ORIGINAL PAPER

Angiogenesis and tumor grading in primary breast cancer patients: an analysis of 158 needle core biopsies

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Abstract

Angiogenesis, the formation of new blood vessels from a preexisting vascular bed, is a complex multistep process. To investigate how tumor angiogenesis correlates with tumor grading in breast carcinoma diagnosed on core biopsy, microvessels were counted (and graded the density of microvessels) within the initial invasive carcinomas of 158 patients. Using light microscopy, the number of microvessels was counted manually in a subjectively selected hot spot (in the most active areas of neovascularization per 400× field), and their values were separated as above or below median (low and high), without knowledge of the outcome in the patient or any other pertinent variable. When the mean values of MVD of the various groups defined by tumor grading were compared, significant difference was noted (P = 2.61E–17). When the mean values of MVD of the groups defined by tumor grading risk were compared, significant difference was noted (P = 7.68E–05). When tumors were classified as high or low MVD, based on a cut-off value (30.70175 microvessels/mm^2), cases with high MVD were significantly more numerous in MSBR four patients. MVD did show a relationship with groups defined by MSBR grade (P = 3.25209E–07) or with tumor grading risk (P = 2.54181E–06). Assessment of tumor angiogenesis may therefore prove valuable in selecting patients with early breast carcinoma for aggressive therapy.

***Keywords*:** angiogenesis, breast carcinoma, MSBR, needle breast core biopsy.

Introduction

While breast cancer remains a leading cause of death in women, particularly in women 40–55 years of age [1], about 70% of women present with axillary lymph-node negative disease, and only about 30% of them will ever develop distant metastases; the other 70% are essentially "cured" of their disease by surgical excision of the primary tumor [2–4].

Clinical and histopathologic characteristics of the primary tumor are used to stratify patients into groups having different outcomes, but they do not predict accurately the outcome for any individual patient. Thus, there is need to identify additional tumor characteristics that are able to predict more accurately the outcome for an individual patient with breast cancer, especially if the disease is clinically node negative.

Despite a considerable body of evidence that the Bloom–Richardson grading system (BRG), which is based on the assessment of tubule formation, nuclear pleomorphism, and mitotic activity, provides important independent prognostic information in patients with breast cancer, this system has not been universally accepted, mainly because of its subjective nature and apparently poor reproducibility. A major improvement has been provided by Elston CW and Ellis IO [5] who have clearly defined the criteria, particularly by applying numerical limits to the measurement of tubule formation and mitotic counts. Whereas the relative numbers of both hyperchromatic nuclei and mitotic figures were analyzed in the original BRG, only clearly identifiable mitotic figures are evaluated in the new system. In addition, the size of the high power field, which may vary greatly from one microscope to another, is taken into account.

Angiogenesis, the growth and proliferation of blood vessels from existing vasculature, is a complex multistep process involving extracellular matrix remodeling, endothelial cell migration and proliferation, microvessel differentiation, and anastomosis. This process is quiescent in normal tissues and becomes active in rapidly growing tissues – including solid tumors. It has been shown that, in order to overcome tissue death by hypoxia, tumor growth beyond 1–2 mm^3 is dependant upon the formation of new vasculature [6, 7]. Angiogenesis is, thus, an established step in solid tumor progression.

Most assessments of angiogenesis in female breast carcinoma have shown it to be of significant prognostic value [8–12]. However, not all studies in this field have observed such important clinical correlations to MVD [13, 14]. The reason for this discrepancy is not known.

Stereotactic core needle biopsy (SCNB) is a faster, less invasive, and less expensive alternative to surgical biopsy for the diagnosis of breast lesions, and its results have high concordance (87–96%) with those of histopathological findings at surgery [15–19].

Purpose

This retrospective study was to evaluate the correlations between intratumoral microvessel density (MVD) and tumor grading, in order to identify those

tumours with a prominent angiogenic phenotype. It would be an important advance if high MVD could be used to help in predicting the prognosis of patients, particularly in high risk individuals.

Patients and methods

Selections of cases

The histological slides of non-palpable, mammographically detected lesions in which percutaneous stereotactic biopsies were performed from January 2004 until December 2004, in SAPAG Hautepierre, Strasbourg (France), were retrospectively reviewed. Lesions were defined as non-palpable when patients, surgeons, and the SCNB examiner (a radiologist) could not palpate any breast lesion during physical examination.

For all cases, mammography and ultrasonography reports and films were collected for review. In addition, medical charts were reviewed to verify that none of the patients included in the study had clinical evidence of malignancy or a history of ipsilateral breast carcinoma and also to collect clinical information, such as age, family history of breast carcinoma, parity, hormone replacement therapy received, and history of contralateral breast carcinoma.

To be eligible for this retrospective study, women had to have undergone a SNCB of a primary breast cancer. The criteria of inclusion or exclusion are listed in Table 1.

Table 1 – ***Inclusion and exclusion criteria of the study***

Inclusion criteria	Exclusion criteria
Female sex	Pregnancy
Age older than 21 years	Recurrent disease
Not pregnant	Previous mastectomy
Suspicious lesion of the breast (mammography)	Fine needle aspiration within one week prior to scintimammography
Recommendation for excisional after mammography	Core biopsy during the previous four weeks
Patient with node-negative breast cancer	Previous chemotherapy
Unicentric tumor	Medically unstable patient (severe arrhythmia, heart failure or recent surgery)

Mammographic lesions were divided into two groups: calcifications (without masses) and masses. Masses included asymmetric densities, areas of architectural distortion, and other space-occupying lesions (all with or without associated calcifications).

The lesions were categorized according to the Breast Imaging Reporting and Data System (BI–RADS) developed by the American College of Radiology [20].

Biopsy procedure

Stereotaxic localization was performed by radiologists trained in mammography using a dedicated stereotactic breast biopsy system, an automatic biopsy gun, and a 14-gauge biopsy needle with a long throw (2.3 cm excursion).

The core needle biopsy was performed by first cleansing the skin overlying the lesion with alcohol; this has been followed by skin and subcutaneous infiltration with approximately 1–2 ml of 1% lidocaine.

Usually one to three biopsies were taken from different areas in each lesion utilizing the same biopsy instrument. The core needle biopsy specimens were removed from the trough in the stylet by rinsed in a container filled with sterile saline. This technique was preferred because less tissue manipulation was involved and the adequacy of the tissue could be judged visually by inspecting the container to determine whether the specimen sank or floated in the saline. The final pass into the lesion was then made and, because sterile technique was no longer necessary, a touch preparation was made in some cases before removing the specimen from the needle. The specimens were then transferred into formalin and processed in the histology laboratory.

Surgical clip was placed in patients when the entire lesion was removed by the needle core biopsy.

Tissue specimens

It was obtained a mean of 2.6 specimens (range, one to 8) per lesion. Core specimens were radiographed to document the presence of calcification. Core specimens were fixed in 10% formalin; paraffin embedded, sectioned, leveled ×3, and stained with Hematoxylin–Eosin. Additional levels were requested, if necessary, for histologic documentation of calcification. The use of a polarizing lens assisted in the microscopic identification of microcalcification in some cases. Two pathologists retrospectively reviewed the histologic slides. At the retrospective review, the pathologists knew each lesion was later excised but did not know the excisional diagnosis.

Histological review

The original diagnosis of invasive malignancy was made by the same senior pathologist (SAPAG) in almost all cases. For these cases, Hematoxylin–Eosin stained slides of core biopsy samples were retrieved from the pathology archives and reviewed by a second pathologist (S.V.) to confirm the diagnosis of invasive malignancy. Diagnoses were confirmed in all cases.

The MSBR grading system

The Scharff–Bloom–Richardson (SBR) grading system is applied most successfully to cases of invasive ductal carcinoma NOS. Usually, this histoprognostic grading is not considered appropriate for lobular invasive carcinoma.

Pathology reports from core biopsies were evaluated using the histological grading system by LeDoussal V *et al.* [21] This is a modified Scharff–Bloom–Richardson (MSBR) system that classifies the tumors into one of five grades (grades 1–5) by adding the nuclear pleomorphism (grade 1–3) and the mitotic index (grade 1–3).

In the SBR grading system, grades I and III clearly have defined those patients with low and high risk for relapse, respectively. However, it is well known that more than 50% of the patients fall into the intermediate risk category, grade II, which provides essentially no useful prognostic information for those patients.

To improve the assignment of patients to specific risk groups, a modified grade (MSBR), with five categories ordered according to the degree of malignancy, has been built from the nuclear pleomorphism and the mitotic index of the SBR grade. In combination with clinical stage, MSBR was found to be a prognostic indicator with high discriminatory power and caused the SBR grade to lose its significance.

The first three categories of this MSBR may be gathered to designate low risk patients, whereas the last two categories, once combined, contain the entire SBR grade III plus 57% of the SBR grade II tumors, and reliably identify high risk node negative patients [22].

The MSBR grade (modified Scharff–Bloom–Richardson) is arrived by evaluating the cancer for the following two parameters in order to sum or add-up the designated points in order to produce an Arabic numerical sum score [23, 24].

Tubules formation (a pattern or architectural parameter assessing cellular organization) is not a factor in this system.

Nuclear (size/shape) variation (a cytological/cellular parameter, Bloom–Richardson grade):

- 1 point, if only mild nuclear enlargement, no/mild darkening of chromatin (nuclear DNA/chromosomes), no/mild variation of nuclear shapes and sizes;
- 2 points, if moderate such changes;
- 3 points, if nuclei quite large, or bizarre, or have prominent nucleoli, and are quite dark (hyperchromatic).

Mitotic activity (a growth rate parameter, which is determined in the tumor area showing the fastest growth rate, usually the tumor periphery, counting the number of mitotic figures in ten high-power microscopic fields –400×).

- 1 point, 0–1 mitotic figure per high-power field (400×);
- 2 points, 2 mitotic figures per high-power field (400×);
- 3 points, 3 mitotic figures per high-power field (400×).

Quantification of tumor vascularity

Microvessel counts and density scoring were performed manually as a single microvessel count by light microscopy in areas of invasive tumor, without any knowledge of the subjects' previous investigations or clinical outcome, using a procedure on the basis of a modification of the method by Weidner N *et al.* [9]

The slides from each tumor were at first scanned at 40× magnification, using a light microscope Olympus BX60 to select areas with the densest vascularization (hot spots). Normal mammary tissue, large areas of inflammation, granulation tissue, and tumor necrosis were excluded.

Vascularity was defined by the number of microvessels (capillaries and small venules) per area counted in the fields of highest vascular density ("hot spots") at 400× magnification.

After the individuation of the hot spots within the tumor, three adjacent, non-overlapping fields from each section were selected using a high-power magnification (×40 objective, and ×10 ocular, 0.152 mm^2 per field).

The count performed was the field thought to contain the highest number of microvessels found at low magnification, and each subsequent count was the field thought to be the next highest. MVD was quantified as the sum vessel count of the three fields (3 × 0.152 mm^2) from each tumor.

Microvessel counts and density scoring were repeated "blind" four months later and no discrepant results were found.

All microvessel counts were standardized. The standardized microvessel score was expressed as counts per square millimeter and was obtained by dividing the actual count by the size of three microscope field (0.456 mm^2).

Statistical analysis

Descriptive statistics compared the microvesel density between groups defined by MSBR grade (1, 2, 3, 4, 5) or by risk – low risk patients (MSBR 1, 2, 3) *versus* high risk patients (MSBR 4, 5).

Results are reported as mean ± standard deviation, medians and ranges for the microvessel counts performed for each subsets.

A P-value equal to or less than 5% was considered statistically significant.

Independent group *t*–tests were used to compare the two patient groups on both the continuous and the ordinal measures. χ^2 tests of independence or Fisher's exact test was used to compare the two groups in regard to the categorical data. *One-way ANOVA* was used when more than two groups of microvessel counts were compared.

If the *t* value that is calculated is above the threshold chosen for statistical significance (usually the 0.05 level), the null hypothesis that the two groups do not differ is rejected in favor of an alternative hypothesis, which typically states that the groups do differ.

Results

A total of 158 women met the eligibility criteria for this report. The mean age at diagnosis of breast cancer was 59.5 (SD: ± 12.27481; range 26 to 93 years) for all patients.

The MVD ranged from 19.73684 to 72.36842 microvessels per mm^2 (median 30.70175, mean ±SD: 35.29591 ± 11.52149) for all patients. Thus, the cutoff was defined to be less than 30.70175 microvessels per mm^2 at 400× magnification. In this study low-MVD was defined as less than 30.70175 microvessels per mm^2 and high-MVD at least 30.70175 microvessels per mm^2.

In total, there were 53 (33.55%) patients in the low-MVC group and 105 (66.45%) in the high-MVC group, nine case in the low-MVC group and five in the high-MVC group in MSBR one patients, 39 cases in the low-MVC group and 39 in the high-MVC group in MSBR two patients, five cases in the low-MVC group and 49 in the high-MVC group in MSBR three patients, 0 cases in the low-MVC group and 10 in the high-MVC group in MSBR four patients, 0 cases in the low-MVC

group and two in the high-MVC group in MSBR five patients.

The median microvessel density was 26.31579 microvessels per mm^2 (range: 19.73684–30.70175 microvessels per mm^2, mean ±SD: 26.62907 ± 3.634831) in MSBR one patient (Figure 1).

The median microvessel density was 29.60526 microvessels per mm^2 (range: 19.73684–63.59649 microvessels per mm^2, mean ±SD: 29.88641 ± 8.534531) in MSBR two patients (Figure 2).

The median microvessel density was 41.66667 microvessels per mm^2 (range: 24.12281–63.59649 microvessels per mm^2, mean ±SD: 41.82911 ± 9.989301) in MSBR three patients (Figure 3).

The median microvessel density was 50.4386 microvessels per mm^2 (range: 43.85965–72.36842 microvessels per mm^2, mean ±SD: 52.41228 ± 8.979632) in MSBR four patients (Figure 4).

The median microvessel density was 44.95614 microvessels per mm^2 (range: 32.89474 and 57.01754 microvessels per mm^2, mean ±SD: 44.95614 ± 17.0574) in MSBR five patients (Figure 5).

When the mean values of MVD of the various groups defined by MSBR grade were compared, significant difference has been noted (P = 2.61E–17, *One-way ANOVA test*) (Table 2 and Figure 6).

Table 2 – *Correlation of groups defined by MSBR grade with MVD in 158 patients with breast carcinoma*

MVD	MSBR grade					Total	*P* value*
	1	*2*	*3*	*4*	*5*		
Low	9	39	5	0	0	53	3.25209E–07
High	5	39	49	10	2	105	
Total	14	78	54	10	2	158	

**The χ^2 was used to evaluate the correlation between MSBR grade and MVD.*

When tumors were classified by risk, 53 cases in the low-MVC group and 93 in the high-MVC group in low-risk patients (MSBR 1, 2, 3) and 0 cases in the low-MVC group and 12 in the high-MVC group in high risk patients (MSBR 4, 5).

When tumors were classified by low and high-risk patients the median microvessel density was 30.70175 microvessels per mm^2 (range: 19.73684–63.59649 microvessels per mm^2, mean ±SD: 33.99123 ± 10.65425) in low-risk patients (MSBR 1, 2, 3), and 50.4386 microvessels per mm^2 (range: 32.89474–72.36842 microvessels per mm^2, mean ±SD: 51.16959 ± 10.04225) in high-risk patients (MSBR 4, 5).

When the mean values of MVD of the groups defined by risk were compared, significant difference was noted (P = 7.68E–05, t-Test: *Two-Sample Assuming Unequal Variances*).

When tumours were classified as high- or low-MVD, based on a cut-off value (30.70175 microvessels per mm^2), cases with high MVD were significantly more numerous in MSBR four patients.

MVD did show a relationship with groups defined by MSBR grade (P = 3.25209E–07, χ^2 test) or with tumor grading risk (P = 2.54181E–06, χ^2 test).

Discussions

Several studies have shown that the mitotic count is the most important constituent of histological grade [21, 25], but there are well known problems with reproducibility of grading because of the lack of strict protocols [26–29]. In different studies, a highly standardized way of assessing the mitotic activity index (MAI; counting at ×400, magnification in an area of 1.6 mm^2, in the highest proliferative invasive area in the periphery of the tumor) provided a very strong prognostic factor, with additional prognostic value to tumor size and lymph node status in several retrospective studies [30–33] and two prospective studies [34, 35].

Several other groups from different countries have confirmed the prognostic value of mitosis counting in primary invasive breast cancer [36–38], including prospective studies [39]. Elkhuizen PH *et al.* found that patients who had undergone breast conserving treatment and had a recurrence after an interval of more than two years, but who had a high mitotic count, had an equally poor prognosis as those patients with local recurrence detected after a short interval [40]. The threshold in the different studies varies slightly, but there seems to be a consensus threshold at about 10–12 mitosis/2 mm^2, as in histological grading. Mitosis counting in lymph node metastases also provides some prognostic value [41].

Only a few smaller studies failed to reveal prognostic value [42, 43]. In several studies, mitotic count has been shown to have additional prognostic value to tumor size and lymph node status; a combination denoted the multivariate prognostic index [31, 44–48].

Lymph node negative patients with breast cancer who have tumours between 1 and 3 cm and a MAI lower than 10/1.6 mm^2 receive no adjuvant treatment, whereas patients with a MAI ≥10/1.6 mm^2 receive adjuvant chemotherapy and/or endocrine treatment, depending on their steroid receptor status. In addition, the *College of American Pathologists*' consensus statement 1999 mentions mitotic figure counting as a category I prognostic factor for breast cancer, [49] and the mitotic count has also been recognized by the UICC as an "essential prognostic factor" [50].

The onset of angiogenic activity does not require that all tumor cells become angiogenic. In fact, tumors appear to be heterogeneous for angiogenic activity. Consequently, it is imperative to determine microvessel density in the areas of the most intense neovascularity (i.e., at the neovascular "hotspot"). The highly angiogenic tumor cells are the likely source of growing metastatic foci. Also, an increase in the proportion of angiogenic tumor cells within a primary tumor is one mechanism by which overall neovascularization could increase. Indeed, those breast carcinomas from the current study showing the highest microvessel counts likely contained higher percentages of angiogenic cells than the tumors with low counts. Moreover, this mechanism provides an explanation for the strong association of increasing neovascularization with increased risk of metastasis [6].

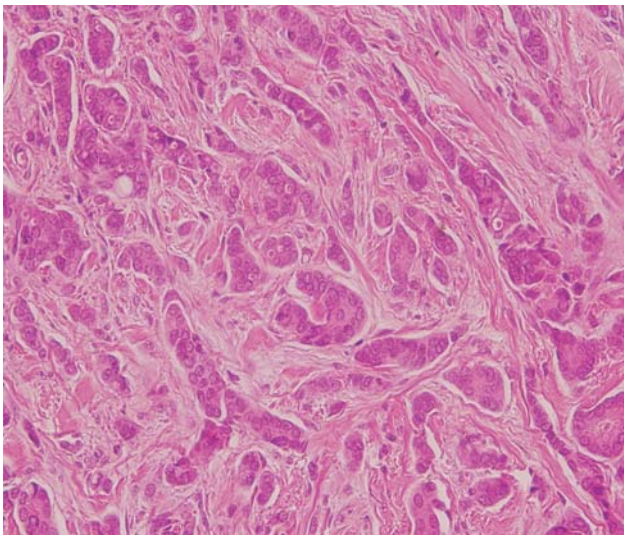

Figure 1 – *80-year old women with invasive ductal carcinoma, MSBR 1 (HE stain, ×400)*

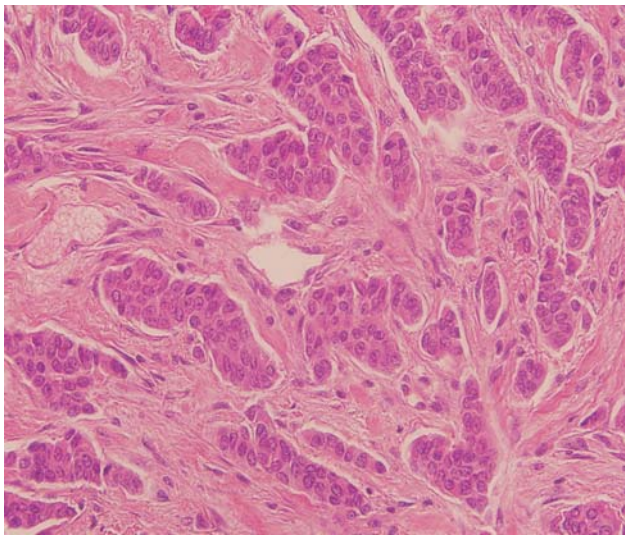

Figure 2 – *74-year old women with invasive ductal carcinoma, MSBR 2 (HE stain, ×400)*

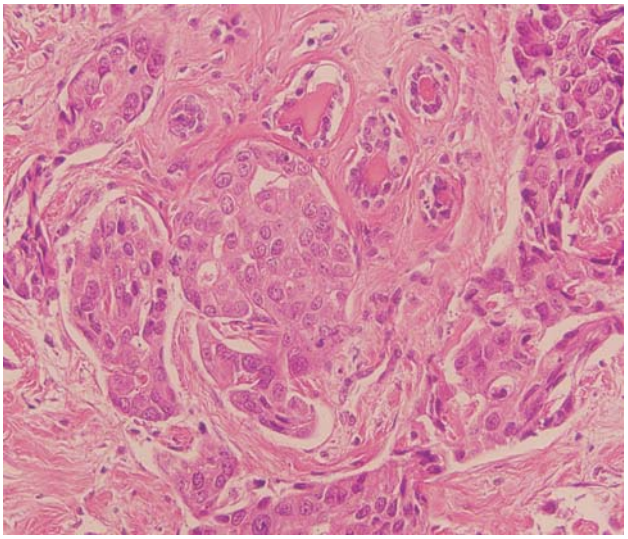

Figure 3 – *47-year old women with invasive ductal carcinoma, MSBR 3 (HE stain, ×400)*

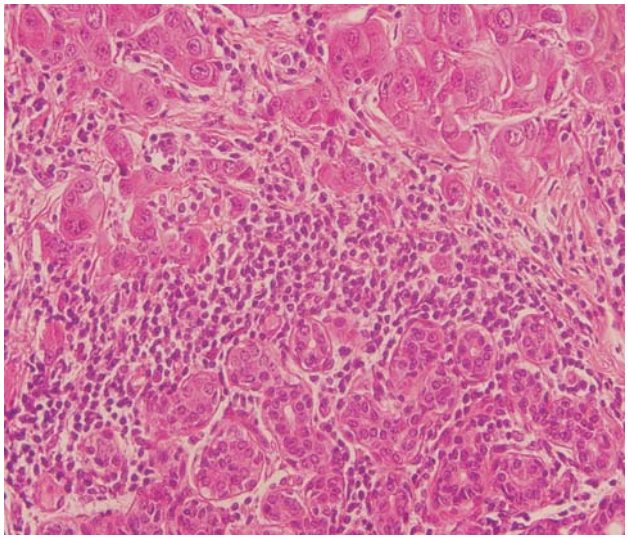

Figure 4 – *38-year old women with invasive ductal carcinoma, MSBR 4 (HE stain, ×400)*

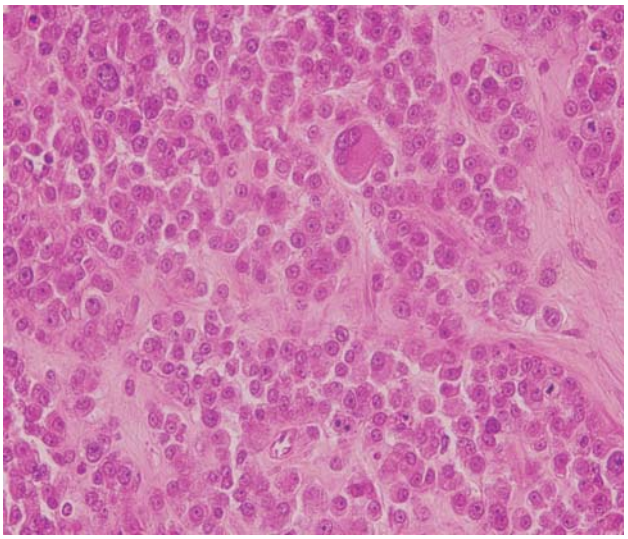

Figure 5 – *65-year old women with invasive atipical lobular carcinoma, MSBR 5 (HE stain, ×400)*

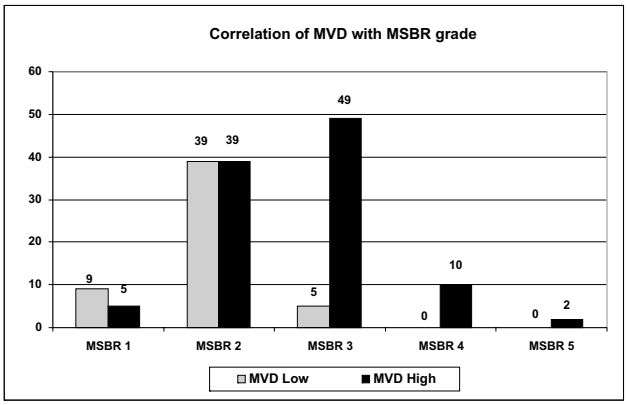

Figure 6 – *Number of tumors with low and high microvessel density as a function of MSRB grade*

Furthermore, tumor angiogenesis can facilitate metastatic spread in other ways. For example, newly proliferating capillaries have fragmented basement membranes and are leaky, making them more accessible to tumor cells than mature vessels. In addition, the invasive chemotactic behavior of endothelial cells at the tips of growing capillaries is facilitated by their secretion of collagenases and plasminogen activator. These degradative enzymes may also facilitate the escape of rumor cells into the tumor neovasculature. And, it has been shown that greater numbers of tumor vessels increase the opportunity for tumor cells to enter the circulation [51].

The results reported here indicate that the extent of angiogenesis in human breast carcinoma correlates with tumoral grade. The present findings show that the quantitation of tumor angiogenesis in the primary tumor at the time of first diagnosis may be useful in predicting the prognosis of patients. Such information might prove valuable in deciding whether to administer adjuvant therapy to node-negative patients with breast carcinoma, a subject of considerable controversy.

Conclusions

Neovascularization permits, but does not guarantee, progressive tumor spread. Angiogenesis and proliferation plays an important role in the clinical behavior of invasive breast cancer. Increased MVD and proliferation correlates strongly with poor prognosis, irrespective of the methodology used. In general, however, little attention has yet been paid to the value of MVD and proliferation in predicting response to treatment. A prospective comparison between the prognostic and predictive value of microvessel density and mitosis counting would be of great interest.

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