TUMOR BIOLOGY

Cellular Changes on the Route to Metastasis

Before marching his army into battle, any good general wants to gather as much intelligence as possible about the enemy. The goal, of course, is to find the weak points—the Achilles' heel—where the opposing forces are most likely to succumb to an attack. That's the strategy now being pursued by the scientific generals waging war on one of the body's most insidious and dangerous enemies—breast cancer. By comparing breast cancer cells with their normal counterparts, they are trying to pick up the biological changes that cause the cancer cells not only to grow out of control in their original site but, worse, to spread and form tumors at distant organs, including the lung, bone, and brain.

While the researchers on the front lines would be the first to admit that their knowledge of the adversary is by no means complete, they have identified a few promising points of possible tumor vulnerability. In particular, they've found that breast cancer cells change their patterns of protein expression in two key ways: They make increased amounts of proteins that stimulate growth and help the diseased cells metastasize to distant organs, and at the same time they decrease the production of proteins that put the brakes on growth and metastasis.

Identification of these protein changes could provide a route to successful attacks on the cancerous cells. In fact, trials based on blocking the action of growth- or metastasis-enhancing proteins are already getting under way. And that could be of help to a huge number of women, since only 7% of women with breast cancer have full-blown metastases in distant organs when their cancer is first detected. Which tells researchers, says molecular biologist Patricia Steeg of the National Cancer Institute (NCI), that "a tremendous therapeutic window" is still open at the time of diagnosis. (See Perspective by Marc Lippman on "The Development of Biological Therapies for Breast Cancer.")

But finding out what makes tumor cells become metastatic isn't just the equivalent of finding an Achilles' heel in a powerful opponent's defenses. It's also analogous to intercepting the same enemy's coded signals. The reason is that some of the same protein changes may be used to identify patients whose cancers are most likely to metastasize and are therefore most in need of aggressive chemotherapy or other systemic therapy once their primary breast tumors have been surgically removed. There's now a desperate need for such prognostic indicators, especially for "node-negative" women—the roughly 50% of breast cancer patients whose lymph nodes show no sign of cancer spread at the time of their original surgery.

Although these women have a much better chance of remaining disease-free than node-positive women, they nonetheless must face a daunting fact: There's still a 30% chance that their cancer will return. Some indicators, such as loss of receptors for the hormones estrogen and progesterone (see box), have been in use for several years, but none of them predict with complete reliability which node-negative patients are likely to relapse. These women are therefore forced to make an agonizing choice between undergoing chemotherapy, which often has debilitating side effects—and which may well be unnecessary—and taking their chances that they won't be one of the unfortunate 30%. Hence current efforts to understand the biology of breast cancer cells could not only point the way to better breast cancer therapies, it could also help women decide how much treatment they need.

Increased supply lines

Several cellular changes have already emerged that might fit the bill as good predictors of metastasis. Many appear to be working to increase the proliferation of tumor cells. In work done a few years ago, for example, tumor biologist Robert Kerbel of the University of Toronto found that metastatic breast cancer cells can outgrow nonmetastatic cells both in lab cultures and in a mouse model. "Cells that have the capacity to metastasize may have a growth advantage that not only allows them to grow at the distant sites, but also in the primary tumor," concludes Kerbel.

But simply having a proliferative advantage isn't sufficient for tumor cells to form a tumor of more than minimal size. Like an invading army, a tumor's ability to grow, and then to metastasize, is intimately linked to its ability to expand its lines of supply—in this case to grow new blood vessels. Indeed, from the clinical point of view, one of the more promising biological changes researchers are focusing on is a tumor's capacity to stimulate vascular growth, a process known as angiogenesis.

At some point in their development, tumor cells begin putting out several angiogenic proteins, such as basic fibroblast growth factor and vascular-endothelial cell growth factor. Although it's not yet clear exactly what triggers the factors' release, an accumulation of two decades worth of evidence shows that tumors need their own blood supply to grow beyond a size of 2 to 3 millimeters. And since small, nonvascularized tumors rarely metastasize, it is a good bet that the blood vessels provide an opening—the "hole in the dam," as molecular biologist Adrian Harris of the Imperial Cancer Research Fund's lab in Oxford, England, describes it—through which metastatic cells can escape and spread to distant sites in the body.

About 3 years ago, these observations led angiogenesis researcher Judah Folkman of Harvard Medical School, and pathologist Noel Weidner, then also at Harvard, to see if they could find any correlation between the extent of angiogenesis in breast cancers and metastasis. Their approach was relatively straightforward: They stained tumor tissue obtained from women treated for primary invasive breast cancer at Brigham and Women's Hospital in Boston between 1978 and 1983 with an antibody to clotting factor VIII, which is made exclusively by the endothelial cells lining blood vessels. The result: As the number of vessels counted per high power microscope field went up, so did the likelihood of metastasis, Folkman says. Only

Some Tumor Changes Linked to Poor Breast Cancer Prognosis

Characteristic	Change	Effect	Status as prognostic marker
Estrogen receptor	Lost	Loss of normal growth control	In use
Progesterone receptor	Lost	Loss of normal growth control	In use
Angiogenesis	High blood vessel count	Blood vessel proliferation promotes tumor growth and escape of metastatic cells	Experimental
erbB2/HER2/neu gene	Amplified	Increased number of growth factor receptors	Experimental
nm23 gene	Activity decreased	Increased tumor cell motility? Other?	Experimental

one of seven women with a count below 33 developed metastases, compared to all 15 women with counts above 100. Those with intermediate counts had intermediate metastasis rates. The extent of angiogenesis "predicted the occurrence of metastasis better than other indicators," Folkman concludes.

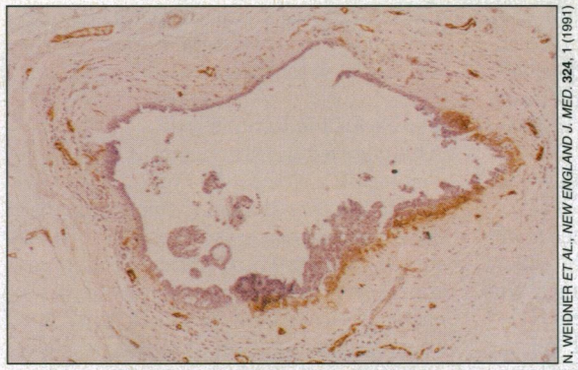
Since then, the result has been confirmed in reports from four other groups, including Harris,' as well as in a second and larger study by the team led by Weidner, who is now at the University of California, San Francisco, and Folkman. In a particularly encouraging development, the researchers are finding that measuring the extent of angiogenesis in breast tumors may help to identify the node-negative women who are likely to relapse. "It's good for picking out prognosis in node-negative as well as in node-positive women," Harris says.

Because the growth of both the primary tumor and metastatic tumors depends on their ability to stimulate new blood vessel growth, angiogenesis may also be a good therapeutic target for breast and other cancers. Finding drugs that block blood vessel growth into tumors may not be an easy job, however, since tumors produce not just one, but a range of angiogenic factors. Indeed, the Harris group has found that one line of cultured breast cancer cells produces at least four angiogenic proteins: basic fibroblast growth factor, vascular-endothelial growth factor, pleiotrophin, and platelet-derived endothelial cell growth factor. Nonetheless, researchers are sufficiently hopeful about the anti-angiogenesis approach to cancer therapy that they are beginning clinical trials. For example, Marc Lippman's group at Georgetown University School of Medicine in Washington is testing pentosan polysulfate, a drug that blocks the activities of basic fibroblast growth factor and related angiogenesis-stimulating proteins, in women with breast cancer.

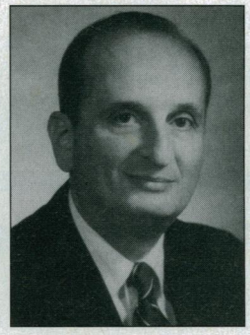
Two trials have also recently gotten under way in patients with Kaposi's sarcoma, a cancer that frequently afflicts people with AIDS. One of these is being conducted at NCI with a drug originally isolated from a fungus by the Folkman group and the other is being carried out at San Francisco General Hospital with platelet factor IV, an angiogenesis-inhibiting protein produced by the biotech firm Repligen Corp. of Cambridge, Massachusetts. All the trials are still in the early stages and it's too soon to tell how effective anti-angiogenic treatments will be.

Altered growth control patterns

In addition to studying and attacking a tumor's supply lines, cancer researchers are focusing on cancer cells' ability to stimulate their own growth by releasing growth factors (although this direct growth stimulation is not completely independent from angiogenesis, since some of the same factors participate in both).



N. WEIDNER *ET AL., NEW ENGLAND J. MED.* 324, 1 (1991)



Danger sign. The small cancer at the bottom of this breast duct is already inducing angiogenesis *(indicated by orange staining).* At right is angiogenesis researcher Judah Folkman of Harvard.

Take, for example, Toronto's Kerbel. To follow up on his observation that metastatic breast cancer cells grow faster than nonmetastatic cancer cells, Kerbel switched to melanoma, which, because it is a skin cancer, can be followed throughout all its developmental stages, something that's not been possible with breast cancer.

The Kerbel group showed that as the skin lesions progress from the early premalignant state to advanced metastatic melanomas, their properties change markedly. In particular, cells from advanced melanomas produce growth factors, including the angiogenesis-stimulating basic fibroblast growth factor, that are not made by normal cells or those of the early lesions. What's more, Kerbel and his colleagues came up with a curious finding: Proteins such as transforming growth factor beta and interleukin 6 that *inhibit* the growth of normal and early lesion cells *stimulate* the growth of cells from advanced melanomas—another change that may drive the cells to increased growth.

This turnaround in the cells' responses took some observers, including NCI's Steeg, by surprise. "When I first saw some of the papers I thought they were wrong. This is a night and day difference in cell behavior, and biology usually doesn't work this way," she says. Steeg has since changed her mind about the idea, however, as further work from Kerbel's group and others, including her own, has provided continued support. "What we're coming on is that in the final colonization response [when metastatic tumors form] metastatic cells may be inappropriately responding to negative signals."

Kerbel's melanoma model seems to apply to breast cancer cells as well. Lippman's group, for example, has shown that breast cancer cells pour out several growth-stimulatory proteins, including epidermal growth factor, fibroblast growth factor, and the insulin-like growth factors. The cells may also become more responsive to the growth factors they are producing by making more of the receptors through which the proteins act.

Receptor gene targeted

One of those receptors, in particular, has gained a lot of attention recently—because of its potential both as a prognostic indicator and a therapeutic target. About 5 years ago, Dennis Slamon's group at the University of California, Los Angeles (UCLA), School of Medicine found that breast cancer cells from some women have extra copies of a gene variously known as *erbB2*, *HER2*, or *neu*. As a result, the cells make greater than normal amounts of the *erbB2* protein, which appears to be the receptor for a growth factor.

Slamon and his colleagues went on to show that women whose tumors have this gene amplification have a worse prognosis, with a shorter disease-free interval and lower survival rates, than those whose tumors don't have it. Although there was controversy at first as some groups were not able to detect the correlation, larger studies done 3 years ago by Slamon's group and also by those of Lippman and Harris (*Science*, 12 May 1989, pp. 654 and 707) have borne out the original conclusion. The later studies also suggest that *erbB2* amplification may be used to predict the prognosis of node-negative, as well as node-positive, women.

Additional evidence that *erbB2* amplification may enhance the ability of breast cancer cells to grow and spread comes from animal experiments in which Slamon's group introduced extra copies of the normal human *erbB2* gene into cultured human breast cancer cells. Not only did the growth rate of the cells go up, but their ability to form tumors in nude mice also increased "dramatically."

The possibility that *erbB2* amplification and over-expression contribute to the pathogenesis of breast cancer makes the *erbB2* receptor protein another tempting target for therapy. Test-tube and animal studies have been encouraging, Slamon says. Antibodies directed against the receptor protein inhibited the growth of breast cancer cells in culture and in nude mice.

Buoyed by these results, the Slamon group has gone on to conduct a "phase I" clinical trial of a monoclonal antibody against the *erbB2* receptor in 10 women who have advanced breast cancer and whose tumors overexpress the *erbB2* gene. The trial was not designed to find out if the antibody, which was supplied by the biotech firm Genentech Inc. of South San Francisco, is effective. In-

stead its goal was to determine how the body handles the antibody and to assess its toxicity. And it's passed these tests. The antibody localized in the patients' tumors, Slamon says, and showed no toxic side effects in doses up to 500 milligrams.

Because the particular antibody used for this trial was of mouse origin, however, it cannot be given more than once since it will induce antibodies against itself. More recently, the Slamon group has been testing a "humanized" antibody, made by Genentech researcher Paul Carter, in which all but the antigen-recognition portions of the molecule are replaced by human antibody sequences. This antibody worked about as well as the original one in inhibiting breast tumor growth in mice, Slamon says, and the 16 patients who received single doses tolerated it well. The UCLA workers are currently beginning a multiple-dose toxicity trial in which they will also look for therapeutic effects.

Gene's loss is tumor's gain

Although for growth factors and their receptors, such as the *erbB2* protein, it's overproduction that's linked to tumor growth, in other cases researchers have found that it's the loss of a particular protein product that correlates with a poor prognosis. A recent example comes from Steeg. In work done about 6 years ago, she and her colleagues identified a gene whose activity suppresses the metastatic capabilities of melanoma cells. The NCI workers subsequently showed that the activity of the gene, which they named *nm23* (where nm stands for non-metastatic), was low in breast tumors from women with positive lymph nodes and other signs of poor prognosis. And just last year, they took the observation a step further, showing that low production of the protein encoded by *nm23* correlates with reduced survival of breast cancer patients. Additional studies buttressing the idea that loss of *nm23* activity might contribute to a poor breast cancer prognosis come from groups led by Colm Hennessy of the University of Newcastle on Tyne in England, Katsuiku Hirokawa of the Tokyo Metropolitan Institute of Gerontology, and Robert Rees of the University of Sheffield in England.

While these correlations suggest that measuring *nm23* gene activity might give another indication of breast cancer prognosis, Steeg herself concedes that by themselves they can't prove that *nm23* is a metastasis suppressor. Indeed, loss or inactivation of the gene might be merely a "bystander effect," caused by an alteration in another gene that is the true culprit. So the NCI group submitted their hypothesis to an acid test. They transferred the normal *nm23* gene into highly metastatic melanoma or human breast cancer cells, both of which had the expected low *nm23* protein production. The result: By boosting the production of the protein, the transferred gene greatly reduced the number of metastases the recipient cells form in experimental animals.

Exactly how *nm23* might suppress metastasis is unclear, although a possible clue comes from experiments done by Bruce Zetter's group at Harvard Medical School. Steeg supplied Zetter and his colleagues with the mouse melanoma and human breast cell lines with the transferred *nm23* gene and also unaltered control cell lines. Without knowing which were which, the Zetter group then measured the cells' ability to migrate in response to several different substances, including platelet-derived growth factor and insulin-like growth factor-1, that stimulate the motility of the unaltered cancer cells. When the researchers broke the code, they found that in every case, the acquisition of the *nm23* gene blocked the migration response. "The metastatic cell gets out of the primary tumor and into the circulation where it has a very short half-life—about 30 to 60 minutes," explains Zetter. "Therefore the one that migrates out of the circulation most rapidly is the one that will form a metastasis."

Because *nm23* suppressed responses to several different chemoattractants, Zetter suggests that it exerts its effect through a signaling pathway common to them all. Steeg agrees: "If I had to guess at what *nm23* is doing, I would guess it is modulating signal transduction, although I don't know how."

If so, then it might be possible to find drugs that fight metastasis by mimicking *nm23*'s effects in the cell, although that's not likely to happen any time soon.

Hormone Receptors: Dangerous Loss

Among the many changes in breast tumors that have been linked to poor patient survival is loss of the receptors for the hormones estrogen and progesterone. Indeed, the absence of these receptors on the surface of tumor cells is already in wide use as a prognostic indicator, with chemotherapy generally prescribed for such patients even if there is no sign of spread to the lymph nodes. Only recently, however, have researchers begun to get an idea of how the loss of these receptors causes cancer cells to become more aggressive.

At first glance, it might seem that the receptor loss might be a good thing since estrogen and progesterone are growth stimulators for normal breast cells. Indeed, exposure to the hormones is considered to be an important factor contributing to the onset of breast cancer. Noting that women who have their ovaries, the major source of estrogen in the body, removed before age 35 rarely get breast cancer, endocrinologist Kate Horowitz of the University of Colorado Medical Center in Denver says: "The reason that women rather than men get breast cancer is not that women have breasts, but that they have ovaries."

Losing the estrogen and progesterone receptors is bad, however, because when that happens breast cancer cells apparently gain the ability to grow even in the absence of the hormones. Not only does that make them very aggressive and dangerous, it also makes them resistant to therapy. When breast cancer cells lose the receptors, for example, the cancers can't be treated with drugs that bind to the receptors and prevent the hormones themselves from binding. The anti-estrogen tamoxifen, which is already widely used, is one such drug. And RU-486, the progesterone-blocking drug best known for its use in inducing abortions, is another candidate for breast cancer therapy. "If you could block progesterone receptors, you could get a double-whammy," says Horowitz, who is herself studying RU-486.

As part of an effort aimed at understanding why tumors lose their sensitivity to tamoxifen, molecluar biologist Suzanne Fuqua of the University of Texas Health Science Center in San Antonio has found mutations in the estrogen receptor gene in breast cancers that are estrogen receptor negative that may help explain why these cells are so aggressive. In tumors from about half of the patients, the gene is missing the region that encodes the portion of the receptor that binds estrogen. But the receptor's inner portion, which is that part that transmits the growth signal to the cell nucleus, remains intact and capable of functioning.

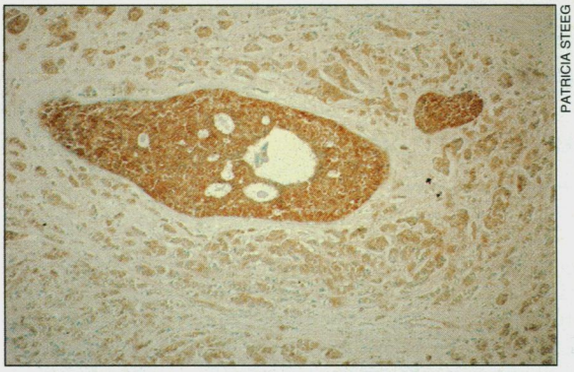
This mutation is thus analogous to the one that converts the gene for the epidermal growth factor receptor into a cancer-causing oncogene. The supposition is that with the hormone- or growth factor-binding segment of the receptor gone, the inner portion becomes locked in the active position. Or as Fuqua puts it, " The gas pedal is on all the time, and there's no brake." Fuqua cautions that she hasn't done the clinical trials needed to see if the patients who have the mutation are in fact resistant to tamoxifen, although she has found that putting the mutant gene into breast cells makes them resistant to the drug.

–J.M.

Genetic master switches?

As researchers identify more and more changes as breast cells undergo their insidious progression to a malignant state, a central question keeps coming up: What causes all those changes? The leading hypothesis right now is that it's due to the loss of one or more "tumor suppressor" genes, which are thought to keep cell growth in check by regulating other genes, possibly including those encoding growth factors and their receptors. The breast cancer susceptibility gene that Berkeley geneticist Mary-Claire King and several other groups are now homing in on (see preceding article) may be one such tumor suppressor gene. Another is the p53 gene, which has already been implicated in breast cancer, as well as several other types of cancer.

Stephen Friend's group at Massachusetts General Hospital has found, for example, that inactivation of the p53 gene may account for perhaps 10% of hereditary breast cancer cases. And in an intriguing related development last fall, teams led by Thea Tlsty of the University of North Carolina in Chapel Hill and Geoffrey Wahl of the Salk Institute in San Diego reported results showing that p53 gene inactivation not only causes a loss of normal cell cycle control but also an increased frequency of gene amplification—the same kind of defect seen with *erbB2*. Suppressor gene inactivation may therefore produce the myriad changes contributing to the formation of malignant tumors in at least two ways: by unleashing the activity of growth stimulatory genes and by causing genetic instability, thereby producing gene amplifications and other kinds of mutations.



PATRICIA STEEG

BILL BRANSON

Metastasis suppressor? The noninvasive tumor cells within the breast duct have higher *nm23* gene activity than the tumor cells invading the surrounding tissue. Patricia Steeg, *nm23* discoverer.

One development that may help in working out the contributions that all these genetic alterations make to breast cancer is a new system for culturing human breast cells devised by Ole Petersen of the University of Copenhagen, Mina Bissell of the Lawrence Berkeley Laboratory, and their colleagues. The work also points up the importance of the basement membrane, the layer of protein and carbohydrate that separates mammary epithelial cells, which are the ones that give rise to breast cancers, from underlying cells.

Molecular and genetic studies of breast cancer development have been handicapped, Bissell says, because "people have not been able to culture normal and tumor cells under conditions where they can compare them." But Petersen and Bissell found that if they culture normal mammary cells *in* basement membrane material, rather than *on* it as most researchers do, the cells will form tiny, sac-like structures that look very much like the sac-like alveoli of breast tissue. Under the same circumstances, Bissell says, breast cancer cells form structures that are three to four times bigger than normal and "completely disorganized." By allowing direct comparisons between the normal and cancer cells, the culture system should help find out just what goes wrong during cancer development.

But even if all the important changes can be identified, Kerbel cautions that that still might not enable physicians to predict a woman's prognosis with 100% accuracy. He notes that about 15 prognostic indicators have been identified so far. "They always start with encouraging results. But once the more rigorous trials are done, they look less good," Kerbel says. "I'm worried that we won't ever be able to clearly separate those who will progress from those who won't." Still, he says, 95% to 99% might be possible. "And then women will be able to make an informed choice" about therapy.

–Jean Marx

Additional Reading

Epidemiology

1. R. Harris, M.E. Lippman, U. Veronesi, and W. Willett, "Breast Cancer," *New England Journal of Medicine* **327**, 319, 390, and 473 (1992).
2. L.G. Kessler, E.J. Feuer, and M.L. Brown, "Projections of Breast Cancer Burden to U.S. Women: 1990-2000," *Preventive Medicine* **20**, 170 (1991).
3. B.A. Miller, E.J. Feuer, and B.F. Hankey, "The Increasing Incidence of Breast Cancer Since 1982: Relevance of Early Detection," *Cancer Causes and Control* **2**, 67 (1991).
4. J.L. Kelsey and M.D. Gammon, "The Epidemiology of Breast Cancer," *CA-A Cancer Journal for Clinicians* **41**, 146 (1991).
5. M.C. Pike and D.V. Spicer, "Oral Contraceptives and Cancer," in *Contraception*, D. Shoupe and F. Haseltine, eds., (in press) Springer Verlag (1992).
6. A. Ekbom, *et al.*, "Evidence of Prenatal Influences on Breast Cancer Risk," *Lancet* **340**, 1015 (1991).

Genetics

1. M.-C. King, "Linkage of Early-Onset Familial Breast Cancer to Chromosome 17q21," *Science* **250**, 1684 (1990).
2. S.A Narod, *et al.*, "Familial Breast-Ovarian Cancer Locus on Chromosome 17q12-q23," *Lancet* **338**, 82 (1991).
3. A. Bowcock *et al.*, "Polymorphisms in THRA1 and D17S183 Flank an Interval of Less Than 4 cm for the Breast-Ovarian Cancer Gene on Chromosome 17q," *American Journal of Human Genetics*, in press. (See also collection of papers on breast cancer genetics in the same issue.)
4. M.-C. King, "Breast Cancer Genes: How Many, Where, and Who Are They?" *Nature Genetics* **2**, 125 (1992).

Tumor Biology

1. R. Barnes *et al.*, "Low *nm23* Protein Expression in Infiltrating Ductal Breast Carcinomas Correlates With Reduced Patient Survival," *American Journal of Pathology* **139**, 245 (1991).
2. K. De Santes and D. Slamon, "Radiolabeled Antibody Targeting of the *HER2/neu* Oncoprotein," *Cancer Research* **52**, 1916 (1992).
3. E.R. Horak *et al.*, "Angiogenesis, Assessed by Platelet/Endothelial Cell Adhesion Molecule Antibodies, as Indicator of Node Metastases and Survival in Breast Cancer," *Lancet* **340**, 1120 (1992).
4. R.S. Kerbel, "Expression of Multi-Cytokine Resistance and Multigrowth Factor Independence in Advanced Stage Metastatic Cancer," *American Journal of Pathology* **141**, 519 (1992).
5. O.W. Petersen, L. Rønnov-Jessen, A.R. Howlett, and M.J. Bissell, "Interaction with Basement Membrane Serves to Rapidly Distinguish Growth and Differentiation Pattern of Normal and Malignant Human Breast Epithelial Cells," *Proceedings of the National Academy of Sciences* **89**, 9064 (1992).
6. N. Weidner *et al.*, "Tumor Angiogenesis: A New Significant and Independent Prognostic Indicator in Early-Stage Breast Carcinoma," *Journal of the National Cancer Institute* **84**, 1875 (1992).