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Profiling breast cancer by array CGH

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Summary

Breast tumors display a wide variety of genomic alterations. This review focuses on DNA copy number variations in these tumors as measured by the recently developed microarray-based form of comparative genomic hybridization. The capabilities of this new technology are reviewed. Initial applications of array CGH to the analysis of breast cancer, and the mechanisms by which the particular types of copy number changes might arise are discussed.

Introduction

The development of solid tumors involves acquisition of genetic and epigenetic alterations, and the concomitant changes in gene expression, that modify normal growth control and survival pathways. One of the characteristics of breast tumors is the great heterogeneity in aberrations that are found. The tumor genomes may have nearly normal or highly abnormal karyotypes; they may or may not contain point mutations, or epigenetic modifications, such as methylation. In different tumors, expression of the same gene may be altered in multiple ways, or particular functional pathways may be affected at different locations. It is now generally accepted that in order for a sufficient number of alterations to accumulate to cause a malignancy, one or more mechanisms that work to maintain genetic integrity in cells and/or to regulate cell cycle progression must be compromised, presumably through mutations that occur early in tumorigenesis [1]. Here, we focus on DNA copy number alterations in breast tumors, first reviewing the capability of the recently developed microarray-based form of comparative genomic hybridization (array CGH) to measure and map these aberrations, and then discussing the mechanisms by which the particular types of copy number changes might arise.

Array CGH

As originally described, CGH detects and maps DNA sequence copy number variation throughout the entire genome onto a cytogenetic map supplied by metaphase chromosomes [2]. The use of metaphase chromosomes as the hybridization target has previously limited the resolution of CGH to 10–20 Mb, prohibited resolution of closely spaced aberrations, and only allowed linkage of CGH results to genomic information and resources with cytogenetic accuracy. Array-based CGH, on the other hand, provides the capability to map copy number aberrations relative to the genome sequence, with the resolution being determined by the spacing of the clones. In array CGH, arrays of genomic BAC, P1, cosmid or cDNA clones are used as the hybridization target in place of the metaphase chromosomes [3–6]. Relative copy number is then measured at these specific loci by hybridization of fluorescently labeled test and reference DNAs as in conventional CGH [4]. Since the clones used on the array contain sequence tags, their positions are accurately known relative to the genome sequence, and genes mapping within regions of copy number alteration can be readily identified using genome databases.

Arrays comprised of large insert genomic clones such as BACs, P1's and cosmids provide reliable

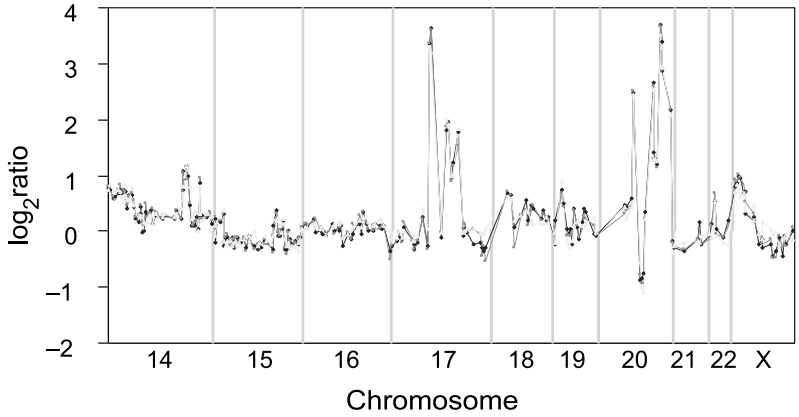

Figure 1. Comparison of three hybridizations with BT474 DNA. Test BT474 DNA and male reference DNAs were labeled either by nick translation or random priming and hybridized to an array of 1777 clones (HumArray 1.11). The $\log_2$ratios for three hybridizations are shown together, plotted according to order on the draft sequence for chromosomes 14–22 and the X. The complete data sets are available in the web supplement to Snijders et al. [31].

copy number measurements on individual clones, and thus have potential utility for both research and clinical applications. However, preparation and spotting of BAC DNA is problematic. BACs are single copy vectors so the yield of DNA from cultures is low compared to yields from plasmid vectors, and spotting high molecular weight DNA at sufficient concentration to obtain good signal to noise in the hybridizations may be difficult. Therefore we have used a ligation-mediated PCR procedure [7] to make a complex representation of the BACs for spotting on the array [6]. The procedure yields sufficient spotting solution (0.8 μg/μl DNA in 20% DMSO) from 1 ng of BAC DNA to make tens of thousands of arrays, and the ratios measured on arrays comprised of BAC representations are essentially identical to ratios previously reported on DNA from the same whole BAC [6]. Detailed protocols for array production and hybridization are available elsewhere [6, 8].

We have assembled arrays of ∼2500 BACs and P1's for scanning the entire genome for copy number changes. These arrays provide resolution of ∼1.4 Mb. Each clone is printed in triplicate in a 12 mm × 12 mm square area. A typical hybridization requires 200–300 ng of tumor DNA, although dilution tests have produced successful hybridizations with as little as 3 ng of DNA. Best results have been obtained with DNA extracted from frozen specimens by conventional methods. It is also possible to use DNA extracted from paraffin embedded specimens, as well as DNA obtained by back extraction after Trizol purification of RNA, but results are more variable. Higher resolution arrays have also been assembled across regions of particular interest [9]. By using overlapping clones from regions of contiguous clone coverage copy number changes can be mapped with a genomic resolution less than the clone length (<50 kb).

Validation of the capability of array CGH to measure single copy gains and losses has been carried out using cell strains with known monosomies or trisomies [6]. The measured ratios closely approximated the ideal ratios. For example, 13 different regions of trisomy were measured in this set of cells, and the mean of the $\log_2$ratios of these trisomic chromosomal regions was 0.49 ± 0.05. This value is close to, but slightly less than, the expected $\log_2$ratio = 0.58 for the 3/2 copy number ratio that results from a trisomy. In female/male comparisons, the mean $\log_2$ratios on the X chromosome were 0.72 ± 0.08, compared to the expectation of 1.0. These underestimations of the magnitude of copy number deviations are most likely due to incomplete suppression of repetitive sequences or errors in background subtraction [4].

Three replicate hybridizations with BT474 cell line DNA were carried out to assess the reproducibility of array CGH measurements [6]. In two of the hybridizations, test and reference DNAs were labeled by random priming, while the third was labeled by nick translation. Figure 1 shows the results for chromosomes 14–22 and the X. Nearly identical ratios were obtained on each clone (average S.D. of the $\log_2$ratio = 0.08), including those clones reporting high level amplifications (chromosomes 17 and 20) and losses (chromosome 20). Thus, it is generally

not necessary to perform the array measurement more than once on a given specimen.

The ratios measured in tumor cells are likely to reflect the presence of admixed normal cell DNA, tetraploid DNA content, and/or tumor heterogeneity. Therefore to make copy number measurements on tumor specimens, it is necessary to detect alterations that are lesser in magnitude than one expects for single copy changes in diploid genomes. In order to assess the capability to detect small ratio changes, a series of measurements was carried out on dilutions of a trisomic cell strain with normal DNA. Copy number aberrations were reliably detected in mixtures comprised of >50% normal DNA, indicating the capability to detect ratios much less than 1.5 expected for a trisomy ($\log_2$ratio = 0.58). Furthermore, as expected from these experiments, $\log_2$ratios of 0.47 ± 0.08 and 0.32 ± 0.07 are clearly visible in the tumor samples shown in Figure 3(c) and (d), respectively.

Copy number alterations in breast tumor genomes

Studies of breast tumors by conventional karyotyping, fluorescent *in situ* hybridization (FISH), spectral karyotyping or MFISH, chromosomal CGH and array CGH have found a wide range in the number and variety of types of chromosome level alterations in different tumors. Representative copy number aberrations detected by array CGH are shown in Figure 2. Often net gain or loss of whole chromosomes or parts of chromosomes is observed (Figure 2(a)). More focal aberrations are also seen, including gene amplifications, defined as high copy number increases of a restricted region of a chromosome arm (Figure 2(b)) or deletions (Figure 2(c)). Examples of some of the variety observed in breast tumors and cell lines can be seen in the whole genome copy number profiles shown in Figure 3. The *BRCA1* deficient cell line HCC1937 [10] shows many low level copy number alterations and no amplifications, while amplification of chromosome 11 was observed in 600 MPE along with relatively few low level changes (Figure 3(a) and (b)). The two tumors shown in Figure 3(c) and (d) also showed different spectra of copy number alterations. Both tumors showed copy number changes involving chromosome arms. One tumor (Figure 3(c)) showed only alterations involving extended chromosomal segments, including gains of 1q and 16p and losses of a small portion of distal 2q, a portion of 7q and all of 16q. In the other breast tumor (Figure 3(d)), a greater variety of aberrations was seen. Alterations involving chromosome arms included gain of 3q, gain of 5q and a higher level gain of 5p. In addition, amplification on chromosome 20q was observed, as were losses of whole chromosomes (chromosomes 13, 14 and 22). This wide variety of tumor genomic alterations is likely to reflect the many different solutions adopted by individual tumors to escape normal protective mechanisms for growth control and senescence and will be discussed further below.

Array CGH provides a higher resolution mapping of amplicons and indicates that amplicons may be simple or highly complex. In some cases, the identification of oncogenes has been facilitated, because they have been found to be up-regulated when present at elevated copy number in very focal amplicons (e.g., *CMYC* in COLO320, Figure 2(b)). The amplicon encompassing *ERBB2* frequently appears as a simple peak in the copy number profile, while others such as those on 11q including *CCND1* are often highly complex (Figure 4). Amplification of *CCND1* is generally accompanied by amplification of several distinct adjacent copy number peaks, as well as loss of copy number on distal 11q. In breast cancer, overexpression of *ERBB2* is almost always associated with amplification of the gene [11]. For other well-characterized oncogenes such as *CCND1*, however, multiple mechanisms for increasing expression occur frequently in addition to amplification [12], raising the possibility that amplification of the *CCND1* region may be selected coordinately with the other regions due to properties of the genome, or that selection for overexpression of other oncogenes mapping near *CCND1* and/or loss of distal 11q was the driver for the chromosomal aberrations.

Previous studies comparing tumor recurrences with the corresponding primaries by chromosome CGH showed that the genomes of established tumors are remarkably stable [13]. In agreement with these earlier observations, the higher resolution comparison of a tumor and recurrence by array CGH showed striking similarity of the two specimens. Copy number differences involving two chromosome regions were noted, gain of 7p and loss of 10q, whereas almost all other aberrations were identical, even ratios on individual BACs (Figure 5).

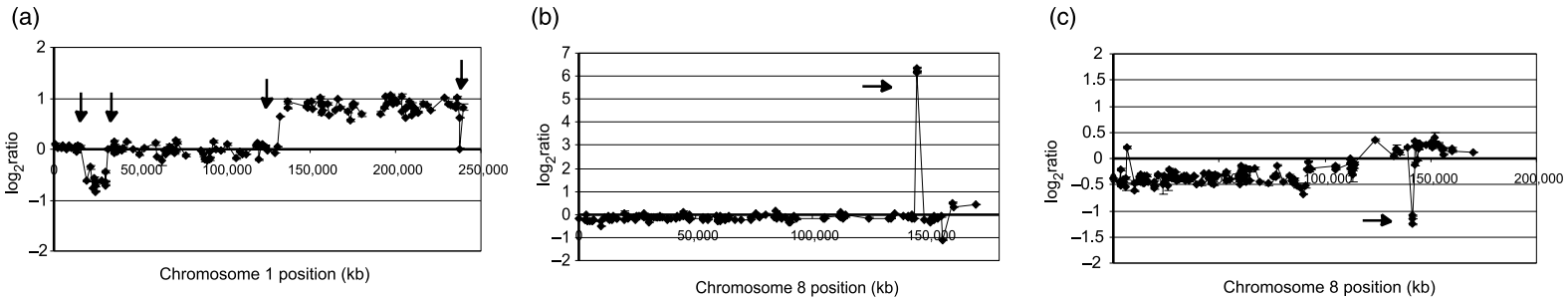

Figure 2. Copy number aberration types. The $\log_2$ratios are plotted on individual chromosomes according to the positions of the clones on the UCSC August 2001 freeze of the genome sequence (http://genome.ucsc.edu). (a) Copy number profile for chromosome 1 from 600 MPE showing a low level copy number loss on 1p and gain of 1q. The arrows indicate positions of transition in the copy number profile. Copy number transition points are likely to reflect breakage events that lead to chromosome rearrangements that underlie copy number abnormalities. (b) Copy number profile for chromosome 8 in COLO320 showing focal, high level (60–70-fold) amplification of the *CMYC* region (arrow). (c) Copy number profile for chromosome 8 in MDA-MB-231 showing loss of 8p and proximal 8q, gain of distal 8q and a focal deletion (arrow). The complete data sets are available in the web supplement to Snijders et al. [6].

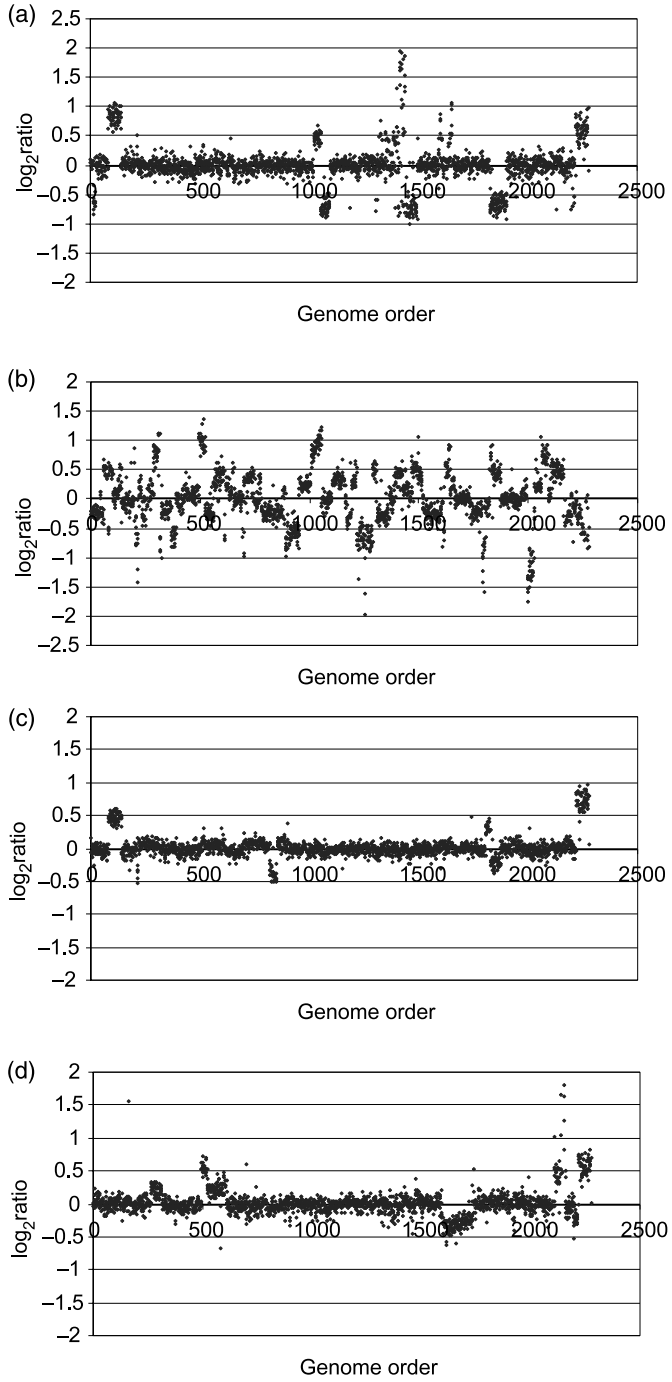

Figure 3. Whole genome copy number profiles. Hybridizations were carried out to HumArray1.14 arrays comprised of 2463 clones mapping to chromosomes 1–22 and the X. The $\log_2$ratios for each chromosome in order from 1p to Xqter are plotted in order according to the positions of the clones on the UCSC August 2001 freeze of the genome sequence (http://genome.ucsc.edu). (a) Copy number profile of 600 MPE showing low level copy number gains and losses in addition to amplification. The reference DNA was male. The data set is available in the web supplement to Snijders et al. [6]. (b) Copy number profile of HCC1937 showing low level copy number aberrations involving all chromosomes. The reference DNA was female. (c) and (d) Copy number profiles of breast tumors showing low level copy number gains and losses and amplification. The reference DNA was male. The data sets for these tumors are available in the web supplement to Snijders et al. [6].

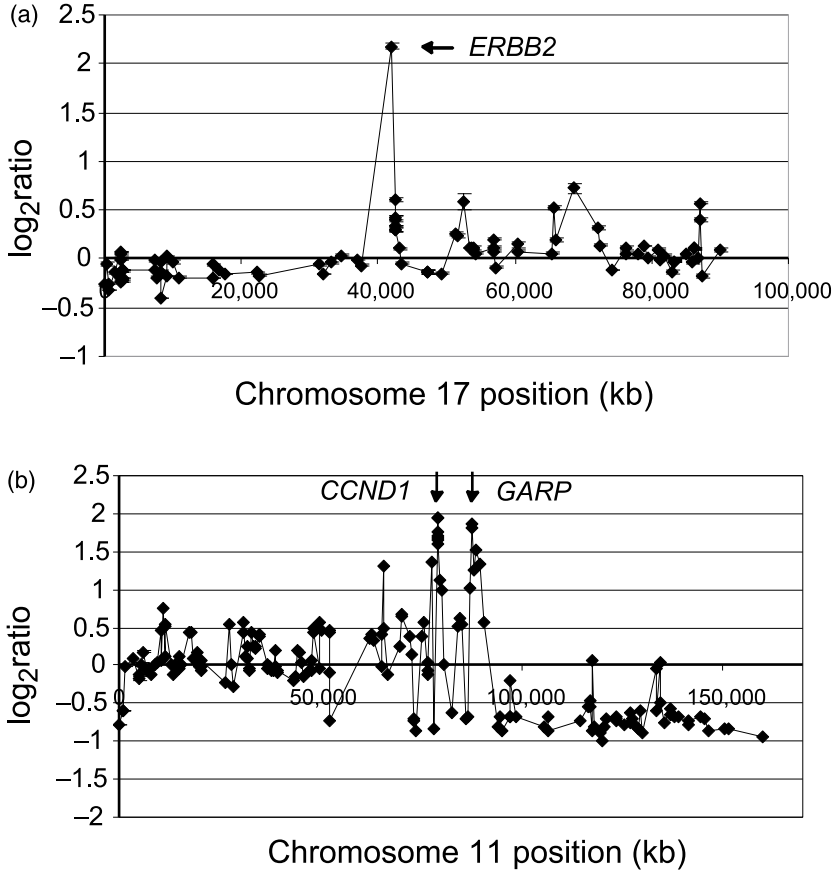

Figure 4. Copy number profiles of chromosomes with amplifications. The $\log_2$ratios are plotted for individual chromosomes according to the positions of the clones on the UCSC August 2001 freeze of the genome sequence (http://genome.ucsc.edu). (a) Amplification of *ERBB2* in a breast tumor. The DNA was isolated from the Trizol pellet remaining after extraction of RNA from the frozen tumor specimen. (b) Amplification on chromosome 11 in 600 MPE showing a number of individual copy number peaks and loss of distal 11q. The amplified regions encompassing *CCND1* and *GARP* are indicated. The data set is available in the web supplement to Snijders et al. [6].

Genesis and evolution of copy number aberrations in tumor genomes

Some of the variety in the complexity of the copy number profiles seen in breast tumors may be attributed to the underlying defects in maintenance of genome stability in the tumors. This relationship of mechanistic defect to aberration type is most clearly established in tumors with defects in mismatch repair, in which there are frequent nucleotide level changes and relatively few chromosome level changes [14]. Thus, the spectrum of alterations that one sees in fully developed breast tumors is likely to reflect selection acting on the variation that is permitted to arise by the particular failures in genomic surveillance mechanism(s) present in the tumor. Since certain genes will be more susceptible to alteration by a particular failure in genome surveillance, changes in expression of genes may be brought about in multiple different ways in different tumors, or particular functional pathways may be affected at different points.

Aberrations revealed in breast tumors by array CGH frequently involve whole or partial chromosome arms and gene amplification. Gene amplification has been studied *in vitro* in a variety of systems by selection for cells capable of growth in the presence of certain drugs. Studies using cultured mammalian cells indicate that gene amplification is initiated by a DNA double strand break, possibly at a common chromosomal fragile site, and that it can occur only in cells that are able to progress inappropriately through the cell cycle with this damaged DNA [15–20]. Abrogation of cell cycle checkpoints and failures in the DNA damage response [21] are also likely to contribute to

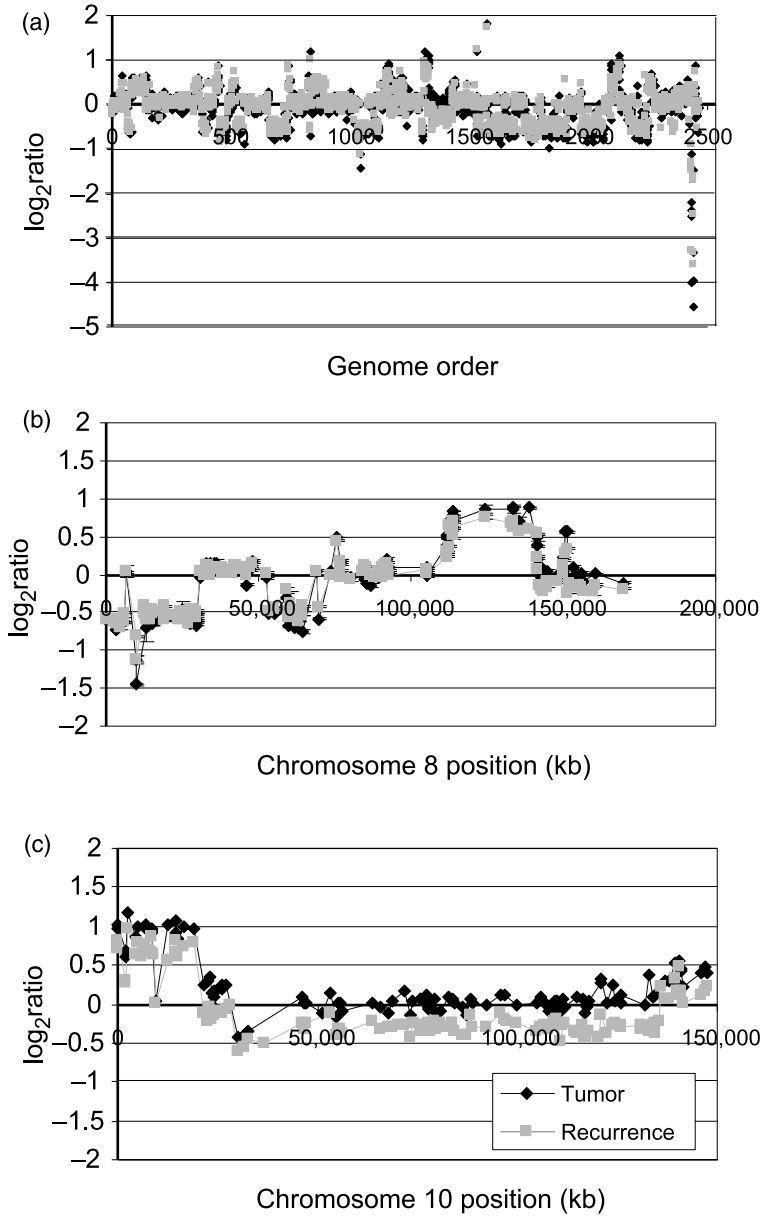

Figure 5. Copy number profiles of a primary tumor and corresponding recurrence. Tumor and male reference DNAs were labeled and hybridized to HumArray2.0 comprised of 2464 clones representing all chromosomes. The $\log_2$ ratios are plotted in order from 1p to Y according to the positions of the clones on the UCSC August 2001 freeze of the genome sequence (http://genome.ucsc.edu). (a) Whole genome copy number profiles of the tumor (black) and recurrence (gray). Two regional copy number differences, gain of 7p and loss of a portion of 10q distinguish the tumor and the recurrence. The set of very low ratios on the Y chromosome clones at the right of the figure reflect the absence of these sequences in the female test DNA and therefore are equivalent to the ratios expected for a homozygous deletion. (b) Copy number ratios for chromosome 8 showing nearly identical ratios on all clones. (c) Copy number ratios for chromosome 10 showing a copy number difference between the tumor and recurrence on 10q.

the formation of low level copy number alterations (non-reciprocal translocations).

Telomere erosion is another mechanism by which copy number abnormalities may be generated. Hyperplastic lesions are thought to provide the expanded population of proliferating cells necessary for tumor evolution. Extra rounds of cell division in already compromised cells provide greater opportunity for

accumulation of additional errors, resulting frequently in loss of function of genes that regulate the cell cycle and/or cellular lifespan (e.g., methylation of *CDKN2A*, inactivation of *RB1*) and enhanced expression of genes promoting transit through the cell cycle (e.g., *E2F1*, *CCND1*). However, most human cells, other than stem cells do not express telomerase [22]. Therefore, at each cell division telomere erosion takes place due to incomplete replication of the chromosomal ends and in cells with intact checkpoints leads eventually to cell death when telomeres reach a critically short length [23]. If cells survive inappropriately with short telomeres, then aberrant cell divisions are likely to occur due to end-to-end fusions of chromosomes. They may give rise to non-reciprocal translocations [24] and/or to amplifications by repeated cycles of aberrant segregation involving breakage-fusion-bridge cycles [25]. If cells continue to divide with critically shortened telomeres, then the genome is likely to accumulate more aberrations through the repeated cycles of chromosome rearrangement and mis-segregation. These events are likely to be lethal, necessitating stabilization, which is often accomplished by re-activation of telomerase.

Other recurrent abnormalities involve low level gains or losses of particular chromosome arms due to breaks occurring preferentially at the centromere. For example, gain of 1q and loss of 16q has been reported to occur frequently in lobular and tubular breast tumors [26–28]. Cytogenetic analysis indicates that these copy number changes can be attributed to rearrangements involving the pericentromeric regions of chromosomes 1 and 16 [29]. Hypomethylation of these regions, which contain type 2 satellite sequences causes de-condensation of the chromatin and is associated with rearrangements involving these loci in the rare inherited ICF syndrome [30]. Aberrant hypomethylation of chromosomes 1 and 16 in breast tumors has been reported [31], suggesting it may contribute to initiation of similar rearrangements during evolution of these tumors.

In addition to alterations likely to derive from chromosome breakage, tumor genomes frequently deviate significantly from diploid copy number. They may contain gains or losses of copies of entire chromosomes and may display bimodal numbers of chromosome sets. Some of these aberrations may arise due to defects in cell division. Morphologically and functionally aberrant centrosomes have been observed frequently in tumor cells. This phenomenon, referred to as 'centrosome amplification' appears to reflect aberrant segregation of centrosomes and has been associated with formation of multi-polar spindles and failures in cytokinesis and karyokinesis [32]. They have also been found to be associated with those tumor genomes that show greater deviation from normal [33]. Centrosome amplification and extended periods of telomere erosion provide mechanisms for generating many chromosome level aberrations, most of which are likely to be lethal. Thus, both of these processes appear to be stabilized in established tumors. In contrast, cell cycle defects and other failures in DNA repair continue to malfunction, as evidenced, for example, by the presence of microsatellite instability in mismatch repair deficient cell lines [34] and the capability of tumor cell lines to amplify drug resistance genes when challenged [35]. The capability of telomere erosion and centrosome amplification to generate many copy number changes suggests that they contribute to formation of tumor genomes with the greater number of copy number aberrations. On the other hand, tumor genomes with few copy number changes (including those with mismatch repair defects) are likely to have followed different evolutionary routes. They may avoid telomere erosion by stabilization of telomeres by other means, including mismatch repair deficiency [36] and ALT [37] or indeed may arise from cells in which telomerase remained active.

Clinical significance of copy number aberrations

Copy number aberrations are proving to be useful diagnostic markers. The tight correlation of amplification of *ERBB2* with increased expression of this gene provides the basis for the improved performance of tests that measure the DNA copy number of *ERBB2* compared to immunohistochemical detection of protein levels for predicting response to Herceptin [11, 38]. Similarly, combinations of FISH probes for regions of recurrent copy number aberration in other tumor types are proving useful for monitoring disease progression [39] or for distinguishing benign lesions from metastatic ones [40]. Array CGH can be performed at a much higher throughput than chromosome CGH and provides much higher resolution information. Applications of this technology in larger studies should help to reveal additional regional copy number markers of utility for prediction and prognosis in breast cancer.

Classification of breast tumors based on the types of copy number aberration they display may also be facilitated by the enhanced capabilities of this technology. Genetic instability is an on-going process in tumors that allows them to evolve and survive therapeutic challenge. Amplification, in particular, is often associated with poor prognosis. Thus, identifying and understanding the involved mechanisms will be important for the design of therapies that target the dysfunctional genes (e.g., *TP53* by Onyx-015), or to avoid therapies for which the dysfunctional genes would render the tumor resistant (e.g., cisplatin resistance associated with defects in mismatch repair genes).

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