

Endothelin-1-, Endothelin-A-, and Endothelin-B-Receptor Expression Is Correlated with Vascular Endothelial Growth Factor Expression and Angiogenesis in Breast Cancer

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ABSTRACT

Purpose: Endothelin-1 (ET-1) and its receptors (ET_AR and ET_BR), referred to as the endothelin (ET) axis, are overexpressed in breast carcinomas, and influence tumorigenesis and tumor progression by various mechanisms, including angiogenesis. The objective of the study was to clarify if expression of the ET axis participates in angiogenesis of breast carcinoma

Experimental Design: We analyzed expression of ET-1, ET_AR, ET_BR, and vascular endothelial growth factor (VEGF) immunohistochemically in 600 tissue array specimens from 200 paraffin-embedded breast carcinomas performing tissue microarray technology. Microvessel density (MVD) was determined by counting microvessels (identified by factor VIII) in each core specimen.

Results: Moderate or strong immunostaining was observed for ET-1 in 25.4%, for ET_AR in 43.7%, and for ET_BR in 22.2% of breast carcinomas. Of all cases, 44.7% showed significant expression of VEGF. MVD varied between different tumor specimens (range, 0–80; median, 17). We observed a statistically significant correlation between MVD and ET expression status with higher MVD in ET-positive tumors. Moreover, expression of VEGF was found more frequently in tumors with overexpression of the ET axis (each $P < 0.001$). Staining of VEGF was correlated positively with MVD

Conclusions: These results indicate that increased ET-1, ET_AR, and ET_BR expression is associated with increased VEGF expression and higher vascularity of breast

carcinomas and, thus, could be involved in the regulation of angiogenesis in breast cancer. Our findings provide evidence that the expression pattern of the ET-axis and in particular of ET_AR may have clinical relevance in future antiangiogenic targeted therapies for breast cancer.

INTRODUCTION

Angiogenesis is a multistep process that is regulated by a combination of paracrine, autocrine, and localized environmental stimuli. This regulation involves endothelial cell activation, migration, and proliferation, as well as the extracellular matrix and cells of the target tissue (1). Because angiogenesis is necessary for tumor growth and metastasis, antiangiogenic strategies for treatment of cancer are currently a focus of major scientific interest.

Several studies have shown that the angiogenic potential of breast carcinomas as assessed by tumor microvessel density (MVD) correlates with tumor progression and metastasis, and, thus, predicts a poor clinical outcome in breast cancer patients (2–4). Expression of vascular endothelial growth factor (VEGF), a multifunctional glycoprotein, which is inducible by hypoxia, growth factors, and oncogenes, has shown significant correlations with MVD (5, 6). VEGF and its receptors are considered to be one of the most crucial regulatory pathways in angiogenesis, because they act as specific growth factors for endothelial cells inducing their proliferation and migration (7, 8). Most studies on VEGF in breast cancer patients reported that VEGF expression in breast cancer tissues is positively associated with relapse-free survival, overall survival, or both (reviewed in Ref. 9).

Endothelin (ET)-1, a vasoactive peptide, is produced primarily in endothelial, vascular smooth muscle, and epithelial cells (10, 11). Increased ET-1 expression has been demonstrated in several human malignancies including ovarian, prostate, and colorectal cancer (12–15). Conditions of stress such as hypoxia lead, in combination with growth factors and cytokines, to an enhanced production of various angiogenic mediators such as ET-1 (16, 17). ET-1 effects are mediated through two distinct subtypes of G protein-coupled receptors, ET_AR and ET_BR, which are expressed in several tissues (12, 18). Recent studies have suggested that ET receptor activation promotes tumorigenesis and tumor progression by various mechanisms, including proliferation (19), invasion (20), angiogenesis (17, 21, 22), and inhibition of apoptosis (23). ET-1 contributes to the process of angiogenesis, stimulating endothelial cell growth predominantly through ET_BR and inducing vascular smooth muscle cell and pericyte mitogenesis mediated through ET_AR (21, 24). The hypothesized modulating effect of ET-1 on angiogenesis is supported by the finding of ET-1 expression in ovarian carcinoma, which correlates significantly with neovascularization and VEGF expression (21). As for ovarian carcinoma, in colo-

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rectal cancer (25) and brain tumors (26), tumor vascularity correlates with ET-1 expression. Additional studies have suggested that activation of ET_AR by ET-1 stimulates the production of VEGF, which in turn stimulates tumor growth and angiogenesis by increasing the levels of hypoxia-inducible factor-1 in a time- and dose-dependent manner (17).

There is also evidence for involvement of the ET axis in blood perfusion of breast tumors, because in a rodent model blood flow to the tumor tissue increased significantly in response to ET-1 mediated through ET_BR (27). Another study reported a colocalization of ET expression with areas of hypoxia in a murine breast cancer model, HTH-K, and, *in vitro*, an up-regulation of ET-2, ET_AR, and ET_BR mRNA induced by hypoxia (28).

We have demonstrated recently an increased ET-1, ET_AR, and ET_BR expression in breast carcinomas. Overexpression of ET-1, ET_BR, and especially of ET_AR correlated with aggressive types of breast cancer and with poor clinical outcome, indicating that ET expression may contribute to disease progression (29). Because angiogenesis of breast carcinomas as assessed by MVD, and VEGF expression is also associated with tumor progression, metastasis, and reduced survival (2–4, 9), the objective of the present study was to assess whether ET expression is related to angiogenesis in breast cancer. Therefore, this study examined for the first time both the angiogenic markers, MVD and VEGF, and the expression of ET-1, ET_AR, and ET_BR in corresponding areas of breast carcinomas using a tissue microarray (TMA) with 600 core biopsies of 200 breast cancer specimens.

MATERIALS AND METHODS

Patients. This study was conducted on a series of 600 tumor samples from 200 patients with invasive breast cancer diagnosed between 1993 and 1995 at the Department of Gynecology, University of Münster (Münster, Germany). A database comprising detailed clinical data regarding diagnosis and histopathological variables was created. Routinely fixed paraffin-embedded tumor samples of patients were obtained from the archives of the Gerhard-Domagk-Institute of Pathology, University of Münster. Among them were 108 (54%) ductal invasive, 46 (23%) lobular, 13 (6.5%) tubular, 4 (2%) mucinous, 4 (2%) medullary, and 25 (12.5%) of mixed histological differentiation. Tissue specimens were classified according to the Tumor-Node-Metastasis classification of the International Union Against Cancer, and tumor grade was assigned based on the criteria of Elston and Ellis (30). Table 1 summarizes the distribution of Tumor-Node-Metastasis stages and histological grade. Mean tumor size, which was recorded in the initial pathology description, ranged from 3 mm to 190 mm (median, 20 mm; mean, 27.4 mm; SD, 23.9 mm).

Breast Cancer TMA. For each of the 200 cases we selected a representative tumor block as donor block for the TMA. Using an H&E-stained slide, three morphologically representative regions were defined for each of the 200 tumor samples. We acquired from these regions cylindrical core tissue specimens (diameter = 0.6 mm) and arrayed them precisely into a new recipient paraffin block (20 × 35 mm) using a custom-built precision instrument (Beecher Instruments, Silver Spring,

Table 1 Distribution of TNM^a stages and histological grade in the reported series of breast cancer patients (n = 200)

Clinicopathological parameter	n (%)
Tumor stage ^b	
pT1	84 (42.4%)
pT2	61 (30.8%)
pT3	15 (7.6%)
pT4	38 (19.2%)
Lymph nodes ^b	
pN0	105 (54.7%)
pN1	72 (37.5%)
pN2	14 (7.3%)
pN3	1 (0.5%)
Metastases at diagnosis	
No	176 (88%)
Yes	24 (12%)
Histological grade	
G1	20 (10%)
G2	108 (54%)
G3	72 (36%)

^a TNM, Tumor-Node-Metastasis.

^b Information on pT stage was available in 198 of 200 (99%), on pN stage in 192 of 200 (96.5%) patients.

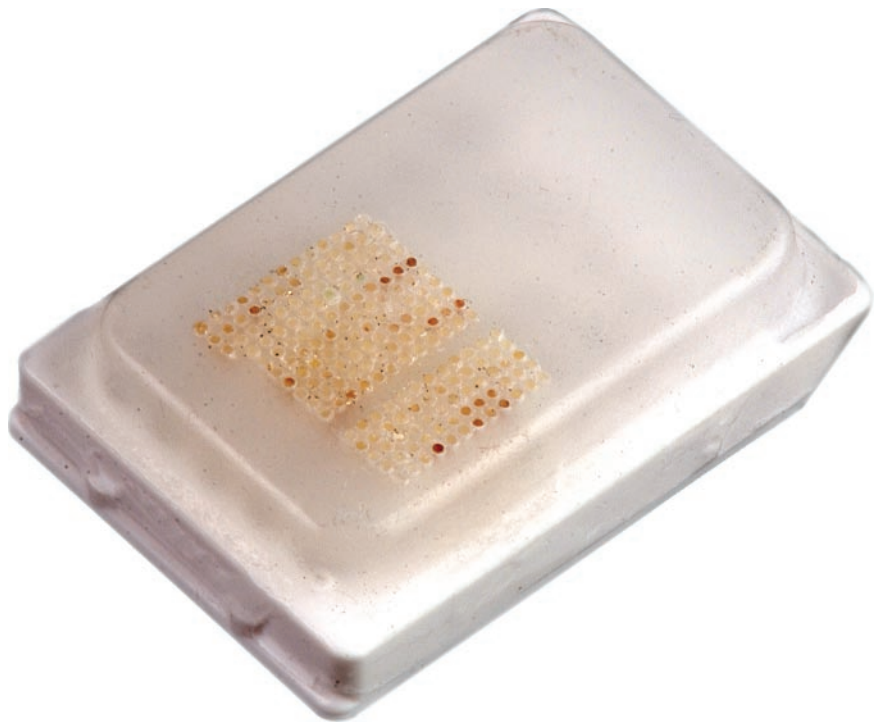
MD). From the 600 tumor samples available, four tissue array blocks were prepared, each containing 72–180 tumor sample cores (Fig. 1).

Immunohistochemistry for ET-1, ET_AR, and ET_BR.

Consecutive sections of 2–3 μm were cut from the TMAs and processed for immunohistochemistry. Before ET-1 staining, specimens were subjected to heat-induced antigen retrieval in a steamer (Type 3216, Braun, Kronberg, Germany). Immunohistochemical staining for ET-1, ET_AR, and ET_BR was performed in a multistep semiautomatic procedure (Ventana NexES automated immunohistochemistry system) as described previously (29). For ET-1 a monoclonal mouse antibody at a 1:500 dilution (25 min), and for ET_AR and ET_BR sheep polyclonal antibodies at a 1:100 dilution (30 min) were used (Alexis Biochemicals Corporation, Lausen, Switzerland). Ovarian cancer tissue served as positive control for ET-1 staining, prostate cancer tissue for ET_AR, and smooth muscle tissue for ET_BR. Specificity of the antibodies was confirmed using omission of the primary antibodies and replacement of the primary antibodies by IgG of the respective species as negative controls. Immunohistochemical staining was independently scored by two investigators from 600 array cores. According to the literature (21), cytoplasmic staining intensity was scored semiquantitatively into different grades on an arbitrary four-tiered scale of 0 to 3+. The following scoring criteria were agreed upon before the analysis: grade “0,” no detectable immunostaining of tumor cells; “1+,” weak staining of the majority of tumor cells; “2+,” moderate staining intensity of tumor cells; and “3+,” strong staining intensity of tumor cells. We defined tumor samples with a moderate (2+) or strong (3+) cytoplasmic immunostaining intensity to have an elevated ET-1, ET_AR, or ET_BR expression and thus to be “positive” (Fig. 2).

Immunohistochemistry for VEGF, Factor VIII, and CD31. Antibodies used were polyclonal rabbit antibodies directed against VEGF (Ab-1; Oncogene, San Diego, CA) and factor VIII (FVIII), and mouse monoclonal anti-CD31 (both,

Fig. 1 Tissue microarray block.



Dako Cytomation, Glostrup, Denmark). Tissue sections from the TMA blocks were dewaxed and rehydrated. For immunolabeling, an avidin-biotin-phosphatase technique (Dako ChemMate Detection kit) was used. Antigen retrieval for VEGF and CD31 was performed by hot water steaming in citrate buffer (pH 6; 30 min). Before staining for FVIII, slides were pretreated with Proteinase K (Dako; 10 min). VEGF antibody was applied at a dilution of 1:15, FVIII at a dilution of 1:1000, and CD31 at a dilution of 1:400. After incubation with primary antibodies for 25 min at room temperature, secondary antibodies and streptavidin complex were applied for 20 min each (Dako TechMate). Development of immunostaining was achieved with modified new fuchsin, and finally sections were counterstained with hematoxylin. Kidney tissue was used as a positive control for VEGF, inflammatory tissue for FVIII, and tissue from a hemangioma for CD31. Omission of the primary antibodies and replacement of the primary antibodies by IgG of the respective species served as negative controls.

Expression of VEGF was characterized as a negative or positive reaction according to both the intensity of the immunostaining and the percentage of stained tumor cells. Samples were judged to be VEGF-positive if >10% of the tumor cells showed moderate or strong cytoplasmic immunoreaction (Fig. 2; Ref. 31). Due to the TMA technique, which naturally focuses on morphologically representative preselected tumor areas, MVD was defined as the number of microvessels identified within an array core containing tumor tissue (0.6-mm diameter). Microvessels were visualized by staining endothelial cells for FVIII and counted by light microscopy at a $\times 200$ magnification (Fig. 2). Any stained endothelial cell or endothelial cell cluster clearly separated from adjacent vessels or other stromal ele-

ments was considered a single countable microvessel. The presence of a vessel lumen was not necessary for an element to be defined as a microvessel (32). MVD was investigated by one observer (C. K.). Each core was counted twice, and the mean value of these two counts was used for additional analysis. To confirm findings on MVD as defined by FVIII immunolabeling, we also assessed MVD by CD31 immunostaining and compared both results.

Data Analysis. Staining intensity was evaluated semi-quantitatively in a blind fashion. For statistical analysis SPSS for Windows (Version 10.0) was used. Correlations between MVD or VEGF and ET expression were analyzed by Kruskal-Wallis and Mann-Whitney test, and by cross-tables applying χ^2 and Fisher's exact test. Correlations between the three different samples from identical tumors were tested to investigate variance of expression. Also, associations between expression of different factors within the same tissue samples were examined. $P < 0.05$ was considered statistically significant.

RESULTS

Immunohistochemistry for ET-1, ET_AR, and ET_BR.

Immunostaining for ET-1, ET_AR and ET_BR could be evaluated in 472 (78.7%), 481 (80.2%), and 478 (79.7%) from 600 tissue array cores, respectively, because several core samples were either detached or did not contain a sufficient number of tumor cells. Because we wanted to assess the differences or correlations among expression of ET, VEGF, and FVIII in corresponding areas of breast carcinomas as defined by single core specimens, each of the three samples from every tumor on the TMA was scored separately. Immunolabeling for ET-1, ET_AR, and

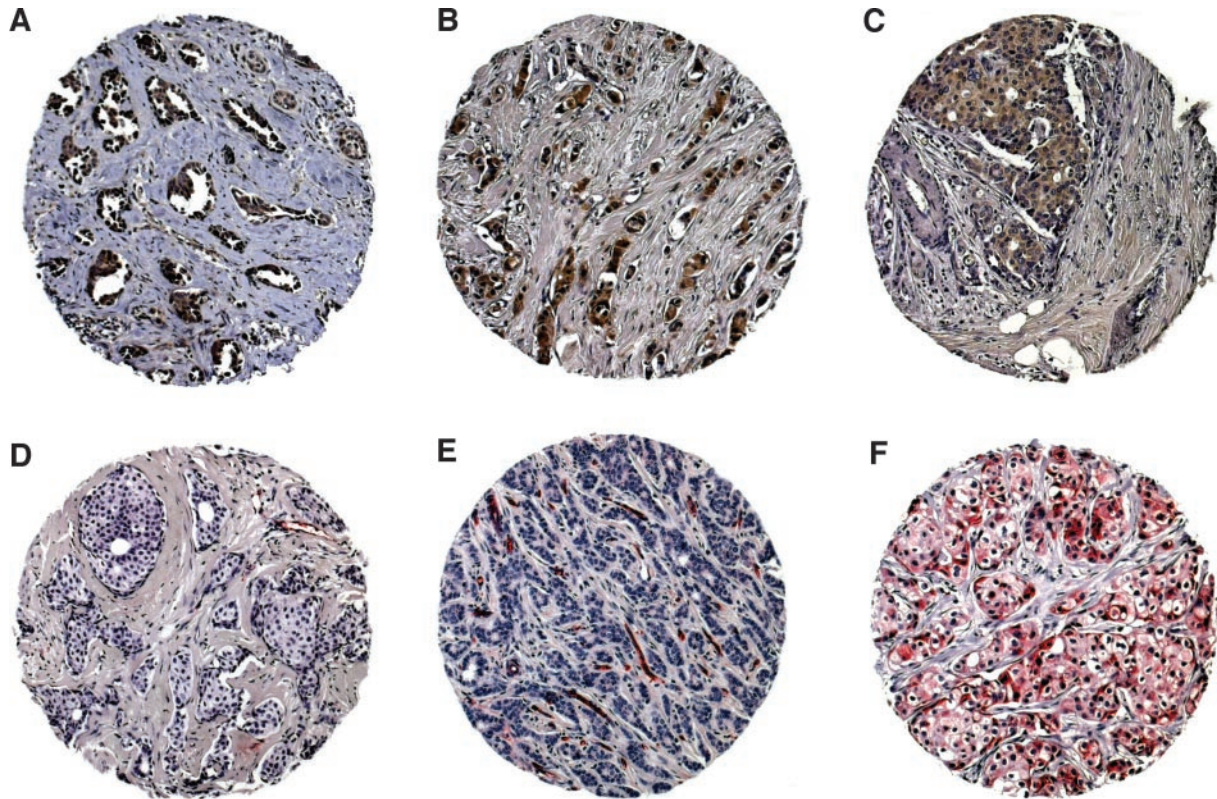


Fig. 2 A–F, representative examples of breast carcinoma tissue array specimens with strong immunohistochemical expression (3+) of endothelin (ET) 1 (A), ET receptor A (B), and vascular endothelial growth factor (F) and moderate expression (2+) of ET receptor B (C). Breast tissue samples with low (D) and high (E) microvessel density as assessed by staining endothelial cells for factor VIII related antigen.

ET_BR presented as homogeneous cytoplasmic staining of epithelial tumor cells.

Staining intensity of ET-1, ET_AR, and ET_BR among different tumors varied from complete absence of staining to strong staining. Moderate or strong immunoreaction defined as “posi-

tive” staining (Fig. 2) was observed for ET-1 in 120 of 472 (25.4%), for ET_AR in 210 of 481 (43.7%), and for ET_BR in 106 of 478 (22.2%) evaluable breast carcinoma samples. Fig. 3 provides detailed information on gradual assessment of ET staining. Overall, we observed a statistically significant corre-

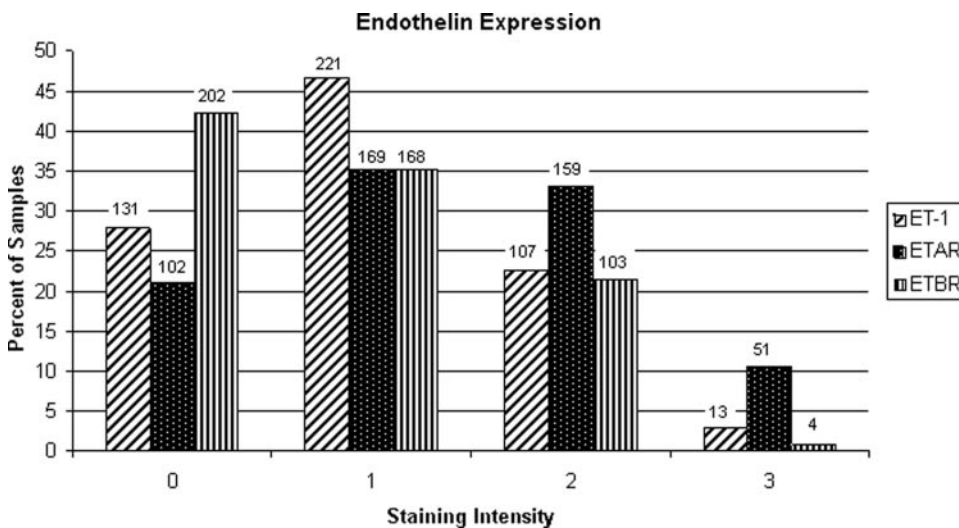


Fig. 3 Expression of endothelin (ET) 1, ET receptor A (ET_AR), and ET receptor B (ET_BR) as graded for staining intensity. Scoring criteria: grade “0” = no detectable immunostaining of tumor cells; “1” = weak staining of the majority of tumor cells; “2” = moderate staining intensity of tumor cells; “3” = strong staining intensity of tumor cells. Numbers above columns represent the absolute numbers of core samples. Total numbers of core specimen that were evaluable for ET-1 staining were n = 472; for ET_AR, n = 481; and for ET_BR, n = 477.

lation of ET expression within different samples from identical tumors ($P < 0.001$ for each factor). For ET-1, 90.5% of cases showed no discordance between ET scoring within the three core samples per case. This was also observed for ET_AR (82.6% of cases) and for ET_BR (81.1% of cases). Comparing serial sections of single array cores a close concordance between expression of ET-1 and ET_AR, between ET_AR and ET_BR (for each correlation, $P < 0.001$), and between ET-1 and ET_BR ($P = 0.012$) was found.

VEGF Immunohistochemistry and MVD. VEGF expression could be analyzed in 479 of 600 cases (79.8%) of the breast cancer TMA. Among these, 214 (44.7%) cases showed positive VEGF immunostaining (Fig. 2). VEGF immunolabeling was heterogeneous across different tumors, ranging from very intense to pale staining in various numbers of tumor cells per core. We did not observe any staining of adjacent normal breast tissue, and no stromal staining was seen. MVD as defined by FVIII immunostaining was evaluable in 421 of 600 core samples (70.2%). The median number of small blood vessels per core specimen was 17 (range, 0–80). Expression of VEGF correlated positively with MVD ($P = 0.028$). Staining of the TMA with CD31 showed comparable results for MVD, with a positive correlation (>90%) between FVIII and CD31 (Fig. 4). Because of this close concordance and our experience of good reproducibility of FVIII staining results, we have used the MVD data based on FVIII-immunostaining for all of the correlations between MVD and the ET-axis or other tumorbiological factors.

Correlation of VEGF and MVD with Expression of ET Axis. Table 2 shows the correlations and P s between expression of ET-1, ET_AR, and ET_BR on the one hand and VEGF on the other. ET-1, ET_AR, and ET_BR expression correlated significantly with the VEGF status. All of the factors of the ET axis were also positively correlated with MVD (Kruskal-Wallis test). Table 3 lists the corresponding P s. When MVD per core was classified into three groups, (1) 0–8 vessels per core, (2) 9–16 vessels per core, and (3) ≥ 17 vessels per core, we observed that with respect to ET receptor status the most obvious differences in MVD were observed between group 1 and 2 (Table 4). Differences in MVD depending on ET status were statistically significant (ET-1, $P = 0.008$; ET_AR, $P = 0.012$; and ET_BR, $P = 0.006$).

As to correlations between possible autocrine loops in the ET axis and VEGF expression or MVD, tumors with ET-1/ET_AR and ET-1/ET_BR coexpression showed positive correlations with VEGF expression ($P < 0.001$ for both correlations) comparable with those for individual factors. In contrast, correlations of coexpressing tumors with MVD were less strong than for each factor alone ($P = 0.078$ and $P = 0.099$, respectively).

DISCUSSION

In this study we have shown a close correlation between ET-1, ET_AR, and ET_BR expression in breast carcinomas and angiogenesis as assessed by MVD and VEGF expression indicating a potential role of the ET axis in neovascularization of breast cancer.

We demonstrated recently that the expression of the ET axis is associated with aggressive types of breast cancer and

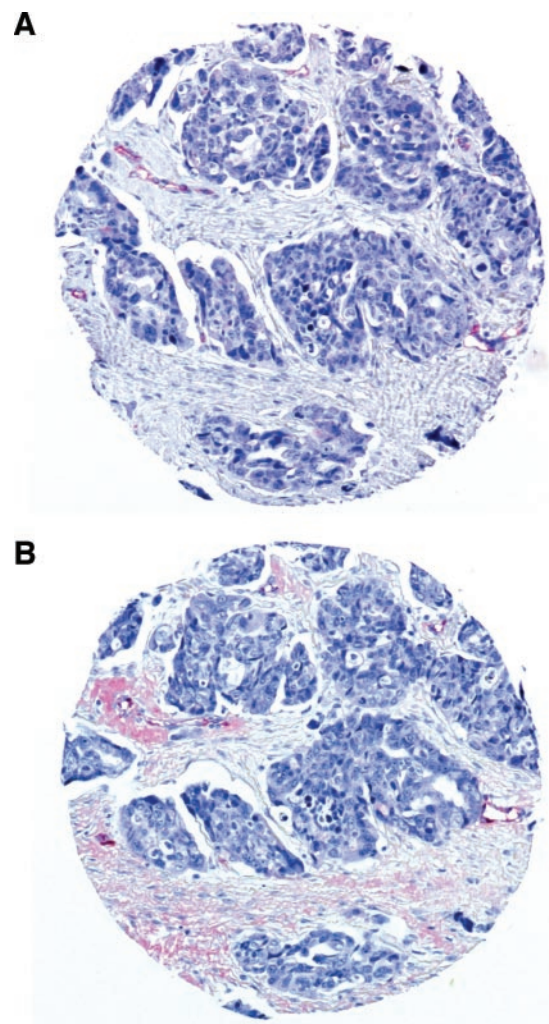


Fig. 4 For both FVIII (A) and CD31 (B) immunohistochemistry reveals similar staining patterns of the endothelial cells. The breast cancer tumor cells are negative.

reduced disease-free and overall survival. Interestingly, increased expression of the ET axis was more common in inflammatory carcinomas and in tumors with lymphovascular invasion (29). In the present study, we sought to determine whether the expression of the ET axis is related to angiogenesis in breast carcinomas.

Analysis of the immunohistochemical ET-1, ET_AR, ET_BR, VEGF, and FVIII expression in our breast cancer TMA confirmed a close correlation of MVD and VEGF expression as described by others (34, 35). Expression of ET-1, ET_AR, and ET_BR correlated significantly with a positive VEGF status. Furthermore, all of the factors of the ET axis were positively correlated with MVD. Interestingly, the threshold for changing ET receptor status (from negative to positive) with respect to MVD appears to lie between the groups of tumors with little vascularity and those with moderate vascularity. There was no additional increase of ET_AR or ET_BR positivity in highly vascularized tumors. These

Table 2 Correlations between VEGF^a and expression of ET-1, ET_AR, and ET_BR in breast cancer specimens

Variable	ET score	VEGF		P ^b
		Negative	Positive	
		n (% of score)		
ET-1 (n = 458)	0	94 (72.9%)	35 (27.1%)	P<0.001
	1	114 (53.0%)	101 (47.0%)	
	2	37 (36.3%)	65 (63.7%)	
	3	3 (25.0%)	9 (75.0%)	
ET _A R (n = 465)	0	80 (82.5%)	17 (17.5%)	P<0.001
	1	110 (66.7%)	55 (33.3%)	
	2	56 (35.9%)	100 (64.1%)	
	3	9 (19.1%)	38 (80.9)	
ET _B R (n = 462)	0	138 (71.1%)	56 (28.9%)	P<0.001
	1	88 (53.3%)	77 (46.7%)	
	2	25 (25.3%)	74 (74.7%)	
	3	1 (25.0%)	3 (75.0%)	

^a VEGF, vascular endothelial growth factor; ET, endothelin; ET_AR, endothelin-A receptor; ET_BR, endothelin-B receptor.

^b Kruskal-Wallis test.

Table 3 Associations of MVD^a with expression of ET-1, ET_AR, and ET_BR in breast cancer specimens

Factor	n	MVD P ^b
ET-1	386	0.039
ET _A R	391	0.037
ET _B R	389	0.019

^a MVD, microvessel density; ET, endothelin; ET_AR, endothelin-A receptor; ET_BR, endothelin-B receptor.

^b Kruskal-Wallis test.

findings suggest that ET receptors may play a role in early neovascularization of breast carcinomas. Comparing serial sections of array cores, a close concordance between all three factors, ET-1, ET_AR, and ET_BR, was found. Thus, we extended the analysis to take possible autocrine loops in the ET axis (ET-1/ET_AR and ET-1/ET_BR) into consideration, demonstrating that correlations with VEGF expression were comparable with those observed for individual factors. In contrast, correlations of coexpressing tumors with MVD tended to be less strong than for each factor alone.

However, we observed a much lower amount of ET-1- and ET_BR-positive breast carcinomas in this study (25.4% and 22.2% versus 43.1% and 53.4%, respectively) in comparison to

our previous results (29). This result may be explained by the different series and higher number of patients investigated in our present work. ET_AR, which was shown previously to have the most important prognostic value, and clinically may be most relevant as a potential antiangiogenic target (29), showed no differences concerning the percentage of ET_AR-positive carcinomas.

Our data are consistent with recent findings in ovarian cancer in which increased expression of ET-1 and ET_A/ET_B receptors is associated significantly with increased VEGF expression and MVD (17, 22). Furthermore, activation of ET_AR by ET-1 stimulates the production of VEGF, which induces proliferation of endothelial cells and vascular permeability (17).

Similarly, in human gliomas ET-1 expression was correlated with transforming growth factor β1 expression and tumor vascularity (26). These preclinical data indicate that the ET axis is involved in the process of angiogenesis and may accelerate neovascularization in malignancies. For breast cancer, there are no comparable data available investigating a potential role of the ET axis in angiogenesis. Two studies in rodent models of breast cancer showed that blood flow to the tumor is increased in response to ET-1 (27), and that hypoxia leads to an up-regulation of ET-2, ET_AR, and ET_BR mRNA (28).

Thus far, the exact biochemical link between the ET axis and angiogenesis remains unclear. With respect to our finding of a higher MVD in ET-positive tumors, a possible explanation could be that the ET system has an impact on the serine proteinase inhibitor plasminogen activator inhibitor type-1. Plasminogen activator inhibitor type-1 expression plays a major proangiogenic role in tumor progression (35, 36) and is in particular essential for neovessel stability (37). Recent data strongly implicate that ET-1/ET_AR stimulates plasminogen activator inhibitor type-1 secretion, possibly leading to tumor growth and metastasis through degradation of the extracellular matrix (38). Thus, the tumor-promoting, proangiogenic activity of the ET axis may presumably be mediated through the serine proteinase inhibitor plasminogen activator inhibitor type-1. ET_AR blockade by selective ET_AR antagonists may, therefore, provide an additional antiangiogenic approach to the treatment of breast cancer.

Most studies investigating angiogenesis in tumors have determined MVD by estimating the number of microvessels in the most vascular areas (so-called "hot spots") of the tumor as described by Weidner *et al.* (32), or applied the Chalkley counting using the mean of three Chalkley counts for the individual

Table 4 Changes in ET^a status with respect to MVD were statistically significant (ET-1, n = 386, P = 0.008; ET_AR, n = 391, P = 0.012; ET_BR, n = 389, P = 0.006; χ² test)

MVD ^b	ET-1		ET _A R		ET _B R	
	Negative	Positive	Negative	Positive	Negative	Positive
	% of MVD grade					
(1) 0–8	84.4%	15.6%	69.4%	30.6%	90.8%	9.2%
(2) 9–16	79.8%	20.2%	50.4%	49.6%	72.3%	27.7%
(3) ≥17	67.8%	32.2%	48.1%	51.9%	71.7%	28.3%

^a ET, endothelin; MVD, microvessel density; ET_AR, endothelin-A receptor; ET_BR, endothelin-B receptor.

^b MVD, vessels per core specimen (0.6 mm diameter).

tumor (39, 40). Because the main goal of our present study was to compare the local expression of the five investigated markers within a certain area of a tumor, we chose the TMA technique, which naturally focuses on preselected tumor areas. Thus, we assessed MVD within the three arrayed cores from distinct morphologically representative areas of each breast carcinoma. All of the core specimens had exactly the same size (0.6-mm diameter) and, thus, the MVD was well comparable. Therefore, we might not have evaluated those areas of a tumor containing the maximum number of microvessels. However, as serial sections of tissue cores represent the same spot of a tumor, our method allowed exact comparison of ET and VEGF expression and MVD. Nevertheless, additional studies (*in vitro*) are ongoing to investigate how the ET axis and VEGF are exactly linked in breast cancer.

In summary, we have shown that ET-1 and its receptors are expressed by breast carcinoma cells, and that expression of the ET axis positively correlates with VEGF expression and degree of intratumoral vascularization. Thus, influence of the ET axis on tumor progression of breast carcinomas may occur through angiogenic effects on endothelial cells and through stimulation of VEGF as described for ovarian carcinoma cells (21). Therefore, the ET axis and in particular ET_AR may have clinical relevance representing potential targets for an antiangiogenic therapy in breast cancer using selective ET receptor antagonists.

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