Chromosomal Rearrangements and Oncogene Amplification Precede Aneuploidization in the Genetic Evolution of Breast Cancer

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ABSTRACT

Breast carcinoma is thought to arise because of multiple successive changes in the genome of the normal epithelial cells. However, little is known of the order of appearance of different types of genetic aberrations. We studied the ERBB2 (Her-2/neu) and CCND1 (cyclin D1) oncogene amplification in flow cytometrically sorted diploid and nondiploid tumor cell populations by fluorescence in situ hybridization (FISH). The purity of the cell sorting was confirmed by static DNA image cytometry. Spectral karyotyping was used to define differences in a genome-wide manner between two distinctly different aneuploid cell clones found in each of two breast cancer cell lines. FISH indicated the presence of gene amplification both in diploid and nondiploid cell clones in 17 of the 21 amplification-containing tumors analyzed. The oncogene copy numbers remained unchanged throughout aneuploidization in 11 of 17 tumors. The remaining six tumors showed an increase in oncogene copy number as well as the number of chromosome 11 or 17 centromeres (the original location of CCND1 and ERBB2, respectively). Breast carcinoma cell lines MDA-157 and MDA-436 showed a significant number of chromosomal rearrangements in the near-diploid clones, which were present in duplicate in the corresponding aneuploid (polyploid) clones. These results indicate that ploidy shift, i.e., aneuploidization, in breast cancer is a late genetic event, which is preceded by both oncogene amplifications as well as many chromosomal rearrangements.

INTRODUCTION

Breast carcinoma, as well as other carcinomas, arises because of multiple changes in the genome of the normal epithelial cells. These changes include single nucleotide point mutations, amplifications, or deletions of single genes, insertions and translocations, gains and losses of entire, or parts of, chromosomes and chromosome arms, and eventually gross changes in chromosome number (aneuploidization; Refs. 1–3). A widely accepted model depicted from colorectal carcinoma suggests that genetic aberrations occur in stepwise manner, correlating well with morphological change from adenoma to carcinoma (4). However, studies in early breast lesions are not fully consistent with this model. Atypical ductal hyperplasia and DCIS have repeatedly been shown to contain numerous genetic aberrations, similar to invasive carcinomas (5, 6). Thus, the relationship between genetic pathogenesis and the morphological progression in breast cancer has remained obscure.

...do not hallucinate.
and counterstained with 0.2 M SSC for 1 min). After a short rinse in distilled water, the slides were air dried.

Three-color FISH was carried out as described previously (8) with minor modifications. Slides were denatured in a 70% formamide-2 M NaCl solution, 10 min each. Samples were then further fixed with 1% paraformaldehyde in PBS (10 min at 4°C), dehydrated in graded ethanol series (70, 85, and 100%), air dried, and baked at 80°C for 30 min in a hybridization oven.

DNA Image Cytometry. Before analysis, the slides with flow cytometrically sorted cells were fixed in 4% phosphate-buffered formaldehyde for at least 30 min and then Feulgen stained as described earlier (21, 22). Integral optical densitometric measurement of nuclear DNA content was performed with a LabEye 3PC image analysis system (Innovate Vision AB, Linköping, Sweden). For each specimen, well-preserved nuclei were selected randomly, and integrated absorbance was measured at a wavelength of 540 nm. Nuclei of diploid cells were collected onto microscope slides for each peak. Slides were immediately fixed in 50% Carnoy’s solution (3:1 methanol:acetic acid in water) and air dried.

Flow Cytometric Sorting. Simultaneous with the DNA analysis of the tumors, nuclei from the two different cell populations were separated by flow cytometric sorting. As sorting criteria, narrow electronic gates were set in the DNA histogram around the G0-G1 peaks, defined by the CVs. PBS (pH 7.0) was used as sheath fluid for sorting. The analysis rate was ~150 nuclei/s. Electronic controls for the sorting were set as follows: droplet frequency, 30 KHz; three droplets/sorted event; and coincidence check of five droplets, this yielding an efficiency close to 100% and purity ~95%. Up to 300,000 sorted nuclei was collected onto microscope slides for each peak. Slides were immediately fixed in 50% Carnoy’s solution (3:1 methanol:acetic acid in water) and air dried.

FISH. After air drying, slides were fixed with 50, 70, and 100% Carnoy’s solution, 10 min each. Samples were then further fixed with 1% paraformaldehyde in PBS (10 min at 4°C), dehydrated in graded ethanol series (70, 85, and 100%), air dried, and baked at 80°C for 30 min in a hybridization oven. Two-color FISH was carried out as described previously (8) with minor modifications. Slides were denatured in a 70% formamide-2× SSC at 72°C for 3 min. The directly labeled dual-color probes for CCND1 (and chromosome 11 centromere) and ERBB2 (and chromosome 17 centromere) were obtained from Vysis, Inc. (Downers Grove, IL). The hybridization mixture for each slide contained 3.4 µl of master mix (70% formamide and 10% dextran sulfate in 2× SSC), 0.4 µl of placental DNA, and 0.25 µl of the probe solution. The probe mixture was denatured at 75°C for 5 min and applied onto slides. The hybridization was carried out overnight at 42°C. Posthybridization stringency washes were done at 72°C (0.4× SSC for 2 min) and room temperature (2× SSC for 1 min). After a short rinse in distilled water, the slides were air dried and counterstained with 0.2 µM DAPI in an antifade solution (Vectashield; Vector Laboratories, Burlington, CA).

Hybridization signals were analyzed using a Zeiss Axioplan 2 epifluorescence microscope equipped with dual band-pass fluorescence filter (Chromatopan, Braintree, NV), which enables simultaneous detection of both green (500–600 nm) and red (600–700 nm) fluorescence. Hybridization signals from at least 50 nuclei were scored to assess the chromosome centromere and oncogene copy numbers. The nuclei was determined to carry an amplification if the number of gene probe signals divided by the number of centromere signals was ≥1.5. Digital images were taken with a cooled CCD camera (Sensys; Photometrics, Tucson, NV) operated via Quips FISH image analysis software (Vysis, Inc.).
**CGH.** CGH was done according to a protocol published previously (16, 24). Briefly, tumor DNA was extracted from freshly frozen tumor tissue (Qiagen, Hilden, Germany) and labeled with FITC-dUTP and FITC-dCTP (DuPont, Boston, MA) using standard nick translation. Labeled DNAs (400–800 ng each, labeled reference DNA; Vysis) and 10 μg of unlabeled Cot-1 DNA (Life Technologies, Inc., Gaithersburg, MD) were hybridized onto commercially available normal metaphase chromosomes (Vysis, Inc.). The hybridizations were evaluated using the QUIPS digital image analysis system (Vysis, Inc.). At least five metaphases from each tumor were analyzed.

**SKY.** The probe mixture containing 24 differentially labeled, chromosome-specific painting probes and Cot-1 blocking DNA (SKY kit; ASI Applied Spectral Imaging, Migdal Ha’Emek, Israel) was denatured and hybridized to denatured tumor metaphase chromosomes according to the protocol recommended by ASI. After hybridization and washing, the chromosomes were counterstained with DAPI. Image acquisitions were performed using a SD200 Spectracube system (ASI) mounted on a Zeiss Axioskop microscope with a custom designed optical filter (SKY-1; Chroma Technology, Brattleboro, VT). The emission spectra were then converted to the pseudocolors matching the fluorochrome combinations of each chromosome. For each cell line and clone, at least seven metaphases were analyzed (25).

### RESULTS

This study was initiated by our CGH findings of breast tumors containing only a small proportion of aneuploid cells when studied by flow cytometry. An example of such a tumor is given in Fig. 1. The flow cytometric DNA histogram (Fig. 1A), shows only a small proportion (23%) of nondiploid cells (DNA index, 2.8); yet, the CGH made from the same tumor specimen reveals a large number of chromosomal gains and losses (Fig. 1B). CGH typically detects clonal chromosomal copy number imbalances (gains and losses) when present in at least 60% of the cells from which the DNA is extracted. This suggests the presence of genetically deviant diploid cells, because the small fraction of nondiploid cells could not possibly be detected by CGH.

**Analysis of ERBB2 and Cyclin D1 Copy Numbers in Flow Cytometrically Sorted Diploid and Nondiploid Tumor Cell Populations.** To study diploid and nondiploid tumor cell clones separately, the propidium iodide-stained tumor cell suspensions were sorted by their DNA content with a flow cytometer and collected on a small area of the flow cytometer’s detection window. After sorting, the samples were fixed with ethanol and stained with FITC- or Cy3-labeled chromosomal painting probes. The DNA content of the sorted cells was then determined by flow cytometry using propidium iodide as a DNA stain. The results of this analysis are shown in Table 1.

### Table 1  Amplification of CCND1 oncogene in flow cytometrically sorted diploid and nondiploid tumor cells

<table>
<thead>
<tr>
<th>Tumor no.</th>
<th>DNA indexes by FCM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DNA indexes by image cytometry</th>
<th>Mean CCND1/cen 11 in diploid sort</th>
<th>Mean CCND1/cen 11 in nondiploid sort</th>
<th>Clones amplified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.03/2.11</td>
<td>~1.0–2.1</td>
<td>9/3</td>
<td>10/3.5</td>
<td>Both</td>
</tr>
<tr>
<td>2</td>
<td>1.03/1.35</td>
<td>~1.0–1.7</td>
<td>7/2.6</td>
<td>8/3</td>
<td>Both</td>
</tr>
<tr>
<td>3</td>
<td>1.00/1.59</td>
<td>~1.0–1.7</td>
<td>4/2</td>
<td>9/4</td>
<td>Both</td>
</tr>
<tr>
<td>4</td>
<td>1.00/2.15</td>
<td>ND</td>
<td>8/2</td>
<td>11/3</td>
<td>Both</td>
</tr>
<tr>
<td>5</td>
<td>0.98/1.72</td>
<td>ND</td>
<td>2/2</td>
<td>7/2</td>
<td>Nondiploid</td>
</tr>
<tr>
<td>6</td>
<td>1.00/1.96</td>
<td>~1.0–1.9</td>
<td>3/2</td>
<td>7/4</td>
<td>Both</td>
</tr>
<tr>
<td>7</td>
<td>0.99/1.48</td>
<td>~1.0–1.5</td>
<td>4/2</td>
<td>6/3</td>
<td>Both</td>
</tr>
<tr>
<td>8</td>
<td>0.99/2.22</td>
<td>ND</td>
<td>10/2</td>
<td>9/3</td>
<td>Both</td>
</tr>
<tr>
<td>9</td>
<td>0.99/2.22</td>
<td>ND</td>
<td>2/2</td>
<td>9/2.3</td>
<td>Nondiploid</td>
</tr>
<tr>
<td>10</td>
<td>1.00/1.51</td>
<td>ND</td>
<td>11/3</td>
<td>7/3.6</td>
<td>Both</td>
</tr>
</tbody>
</table>

<sup>a</sup> FCM, flow cytometric analysis; cen; centromere; ND, not determined.

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Fig. 3. A (diploid) and B (nondiploid) show FISH of ERBB2 oncogene (red) and chromosome 17 centromere (green; tumor 14). C (diploid) and D (nondiploid) show FISH of CCND1 oncogene (red) and chromosome 11 centromere (green; tumor 4). The amplifications of both oncogenes are clearly distinguishable in both cell clones when dividing the number of gene probe signals (red) by the number of centromere probes (green; amplification if ≥1.5). Lower right corners, average probe:reference ratio for each sample. DAPI (blue) was used as a nuclear counterstain.
microscope slides. Static DNA image cytometry was performed to confirm the purity of sorted cell clones. The cell clones with the DNA indexes matching flow cytometry were found by image cytometry, and no evidence for significant impurity or contamination was found (Fig. 2 and Table 1).

The FISH hybridizations, performed on the sorted cells, indicated that both diploid and nondiploid cell clones often showed amplification of ERBB2 and CCND1 (Fig. 3). The gene copy numbers from 21 sorted tumors are presented in Tables 1 and 2. Eight of 10 tumors (80%) with CCND1 amplification were amplified in both diploid and nondiploid cell (copy number relative to 11 centromeres, $\geq 1.5$). In two tumors (18%), only the nondiploid clone was found amplified (Table 1). In the 11 tumors previously determined as having ERBB2 oncogene amplification, 9 (82%) showed amplification in both clones and two in the nondiploid clone only (18%; Table 2). Tumors with amplification in the diploid clone only were never seen.

The mean copy number of CCND1 and ERBB2 (per cell) remained approximately the same in diploid and nondiploid cells in 11 of 17 tumors with amplification in both clones (tumors 1, 2, 8, 10, 15, 16, 17, 18, 19, 20, and 21; see Tables 1 and 2). In the remaining cases (tumors 3, 4, 6, 7, 13, and 14; see Tables 1 and 2), the gene copy number, as well as the chromosome copy number of the respective reference centromere, increased during aneuploidization. When counting the ratio between gene copy number and chromosome copy number, we found that it remained stable throughout aneuploidization.

We analyzed the copy numbers of the reference probes (pericentromeric probes for chromosomes 11 and 17, analyzed separately). Surprisingly, multiple signals were found in the diploid clones of 7 tumors (tumors 1, 2, 10, 16, 17, 18, and 21; see Tables 1 and 2). All of these tumors showed ERBB2 or CCND1 amplification both in diploid and nondiploid clones. The corresponding flow cytometric DNA histograms showed a small CV (<5.2% in all but one tumor) for the diploid DNA peaks, which is generally considered as a sign of little or no genetic instability.

Duplication of Chromosomal Changes as Evidenced by SKY. The order of appearance of chromosomal rearrangements was further studied with breast cancer cell lines MDA-157 and MDA-436, which both contain two distinct nondiploid cell clones despite tens of passages in culture. The flow cytometric DNA indexes were 1.31 and 2.50 (79 and 21% of cells in G0 -G1 peaks) for MDA-157 and 0.85 and 5.2% in all but one tumor) for the trout erythrocytes. In

For example, derivative chromosomes containing material from chromosomes 3, 5, 12, and 20 in MDA-157 and chromosomes 1, 7, 8, and 21 in MDA-436 (Fig. 5) were duplicated in the polyploid clone. Several "new" aberrations (not present in the near-diploid clone) were also found in the polyploid clones. These include chromosomes 1, 4, and 9 in MDA-157 and chromosomes 2, 3, and 6 in MDA-436.

**DISCUSSION**

This study was initiated by our findings made by CGH in which we were able to show copy number imbalances in breast tumor samples

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### Table 2: Amplification of ERBB2 oncogene in flow-cytometrically sorted diploid and nondiploid tumor cell clones

<table>
<thead>
<tr>
<th>Tumor no.</th>
<th>DNA index by FCM(a)</th>
<th>Mean ERBB2/cen 17 in diploid sort</th>
<th>Mean ERBB2/cen 17 in nondiploid sort</th>
<th>Clones amplified</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>0.99/1.77</td>
<td>2/2</td>
<td>30/3</td>
<td>Nondiploid</td>
</tr>
<tr>
<td>12</td>
<td>0.99/1.64</td>
<td>2/2</td>
<td>18/2</td>
<td>Nondiploid</td>
</tr>
<tr>
<td>13</td>
<td>0.99/1.48</td>
<td>4.3/1.4</td>
<td>18/4.4</td>
<td>Both</td>
</tr>
<tr>
<td>14</td>
<td>0.97/1.64</td>
<td>15/2</td>
<td>24/3.7</td>
<td>Both</td>
</tr>
<tr>
<td>15</td>
<td>1.00/1.58</td>
<td>16/2</td>
<td>18/2</td>
<td>Both</td>
</tr>
<tr>
<td>16</td>
<td>1.00/1.90</td>
<td>12/5.2</td>
<td>10/4.6</td>
<td>Both</td>
</tr>
<tr>
<td>17</td>
<td>1.01/2.80</td>
<td>18/3</td>
<td>10/3.5</td>
<td>Both</td>
</tr>
<tr>
<td>18</td>
<td>1.01/2.93</td>
<td>11/4.5</td>
<td>11/5.3</td>
<td>Both</td>
</tr>
<tr>
<td>19</td>
<td>1.00/1.86</td>
<td>19/2</td>
<td>17/2</td>
<td>Both</td>
</tr>
<tr>
<td>20</td>
<td>0.99/1.78</td>
<td>10/4.7</td>
<td>18/2.3</td>
<td>Both</td>
</tr>
<tr>
<td>21</td>
<td>0.99/0.26</td>
<td>14/3</td>
<td>18/3</td>
<td>Both</td>
</tr>
</tbody>
</table>

\(a\) FCM, flow cytometric analysis; cen, centromere.
containing only a small fraction of aneuploid cells, as evidenced by DNA flow cytometry (26). CGH generally requires the sample to contain at least 60–70% of genetically deviating cells to detect aberration (16). We have found several tumors with an aneuploid cell fraction of <30%, and yet they show multiple chromosomal aberrations by CGH. This suggests indirectly that a significant fraction of the diploid cells must be genetically deviant and contain at least partly the same genetic aberrations as the aneuploid cells.

To explore the order of appearance of genetic aberrations directly, we sorted tumor cells for their DNA content by flow cytometry and analyzed the different clones by FISH. In 17 of 21 tumors, the amplification of ERBB2 and CCND1 was present both in DNA-diploid and nondiploid cell clones. When the diploid clone was found amplified, typically only 25–50% of the cells showed oncogene amplification. The presence of cells without amplification can be best explained by the presence of nonmalignant cells, i.e., stromal, inflammatory, and benign epithelial cells that are present in every breast tumor. The presence of nonepithelial cells in breast carcinomas has been shown previously (27). The contamination of the diploid cell sort by nondiploid cells was excluded by analyzing the DNA content of the cells after sorting, using DNA image cytometry. These experiments showed clearly that there was no contamination of nondiploid cells on the diploid sorted slides (or vice versa). On the basis of the image cytometry data, we feel that the relatively high proportion of cells with oncogene amplification among the diploid cells (25–50%) is very unlikely due to nondiploid cell contamination. Thus, we conclude that aneuploid primary breast tumors often contain DNA diploid cell clone(s) that have undergone oncogene amplification.

If the gene amplification takes place already in diploid state (before aneuploidization), one would expect that the number of oncogene
copies becomes multiplied along with chromosome multiplication. This was seen only in 6 of the 17 tumors where both diploid and nondiploid cells were found amplified. Thus, in these 6 tumors, the amplicon-containing chromosomes have multiplied during aneuploidization. In the remaining 11 tumors, the mean number of copies of ERBB2 or CCND1 was almost the same in the DNA diploid and nondiploid cells. The same was true also for chromosome 11 and 17 centromere counts, which neither showed any clear increase. In these tumors, the diploid clones already showed unexpectedly more than two centromere signals/cell. It is, therefore, possible that the multiplication of amplification-carrying chromosomes has occurred already before gross polyplidization. The preserved diploid DNA content by flow cytometry can be explained by losses of other chromosomes or chromosome arms. An alternative explanation for unaltered oncogene copy numbers is that the amplified gene copies are present in double minute chromosomes. However, according to cytogenetic literature, double minute chromosomes are considered to be rare in primary breast cancer (28).

The endoreduplication after multiple chromosomal rearrangements was best visualized by our data from SKY. The flow cytometry analysis confirmed that the two different clones analyzed were not artifacts caused by induction metaphase cells in culture (by Colcemid analysis confirmed that the two different clones analyzed were not breast cancer (28).

double minute chromosomes are considered to be rare in primary chromosome arms. An alternative explanation for unaltered oncogene flow cytometry can be explained by losses of other chromosomes or before gross polyploidization. The preserved diploid DNA content by multiplications become multiplied along with chromosome multiplication.

ACKNOWLEDGMENTS

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