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Molecular Cytogenetics of Primary Breast Cancer by CGH

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Comparative genomic hybridization (CGH) reveals DNA sequence copy number changes that are shared among the different cell subpopulations present in a tumor and may help to delineate the average progression pathways of breast cancer. Previous CGH studies of breast cancer have concentrated on selected subgroups of breast cancer. Here, 55 unselected primary breast carcinomas were analyzed using optimized quality-controlled CGH procedures. Gains of 1q (67%) and 8q (49%) were the most frequent aberrations. Other recurrent gains were found at 33 chromosomal regions, with 16p, 5p12–14, 19q, 11q13–14, 17q12, 17q22–24, 19p, and 20q13 being most often (>18%) involved. Losses found in >18% of the tumors involved 8p, 16q, 13q, 17p, 9p, Xq, 6q, 11q, and 18q. The total number of aberrations per tumor was highest in poorly differentiated ($P = 0.01$) and in DNA aneuploid ($P = 0.05$) tumors. The high frequency of 1q gains and presence of +1q as the sole abnormality suggest that it is an early genetic event. In contrast, gains of 8q were most common in genetically and phenotypically advanced breast cancers. The vast majority of breast cancers (80%) have gains of 1q, 8q, or both, and 3 changes (+1q, +8q, or −13q) account for 91% of the tumors. In conclusion, CGH results indicate that certain chromosomal imbalances are very often selected for, sometimes in a preferential order, during the progression of breast cancer. Further studies of such common changes may form the basis for a molecular cytogenetic classification of breast cancer. *Genes Chromosomes Cancer 21:177–184, 1998.*

INTRODUCTION

Although breast cancer is the most common cancer in females, only about 500 karyotypes (Mitelman, 1994; Pandis et al., 1996) have been published in the literature. Several recurrent clonal structural and numerical chromosomal abnormalities have been detected in primary breast cancer by cytogenetic analysis. Of these, the following appear particularly important based either on their frequency or on their involvement in the early stages of tumor progression: i(1q), der(1q;16p), del(1q), del(1p), del(3p), del(6q), del(11q), del(17p), i(6p), as well as −2, +7, −15, and +20 (Dutrillaux et al., 1990; Thompson et al., 1993; Trent et al., 1993; Pandis et al., 1995; Steinarsdottir et al., 1995). Although the success rate and sensitivity of G-banding analysis have dramatically improved during the last years, the frequency of aberrations may still be underestimated because of the inability to obtain analyzable metaphase cells from some tumors. Furthermore, while karyotyping is ideal in revealing the tremendous genetic heterogeneity of cancer (Dutrillaux et al., 1990; Pandis et al., 1995), the identification of all clonal genetic aberrations is sometimes difficult because of the overwhelming complexity of changes.

The molecular cytogenetic method of comparative genomic hybridization (CGH) may supplement information from karyotyping (du Manoir et al., 1993; Kallioniemi et al., 1994a; Speicher et al., 1995; Waldman et al., 1996). CGH analysis is applicable to all uncultured tumors regardless of their mitotic activity or the complexity of chromosomal changes, thereby limiting possible selection biases. While the technique does not detect all structural rearrangements, it offers an overview of those DNA sequence copy number changes that are present in most of the tumor cells, i.e., those changes that are most likely to be clonal in origin. Many of the previous CGH studies of breast cancer were performed during the early phases of CGH technology development. Therefore, only gains and amplifications were considered able to be evaluated reliably (Kallioniemi et al., 1994a), and aberrations affecting certain chromosomes and chromosomal regions such as 1p32-pter, 16p, 17p, 19, and 22 were deemed unable to be evaluated with indirectly labeled genomic DNAs (Kallioniemi et al., 1994a; Isola et al., 1995). Most studies are also based on highly selected patient materials, such as node-negative patients selected by disease outcome (Isola et al., 1995), metastatic tumors (Kuukas-

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järvi et al., 1996), tumors with homogeneously staining chromosomal regions (Muleris et al., 1995), or tumors selected by DNA ploidy pattern (Ried et al., 1995).

Already there are over 100 publications of CGH analyses of various tumor types, altogether comprising over 1,500 tumor specimens (Kallioniemi et al., personal communication). However, studies of unselected primary breast cancers with modern CGH techniques are lacking. Also, recent developments in directly conjugated CGH and rigorous quality control have improved the reliability and accuracy of CGH analysis (Karhu et al., 1997).

Here, 55 breast carcinomas were analyzed with optimized, quality-controlled CGH using direct fluorescent label conjugation. The aim was to define the most common genomic imbalances in an unselected series of breast cancers, to correlate CGH findings with clinicopathological features, as well as to distinguish possible early genetic aberrations and changes that could be important in the classification of breast cancer. Finally, CGH copy number profiles of 20q, a genomic region often undergoing amplification, were compared with FISH copy number profiles generated from the same tumors with a large collection of specific probes.

MATERIALS AND METHODS

Tumor Specimens

Fifty-five consecutive primary breast carcinomas diagnosed at the Tampere University or City Hospital during 1988–1991 were included in this study. The mean age of the patients was 62 years (range 34–92). Twenty-eight tumors were node-negative, 26 node-positive, and 1 unknown. Fifty-three were invasive ductal carcinomas and 2 invasive lobular. The distribution of tumor grades (according to the WHO system) was grade I (13 cases), grade II (27), and grade III (15). None of the patients received any therapy prior to specimen collection. High-molecular-weight tumor DNA was isolated according to standard methods from freshly frozen breast tumor samples and, for reference female DNA, from peripheral blood. Nuclei for FISH from all the tumors were isolated from 100 μm sections according to the Vindelov procedure (Hyytinen et al., 1994).

Comparative Genomic Hybridization

CGH was performed using directly fluorochrome-conjugated DNAs, as described previously (Kallioniemi et al., 1994b; Isola et al., 1995; Visakorpi et al., 1995). Briefly, the metaphase spreads were denatured at 72–74°C for 2.5–3 minutes in a formamide solution (70% formamide, 2XSSC, pH 7) and dehydrated in a series of 70, 85, and 100% ethanols. Tumor DNAs were labeled with FITC-dUTP (DuPont, Boston, MA) and normal female reference DNA with Texas red-dUTP (DuPont) using nick translation. Labeled tumor and normal DNAs (400 ng each), together with 10 μg of unlabeled Cot-1 DNA (Gibco BRL, Gaithersburg, MD), were dissolved in a hybridization mixture consisting of 50% formamide, 10% dextran sulfate, and 2XSSC (1XSSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7). This mixture was denatured at 75°C for 5 minutes and applied on normal lymphocyte metaphase preparations. The hybridization was done at 37°C for 2 days. After hybridization, the slides were washed 3 times in 50% foramide/2XSSC (pH 7), twice in 2XSSC, and once in 0.1XSSC at 45°C, followed by 4XSSC and distilled water at room temperature for 10 minutes each. After air drying, the samples were counterstained with 4,6-diamino-2-phenylindole (DAPI) in an antifade solution.

Digital Image Analysis

CGH hybridizations were analyzed with a Nikon SA (Nikon Corp., Tokyo, Japan) or Olympus BX50 (Olympus Corp., Tokyo, Japan) epifluoresence microscope. Three single-color images corresponding to the DAPI, FITC, and Texas Red emissions were obtained from each metaphase by a Xillix CCD camera (Xillix Technologies Corp., Vancouver, BC, Canada) interfaced to a Sun LX workstation (Sun Microsystems Computer Corp., Mountain View, CA). Five to eight metaphase cells were analyzed from each tumor sample. Analysis and interpretation of the hybridization was done as described previously (Kallioniemi et al., 1994b; Piper et al., 1995) using Scilimage software with Resource for Molecular Cytogenetic extensions (from Damir Sudar and Joe Gray, UCSF). In brief, the green and red fluorescence intensities were determined from p-telomere to q-telomere for each chromosome by integrating intensities across the medial axis. The absolute fluorescence intensities were normalized so that the average green-to-red ratio of all chromosome objects was 1.0. The mean intensity profile and its standard deviation were plotted for all chromosomes from pter to qter. As a negative control, FITC-labeled normal male DNA was hybridized with Texas red-labeled normal female DNA; the mean green-to-red ratio and standard deviation for all autosomes remained between 0.9 and 1.1, whereas for the X chromosome it was close

to 0.5. Chromosome regions where the mean and the SD were over 1.15 were considered gain in tumor genome, and all regions where the mean and SD were under 0.85 were considered loss. As a positive control, DNA from breast cancer cell line MCF-7 with previously known aberrations was also used in each hybridization batch.

FISH

Two-color FISH was performed for all tumor samples with 10–15 probes (RMC20C038 and RMC20C041 for controls; *AIB 3–4* and RMC20C041 for 20q11; and RMC20C002, *PTPN1*, RMC20C026, *MC3R*, RMC20C001, *CYP24*, *PCK1*, RMC20C030, cK20.10e9, *GNAS1* for 20q13) mapped to chromosome 20 to ascertain the degree of correlation between the 2 methods. In this procedure, a biotin-14-dATP-labeled 20q-probe and a digoxigen-11-dUTP-labeled 20p reference probe were hybridized as described (Tanner et al., 1994). Tumor nuclei were isolated and dropped on slides as described earlier (Hyytinen et al., 1994), postfixed in 4% paraformaldehyde-PBS for 5 minutes at 4°C prior to hybridization, dehydrated in graded ethanols, air-dried, and incubated at 80°C for 30 minutes. Slides were denatured in 70% foramide, 2XSSC solution at 72–74°C for 3 minutes, followed by proteinase K digestion (0.5 μg/ml). The hybridization mixture contained 18 ng of each labeled DNA and 10 μg of human placental DNA. After 24-hour hybridization, the probes were detected immunochemically with avidin-FITC and antidigoxigen rhodamine. Slides were counterstained with 0.2 μM DAPI in an antifade solution. For analysis of FISH results, at least 50 intact and nonoverlapping nuclei were scored to examine the actual copy number at chromosome 20.

Statistical Methods

Association between clinopathological features and CGH results were analyzed with Fisher's exact test. The correlation between genetic aberrations as well as comparisons of simple and complex cancer types were analyzed with the Mann–Whitney U test. All *P*-values were two-tailed.

RESULTS

Overview of Copy Number Aberrations in 55 Unselected Breast Cancers

All tumors contained genetic aberrations by CGH. The total number of copy number aberrations (CNAs) was on average 7.7±5.0 per tumor (range 1–22). The total number of gains was 4.1±2.8, and that of losses 3.6±4.0. A summary of the prevalence of chromosomal gains and the minimal regions of involvement are shown in Table 1, and the most frequent losses in Table 2.

TABLE 1. The Most Common Gains in 55 Primary Breast Cancers by CGH

Gains	n	%	95% C.I.	Minimal region of involvement
1q	37	67	53–79	1q24–32
8q	27	49	35–63	8q22-qter
16p	21	38	25–52	—
5p	13	24	13–37	5p12–14
19q	11	20	10–33	—
11q	10	18	9–31	11q13–14
17q	10	18	9–31	17q12 and 17q23 equally
19p	10	18	9–31	—
20q	10	18	9–31	20q13
3q	9	16	9–29	3q24-qter
7p	7	13	5–24	—
20p	6	11	4–22	—
12q	6	11	4–22	12q14–21

TABLE 2. The Most Frequent Losses in 55 Primary Breast Cancers by CGH

16q	21	38	25–52	16q21-qter
8p	16	29	18–43	—
13q	14	25	15–39	13q12–31
17p	12	22	12–35	—
9p	11	20	10–33	—
Xq	11	20	10–33	Xq22-qter
6q	10	18	9–31	6q22-qter
11q	10	18	9–31	11q14-qter
18q	10	18	9–31	—
1p	9	16	9–29	1cen-p31
2q	7	13	5–24	2q22–34
3p	7	13	5–24	3cen-p21
12q	7	13	5–25	12q21-qter
4p	6	11	4–22	—
4q	6	11	4–22	—
5q	6	11	4–22	5q12–31

The most frequent gains were 1q, 8q, and 16p (Figs. 1 and 2). At 1q and 16p, usually the whole arms were involved in gains, whereas at 8q, the overrepresented DNA sequences were limited to 8q22–24 in ⅓ of the 8q-positive tumors (Fig. 1). The most common whole chromosome gains affected chromosomes 7 and 19. Subregional gains seen in at least 2 or more cases were found at 31 different chromosomal regions. A systematic distinction between high-level DNA amplifications and low-level gains was not attempted, as CGH cannot reliably discriminate between such events (see results below, and Tanner et al., 1994). However, a few unambiguous DNA amplifications were seen. These mapped to 4q21, 8q22–23, 16p, 17q22–24, and 20q13.

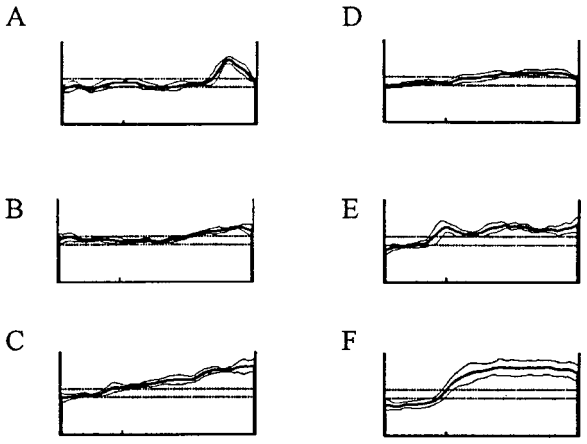

Figure 1. CGH copy number profiles of chromosome 8 from 6 breast carcinomas illustrating overrepresentation of different regions along 8q.

Of the DNA copy number losses, 16q was the most common (38%), followed by 8p (29%), 13q12–31 (26%), and 17p (22%). Altogether, 33 separate regions of recurring loss (seen in 2 or more cases) were detected.

Validation of Increased Copy Number at 20q by Interphase FISH

The copy number of specific loci along 20q was independently established from 51 of the tumors by interphase FISH with at least 10 different chromosome 20-specific probes. Copy number comparisons were limited to alterations along the length of chromosome 20. Six cases showed a copy number increase at 20q by CGH. Two of these were gains of 20q13, one 20q11 and 20q13, and in 3 cases, the amplified region spanned the entire long arm of chromosome 20. Three of the tumors showed increases also by FISH with 1 or more 20q probes. FISH studies recognized 2 independent regions of amplification at 20q13 (RMC20C001 and *PTPN1*), and 1 at 20q11 (*AIB 3–4*). Because of the limited resolution of CGH, involvement of these different regions could not be anticipated from the CGH results, except for 1 case which showed both 20q11 and 20q13 amplification by FISH, as well as by CGH (Fig. 3). Furthermore, in several cases, FISH revealed low-level (1.5–3-fold) overrepresentation of narrow regions which CGH could not detect.

Correlations Between the Genetic Aberrations

The overall number of DNA losses per tumor correlated with that of DNA gains ($P = 0.03$). A few significant correlations between the 10 most common losses and 10 most common gains (aberrations whose prevalence exceeded 15%) were also found. For example, 19q gain and 9p loss were significantly associated with one another ($P = 0.0004$), as were 17q22–q24 gain and 13q loss ($P = 0.01$). At 11q, gain of the 11q13 region was often associated with loss of the distal 11q.

Association of CGH Findings With Clinicopathological Features and DNA Ploidy

The total number of genetic aberrations was significantly associated with the histological differentiation (WHO grade) of the tumor (Table 3). The average copy number aberration (CNA) in grade III tumors was 2.3 times higher than that seen in grade I tumors ($P = 0.008$). DNA aneuploidy was also associated with a high CNA ($P = 0.02$). A high number of gains correlated with negative progesterone receptor status and S-phase fraction (Table 3).

The most frequent gains and losses were tested for correlation with clinicopathological features of the disease. The most common aberration, 1q gain, showed no association with any clinicopathological features. In contrast, gains of 8q correlated with DNA index ($P = 0.03$) and high S-phase fraction ($P = 0.02$). 3q gain was associated with high grade ($P = 0.01$) and the Xq loss with estrogen receptor negativity ($P = 0.01$). *P*-values were not adjusted for multiple comparisons.

Comparison of Changes in Genetically Simple and Highly Complex Breast Cancers

Two strategies were used to identify genetic changes possibly involved in the early stages of progression. First, 7 tumors were identified that contained only 1–2 aberrations per tumor by CGH. These tumors were either diploid (4 cases) or near-tetraploid (3 cases) by DNA flow cytometry. Gain of 1q was most common in these tumors (5 cases), with other regions (9p gain, 20 gain, 6q22–q24 loss, 8 gain, 12q21-qter loss) each being found in only a single tumor. In 2 tumors, 1q gain was the sole genetic aberration. We also compared the prevalence of CNAs in groups of tumors defined by each specific abnormality. The average CNA for tumors with 1q gains was equally high (7.5±4.8) as for those with no 1q gain (8.2±5.7). In contrast, tumors with 8q gains were genetically more complex (average CNAs 9.1±4.4 per tumor) than those with no 8q gains (average CNAs 6.4±5.4, $P = 0.01$), suggesting that 8q gains occurred either later during tumor progression or in conjunction with the development of genetically more advanced tumors. A similar association was obtained for many other DNA gains, such as of 19q, 11q, 17q12, 19p, and 3q. Losses of most chromosomal regions were also

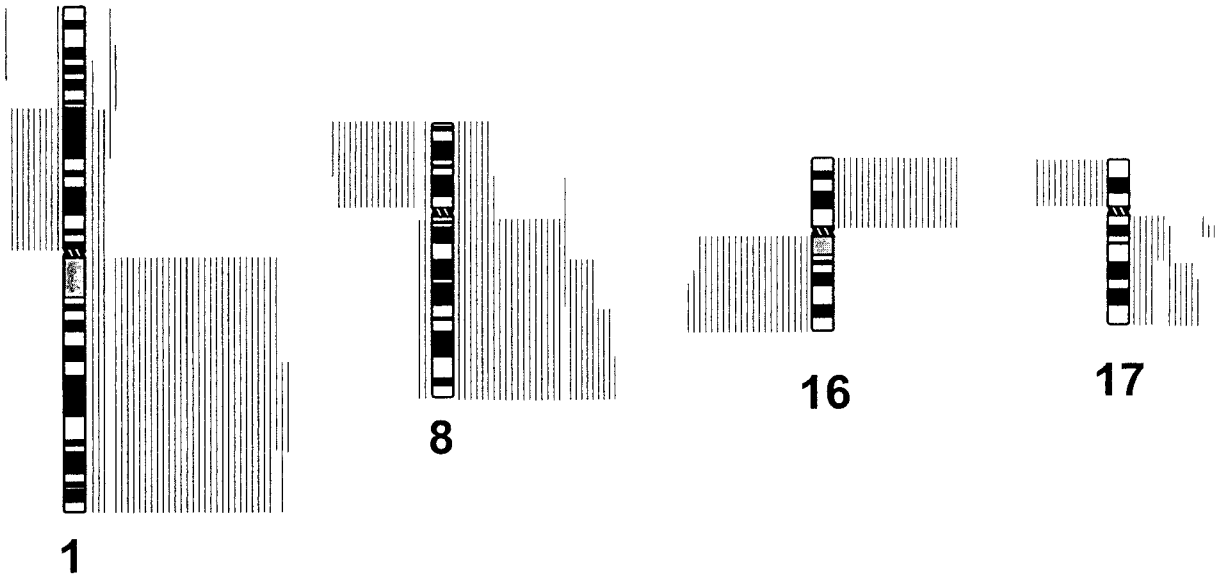

Figure 2. Summary of copy number aberrations involving chromosomes 1, 8, 16, and 17 in 55 primary breast cancers. Gains are represented on the right side of the chromosome ideogram and losses on the left.

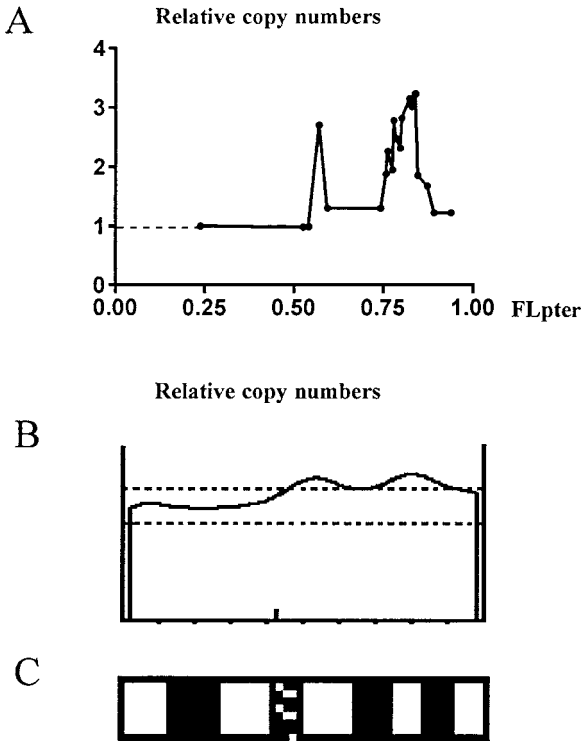

Figure 3. Comparison of CGH and FISH copy number profiles of chromosome 20 in a primary breast cancer.

TABLE 3. Correlation of CNAs and Clinicopathological and Biological Features of 55 Breast Carcinomas

		Number of all aberrations	Number of gains	Number of losses
All tumors	55	7.7 ± 5.0	4.1 ± 2.8	3.6 ± 4.0
Tumor size				
T1	20	6.7 ± 5.1	4.0 ± 3.1	2.8 ± 3.5
T2–T4	34	8.5 ± 5.1	4.1 ± 2.7	4.2 ± 4.4
Nodal status				
N0	28	7.1 ± 4.8	4.0 ± 3.2	3.2 ± 3.4
N1	26	8.5 ± 5.4	4.1 ± 2.4	4.2 ± 4.7
Grade				
I	13	5.1 ± 3.1	3.7 ± 2.8	1.4 ± 1.4
II	27	7.5 ± 5.5**	3.6 ± 2.8*	3.9 ± 4.1*
III	15	10.5 ± 4.6	5.4 ± 2.7	5.1 ± 5.0
ER status				
Positive	34	8.0 ± 4.8	3.8 ± 2.8	3.7 ± 3.9
Negative	18	7.4 ± 5.3	4.7 ± 3.0	3.4 ± 4.5
PR status				
Positive	17	6.1 ± 4.5	2.9 ± 2.3**	3.6 ± 3.8
Negative	35	8.4 ± 5.2	4.8 ± 3.0	3.6 ± 4.6
DNA index				
Diploid	27	6.0 ± 4.3*	3.4 ± 2.9	2.7 ± 3.2
Aneuploid	26	9.1 ± 5.5	4.8 ± 2.7	4.3 ± 4.7
S-phase fraction				
<8%	22	6.1 ± 4.7	3.2 ± 2.9*	3.3 ± 3.6
>8%	25	8.8 ± 5.4	4.5 ± 2.7	3.8 ± 4.7

*$P \leq 0.05$, **$P \leq 0.01$.

clearly associated with high overall number of genetic aberrations. However, 16q and 17p losses showed no such association, suggesting that these aberrations may also occur during early tumor progression.

Molecular Cytogenetic Classification of Breast Cancers

The high frequency of 1q gains (67%) suggests that it may be one of the early and probably essential steps in the multistep progression of most

breast cancers. 8q gains occurred independently of 1q, with 80% of tumors showing either or both of these aberrations. Of the 55 tumors, 31% showed 1q only, 13% 8q only, 36% both, and 20% showed none of these changes. Ninety-one percent of the tumors contained 1 of just 3 common genetic changes, +1q, +8q, or −13q.

DISCUSSION

The importance of genetic aberrations in the progression of breast cancer is reflected in the fact that all primary breast tumors studied by CGH contained DNA sequence copy number aberrations. This is higher than the frequency of chromosome abnormalities previously reported (range 25–81%) in most studies by classical cytogenetic techniques (Thompson et al., 1993; Trent et al., 1993; Pandis et al., 1995; Steinarsdottir et al., 1995) or by measurement of the total cellular DNA content (60–80%) by DNA flow cytometry (Devilee and Cornelisse, 1994). While DNA copy number aberrations are characteristic for virtually all breast cancers, the number of such changes per tumor increases significantly with the loss of the differentiation status of the tumor cells.

Gains of 1q and 8q were the most common genetic changes. Either 1 or both of these changes were found in 80% of unselected breast cancers. Despite the fact that both changes were very common, there appeared to be major differences in the representation of these changes at different stages of the accumulation of genetic changes. The fact that 1q gain appeared as the sole genetic event, and that it was already very common in the genetically less complex tumors, supports the idea that 1q gain is a relatively early event. 1q gains resulting from either isochromosome formation of 1q or an unbalanced t(1;16)(q10;p10) translocation have also been reported as sole or very early changes in cytogenetic studies (Dutrillaux et al., 1990; Pandis et al., 1995). In contrast, 8q gains were usually seen in tumors with a large number of other aberrations. This suggests that 8q is more likely to be associated with a later progression step or that tumors having this aberration become unstable and acquire other aberrations. Almost half of the breast cancers contained 8q gains, suggesting that genes in the 8q region are likely to provide a critical advantage for tumor progression. The fact that 8q gains were also associated with high S-fraction and that previous studies have linked high-level 8q gains with aggressive disease type (Ried et al., 1995) or poor prognosis in breast cancer (Isola et al., 1995) support this conclusion. Although 8q24 contains the *MYC* gene (Devilee and Cornelisse, 1994; Bieche and Lidereau, 1995), most gains are not limited to this region, and may affect other genes along the long arm of chromosome 8.

Gains of 1q, 8q, or both were found in 80% of breast cancers. These 2 gains and the loss of 13q together accounted for 91% of all tumors. Thus, in spite of the substantial complexity of breast cancer and the large overall number of changes involved, a few aberrations are selected for in most cases. Since the 1q and 8q gains appeared to have different phenotypic consequences and possibly are acquired (on average) at different phases of the progression pathway, such critical common chromosomal aberrations, and combinations thereof, may be helpful to formulate potential as a molecular cytogenetic classification of breast cancer.

Besides 1q and 8q gains, gains of 16p and a number of other defined regions of increased copy number may also be significant. Most of them have not been found by cytogenetic analyses (Dutrillaux et al., 1990; Kallioniemi et al., 1994a; Isola et al., 1995; Pandis et al., 1995; Ried et al., 1995). In this material, high-level amplifications were not as frequent as in previous CGH studies, many of which were enriched in aggressive or more advanced breast cancers (Kallioniemi et al., 1994a; Isola et al., 1995; Muleris et al., 1995; Ried et al., 1995). Some of the common regions of involvement found here, such as 12q14–21, 17q23, and 20q13, were also previously reported, while others such as 3q24-qter, and 5p have not previously received much attention as harboring amplified breast cancer genes. Examples of genes whose amplification in cancer was initially discovered by CGH analysis and subsequently ascertained by positional candidate studies include the androgen receptor gene in recurrent prostate cancer (Visakorpi et al., 1995), *REL* in non-Hodgkin lymphomas (Houldsworth et al., 1996; Joos et al., 1996), and *BCL2* in recurrent B-cell lymphomas (Monni et al., 1996). No such positional candidate genes have yet been implicated in the regions of amplification found in breast cancer by CGH. However, only the 20q13 region has been thoroughly studied (Guan et al., 1996; Tanner et al., 1996). Definition is important of which of the many regions of increased copy number reported in CGH studies represent true amplifications, and which are low-level gains. As indicated by the present comparison of FISH and CGH copy number profiles, such a distinction is difficult to make based on CGH results alone (Tanner et al., 1996).

The localization of frequently lost chromosome regions by CGH analysis (8p, 16q, 13q, 17p, 6q, 9p,

and 11q) coincides with areas showing frequent loss of heterozygosity (LOH) (Devilee and Cornelisse, 1994; Bieche and Lidereau, 1995). Physical loss is thus a prominent mechanism of loss at these regions. The frequency of CGH loss was, in general, lower than that reported by LOH studies. This indicates that small regions of loss are missed by CGH, as are losses caused by mitotic recombinations or other mechanisms that do not involve copy number alterations. Since CGH does not distinguish between alleles, a loss of 1 copy over a diploid background results in a 50% decrease of the CGH ratio, whereas LOH studies would reveal a complete loss of 1 allele. Thus, LOH would be more easily detectable than the corresponding CGH loss, especially in the presence of normal cell contamination and intratumor heterogeneity. On the other hand, CGH is more informative than LOH studies in that copy number gains are easily distinguished from losses. PCR-based LOH studies measure allelic imbalance, which may be caused by both deletions and amplifications (Devilee and Cornelisse, 1994). For example, while LOH at 11q13 and 17q23 (Cropp et al., 1993; Godwin et al., 1994; Kirchweger et al., 1994; Zhuang et al., 1995) has been reported to be common in breast cancer, CGH analysis found exclusively gains and amplifications at these regions, suggesting that amplification of 1 allele is the predominant mechanism of allelic imbalance at these regions.

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