

ORIGINAL PAPER

Angiogenesis and tumor histologic type in primary breast cancer patients: an analysis of 155 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, which may also permit metastasis. To investigate how tumor angiogenesis correlates with tumor histologic type in breast carcinoma diagnosed on core biopsy, microvessels were counted (and graded the density of microvessels) within the initial invasive carcinomas of 155 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 groups defined by histological type were compared, no significant difference was noted ($P = 0.060253$). When tumors 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. MVD did show a relationship with groups defined by tumor histological type ($P = 0.003101$). Assessment of tumor angiogenesis may therefore prove valuable in selecting patients with early breast carcinoma for aggressive therapy.

Keywords: angiogenesis, breast carcinoma, histologic type, needle breast core biopsy.

Introduction

Invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) are the major histological types of invasive breast cancer among women of different races worldwide, ranging from 47 to 79% and 2 to 15%, respectively [1].

The two tumor subtypes are distinguished based on their histology, with ductal tumors tending to form glandular structures, whereas lobular tumors are less cohesive and tend to invade in single file [2, 3].

Although histologically disparate, these tumor types show clinical similarities and differences. Characteristics such as tumor site, size, grade, and stage at presentation are similar for both types [4]. ILCs often present with subtler signs on physical examination and mammography due to their characteristic histology and absence of a sclerotic tissue reaction. In contrast to a mammographic mass, asymmetric density or architectural distortion are the predominant mammographic signs in more ILCs than IDCs, whereas malignant calcifications are less frequent in ILCs [5].

Although treatment for stage-matched ductal *versus* lobular tumors is similar [6, 7], some studies suggest that metastatic patterns differ between lobular and ductal tumors [8, 9], and lobular tumors may be less responsive to neoadjuvant therapy [10].

The metastatic patterns of IDC and ILC are clearly different, with gastrointestinal, gynecologic, and peritoneal-retroperitoneal metastases, particularly to

endocrine-related sites such as adrenal glands and ovaries, markedly more prevalent in ILCs [9, 11–13].

IDC and ILC are managed similarly, but whether overall survival rates of patients differ is controversial [4, 14, 15]. Such studies suggest that lobular tumor development and progression may follow a distinct pathway from ductal tumors.

Unfortunately, most breast cancer researches has focused almost exclusively on the ductal subtype.

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 [16, 17]. 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 [18–22]. However, not all studies in this field have observed such important clinical correlations to MVD [23, 24]. 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 histopathologic findings at surgery [25–29].

Purpose

This retrospective study was to evaluate the correlations between intratumoral microvessel density (MVD) and histological type, 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

Selection of cases

The histologic slides of nonpalpable, mammographically detected lesions in which percutaneous stereotactic biopsy was performed from January 2004 until December 2004 in SAPAG HautePierre, Strasbourg, France, were retrospectively reviewed.

Lesions were defined as nonpalpable 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 in this study was: female sex, age older than 21 years, not pregnant, suspicious lesion of the breast (mammography), patient with node-negative breast cancer, recommendation for excisional after mammography.

Mammographic lesions were categorized according to the Breast Imaging Reporting and Data System (BI-RADS) developed by the American College of Radiology [30].

Biopsy procedure

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) performed stereotaxic localization.

The core needle biopsy was performed by first cleansing the skin overlying the lesion with alcohol; this was 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. 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 eight) per lesion. To document the presence of calcification the core specimens were radiographed. Then the core specimens were fixed in 10% formalin, paraffin embedded, sectioned, leveled $\times 3$, and stained with Hematoxylin and 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 same senior pathologist (SAPAG) in almost all cases made the original diagnosis of invasive malignancy. For these cases, Hematoxylin and 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. Invasive tumors were classified by histologic type, according to the criteria outlined in the *World Health Organization Classification of Tumours* [31].

Immunohistochemical evaluation and scoring

Many investigations suggest that E cadherin (E-CD) protein expression is lost in ILC but not IDC of the breast [32–35]. E-CD is a calcium-dependent, epithelial-specific cell-cell adhesion molecule who is reduced or lost expression is associated with tumor dedifferentiation and increased metastatic potential in human carcinomas [36]. Lehr HA *et al.* found that IDC express E-CD in a similar peripheral-predominant immunostaining pattern, while all ILCs are negative for E-CD, suggesting a role for E-CD in the architectural organization of the cytoskeletal scaffolding within the tumor cells [37]. Acs G *et al.* described E-CD as a useful diagnostic tool strongly specific for tumors of ductal origin. They found that all in situ carcinomas with mixed ductal and lobular features demonstrated complete loss of staining [38].

A small proportion of intraepithelial neoplasias cannot be easily separated into ductal or lobular subtypes based on pure H&E morphology. Using immunostains for E-cadherin and ck34 β E12, some of these will qualify as ductal (E-cadherin+, ck34 β E12-), some as lobular (E-cadherin-, ck34 β E12+), while others are either negative for both markers (negative hybrid) or positive for both (positive hybrid) [39]. This group of lesions requires further evaluation as it may reflect a neoplasm of mammary stem cells or the immediate post-stem cells with plasticity and potential to evolve into either ductal or lobular lesion.

Invasive carcinomas with ductal and lobular features showed three staining patterns: complete or almost complete lack of membrane staining, uniform

membrane expression throughout the tumor, and focal loss of E-CD staining, which correlated with the histologic impression of focal lobular features [38].

Antibodies

For the detection of E-cadherin and Ck34 β E12 the mouse monoclonal antibodies (Novocastra, UK) were used. All the dilutions were done in phosphate buffered saline (PBS).

The antibodies, clones, dilutions, pretreatment conditions, and sources for immunohistochemical studies are listed in Table 1.

Table 1 – Antibodies used for immunohistochemistry

Antibodies	Clone	Source	Dilution	Staining	Pretreatment
E-cadherin	NCH-38	Novocastra	1:50	M	H
HMW Cytokeratin (human cytokeratins 1, 5, 10, and 14 in the Moll catalog)	34 β E12	Novocastra	1:200	Cyto	H

Cyto – cytoplasmic staining, M – membranous staining, H – heating, 0.01M citrate buffer (pH 6.0)

Immunohistochemical staining

Immunohistochemical staining was performed on 10% formalin-fixed deparaffinized sections using the streptavidin–biotin method.

Immunohistochemistry was performed using an automated immunostainer VENTANA (NexES) according to the manufacturer's instructions. This system uses capillary action to draw up reagents to cover the specimens on the specially prepared slides.

Briefly, 4 μ m sections were cut from the paraffin embedded blocks using a microtome. The glass slides were previously coated with poly-L-lysine. The sections were then incubated at 37°C overnight.

Thereafter, the sections were deparaffinized in xylene (30 minutes, twice), sequentially dehydrated by incubating in 1:1 xylene-alcohol mixture, 100% alcohol, 90% alcohol, 70% alcohol, 50% alcohol, 30% alcohol and 1 \times PBS (10 minutes each).

The slides were subjected to heat-induced epitope retrieval by immersing them in 0.01 M boiling citrate buffer (pH 6) in a pressure cooker for 3 minutes. They were subsequently cooled with the lid on for an additional 10 minutes.

After removing the lid, the entire pressure cooker was filled with cold running tap water for 2 to 3 minutes or until the slides were cool.

At 36°C, the stainer sequentially added an inhibitor of endogenous peroxidase, the primary antibodies (32 minutes), a biotinylated secondary antibody, an avidin-biotin-complex with horseradish peroxidase (30 minutes), 3,3'-diaminobenzidine (3,3'-diaminobenzidine tetrahydrochloride) (15 minutes).

The sections were counterstained with Mayer Hematoxylin, dehydrated, cleared in xylene, and mounted. The normal breast tissues adjacent to the tumor areas served as an internal control. Negative controls were obtained by staining protocols omitting

the first antibody, or by using nonimmune mouse sera in place of the first antibody.

Interpretation of staining results

In almost all cases immunoreactivity was evaluated semiquantitatively by the same senior pathologist (SAPAG). Immunoreactivity was re-evaluated semiquantitatively by one pathologist (SV) the interobserver concordance was more than 90%. Both pathologists were blinded to the clinicopathologic data and patients' outcome.

The type and distribution of immunostaining for E-cadherin and ck34 β E12 were recorded and compared to normal ductal breast epithelium present on the same slide.

The number of positive cells in 500 tumor cells within 4–6 microscopic fields at 400 \times magnification was counted.

Staining results were classified into four grades depending on the percentage of E-cadherin or ck34 β E12 positive cells; negative = 0 (no positive cells), low = 1+ (<15% positive tumor cells), moderate = 2+ (15–50% positive tumor cells), and diffuse = 3+ (>50% positive tumor cells).

Cells with clear intercellular and contiguous membranous (E-cadherin) or cytoplasmic (ck34 β E12) staining were scored as positive cells.

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.* [19].

The slides from each tumor were at first scanned at 40 \times 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 \times 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 \times objective and 10 \times ocular, 0.152 mm² 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 \times 0.152 mm²) 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 mm²).

Statistical analysis

Descriptive statistics compared the microvessel density between different histologic types. Results are reported as mean \pm 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 histological type of the breast primary tumor was recorded in Table 2.

Table 2 – Distribution of cases according to histologic type

Histologic type	Frequency
Invasive ductal carcinoma (IDC)	112 (70.89%)
Invasive lobular carcinoma (ILC)	30 (18.99%)
Invasive carcinoma with mixed duct and lobular features (IDLC)	13 (8.23%)
Tubular carcinoma (TC)	1 (0.63%)
Tubulo-lobular carcinoma (TLC)	2 (1.26%)

Of the entire study sample, three patients were excluded from further analysis because their number was insufficient: tubular carcinoma (1) and tubulo-lobular carcinoma (2).

E-cadherin protein expression is lost in ILC but not IDC of the breast (Figure 1). Double immunostain is positive for both E-cadherine (Figure 2) and ck34 β E12 (Figure 3), qualifying the lesion as a hybrid positive type that may suggest the diagnosis of invasive carcinoma with mixed duct and lobular features

The distribution of histologic types in this study was as follows: 112 (70.89%) invasive ductal carcinoma, 30 (19%) invasive lobular carcinoma and 13 (8.23%) invasive carcinoma with mixed duct and lobular features.

The MVD ranged from 19.73684 to 72.36842 microvessels per mm² (median 30.70175, mean \pm SD: 35.58291 \pm 11.44306) for all patients. Thus, the cutoff was defined to be less than 30.70175 microvessels per mm² at 400 \times magnification. In this study low-MVD was defined as less than 30.70175 microvessels per mm² and high-MVD at least 30.70175 microvessels per mm².

The median microvessel density was 35.08772 microvessels per mm² (range: 19.73684 – 63.59649 microvessels per mm², mean \pm SD: 36.90868 \pm 11.2896) in patients with invasive ductal, 30.70175 microvessels per mm² (range: 19.73684 – 72.36842 microvessels per

mm², mean \pm SD: 32.60234 \pm 12.50833) in patients with invasive lobular, 30.70175 microvessels per mm² (range: 21.92982 – 46.05263 microvessels per mm², mean \pm SD: 31.03913 \pm 7.89911) in patients with invasive carcinoma with mixed duct and lobular features.

In total, there were 50 (32.26%) patients in the low-MVC group and 105 (67.74%) in the high-MVC group, 31 cases in the low-MVC group and 81 in the high-MVC group in patients with invasive ductal carcinoma, 14 cases in the low-MVC group and 16 in the high-MVC group in patients with invasive lobular carcinoma and five cases in the low-MVC group, and eight cases in the high-MVC group in patients with invasive carcinoma with mixed duct and lobular features (Table 3, Figure 4).

Table 3 – Correlation of groups defined by tumor histologic type with MVD in 155 patients with breast carcinoma

MVD	Histologic type			Total (%)	P value*
	IDC	ILC	IDLC		
Low (%)	31 (27.68)	14 (46.67)	5 (38.46)	50 (32.26)	0.003101
High (%)	81 (72.32)	16 (53.33)	8 (61.54)	105 (67.74)	
Total (%)	112 (70.89)	30 (19)	13 (8.23)	155	

NOTE: Data are number of patients.

*The χ^2 was used to evaluate the correlation between histologic type and MVD. *P* < 0.05 indicates statistical significance.

When the mean values of MVD of the various groups defined by tumor histological type were compared, no significant difference was noted (*P* = 0.060253, One-way ANOVA test).

MVD did show a relationship with groups defined by tumor histological type (*P* = 0.003101, χ^2 test).

Discussions

The goal was to study the relationship between angiogenesis and tumor histologic type, which is in contrast to other studies that assessed angiogenesis as a prognostic factor.

It is believed that all breast carcinomas, including both IDC and ILC, start in the terminal ductal lobular unit (TDLU) [40–44]. The malignant epithelial cells in IDC or ILC may represent differences in cell of origin within the TDLU (progenitor cell differences) or differences in point when the cancer started during the TDLU lobular maturation process (type 1 lobule for IDC versus type 2 lobule for ILC). This might explain why we see some lobular carcinomas as a distinct subtype and others with more similar gene expression to ductal carcinoma – there may be a continuum in the occurrence of epithelial carcinomas within the TDLU or from cells derived during the continuum of the TDLU maturation process.

Recent research reported a disproportionate increase of ILCs in the United States and Europe, possibly associated with increased usage of combined hormone replacement therapy [45–49]. In the United States, ductal carcinoma incidence rates remained essentially constant from 1987 to 1999, whereas lobular carcinoma rates increased steadily, significantly increasing the proportion of breast cancer with a lobular component from 9.5 to 15.6% during that period.

Figure 1 – 56-years-old women with invasive lobular carcinoma and LIN2 (inset). Complete absence of E-cadherin immunoreactivity (immunoperoxidase stain for E-cadherin, 400×)

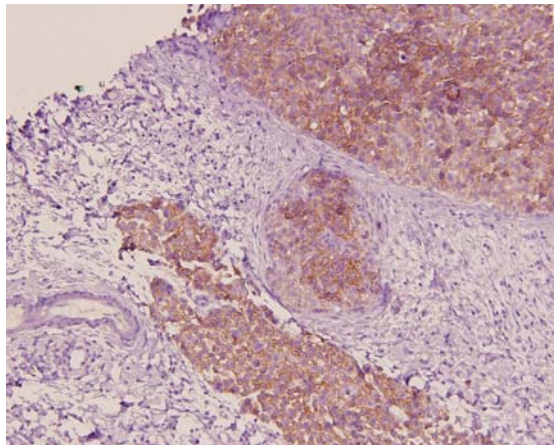
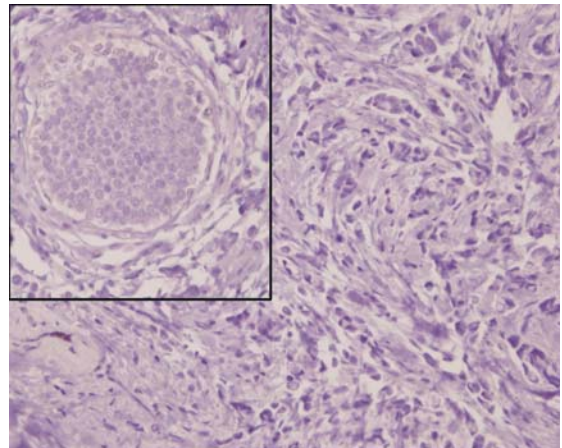


Figure 2 – 63-years-old women with invasive carcinoma with mixed duct and lobular features. Strong cell membrane immunoreactivity for E-cadherin (immunoperoxidase stain for E-cadherin, 200×)

Figure 3 – 63-years-old women with invasive carcinoma with mixed duct and lobular features. Strong cell cytoplasm immunoreactivity for 34βE12 (immunoperoxidase stain for 34βE12, 200×)

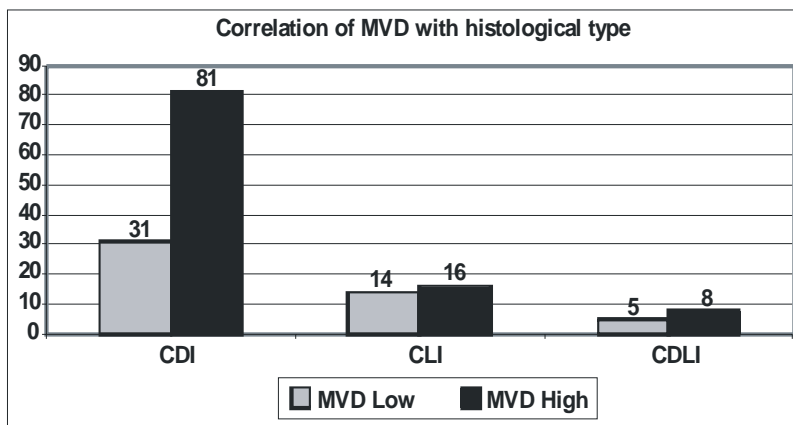
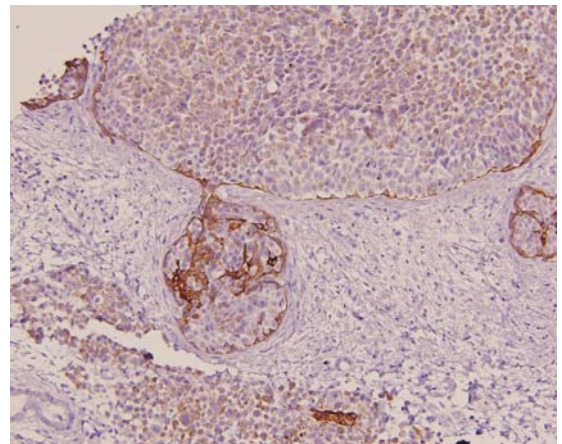


Figure 4 – Number of tumors with low and high microvessel density as a function of tumor histological type

In Switzerland, there has been a mean annual increase in the incidence of IDC of 1.2% compared with a mean annual increase of 14.4% for ILC during the period 1976–1999. Use of combined hormone replacement therapy, but not estrogen replacement therapy alone, seems to increase the risk of developing ILC by 2.7-fold, whereas the increase in IDC risk is only 1.5-fold [47].

Because ILC is the most rapidly increasing breast cancer phenotype, more difficult to diagnose than IDC, and yet is treated similarly to IDC, it is imperative to determine whether the clinical treatment of ILC should differ from IDC.

To individualize breast cancer treatment, a molecular understanding of the mechanisms that underlie the development of these two phenotypes is crucial.

The differential expression of cell adhesion molecules may account for some of the differences observed in invasion patterns of ILCs and IDCs. Single files or cords of small cohesive cells that diffusely infiltrate the stromal tissues characterize the classical invasion pattern of ILCs. In contrast, tubule formation or solid sheets of tumor cells characterize IDCs. Different morphological patterns of invasion may be associated with different adhesive properties between the malignant epithelial cells themselves and with surrounding tissues.

A recent study [50] analyzing IDCs with and without lymphovascular tumor emboli, assessed by E-cadherin immunostaining, suggested that, although this cell adhesion molecule is characteristically lost in ILCs and may even show loss in some high grade IDCs, observation of diffuse strong E-cadherin expression in IDCs may play a role in tumor growth as intravascular nests or emboli within lymphatics when lymphovascular invasion exists. In E-cadherin negative tumors that metastasize, individual cells may be able to migrate and travel in the vasculature and lymphatics differently than tumor emboli, which are composed of clusters of cells, potentially explaining the different patterns of distant metastatic spread in ILCs and IDCs.

Further studies would be required to explore whether the ductal-like ILCs should be treated similarly to other IDCs of their particular molecular phenotype (basal-like, luminal A or B, and ERBB2 expressing), and if different and type-specific treatment may be indicated for the typical ILCs.

At present, few papers report immunohistochemical markers useful for differentiation of lobular and ductal carcinomas of the breast or for differentiation of carcinomas derived from luminal and myoepithelial cells. Infiltrating lobular carcinoma (ILC) and infiltrating ductal carcinoma (IDC) are similar in many respects and their histologic features occasionally overlap [51–53]. Despite the many similarities, some clinical follow-up data and the patterns of metastasis suggest that ILC and IDC are biologically distinct [15, 54].

Tumorigenesis is a multistep process that requires the acquisition of certain properties common to all

tumors. These properties include uncontrolled cell division, suppression of senescence, inhibition of apoptosis and induction of angiogenesis [55]. The role of angiogenesis in the development and progression of human cancers has been widely studied [56]. New blood vessels can be stimulated to grow when factors that promote angiogenesis are up-regulated or those that inhibit angiogenesis are down-regulated [16, 57]. This investigation was stimulated by the conflicting conclusions of some studies.

In this study, cases with high MVD were significantly more numerous.

The *College of American Pathologists* considers angiogenesis to be a Category III breast cancer prognostic factor, meaning that it is a factor not sufficiently studied to demonstrate their prognostic value [58].

Further studies to determine whether a specific number of microvessels within the primary tumors of patients with breast carcinoma are predictive of occult metastasis is warranted, because this information could improve selection of patients for elective lymph node dissection and adjuvant chemotherapy.

☐ Conclusions

Neovascularization permits, but does not guarantee, progressive tumor spread. 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.

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