 3 high power fields. Any endothelial cell or endothelial cell cluster positive for CD31 and separate from an adjacent cluster was considered a single countable microvessel.

Statistical analysis

Statistical analysis was performed with SPSS13.0 soft, and included Pearson's Correlation test, $p<0.05$ being considered as significant.

RESULTS

Ductal invasive carcinoma was diagnosed in 30 cases, lobular invasive carcinoma was found in 2 cases and papillary carcinoma in 2 cases. Associated ductal *in situ* carcinoma (DCIS) was identified in 18 cases. Three cases were well differentiated (G1), 16 cases were moderately differentiated (G2), and 15 were undifferentiated (G3). Peritumoral invasion of the mammary tissue was observed in 14 cases, perineural invasion in 17 cases and fat tissue invasion in 24 cases. Calcification of the tumor lesions was noticed in 10 cases, tumor necrosis in 17 cases and inflammation in 29 cases.

We also evaluated the tumor lesions according to the TNM staging system. Taking into account the tumor dimensions, 12 cases were staged as T1c, 15 as T2, 2 as T3, 2 as T4a and 3 as T4b. After having evaluated an average of 11 regional lymph nodes in each patient, we staged the patients as follows: pN0 – 11 cases; pN1a – 19 cases; pN2a – 5 cases; pN3a – 5 cases; pNx – 4 cases (Table 1).

VEGF was positive in 30 cases (88.23%), in the cytoplasm of tumor cells with granular pattern (Fig. 1). Using the VEGF scoring described above, we found 18 cases with a +3 pattern, 7 cases with +2, and other 5 cases with +1. The intensity of staining was heterogeneous, but most of the cases showed moderate to intense expression of VEGF. Expression of VEGF was significantly associated with the number of lymph nodes with metastasis (p=0.021),

Table 1. Patients' distribution depending on age, sex, histological grading, T stage, N stage and VEGF expression. The majority of the patients were over 46 years, female, in the histological grading classes II and III, T1-T2, N-positive and with a +3 VEGF expression.

	Cases	**VEGF expression**			
		0	1	2	3
	n= 34	n=4	n=5	n=7	n=18
Age					
>46	24	4	3	6	11
<46	10	0	2	1	7
Sex					
Masculie	2	0	0	1	1
Feminie	32	4	5	6	17
Histological grading					
I	3	0	2	1	0
II	16	2	1	5	8
III	15	2	2	1	10
T stage					
T1-T2	27	1	4	7	15
T3-T4	7	3	1	0	3
N stage					
Nx	4	1	1	1	1
N0	11	1	2	2	6
pN1a-pN3a	19	2	2	4	11

the number of cells in mitosis (p=0.027), the presence of necrosis (p=0.036) and the T-stage (p=0.012, inverse correlation).

The microvessel density (MVD) was appreciated according to the hot-spot method, by choosing three fields of maximum vascular density from the tumor area (Fig. 1). The evaluation of MVD was made according to the above-described methodology, on sections immunostained with CD31. Only the structures with lumen presenting a positive reaction at endothelial level were taken into consideration. MVD correlated very well with the histological grading (p=0.019), the number of cells in mitosis (p=0.025), the presence of inflammation (p=0.024) and the presence of necrosis (p=0.024).

No correlation was observed between VEGF expression and MVD, age, histological type, presence of lymph node metastasis, peritumoral, perineural invasion or invasion of the adipose tissue (p>0.05). Interestingly, we obtained an inverted correlation between VEGF expression and the histological grading in the N-negative patients (p=0.0073).

MVD did not correlate with age in the general population, but it presented a significant correlation in the N-negative patients (p=0.035). In addition, there was no correlation between MVD and T, N, number of metastasized axilar nodules, peritumoral, perineural or fat tissue invasion.

DISCUSSION

It has been more than 35 years since Judah Folkman suggested that the tumor vasculature would be a target for anticancer therapy. In the interim there has been a huge increase in our understanding of the biology underlying tumor angiogenesis (Fox et al., 2007), so that in the past 20-30 years, new clinically relevant variables have emerged to integrate traditional anatomopathologic factors for the prognosis and treatment of breast cancer (De Paola et al., 2002).

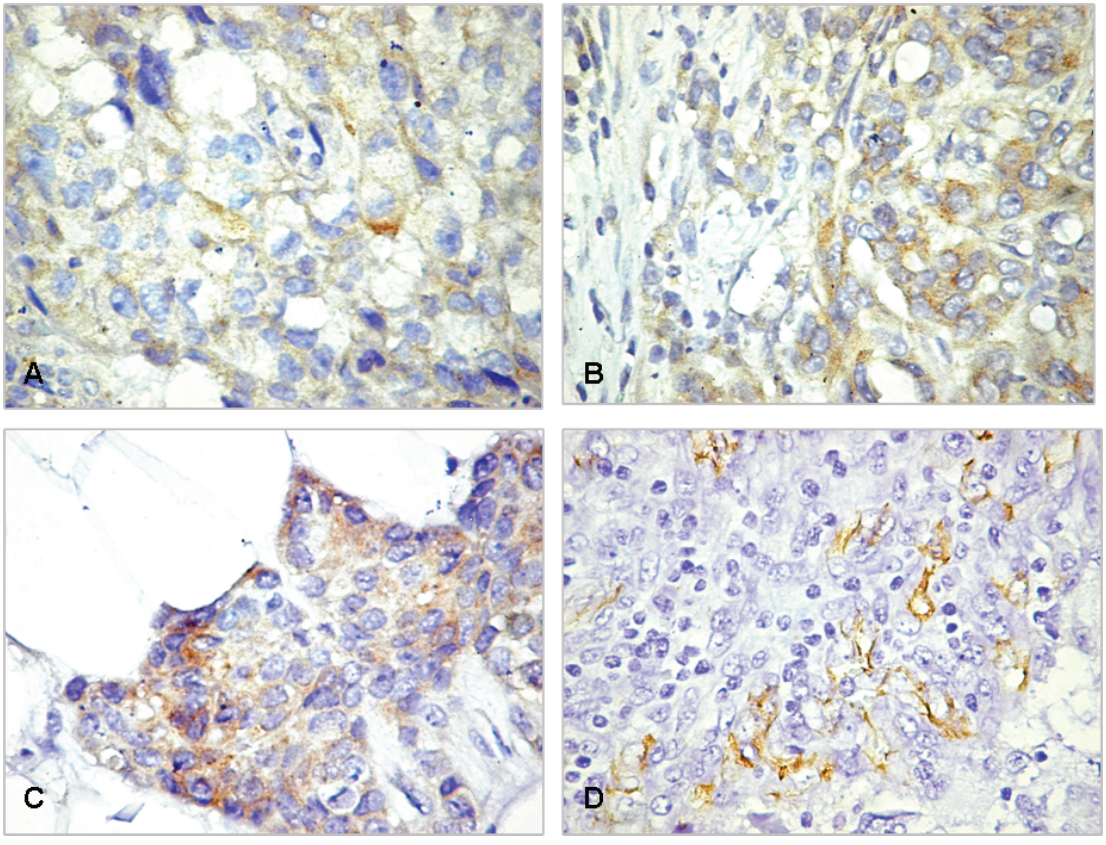

Fig. 1. Immunohistochemical expression of VEGF and CD31 in breast cancer specimens. A. VEGF expression 1+; B. VEGF expression 2+; C. VEGF expression 3+; D. CD31 expression.

On the other hand, it has been reported that the capacity of tumor cells to induce angiogenesis does not always correlate with malignancy, proving that the onset of angiogenic activity in tumor cells is an independent event that may be expressed at very different times during neoplasia (Folkman, 1990).

Most of the DCIS cases showed significant expression of various angiogenic growth factors, indicating that *in situ* carcinomas are capable of inducing angiogenesis (Wülfing et al., 2005). As the disease stage of cancer seems to be a late event in tumor development (Almog et al., 2009), it would be important to determine these angiogenic markers in pure DCIS in order to facilitate discrimination of a more angiogenic subset with a potentially higher risk of progression (Wülfing et al., 2005).

Primary breast cancers have proved to express multiple angiogenic factors (Relf et al., 1997) and, among them, VEGF represents a biologic marker of breast tumor angiogenesis (Manders et al., 2002). Although some authors maintain that VEGF expression is not a prognostic marker either in terms of disease-free or overall survival (De Paola et al., 2002), many others report a significant relationship between the clinical stage of disease advancement and VEGF expression (Gasparini, 2000; Relf et al., 1997; Thielemann et al., 2008). From the data currently available on the role of VEGF expression in breast cancer, it must be concluded that results are contradictory and still inconclusive (De Paola et al., 2002). In addition to VEGF expression, MVD also has roles in the promotion of the process of angiogenesis within the limits of malignant tumors (Thielemann et al., 2008).

A meta-analysis found a statistically significant inverse relationship between angiogenesis, assessed by MVD, and survival, confirming that human invasive breast cancer is an angiogenesis-dependent malignancy (Uzzan et al., 2004).

We demonstrated in our study that early T-stage tumors overexpress VEGF, compared to late stage-tumors, in which VEGF expression is decreased, but we could not find any correlation between MVD and the T-stage. On the other hand, Adams et al. (2000) and Gasparini et al. (1994) reported that MVD presents a significant association with tumor size, while Thielemann et al. (2008) showed statistical relationships between VEGF concentration in the plasma and the size of the primary tumor and also between MVD and the stage of clinical disease advancement and tumor size. In addition, Linderholm et al. (2008) say that high VEGF expression was significantly associated with larger tumor size, while Manders et al. (2002) conclude that VEGF levels were found to be positively associated with tumor size in node-negative patients with primary breast cancer. Interestingly, Valković et al. (2002) reported no significant correlation between tumor size and VEGF or MVD in invasive ductal breast carcinoma, suggesting that the biological phenomena of neovascularization, tumor proliferation and growth are not permanently mutually dependent processes. As a conclusion for this debate, Gasparini et al. (1994) suggest that the association of intratumoral VEGF with other clinical prognostic factors is unresolved.

In our study, VEGF expression did not correlate with the lymph node status, but, interestingly, it correlated with the number of metastasized lymph nodes, while MVD presented no correlation with either the lymph node status, or the number of metastasized lymph nodes. Valković et al. (2002) proved that VEGF expression and MVD were significantly associated with the lymph node involvement in invasive ductal breast carcinoma. Thielemann et al. (2008) discovered, in their study, an almost threefold increase in VEGF concentration in the plasma of women with breast cancer, which showed involvement of the axillary lymph nodes, compared to levels in women without metastases, thereby demonstrating the prognostic value for the assessment of this marker. However, no statistical relationship was found between the tumor vessel densities of women with metastases to the lymph nodes and those without (Thielemann et al., 2008).

Interestingly, we obtained an inverted correlation between VEGF expression and the tumor grade in N-negative patients. In addition, we proved that both VEGF expression and MVD are indicators for the proportion of cells in mitosis. Our results are in agreement with Adams et al. (2000) and Gasparini et al. (1994) who also demonstrated that VEGF expression was inversely correlated with tumor grade. Interestingly, Thielemann et al. (2008) obtained a totally different result, showing a statistical relationship between VEGF concentration in the plasma and the histological grade of malignancy. Linderholm et al. (2008) had a comparable experience, concluding that high VEGF expression was significantly associated with histological grade III. Similar to the results of Valković et al. (2002) who proved a significant increase in the number of small blood vessels in grade III invasive ductal breast carcinoma, we demonstrated that MVD correlated with the histological grade (Valković et al., 2002). Gasparini et al. (1994) had similar results, suggesting that intratumoral MVD correlated with tumor grade.

In our work, we found a correlation between MVD and age but only in the case of node-negative patients, but VEGF expression did not correlate with age at all. Gasparini et al. (1994) also reported that intratumoral MVD correlated with age in the node-negative patients, while Linderholm et al. (2008) stated that VEGF expression was not significantly associated with age.

We observed that VEGF overexpression and high MVD were associated with tumor necrosis. Our results are in agreement with Brown et al. (1993) who demonstrated that VEGF expression was accentuated in tumor cells close to areas of necrosis. According to Du et al. (2003), this may be explained by hypoxia, which can stimulate VEGF expression and its bio-

logical activity. In addition, Leek et al. (1999) suggest that an association was noted between higher vascular density and increasing necrosis.

The relationship between VEGF expression and MVD represents a subject of debate. Adams et al. (2000) agree with our results, concluding that no significant correlation between VEGF staining intensity and Chalkley count was seen. On the contrary, Valković et al. (2002) demonstrated a clear positive correlation between parenchymal VEGF expression and MVD in invasive ductal breast carcinoma, while Pavlakis et al. (2008) obtained the same results, but in hyperplastic and pre-invasive breast lesions.

CONCLUSIONS

Although their relationship with the TNM staging remains unclear, VEGF expression and MVD proved to be important indicators of the malignant status in breast cancer, confirming the major involvement of angiogenesis in this type of cancer.

VEGF expression and MVD possess an undoubtable prognostic value, but it seems they are not entirely independent of the TNM staging system, suggesting that "biological determinism" represents a tool for improving the "anatomic model" in establishing the evolution and appropriate therapy in breast cancer.

On the other hand, VEGF expression and MVD are valuable markers of the breast tumors' angiogenic profile; however, the pattern of their relationship needs further analysis of the VEGF receptors in order to be fittingly described.

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