

Biological Behavior of Human Breast Cancer Micrometastases¹

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ABSTRACT

Purpose: Clinically undetectable micrometastases may account for disease recurrence in breast cancer patients after variable disease-free intervals. However, little is known about the cellular mechanisms controlling human breast cancer micrometastases. We compared tumor proliferation rate, apoptotic index, and angiogenesis in human breast cancer micrometastases with those of macroscopic axillary lymph node metastases.

Experimental Design: Seven breast cancer micrometastases (<2 mm) obtained from the sentinel nodes of seven patients were compared with 13 macrometastases (lymph node replaced with tumor) obtained from 13 patients. The tissue was fixed in formalin, embedded in paraffin, serially sectioned, and evaluated by H&E and immunohistochemistry for cytokeratin. Tumor proliferation rate was assessed as the number of Ki-67-positive nuclei/total number of tumor nuclei. Tumor vascularity was quantified using antibody to factor VIII to identify microvessels per high-power field (at $\times 400$). Apoptosis was quantified using the terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling method. Results were analyzed with the Wilcoxon rank-sum test.

Results: Median size of micrometastases was 0.5 mm (range, 0.4–1.0), and the median number of tumor nuclei/section was 143 (range, 90–312). Median proliferation rate for macrometastases was greater than for micrometastases (35% versus 12%; $P = 0.003$). Median microvessel density/high-power field for macrometastases was greater than for micrometastases (17 versus 1; $P < 0.001$). There was no difference in apoptotic index between macrometastases and micrometastases (1.1% versus 0.7%; $P =$ not significant).

Conclusions: Human breast cancer micrometastases have lower tumor proliferation rates and angiogenesis than breast cancer macrometastases. These characteristics may explain their differential growth patterns.

INTRODUCTION

Tumor metastasis is the major cause of mortality in breast cancer patients, but only a minority of patients exhibits clinically detectable metastases at diagnosis (1). The long-term risk of tumor recurrence is well recognized: metastases may appear even 10–20 years after curative primary tumor removal (1–4). Clinical studies of tumor metastasis in breast cancer patients show that tumor cells can undergo a period of dormancy followed by rapid growth during relapse (2, 5). Thus, a dormant tumor population remains clinically undetectable for months or years and consequently poses a continuous risk of recurrence.

The metastatic cascade is known to involve multiple steps. For a tumor cell to metastasize effectively, it must penetrate several barriers and be able to respond to specific growth factors. Tumor cells must first obtain entrance to the vasculature in the primary tumor, survive the circulation, halt in the microvasculature of the target organ, depart from this vasculature, grow in the target organ, and induce angiogenesis (6). Micrometastases, which are microscopic (<2 mm) deposits of malignant cells (7), provide an opportunity to study tumor cells at the earliest stages of the process where they have acquired some but not all of the necessary properties required in the metastatic cascade.

In the past decade, our ability to detect occult breast cancer micrometastases has been enhanced by the advent of the SLN³ biopsy. This technique focuses on the first node in the lymphatic basin, the “sentinel” node, which, as the first node to receive drainage from the tumor site, is the regional node most likely to harbor metastatic disease (8, 9). The technique of serial sectioning and immunohistochemical staining is used to increase the detection of micrometastatic disease found in the SLN. This approach has been shown to increase the detection rate of micrometastatic disease by 9–31% (10–14). Therefore, sentinel lymphadenectomy provides a novel resource for the study of breast cancer micrometastases.

In this study, breast cancer micrometastases in SLNs from seven patients and macroscopic axillary lymph node metastases from 13 patients were used to compare tumor vascularity, tumor proliferation rate, and apoptotic index.

MATERIALS AND METHODS

Sentinel Lymphadenectomy and Axillary Lymph Node Specimens. This research was conducted under an Institutional Review Board-approved protocol at Memorial Sloan-Kettering Cancer Center. Intraoperative lymphatic mapping with SLN biopsy was performed using blue dye injected peritumorally and radioisotope injected intradermally following our institutional protocol (15) as described. Completion axillary

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³ SLN, sentinel lymph node; HPF, high-power field; IHC, immunohistochemistry; NS, not significant; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor.

lymph node dissection was performed if the SLN was positive for carcinoma.

All of the patients who underwent SLN biopsy between September 1996 and October 2000 were prospectively entered into a database, where the number of positive nodes and the presence of micrometastases were recorded. Patients included in this study were selected from this database. We chose to study micrometastases that were identified by H&E staining and between 0.4 and 2 mm in size. Patients with micrometastases seen only with IHC with antibodies to cytokeratin were excluded. Patients with SLN micrometastases were excluded from this study if additional lymph nodes in the axillary lymph node dissection contained disease. Lymph node macrometastases were selected from patients with >15 positive axillary lymph nodes, where the lymph nodes were replaced with tumor. By choosing patients with large numbers of positive nodes, we ensured that we would be studying macrometastases from patients with aggressive disease.

IHC with Factor VIII-related Antigen and Ki-67 Antibody. IHC for factor VIII-related antigen and Ki-67 antibody was performed on formalin-fixed, paraffin-embedded tissue sections using avidin-biotin-peroxidase as described (16). Pretreatment was microwave heating for 10 min in 0.01 M citric-acid buffer (pH 6). The buffer is prepared by dissolving 2.1 g of citric acid in 990 ml of distilled water. After dissolving the salt, 5 N NaOH is added until the solution reaches pH 6.0 (~10 ml of 5 N NaOH). For Ki-67 analysis, 150 μ l of Ki-67 (1:50) monoclonal antibody (DAKO, Carpinteria, CA) was used. For factor VIII analysis, 150 μ l of factor VIII (1:20) polyclonal antibody (DAKO) was used. Negative control sections substituted normal serum for the primary antibody. Wilm's tumor-expressing factor VIII and Ki-67 were used as positive controls.

Apoptosis. Apoptosis was quantified using the method of terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling. Terminal deoxynucleotidyl transferase-labeling of formaldehyde-fixed tissue sections was performed according to Gavrieli *et al.* (17). Positive reactions were detected with a peroxidase-labeled antibody against deoxyuridine triphosphate-digoxigenin.

Quantitation of Microvessel Density, Proliferation Rate, and Apoptotic Index. Tumor vascularity was quantified as described previously using antibody to factor VIII-related antigen to identify the number of microvessels/unit area ($\times 400$; Refs. 18, 19). For macrometastases, three fields judged to have the greatest numbers of microvessels were used to record the number of microvessels/unit area. For micrometastases, the whole tumor fell within the $\times 400$ field, and, therefore, only one field was counted to determine the number of microvessels/unit area.

The breast cancer cell-proliferation rate was assessed with the MIB-1 antibody (Ki-67) as the number of positive nuclei/total number of tumor nuclei counted (18). Apoptotic index was quantitated as the number of apoptotic nuclei/total tumor nuclei (18). To quantify both proliferation rate and apoptotic index for macrometastases, ≥ 1000 tumor cells were counted/lymph node at $\times 400$. For micrometastases, the whole tumor fell within the $\times 400$ field, and therefore, all of the tumor cells within the micrometastases were counted (median number of tumor nuclei/section was 143; range, 90–312).

Statistics. Results for proliferation rate, microvessel density, and apoptotic index were analyzed with the Wilcoxon rank-sum test. Results for patient characteristics were analyzed with the χ^2 test.

RESULTS

Patient and Tumor Characteristics. We studied seven patients with solitary lymph node micrometastases and 13 patients with lymph node macrometastases. One lymph node/patient was used for analysis. The median age of patients with micrometastases was 48 years (range, 39–68 years) and for those with macrometastases, 50 years (range, 38–79 years; $P = \text{NS}$). The median primary tumor size for patients with micrometastases was 1.8 cm (range, 1.1–3.5 cm); for patients with macrometastases, it was 1.9 cm (range 1.3–2.9 cm; $P = \text{NS}$). Of patients with micrometastases, six had T1c, stage IIA breast cancer and one had T2, stage IIB breast cancer, according to the 1997 American Joint Committee on Cancer staging system (20). Of patients with macrometastases, eight patients had T1c, stage IIA breast cancer, and five patients had T2, stage IIB breast cancer. There was no statistically significant difference in the following characteristics of the primary tumor between patients with micro- and macrometastases: presence of lymphovascular invasion, HER-2/*neu* amplification, estrogen-receptor positivity, and progesterone-receptor positivity. Median size of lymph node micrometastases was 0.5 mm (range, 0.4–1.0 mm), and median number of tumor nuclei/section was 143 (range, 90–312).

Tumor Vascularity. The median microvessel count in micrometastases was 1 microvessel/ $\times 400$ (range, 0–5; $n = 7$). The macrometastases exhibited significantly greater numbers of tumor microvessels (median, 17 microvessel/ $\times 400$; range, 10–40; $n = 13$; $P < 0.001$; Fig. 1, C and D and Fig. 2A).

Tumor-Cell Proliferation Rate. The median proliferation rate for micrometastases was 12% (range, 0.3–33%; $n = 7$) and was significantly lower than those from macrometastases (median, 35%; range, 21–80%; $n = 13$; $P = 0.003$; Fig. 1, E and F and Fig. 2B).

Apoptotic Index. The apoptotic index was low in both micrometastases and macrometastases. The median apoptotic index for micrometastases was 0.7% (range, 0–3.8%; $n = 7$), while the median apoptotic index for macrometastases was 1.1% (range, 0.2–4.9%; $n = 13$; Fig. 2C).

DISCUSSION

Despite apparent curative surgery in the treatment of breast cancer, 21% of node-negative patients still develop lymph node and distant metastatic disease at 18 years (21). It is likely that this group of patients has occult micrometastatic disease at the time of initial surgery and that they are understaged. Recent studies have established that current routine histological assessment of regional lymph nodes underestimates breast cancer metastases. Use of serial sectioning and IHC for the evaluation of routine histology-negative lymph nodes in patients with breast cancer has been performed in multiple studies and has been shown to increase the detection rate of micrometastatic disease by 9–31% (10–14). However, the growth properties of human breast cancer micrometastases have not been elucidated.

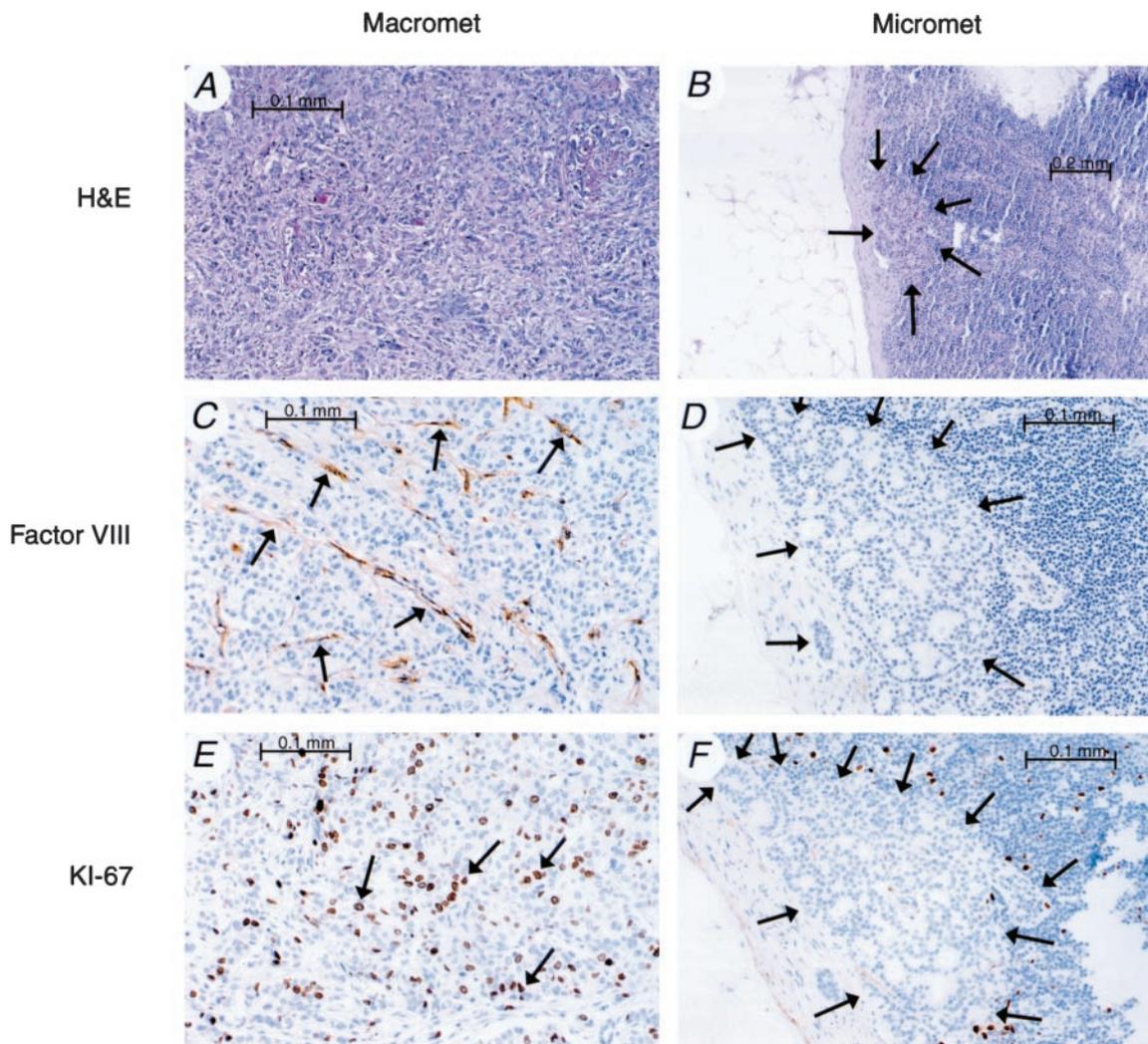


Fig. 1 H&E staining of breast cancer lymph node macrometastases (A, $\times 400$) and micrometastases (B, arrows, tumor-lymph node interface, $\times 200$). Immunohistochemical analysis of vascularization of human breast cancer lymph node macrometastases (C, $\times 400$) and micrometastases (D, $\times 400$). Tumor vascularization was analyzed by staining with polyclonal antibody against factor VIII, an endothelial-specific marker. In breast cancer macrometastases (C), there was marked neovascularization (brown stain; arrows, representative blood vessels). In contrast, breast cancer micrometastases (D) had a marked decrease in tumor microvessel density. Arrows, tumor-lymph node interface. Immunohistochemical analyses of proliferation of breast cancer macrometastases (E, $\times 400$) and micrometastases (F, $\times 400$). Tumor proliferation was analyzed by staining with antibody against Ki-67. In breast cancer macrometastases, there was a much higher rate of proliferation (E, red/brown stain; arrows, representative proliferating cells) compared with micrometastases (F, arrows, tumor-lymph node interface).

The commonly accepted theory of breast cancer metastatic development, Gompertzian growth kinetics, assumes that neoplastic growth begins with tumor seeding and continues until clinical recurrence is documented (22–24). This theory postulates that near-regular exponential growth occurs at small cell numbers with decelerated growth at larger cell numbers. Therefore, small tumors grow faster than larger tumors but also have more cells in their mitotic cycle and are, thus, more susceptible to chemotherapy. This model has been the basis for conventional chemotherapy. Our data in human breast cancer shows that micrometastases have a low proliferation rate and apoptotic index, while macrometastases have a significantly higher proliferation rate with no difference in apoptosis. This finding is not consistent with the Gompertzian hypothesis.

An alternative hypothesis for breast cancer metastatic development is the tumor dormancy hypothesis (1–4, 25, 26). This hypothesis assumes that for some patients, micrometastases do not grow for a given period of time during the preclinical phase depending on tumor and/or host factors. The immune system and suppression of angiogenesis are the most likely of various possible sources of this phenomenon. Micrometastases may eventually escape dormancy by means of a subset of tumor cells within the micrometastases switching to an angiogenic phenotype (27, 28). This process involves multiple regulatory factors produced by tumor cells, host stromal cells, and/or infiltrating leukocytes (29–32). Among these are angiogenic stimulators such as VEGF (33, 34) and acidic fibroblast growth factors and bFGFs (35). Once angiogenesis occurs, dormancy is broken,

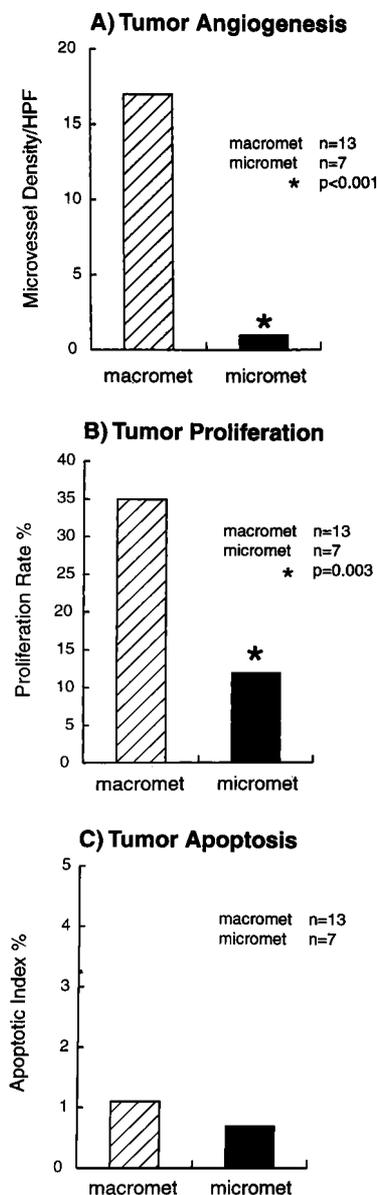


Fig. 2 Quantitation of the microvessel density/HPF, proliferation rate, and apoptotic index in breast cancer macrometastases and micrometastases. *A*, lymph node macrometastases had significantly more factor VIII-stained microvessels/HPF than lymph node micrometastases. *B*, lymph node macrometastases had a significantly higher proliferation rate, as assessed with Ki-67, than lymph node micrometastases. *C*, no significant difference in apoptotic index between lymph node macrometastases and micrometastases.

tumor proliferation increases, and clinically evident metastatic disease develops.

Tumor dormancy has been documented in animal tumor models where micrometastatic foci have been directly observed and studied (28, 36). These studies have shown that inhibition of vascularization of these micrometastases might explain how some tumor cells remain dormant. Animal studies have demonstrated that micrometastatic cells are dependent on passive diffusion for oxygen and nutrient supply, and this may limit their

growth to 2–3 mm (37). To grow larger than a few cubic millimeters, solid tumors must generate new vasculature through the process of angiogenesis (38). In mice, lung metastases, dormant during angiogenesis suppression with angiostatin, exhibited rapid growth when the inhibition of angiogenesis was removed (36).

Arbiser *et al.* (28) developed previously a model of the angiogenic switch by sequential introduction of SV40 large tumor antigen and H-ras into murine endothelial cells. When endothelial cells expressing the SV40 large tumor antigen were injected into mice, they formed dormant hemangiomas with low proliferative and apoptotic indices and had low production of VEGF. When H-ras was introduced into these cells, they formed rapidly proliferating angiosarcomas *in vivo* and had high VEGF production levels. This study demonstrated that introduction of a single gene resulted in a switch to the angiogenic phenotype and broke tumor dormancy.

In humans, Barnhill *et al.* (18) demonstrated that melanoma micrometastases lack significant tumor vascularity and have low rates of proliferation and apoptosis when compared with melanoma macrometastases. Our findings in human breast cancer micrometastases were similar to the findings of Barnhill *et al.* (18) in that the micrometastases lacked angiogenesis and had a low proliferation rate and apoptotic index, while macrometastases had a significantly higher proliferation rate with no difference in apoptosis. Therefore, the two biological studies of human micrometastases, although both limited in terms of the number of specimens, support the tumor-dormancy theory and are inconsistent with the Gompertzian hypothesis.

Understanding the biological mechanisms of micrometastases will contribute significantly to the design of treatment strategies for patients with micrometastatic disease. Conventional chemotherapy, which is based on the Gompertzian hypothesis, has been shown to increase survival by ~30% in patients with primary tumors >1 cm in size and node-negative disease (39). A potential strategy to improve upon this result for adjuvant treatment of patients with breast cancer micrometastases would be to administer antiangiogenic treatment to maintain dormancy of micrometastatic tumor cells.

A number of antiangiogenic agents have been developed as pharmaceuticals and are currently being tested in clinical studies. Two of these agents, cyclophosphamide and paclitaxel, are anticancer drugs that are used in the current treatment of breast cancer. We and others have shown previously that paclitaxel is an *in vivo* inhibitor of bFGF- and VEGF-induced angiogenesis (40–42). Cyclophosphamide also directly inhibits bFGF-induced corneal neovascularization (43).

To reveal the antiangiogenic capability of cancer chemotherapy, Browder *et al.* (43) developed an alternative antiangiogenic schedule (shorter intervals without disruption) for administration of cyclophosphamide. When EMT-6 breast cancer and Lewis lung carcinoma cells lines were made drug resistant to cyclophosphamide in mouse models, the antiangiogenic schedule suppressed tumor growth 3-fold more effectively than the conventional schedule. Cyclophosphamide administered via the antiangiogenic schedule induced apoptosis of the endothelial cells within the tumor, and endothelial-cell apoptosis occurred before apoptosis of the drug-resistant tumor cells (43).

Our findings in human breast cancer micrometastases and

those of Barnhill *et al.* (18) in human melanoma micrometastases show that micrometastases lack angiogenesis and have a low proliferation rate. Therefore, in an attempt to improve upon the benefit of conventional chemotherapy for patients with micrometastatic disease, antiangiogenic scheduling of cyclophosphamide, paclitaxel, or other angiogenesis inhibitors may have the potential for prolonging tumor dormancy in breast cancer patients with micrometastatic disease and warrants additional investigation.

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