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 (pseudoploid) 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 concordant 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.

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Tumor Samples. Tumor specimens from 21 primary invasive breast cancers were obtained from the frozen tumor tissue bank. The specimens have been collected for the routine analysis of steroid hormone receptors, and DNA flow cytometry was performed at the Department of Oncology, Lund University, Sweden. The tumors selected had all been determined previously as having amplification of the 11q13 region (tumors 1–10) or ERBB2 oncogene (tumors 11–21) by Southern blot hybridization (17, 18). For the SKY analysis, two breast cancer cell lines (MDA436 and MDA157) were obtained from American Type Culture Collection (Rochester, Michigan) and used in the SKY preparations.

DNA Flow Cytometry. Freshly frozen tumor samples (100–200 mg) and cell lines were prepared for flow cytometric analysis as described previously (19). The DNA histograms were obtained using the modal values of chicken and trout RBCs. The mean channel numbers of G0–G1 peaks were then used for the calculation of the DNA index, with chicken and trout RBCs as reference standard (19). The
and counterstained with 0.2 M SSC for 1 min). After a short rinse in distilled water, the slides were air dried.

The nuclei was determined to carry an average of 500 – 600 nm green and 600 – 700 nm red fluorescence. Hybridization signals from at least 50 nuclei were scored to assess the chromosome centromere.

Two-color FISH was carried out as described previously (8) with minor modifications. Slides were denatured in a 70% formamide-2 M DAPI in an antifade solution (Vectorshield; Vector Laboratories, Burlingame, CA).

Hybridization signals were analyzed using a Zeiss Axioplan 2 epifluorescence microscope equipped with dual band-pass fluorescence filter (Chromatechology, Brattleboro, 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 average of 500–600 nm green and 600–700 nm red fluorescence. Hybridization signals from at least 50 nuclei were scored to assess the chromosome centromere.

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.).

Fig. 1. A flow cytometric DNA histogram (A) and the CGH copy number profiles of chromosomes showing gains and losses (B) from a primary breast tumor that contained only a small proportion (23%) of nondiploid cells of the total cell count (nondiploid DNA index, 2.8). In panel A, C and T refer to chicken and triton erythrocytes, respectively, used as internal controls. D and Non-D, diploid and nondiploid cell populations, respectively. In panel B, the curves shown are mean values of gains/losses from at least six chromosomes (bold) ± 95% confidence interval. The threshold values for losses and gains were set to 0.85 and 1.15, respectively. This tumor was not further analyzed by FISH.

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.

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 from human cerebellum (fresh autopsy material) were used as diploid external reference cells for ploidy assessment and as a control for Feulgen staining (23).

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-colored 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 (Vectorshield; Vector Laboratories, Burlingame, CA).

Hybridization signals were analyzed using a Zeiss Axioplan 2 epifluorescence microscope equipped with dual band-pass fluorescence filter (Chromatechology, Brattleboro, 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 average of 500–600 nm green and 600–700 nm red fluorescence. Hybridization signals from at least 50 nuclei were scored to assess the chromosome centromere.

Fig. 2. Confirmation of the purity of the flow cytometrically sorted diploid and nondiploid cell clones (tumor 1). A, the original DNA flow cytometry histogram with clearly distinguishable diploid and nondiploid cell populations. B and C, the DNA image cytometry histogram from the flow cytometrically sorted diploid and nondiploid clones, respectively. Insets, histograms of diploid reference cells (nonsorted cells from human cerebellum). Note that the propidium iodide staining made for sorting has impaired the Feulgen staining, thereby shifting both peaks to the left in the image cytometry histograms. The diploid DNA index was 1.03 by flow cytometry and ~1.0 by image cytometry. The nondiploid DNA index was 2.11 by flow cytometry and ~2.1 by image cytometry.
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 onto

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 in diploid sort</th>
<th>Mean CCND1/cen 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.
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, >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 1.70 for MDA-436 (77 and 23% of cells in G0-G1 peaks; Fig. 4). The modal chromosome numbers in these clones when analyzed by SKY where 54 and 95 for MDA-157 and 39 and 80 for MDA-436, respectively.

The different clones were analyzed separately by SKY, as shown in Fig. 5. In both cell lines, the aneuploid (polyploid) clone contains a majority of the aberrant chromosomes of the near-diploid clone in duplicate. 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...
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 polyploidization. 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 flow cytometry can be explained by losses of other chromosomes or chromosome arms. The preserved diploid DNA content of nondiploid cells. The same was true also for chromosome 11 and 17.

REFERENCES


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REFERENCES

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