American Journal of Pathology, Vol. 161, No. 2, August 2002

Short Communication

Variable Levels of Chromosomal Instability and Mitotic Spindle Checkpoint Defects in Breast Cancer

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Cytogenetic analyses have revealed that many aneuploid breast cancers have cell-to-cell variations of chromosome copy numbers, suggesting that these neoplasms have instability of chromosome numbers. To directly test for possible chromosomal instability in this disease, we used fluorescent *in situ* hybridization to monitor copy numbers of multiple chromosomes in cultures of replicating breast cancer-derived cell lines and nonmalignant breast epithelial cells. While most (7 of 9) breast cancer cell lines tested are highly unstable with regard to chromosome copy numbers, others (2 of 9 cell lines) have a moderate level of instability that is higher than the "background" level of normal mammary epithelial cells and MCF-10A cells, but significantly less than that seen in the highly unstable breast cancer cell lines. To evaluate the potential role of a defective mitotic spindle checkpoint as a cause of this chromosomal instability, we used flow cytometry to monitor the response of cells to nocodazole-induced mitotic spindle damage. All cell lines with high levels of chromosomal instability have defective mitotic spindle checkpoints, whereas the cell lines with moderate levels of chromosomal instability (and the stable normal mammary cells and MCF10A cells) arrest in G_2 when challenged with nocodazole. Notably, the extent of mitotic spindle checkpoint deficiency and chromosome numerical instability in these cells is unrelated to the presence or absence of *p53* mutations. Our results provide direct evidence for chromosomal instability in breast cancer and show that this instability occurs at variable levels among cells from different cancers, perhaps reflecting different functional classes of chromosomal instability. High levels of chromosomal instability are likely related to defective mitotic checkpoints but not to *p53* mutations. ***(Am J Pathol 2002, 161:391–397)***

Abnormal numbers of chromosomes (aneuploidy), structurally rearranged chromosomes, and nucleotide-level mutations are characteristic of most human cancers. In fact, the numbers of genomic alterations in cancer cells appear to exceed the level possible from a stepwise accumulation of mutations in cells with normal mutation rates, leading to the proposition that cancer cells have genomic instability.[1,2]

One form of genomic instability found in cancer cells, chromosomal instability, is characterized by losses or gains of chromosomes during cell replication. Much of our understanding of chromosomal instability is based on cell culture experiments that monitored chromosome numbers of replicating cultures of colorectal cancer derived cell lines.[3,4] These studies have shown a consistent relationship between instability of chromosome numbers in replicating cultures of the colorectal cancer cells and a defect in a checkpoint that normally arrests cells in mitosis when agents such as nocodazole disrupt the mitotic spindle,[5] suggesting that chromosomal instability results from a defective mitotic spindle mechanism that allows segregation of improperly aligned chromosomes during mitosis. Some colorectal cancer cell lines with chromosomal instability have also have been found to have mutations of the *BUB1* mitotic checkpoint gene,[5] providing genetic evidence to strengthen this link between the

Supported by the National Cancer Institute Breast Cancer SPORE Program (5P50CA088843).

Accepted for publication May 16, 2002.

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mitotic spindle checkpoint defect and the unstable phenotype.

Studies of breast cancer karyotypes have demonstrated that this type of cancer frequently has the structural genomic aberrations seen in other forms of human cancer, including aneuploidy.[6–8] Furthermore, several recent studies have shown variability of chromosome numbers from cell-to-cell within a given breast cancer,[7,9–11] suggesting that breast cancers also have chromosomal instability. The present study was undertaken to characterize chromosomal instability of breast cancer by measuring chromosome numerical changes in replicating cultures of breast cancer cell lines, and to study the possible mechanistic role of defective mitotic spindle checkpoints in causing chromosomal instability in breast cancer. In addition to the expected finding of some breast cancer cell lines with high levels of chromosomal instability and defective mitotic checkpoints, we observed other cell lines with moderate levels of chromosomal instability and intact mitotic spindle checkpoints. These results suggest that there are multiple mechanisms for generating chromosomal instability in breast cancer.

Materials and Methods

Breast Cancer Cell Lines, Culture Conditions, and Treatments

Nine human breast cancer cell lines (MCF7, MDA-MB-361, MDA-MB-231, T47D, BT-549, HCC38, HCC1806, HCC1143, HCC1937), and the non-tumorigenic breast epithelial cell line MCF10A were purchased from ATCC (Rockville, MD) and cultured in recommended media at 37°C/5% CO_2. Primary cultures of mammary epithelial cells were established and maintained in MEGM (Clonetics, San Diego, CA) using previously described methods.[12] All cell types proliferated well in cell culture, with doubling times ranging from 0.8 days (for HCC1143 cells) to 1.2 days (for MCF10A cells).

Nocadazole treatment of cells (0.2 or 2.0 μg/ml for 24 hours) was used to disrupt the mitotic spindle and induce mitotic arrest. Treated and untreated cell cultures were harvested with trypsin for cell cycle analysis or stained *in situ* for fluorescent *in situ* hybridization (FISH) analysis.

Cell Cycle Analysis

Single parameter cell cycle was measured using a FACScan (Becton-Dickinson Immunocytometry Systems, San Jose, CA) on propidium iodide stained nuclei, prepared with the nuclear isolation technique previously described.[13] Cell cycle compartments were deconvoluted from single-parameter histograms of 10^4 cells by Multicycle (Phoenix Flow Systems, San Diego, CA) and debris and doublets were removed *via* software algorithms.

Because single-parameter DNA histograms cannot differentiate G_2 from M cells, immunofluorescent staining with the mitotic protein monoclonal #2 antibody (MPM-2, Upstate Biotechnology, Lake Placid, NY) was used as previously described[14] to distinguish these phases of cell cycle. In addition, cycling and non-cycling cells in the G_0/G_1 peak were differentiated pulse-labeling cultures for 24 hours with 10 μmol/L 5-bromodeoxyuridine (BrdU; Sigma Chemical Co., St. Louis, MO) as previously described.[15] Cells were harvested at 24 hour intervals after removal of BrdU (chase periods) for analysis and incorporated BrdU was detected using a fluorescein (FITC) conjugated antibody to BrdU (clone B44, Becton-Dickinson Immunocytometry Systems, San Jose, CA).

Fluorescence in Situ *Hybridization Analysis of Chromosome Copy Numbers*

To determine chromosome copy numbers in proliferating cell cultures, cells were subcultured on chamber slides at low density and allowed to proliferate until discrete colonies of approximately 100 to 200 cells formed (ie, 6 to 8 generations). FISH was then performed with centromeric probes specific for chromosomes 4, 6, 7, 9, 10, 11, 15, 16, 17, and 18 (Vysis, Downers Grove, IL), using protocols recommended by the manufacturer. Individual representative colonies were selected for each experiment and chromosome copy numbers were counted for all cells within the colony using appropriate excitation light sources to visualize the probes.

Results

Infidelity of Chromosome Copy Numbers in Replicating Breast Cancer Cell Cultures

Using centromeric fluorescent FISH probes, we counted copy numbers for up to 10 different chromosomes in at least 100 cells from each of several colonies[2–5] in the 11 cell types examined. Examples of the results of the FISH experiments are shown in Figure 1. Little variation was seen for counts between different colonies using the same probe (even when colonies from different original platings of cells were counted) and we combined all counts to calculate a mode (the most common copy number) and variability (percentage of cells with copy number different from the mode) for each chromosome of each cell type and we also calculated a mean variability index for all chromosomes counted in each cell type. These modes were remarkably consistent among the multiple colonies examined to count individual chromosomes. Even for situations where the average variability of chromosome counts within individual colonies frequently exceeded 50% (eg, HCC1143), only two colonies had a mode different (by a value of 1) than the overall mode. These results are summarized in Table 1.

Some variability in the extent of instability among different individual chromosomes within particular cell types is seen, the most striking example being in the MCF10A cells, where 22% of the cells showed deviation from the mode for chromosome 17 in contrast to 3 to 4% deviation for other chromosomes. No particular chromosome showed a consistently increased level of variability com-

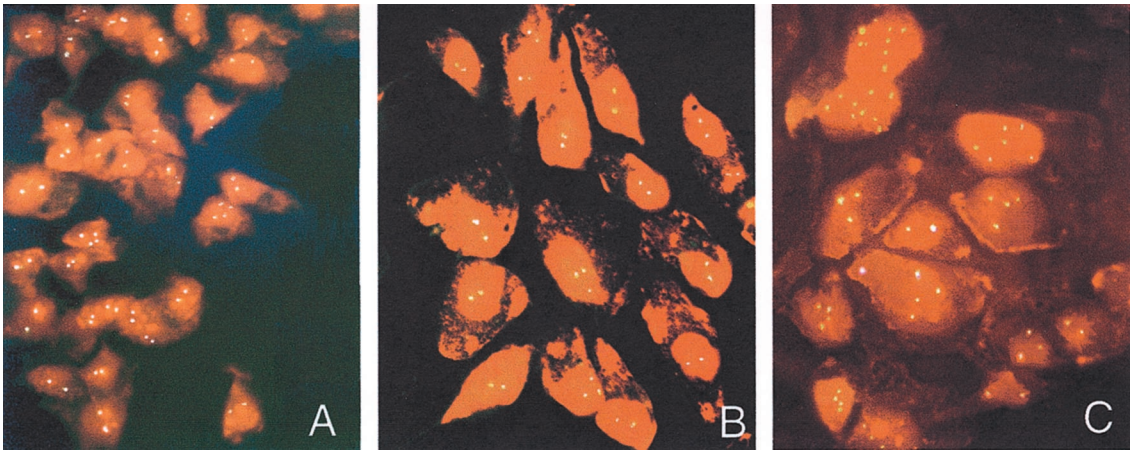

Figure 1. Chromosomal instability in breast cancer cells. Fluorescent *in situ* hybridization measurement of centromeric probes was used to measure chromosome copy numbers in colonies of cultured cells. Results shown are for probes to chromosome 11 in normal mammary epithelial cells (**A**), chromosome 16 in HCC1806 cells (**B**), and chromosome 7 in HCC1143 cells (**C**) are shown. Significant cell-to-cell variability in numbers of chromosomes is seen only in the HCC1143 cells.

pared to other chromosomes across the different cell types, and thus an explanation for this chromosome-specific instability is not readily apparent. Notably, this variability for individual chromosomes does not affect the general classification of the cell lines with regard to the overall level of chromosome numerical instability, as described below.

Considering overall chromosomal instability, seven of the breast cancer cell lines (MCF7, MDA-MB-361, T47D, BT-549, HCC38, HCC1143, and HCC1937) showed highly variable copy numbers among different cells in the culture for all of the chromosomes counted, with numbers of specific chromosomes varying from the mode in over 40% of the cells. For example, the highest level of variability, 62.6%, was seen for the HCC1143 cell line. The differences in the level of variability were not significantly different among these seven cell lines (using two-tailed *t*-test with unequal variance, $P > 0.25$ for all comparisons).

Two of the breast cancer cell lines, MDA-MB-231 (18.1%) and HCC1806 (26.6%), were found to have significantly lower levels of chromosome numerical variability than the seven most unstable cell lines ($P < 0.001$ for all pairwise comparisons of MDA-MB-231 or H1806 to other cell lines). However, the MDA-MB-231 and HCC1806 cell lines do have significantly greater variability than do normal mammary epithelial cells or the non-tumorigenic MCF10A cell line ($P < 0.05$ for all pairwise comparisons), indicating that these two cell lines show a measurable level of instability by this assay. It is uncertain whether the level of variability measured in the normal mammary epithelial cell cultures (10.5%) and MCF10A

Table 1. Summary of FISH Results in Breast Cancer Cell Lines and Normal Mammary Epithelial Cells

Cell line	p53 status		Ch 4	Ch 6	Ch 7	Ch 9	Ch 10	Ch 11	Ch 15	Ch 16	Ch 17	Ch 18	Average variability
MCF-10A	WT	Mode		2	2	2		2			2	2	
		% variable		5	5	4		4			22	5	7.7%
Normal breast	WT	Mode	2	2			2	2		2	2		
epithelial		% variable		12	12			9	14		12	11	11.5%
MDA-MB-231	M	Mode	3	3	4	4	4	4	1		4	3	
		% variable	23	15	18	16	21	15	8		35	12	18.1%
HCC-1806	M	Mode	2	3	2	2	3	3	3	2	2		
		% variable	21	28	36	31	36	31	19	15	23		26.6%
MDA-MB-361	WT	Mode	2	2	5	2		2			3		
		% variable	61	55	47	40		63			61		54.5%
BT-549	M	Mode		2				2	2		2	3	
		% variable		57				67	25		39	60	49.6%
T47D	M	Mode	1	2	2	1	2	2	2	3	3		
		% variable	48	55	60	42	51	74	53	18	47		49.7%
HCC-1937	M	Mode							3	7	4		
		% variable							56	51	44		50.3%
HCC-38	M	Mode	2	1	4	2		4			4		
		% variable	73	40	55	66		63			11		50.3%
MCF-7	WT	Mode	4	3	3	2	3	3	2		3		
		% variable	55	52	65	38	58	73	55		69		58.1%
HCC-1143	WT	Mode	2	2	5	2	4			3	3		
		% variable	57	65	75	58	74			35	73		62.4%

The mode and percentage of cells with copy numbers different than mode are reported for each of the chromosomal probes tested. In addition, for each cell type, the overall variability represents the average of variability for all individual chromosomes.

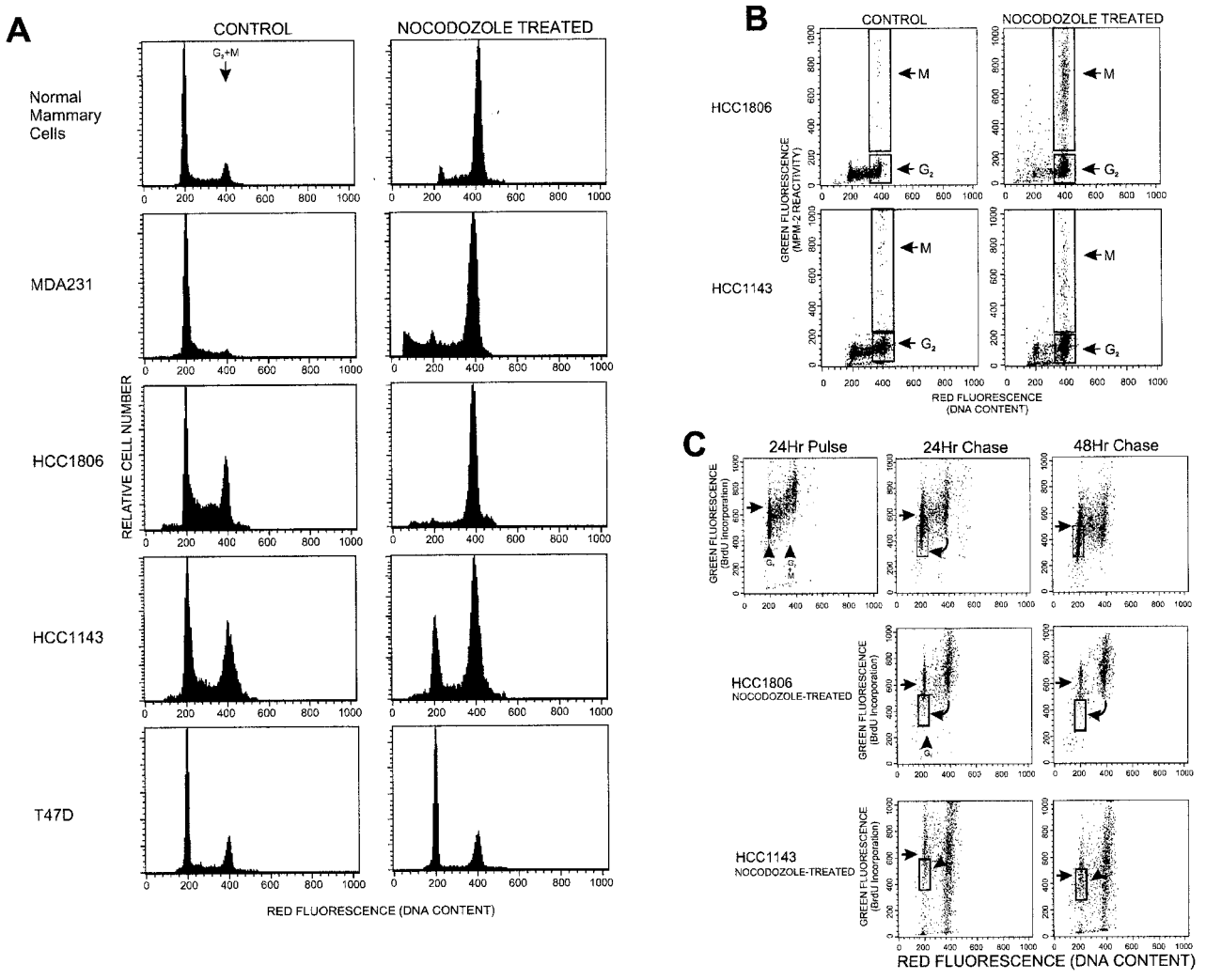

Figure 2. Flow cytometry analysis of mitotic checkpoints in mammary epithelial cells and breast cancer cells. **A:** DNA content histograms are shown for 5 cell types grown in control (log growth) conditions and 24 hours after treatment with nocodazole. Effective block in G/M is seen for normal mammary cells, MDA-MB-231 cells, and HCC1806 cells, but not in HCC1143 or T47D cells. **B:** The increase in the G_2/M peak of the histogram for nocadozole-treated HCC1806 cells is largely due to arrest of cells in mitosis. Cells represented in boxes labeled M have high DNA content (G_2/M peak) and high level of labeling with the MPM-2 antibody. Increased numbers of cells arrested in mitosis are seen in the nocodazole-treated HCC1806 cultures but not the nocodazole-treated HCC1143 cultures. **C:** BrdU pulse-label experiments to demonstrate escape of HCC1143 cells from G_2/M block. The **top row** of panels show distribution of BrdU labeling as a function of DNA content for untreated HCC1806 cultures at end of 24-hour BrdU label (pulse) and 24 hours and 48 hours after removal of BrdU (chase). Increasing numbers of cells in G_1 with low levels of BrdU (box) represent cells that have diluted amounts of BrdU through cell replication. The **second row** shows results for nocodazole-treated HCC1806 cultures. After 24 hours and 48 hours chase, these treated cultures have significantly fewer numbers of cells in $G_1$with reduced levels of BrdU (boxes) when compared to control cultures, consistent with arrest of these cultures in G_2. In contrast, a significant proportion of the nocodazole-treated HCC1143 cells in G_1 have reduced BrdU labeling after 24 hours and 48 hours chase (**third row**), indicating that these cells incorporated BrdU during a previous pulse-label cycle and escaped the G_2/M block during nocodazole treatment. The distribution of cells for untreated HCC1143 cultures was essentially similar to those shown for HCC1806.

cultures (7.7%) reflects the background error in our measurements or a modest level of instability in these cells.

Mitotic Checkpoints Are Defective in Breast Cancer Cell Lines with High Chromosomal Instability

To quantify the ability of nocadazole to block cells in mitosis, we performed flow cytometric analysis and measured the G_0/G_1, S, and G_2/M areas of the DNA labeling histograms (Figure 2A). For normal mammary cells, MCF10A cells, and the MDA-MB-231 and HCC1806 cell lines, nocodazole treatment (at 0.2 μg/ml) results in a nearly complete accumulation of cells in the G_2/M peak. For the other 7 breast cancer cell lines, the accumulation of cells in response to nocodazole treatment is incomplete, and significant numbers of cells are seen in the G_0/G_1 peak. This incomplete block was seen even when higher levels (2 μg/ml) were used.

We further characterized the G_2/M peak of the HCC1806 and HCC 1143 cells using antisera to MPM-2 as a marker to differentiate M phase cells from those in G_2 phase (Figure 2B). These experiments showed that nocodazole treatment leads to a 390-fold increase in the percentage of HCC1806 cells in M phase, but only a 1.33-fold increase in the percentage of HCC1143 cells in

M phase. Thus, quantitative data indicate that the accumulation of nocodazole-treated HCC1806 cells in the G_2/M peak is due specifically to block of cells in M phase. These data corroborate independent observations of increased mitotic figures in DAPI-stained cultures of HCC1806 and MDA-MB-231 cells treated with nocodazole (data not shown).

To determine whether the substantial G_0/G_1 peak seen in most nocodozole-treated breast cancer cultures (eg, HCC1143) represents cells that have escaped from the nocodazole-induced mitotic block or cells that failed to undergo DNA synthesis and cell replication (ie, blocked in G_0/G_1), we pulse-labeled cultures with BrdU for 24 hours before nocodazole treatment. Then, at 24-hour intervals of "chase" (incubation without BrdU), we measured the levels of incorporated BrdU and total DNA content (Figure 2C). In control cultures grown in the absence of nocodazole, transition of cells through the G_2 and M phases of the cell cycle was represented by increasing numbers of cells in G_1 with low levels of BrdU label. Nocodazole treatment of HCC1806 cultures blocked this transition, preventing the emergence of these cells in G_1 with low levels of BrdU label during the 48 hours chase. In contrast, increased numbers of HCC1143 cells in G_1 with low levels of BrdU appeared during the 48-hour chase, indicating that significant numbers of these cells escaped the of nocodazole-induced G_2/M block.

For each cell type, we compared the relative ability of nocodazole to arrest cultures in mitosis (expressed as the ratio of the G_2/M peak in nocodazole-treated cultures to that in control cultures) to the level of chromosome numerical instability. These ratios range from 1.29 in the T47D cells, which show minimal blocking response to nocodazole, to 5.28 in normal epithelial cultures, where essentially all nocodazole-treated cells are blocked in mitosis. Overall, a remarkably strong correlation (correlation = 0.951, $P < 0.001$) was seen between this "mitotic block index" and the measures of variability from the mode numbers of chromosomes.[16] This data for our set of breast cancer and mammary epithelial cell cultures is summarized in Figure 3.

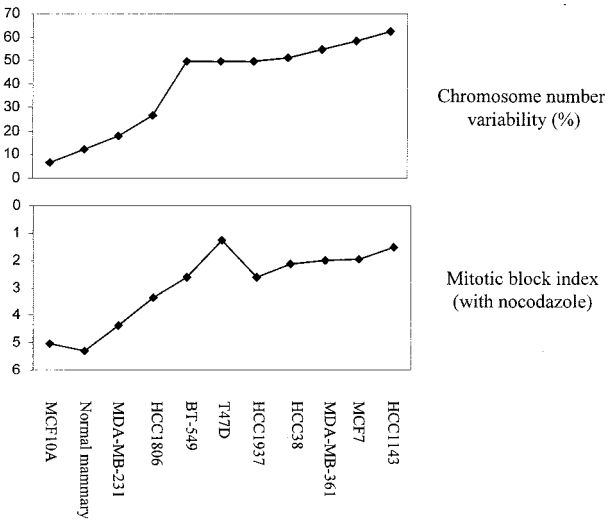

Figure 3. Relationship between chromosome number variability and nocodazole-induced mitotic arrest. Chromosome number variability and mitotic block index (ratio of G_2/M peak in nocodazole-treated cells compared to control cultures) for nocodazole-treated cells are shown for all of the 11 cell types tested. Nearly complete lack of blocking by nocodazole treatment results in a low ratio (eg, 1.29 for T47D cells), whereas higher ratios (eg, 5.28 for normal epithelial cells) are limited by the presence of some G_2/M cells in control cultures. The relationship of chromosome number variability to mitotic block index shows a strong negative (ie, inverse) correlation of −0.951, 95% confidence interval of −0.988 to −0.819, ($P < 0.001$).[16]

Defective Mitotic Checkpoints and Chromosome Numerical Instability Are Unrelated to p53 Mutations in Breast Cancer Cell Lines

Mutations of the p53 gene have been previously proposed to be associated with defective mitotic checkpoints and genetic instability.[17–21] All of the cell types used in these experiments have been characterized with regard to p53 mutations,[22,23] with the exception of the primary mammary cells, which we assume to have wild-type p53. Our data (Table 1) failed to find any relationship between the presence or absence of p53 mutations and chromosome numerical instability. For example, p53 mutations have been described for only 4 of the 7 breast cancer cell lines[22,23] with defective mitotic checkpoints and high levels of chromosome numerical instability. Furthermore, MDA-MB-231 and HCC1806 cell lines, the two breast cancer cell lines with the lowest levels of instability of chromosome numbers during replication and intact nocodazole-induced mitotic checkpoints, also have mutations of the p53 gene.[22,23]

Discussion

As seen in other forms of human cancer, the genetic alterations in breast cancers include chromosome structural alterations as well as specific gene sequence alterations. However, the extent of these structural changes, including variations in chromosome copy numbers, is not uniform among all breast cancers. For example, karyotypic studies of breast cancers have previously shown that while many neoplasms have extensive chromosome structural and numerical changes, other breast cancers have only minimal chromosomal changes.[8,9] These cytogenetic studies demonstrate variable states of chromosome structural variation among different cancers and, with our present investigation, we demonstrate that breast cancer cell lines have vastly different rates by which these chromosome numerical changes occur. Our studies are a thus a more direct measure of chromosome numerical instability, and our results suggest that this form of genetic instability is highly variable among different breast cancers.

Notably, the breast cancer cell lines with the highest levels of chromosome numerical instability all have a defective mitotic spindle checkpoint as reflected by transition of cells through G_2/M during exposure to nocodazole. This situation parallels that reported for

colorectal cancer cells with chromosome numerical instability[3] and is consistent with a defective mitotic spindle checkpoint contributing to the development of chromosome numerical instability in many breast cancers. Remarkably, however, breast cancer cell lines with intact mitotic spindle checkpoints also have measurable levels of chromosome numerical instability, although to a significantly lesser extent than the cell lines with defective mitotic spindle checkpoints. Thus, our data imply that there may be functionally different classes of breast cancers based on different levels of chromosome numerical instability as well as different mechanisms for achieving this instability.

Our data do not, however, appear to be entirely consistent with an alternative hypothesis for chromosomal instability, which proposes that aneuploidy itself destabilizes the symmetry of chromosome segregation, leading to chromosomal instability.[24] The MDA-MB-231 and HCC-1806 cell lines are both highly aneuploid,[22,23] yet both of these cell lines have relatively modest levels of chromosome numerical instability. Furthermore, the fibrocystic-disease derived MCF-10A cell line also has significant aneuploidy,[25] although the level of chromosome numerical instability measured in this cell line using our assay is not significantly different from that of normal mammary epithelial cell cultures.

A number of previous studies have also associated p53 mutations with genetic instability and, in some cases, defective mitotic checkpoints.[17] The functional significance of p53 mutations likely depends on the specific mutation. For example, one class of p53 missense mutation results in a dominant, gain-of-function activity that results in altered spindle checkpoint control and genomic instability.[19] Furthermore, some defects in p53 might be more closely related to mitotic recombination errors than chromosomal losses and gains.[20,26]

Based on our studies, defects in the mitotic spindle checkpoint and high levels of chromosome numerical instability in breast cancer do not appear to be related in any consistent manner to p53 status. While we do not yet understand the molecular mechanisms for defective mitotic spindle checkpoint and associated chromosomal instability in breast cancer, it is notable that several of the breast cancer cell lines with these properties (T47D, MDA-MB-361, and BT-549) have been reported to express relatively decreased levels of the MAD2L1 mitotic spindle checkpoint gene.[27,28] It is also notable that the MDA-MB-231 cell line, which has an intact mitotic checkpoint response to nocodazole and an intermediate level of chromosomal instability, has amplification and overexpression of the STK15 gene that has been previously shown to be associated with centrosomal amplification and aneuploidy.[29] Future investigations may help to determine whether defects of the mitotic spindle checkpoint and defects of centrosomes are independent mechanisms for causing chromosomal instability and differentially involved in different cancers.

Given the extensive chromosomal instability seen in many of the breast cancer cell lines, it is surprising that the modes of chromosomal numbers were remarkably consistent among different colonies of a given cell line, even when less than 50% of the cells within a colony had the modal number of chromosomes. Individual cancer cells undoubtedly had variability for many different chromosomes at the time they were plated to form colonies. Yet, as these cells replicated to from colonies, chromosomal counts apparently tended to return to a particular mode rather than drift toward increasing levels of aneuploidy. The mechanism for this tendency to return to a particular mode and range of chromosome numbers, though clearly not evident from our data, could involve unbalanced distribution of chromosomes in a non-random manner or a selective growth advantage for cells with particular chromosomal content.

In summary, our study indicates that chromosome numerical instability occurs at variable levels in breast cancers, perhaps reflecting functionally different classes of chromosomal instability in breast cancer. Abnormal nocodazole-related mitotic spindle checkpoints probably contribute to high levels of chromosomal instability and might define one of these classes. Other breast cancers, without defective nocodazole-related mitotic spindle checkpoint defects, have more modest levels of chromosome number variability and appear to constitute a different class with respect to chromosomal instability. Finally, it is also important to consider that most breast cancers have structural rearrangements of chromosomes in addition to alterations of chromosome numbers. Thus other mechanisms for disrupting the genome, which might lead to events such as illegitimate recombination, are also likely to contribute to genetic instability in breast cancers.

Acknowledgments

We thank Donald Coffee for the helpful discussions and Christa Morris and Anita Hawkins for technical assistance and advice.

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