

ORIGINAL PAPER

Angiogenesis and ER/PR status in primary breast cancer patients: an analysis of 158 needle core biopsies

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Abstract

Formation of new blood vessels from a preexisting vascular bed (angiogenesis) is a complex multistep process, which may also permit metastasis. Estrogen and progesterone are important in breast tumorigenesis, and their effects on the breast are mediated by their respective receptors, the estrogen receptor (ER) and the progesterone receptor (PR). To investigate how tumor angiogenesis correlates with ER/PR status 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 ER/PR status were compared, significant difference was noted ($P = 1.57E-05$). When tumors were classified as high or low MVD, based on a cut-off value (30.70175 microvessels/mm²), cases with high MVD were significantly more numerous in patients with ER+/PR- status. MVD did show a relationship with groups defined by ER/PR status ($P = 0.000801$). Assessment of tumor angiogenesis may therefore prove valuable in selecting patients with early breast carcinoma for therapeutic hormonal therapy.

Keywords: angiogenesis, breast carcinoma, ER/PR status, needle breast core biopsy.

Introduction

It has been well documented that estrogen and progesterone are important in breast tumorigenesis [1–3] and their effects on the breast are mediated by their respective receptors, the estrogen receptor (ER) and the progesterone receptor (PR) [4–7].

ER and PR status are the only prognostic and predictive biomarkers recommended for routine clinical use in breast cancer by the American Society of Clinical Oncology and the College of American Pathologists [8, 9].

Both are relatively weak *prognostic* factors, but strong *predictive* factors for response to adjuvant and therapeutic hormonal therapy. The primary reason for measuring these biomarkers today is the latter. These recommendations, including the overview analysis of randomized clinical trials in early breast cancer are based on standardized ligand-binding assay methodology [10].

Furthermore, it has been hypothesized that hormone-related risk factors that reflect exposure to estrogen and progesterone may be predominantly associated with breast tumors that express ER and PR, but not with those lacking ER and PR expression [11–17].

Several epidemiological studies have examined this hypothesis by ER and PR status separately or jointly [18, 19] and a review from 2004 [20] concluded that early age at menarche, nulliparity, and delayed childbearing were associated with an increased risk for

receptor-positive breast cancer, but not with receptor-negative breast cancer.

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 mm³ is dependant upon the formation of new vasculature [21, 22]. 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 [23–27]. However, not all studies in this field have observed such important clinical correlations to MVD [28, 29]. 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 [30–34].

This retrospective study was to evaluate the correlations between intratumoral microvessel density (MVD) and steroid hormone receptors profile (ER and PR status jointly), in order to identify those tumors 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.

☐ Material and methods

The histological slides of non-palpable, mammographically detected lesions in which percutaneous stereotactic biopsy 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 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 excision after mammography.

Mammographic lesions were categorized according to the Breast Imaging Reporting and Data System (BI-RADS) developed by the American College of Radiology [35].

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 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, 1 to 8) 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–Eosin. Additional levels were requested, if necessary, for histological documentation of calcification.

The use of a polarizing lens assisted in the microscopic identification of microcalcification in some cases. Two pathologists retrospectively reviewed the histological 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.

Immunohistochemical evaluation and scoring

For the detection of estrogen receptor and progesterone receptor 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
Estrogen receptor	6F11	Novocastra	1 : 20	N	H
Progesterone receptor	1A16	Novocastra	1 : 100	N	H

N – nuclear staining, H – heating, 0.01M citrate buffer (pH 6.0)

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'-diamino-

benzidinetetrahydrochloride) (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 non-immune mouse sera in place of the first antibody.

Interpretation of immunostaining 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 clinico-pathologic data and patients' outcome.

The type and distribution of immunostaining for estrogen and progesterone receptors 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 $\times 400$ magnification was counted.

Immunostained slides were evaluated by light microscopy and the immunohistochemistry signal was scored using the so-called „Allred Score” [36].

Briefly, a proportion score was assigned representing the estimated proportion of positive staining tumor cells (0 = none; 1 = 1/100; 2 = 1/100 to $<1/10$; 3 = 1/10 to $<1/3$; 4 = 1/3–2/3; 5 = $\geq 2/3$). Average estimated intensity of staining in positive cells was assigned an intensity score (0 = none; 1 = weak; 2 = intermediate; 3 = strong). Proportion score and intensity score were added to obtain a total score that ranged from 0–8.

On the basis of these results, tumors were defined as ER(PR)-positive if their total IHC score was greater than 2 (corresponding to as few as 1% to 10% weakly positive cells) and ER (PR)-negative if their score was 0 or 2.

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.* [24]

The slides from each tumor were at first scanned at $\times 40$ 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 $\times 400$ 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 ($\times 40$ objective, and $\times 10$ 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 ($3 \times 0.152 \text{ mm}^2$) 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 and ER/PR status. Results are reported as mean \pm standard deviation, medians and ranges for the microvessel counts performed for each subset. 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 steroid hormone receptors profile of the breast primary tumor was recorded in Table 2.

Table 2 – Distribution of cases according to ER/PR status

Histologic type	Frequency
ER/PR ⁻	25 (15.82%)
ER ⁺ /PR ⁻	65 (41.14%)
ER ⁺ /PR ⁺	68 (43.04%)
ER/PR ⁺	0

Of the entire study sample, patients with ER⁻/PR⁺ were excluded from further analysis because their number was insufficient.

Among the 158 case patients, 25 (15.82%) were ER⁻/PR⁻, 65 (41.14%) were ER⁺/PR⁻ (Figures 1 and 2), and 68 (43.04%) were ER⁺/PR⁺ (Figure 3 and 4).

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

The median microvessel density was 43.85965 microvessels/mm² (range: 28.50877 – 72.36842 microvessels/mm², mean \pm SD: 44.73684 \pm 10.45986) in patients with ER⁻/PR⁻ status, 30.70175 microvessels/mm² (range: 19.73684 – 63.59649 microvessels/mm², mean \pm SD: 34.51417 \pm 10.99418) in patients with ER⁺/PR⁻ status, 30.70175 microvessels/mm² (range: 19.73684 – 63.59649 microvessels/mm², mean \pm SD: 32.57224 \pm 10.72509) in patients with ER⁺/PR⁺ status.

In total, there were 53 (33.54%) patients in the low-MVC group and 105 (66.46%) in the high-MVC group, one case in the low-MVC group and 24 in the high-MVC group in patients with ER⁻/PR⁻ status, 21 cases in the low-MVC group and 44 in the high-MVC group in patients with ER⁺/PR⁻ status and 31 cases in the low-MVC group and 37 in the high-MVC group in patients with ER⁺/PR⁺ status (Table 3 and Figure 5).

Table 3 – Correlation of groups defined by ER/PR status with MVD in 158 patients with breast carcinoma

MVD	Steroid hormone receptors profile			Total (%)	P value*
	ER/PR ⁻	ER ⁺ /PR ⁻	ER ⁺ /PR ⁺		
Low (%)	1 (4.00)	21 (32.31)	31 (45.59)	53 (33.54)	0.000801
High (%)	24 (96.00)	44 (67.79)	37 (54.11)	105 (66.46)	
Total (%)	25 (15.82)	65 (41.14)	68 (43.04)	158	

Note: Data are No. of patients.

*The χ^2 was used to evaluate the correlation between ER/PR status and MVD. $P < 0.05$ indicates statistical significance.

When the mean values of MVD of the various groups defined by ER/PR status were compared, significant difference was noted ($p = 1.57E-05$, One-way ANOVA test).

MVD did show a relationship with groups defined by ER/PR status ($p = 0.000801$, χ^2 -square test).

☒ Discussions

The goal was to study the relationship between angiogenesis and ER/PR status, which is in contrast to other studies that assessed angiogenesis as a prognostic factor. 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 [37].

The role of angiogenesis in the development and progression of human cancers has been widely studied [38]. New blood vessels can be stimulated to grow when factors that promote angiogenesis are up-regulated or those that inhibit angiogenesis are down-regulated [21, 39]. This investigation was stimulated by the conflicting conclusions of some studies.

Nearly 30 years ago, it was recognized that transcription of the progesterone receptor (PR) gene was regulated by estrogen in breast and reproductive tissues and that estrogen receptor positive (ER+) breast tumors that lacked PR expression were less responsive to endocrine therapy than those that express PR.

At that time, Horwitz KB and McGuire WL [40] hypothesized that PR loss was due to loss of ER activity, due to either low circulating estrogen in some older women or a nonfunctioning ER pathway [41].

This hypothesis, however, did not fully explain why some ER⁺/PR⁻ tumors respond to endocrine therapy, albeit at a lower frequency, than tumors that are both ER+ and PR+ (ER⁺/PR⁺). Later, it was recognized that ER and PR status are not always stable phenotypes and that they can in fact change over the natural history of the disease or as a consequence of endocrine treatment [42]. During tamoxifen therapy, levels of both PR and ER decrease but PR levels decrease more dramatically than ER levels, with up to half of the tumors completely losing PR expression as they develop tamoxifen resistance [43].

In patients with such tumors, the loss of PR translates into a more aggressive disease and worse overall survival, suggesting that other alterations in the molecular machinery driving tumor growth accompany the loss of PR receptor expression [44].

It was confirmed that tamoxifen is less efficacious in ER⁺/PR⁻ tumors than in ER⁺/PR⁺ tumors in both the metastatic and adjuvant treatment settings [45–50]. Comparing the efficacy of tamoxifen with that of the aromatase inhibitor anastrozole, showed overall that patients with ER⁺/PR⁺ tumors had a lower recurrence rate than those with ER⁺/PR⁻ tumors (7.6% versus 14.8%) [51].

This difference in overall recurrence was due largely to the lower efficacy of tamoxifen in the subgroup of patients with ER⁺/PR⁻ tumors; there was little difference in the recurrence rate of PR+ versus PR- tumors in patients who were treated with anastrozole.

The observation that patients with ER⁺/PR⁻ tumors respond nearly as well to anastrozole as those with ER⁺/PR⁺ tumors suggests that the ER signaling pathway is functional in many ER⁺/PR⁻ tumors and that these tumors are still dependent on estrogen for growth, despite having somewhat lower ER levels. Thus, the etiology of the ER⁺/PR⁻ phenotype, either de novo or acquired, cannot be attributed entirely to the nonfunctional ER hypothesis. Furthermore, ER activity has been observed in freshly prepared breast lysates of ER⁺/PR⁻ tumors [52].

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

☒ Conclusions

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

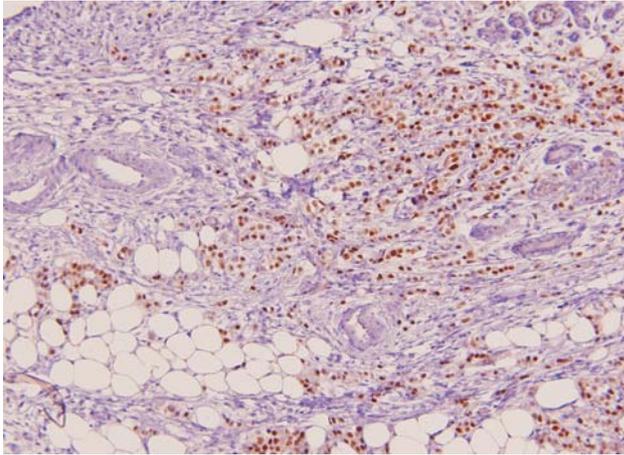


Figure 1 – 50-year old women with invasive lobular carcinoma. ER-positive cells showed a dark brown or black nuclear signal ($\times 200$). This tumor would get a total immunohistochemical score of 8 (proportion score [= 5] + intensity score [= 3])

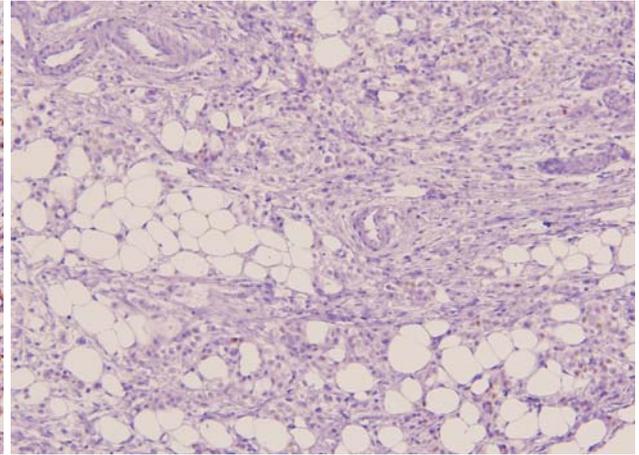


Figure 2 – 50-year old women with invasive lobular carcinoma. Complete absence of PR immunoreactivity ($\times 200$)

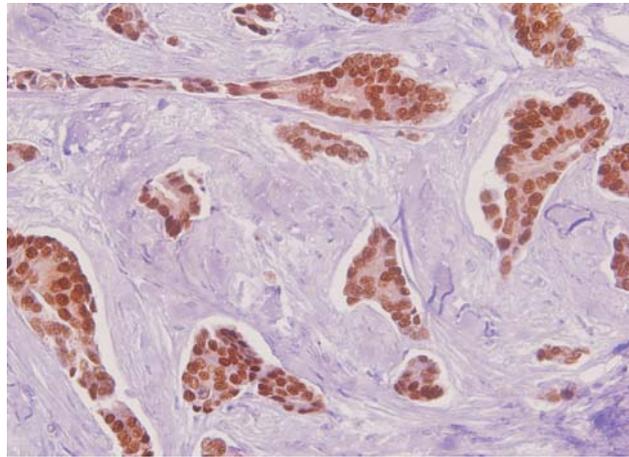


Figure 3 – 62-year old women with invasive ductal carcinoma. ER-positive cells showed a dark brown or black nuclear signal ($\times 400$). This tumor would get a total immunohistochemical score of 8 (proportion score [= 5] + intensity score [= 3])

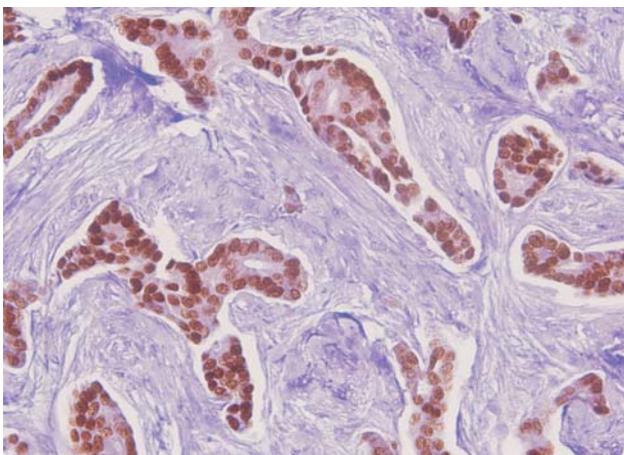


Figure 4 – 62-year old women with invasive ductal carcinoma. PR-positive cells showed a dark brown or black nuclear signal ($\times 400$). This tumor would get a total immunohistochemical score of 8 (proportion score [= 5] + intensity score [= 3])

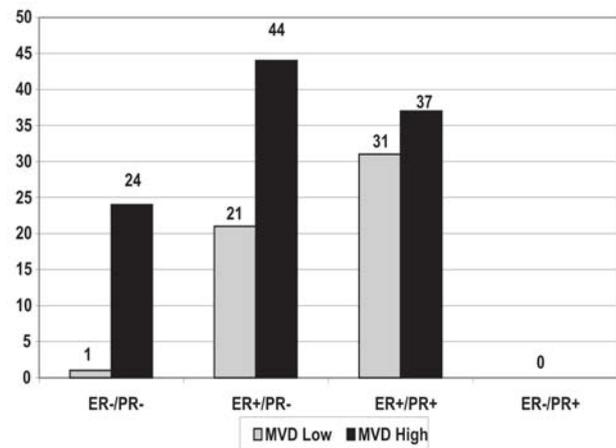


Figure 5 – Number of tumors with low and high microvessel density as a function of ER/PR status

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