Intracellular Patterns of Her-2/neu, ras, and Ploidy Abnormalities in Primary Human Breast Cancers Predict Postoperative Clinical Disease-Free Survival

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ABSTRACT

Purpose: In an earlier study (S. E. Shackney et al., Cancer J. Sci. Am., 2: 106, 1996), the presence of aneuploidy, Her-2/neu overexpression, and ras overexpression in the same cells (triple-positive cells) was of prognostic significance (P < 0.015) in 91 patients with localized breast cancer (median follow up, 32 months). Here, we present results involving a larger group of patients with longer follow-up.

Experimental Design: Fixed cell suspensions prepared from primary tumors of 189 patients with early breast cancer were studied prospectively by multiparameter flow cytometry. Correlated intracellular fluorescence-based measurements of cell DNA content and Her-2/neu and ras protein were obtained on each of >2000 cells in each tumor. Intracellular combinations of abnormalities in these measurements were correlated with subsequent patient disease-free survival (DFS). Median time on study was 54 months (range, 7–128 months).

Results: DFS of patients with ≥5% triple-positive tumor cells was shorter than those who did not meet this criterion (P = 0.004). The difference remained statistically significant after accounting for nodal status, tumor size, and each of the component abnormalities (P = 0.006). Node-negative patients whose tumors had fewer than 2 abnormalities/cell had an especially favorable clinical course, with a 5-year DFS of 96% (lower confidence bound, 86%).

Conclusions: Patterns of accumulated intracellular molecular abnormalities in cells of primary human breast cancers are predictive for subsequent DFS independently of the abnormalities themselves taken individually.

INTRODUCTION

Most breast cancer patients with clinically localized disease at surgery are considered candidates for postoperative adjuvant systemic therapy (1). However, it is troubling that for most of these patients adjuvant therapy is unnecessary because most would not experience tumor recurrences even without treatment. Therefore, there is a need to find better prognostic factors than those currently available to identify patients who are at such low risk for recurrence that they can safely forego the discomfort and potential complications of adjuvant treatment. It has recently been suggested that withholding adjuvant therapy might be acceptable to most patients, to physicians, and to advisory bodies on clinical management if the risk of recurrence might be acceptable to most patients, to physicians, and to advisory bodies on clinical management if the risk of recurrence was <5% (2). To date, no single factor has been validated prospectively in patients with localized breast cancer that can separate high-risk from low-risk patients with sufficient reliability to justify the withholding of therapy from patients in the low-risk group.

A better separation might be achieved by combining multiple prognostic factors. Recent technological developments have enabled the pursuit of several new approaches to the optimization of combinations of prognostic factors. The use of microarrays to identify patterns of gene expression that are of prognostic value in human breast cancer appear promising (3–6), although results of such studies will require methodological and clinical validation in unbiased prospective studies (7).

Flow cytometry and laser scanning cytometry can be used to perform multiple simultaneous measurements on a large number of individual cells in each tumor. The advantages of intact cell-based measurements in heterogeneous human solid tumors include (a) the ability to quantitate the intracellular levels of individual cell constituents, (b) the ability to determine whether multiple molecular abnormalities are present in the same cells or in different cells, and (c) the ability to appreciate the biological/prognostic significance of specific combinations of intracellular molecular abnormalities in different cell subpopulations within a given tumor.

In this article, we report results on DFS for 189 unselected, prospectively studied patients with primary breast cancer in whom aneuploidy and the levels of Her-2/neu protein and ras protein were determined simultaneously in each of several thousand cells in each tumor. In the late 1980s, when this study was initiated, Her-2/neu had just been identified as a prognostic factor in breast cancer (8, 9). Ras was among the first oncogenes to be studied in detail in the early and mid-1980s and was clearly implicated in neoplastic transformation and tumor aggressiveness, particularly in combination with other oncogenes (10–12). DNA aneuploidy was known to be associated with tumor aggressiveness, particularly in human solid tumors. Its independent prognostic role in breast cancer was suggested in some but not all studies. It seemed a good candidate for evalu-
ation in combination with other molecular abnormalities because there was reason to suspect that aneuploid cells might selectively accumulate multiple copies of oncogene-bearing chromosomes (13). Furthermore, there were fluorescent antibodies and fluorescent DNA dyes available with characteristics that made it possible to measure aneuploidy simultaneously with Her-2/neu and ras in the same cells.

Clinical data on the first 91 patients were previously reported (14) and were updated and included in the present study. The presence of triple-positive cells is shown to be an adverse prognostic feature that retains a high degree of statistical significance even after conventional prognostic factors are taken into account. Node-negative patients whose tumors cells had no more than one abnormality/cell among those measured had an especially favorable clinical course. Intracellular patterns of molecular abnormalities in the remaining nontriple-positive tumors can provide useful leads for pursuing a systematic hypothesis-testing strategy to progressively improve prognostic capabilities in future studies.

MATERIALS AND METHODS

Patient Population. This study was approved by the Institutional Review Board of Allegheny General Hospital (Pittsburgh, PA). Fresh tumor samples were obtained from 189 primary breast cancers in patients who underwent surgery at Allegheny General Hospital and other Pittsburgh area hospitals between November 1, 1990 and September 1, 2001, with informed consent.

All patients undergoing surgery stage I–III primary breast cancer were considered eligible for study. Locally recurrent tumors, tumors metastatic to the breast from other sites, non-epithelial primary breast tumors, and breast tumor samples in which only preinvasive tumor was found on histological examination were excluded.

Table 1 summarizes the clinical characteristics of these patients. Patients were followed for disease-free survival (DFS) and overall survival. Median time on study was 54 months. Postoperative patient management was at the discretion of the attending physician. Because the purpose of this study was to correlate laboratory measurements with the degree of tumor aggressiveness rather than with clinical treatment benefit, our focus in this report is on patient DFS rather than overall survival.

Sample Preparation. Freshly obtained tumor samples were disaggregated mechanically and fixed either in methanol for DNA analysis or in paraformaldehyde plus methanol for multiparameter analysis, as described previously (15).

DNA Staining for Flow Cytometry. Methanol-fixed cells were stained with propidium iodide (Sigma, St. Louis, MO) at a final concentration of 50 μg/ml plus RNAase (Sigma) at a final concentration of 1 mg/ml for DNA ploidy determination.

Simultaneous DNA and Quantitative Protein Immunofluorescence Measurements. Simultaneous DNA and quantitative protein immunofluorescence measurements were performed on paraformaldehyde/methanol-fixed cells. The first 76 samples were studied using a Coulter EPICS 753 instrument with an argon laser tuned to a wavelength of 488 nm and a rhodamine dye laser tuned to 585 nm. DNA was measured with propidium iodide, using the argon laser and a 590-nm long pass filter. Her-2/neu was measured with a mouse monoclonal anti-Her-2/neu antibody (Ab-1; Cambridge Research Biochemicals, Wilmington, DE) and Texas Red-conjugated rabbit antimouse IgG (Cappel, Durham, NC), using the dye laser and a 590-nm long pass filter. Ras was measured with an anti-H-ras sheep affinity-purified antibody (Cambridge Research Biochemicals).
and FITC-conjugated goat antiseep IgG (Vector; Burlingame, CA), using the argon laser and a 530-nm band pass filter. The remaining 113 cases were studied using a Coulter Elite instrument equipped with an UV-emitting argon laser (351–364 nm), an argon laser tuned to 488 nm, and a HeNe laser tuned to 633 nm. DNA was measured with 4',6-diamidino-2-phenylindole (Sigma) at a concentration of 0.1 μg/ml, using the UV laser and a 390-nm band pass filter. Her-2/neu was measured with a mouse monoclonal anti-Her-2/neu antibody conjugated directly with FITC (clone CB11; Novocastra Laboratories, Newcastle upon Tyne, United Kingdom) using the argon laser, a 550-nm dichroic filter, and a 535-nm band pass filter. Ras was measured with an anti-v-H-ras rat monoclonal antibody (Ab-5) that recognized H-, K-, and N-ras, conjugated directly to Cy-5 (Amersham Life Sciences, Piscataway, NJ), using the HeNe laser and a 675-nm band pass filter.

For each sample, immunofluorescence measurements were compared with those obtained on standard reference cells (see below) that were stained with the same fluorescent antibodies and run concomitantly. The majority of breast cancer samples reported in this study contained at least 10,000 analyzable cells and all had at least 2,000 analyzable cells. Cell doublets were gated out electronically at the time of data collection.

Controls and Reference Standards. When this study was initiated, peripheral blood lymphocytes were used both as low-level immunofluorescence staining controls and as relative reference cells. Since 1997, studies included reference cells (JC-1939 cells) with each sample run in which Her-2/neu levels were quantitated independently by ELISA (in units of molecules/cell). On the basis of this absolute reference standard, the intracellular levels of Her-2/neu could be calculated for all cells in a concomitantly run tumor sample. Because the ratio of mean Her-2/neu immunofluorescence/reference cell to mean lymphocyte fluorescence is known, the mean level of Her-2/neu/cell in normal lymphocytes could be calculated, and levels of Her-2/neu/cell in tumor cell samples collected before 1997 were recalibrated in units of molecules of Her-2/neu protein/cell. Levels of ras protein/cell were expressed as multiples of the uncorrected mean fluorescence of normal lymphocytes.

Flow Cytometry Data Analysis. Because measurements of cell DNA content by flow cytometry are more reproducible and exhibit lower coefficients of variation in methanol-fixed cells than in paraformaldehyde/methanol-fixed cells (15), the methanol-fixed samples were used for the identification of aneuploid tumor cell populations and the classification of tumors as diploid or aneuploid, using previously described criteria (16). Paraformaldehyde/methanol fixed samples were used for multiparameter analysis. Pre-G1 debris was gated out by inspection. A mean autofluorescence correction was applied cell by cell for each measurement based on corresponding values obtained in normal lymphocytes.

In tumors with aneuploid G1 peak DNA indices that were in the hypotetraploid range, the diploid G1 through G2-M region, which included the overlapping aneuploid G1 peak, was excluded from the analysis (Fig. 1B). To maintain consistency in the analysis, the aneuploid G1 peaks of hypotetraploid cell populations were also excluded from the analysis. Similarly, the G1 peaks in diploid and near-diploid aneuploid samples were also excluded (Fig. 1A).

Cells that contained 150,000 molecules of Her-2/neu protein or more were classified as Her-2/neu overexpressing cells. The mean level of ras in normal lymphocytes was assigned an arbitrary value of 10,000 units/cell. Cells that contained at least 40,000 units of ras protein (>3 SDs above the mean) were classified as ras-overexpressing cells. Aneuploid cells that overexpressed both Her-2/neu protein and ras protein were classified as triple-positive cells.

Cell aggregates (nontriple positive cell doublets, triplets, and larger cell clumps) can be found in the hypertetraploid region of the DNA histogram even after electronic gating. Cell aggregates can mimic triple positive cells and represent a potentially serious confounding factor in the detection and quantitation of the true triple-positive cell fraction. Most of these cell aggregates could be gated out from an aggregate-rich region of the bivariate frequency map of Her-2/neu/cell and cell DNA content, the boundaries of which are determined using a previously described statistical cell aggregate model (14). In our previously published clinical study, we estimated that residual cell aggregate fractions might still be in the range of 2–4%, and we adopted a requirement that triple-positive cell fraction represent at least 5% of all cells in an aneuploid tumor sample for it to be classified as a triple-positive tumor (14). The same 5% threshold requirement was adopted in the present study.

In an aneuploid tumor, the cells that were present in the hypertetraploid region and also overexpressed either Her-2/neu or ras but not both were considered double-positive cells. In an aneuploid tumor, cells excluded from the hypertetraploid region that overexpressed both Her-2/neu and ras were also considered...
double-positive cells. When double-positive cells represented at least 5% of all cells in a nontriple-positive aneuploid tumor, the tumor was classified as a double-positive aneuploid tumor. When the percentage of double-positive cells was <5% of all cells in the sample, the tumor was classified as a single-positive aneuploid tumor.

In a diploid tumor, cells that overexpressed both Her-2/neu and ras were considered double-positive diploid cells. When double-positive cells represented at least 5% of all cells in the sample, the tumor was classified as a double-positive diploid tumor. Cells that overexpressed either Her-2/neu or ras but not both were considered single-positive diploid cells. When single-positive diploid cells represented at least 5% of all cells in a nondouble-positive diploid tumor, the tumor was classified as a single-positive diploid tumor. Otherwise, the tumor was classified as a triple-negative tumor.

**Statistical Analysis.** Effects of prognostic factors were evaluated using the log-rank test; multiple prognostic factors were evaluated using the Cox proportional hazards model. Effects of subpopulation sizes were evaluated using both the quantitation of percent triple-positive cells and the classification described above. The latter results are presented predominantly because they may be more relevant for clinical decision-making. Statistical tests for Cox regression models were based on the likelihood ratio.

**RESULTS**

**Overall and DFS of Triple-Positive versus Nontriple-Positive Tumors.** Fig. 2A compares the overall survival curve of patients whose tumors contained at least 5% triple-positive cells with the overall curve for patients whose tumors did not meet this criterion. The presence of triple-positive cells was a statistically significant adverse prognostic feature (P = 0.002 by log-rank test). The overall survival of patients with node-negative and node-positive tumors are shown in Fig. 2B. The difference in overall survival by nodal status was statistically significant (P < 0.0001).

The DFS curve of patients whose tumors contained at least 5% triple-positive cells is compared with the DFS curve for patients whose tumors did not meet this criterion in Fig. 3A. The presence of triple-positive cells was a statistically significant adverse prognostic feature (P = 0.004). The DFS of patients with node-negative and node-positive tumors are shown in Fig. 3B. The difference in DFS by nodal status was also statistically significant (P < 0.00005). Differences in DFS based on tumor size (Fig. 3C) or hormone receptor status (Fig. 3D) were not statistically significant in this group of patients.

Analysis by the proportional hazards model controlling for nodal status, tumor size, S fraction, and individual components of triple positivity (aneuploidy, Her-2/neu overexpression, or ras overexpression) showed that triple positivity was an independent predictor of high risk (P = 0.006, likelihood ratio test; also see Table 2). This result suggests that the presence of aneuploidy, Her-2/neu overexpression, and ras overexpression in the same cells might be of greater prognostic significance than any combination of all three abnormalities in different cells in the same tumor. Therefore, we compared the DFS of patients with triple-positive tumors with that of patients whose tumors contained at least 5% aneuploid cells, at least 5% of cells with Her-2/neu overexpression, and at least 5% of cells with ras overexpression but in which all three abnormalities were not present simultaneously in the same cells. The results, shown in Fig. 4, indicate that triple-positive tumors were significantly more likely to recur (P = 0.0067, hazard ratio = 2.04).

We found no treatment-related imbalances among triple-positive patients and nontriple-positive patients that might account for the observed differences in DFS.

**Characteristics of the Triple-Positive Cell Fraction.**

The laboratory and clinical features of patients with triple-positive tumors are summarized in Table 3. In this study, for a tumor to qualify as triple-positive tumor, the proportion of triple-positive cells in the hypertetraploid region had to ≥5% of all cells in the sample. This threshold was applied to minimize the potentially spurious effects of cell aggregates present in the sample, as described previously (14). However, in the present study, when a proportional hazards model was fitted treating the proportion of triple-positive cells as a quantitative linear predictor, the hazard ratio was a factor of 1.07 for each percent of

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Fig. 2 A, a comparison of overall survival curves for patients with triple-positive tumors and patients with nontriple-positive tumors. B, overall curves for node-negative patients and node-positive patients.
triple-positive cells \( P = 0.019 \) Wald test, 0.039 likelihood ratio test). This prompted a closer examination of the effect of detection threshold level on the relationship between triple positivity and DFS.

As shown in Fig. 5, the magnitude of the difference in long-term DFS between triple- and nontriple-positive tumors increased roughly monotonically with increasing threshold, up to a threshold of 7%. The difference between the DFS curve pairs was of statistical significance for thresholds in the range 5 through 13%, with the most significant thresholds between 7 and 8% \( P = 3.66 \times 10^{-5} \) unadjusted.

**DFS in Relation to the Number of Accumulated Molecular Abnormalities pr Cell.** The DFS curves for patients whose tumors contained at least 5% of cells with 0, 1, 2, or 3 abnormalities/cell are compared in Fig. 6. DFS for patients with tumors that had triple-positive cells were significantly worse than for patients whose tumors had accumulated 0, 1, or 2 abnormalities/cell \( P \) between 0.0099 and 0.043), which were not significantly different from each other \( P = 0.67 \). The data are consistent with the notion that a critical number and/or a critical mix of accumulated molecular abnormalities/cell may be required for the acquisition of metastatic potential and that the crossing of this threshold is most clearly reflected by the presence of triple-positive cells.

**DFS in Relation to the Number of Accumulated Molecular Abnormalities/Cell and Nodal Status.** The DFS curves of triple-positive and nontriple-positive tumors, examined separately in node-negative and node-positive patients are shown in Fig. 7. Among node-negative patients (Fig. 7A), the difference in DFS between patients with triple-positive tumors and those with nontriple-positive tumors was statistically significant \( P = 0.0077 \). Among node-positive patients (Fig. 7B), the difference in DFS between patients with triple-positive tumors and those with nontriple-positive tumors did not achieve statistical significance \( P = 0.062 \) using the log-rank test, which has poor power when the hazards differ primarily in the early time period, but was significant \( P = 0.036 \) using the Wilcoxon test. When the DFS curves for node-negative triple-positive patients (Fig. 7A) and node-positive triple-positive patients are compared (Fig. 7B), it is apparent that both approach a plateau that is in the range of 0.4, but the times to relapse are much shorter in node-positive triple-positive patients. Using the Cox propor-

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Proportional hazards regression model*</th>
</tr>
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<tbody>
<tr>
<td>Predictor</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>Nodal status ( \geq 1+ )</td>
<td>1.96</td>
</tr>
<tr>
<td>Triple positive ( \geq 5% )</td>
<td>1.85</td>
</tr>
<tr>
<td>Size ( \geq 2.5 \text{ cm} )</td>
<td>1.51</td>
</tr>
<tr>
<td>Hypertetraploidy ( \geq 5% )</td>
<td>1.48</td>
</tr>
<tr>
<td>Ras ( \geq 5% )</td>
<td>0.75</td>
</tr>
<tr>
<td>Her-2/neu ( \geq 5% )</td>
<td>0.85</td>
</tr>
<tr>
<td>S fraction</td>
<td>1.0</td>
</tr>
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</table>

* \( P \)s are for Wald tests.
tional hazard model, the estimated hazard ratio was larger in node-negative patients (2.18 versus 1.49). However, the interaction between nodal status and triple positivity was not significant ($P = 0.30$); thus, it is unclear whether triple positivity is truly a better prognostic factor for node-negative patients than for node-positive patients.

Patterns of Accumulation of Intracellular Abnormalities during Tumor Progression. Although the DFS of patients whose tumors contained 0, 1, or 2 abnormalities/cell was longer than that of patients with triple-positive tumors, some patients with nontriple-positive tumors still developed recurrent disease (Fig. 6). Presumably, there were cells in these nontriple-

### Table 3 Laboratory and clinical features of patients with triple-positive tumors

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>% triple positive cells</th>
<th># positive axillary nodes</th>
<th>Tumor size, cm</th>
<th>Estrogen receptor</th>
<th>Progesterone receptor</th>
<th>S fraction, %</th>
<th>Disease-free survival, months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 IDC</td>
<td>8.0</td>
<td>6</td>
<td>4.0</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>25.0</td>
</tr>
<tr>
<td>2 IDC</td>
<td>13.9</td>
<td>19</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
<td>7.5</td>
<td>10.7</td>
</tr>
<tr>
<td>3 IDC</td>
<td>6.7</td>
<td>0</td>
<td>4.8</td>
<td>+</td>
<td>+</td>
<td>12.0</td>
<td>66.9</td>
</tr>
<tr>
<td>4 Mixed IDC and ILC</td>
<td>9.4</td>
<td>3</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>38.0</td>
<td>32.4</td>
</tr>
<tr>
<td>5 IDC</td>
<td>5.4</td>
<td>0</td>
<td>1.5</td>
<td>+</td>
<td>-</td>
<td>21.0</td>
<td>88.6</td>
</tr>
<tr>
<td>6 IDC</td>
<td>5.0</td>
<td>3</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>26.0</td>
<td>5.6</td>
</tr>
<tr>
<td>7 IDC</td>
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<td>NA</td>
<td>1.0</td>
<td>+</td>
<td>NA</td>
<td>25.1</td>
<td>60.4</td>
</tr>
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<td>NA</td>
<td>30.2</td>
<td>33.4</td>
</tr>
<tr>
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<td>16.2</td>
<td>4</td>
<td>1.3</td>
<td>+</td>
<td>+</td>
<td>24.9</td>
<td>10.9</td>
</tr>
<tr>
<td>10 ILC</td>
<td>8.6</td>
<td>3</td>
<td>2.5</td>
<td>+</td>
<td>+</td>
<td>35.8</td>
<td>4.3</td>
</tr>
<tr>
<td>11 Mucinous</td>
<td>12.0</td>
<td>0</td>
<td>3.0</td>
<td>+</td>
<td>+</td>
<td>36.9</td>
<td>7.2</td>
</tr>
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<td>1</td>
<td>2.2</td>
<td>+</td>
<td>-</td>
<td>19.2</td>
<td>21.6</td>
</tr>
<tr>
<td>13 IDC</td>
<td>18.4</td>
<td>0</td>
<td>4.0</td>
<td>-</td>
<td>-</td>
<td>14.0</td>
<td>7.1</td>
</tr>
<tr>
<td>14 IDC</td>
<td>6.0</td>
<td>4</td>
<td>6.0</td>
<td>+</td>
<td>+</td>
<td>19.3</td>
<td>100.8+</td>
</tr>
<tr>
<td>15 IDC</td>
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<td>NA</td>
<td>1.5</td>
<td>+</td>
<td>+</td>
<td>24.8</td>
<td>88.2+</td>
</tr>
<tr>
<td>16 IDC</td>
<td>12.9</td>
<td>NA</td>
<td>1.5</td>
<td>+</td>
<td>-</td>
<td>53.2</td>
<td>117.7+</td>
</tr>
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<td>2.0</td>
<td>+</td>
<td>+</td>
<td>29.9</td>
<td>87.4+</td>
</tr>
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<td>0</td>
<td>2.7</td>
<td>-</td>
<td>-</td>
<td>49.9</td>
<td>112.2+</td>
</tr>
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<td>+</td>
<td>+</td>
<td>25.4</td>
<td>110.5+</td>
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<td>2.0</td>
<td>-</td>
<td>-</td>
<td>29.5</td>
<td>82.5+</td>
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<tr>
<td>21 IDC</td>
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<td>13.5</td>
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<td>+</td>
<td>20.2</td>
<td>39.4+</td>
</tr>
<tr>
<td>22 IDC</td>
<td>5.8</td>
<td>0</td>
<td>2.4</td>
<td>+</td>
<td>+</td>
<td>15.4</td>
<td>44.1+</td>
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<td>+</td>
<td>-</td>
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<td>55.3+</td>
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<tr>
<td>24 IDC</td>
<td>5.2</td>
<td>0</td>
<td>7.0</td>
<td>-</td>
<td>-</td>
<td>56.6</td>
<td>56.4+</td>
</tr>
<tr>
<td>25 IDC</td>
<td>5.8</td>
<td>0</td>
<td>3.5</td>
<td>+</td>
<td>-</td>
<td>18.2</td>
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<tr>
<td>26 IDC</td>
<td>9.5</td>
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<td>1.5</td>
<td>-</td>
<td>-</td>
<td>47.0</td>
<td>40+</td>
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<td>27 IDC</td>
<td>5.0</td>
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<td>+</td>
<td>+</td>
<td>18.1</td>
<td>39.8+</td>
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<tr>
<td>28 IDC</td>
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<td>+</td>
<td>-</td>
<td>6.5</td>
<td>38.1+</td>
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<td>29 IDC</td>
<td>8.1</td>
<td>0</td>
<td>2.0</td>
<td>-</td>
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<td>12.3</td>
<td>25.1+</td>
</tr>
<tr>
<td>30 IDC</td>
<td>8.3</td>
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<td>0.6</td>
<td>-</td>
<td>-</td>
<td>10.9</td>
<td>28.4+</td>
</tr>
</tbody>
</table>

* IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; NA, not available.
positive primary tumors that had followed alternative evolutionary pathways to aggressive malignancy and had acquired the capacity to metastasize by accumulating molecular abnormalities that were not measured in the present study. As Fig. 6 shows, similar patterns of tumor recurrences are observed in patients with double-positive, single-positive, and triple-negative tumors, suggesting that the convergence of all three abnormalities is required to confer excess risk. To additionally explore this question, we compared DFS of node-positive and node-negative patients in subsets of patients whose tumors contained 0, 1, 2, or 3 molecular abnormalities in the most advanced cells. The results are shown in Fig. 8. Statistically significant differences in DFS between node-positive and node-negative patients were present in the subsets of patients whose tumors had 0 or 1 abnormalities/cell (Fig. 8A and B). Differences in DFS between node-positive and node-negative patients were of borderline statistical significance ($P = 0.052$) in the subset of patients whose tumors had 2 abnormalities/cell (Fig. 8C) but were not significant in patients whose tumors had 3 abnormalities/cell (Fig. 8D). As previously noted (Fig. 7), in the triple-positive group, the plateau regions of the DFS curves were similar in node-negative and node-positive patients, but tumor recurrences developed sooner after surgery in node-positive patients than in node-negative patients (Fig. 8D). This pattern is consistent with the notion that triple-positive tumors had achieved a critical stage of tumor development that was sufficient to confer metastatic potential and that metastases had actually seeded earlier in the clinically more advanced node-positive patients.

Among the 14 node-negative patients with tumors that have no abnormalities/cell, none relapsed during the period of observation (Fig. 8A); 7 patients had been followed for $>5$ years. Assuming an exponential distribution, a 95% confidence lower bound for the 5-year survival is 79%. Among node-negative patients with one abnormality/cell, only 2 of 29 relapsed during the period of observation (Fig. 8B). When the node-negative patients whose tumors had fewer than 2 abnormalities/cell are combined, they had a 5-year DFS of 94% (lower confidence bound, 0.87). However, it should be noted that this group of patients represent only 26% of the patients with known nodal status.

**DISCUSSION**

In this article, we present the results of a prospective study in which intracellular patterns of accumulated molecular abnormalities found in primary breast cancers at the time of surgery are correlated with subsequent clinical outcome. These studies originated with the premise that tumors accumulate multiple genotypic and phenotypic abnormalities as they evolve from the near-normal state to aggressive malignancy in keeping with a model proposed by Nowell (17). We reasoned that the sequence of accumulation of molecular abnormalities in any given tumor would be recorded and preserved in the intracellular patterns of...
molecular abnormalities found in the most advanced cells in that
tumor and in intermediate progenitor cell subpopulations that
might persist in the primary tumor during its biological evolu-
tion. We hypothesized that the co-occurrence of multiple mo-
olecular abnormalities in the same cells might enable synergis-
tic interactions that would not occur if these abnormalities de-
veloped in different cells in the same tumor and that such syn-
ergistic biological effects might have clinical consequences.

The Identification of High-Risk Patients. Our previous
studies of intracellular patterns of molecular changes have con-
firmed that both diploid and aneuploid tumor cells accumulate
multiple molecular abnormalities (18, 19). The clinical rele-
ance of these findings was established early when, among 91
patients followed in the study for a median of 32 months, the
presence of aneuploid cells that simultaneously overexpress
Her-2/neu and ras (triple-positive cells) was found to be of
prognostic significance (14).

We have now accumulated a series of 189 patients with
stage I-III breast cancer whose tumors had been studied pro-
spectively by multiparameter flow cytometry and in which DNA
aneuploidy, Her-2/neu expression, and ras expression were in-
cluded among the measurements performed simultaneously on
each cell. We show here that patients whose tumors contained at
least 5% triple-positive cells had shorter DFSs than those whose
tumors did not (Fig. 2A). This difference was statistically sig-
nificant even after nodal status, tumor size, S-fraction, and the
contribution of each of the individual components of the triple-
positive measurements were taken into account using the Cox
proportional hazard model. At the time of inception of this
study, nuclear grade had not been included among the data to be
gathered prospectively. It has since been appreciated that both
aneuploidy and Her-2/neu overexpression were associated with
high nuclear grade in infiltrating ductal carcinomas (20), and we
have recently performed a retrospective review of nuclear grade
in the tumors reported here. Among 23 triple-positive tumors for
which data on nuclear grade are available, 4 were grade 2-in-
filtrating ductal carcinomas (17%), and 18 were grade 3 (78%),
of which, 17 were infiltrating ductal carcinomas, 1 of which
contained a lobular component; 1 grade 3 triple-positive tumor
was a mucinous carcinoma. When nuclear grade was included as
a covariate in the Cox proportional hazard model, nodal status
and triple positivity still contributed significantly to prognosis,
whereas nuclear grade did not (21).

The presence of triple-positive cells can be obscured by
diploid cells with G2-M cell DNA contents, by the presence of
cell aggregates, and perhaps by other factors. Despite restriction
of the analysis of triple-positive cells to the post-G2-M region of
the DNA histogram and despite gating corrections for cell
aggregates, a thresholding phenomenon was observed such that
the adverse prognostic features of triple-positive cells became
apparent only when their representation in the post-G2-M region
comprised at least 5% of the total cell population. Using laser
scanning cytometry, we have recently found that cell aggregates
with tetraploid and hypertetraploid cell DNA contents persist
even after appropriate corrections are applied, often representing
2–4% of the total cell population (A. A. Pollice, C. A. Smith,
and S. E. Shackney, unpublished observations). However, the
extent to which cell aggregates actually interfered with the
detection of true triple-positive cells was not determined directly
in this study.

As shown in Fig. 4, the DFS of patients with triple-positive
tumors was significantly worse than that of patients who have
all three abnormalities but not simultaneously in the same cells.
Thus, our intact cell-based studies indicate that quantitative
intracellular levels of multiple cell constituents convey prognos-
tic information when they are abnormally elevated in the same
cells. Such information cannot be captured readily by immuno-
histochemical techniques, which are subject to partial cell sec-
tioning effects (22) and which are commonly used to detect
individual cell constituents only one at a time. Techniques that
require that cells be disrupted and the contents of multiple cells
be pooled before analysis can capture neither the intracellular
levels of individual cell constituents nor the correlations among
levels of multiple intracellular constituents in heterogeneous
human tumors.

Although the detection threshold of 150,000 molecules/cell
that was used for flow cytometric detection of Her-2/neu protein
in the present study is well above normal levels (23), it is still
below levels that are generally detectable by immunohistochem-
techniques (24). Immunohistochemical testing for Her-2/
neu overexpression was not included originally among the data
types collected prospectively in study reported here. In a re-
cently performed retrospective immunohistochemical analysis of a subset of our cases (R. Dawson, unpublished observations), among 12 cases that were triple positive by flow cytometry, 9 overexpressed Her-2/neu by immunohistochemistry and 3 cases did not. Of interest, all 3 of these triple-positive, immunohistochemically Her-2/neu-negative cases relapsed. This would suggest that even relatively modest increases in the levels of cellular Her-2/neu expression might convey useful prognostic information when they are associated with other molecular abnormalities in the same cells, even if they might not be predictive for therapeutic response to anti-Her2/neu antibody.

Her-2/neu protein overexpressing tumors, particularly those with low to moderate levels of overexpression, do not always exhibit Her-2/neu gene amplification by fluorescence in situ hybridization (18). This would suggest the potential prognostic relevance of other molecular mechanisms that might affect Her-2/neu overexpression. For example, increased Her-2/neu gene copy number because of chromosome 17 aneusomy alone can be associated with Her-2/neu overexpression by immunohistochemistry (25). In addition, there is a well documented reciprocal relationship between Her-2/neu expression and ER expression in human breast cancer (reviewed in Ref. 24). This might reflect altered regulation of pathways that govern Her-2/neu expression at the transcriptional or translational level.

Conversely, when criteria are adopted for Her-2/neu gene amplification by fluorescence in situ hybridization that permit the systematic inclusion of cases with low to moderate levels of amplification, some tumors exhibit Her-2/neu amplification without Her-2/neu protein overexpression, even at the 150,000 molecule/cell threshold (18).

**Strategies for Identifying Additional Subsets of Low-Risk Patients.** Patients with node-negative nontriple-positive tumors, particularly those with fewer than 2 abnormalities/cell, appear to have a favorable long-term DFS, particularly the patients with triple negative tumors (Fig. 7). None of 14 node-negative patients with triple-negative tumors relapsed during the period of study, 7 of whom were followed for >5 years. However, node-negative patients with triple-negative tumors represent only 8% of all patients in this series. If node-negative patients with single-positive tumors were included in the group of potentially low-risk patients, this group would account for 26% of all patients. The 16% of patients with triple-positive tumors can also be considered to have a well-characterized prognosis (although in this case poor). There remain ~60% of patients who are poorly characterized with regard to prognosis. This group consists of node-negative patients with tumors that are double-positive and node-positive patients with double-positive, single-positive, and triple-negative tumors. In this group of patients, one might probe the biological significance of interrelationships among other potentially relevant intracellular molecular signaling components through the performance of additional combinations of measurements.

Our previously published studies have suggested that p53 dysfunction, which occurs early in the course of breast cancer evolution, often precedes Her-2/neu gene amplification/protein overexpression and aneuploidy, whereas ras gene overexpression is a late development (18, 19, 26). Ras gene mutations,
which often appear early in other tumors, are rare in human breast cancers (27–29). On the basis of these observations and on the findings in the present study that suggest that there are branching molecular evolutionary pathways to aggressive malignancy (Figs. 6 and 8), we surmise that the late appearance of ras overexpression in aneuploid Her-2/neu-overexpressing cells represents a critical transition to aggressive malignancy. In experimental tumor cell systems, ras overexpression (30) and sustained ras/ERK signaling (31, 32) have been shown to be correlated with the acquisition of metastatic properties. Thus, we would entertain the hypothesis that triple positivity may reflect the selection for cells with sustained receptor tyrosine kinase-driven activation of the ras/ERK signaling pathway in a subset of patients with aggressive breast cancer. To test this hypothesis, one might seek to determine whether aneuploid cells that overexpress both Her-2/neu and ras might also overexpress extracellular signal-regulated kinase (ERK)1/2, or exhibit high levels of phosphorylated ERK1/2.

In patients with nontriple-positive tumors who relapsed, one might search for critical transitions to aggressive malignancy in alternative molecular evolutionary pathways to the acquisition of metastatic potential. In the group of tumors in which the most advanced cells were aneuploid and overexpressed Her-2/neu but not ras one might seek to identify deranged intracellular signaling pathways that still rely on membrane-associated receptor tyrosine kinase activation but that bypass a requirement for ras overexpression. These might include Her-2/neu-erbB-3 heterodimer-mediated phosphatidylinositol 3’-kinase pathway activation (33, 34), Her-2/neu/β-catenin-mediated signaling (35–37) or Her-2/neu/src signaling (38, 39) perhaps via signal transducers and activators of transcription 3 (38).

Tumors in which the most advanced cells were aneuploid but overexpressed neither Her-2/neu nor ras might represent intracellular signaling pathways that involve loss or inactivation of retinoblastoma protein (Rb). Loss of Rb obviates the requirement for signaling through cyclin D1 (40, 41), and we would hypothesize that evolutionary selection might not favor cells with receptor tyrosine kinase-driven ras/ERK hyperactivation in this setting. Loss of retinoblastoma function occurs in up to ~40% of human breast cancers (42, 43). Such tumors often exhibit low levels of cyclin D1 expression and high levels of cyclin E expression (43), and the latter is associated with clinically aggressive disease (44–46). Thus, it would be of interest to determine whether in breast tumors with aneuploid cells that exhibit neither Her-2/neu overexpression nor ras overexpression, these same cells might also exhibit low levels of cyclin D1, loss of retinoblastoma, and cyclin E overexpression.

The testing of these hypotheses will require the technical ability to probe simultaneously many more components of the intracellular molecular signaling network in each tumor sample than can be done conveniently by current flow cytometric methods. Laser scanning cytometry can be used to perform multiple study panels on cells obtained from fresh human tumors, each study panel consisting of four or more correlated measurements/cell on small aliquots of fixed tumor cell suspensions. The methodology for performing such studies is well documented (47).

Thus, it is now practical to pursue an intact cell-based approach that preserves quantitative intracellular relationships to investigate correlations among quantitative levels of large numbers of intracellular constituents measured at least four at a time. The data presented here demonstrate that such intracellular relationships are of potential clinical prognostic significance.

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Intracellular Patterns of Her-2/neu, ras, and Ploidy Abnormalities in Primary Human Breast Cancers Predict Postoperative Clinical Disease-Free Survival

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