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ORIGINAL ARTICLE

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Assessment of tumor angiogenesis in invasive breast carcinomas: absence of correlation with prognosis and pathological factors

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Abstract Different retrospective studies have shown that microvessel counting (MVC) is an independent prognostic marker for clinical outcome in breast cancer. The aim of this study was to evaluate the prognostic value of MVC alone or in association with classical clinicopathological parameters, as well as the reproducibility of the technique. We analyzed a retrospective series of 216 cases of breast carcinoma. Tissue sections were stained for Factor-VIII-related antigen. Microvessel quantification was performed at ×400 magnification in the three most vascular areas of the tumors (hot spots). Mean and highest values were studied. Furthermore, a semi-quantitative evaluation of MVC was performed by use of an image-analysis system. The effect of multiple factors on survival was tested under a Cox multivariate proportional hazards model. In ten cases, a study of the reproducibility was done by evaluating MVC in different sections of the same block and in different blocks of a same tumor. There was no association between MVC (determined at a microscopic level or by image analysis) and overall survival or relapse-free survival. No association was found with tumor size, tumor grade, and lymph-node status. The study of reproducibility showed a very high intra-tumoral variation of MVC. The intra-individual coefficient of variation (CV) varied between 20 and 80%. This study did not show any significant correlation between angiogenesis, as assessed by MVC, and relapse-free survival or overall survival in infiltrating breast carcinomas. The low reproducibility of the MVC for the same tumors suggests that this technique must still be optimized before routine application.

Keywords Angiogenesis · Breast carcinoma · Prognostic factors

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Introduction

Classical morphological prognostic factors, such as axillary lymph-node status, tumor size, and histological grade, have a well-established role in the management of breast-cancer-bearing patients. However, they do not allow patients to be stratified for appropriate therapy on an individual basis. It is critical to identify patients not requiring an adjuvant therapy after surgery and those who may benefit from an aggressive complementary treatment. It would also be interesting to identify patients who are likely to respond to or to resist a particular type of hormono- or chemotherapy in order to avoid both under- or over-treatment.

For these reasons, a great number of cell biological markers, such as steroid hormone receptors, oncogene or suppressor-gene mutations and/or expression, growth factors, cell-cycle-associated molecules, and angiogenesis have been extensively studied [9]. There is now considerable experimental evidence that tumor growth and metastasis are dependent upon neovascularisation, which is required for the expansion of the tumor over 2 mm in maximal diameter [11]. Much data suggest that the evaluation of the tumor angiogenic phenotype by immunohistochemical determination of microvessel density could have a prognostic value in different types of cancers [41], including breast cancers [5, 13, 18, 40, 42, 43]. In contrast, other studies failed to demonstrate this hypothesis [1, 2, 8, 16, 17, 26, 28, 29, 33, 37].

The aim of this study was to investigate the value of the microvessel count (MVC) as an independent prognostic factor in predicting survival outcome in patients with invasive breast carcinoma and to evaluate the reproducibility of the MVC technique.

Patients and methods

Patients and tumors

This retrospective study was carried out on 216 breast invasive cancers retrieved from the archival files of the university pathology laboratory at the CHR-Citadelle, Liège, Belgium.

Table 1 Clinicopathological characteristics of the patients

Clinical characteristics	
Age (mean, range)	60±12.9 years
Tumor size (mean, range)	21.7±16.1 mm
Survival follow up (mean, range)	74±21 months
Death	36 patients (17.5%)
Recurrence	34 patients (16.6%)
Surgical therapy	Invasive cancers
Lumpectomy	63 (29.2%)
Mastectomy	153 (70.8%)
Adjuvant therapy	
Chemotherapy	22 (10.2%)
Radiotherapy	51 (23.6%)
Chemotherapy + radiotherapy	34 (15.7%)
Tamoxifen	115 (53.2%)
Pathological characteristics	Numbers (%)
Histology	
Ductal invasive carcinoma N.O.S. type	150 (69.4%)
Lobular invasive carcinoma	14 (6.5%)
Mixed ductal and lobular invasive carcinoma	24 (11.1%)
Others	28 (13%)
Total	216 (100%)
Histological grade	
Grade I	55 (31.8%)
Grade II	67 (38.7%)
Grade III	51 (29.5%)
Total	173 (100%)
Lymph nodes	
Positive	91 (42.1%)
Negative	125 (57.9%)
Total	216 (100%)

The characteristics of surgical treatment and adjuvant therapy are reported in Table 1. Sixty-three patients with invasive cancers were treated by lumpectomy with axillary-node sampling, and 153 underwent modified radical mastectomy. Altogether, 22 were treated with chemotherapy (cyclophosphamide, methotrexate, and fluorouracil), 51 had radiotherapy, and 34 had both radiotherapy and chemotherapy; nine patients followed a therapeutic protocol with neo-adjuvant therapy (cyclophosphamide, mitoxantrone, and fluorouracil). One hundred fifteen patients received hormonal adjuvant therapy (i.e., Tamoxifen). Tumor tissue was routinely processed for (immuno) histological analysis.

Histological study

Tumor size was defined as the largest diameter of the tumor at the time of trimming of the fresh specimens. Breast cancers were classified according to the WHO histological classification, completed with recently described types such as the cribriform carcinoma or mixed types such as the mixed ductal and lobular invasive carcinoma [10]. The histoprognostic grade was evaluated using the Bloom and Richardson method, according to Contesso's recommendations [7] and applied to ductal invasive carcinoma, NOS type, and mixed ductal and lobular invasive carcinoma. In all cases, the axillary-node status was histologically assessed. The characteristics of the series are summarized in Table 1.

Immunohistochemistry

Paraffin wax-embedded tissue sections were mounted on poly-L-lysine-coated slides. Briefly, after pronase pretreatment, the sections were incubated with the polyclonal antibody against Factor-VIII-related antigen (AO82, Dako, Glostrup, Denmark), and then the immunoperoxydase technique was performed with an automatic immunostaining device.

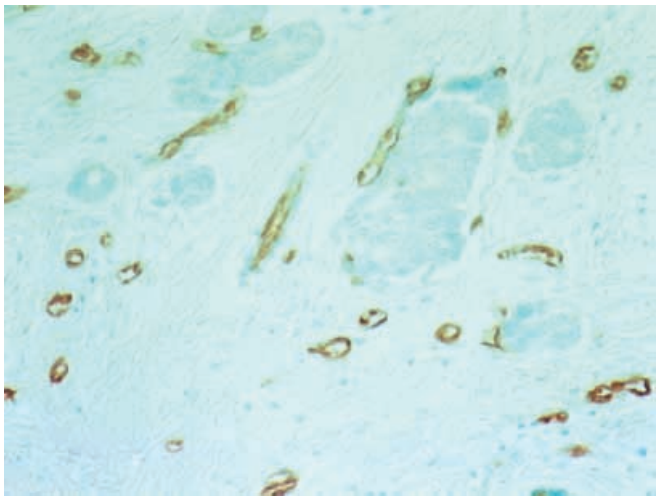

Fig. 1 Example of a representative field of a hot spot in an invasive ductal carcinoma (immunoperoxydase staining for Factor-VIII-related antigen; magnification ×200)

Microvessel count

Two methods were used to evaluate the microvessel count (MVC) in anti-Factor-VIII-related antigen stained slides of invasive carcinoma. MVC was determined by light microscopy in the invasive tumor areas. In each tumor, the three areas with the highest vascularization ("hot spots") were selected (Fig. 1). A ×400 field of these three areas was evaluated. The area of the microscopic field was 0.63 mm^2. Results were expressed as the *mean MVC* per field for the three hot spots and as the *highest MVC* per field observed in the most vascularized hot spot. Furthermore, for each tumor, the same hot spots were evaluated using an image-analysis system. Images were captured using a microscope coupled to a color video camera (Sony Dxc-M3a) and then processed with an image analysis device (Ibas 2000, Kontron Bild Analyse). The parameters automatically detected by the system were the number of microvessels, the area of the microvessels, and the overall area. Results were reported as the mean or highest MVC and as the mean or highest MV areas.

A study of the reproducibility of MVC (mean and highest values) was performed by measurements obtained by microscopic counting and by image analysis from two different blocks of the same tumor in five cases, from three different blocks in two cases, or from two additional histological sections separated by 20 µm in ten cases.

Statistical analysis

Results were expressed as means and standard deviations (SD) for quantitative variables. Means were compared by one-way analysis of variance. Correlations were calculated to measure the association between variables. Survival curves were displayed by the Kaplan-Meier method, and the effect of covariates on lifetime and recurrence was tested by Cox PH regression analysis. All calculations were performed with the SAS (version 6.12) and S-PLUS (version 3.1) statistical packages. Results were considered to be significant at the 5% critical level ($P<0.05$).

Results

Clinicopathological parameters and population survival

Table 1 reports the major characteristics of the patient series, including treatment, age, tumor size, histological type and grade, lymph-node status, death, and recurrence rate. The median age and follow-up time obtained from 216 patients were 60 years and 74.2 months, respectively. Thirty-six patients died (17.5%) and 34 (16.6%) showed local recurrence or metastasis during the follow up. There was a significant difference in both overall survival (OS) and relapse-free survival (RFS) when stratifying patients according to tumor size (P=0.004 and P=0.0001) and histological grade (P=0.001 and P=0.0001). Axillary lymph-nodes status was statistically associated with relapse-free survival (P=0.001) (data not shown).

Prognostic value of MVC

In order to evaluate whether MVC (either the mean or the highest values) determined at a microscopic level or by image analysis (IBAS) was associated with significant differences in terms of both overall and relapse-free survival, the cases were distributed into two groups, with a cut-off count of 19.45 (or 34 by IBAS) and 28 (or 41 by IBAS) for the mean and the highest MVC, respectively. These cut off values were the median of all the individual values obtained in the present case series. As shown in Figs. 2 and 3, no significant differences were observed. Similarly, vascular areas calculated by image analysis were not associated with favorable or pejorative clinical outcome (data not shown).

MVC in relation to the classical pathological factors

As shown in Table 2, there was no relationship between MVC (mean or highest value) and the histological type or grade or the lymph node status.

Reproducibility of MVC measurements

Owing to the lack of correlation between MVC and prognosis or pathological factors, a study on the reproducibility of the microvessel counts was undertaken. Measurements of MVC in different sections and blocks of the same patient were compared. They showed a very high variation of MVC values within an individual tumor. The intra-individual coefficient of variation (CV) varied between 22 and 80% (Tables 3 and 4).

Multivariate analysis

MVC and the clinicopathological factors including histological type and grade, tumor size, and lymph-node status were analyzed by the multivariate Cox proportional hazard model. The most significant predictive parameters were the histological grade and size tumor, both for overall (P=0.001 and P=0.005, respectively) or relapse-free survival (P=0.006 and P=0.006, respectively).

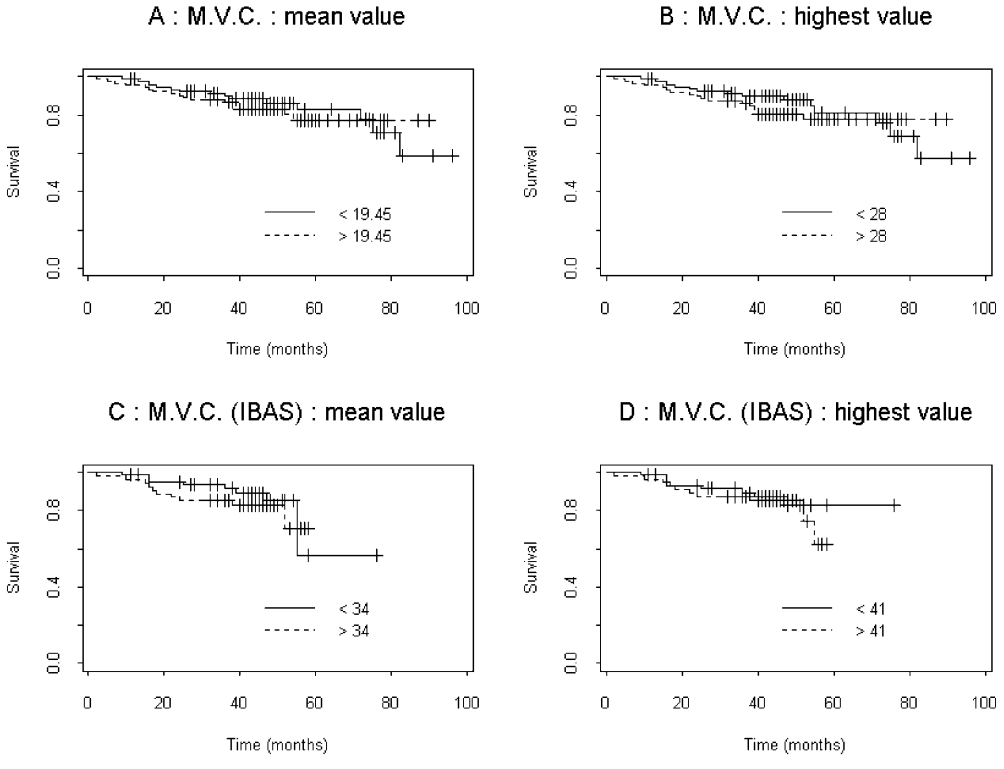

Fig. 2 Absence of correlation between the overall survival of breast-cancer patients and angiogenesis expressed as the mean (**A** and **C**) or the highest (**B** and **D**) values of microvessel counts (*M.V.C.*) determined by microscopic (**A** and **B**) or image analysis (*IBAS*; **C** and **D**). The cut-off values were 19.45 and 28 for the microscopic counts (**A** and **B**, respectively) and 34 and 41 for the image-analysis measurements (**C** and **D**, respectively)

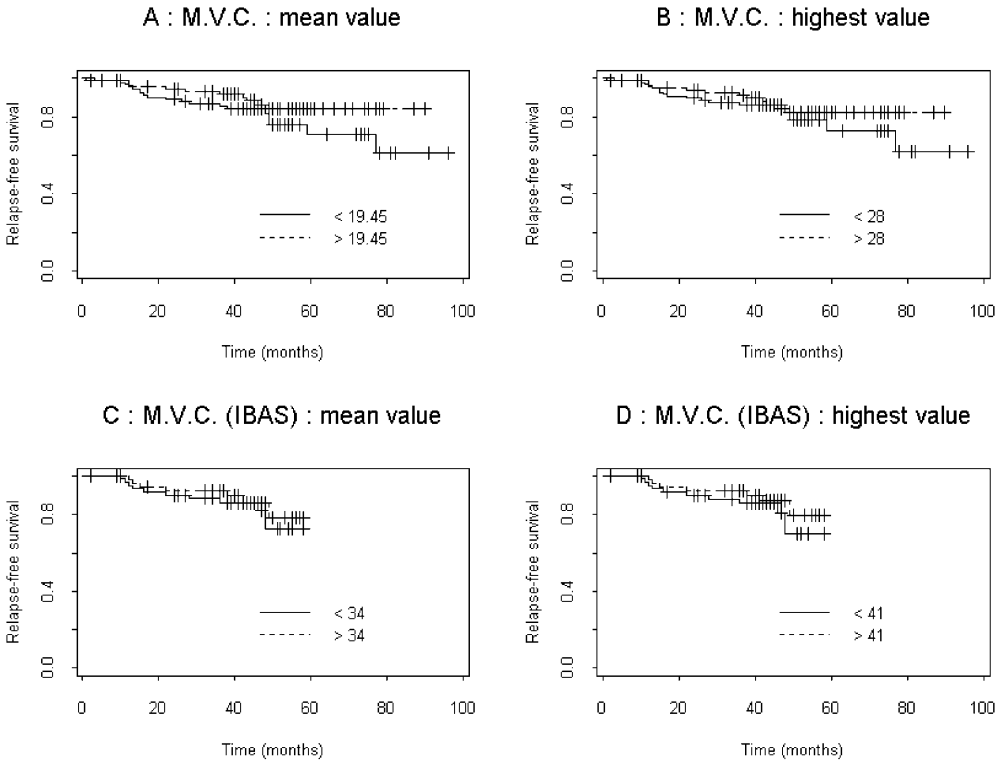

Fig. 3 Absence of correlation between the relapse-free survival of breast-cancer patients and angiogenesis expressed as the mean (**A** and **C**) or the highest (**B** and **D**) values of microvessel counts (*M.V.C.*) determined by microscopic (**A** and **B**) or image analysis (*IBAS*; **C** and **D**). The cut-off values were 19.45 and 28 for the microscopic counts (**A** and **B**, respectively) and 34 and 41 for the image-analysis measurements (**C** and **D**, respectively)

Table 2 Microvessel counts (*MVC*) in relation to pathological factors

Patient characteristics	Number (%)	MVC mean value (*n*)	MVC highest value (*n*)
Histology			
Ductal invasive carcinoma NOS type	136 (68.7)	28.1±24.7	35.7±29.2
Lobular invasive carcinoma	12 (6.1)	23.5±20.7	31.4±27.9
Mixed ductal and lobular carcinoma	24 (12.1)	27.6±25	35±31.4
Others	26 (13.1)	33.8±12.6	46.1±16
		P-value: 0.71	*P*-value: 0.37
Histological grade			
Grade I	52 (32.5)	29.6±24.1	38.2±32.2
Grade II	63 (39.4)	29.2±29.8	36.7±32.4
Grade III	45 (28.1)	24.9±15.9	31.8±20.4
		P-value: 0.59	*P*-value: 0.54
Lymph nodes			
Positive	84 (40.6)	29.9±28.6	37±32.7
Negative	114 (59.4)	26.2±19	34.5±25.5
		P-value: 0.27	*P*-value: 0.54

Table 3 Variations in the microvessel counts (*MVC*, mean value) between different section levels of a block and in different blocks. *CV* Coefficient of variation, *SD* standard deviation

Case number	Section 1 Block 1	Section 2 Block 1	Section 3 Block 1	Section 1 Block 2	Section 2 Block 3	Mean±SD	CV (%)
1	13	21	24	30	26	23±6.2	27
2	63	25.3	31	24		36±18.4	51
3	34	21	19.6			25±8	32
4	30	17	20.3	21		22±5.6	25
5	11.3	19	34.3	35.6	28	26±10.3	40
6	18	11.6	16.3			15±3.3	22
7	13.5	13.3	23.3			18±7	39
8	103	28	30			54±42.7	80
9	16	12.6	6.5			12±4.8	41
10	14.6	13.3	14.6	21.3		16±3.6	23

Table 4 Variations in the microvessel counts (*MVC*, highest value) between different section levels of a block and in different blocks. *CV* Coefficient of variation, *SD* standard deviation

Case number	Section 1 Block 1	Section 2 Block 1	Section 3 Block 1	Section 1 Block 2	Section 1 Block 3	Mean±SD	CV (%)
1	19	21	24	30	42	27±9.2	34
2	77	26	45	30		44± 23.2	52
3	43	28	23			31±10.4	33
4	35	29	34	29		32±3.2	10
5	16	24	34	37	38	30±9.5	32
6	26	16	19			20±5.1	25
7	18	21	25			23±2.8	12
8	140	38	43			74±57.5	78
9	16	18	7			14±5.9	43
10	20	18	18	24		20±2.9	14

Discussion

Breast cancer is a heterogeneous neoplasm, which contains malignant cell clones with different metastatic potentials. The clinical outcome of different patients with equivalent clinico-pathological stage is variable. Prognostic factors, which can be used to predict the aggressiveness of the tumor, are required, and many parameters have been studied. In this context, measuring the MVC as a reflect of tumor angiogenesis has been proposed. Tumor angiogenesis refers to the growth of new vessels from pre-existent vessels and is required for neoplastic growth, progression, and metastasis [11]. Recently, quantification of intratumoral microvessel density in histological specimens, using specific endothelial markers and immunohistochemical techniques, has been found to be of prognostic value in breast cancer [5, 13, 18, 40, 42, 43] and in a variety of other solid tumors [for a review: 41]. Initial studies of breast cancers by Weidner et al. [42], using the immunohistochemical visualization of Factor-VIII-related antigen on the endothelium and specific criteria to count the microvessels, showed that the number of vessels in one selected vascular hot spot in histological sections of invasive cancer correlates with metastases spreading. Several pilot studies [5, 13, 18, 40, 42, 43] showed that microvessel density is an independent prognostic marker for both relapse-free and overall survival. Some other reports gave similar results [3, 4, 30, 34, 36]. These numerous studies suggested that human breast cancers present a heterogeneous level of angiogenic activity and that highly vascularized tumors are biologically more aggressive and associated with poorer outcome. However, in this study, we found no correlation between tumor angiogenesis and prognosis, as others have also found [1, 2, 8, 16, 17, 26, 28, 29, 33, 37].

This discrepancy with previous studies could be due to the biological tumor heterogeneity, which may hinder the reproducibility of the angiogenesis assessment method or result from technical differences with other published studies. Notably, among the reported studies, there was also a high variability in the choice of antibodies, vascular parameters, selection criteria, counting methodology, and quantification of results. In one study, a high coefficient of variation (average 24%, range 6–55%) was found when more than one tissue block was analyzed as compared with 15% (range: 0.5–42%) when counts were made within one section of one block [21]. In another study [24], vessel counts among blocks varied by less than 20% in 14 out of 20 cases when MVC was measured in sections from three different blocks. A concordance rate of 71–78% of MVC when two blocks were evaluated was reported in a study of 41 tumors [37]. The authors pointed out that tumor heterogeneity between blocks might introduce a considerable error in the assessment of vascular density. Axelsson et al. [2], analyzing only one section, showed a variability of the MVD (microvessel density, obtained by dividing the count by size of the microscopic field) from field to field within a single tumor. Our study also confirmed this assay variability. When MVC was measured in different sections of the same block or different sections of different blocks, an intra-individual coefficient of variation (CV) ranging between 22 and 80% was observed. These data support the view that there is an important intratumoral vascular heterogeneity and that the evaluation of the angiogenic phenotype of a tumor by MVC measurement in a single area of one histological section is not representative of the whole tumor, suggesting that separate portions of a tumor should be sampled to get a better overall estimate of tumor microvessel density. Another possible reason for discrepancy of our study with other reports can be related to the heterogeneity of patient databases, which may reflect geographical differences in genetics, variable patient follow-up, and treatment protocols [41]. Axelsson et al. [2] compared the clinico-pathological characteristics of their patient population with those studied by Fox [13] in Great Britain and Gasparini [15] in Italy. They showed important differences in the number of metastases, mortality, tumor grade or size, the proportion of ER-negative tumors, the type of treatment, and the follow-up time. It has been proposed that any prognostic marker should fulfill a minimum set of criteria, including sample size and patient population [27]. Many studies about angiogenesis in breast carcinomas have analyzed a heterogeneous population with respect to histological type, node status, age, menopausal status, and follow-up duration. Morphopoulos et al. [29] examined a series of lobular infiltrative carcinomas. No association between microvessel density and overall survival or relapse-free survival was shown. Axelsson's study [2],

analyzing patients with invasive ductal carcinoma that was not otherwise specified, also did not show any correlation between MVD and clinical outcome. There may be several reasons for the high variability in the quantification of microvessels. The pathologist's experience seems to be of critical importance. It has been reported that microvessel counting by an inexperienced pathologist is 7% higher than that by an experienced pathologist. Furthermore, only the counting made by the experienced observer gave prognostic information [3]. A second reason is that the selection of the analyzed areas is highly subjective. Weidner et al. [42] advocated the counting of vessels in the area with the highest vascularisation. Axelsson et al. [2], even using these guidelines (one of these authors was trained by Weidner), however, found a significant inter-observer variability. In our study, we measured MVC in three hot spots. This method is similar to that used by Bosari et al. [5], but is different from Weidner's method, in which only one hot spot is measured. We think that our highest value among the three counted hot spots and the Weidner's count in one hot spot are superposable. In the present study, counting was made at ×400 magnification, which is higher than in Weidner's studies. This magnification was also used by other authors, such as Vermeulen et al. [39], who noticed a two-fold higher MVD when the magnification was ×400 rather than ×200, provided MVD was expressed as the number of microvessels/mm^2. In that study, angiogenesis quantification gave prognostic information whatever the magnification (×200 or ×400). These authors also indicated that the selection of hot spots is an important cause for the inter-observer variability and may be improved by training. The localization of the zone in the tumor where the hot spot is chosen could also be important. Recently, a study demonstrated a significant association between tumor recurrence and MVC evaluated in a hot spot localized at the periphery of the tumor. MVC in the central and intermediate field was not associated with RFS or OS [20]. In our study, the majority of the hot spots were situated at the periphery of the tumors. Another recent study reported significant inter- and intraobserver variation and difficulties in identifying the hot spots in relation to vascular pattern [19]. The MVC reported in the different studies varied [12]. Expressed as counts per square millimeter, the mean of the highest MVC in the present study was 59; this value was similar to that reported in several papers [22, 30, 31], but smaller than that reported by others [5, 18, 36, 42]. Differences in the choice of antibodies may also partially explain the variability of the results. As did Weidner [42] and other authors [5, 18, 36], we used the anti-Factor-VIII-related antigen polyclonal antibody. Other authors, however, recommend the anti-CD31 antibody, arguing its higher specificity for vascular endothelium [23, 32, 38]. In a recent paper, Martin et al. [25] showed a close association between vessel density and survival rate by using three different antibodies (anti-Factor-VIII-related antigen, anti-CD31, and anti-CD34). In order to provide a more objective method of assessing MVC, semi-automated counting with a computerized image-analysis system has been proposed by several investigators [3, 6, 14, 35]. With this technique, some authors reported a significant correlation between microvessel number or area and survival. By contrast, Goulding et al. [16], who examined tumor vascularity either by using an interactive image-analysis system or by simply counting the microvessels in the zones with the highest vascular density, did not find any correlation with survival.

Conclusions

The role of angiogenesis in tumor growth and development of metastasis is not disputed. However, the prognostic value of its quantification in histological specimens remains unclear. The present study showed that MVC measured in histological sections stained with an anti-Factor-VIII-related polyclonal antibody may be variable, does not correlate with other prognostic factors, and fails to predict clinical outcome. Breast-cancer heterogeneity as well as problems related to the quantification of the vascular density may explain the observed results.

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