VIA MEDICA

ORIGINAL ARTICLE

Folia Morphol.
Vol. 68, No. 3, pp. 144–155

ISSN 0015–5659
www.fm.viamedica.pl

Morphometric and immunohistochemical study of angiogenic marker expressions in invasive ductal carcinomas of the human breast

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[Received 23 March 2009; Accepted 7 May 2009]

Breast cancer is the leading cause of cancer deaths among women. Results from experimental studies suggest that tumour progression and metastasis in breast cancer are angiogenesis dependant. The College of American Pathologists has stated that further study of quantification of tumour angiogenesis is still required to demonstrate its prognostic value in breast cancer.

In this study, not only the microvascular density (MVD), but also the vascular area ratio (VAR), and the vascular count in different grades of invasive ductal breast carcinoma were assessed using a pan-endothelial marker, CD34, and monoclonal antibody to CD105, by employing computer assisted morphometric measurements. In addition, quantitative expression of vascular endothelial growth factor (VEGF) was detected. Correlation of the vascular parameters and VEGF expression with the different grades of invasive ductal breast carcinoma was clarified.

Immunohistochemical staining for the CD105, CD34, and VEGF antibodies were performed in 25 patients with invasive ductal carcinoma in King Fahd Hospital, Saudi Arabia. Normal breast tissue samples comprised 15 specimens detected at the safety margin of the malignant breast cases were collected.

Positive CD34 stained blood-vessel endothelial cells were observed in all normal breast tissues. In contrast, CD105 and VEGF expression were not expressed in the normal breast ducts and lobules. Widespread staining for CD34, to a lesser extent CD105, and VEGF expression were seen in all tumour specimens with different grades. Significant differences in the vascular parameters, stained with antiCD34, were observed between normal breast tissues and invasive ductal carcinoma. In addition, the vascular parameters stained with antiCD34 and antiCD105, and the percentage of VEGF expression in the three grades of invasive ductal carcinomas showed significant differences with positive correlations.

In conclusion, MVD as well as VAR are considered to reflect the final result of the tumour angiogenesis cascade. In addition, VEGF expression was found to be a useful angiogenic marker. However, few cases were VEGF negatively stained. Thus, the expression of MVD, VAR, and to a lesser extent VEGF might be reference predictors for the biological behaviour and prognosis of breast carcinoma. (Folia Morphol 2009; 68, 3: 144–155)

Key words: computer image analysis, CD34, CD105, VEGF, cancer, MVD

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INTRODUCTION

Angiogenesis is the term coined by Folkman in 1971 [17] to identify the complex process leading to the formation of new blood vessels from the pre-existing vascular network. The growth, invasion, and metastasis of many cancers depend on angiogenesis; solid tumours require neovascularization to grow beyond about 1 mm^3. Angiogenesis in the tumour can be evidenced by immunohistochemical labelling techniques [9]. Immunohistochemical analysis has the advantages of being readily available in the clinical setting and of enabling morphological evaluation of expression patterns. Finding the different markers associated with angiogenesis may help to identify patients at increased risk for recurrence and metastasis, and thus those who require more aggressive therapy and closer surveillance [39]. In addition, the quantification of tumour microvasculature is a candidate target for antiangiogenic therapy [10].

To date, however, no such marker has been definitively identified. Discrepancies between different studies may be due to the various methods of staining tissues using different endothelial marker antibodies and different methods of counting microvessels. Since these studies have been done in series of patients having different pathological and clinical characteristics, a proper meta-analysis is not possible.

One method for detection of the degree of neovascularization is the microvascular density (MVD). Several researchers have demonstrated that the MVD is closely correlated with the prognosis of breast cancer [33]. The assessment of microvessel density was developed by Weidner et al. [37], initially using antibodies against factor VIII-related antigen that stain mainly mature vessels. The more recent use of antibodies against CD34 react not only with newly formed vessels but also normal vessels trapped within tumour tissues and are thus referred to as pan endothelial markers [39].

The association of CD105 with angiogenesis initially came from Wang et al. [36] who found that monoclonal antibodies to CD105 reacted strongly with the endothelium in various tumour tissues but only weakly in normal tissues [40]. Endoglin (CD105), a cell membrane glycoprotein predominantly expressed on cellular lineages within the vascular system, and over-expressed on proliferating endothelial cells, is involved in blood vessel development and represents a powerful marker of neovascularization. CD105 binds several factors of the transforming growth factor beta (TGF-β) superfamily, a pleiotropic cytokine that regulates different cellular functions including proliferation, differentiation, and migration [29].

Other methods have been developed to assess the expression or concentrations of certain angiogenic factors. Among these angiogenic factors, vascular endothelial growth factor (VEGF) has been found to be the most essential factor for differentiation and development of the vascular system. Much evidence indicates that VEGF is a key activator of angiogenesis [16]. It is a highly specific and selective mitogen for vascular endothelial cells. *In vivo*, VEGF is necessary for vasculogenesis, promotes angiogenesis, and enhances vascular permeability in breast cancer [22]. Evidence for the pivotal role of this cytokine in tumour angiogenesis includes the observations of increased expression in tumour cells of numerous human cancers together with up-regulation of the receptors on the associated endothelial cells and the inhibitory effect of antiVEGF antibodies on tumour growth *in vivo*. Disruption of the capacity to produce or up regulate these factors should help to control the progression of cancer [1].

Semi-quantitative evaluation of immunohistochemical labelling is easy to perform and cost-effective, but computerized systems of image analysis, developed to quantify positive immunoprecipitates within tissue sections, are more reproducible and therefore more acceptable for clinical and pathological use [11].

Malignant changes of the breast constitute as many as one in ten women, and the incidence continues to rise. Breast cancer is the leading cause of cancer deaths among women aged 20 to 59 years [27]. Results from experimental studies suggest that tumour progression and metastasis in breast cancer are angiogenesis dependant [22]. The College of American Pathologists has stated that further study of quantification of tumour angiogenesis is still required to demonstrate its prognostic value in breast cancer [17].

In this study, not only the MVD, but also the vascular area ratio (VAR), and the vascular count (VC) in different grades of invasive ductal breast carcinoma were assessed using a pan-endothelial marker, CD34, and a more specific monoclonal antibody to CD105, by employing computer assisted morphometric measurements. In addition, quantitative expression of VEGF was detected. Correlation of the vascular parameters and VEGF expression with the different grades of invasive ductal breast carcinoma was clarified.

MATERIAL AND METHODS

Patients and specimens

This study included 25 patients ranging in age from 30 to 60 years old (mean ± SEM = 49.52 ± ± 1.65 years) with invasive ductal carcinoma who underwent modified radical mastectomy surgery in King Fahd Hospital, Saudi Arabia. The patients did not receive chemotherapy or hormone therapy before surgery. Appropriate local ethical committee approval was obtained. Normal breast tissue samples comprised 15 specimens detected at the safety margin of the malignant breast cases. Clinical features evaluated in malignant cases were age and menopausal status. The malignant breast specimens were divided according to menopausal status into 12 pre-menopausal patients (≤ 50 years) and 13 post-menopausal patients (> 50 years) [31].

All tumour samples had been fixed in 10% buffered formalin and then embedded in paraffin. Serial 5-μm sections obtained from each specimen were subjected to routine haematoxylin and eosin staining for pathological diagnosis. Tumours were graded according to a modified version of Scarff-Bloom-Richardson system (based on tubule formation, nuclear grade, and mitotic count) [15]. Grade I, II, and III of invasive ductal carcinomas were detected in 5 cases (20%), 14 cases (56%), and 6 cases (24%), respectively.

Immunohistochemistry

Immunostaining was performed on formalin-fixed, paraffin-embedded tissue sections following the standard streptavidin-biotin-peroxidase 3,3'-diaminobenzidine immunohistochemical technique according to the immunohistochemical staining protocol [34]. Briefly, deparaffinised and rehydrated tissue sections were incubated in 3% hydrogen peroxide diluted with methanol for 30 minutes to block the endogenous peroxidase activity. Antigen retrieval was performed by first pretreating sections in a microwave oven or incubating them in 0.1% trypsin for 10 minutes at 37.0°C, after which nonspecific immunoreactivity was blocked by incubating the tissue in normal goat serum at room temperature for 5 minutes before application of the monoclonal antibody to CD105, CD34, or VEGF (Lab Vision Corporation, USA), which were diluted with phosphate-buffered saline at a ratio of 1:100. The sections were then washed in PBS and sequentially incubated in biotinylated goat anti-mouse immunoglobulin G and a streptavidin-biotinylated horseradish-peroxidase complex, according to the manufacturer's instructions (Lab Vision Corporation, USA). The staining was performed by immersing slides in 0.05% 3,3'-diaminobenzidine tetrahydrochloride. All tissue sections were counterstained with haematoxylin, dehydrated, and mounted. The assessment of immunostaining was made by two independent investigators using light microscope. Positive control slides were included in all cases.

Evaluation of vascularization

The area of most intense microvascularisation was selected by scanning on low magnification (× 40) to identify three areas with the highest density of microvessels ("hotspots"). Each hotspot was then evaluated at high power magnification (× 200) for the number of stained microvessels per field. Any brown-staining endothelial cell containing a visible nucleus, and clearly separate from adjacent microvessels, tumour cells, and other connective-tissue elements, was considered a single, countable microvessel, without requirement for a lumen or the presence of erythrocytes [7]. Larger vessels with muscular walls were excluded from counting. The mean of three fields were chosen from each slide, to best reflect the overall immunostaining of the vessels. Counting was performed by two independent observers.

Immunoreactivity for VEGF was detected in the tumour cells. A tumour area with any degree of staining was scored as VEGF-positive. Tumours with VEGF--positive and VEGF-negative areas were assigned the score of the area with strongest staining [1].

Computer-assisted morphometric analysis

Computer-assisted analysis was performed as previously described [4]. Digitized pictures were visualized on a high-resolution colour display. Measurements and data collection were carried out using digital image processing and analysis software for professional microscopy (Leica QWin microsystem, United Kingdom). The collected measurements were presented as mean ± standard error of mean.

In normal specimens, two parameters were chosen for microvascularisation description: MVD: [microvessel number/(microvessel area + residual stromal area)] × 10000; and VAR: [microvessel area/(microvessel area + residual stromal area)]; in both lobules and ducts. The residual stromal area (RSA) was calculated by subtracting the ductal and the vascular areas from the total ductal area in breast ducts (Fig. 1A, B), or by subtracting the acinar and the vascular areas from the total lobular area in breast lobules (Fig. 2A–C) [25]. The ductal area was measured as the overall area occupied by the duct, in-

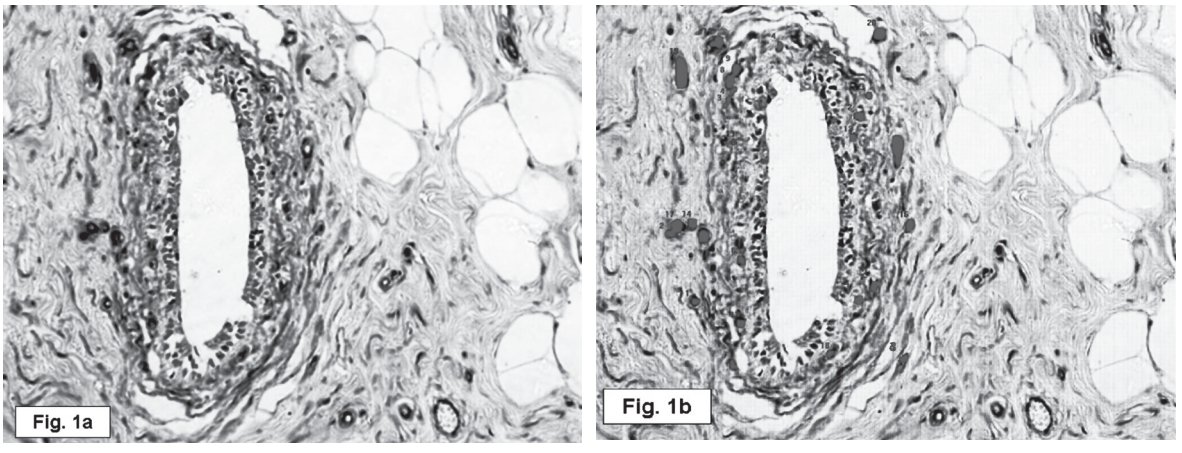


Figure 1. A. Normal breast duct stained with antiCD34 showing microvessels in periductal stroma (× 200); **B.** Computer image processing of the same duct illustrated in Figure 1A, showing the microvessels (black markings) (× 200).

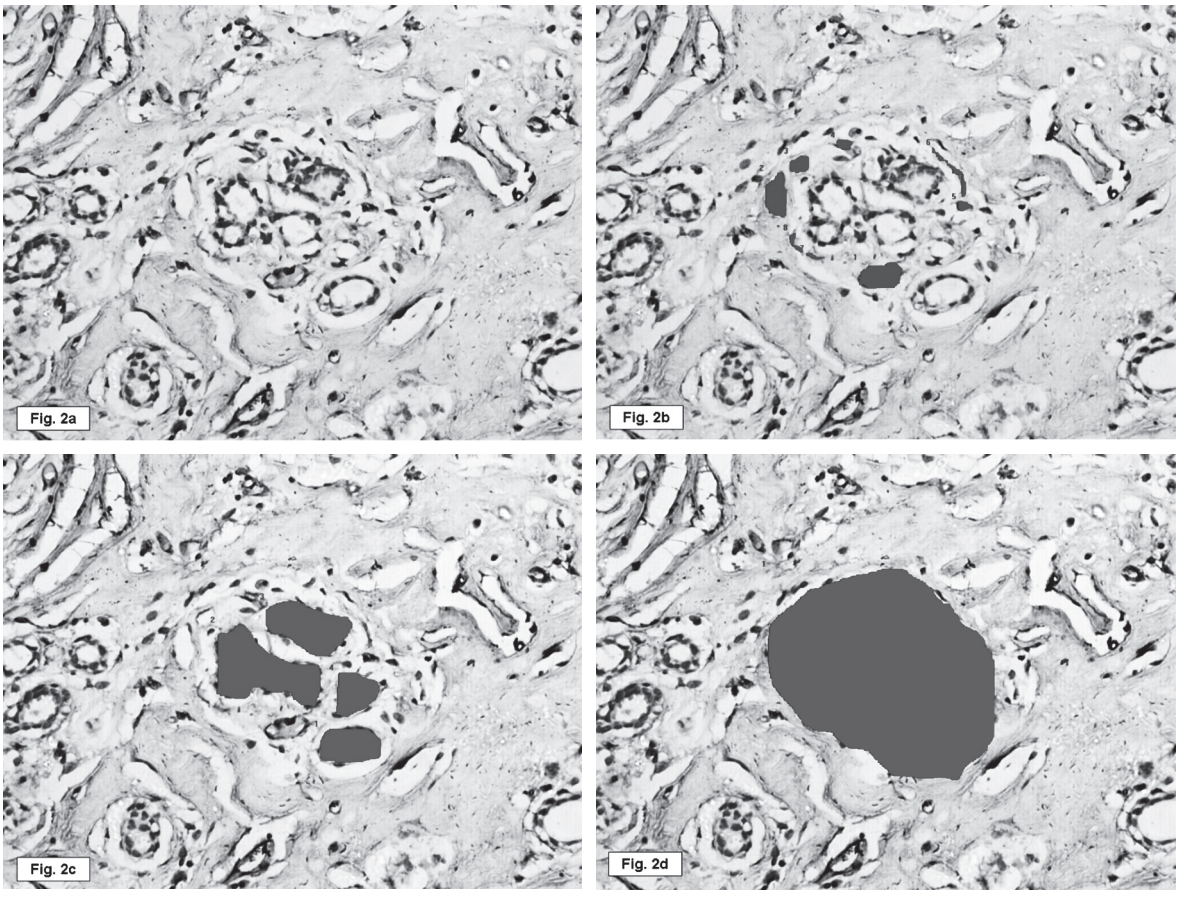


Figure 2. A. Normal breast lobule immunostained with antiCD34 (× 200); **B.** Computer image processing of the same lobule illustrated in Figure 2A, showing the microvessels (black markings) (× 200); **C.** Computer image processing of the same lobule illustrated in Figure 2A, showing the acini (black markings) (× 200); **D.** Computer image processing of the same lobule illustrated in Figure 2A, showing the total breast lobule area (black markings) (× 200).

cluding the lumen, epithelial wall, and a 200-μm rime of stroma around the duct. In invasive ductal carcinomas, the microvascular density was calculated as the (number of vessels/assessed field area) × 10000 and the vascular area ratio as the vessel area/assessed field area [13, 28].

The percentage of cancer cells having cytoplasmic staining for VEGF was recorded. Staining was graded in a four-grade classification as follows: –, for those where reactivity was not detected; 1+, those with positive stain in less than 5% of tumour cells; 2+, a positive stain between 5% and 50%; and 3+, when greater than 50% positive [41].

Staining results were analyzed against histological differentiation. The association between the vascular parameters and various pathological grades were analyzed statistically by one-way analysis of variance (ANOVA) followed by Tukey's HSD (honestly significant difference) pair wise comparisons. The level of significance was set at $p < 0.05$ throughout the study. The correlations between the calculated parameters regarding the microvasculature with CD34 and CD105, and VEGF expressions, were measured by Pearson's correlation coefficient. The current SPSS (version 13) statistical package was used in all statistical analysis [9].

RESULTS

CD34 and CD105 expression

Positive CD34 stained blood-vessel endothelial cells were observed in all normal breast tissues (Fig. 1, 2). Widespread staining for CD34 was also seen in all tumour specimens with different grades as shown in the figures (Fig. 3A–C).

In contrast, CD105 was not expressed in the vascular endothelial cells of the normal breast tissues (Fig. 4, 5), but was expressed in the vascular endothelial cells of invasive ductal carcinoma with different grades (Fig. 6A–C). CD105 expression was mostly observed in the peripheral non- necrotic tumour tissues. It was also observed that the staining of endothelial cells with antiCD34 was intense and easy to visualize, more so than those stained with anti-CD105 antibody.

VEGF expression

VEGF expression was not detected in normal mammary tissues (Fig. 7, 8). Positive staining of tumour cells for VEGF was observed in different grades of invasive ductal carcinomas (Fig. 9A–C). Positive VEGF expression was found in 92% of breast carcinomas. The staining was heterogeneous in most of the tumours evaluated, comprising areas of intense and weak staining. A characteristic granular cytoplasmic staining pattern, independent of vessel proximity, was demonstrated. In some areas, membranous and cytoplasmic positive staining were observed (Fig. 9B). A weak and focal positive reaction of intervening stromal cells (fibroblasts and/or macrophage) was also seen in some cases. Labelling of intratumoural macrophages is shown in Figure 10. Expression of VEGF was found to be negative in 8%, weak in 0%, moderate in 12%, and strong in 80% of breast carcinomas.

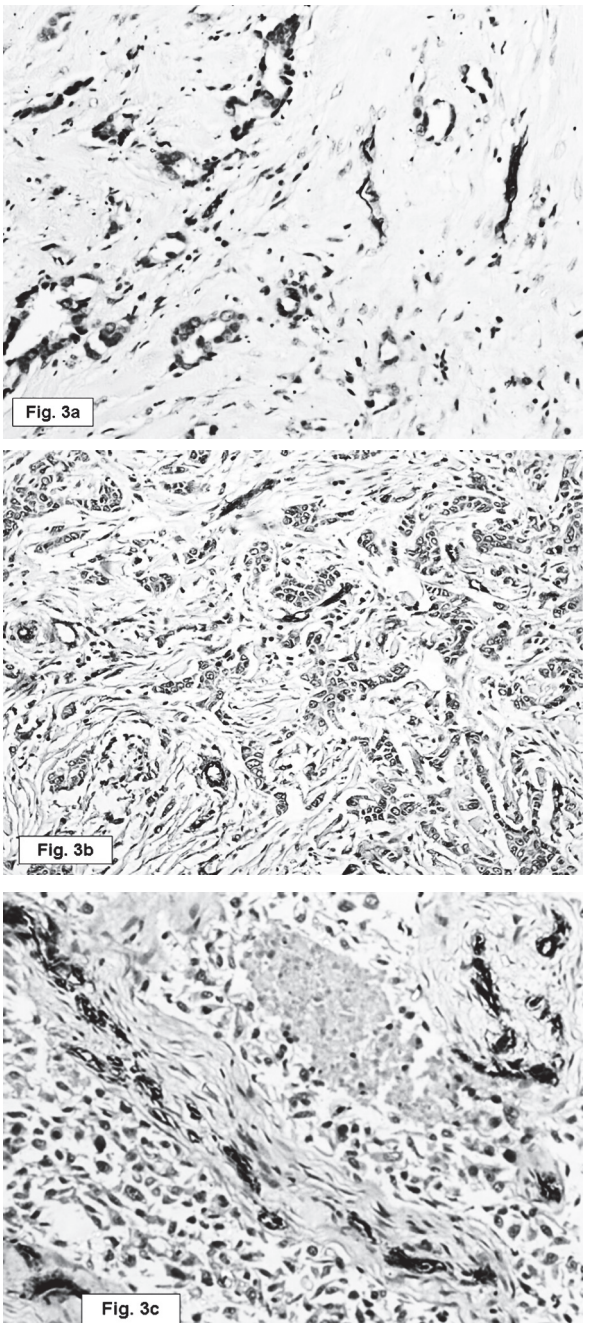


Figure 3. Immunostained grade I (**A**), grade II (**B**), and grade III (**C**) invasive ductal carcinoma of the breast with antiCD34. Note the increased number of microvessels with increased grade (× 200).

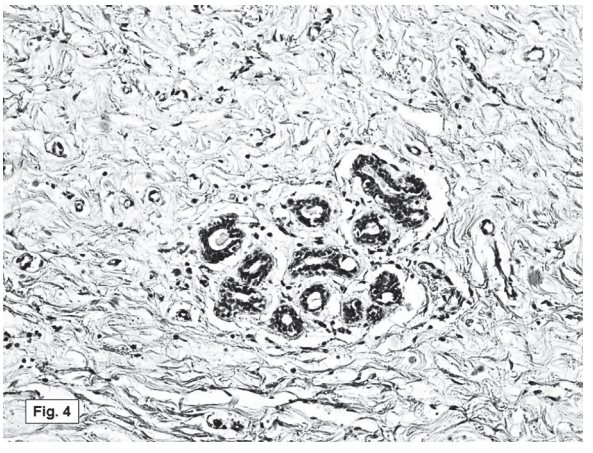


Figure 4. Negative immunostained normal breast lobule with antiCD105 (× 200).

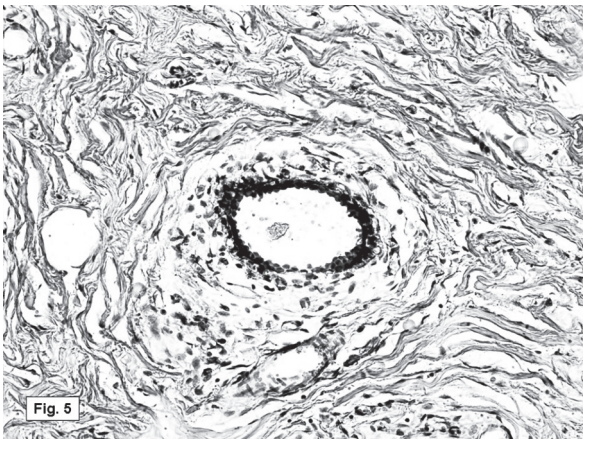
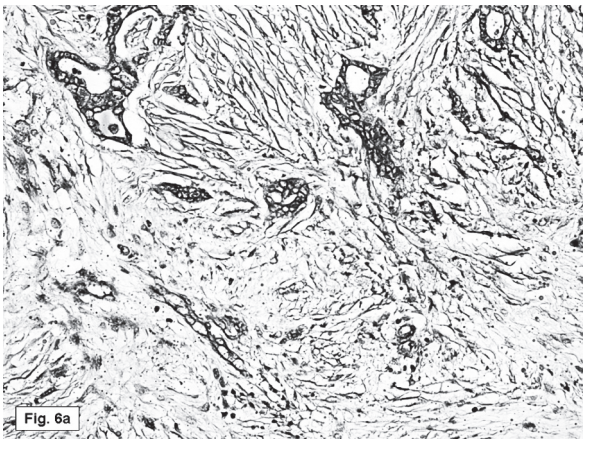
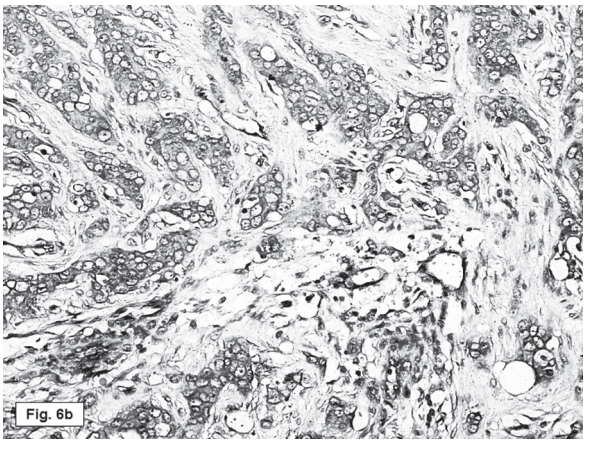


Figure 5. Negative immunostained normal breast duct with antiCD105 (× 200).





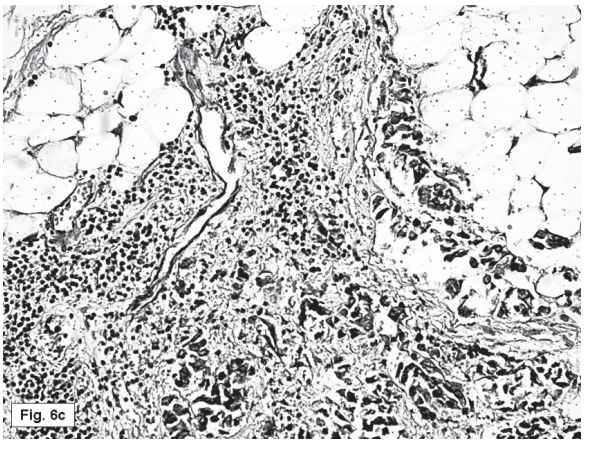


Figure 6. Immunostained grade I (**A**), grade II (**B**), and grade III (**C**) invasive ductal carcinoma of the breast with antiCD105. Note the presence of microvessels near the periphery of tumour tissue in Figure 6C (× 200).

Statistical analysis

The vascular parameters (MVD, VAR, and VC) of normal breast ducts and lobules stained with antiCD34 was measured as shown in Table 1.

Significant differences in the microvascular density, vascular area ratio, and vascular count, stained with antiCD34, were observed between normal breast tissues (lobules and ducts) and invasive ductal carcinoma of the breast (Table 2).

The vascular parameters stained with antiCD34 and antiCD105, and the percentage of VEGF expression in the three grades of invasive ductal carcinomas showed significant differences, being highest in grade III (Table 3). The VAR of antiCD34 and antiCD105, and VEGF expression showed higher statistical significance than the other parameters. No significant differences were detected in these vascular parameters between premenopausal and postmenopausal cancer patients (Table 4).

A positive correlation was observed between the vascular parameters of CD34- and CD105-stained endothelial cells. Figure 11 shows a positive correlation between CD34-MVD and CD105-MVD (r2 = 0.049). In addition, a positive correlation was detected between

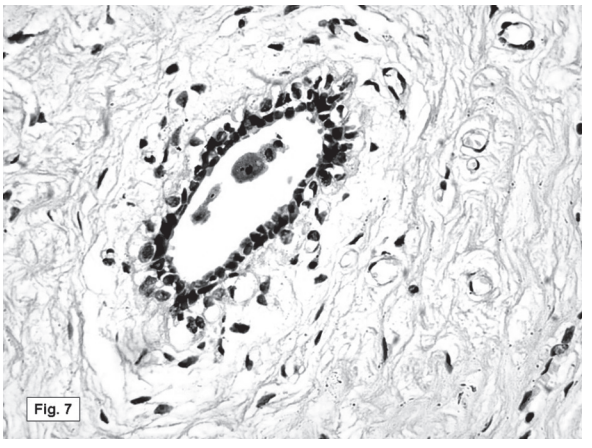


Figure 7. Negative immunostained normal breast duct with antiVEGF (× 400).

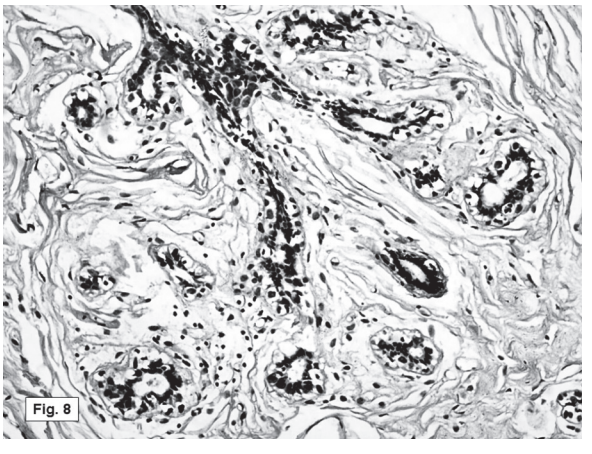


Figure 8. Negative immunostained normal breast lobule with antiVEGF (× 400).

the VEGF expression and the vascular parameters of CD34 and CD105 antibodies. Figure 12 shows the correlation between VEGF expression and CD105-MVD (r2 = 0.176). However, no correlation was found between these vascular parameters or VEGF expressions and the age of cancer patients.

DISCUSSION

Angiogenesis is crucial for tumour development and progression, and antiangiogenetic therapy represents a promising approach for cancer treatment [19, 20]. The original concept of antiangiogenic therapy as an alternative adjuvant to traditional anti-cancer therapies has attracted enormous attention for the past three decades. Equally important to antiangiogenic therapy is the search for markers that can be used for monitoring the efficacy of antiangiogenic therapy and predicting tumour progression [6].

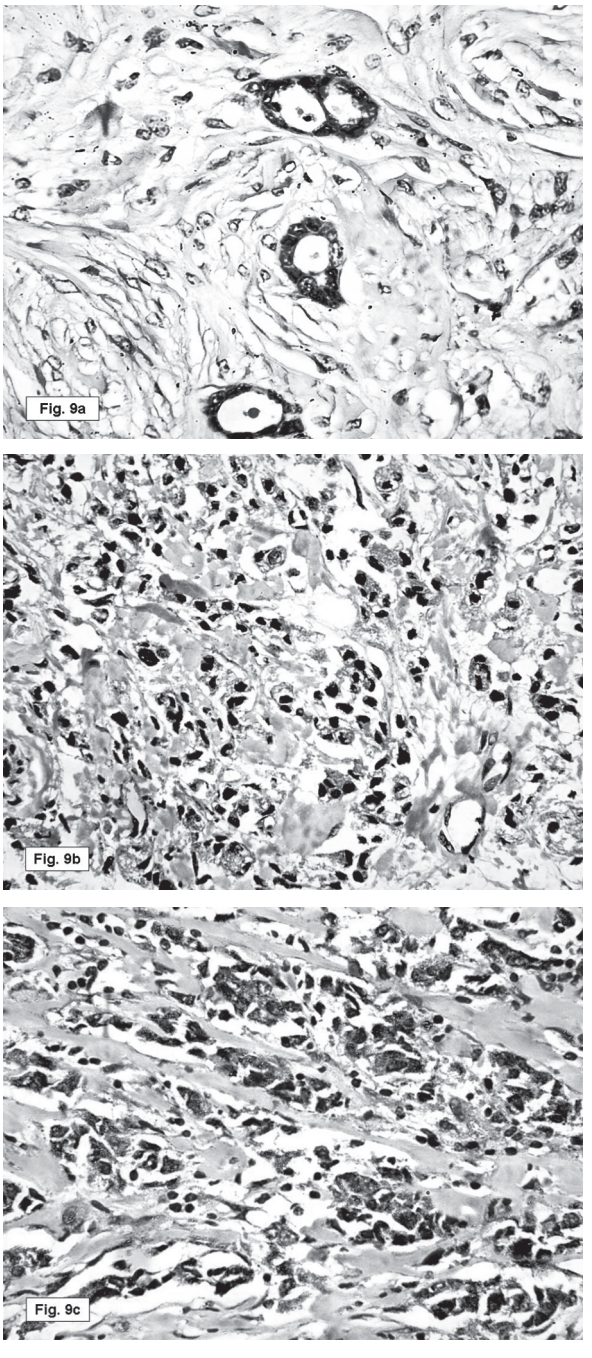


Figure 9. Immunostained grade I (**A**), grade II (**B**), and grade III (**C**) invasive ductal carcinoma of the breast with antiVEGF. Note the membranous and cytoplasmic positive staining in Figure 9B (× 400).

In the present study, the mean of microvascular density of normal breast ducts and lobules stained with antiCD34 antibody was 1.05 ± 0.21 and 1.09 ± 0.12, respectively. However, Naccarato et al. [25] found it to be 2.95 ± 0.16 in ducts and 2.48 ± 0.14 in lobules, using antibody against CD34. Additionally, the mean of the vascular area ratio in the present study was

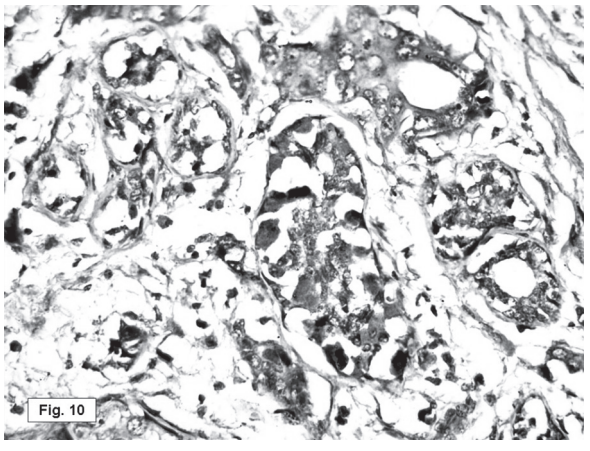


Figure 10. Labelling of intratumoural macrophages with antiVEGF (× 400).

Table 1. Morphometric vascular parameters* of normal breast tissues (ducts and lobules) using CD34 antibody

Vascular parameters	Normal ducts (n = 15)	Normal lobules (n = 15)
VC	12.07 ± 2.06	14.67 ± 1.95
MVD	1.05 ± 0.21	1.09 ± 0.12
VAR	0.04 ± 0.01	0.05 ± 0.01

*Values are presented as means ±SEM; VC — vascular count, MVD — microvascular density, VAR — vascular area ratio

0.04 ± 0.01 in ducts and 0.05 ± 0.01 in lobules. These findings are in-contrast to those of Naccarato et al. [25], who reported that the mean vascular area ratio was 0.29 ± 0.03 in ducts and 0.33 ± 0.02 in lobules.

The vascular parameters measured (MVD, VAR, VC) in this study, using antibodies directed against CD34, were significantly enhanced in malignant lesions as compared to normal breast specimens. The same finding was reported by Teo et al. [32], who found that pure ductal carcinoma in situ exhibited a greater density of $CD34^+$ and $CD31^+$ vessels, compared to normal lobules. The microvessel density was significantly increased in the present study in high-grade carcinomas compared to low grades. In addition, some authors [35] have reported that the mean vascular density increased with lesion severity.

In the present work, the mean microvessel density was 84.12 ± 5.49, and the vascular area ratio was 9.18 ± 1.29 in cases of invasive ductal carcinomas stained with antiCD34 antibody. Dromain et al. [13] reported that the median values of the $CD34^+$ intratumoural microvessel density and the intratumoural microvessel surface area in breast carcinomas were 79.2 and 2.6 microvessels per square millimetre, respectively.

Table 2. Vascular parameters* of normal breast tissues and malignant breast lesions using CD34 antibody

Vascular parameters	Normal specimens (n = 15)	Malignant breast lesions (n = 25)	Significance
Vascular count	13.37 ± 1.41	31.36 ± 2.05	0.00
Microvascular density	1.07 ± 0.12	84.12 ± 5.49	0.00
Vascular area ratio	0.05 ± 0.01	9.18 ± 1.29	0.00

*Values are presented as means ±SEM

Table 3. Vascular parameters* of the three grades of invasive ductal breast carcinomas using CD34, CD105, and vascular endothelial growth factor (VEGF) antibodies

Angiogenic markers	Grade I (n = 5)	Grade II (n = 14)	Grade III (n = 6)	Significance
VC (CD34)	21.00 ± 2.85	33.14 ± 2.81	35.83 ± 2.68	0.03
MVD (CD34)	56.33 ± 7.63	88.90 ± 7.53	96.12 ± 7.18	0.03
VAR (CD34)	4.29 ± 1.03	8.40 ± 0.54	15.07 ± 4.43	0.01
VC (CD105)	4.20 ± 1.24	7.00 ± 0.87	9.83 ± 1.54	0.03
MVD (CD105)	11.27 ± 3.33	18.78 ± 2.34	26.38 ± 4.12	0.03
VAR (CD105)	3.44 ± 1.82	8.76 ± 1.37	19.13 ± 2.34	0.00
VEGF	22.40 ± 9.82	78.56 ± 3.48	85.85 ± 5.19	0.00

*Values are presented as means ±SEM; VC — vascular count, MVD — microvascular density, VAR — vascular area ratio

Table 4. Vascular parameters* of premenopausal and postmenopausal women with invasive ductal breast carcinomas using CD34, CD105, and vascular endothelial growth factor (VEGF) antibodies

Angiogenic markers	Premenopausal (n = 12)	Postmenopausal (n = 13)	Significance
VC (CD34)	34.17 ± 3.55	28.77 ± 2.07	0.19
MVD (CD34)	91.65 ± 9.52	77.17 ± 5.54	0.19
VAR (CD34)	9.91 ± 2.28	8.50 ± 1.39	0.60
VC (CD105)	8.33 ± 0.91	6.00 ± 1.09	0.12
MVD (CD105)	22.35 ± 2.43	16.09 ± 2.91	0.12
VAR (CD105)	11.51 ± 1.82	8.96 ± 2.30	0.40
VEGF	69.33 ± 7.86	68.84 ± 8.26	0.97

*Values are presented as means ±SEM; VC — vascular count, MVD — microvascular density, VAR — vascular area ratio

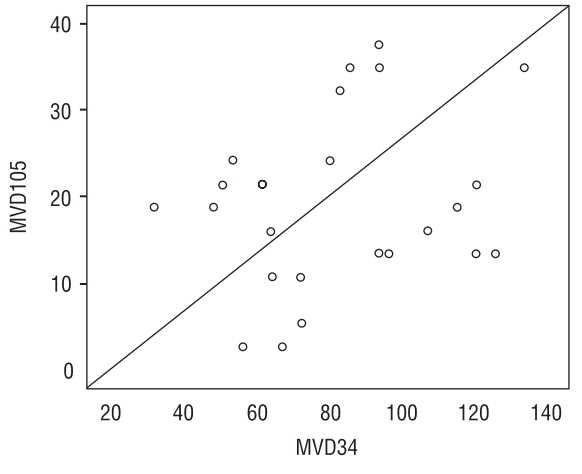


Figure 11. Correlation between CD34 MVD and CD105 MVD.

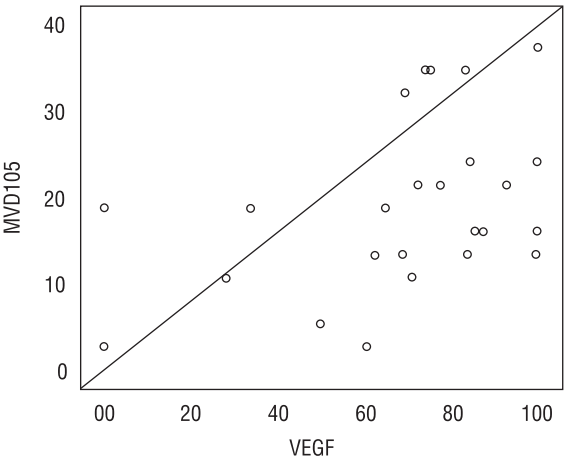


Figure 12. Correlation between VEGF expression and CD105 MVD.

In this study, statistical analysis of the 25 cancer patients showed that CD105 expression was increased in higher grade carcinomas. This finding was in accordance with Gabriel et al. [21] who found that MVD-CD105 was greater in grade III than in grade II, indicating an increase in the vascular neoformation, something which must be evaluated as a possible prognostic factor in breast carcinomas. Also, Zhou et al. [41] observed that increasing MVD was linked with increasing tumour grade and stage.

In the present findings, the mean value of MVD of CD105 expression on endothelial cells of invasive ductal carcinomas was 10 ± 3.92 in grade I, and 18.78 ± 2.34 in grade II, in comparison to 26.38 ± ± 4.12 in grade III, with a total mean of 19.10 ± ± 1.98. Zhou et al. [41] reported the mean of MVD to be 10.63 ± 7.54 in grade I, 14.98 ± 5.72 in grade II, and 18.36 ± 6.01 in grade III, with a total mean value of MVD of 15.22 ± 6.93. Beresford et al. [3] mentioned that the median CD105-positive vessel counts per field ranged from 0 to 18.5 (median 4), a finding that was lower than that detected in this study (median 7). In this research, the CD105 immunoexpression in cancer specimens was mainly at the periphery of the tumour. However, Fonsatti et al. [19, 20] mentioned that Endoglin is mainly present on endothelial cells of both peri- and intratumoural blood vessels, while it is weakly expressed or absent on neoplastic cells.

The present work also showed that CD34 expression was universally expressed within normal and malignant tissues, whereas CD105 expression was absent in normal tissues but positively expressed in breast carcinoma. These data suggest that both CD105 and CD34 could be used for quantification of angiogenesis, but preference should be given to CD105 in the evaluation of prognosis in breast car-

cinoma. This was in accordance with the work of Ding et al. [12], Dales et al. [11], and Beresford et al. [3]. Unlike CD34, which stains both mature and immature vessels, CD105 appears to be much more specific for new, immature vessels. Moreover, Charpin-Taranger et al. [10] and Fonsatti et al. [19, 20] mentioned that CD105 immunodetection may also be considered as a potential tool for selecting patients that could benefit from specific antiangiogenic therapy, using antiCD105 conjugates. Thus, CD-105 can be used as a potential target of therapy in breast carcinoma. Destroying the tumour-associated microvasculature without severely damaging normal tissues or causing major adverse effects is an appropriate goal for biological therapy.

In the present study, immunohistochemical staining for endothelial-specific markers CD34 antigen, or CD105 antigen were clearly highlighted in the vascular endothelium, with a strong staining reaction in the former. However, methodological problems such as inter- and intra-observer variability, the heterogeneity of the tumour, and selection of the area of most intense neovascularization (“hotspot”) remain unsolved. Absolute vessel counts are also influenced by total magnification, selection of the examination area, and the skill and experience of the investigator. Extensive efforts have been made to assess angiogenesis objectively. Calculating the vascular area by computer-assisted methods, e.g., image analysis, was reported to be useful in prognosis [30]; however, another study failed to demonstrate any prognostic significance [24]. In the present study, VAR was significantly increased with tumour grade by immunohistochemical staining with antiCD34 and anti CD105, a finding which should be taken into consideration. Charpin et al. [9] mentioned that to provide more standardized data for the quantification of immunocytochemical studies, diverse computerized image analysis systems have been employed and were found to correlate well with semi-quantitative histological scoring methods and with biochemical data.

VEGF is over-expressed in several malignant tumours as well as in healing wounds, in rheumatoid arthritis, and in delayed hypersensitivity skin reactions. It stimulates angiogenesis by increasing vascular permeability and by acting as an endothelial-cell mitogen [14]. In the present study, 92% of invasive ductal carcinomas were found to be VEGF positive. This was in agreement with Adams et al. [1] who mentioned that the VEGF positivity was 80%. Nieto et al. [26] detected intratumoural VEGF expression in 51% of patients, and Al-Harris et al. [2] observed VEGF positivity in 61.5% of malignant breast lesions. However, Karavasilis et al. [23] found VEGF-positive expression in all cases of cancers of unknown primary, and VEGF was overexpressed in the majority. The pattern of VEGF immunohistochemical staining in the present research was in accordance with Adams et al. [1] and Karavasilis et al. [23]. Both membranous and cytoplasmic staining was observed, a finding which was also reported by Karavasilis et al. [23].

In the present work, VEGF was not detected in normal breast ducts and lobules, a finding similar to that reported by Zhou et al. [41]. Al-Harris et al. [2] observed no over expression of VEGF in normal breast tissues. In addition, occasionally normal epithelial cells and stromal components showed faint staining for VEGF, particularly adjacent stromal endothelial cells, as was reported by Viacava et al. [35]. In normal organisms, angiogenesis is strictly controlled, but in tumours, angiogenesis is uncontrolled and immature.

In the present work, expression of VEGF was found to be negative in 8%, weak in 0%, moderate in 12%, and strong in 80% of breast carcinomas. Also, Zhou et al. [41] found the expression of VEGF negative, weak, moderate, and strong in 29 (23.8%), 38 (31.1%), 34 (27.9%), and 21 (17.2%) of tumour tissues, respectively.

The prognostic importance of VEGF in invasive breast cancer is associated with tumour grade. It was higher in grade III than in grade I. This is in agreement with Callagy et al. [8] and Zhou et al. [41]. In addition, Al-Harris et al. [2] reported that vascular endothelial growth factor immunostaining was positively correlated with tumour grade and stage. Gasparini [22] stated that VEGF could be an important marker of angiogenic activity for prognostic purposes as well as for targeting inhibition of angiogenesis as a novel therapeutic strategy against cancer. However, Nieto et al. [26] did not find any correlation between VEGF expression and histological grade

In the present work, assessment of VEGF expression by immunohistochemistry in invasive ductal carcinoma has shown significant correlation with microvessel counts or density, indicating that both are markers of the degree of angiogenesis, a finding which was also reported by Adams et al. [1], Karavasilis et al. [23], Bolat et al. [5], and Wei-guo et al. [38]. However, other researches showed no correlation between VEGF expression and the degree and/or type of vascularization [26, 34]. Callagy et al. [8] mentioned that tumour stage correlated

with tumour VEGF, but not with microvessel "hot-spot" or vessel counts, and routine measurement of microvessel density in breast cancer is less reliable. Their findings may reflect the relatively small sample size, the relatively uniform patient populations examined in many earlier studies, antibody specificity, or variability in the techniques used to assess immunohistochemical staining or angiogenesis.

No correlation was found between intratumoural VEGF expression and vascular parameters with patient's age. Also, no significant differences in the vascular parameters and VEGF were observed with the menopausal status of cancer patients. This was in accordance with other studies [1].

In conclusion, MVD and VAR are considered to reflect the result of the tumour angiogenesis cascade. In this work, we found that angiogenesis is very active in breast cancer, and is increasing with cancer grade. Several researchers have evaluated the MVD in invasive ductal carcinomas of the breast, with very limited reports regarding the VAR of CD105 or CD34 expressions. In this research, it was found that VAR gave the same prognostic value as that of MVD in cancer breast. In addition, VEGF expression was found to be a useful angiogenic marker. However, few cases were negatively stained. Due to the heterogeneity of staining reaction, the counting of positive cells may be subjected to personal variability. Thus, the expression of MVD, VAR, and to a lesser extent VEGF might be reference predictors for the biological behaviour and prognosis of breast carcinoma.

These conclusions may provide important theoretical evidence for cancer therapy through antiangiogenesis. The results of this work also indicate the possible value of using these biological markers to predict the risk of micro-metastasis in breast cancer. Our results require confirmation in a greater number of patients with a long follow up.

ACKNOWLEDGEMENTS

This work was supported by grant No. A.T: 27/13 from King Abdulaziz City for Science and Technology, Riyadh, Kingdom of Saudi Arabia.

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