

Biologic Markers as Predictors of Clinical Outcome From Systemic Therapy for Primary Operable Breast Cancer

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Purpose: To determine whether pretreatment clinical features and molecular markers, together with changes in these factors, can predict treatment response and survival in patients with primary operable breast cancer who receive neoadjuvant therapy.

Patients and Methods: Mitoxantrone, methotrexate (with or without mitomycin), and tamoxifen chemoendocrine therapy was administered to 158 patients before surgery. Clinical response was assessed after four cycles of treatment. Fine-needle aspiration cytology for estrogen receptor (ER), progesterone receptor (PgR), *c-erbB-2*, *p53*, *bcl-2*, Ki67, S-phase fraction (SPF), and ploidy were performed pretreatment and repeated on day 10 or day 21 after the first cycle of treatment.

Results: Good clinical response (GCR, defined as complete response or minimal residual disease) was achieved in 31% of patients (49 of 158). Tumor size, nodal disease, response, ER, PgR, *c-erbB-2*, *p53*, *bcl-2*,

Ki67, SPF, and ploidy were analyzed as predictors of survival. By univariate analysis, node-positive disease ($P = .05$), lack of ER ($P < .05$) and PgR ($P < .05$), and failure to attain GCR ($P = .008$) were associated with a significantly increased risk of relapse. A significantly increased risk of death was associated with node-positive disease ($P = .02$), lack of ER expression ($P = .04$), and failure to attain GCR. By multivariate analysis, GCR was an independent predictor for survival ($P = .05$). ER expression ($P = .03$), absence of *c-erbB-2* ($P = .03$), and a decrease in Ki67 on day 10 or day 21 of the first cycle ($P < .05$) significantly predicted for subsequent GCR.

Conclusion: Molecular markers may be used to predict the likelihood of achieving GCR, which seems to be a valid surrogate marker for survival.

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SYSTEMIC CHEMOTHERAPY and endocrine treatment for operable breast cancer significantly decrease the risk of relapse and death.¹ However, it is not possible to identify those patients at the outset who are likely to respond to adjuvant treatment and determine which type of treatment should be used. Adjuvant treatment given before surgery (neoadjuvant therapy) has a number of theoretical advantages in breast cancer, including a reduction in the requirement for mastectomy. A decrease in the size of the primary tumor, indicating response to therapy, has been proposed as a surrogate marker of response in micrometastatic sites.

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Furthermore, access to the primary tumor during early treatment allows for in vivo testing for changes in molecular markers that may occur with successful treatment.

Established prognostic factors such as tumor size and nodal involvement are important indicators for breast cancer relapse and survival but have not been shown to be predictive of sensitivity to treatment. Estrogen receptor (ER) and progesterone receptor (PgR) expression predict for response to tamoxifen and endocrine treatment.^{2,3} The *bcl-2* proto-oncogene, which encodes for a protein that inhibits programmed cell death (apoptosis), has been shown to predict for efficacy of adjuvant hormonal treatments.⁴ However, predictive markers for chemosensitivity are less well established. The proto-oncogene *c-erbB-2* (*HER-2/neu*) encodes for a transmembrane glycoprotein, the expression of which reflects proliferative activity of the tumor. Overexpression of *c-erbB-2* has been associated with decreased response to cyclophosphamide, methotrexate, and fluorouracil chemotherapy.^{5,6} Accumulation of aberrant protein expressed by the mutated tumor suppressor gene *p53* product is associated with relative resistance to cytotoxic therapy.⁷ Increased DNA content (ploidy) and increased tumor proliferation as measured by S-phase fraction (SPF) and Ki67 antigen have been shown to predict for relapse and survival⁸ and may also be important markers of response to chemoendocrine treatment.⁹

Measurement of these biologic molecular markers, together with tumor size and lymph node involvement, may therefore indicate the likelihood of response of the primary tumor to neoadjuvant therapy. Changes in these biologic markers, together with clinical tumor response, may allow for early prediction of relapse and survival. To test these hypotheses, the following biologic markers were measured from needle biopsy material of primary breast cancers obtained before and after exposure to chemotherapy: ER, PgR, *c-erbB-2*, *p53*, *bcl-2*, Ki67, SPF, and ploidy. Initial clinical features and measurement of these predictive molecular markers, together with changes in these factors, were then analyzed as early predictors of response, relapse, and survival.

PATIENTS AND METHODS

Clinical Methods

Patient population. From February 1990 to August 1995, patients with primary operable breast cancer were considered for a randomized trial of neoadjuvant chemoendocrine therapy at the Royal Marsden Hospital (Protocol 561). The results of this clinical trial have been previously published.¹⁰ In brief, the inclusion criteria were (1) age younger than 70 years and diagnosis of breast cancer confirmed by fine-needle aspiration (FNA) cytology, (2) physical and psychologic suitability for all treatment options, (3) informed consent, and (4) no evidence of metastases at initial assessment. Exclusion criteria included (1) evidence of metastases at initial assessment, (2) premenopausal status accompanied by a desire to consider further pregnancy, (3) presence of inoperable (including T4) tumors, with chemotherapy or hormonal therapy as the initial treatment of choice, and (4) any evidence of myocardial dysfunction.

The evaluation of predictive molecular markers in primary operable breast cancer was undertaken in a separate protocol, which had been approved by the local ethics committee (Protocol 669). One hundred fifty-eight patients who had received neoadjuvant chemotherapy and who had repeat FNA for measurement of predictive molecular markers were evaluated in this biomarker study. These time points were chosen because they coincided with routine hospital visits: day 10 for nadir blood counts and day 21 for the second cycle of chemotherapy. Of these patients, 131 patients were recruited from the neoadjuvant arm of the clinical randomized trial (Protocol 561), and an additional 27 patients with similar inclusion and exclusion criteria who had also received neoadjuvant treatment and repeat FNA were evaluated after completion of the clinical study.

Patients received neoadjuvant chemotherapy with mitoxantrone 7 to 11 mg/m² and methotrexate 35 mg/m² every 3 weeks, with or without mitomycin 7 mg/m² every 6 weeks, for four cycles before surgery/radiotherapy. A further two to four cycles of chemotherapy were given postsurgery. All patients also received tamoxifen 20 mg/d for 5 years regardless of ER status; tamoxifen therapy was started at the time of chemotherapy for patients who were part of the clinical randomized study (131 patients) or 21 days later (27 patients). Mastectomy (usually with immediate latissimus dorsi reconstruction) was considered neces-

sary if the tumor was located within 2 cm of the nipple or was too large in comparison to breast size. Otherwise, breast-conserving surgery was performed with complete surgical excision. Axillary lymph nodes were excised to level 2 clearance if palpable at initial presentation, usually through a separate axillary incision. The influence of systemic therapy on clinically undetectable nodes is unknown. Because all patients had received neoadjuvant treatment, axillary node biopsy was not required as an indicator for systemic therapy. In patients who achieved clinical complete response (CR), the location of the surgical excision was determined by mammographic or ultrasound identification of residual scarring or according to location before treatment.

All patients who did not require a mastectomy were given postoperative radiotherapy to the breast at a dose of 54 Gy in 27 fractions over 5.5 weeks using two tangential beds, followed by a boost of 10 Gy in five fractions to the tumor bed. Radiotherapy to the axilla (50 Gy in 25 fractions) was administered to patients who did not undergo axillary dissection.

Staging and response. Clinical staging and size of the primary tumor were recorded by two clinicians at the start of treatment, at each cycle, and after completion of four cycles of chemotherapy. Response was defined according to International Union Against Cancer criteria. CR was defined as no residual palpable disease, partial response (PR) was defined as more than 50% reduction in bidimensional measurements, stable disease (SD) was defined as between 50% reduction and 25% increase in tumor size, and progressive disease was defined as more than 25% increase in tumor size. As previously published, we had defined minimal residual disease (MRD) as a residual palpable irregularity at the site of the primary tumor that was too small to be measured, representing an almost CR to treatment.¹⁰ Tumor response was defined as CR, MRD, or PR. A good clinical response (GCR) to treatment was defined as CR or MRD.

Collection of samples. FNA of the primary breast tumor using a 23-gauge needle was performed twice before neoadjuvant chemotherapy and was repeated on day 10 or day 21 of the first cycle of treatment. We had previously demonstrated accurate diagnosis of primary carcinoma in 85% of our patients, with no false-positive results (T.J. Powles, unpublished data). We had also previously shown that cellular aspirates can be obtained from 80% of cancers on day 10 and 50% of cancers on day 21 (unpublished data). However, FNA cannot exclude the 1% to 4% of patients with noninvasive intraductal carcinoma, which is usually curable by surgery alone.

Laboratory Methods

Preparation of specimens for cytopspins and flow cytometric analysis. From each aspirate, a 2-mL single-cell suspension with minimal essential medium was made. Aliquots of 100 μ L were placed in 12 Shandon Cytospin chambers (Shandon, Pittsburgh, PA) and centrifuged at 500 rpm for 5 minutes on 3-aminopropyltriethoxysilane slides. These slides were then stained with May-Grünwald-Giemsa for cytodiagnosis or air dried and stored at -80°C until immunocytochemical analysis. The remaining cell suspension was snap-frozen in liquid nitrogen for flow-cytometric cell-cycle analysis.

Immunocytochemical analysis. Standard methods for immunocytochemical analysis have been described in detail elsewhere.¹¹ Briefly, the thawed cytopsin slides were washed in phosphate buffered saline (PBS) and fixed with acetone, methanol, methanol/acetone, or acetone/methanol. The endogenous peroxidase was then blocked by 0.1% sodium azide in 3% or 10% H₂O₂. For ER and PgR staining, slides were incubated with ER antibody (ER-ICA monoclonal antibody, 1:40 dilution; Abbott Laboratories, Abbott Park, IL) or KD68 antibody

(Abbott PR-ICA monoclonal kit). For Ki67, the slides were incubated with rabbit serum (1:5 dilution) before addition of MIB1 antibody (Dako, Carpinteria, CA). For *p53* and *bcl-2* immunostaining, the slides were blocked with 10% ovalbumin before incubation with an antibody cocktail of *p53*-1801 at 1:20 dilution and *p53*-240 at 1:10 dilution (Novocastra, Newcastle, United Kingdom) or *bcl-2* antibody at 1:200 dilution (Dako), respectively. For *c-erbB-2* immunostaining, rat monoclonal antibody, ICR12 (0.42 µg/mL) was applied for 30 minutes. Secondary antibody was then applied (biotinylated antirat IgG for ER/PgR, rabbit antimouse IgG for *p53* and *bcl-2*, and Ki67 and antirat F(ab) 2 peroxidase conjugate for *c-erbB-2*). After rinsing, the slides were incubated with streptavidin horseradish peroxidase at 1:100 for 30 minutes or ABC horseradish peroxidase (for Ki67) for 20 minutes, rinsed with PBS, exposed to diaminobenzidine tetrahydrochloride chromogen for 10 minutes, rinsed with autobuffer and PBS, counterstained with 1% methyl green, rinsed with deionized water, and then mounted.

ICC scoring. Subjective estimation as previously described¹¹ of the proportion of positive-staining cells on the entire slide (0, none; 1, < one-hundredth; 2, one-hundredth to one tenth; 3, < one tenth to one third; 4, one third to two thirds; and 5, > two thirds) and the intensity of positive signals (0, none; 1, weak; 2, intermediate; and 3, strong signal) of all slides were evaluated by light microscopy semiquantitatively by one author (D.C.A.) without any knowledge of the patient's clinical data. The overall score was expressed as the summation of the proportion and intensity scores. Tumors were regarded as expressing the particular molecular marker if the overall score was ≥ 3 for ER and PgR, ≥ 2 for *p53*, greater than 0 for *bcl-2*, greater than 0 for *c-erbB-2*. For Ki67, the percentage of positive cells was determined by direct counting.

DNA flow cytometry. The details of DNA flow cytometry have been described elsewhere.¹² In brief, the cell suspension was thawed, centrifuged, then lysed and stained for DNA by incubating in a stain detergent solution (nonidet P-40, Sigma, Poole, United Kingdom) containing propidium iodide as the DNA fluorochrome. DNA-stained nuclei were run on a Coulter Elite ESP flow cytometer (Coulter Electronics, Hialeah, FL). Fifty thousand tumor events were acquired on a single-parameter, 256-channel fluorescence histogram, and the cell cycle distributions (G_0 - G_1 , presynthetic; S, synthetic, G_2 -M, postsynthetic and mitotic phase) were analyzed by multicycle software programs (Phoenix Flow Systems, Inc). DNA content was regarded as diploid if G_0 - G_1 peaks were superimposed and aneuploid only if separate peaks were seen.

Statistical Methods

The tumor markers ER, PgR, *p53*, *bcl-2*, Ki67, *c-erbB-2*, SPF, and ploidy were analyzed as continuous variables in this analysis. A univariate logistic regression analysis was used to identify those factors having a predictive influence on the likelihood of response. A multivariate analysis was conducted on the available factors for each patient. Some patients did not have measurements for all of the markers, which reduced the power in the multivariate analysis. Sequential measurements were conducted in a subset of women. Changes in expression of the molecular markers (ER, PgR, *p53*, *bcl-2*, Ki67, *c-erbB-2*, SPF, and ploidy) were calculated by comparing the pretreatment level with the level at day 10 or day 21 after the first course of chemotherapy. The changes in marker levels were then considered, as in the univariate analysis, as possible predictive variables for response.

Overall survival was defined as time from diagnosis to death. Relapse-free survival was defined as time to documented relapse; deaths without relapse were censored. The significance of tumor

markers as predictors of relapse-free or overall survival was analyzed by means of a univariate proportional hazards model, and the relative risks of relapse or death were calculated.

RESULTS

Clinical Outcome

Pretreatment and sequential clinical staging together with biologic markers were assessed in 158 women who received neoadjuvant chemoendocrine therapy. In 27 patients, tamoxifen was started after repeat samples had been performed on day 21. Overall response (CR, MRD, and PR) to neoadjuvant treatment was 69% (110 of 158 patients), and 31% (49 of 158 patients) achieved CR or MRD (GCR). The median follow-up for the patients in this evaluation is 48 months, with 17 metastatic relapses and 19 deaths. The demographic characteristics are listed in Table 1.

Table 1. Patient Demographics

	No. of Patients
Total patients	158
Age, years	
Median	54
Range	28-70
Menopausal status	
Premenopausal	52
Perimenopausal	12
Postmenopausal	94
Clinical staging	
Tumor size	
T1	14
T2	116
T3	28
Nodal status	
Node-negative	114
Node-positive	44
Response*	
CR	18
MRD	31
PR	61
SD	43
PD	5
Pretreatment markers	
ER	127
PgR	135
<i>p53</i>	126
<i>c-erbB-2</i>	100
<i>bcl-2</i>	86
Ki67	109
SPF	106
DNA content	123
Survival	
Relapses	17
Deaths	19

*Overall response was 69% (CR, MRD, and PR); 31% of patients achieved CR or MRD.

Predictors of Relapse-Free and Overall Survival

The following variables were analyzed in 158 patients for relapse-free and overall survival: pretreatment tumor size and nodal disease, clinical response at 3 months, and pretreatment ER, PgR, *c-erbB-2*, *p53*, *bcl-2*, Ki67, SPF, and ploidy. By univariate analysis, node-positive disease ($P = .05$), lack of ER ($P \leq .05$) and PgR expression ($P \leq .05$), and failure to attain GCR at 3 months ($P = .008$) were associated with significantly increased risk of relapse (Table 2). A significantly increased risk of death was associated with node-positive disease ($P = .02$), lack of ER expression ($P = .04$), and failure to attain GCR ($P = .01$). Tumor size, response other than GCR, and the remaining biologic markers were not significant predictors of relapse and death. By multivariate analysis, failure to achieve GCR was the only independent variable associated with increased relative risk of death ($P = .05$) (Table 3). Figure 1 shows the Kaplan-Meier graph of overall survival in patients who attained GCR *versus* patients who did not attain GCR.

Pretreatment Predictors of GCR

Because GCR was the independent predictor for survival, we next analyzed pretreatment characteristics in 158 women for likelihood of achieving GCR using univariate logistic regression. Tumor size and nodal status did not significantly predict for primary tumor response to treatment. Positive ER was associated with a 2.6-fold increase in the likelihood of achieving GCR at 3 months ($P = .03$; 95% confidence interval [CI], 1 to 1.3) whereas *c-erbB-2* expression was associated with a decreased likelihood of 0.5-fold of attaining this response ($P = .03$; 95% CI, 0.3 to 1.0). These results are listed in Table 4.

Table 3. Multivariate Analysis on Pretreatment Prognostic Indicators, Pretreatment Markers, and GCR as Predictors of Relapse and Survival

Variable	Relapse			Death		
	Relative Risk	95% CI	P	Relative Risk	95% CI	P
Clinical						
T size	1	0.5-2.4	.8	1.5	0.8-3.9	.4
Nodes	1.6	0.7-3.7	.2	1.4	0.5-3	.5
Markers						
ER	0.6	0.2-2.7	.6	0.7	0.2-3	.7
PgR	0.8	0.1-3.6	.8	0.9	0.2-4	.9
<i>p53</i>	1.5	0.4-5.2	.5	1.4	0.5-5	.5
Clinical response						
GCR	0.3	0.07-1.5	.2	0.15	0.02-1.0	.05

Prediction of GCR by Changes in Biologic Markers With Chemotherapy

Early changes in markers on day 10 or day 21 from the start of treatment that predict subsequent GCR were next analyzed in all patients. By univariate regression analysis, tumors that showed a decrease in Ki67 antigen were significantly associated with a 2.3-fold (95% CI, 0.9 to 6) increased likelihood of achieving GCR at 3 months ($P < .05$) (Table 5). Subset analysis was performed to assess whether the timing of tamoxifen administration altered these results. In patients who began tamoxifen therapy after repeat biopsies were performed, similar results were observed: Ki67 decreased in 14 of 16 responders, whereas an increase in eight of nine nonresponders ($P = .003$) was observed. Likewise, in patients who were started on tamoxifen at the same time as chemotherapy, a decrease in Ki67 was observed in 27 of 36 responders, compared with an increase in 10 of 17 nonresponders ($P = .03$).

Table 2. Univariate Analysis of Pretreatment Clinical Prognostic Indicators, Pretreatment Molecular Markers, and Clinical Response at 3 Months to Neoadjuvant Chemoendocrine Therapy in 158 Patients as Predictors of Relapse and Death

Variable	Relapses/Total No.	Relapse			P	Deaths/Total No.	Death		P
		Relative Risk	95% CI	Relative Risk			95% CI		
Clinical stage									
T size	17/158	1.5	0.7-2.9	.3	19/158	1.5	0.8-3.0	.2	
Nodes	17/158	1.9	1-3.7	.05	19/158	1.8	1-3.5	.02	
Markers									
ER	14/127	0.7	0.5-0.9	< .05	14/127	0.75	0.4-0.9	.04	
PgR	15/135	0.8	0.5-0.9	< .05	15/135	0.6	0.2-1.6	.3	
<i>c-erbB-2</i>	9/100	1	0.3-3.8	.1	8/100	0.7	0.1-2.7	.5	
<i>p53</i>	15/126	2.6	0.9-7.3	.07	17/126	1.9	0.7-5	.2	
<i>bcl-2</i>	10/86	0.6	1.2-2.6	.6	9/86	1.4	0.4-5.4	.6	
Ki67	13/109	1.7	0.6-5.2	.6	10/109	2	1-3.8	.07	
SPF	12/106	0.9	0.5-1.7	.8	12/106	0.7	0.4-1.4	.5	
DNA	16/123	1.5	0.5-4.7	.4	16/123	0.8 1.1	0.4-3	.8	
Clinical response									
CR, MRD, PR	5/110	0.8	0.6-2	.2	5/110	0.7	0.5-2	.3	
GCR (CR, MRD)	0/49	0.25	0.05-0.9	.008	0/49	0.28	0.08-0.9	.01	

Abbreviation: CI, confidence interval.

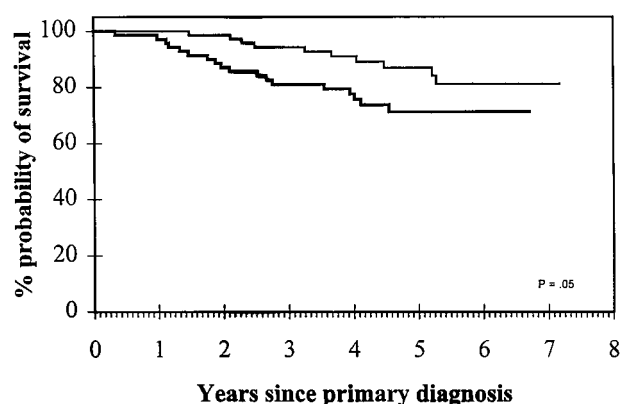


Fig 1. Survival of patients who received neoadjuvant therapy: CR/MRD versus PR/NR.

Decrease in DNA content (change from aneuploidy to diploid tumors) together with increases in *bcl-2* and PgR expression were associated with a nonsignificant trend towards increased likelihood of GCR at 3 months (Table 5). By multivariate analysis of the significant variables predictive of GCR (ER, *c-erbB-2*, change in Ki67), none were independent predictors of this response.

DISCUSSION

Studies have demonstrated improved survival in women whose breast cancers respond to primary systemic therapy.¹³⁻¹⁵ Our results indicate that molecular marker expression may predict for GCR. This response was found to be an independent predictor for survival on multivariate analysis.

Only a few *in vivo* studies have reported changes in proliferation fraction with chemotherapy.¹⁶⁻²¹ We found that a decrease in Ki67 antibodies on day 10 or day 21 of treatment significantly predicted for subsequent objective response. This has been described in other studies with chemotherapy and tamoxifen.^{9,22} Ki67 monoclonal antibody is a specific nuclear antigen that is expressed only on

Table 4. Univariate Analysis of Pretreatment Determination of ER, PgR, *c-erbB-2*, *bcl-2*, *p53*, Ki67, SPF, and Ploidy as Predictors of GCR to Neoadjuvant Chemoendocrine Treatment (2MT)

Variable	No./Total	GCR		P
		Relative Likelihood	95% CI	
ER	38/127	2.6	2-3.1	.03
PgR	41/135	1.0	0.9-1.3	.4
<i>c-erbB-2</i>	34/100	0.5	0.3-1.0	.03
<i>p53</i>	39/126	0.9	0.7-1.3	.3
<i>bcl-2</i>	40/86	1.0	0.8-1.2	.8
Ki67	33/109	1.0	0.8-1.2	.4
SPF	34/106	0.9	0.7-1.4	.2
Ploidy	38/123	0.6	0.4-0.8	.2

Table 5. Changes in Tumor Markers Between Pretreatment and Posttreatment (day 10 or 21) as Predictors of GCR at 3 Months to Neoadjuvant Chemoendocrine Treatment (2MT) by Univariate Analysis

Variable	No.	GCR		P
		Relative Likelihood	95% CI	
Increase in ER	88	1.3	1-1.7	.4
Increase in PgR	73	2.5	0.8-5	.05
Change in <i>p53</i>	61	1.0	0.8-1.2	1.0
Increase in <i>bcl-2</i>	60	0.6	0.5-0.9	.05
Decrease in Ki67	78	2.3	0.9-6	< .05
Change in SPF	60	1.2	1-1.5	.4
Decrease in DNA	61	9.8	1-93	.07

proliferating cells (late G₁, S, M, and G₂ phases of the cell cycle).²³ As a measure of the proliferative fraction of tumor cells, Ki67 correlates well with thymidine-labeling index^{23,24} but not with SPF and proliferating-cell nuclear antigen.^{16,18} Discrepancy in the predictive value of Ki67 and SPF may be due to several reasons, including technical differences and measurement of different phases of the cell cycle.¹⁷ Those women whose tumors do not show this decrease in Ki67 within the first 21 days are less likely to respond and may theoretically benefit from early switching to alternative treatments in the context of a clinical trial.

The relationship between ER and benefit from chemoendocrine treatment most likely reflects response to systemic tamoxifen and is well established in the literature.^{25,26} A few previous studies have reported an association between overexpression of the proto-oncogene *c-erbB-2* and resistance to the cyclophosphamide, methotrexate, and fluorouracil chemotherapy regimen.^{5,6} Other studies show an association between increased responsiveness to high-dose doxorubicin-containing regimens in tumors that overexpress *c-erbB-2*.²⁷ Reduced benefit from tamoxifen for women whose tumors express *c-erbB-2* have been published,²⁸ but these results are not conclusive.²⁹ By univariate analysis, these predictive markers were predictive of GCR. However, because of the number of biomarkers measured, this study had insufficient statistical power to determine independent predictors of GCR.

Standard adjuvant treatment for breast cancer prolongs survival in some patients, whereas other patients with chemoresistant disease may benefit from novel treatments. An ongoing National Surgical Adjuvant Breast and Bowel Project trial (B-27) incorporates molecular markers like *c-erbB-2*.³⁰ Our preliminary study suggests that selection of treatment may be influenced by biologic marker expression. Future clinical studies may appropriately examine whether making initial choices of systemic treatment on the basis of pretreatment marker expression and earlier alteration of therapies on the basis of marker changes could improve clinical response and, ultimately, survival.

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