ORIGINAL PAPER

Angiogenesis and c-*erb*B-2 (HER2/*neu*) overexpression status in primary breast cancer patients: an analysis of 158 needle core biopsies

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Abstract

Angiogenesis, formation of new blood vessels from a preexisting vascular bed is a complex multistep process, which may also permit metastasis. The subset of patients with breast cancer demonstrating a c-*erb*B-2 (HER2/*neu*)-positive status has aggressive tumors and a poor prognosis. To investigate how tumor angiogenesis correlates with c-*erb*B-2 (HER2/*neu*) overexpression 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 c-erbB-2 (HER2/neu) overexpression were compared, significant difference was noted (P = 0.014818). 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. MVD did show a relationship with groups defined by c-*erb*B-2 (HER2/*neu*) status (P = 0.002699) or c-*erb*B-2 (HER2/*neu*) score (P = 0.027589). The correlation of angiogenesis with c-*erbB*-2 (HER2/*neu*) overexpression may be a potential therapeutic target for the treatment and prevention of breast cancer, using antiangiogenic molecules.

***Keywords*:** angiogenesis, breast carcinoma, c-*erb*B-2 (HER2/*neu*), 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.

Progress in molecular biology has resulted in the identification and greater understanding of molecular markers that may have prognostic and predictive value for breast cancer patients. The human epidermal growth factor receptor-2 (c-*erb*B-2/HER2/*neu*) is one of the best characterized of such markers.

The c-*erb*B-2 (HER2/*neu*) gene is the human analog of the rat *neu* gene identified in rat neuroblastomas in the early 1980s [5–9]. This gene has been found to be amplified and/or overexpressed in approximately 25 to 30% of invasive breast cancers in humans, most commonly in invasive ductal carcinomas.

Located on chromosome 17q21, the c-*erb*B-2 (HER2/*neu*) gene encodes a 185 kD transmembrane glycoprotein with tyrosine kinase activity that functions as a growth factor receptor. This protein is a member of the epidermal growth factor receptor family of receptor tyrosine kinases, which includes epidermal growth factor receptor, HER2, HER3, and HER4. Ligand binding to one of these receptors results in the formation of homodimers and heterodimers. Dimerization is followed by phosphorylation, which, in turn, results in a cascade of downstream signaling events that are important for cell growth and maintenance of the transformed state.

Of note, although HER2 forms heterodimers with other members of the family on interaction with their ligands, it has no known ligand of its own and is therefore considered an orphan receptor. However, at very high levels of overexpression, c-*erb*B-2 (HER2/*neu*) may undergo spontaneous homo-dimerization, which can initiate downstream signaling, stimulating cell growth and maintaining cellular transformation. This may be clinically important in c-*erb*B-2 (HER2/*neu*)-overexpressing tumors.

Angiogenesis is the growth of new vessels from existing vasculature that occurs during development and in vascular remodeling in the adult [10, 11]. The microvasculature is a dynamic system that plays an important role in a variety of physiological and pathological processes and it switches between

quiescent and activated states. New vessels can arise by several processes: angiogenesis, vascular remodeling, and the recruitment of endothelial precursor cells from bone marrow and blood vessels [12].

Angiogenesis is a multistep process that depends upon cooperation and interaction between a variety of cells, growth factors, and components of the extracellular matrix. It requires the destabilization of existing vessels, increased permeability with extravasation of plasma proteins and enzymes into the surrounding stroma, changes in endothelial cell adhesion with endothelial cell migration, proliferation, survival, and stabilization of newly formed vascular channels. Vascular remodeling describes new vessel formation by the insertion of interstitial tissue columns into the vessel lumen, with subsequent growth of these columns and partitioning of the vessel. This process of intussusception occurs in normal developing organs and has been shown to occur in some carcinoma [13].

Co-option of endothelial progenitor cells from the circulation into new vessels is known to occur in development, but may also occur in adults. Angiogenesis is recognized as a key factor in the progression of invasive tumors, as enunciated in the "angiogenesis progression" hypothesis [14].

The pathways controlling the switch to an angiogenic phenotype in tumors are complex and poorly characterized but include hypoxia, genetic mutation, and stromal and inflammatory cell responses. There is evidence that changes in oncogene and tumor suppressor gene expression influence new vessel growth during tumor progression [15].

In many tumors, including breast cancer, areas of increased tumor cell proliferation are associated with areas of increased microvessel density ("hot spots") [16]. Not only do new vessels supply oxygen and nutrients to metabolically active tumor cells, but also there is strong evidence for the presence of reciprocal interactions between tumor cells and endothelial cells.

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 [17–21].

Purpose

This retrospective study was to evaluate the correlations between intratumoral microvessel density (MVD) and c-*erb*B-2 (HER2/*neu*) overexpression, 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.

Patients and methods

Selection of cases

The histologic slides of nonpalpable, mammographically detected lesions in which percutaneus 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 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 [22].

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 ×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.

Immunohistochemical evaluation and scoring

Antibody

Immunostaining for c-*erbB*-2 (HER2/*neu*) in primary carcinomas was performed using CB-11 (Novocastra, UK), a monoclonal antibody to the intracellular domain of the protein, and a streptavidin–biotin detection system (kit LSAB2; DAKO).

The antibody, clone, dilution, pretreatment conditions, and source for immunohistochemical studies are listed in Table 1.

Table 1 – ***Antibody used for immunohistochemistry***

Antibody	Clone	Source	Dilution	Staining	Pretreatment
c-*erb*B-2 (HER2/*neu*)	CB11	Novocastra	1:400	M	H

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

Immunohistochemical staining

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

Briefly, 4 μm 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 deparaffinised 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 × PBS (10 minutes each).

The slides were subjected to heat-induced epitope retrieval by immersing them in 0.01 M boiling citrate buffer (pH 6.0) 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 cooled.

Slides were put into the sequence immunostaining system (Shandon, Uncorn, United Kingdom) and rinsed with APK Wash. Slides were then incubated overnight with the monoclonal antibody directed against HER-2 protein (diluted 1:400) at room temperature. During day two, slides were incubated for 20 minutes at room temperature with biotinylated goat anti-mouse and goat anti-rabbit immunoglobulin (Dako), followed by another 20-minutes incubation with horseradish-peroxidase:streptavidin–biotin complex (Dako). Between the subsequent steps, the slides were rinsed with APK Wash for 6 minutes.

3,3'-Diaminobenzidine (Dako) was used as the chromogen. Slides were rinsed extensively in tap water and, finally, were counterstained in Harris's Hematoxylin, dehydrated through a series of alcohols, and mounted. For negative control, the tissue was processed in the same way, except that the primary antibody was omitted (buffer substitution).

Negative (normal breast tissue) and positive (strongly positive carcinoma) control slides were included with each assay.

Under these conditions, normal epithelial cells were not stained, and thus represented an internal negative control (Figure 1).

Interpretation of staining results

In almost all cases the same senior pathologist (SAPAG) evaluated immunoreactivity semiquantitatively. Immunoreactivity was re-evaluated semiquantitatively by one pathologist (S.V.); the interobserver concordance was more than 95%. Both pathologists were blinded to the clinicopathologic data, patients' outcome and status of the gene amplification.

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

For the determination of c-*erbB*-2 (HER2/*neu*) protein overexpression, only the membrane staining intensity of the invasive component in hot spot areas was considered. Cytoplasmic staining was considered nonspecific staining and was not included in the assessment of membrane staining intensity.

Either the percentage of tumor cells showing a complete or a partial membranous pattern was scored in each case. The intensity of membrane staining was assessed as strong, moderate or weak.

The score system used for the interpretation of staining is listed in Table 2.

Table 2 – ***Score system used to assess c-erbB-2 (HER2/neu) status***

c-*erbB*-2 (HER2/*neu*) status	Staining pattern	Score
Negative	No staining or weak membrane staining in <10% of tumor cells	0
Negative	Weak membrane staining in >10% of tumor cells	1+
Positive	Moderate membrane staining in >10% of tumor cells	2+
Positive	Strong membrane staining in >10% of tumor cells	3+

Samples were classified as positive if they had a score of 2+ or 3+ and as negative if they had a score of 0 or 1+.

Strong membranous staining – c-*erbB*-2 (HER2/*neu*) protein high overexpression (3+) – was easily seen at low magnification (using 10× or 20× objectives) (Figure 2).

Moderate staining – c-*erbB*-2 (HER2/*neu*) protein weak overexpression (2+) – was less intensive than strong staining but easily visible using 20× and 40× objectives (Figure 3).

Weak staining – c-*erbB*-2 (HER2/*neu*) protein no overexpression (0, 1+) – was pale and not clearly visible at 400× magnification (Figure 4). Partial membrane staining was defined as incomplete positivity around the membrane of a cell.

To aid in the differentiation of 1+, 2+, and 3+ staining, the Dako's Atlas for the Interpretation of HercepTest, with representative pictures of the staining intensities was used.

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 based on a modification of the method by Weidner N *et al.* [23].

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, nonoverlapping 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 c-*erb*B-2 (HER2/*neu*) status or by c-*erb*B-2 (HER2/*neu*) score (0, 1+, 2+, 3+).

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 c-*erb*B-2 (HER2/*neu*) profile and score of the breast primary tumors were recorded in Tables 3 and 4.

Table 3 – ***Distribution of cases according to c-erbB-2 (HER2/neu) status***

c-*erb*B-2 (HER2/*neu*) status	Frequency
Negative	137 (86.71%)
Positve	21 (13.29%)

Table 4 – ***Distribution of cases according to c-erbB-2 (HER2/neu) score***

c-*erb*B-2 (HER2/*neu*) score	Frequency
0	125 (79.11%)
1+	12 (7.60%)
2+	4 (2.53%)
3+	17 (10.76%)

Among the 158 case patients, 21 (13.29%) were c-*erbB-2* (HER2/*neu*)-positive status (Figures 2 and 3), and 137 (86.71%) were c-*erbB-2* (HER2/*neu*)-negative status (Figures 4 and 5).

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. The median microvessel density was 30.70175 microvessels per mm^2 (range 19.73684 – 63.59649 microvessels per mm^2, mean ± SD 34.39941±11.37256) in patients with c-*erbB-2* (HER2/*neu*)-negative status, 39.47368 microvessels per mm^2 (range 19.73684 – 72.36842 microvessels per mm^2, mean ± SD 41.14453±11.0066) in patients with c-*erbB-2* (HER2/*neu*)-positive status.

In total, there were 53 (33.54%) patients in the low-MVC group and 105 (66.46%) patients in the high-MVC group, 52 cases in the low-MVC group and 85 cases in the high-MVC group, in patients with c-*erbB-2* (HER2/*neu*)-negative status, one case in the low-MVC group, and 20 cases in the high-MVC group, in patients with c-*erbB-2* (HER2/*neu*)-positive status (Table 5, Figure 6).

Table 5 – ***Correlation of groups defined by c-erbB-2 (HER2/neu) status with MVD in 158 patients with breast carcinoma***

MVD	c-*erb*B-2 (HER2/*neu*)		Total (%)	*P* value*
	negative	*positive*		
Low (%)	52 (37.96)	1 (4.76)	53 (33.54)	0.002699
High (%)	85 (62.04)	20 (95.24)	105 (66.46)	
Total (%)	137 (86.71)	21 (13.29)	158	

Data are no. of patients. *The χ^2 was used to evaluate the correlation between c-*erb*B-2 (HER2/*neu*) status and MVD. $P < 0.05$ indicates statistical significance.

When the mean values of MVD of the groups defined by c-*erb*B-2 (HER2/*neu*) status were compared, significant difference was noted (P = 0.014818, t-Test: Two-Sample Assuming Unequal Variances).

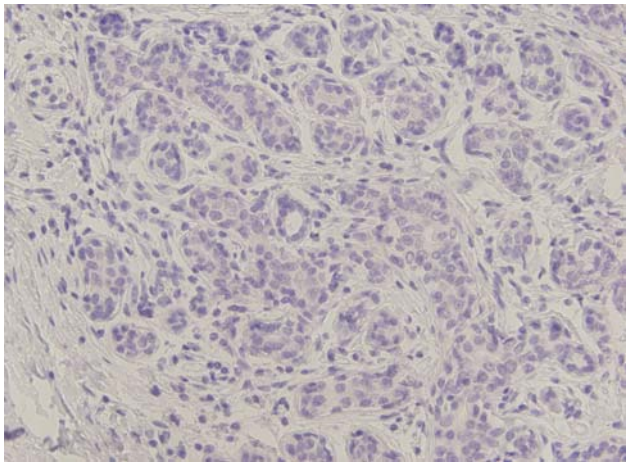

Figure 1 – ***44-years old women (B.E.): normal epithelial cells. Complete absence membranous pattern, internal negative control (c-erbB-2 mouse monoclonal antibody, 400×)***

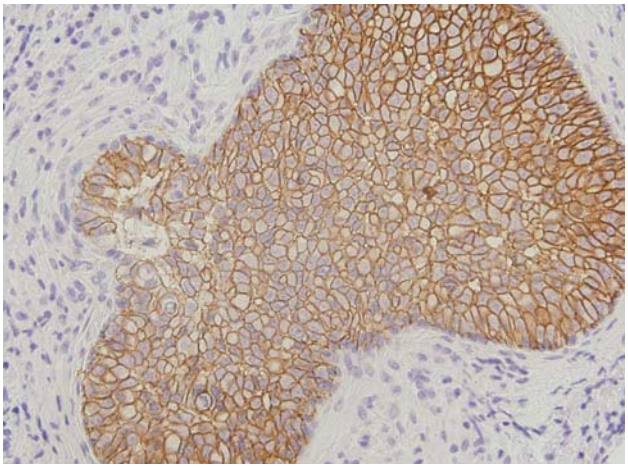

Figure 2 – ***44-years old women (B.E.) with invasive ductal carcinoma. 3+ score. Strong, complete membranous pattern (cerbB-2 mouse monoclonal antibody, 400×)***

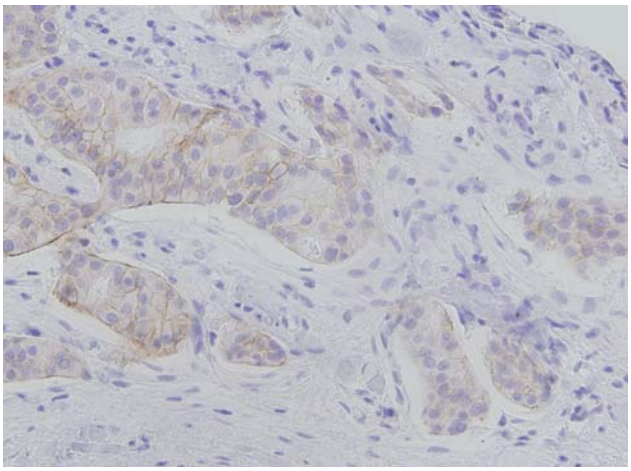

Figure 3 – ***67-years old women (C.M.) with invasive ductal carcinoma. 2+ score. Complete membranous pattern, moderate intensity (c-erbB-2 mouse monoclonal antibody, 400×)***

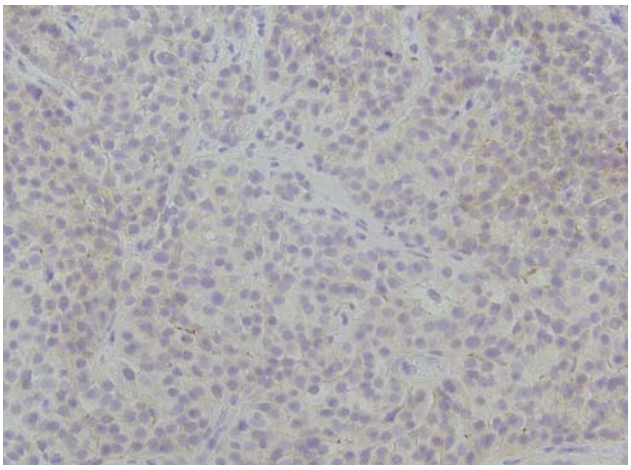

Figure 4 – ***88-years old women (L.A.) with invasive ductal carcinoma. 1+ score. Faint, focal, and incomplete membranous pattern (c-erbB-2 mouse monoclonal antibody, 400×)***

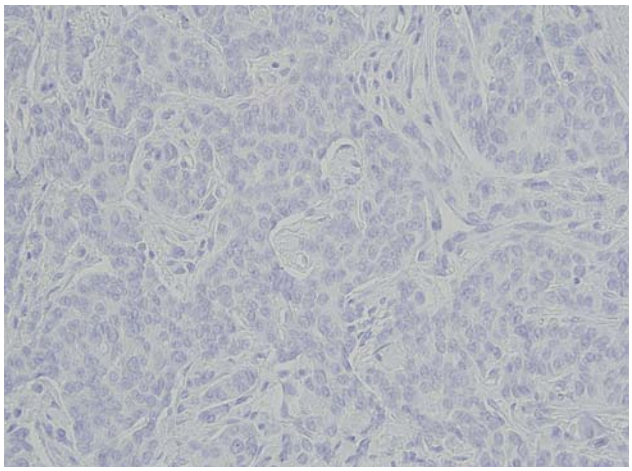

Figure 5 – ***62-years old women (S.A.) with invasive ductal carcinoma. 0+ score. Complete absence membranous pattern (c-erbB-2 mouse monoclonal antibody, 400×)***

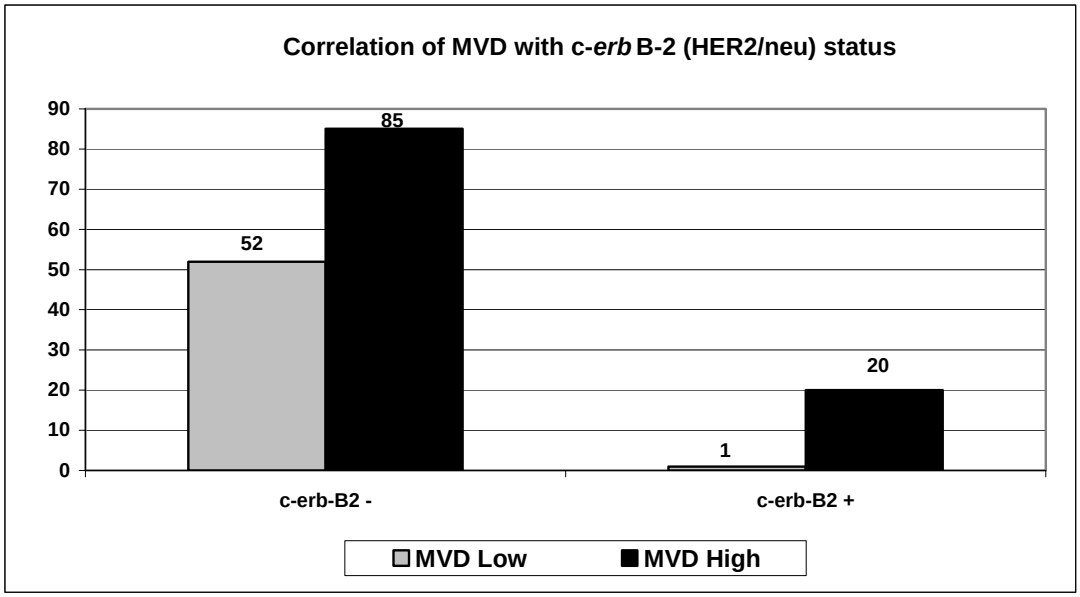

Figure 6 – ***Number of tumors with low and high microvessel density as a function of c-erbB-2 (HER2/neu) status***

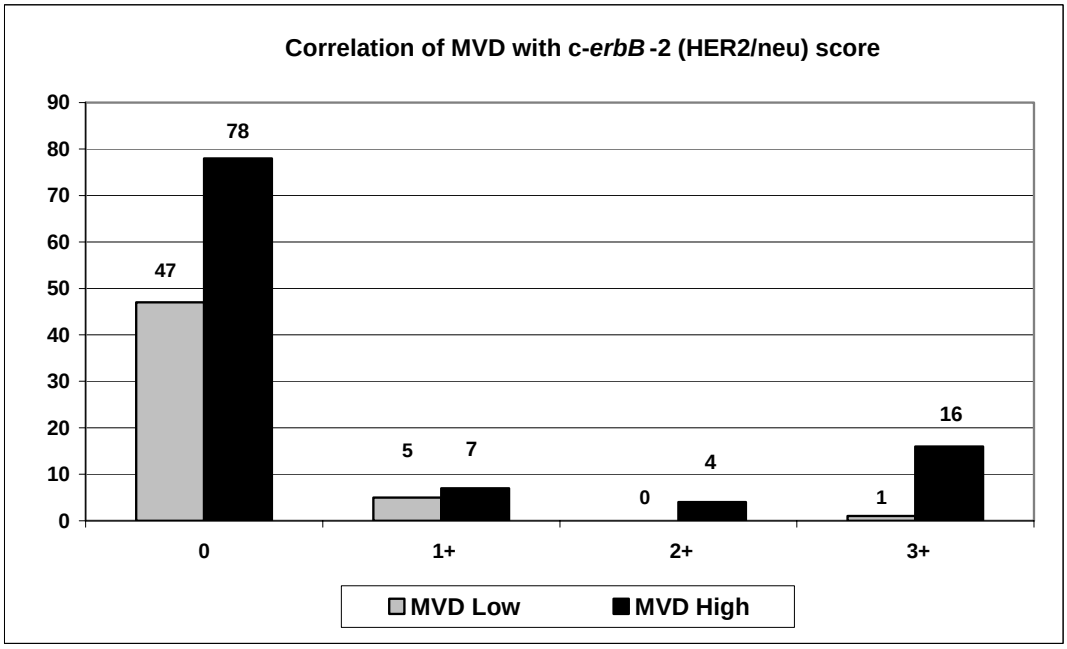

Figure 7 – ***Number of tumors with low and high microvessel density as a function of c-erbB-2 (HER2/neu) score***

MVD did show a relationship with groups defined by c-*erb*B-2 (HER2/*neu*) status (P = 0.002699, Chi-square test).

When tumors were classified by c-*erbB-2* (HER2/*neu*) score there were 47 cases in the low-MVC group and 78 cases in the high-MVC group in score 0 patients, five cases in the low-MVC group and seven cases in the high-MVC group in score 1+ patients, zero cases in the low-MVC group and four cases in the high-MVC group in score 2+ patients, one case in the low-MVC group and 16 cases in the high-MVC group in score 3+ patients.

The median microvessel density was 30.70175 microvessels per mm^2 (range 19.73684 – 63.59649 microvessels per mm^2, mean ± SD 34.40351±11.30766) in score 0 patients. The median microvessel density was 33.99123 microvessels per mm^2 (range 21.92982 – 63.59649 microvessels per mm^2, mean ± SD 34.35672±12.55717) in score 1+ patients. The median microvessel density was 32.89474 microvessels per mm^2 (range 30.70175 – 52.63158 microvessels per mm^2, mean ± SD 37.2807±10.44069) in score 2+ patients. The median microvessel density was 39.47368 microvessels per mm^2 (range 26.31579 – 72.36842 microvessels per mm^2, mean ± SD 42.05366±11.24198) in score 3+ patients.

When the mean values of MVD of the various groups defined by c-*erbB-2* (HER2/*neu*) score were compared, no significant difference was noted (P = 0.077262, One-way ANOVA test) (Figure 7).

MVD did show a relationship with groups defined by c-*erb*B-2 (HER2/*neu*) score (P = 0.027589, Chi-square test).

Discussions

The goal was to study the relationship between angiogenesis and c-*erb*B-2 (HER2/*neu*) overexpression, which is in contrast to other studies that assessed angiogenesis as a prognostic factor. The traditional approaches to treating breast cancer include surgery, radiation therapy, chemotherapy and hormonal therapy. Although these therapeutic modalities, singly and in various combinations, are effective in many patients, they do not specifically target the tumor.

Recent advances in the understanding of the molecular and genetic alterations underlying breast cancer development and progression have initiated a paradigm shift in the treatment of breast cancer. In this new paradigm, the development of new treatments for breast cancer will result from the identification of specific molecular targets that are discovered in studies designed to elucidate the genes and molecules involved in breast tumorigenesis [24–26]. This in turn will result in the identification of molecularly defined patient subgroups. Of particular importance to pathologists, the integration of diagnostic tests and therapeutics will be a critical element in this scenario.

None of the recent developments in the understanding of the molecular events underlying breast tumorigenesis has had a greater immediate impact on both clinicians and pathologists than the recognition of the importance of the c-erbB-2 (HER2/*neu*) oncogene in breast cancer [5, 6]. In particular, the recent development of a therapeutic agent that directly targets the HER2 protein (Herceptin, trastuzamab), represents a model for the future of breast cancer treatment directed toward a specific molecular target.

The College of American Pathologists considers c-*erb*B-2 (HER2/*neu*) to be a Category II breast cancer prognostic factor, meaning that it is a factor that had been extensively studied biologically and clinically, but whose import remains to be validated in statistically robust studies and angiogenesis to be a Category III breast cancer prognostic factor, meaning that it is a factor not sufficiently studied to demonstrate their prognostic value [27].

A prognostic factor is one that provides information regarding patient outcome at the time of diagnosis. The clinical significance of c-*erb*B-2 (HER2/*neu*) overexpression/amplification in invasive breast cancer is considerable and compelling. The initial study by

Slamon DJ *et al.*, in 1987 [28], found a 30% incidence of c-*erb*B-2 (HER2/*neu*) overexpression/ amplification in women with lymph node-positive breast cancer, and this was associated with a significantly poorer outcome, increased risk of recurrent disease, and shorter overall survival. Since that time, there have been numerous studies assessing the prognostic significance of c-*erb*B-2 (HER2/*neu*) amplification and overexpression in patients with breast cancer.

Most studies have shown that c-*erb*B-2 (HER2/*neu*) amplification and overexpression is a significant adverse prognostic factor in patients with node-positive disease [29, 30]. The prognostic role of c-*erb*B-2 (HER2/*neu*) in lymph node-negative breast cancer has been controversial. There are many issues that have contributed to this controversy including small patient numbers in many of the studies, patient selection, variations in treatment, variations in length of follow-up, variations in statistical analysis, and variations in methodology used to assess c-*erb*B-2 (HER2/*neu*) status [31–38]

A predictive factor is one that provides information regarding the likelihood of response to a given therapeutic modality. In recent years, there has been great interest in the interactions between c-*erb*B-2 (HER2/*neu*) overexpression and various forms of systemic therapy in patients with breast cancer because this could provide information that helps in the determining the most suitable systemic therapeutic regimen in a given patient [6, 39].

One practical method in assessing c-*erb*B-2 (HER2/*neu*) status in routine clinical setting is by immunohistochemistry, although fluorescence *in situ* hybridization (FISH) for Her2-neu gene amplification may offer more information on disease prognosis and predicting treatment response [40, 41]. On immuno-histochemistry, a distinct, membranous staining pattern is seen when the receptor is present.

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 [42]. The role of angiogenesis in the development and progression of human cancers has been widely studied [43]. New blood vessels can be stimulated to grow when factors that promote angiogenesis are up-regulated or those that inhibit angiogenesis are down-regulated [44, 45]. This investigation was stimulated by the conflicting conclusions of some studies.

Metastasis is a multistep process in which tumor cells gain access to the vasculature in the primary tumor, survive the circulation, nest in the microvasculature of the target organ, exit from this microvasculature, and proliferate in the target tissues. Tumor cells rarely shed into the circulation before the primary tumor is vascularized, and micrometastases cannot grow to a detectable size until after they have become vascularized. Thus, angiogenesis is necessary at the beginning as well as at the end of the metastatic cascade. It is likely that a primary tumor containing a high proportion of angiogenic tumor cells will generate metastases that are already angiogenic when they begin their growth in the target organ [46].

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 [47, 48]. 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 [49, 50]. These degradative enzymes may also facilitate the escape of rumor cells into the tumor neovasculature. In addition, it has been shown that greater numbers of tumor vessels increase the opportunity for tumor cells to enter the circulation.

In this study, cases with high-MVD were significantly more numerous. Further studies to determine whether a specific number of microvessels within the primary tumors of patients with c-*erb*B-2 (HER2/*neu*) + or c-*erb*B-2 (HER2/*neu*) - status is predictive of occult metastasis is warranted, because this information could improve selection of patients for elective lymph node dissection and adjuvant therapy.

Conclusions

The quantitation of tumor angiogenesis and c-*erb*B-2 (HER2/*neu*) overexpression in the primary tumor at the time of first diagnosis may be useful in the management of breast cancer and open the door to the development of other novel approaches to the treatment of breast cancer using antiangiogenic molecules. Neovascularization permits, but does not guarantee, progressive tumor spread.

Acknowledgements

The author thank to the technical staff of the SAPAG Hautepierre, Strasbourg, France, for help with obtaining data requested for this study. I am particularly grateful to Prof. J. P. Bellocq and to Prof. M. P. Chenard for their excellent assistance.

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Received: March 5th, 2007

Accepted: April 10th, 2007