Genetic Aberrations in Hypodiploid Breast Cancer

Frequent Loss of Chromosome 4 and Amplification of Cyclin D1 Oncogene

Minna M. Tanner,* Ritva A. Karhu,*
Nina N. Nupponen,* Åke Borg,[†] Bo Baldetorp,[†]
Tanja Pejovic,[†] Mårten Fernö,[†] Dick Killander,[†]
and Jorma J. Isola*

From the Laboratory of Cancer Genetics,* University and
University Hospital of Tampere, Tampere, Finland, and the
Department of Oncology,[†] University of Lund, Lund, Sweden

The evolution of somatic genetic aberrations in breast cancer has remained poorly understood. The most common chromosomal abnormality is hyperdiploidy, which is thought to arise via a transient hypodiploid state. However, hypodiploidy persists in 1 to 2% of breast tumors, which are characterized by a poor prognosis. We studied the genetic aberrations in 15 flow cytometrically hypodiploid breast cancers by comparative genomic hybridization (CGH) and fluorescence *in situ* hybridization (FISH). Surprisingly, numerous copy number gains were detected in addition to the copy number losses. The number of gains per tumor was 4.3 ± 3.2 and that of losses was 4.5 ± 3.3 (mean $\pm$ SD), which is similar to that previously observed in hyperdiploid breast cancers. Gains at chromosomes or chromosomal regions at 11q13, 1q, 19, and 16p and losses of 2q, 4, 6q, 9p, 13, and 18 were most commonly observed. Compared with unselected breast carcinomas, hypodiploid tumors showed certain differences. Loss of chromosome 4 (53%) and gain of 11q13 (60%) were significantly more common in hypodiploid tumors. The gain at 11q13 was found by FISH to harbor amplification of the *Cyclin D1* oncogene, which is therefore three to four times more common in hypodiploid than in unselected breast cancers (15 to 20%). Structural chromosomal aberrations (such as *Cyclin D1* amplification) were present both in diploid and hypodiploid tumor cell populations, as assessed by FISH and CGH after flow cytometric sorting. Together these results indicate that hypodiploid tumors form a distinct genetic entity of invasive breast cancer, although they probably share a common genetic evolution pathway where structural chromosomal aberrations precede gross DNA ploidy changes. (*Am J Pathol* 1998; 153:191-199)

Over the past decade a substantial amount of evidence has been collected to indicate that the development of malignant tumors requires the sequential accumulation of somatic genetic aberrations.^{1,2} These aberrations range from single nucleotide point mutations and structural chromosomal changes to gross change in chromosome copy number, called aneuploidy.^{1,2} Approximately 60 to 80% of breast cancers show clear evidence of an aneuploid DNA content by image and flow cytometry (FCM).³⁻⁵ Usually a single aneuploid stemline is observed by FCM, most frequently showing a hypotetraploid DNA content (DNA index, 1.7 to 1.8).^{1,3} Near-diploid (DNA index, 1.1 to 1.4), triploid (DNA index, 1.4 to 1.6), tetraploid (DNA index, 1.9 to 2.1), and hypertetraploid tumors (DNA index, >2.1) each constitute 5 to 15% of aneuploid breast cancers.^{1,3} Multiple DNA aneuploid stemlines have been observed in 5 to 10% of cases.^{1,3} An infrequent (incidence, 1 to 2%) type of nondiploid DNA content is hypodiploidy, defined as a cellular DNA content less than in normal cells (DNA index, <0.95).^{3,6,7} The prognosis of patients with different types of aneuploid tumors have been studied extensively (reviewed in Refs. 1 and 2). In one study, hypodiploid aneuploidy was associated with the worst clinical outcome of all types of aneuploidy, even after adjustment of other prognostic factors.³

Apart from its prognostic clinical utility, DNA ploidy analysis may provide interesting global information on the genetic evolution of breast cancer. Because little information is available from premalignant (atypical hyperplasia) and preinvasive (ductal carcinoma *in situ* (DCIS)) breast lesions, the evolution of somatic genetic aberrations has been difficult to assess. Based on a flow cytometric and cytogenetic study,⁸ structural chromosomal aberrations and losses of entire chromosomes have been suggested to occur first during genetic evolution of

Supported by the Finnish Cancer Foundation, the Scientific Foundation of Tampere University Hospital, the Swedish Cancer Society, the Mrs. Berta Kamprads Foundation, the Gunnar, Arvid, and Elisabeth Nilsson Foundation, the John and Augusta Persson Foundation, the Inga-Britt and Arne Lundberg Foundation, the Franke and Margareta Bergquist Foundation, the Anna-Lisa and Sven-Erik Lundgren Foundation, and the Hospital of Lund Foundation.

Accepted for publication April 10, 1998.

Address reprint requests to Prof. Jorma Isola, University of Tampere, P.O. BOX 607 FIN-33101 Tampere, Finland. E-mail: bljois@uta.fi.

Table 1. Clinicopathological and DNA Flow Cytometric Characteristics of 15 Hypodiploid Cancers Studied by CGH and FISH

Case	Age	Stage	ER/PR status	DNA index	% hypodiploid cells	S-phase fraction
A	32	I	-/-	0.87	15	
B	60	IIb	+/+	0.79	90	8.2
C	44	IIIb	-/+	0.90	65	17.0
D	75	IIb	-/-	0.89	40	15.0
E	52	IIa	+/+	0.77	65	9.2
F	87	IIa	+/+	0.83	85	6.2
G	79	Ia	+/+	0.95	40	5.3
H	61	IIa	+/+	0.88	35	3.6
I	49	IIIa	+/+	0.70	50	5.1
J	71	IIb	+/+	0.76	75	7.6
K	51	IIa	+/+	0.93	50	5.9
L	65	IIa	+/+	0.93	85	4.4
M	53	IIa	-/-	0.87	65	14.0
N	56	I	+/+	0.92	60	7.0
O	47	IIb	-/-	0.85	60	14.0

breast tumors. These would lead to a transient hypodiploid cell clone, the DNA of which is then doubled by endoreduplication. This pathway would explain the most commonly observed hypotetraploid DNA content (DNA index of 1.7 to 1.9).^{1,8,9} Alternatively, it has been proposed that endoreduplication is an early event in the genetic cascade, which is followed by successive chromosomal changes that give additional growth advantage to the tumor cells.¹⁰ However, both theories have little direct empirical evidence, and the molecular pathogenesis of breast cancer has therefore remained poorly understood.

Comparative genetic hybridization (CGH) is a new molecular cytogenetic method that can detect unbalanced structural chromosomal aberrations anywhere in the genome in a single hybridization.¹¹ So far, CGH has been used to characterize primary breast tumors,¹²⁻¹⁵ preinvasive intraductal cancers,^{16,17} and primary tumors and their metastases.¹⁸ Chromosomal aberrations by CGH have been correlated to DNA ploidy in two studies,^{14,15} both showing that aneuploid tumors contain a higher number of copy number aberrations than diploid tumors.^{14,15} We combined flow cytometric ploidy analyses with CGH and fluorescence *in situ* hybridization (FISH) to characterize genetic aberrations in hypodiploid breast cancer, an interesting tumor entity associated with genetic evolution and aggressive clinical course.

Materials and Methods

Patients and Tumor Material

Fifteen freshly frozen primary breast cancer samples were selected from a tumor bank of over 10,000 primary breast carcinomas at the Department of Oncology, University of Lund (Lund, Sweden). Air-dried imprint touch preparations were available from 10 of these tumors for FISH. The clinical features of the patients are shown in Table 1. The mean age of patients was 58.8 years.

Figure 1. Flow cytometric DNA histogram demonstrating hypodiploidy in a breast cancer sample. A small DNA diploid G0/G1 stemline with a DNA index of 1.00 and a larger DNA hypodiploid stemline with a prominent G0/G1 peak with a DNA index of 0.93 (corresponding to 85% of all nuclei analyzed). The S-phase fraction (S) was 4.4%, indicating a low tumor proliferation rate. G2 refers to G2/M phase of the cell cycle for the hypodiploid stemline. C and T stand for chicken and trout red blood cells, which were used as internal controls.

DNA Flow Cytometry

Freshly frozen samples (100 to 200 mg) were prepared, and flow cytometric (FCM) analysis was performed as previously described.¹⁹ Calculation of the DNA index was done after zero-point adjustment of the DNA histogram using the modal values of trout and chicken red blood cells. The mean channel numbers of all G0/G1 peaks were then used for the calculation of the DNA index with trout red blood cells as reference standard. A hypodiploid cell population was defined as a clearly detectable subpopulation (at least 15%) of cells having a DNA index less than 0.95.³ An example of a hypodiploid FCM histogram is shown in Figure 1.

Simultaneously with the DNA analysis of tumors B and E, nuclei from the two different G0/G1 peaks were separated by sorting into small glass vials. Electronic gates were set in the DNA histogram around the stemline peaks as sorting criteria. Phosphate-buffered saline was used as sheath fluid for sorting. The analysis rate was approximately 150 nuclei per second. The electronic controls for the sorting were set as follows: droplet frequency, 30 kHz; three droplets per sorted event; and coincidence check of five droplets, yielding an efficiency close to 100% and purity over 95%. Up to 100,000 sorted nuclei were collected for each peak and stored until DNA was extracted for CGH.

Comparative Genomic Hybridization

CGH was done according to a published protocol.^{20,21} Briefly, tumor DNA and normal female reference DNA were extracted using a standard protocol and labeled with fluorescein isothiocyanate (FITC)-dUTP and Texas-Red-dUTP (DuPont, Boston, MA) using standard nick

Table 2. Gains and Losses in 15 Hypodiploid Breast Cancers by CGH

Case	Gains, entire chromosome	Gains, regional	Losses, entire chromosome	Losses, regional
A		3q24-qter+, 7p14-p21+, 10p+, 15q22-qter+		
B	19+	1q+, 3pter-p21+, 3q26-qter+, 7q+, 11q13+++, 16p+	2-, 8-, 13-, 14-, 18-	3p14-q25-, 4p15-pter-, 6cen-q21-, 9p-, 11q14-qter-
C	19+	1q+, 8q+, 9q+, 12q22-qter+, 17q23-qter+, 20p12-pter+	4-	6q-, 7q-, 9p-, 10q-
D		5p+, 5q22-qter+, 7p+, 8q+, Xp+		
E		1q+, 11q13+, 20q13+	4-, 11-, 13-, 18-	2q-, 3p-, 5q-, 8p-
F		5p+, 11q13++	9-, 13-, 14-, 15-	1p-, 2q-, 5q-, 8p-, 11q14-qter-
G				6q-
H		1q+	13-	12p-, 16q-, 17p-
I	10+	16p+, 20q+	2-, 4-, 18-	1p-
J	19+	1q+, 9q34+, 11q13+, 16p+	2-, 4-, 18-	
K		11q13+	2-, 4-, 5-, 11-, 13-, 18-, X-	6q-, 8q-, 9p-
L	17+, 19+	11q13+, 12q23-qter+, 16p+, 22q+	4-, 5-	9p-,
M	19+	1q+, 8q22-qter+, 11q13+	4-, 5-	12cen-12q23-, 17p-
N	17+, 19+	11q13+, 16p+,	13-	6q-
O	16+, 19+	1q+, 2q33-qter+, 3pter-p21+, 6p+, 7q+, 11q13+, 12q23-qter+, 15q22-qter+, 17q+, 18q21-qter+, 22q12+	4-, 5-, 13-, 14-	1p32-cen-, 3p14-q21-, 18cen-q12-

translation. DNA from FCM-sorted nuclei was first amplified with universal primer polymerase chain reaction (DOP-PCR) to obtain a sufficient amount of starting material for CGH.²² Labeled DNAs (400 ng each) and 10 μ g of unlabeled Cot-1 DNA (Gibco BRL, Gaithersburg, MD) were hybridized onto commercially available normal metaphase chromosomes (Vysis, Downers Grove, IL). The hybridizations were evaluated using a commercial digital image analysis systems (Vysis and SCILImage software package, TNO, Delft, The Netherlands).

DNA Probes for Fluorescence in Situ Hybridization

The following plasmid probes were used to detect α -satellite repeat sequences of each chromosome-specific pericentromeric region: pUC177 (for the centromere of chromosome 1), p α 3.5 (chromosome 3), pJM128 (chromosome 8), pHuR98 (chromosome 9), pLC11A (chromosome 11), pA12H8 (chromosome 12), pHUR195 (chromosome 16), p17H8 (chromosome 17), p18R

Figure 2. Summary of the gains and losses in 15 hypodiploid breast cancers by CGH. Lines on the right side of the chromosome indicate copy number gains, and those on the left side indicate copy number losses. Thick lines indicate high-level amplification.

Figure 3. The comparison of the frequencies of the most common gains (A) and losses (B) in 15 hypodiploid (black columns) to 55 unselected (gray columns) breast cancers (Tirkkonen et al¹⁵). Only aberrations common (>10%) for either type of tumor population are shown. Hypodiploid breast tumors show distinct pattern of genetic aberrations compared with unselected tumors. The frequency of loss of chromosome 4 and amplification of 11q13 (11q) are statistically significant.

(chromosome 18), and pBAMX7 (chromosome X). In addition, commercial centromeric probes for chromosomes 2 (locus D2Z), 4 (D4Z1), and 10 (D10Z1) (Oncor, Gaithersburg, MD) were also used. A gene-specific P1 probe for the *MYC* oncogene (obtained from Resource for Molecular Cytogenetics, Berkeley, CA) at 8q24 and a PAC probe for the *Cyclin D1* oncogene (at locus 11q13) were used to determine the gene copy numbers of these genes. The PAC probe for *Cyclin D1* was obtained by screening a PAC library by PCR using specific primers for *Cyclin D1*.²³ Probes were labeled either with biotin-14-dATP (Gibco BRL) or digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN) using nick translation.

Fluorescence in Situ Hybridization

Imprint touch preparations were done by lightly pressing a semi-thawed frozen tumor onto cleaned and Vecta-bond-coated (Vector Laboratories, Burlingame, CA) microscope slides. After air drying, slides were fixed with 50%, 70%, and 100% Carnoy's solution (3:1 methanol/acetic acid, 10 minutes each). Samples were then fixed with 4% paraformaldehyde in PBS (5 minutes at 4°C), dehydrated in graded ethanols, air dried, and baked at 80°C for 30 minutes in a hybridization oven. Two-color FISH was carried out as previously described²⁴ with minor modifications. Slides were denatured in a 70% form-

amide/2X SSC at 72°C to 74°C for 3 minutes followed by a proteinase K digestion (0.65 µg/ml, 5 minutes). Five nanograms of differentially labeled pericentromeric probes, 20 ng *Cyclin D1* and *MYC* probes (when appropriate) and 10 µg human placental DNA were hybridized onto in a hybridization mixture described previously.²⁴ After standard post-hybridization washes, bound probes were detected immunohistochemically with avidin-FITC (Vector Laboratories, Burlingame, CA) and anti-digoxigenin rhodamine (Boehringer Mannheim).²⁴ Slides were counterstained with 0.2 µmol/L 4,6-diamino-2-phenylindole in an anti-fade solution (Vectashield, Vector Laboratories). Hybridization signals were scored using a Zeiss Axioplan 2 epifluorescence microscope equipped with dual band-pass fluorescence filter (Chromatechnology, Brattleboro, NV), which enables simultaneous detection of both fluorescein and Texas Red fluorescence. Hybridization signals from at least 300 nuclei were scored to assess the chromosome copy numbers and amplification status of *Cyclin D1* and *MYC*. Digital images were taken with a Hamamatsu 9585 camera (Hamamatsu, Hamamatsu City, Japan) operated via ISIS image analysis software (MetaSystems, Altlusheim, Germany). Control hybridizations to normal fibroblasts were done to ascertain that the hybridization efficiencies of the test and reference probes were similar.

Results

Overview of Copy Number Aberrations in 15 Hypodiploid Breast Cancers by CGH

Fourteen tumors contained copy number gains and thirteen contained losses by CGH (Table 2). Gains and losses of entire chromosomes were detected in 8 and 12 tumors, respectively. Smaller subregional gains were detected in 14 tumors and losses in 11 tumors (Table 2). The total number of gains per tumor was 4.3 ± 3.2 and that of losses was 4.5 ± 3.3 (mean $\pm$ SD). The most common gains were at 11q13 (9 of 15), 1q (7 of 15), 19 (7/15), and 16p (6/15), whereas the most frequent losses were entire chromosomes 4, 13, and 18 and subregional losses of 5p, 5q, 2q, 6q, and 9p (Figures 2 and 3).

Comparison of hypodiploid tumors with 55 unselected invasive ductal breast cancers (Tirkkonen et al¹⁵) revealed a partly distinct pattern of genetic aberrations in hypodiploid tumors (Figure 3). Gain at 11q13 (60%; 95% confidence interval (CI), 38 to 88%) was more common in hypodiploid than in unselected tumors (18%; 95% CI, 9 to 31%; Figure 3A). Similarly, loss of entire chromosome 4 (53%; 95% CI 27 to 79%) was particularly characteristic for hypodiploid tumors compared with unselected tumors (frequency, 11%; 95% CI, 4 to 22%; Figure 3B). In other aberrations, especially for copy number gains, a rather similar frequency of aberrations was found.

Validation of CGH Findings

Seven tumors were analyzed twice by CGH in two different laboratories with virtually identical findings (second

Figure 4. Examples of two-color FISH in hypodiploid tumors. Hybridized signals are visualized either in green or red fluorescence, and nuclei are counterstained with DAPI (blue). A tumor sample (I) shows loss of one copy of chromosomes 2 (green) and 18 (red) and a tumor (J) shows loss of one copy of chromosome 4 (green), whereas chromosome X (red) has retained both copies. DAPI (blue) was used for counterstaining of the nuclei. The corresponding CGH copy number profiles of chromosomes 2, 4, 18, and X are shown in below the FISH images.

analyses were done by T. Pejovic at the University of Yale). Furthermore, copy numbers for chromosomes 1, 2, 3, 4, 8, 9, 10, 11, 16, 17, 18, and X were established independently by FISH with centromere-specific probes. Among the eight tumors tested, 75% concordance (18 of 24 loci confirmed in eight tumors) was found between CGH and FISH. Representative examples are shown in Figure 4.

Amplification of Cyclin D1 Oncogene in Hypodiploid Tumors

CGH revealed a regional copy number gain at 11q13 in 9 of 15 cases (60%; Table 3 and Figure 5A). To ascertain whether the gain was due to the well characterized *Cyclin D1* gene amplification, imprint touch preparations were

Table 3. Summary of CGH at 11q13 and FISH of Cyclin D1 Oncogene in 15 Hypodiploid Breast Cancers

Case	CGH at 11q13	<i>Cyclin D1</i> copy number by FISH	Chromosome 11 centromere copy number by FISH	Level of amplification (cyclin D1/11cen ratio)
A	Normal	NT	NT	NT
C	Normal	2	2	No amplification
D	Normal	1–2	2	No amplification
G	Normal	NT	NT	NT
I	Normal	2	2	No amplification
B	Gain*	NT	NT	NT
E	Gain*	1–10	1–2	2–10
F	Gain	50–100	2	25–50
H	Normal	6–10**	2	3–5
J	Gain	2–10	2	2–5
K	Gain	NT	NT	NT
L	Gain	8–10	2	4–5
M	Gain	8–10	2–3	3–4
N	Gain	NT	NT	NT
O	Gain	4–8	2	2–4

*Found in cells with both diploid and hypodiploid total DNA content sorted by flow cytometry.
**Seen in a subpopulation (20 to 30%) of cells.
NT, not tested.

Figure 5. High-level amplification of *Cyclin D1* oncogene at 11q13 in a hypodiploid breast tumor. **A:** CGH green-to-red fluorescence profile showing a copy number increase at 11q13. **B:** FISH of the same tumor demonstrating amplification of the *Cyclin D1* gene (numerous green signals). A chromosome 11 centromere probe was as a reference red signals. DAPI blue was used as a counterstain.

analyzed by two-color FISH. A PAC probe for *Cyclin D1* was hybridized together with a centromere probe to chromosome 11 (Figure 5B). An increased copy number indicating *Cyclin D1* amplification was found in seven cases, all except one showing a regional gain at 11q13 by CGH (Table 3). In one case (H), CGH showed no gain at 11q13, although amplification of *Cyclin D1* was found in a small subpopulation of cells by FISH. Thus, the overall frequency of *Cyclin D1* amplification was 67% (10 of 15).

Evidence that Subregional Copy Number Aberrations Precede Hypodiploidization

Because several copy number aberrations involving chromosome arms and smaller regions (such as gain at 11q13) were observed, we studied next whether chromosomal losses leading to hypodiploidy precede subregional aberrations or vice versa. For this, nuclear suspensions from two tumors (B and E) were flow cytometrically sorted into diploid and hypodiploid cell populations, which were then analyzed by CGH after amplifying the extracted genomic DNA by DOP-PCR. As illustrated in Figure 6, genetic aberrations, such as gains of 11q13 and 16p, were present in cells with both diploid and hypodiploid DNA content. The hypodiploid tumor population showed additional losses of entire chromosomes (chromosome 8 in Figure 6).

In addition to CGH performed from FCM-sorted cells, evidence that structural chromosomal aberrations precede hypodiploidization was also obtained by FISH. As illustrated in Figure 7, amplification of *Cyclin D1* oncogene was seen in cells with either one or two copies of chromosome 11. Similarly, FISH with probes to the *MYC* oncogene and chromosome 8 centromere revealed a mixture of cells with 2:1 and 4:2 ratios of *MYC* and centromere 8 probes, indicating that duplication of *MYC* has occurred before losing one copy of chromosome 8.

Discussion

The present results provide for the first time a detailed genetic characterization of a subgroup of breast tumors defined as hypodiploid by DNA FCM. Our goal was to reveal the chromosomal losses that may be critical for development of the known aggressive phenotype.³ Surprisingly, we found numerous gains in 14 of 15 tumors studied. The gains included those of entire chromosomes (such as chromosome 19), chromosome arms (1q, 8q), and smaller regional copy number gains. Nevertheless, an estimation of a quantitative net effect of gains and

Figure 6. Comparison of averaged CGH profiles from flow cytometrically sorted diploid and hypodiploid subpopulations of tumor B. Green-to-red fluorescence ratio profiles from chromosomes 8, 11, and 16 demonstrate that gain of 11q13 and 16p are present both in diploid and hypodiploid populations and that the latter shows an additional loss of the entire chromosome 8.

Figure 7. Evidence by FISH that subregional genetic aberrations are present in both diploid and hypodiploid tumor cell populations. **A:** Tumor C showing nuclei with a single copy of chromosome 8 (green) and a duplication of *MYC* oncogene (red) and cells with two copies of chromosome 8 and four copies of *MYC*. **b:** Tumor E showing amplification of *Cyclin D1* gene (numerous green signals) in cells with a single copy and those with two copies of chromosome 11 (red signals).

losses on DNA content indicated predominance of losses in all but two tumors (data not shown). The estimation was done by measuring the size of each aberration in megabases and assuming losses and gains to be due to increase or decrease of one copy (2 to 3 for gains and 2 to 1 for losses). The overall loss of DNA material can be explained by the relatively small size of gains, which was overcompensated by losses of chromosome arms and entire chromosomes. Thus, the CGH results are quantitatively in agreement with FCM in hypodiploid breast tumors.

The hypodiploid tumors had on average 4.3 gains and 4.5 losses per tumor. These numbers are equal to those in unselected hyperdiploid breast carcinomas (4.8 and 4.3, respectively),¹⁵ and only slightly higher than in DNA diploid tumors (3.4 and 2.7).¹⁵ This similarity of the CGH aberration patterns in diploid and nondiploid breast cancers (Ref. 15 and current data) suggests that ploidy changes are secondary to the relative chromosomal copy number aberrations detected by CGH. This concept is supported by direct evidence from CGH analyses of FCM-sorted cell subpopulations from the same tumor showing that copy number aberrations (such as gain of 11q13 and 16p) were present already in diploid cells. Additional chromosome losses were detected in a hypodiploid subpopulation, indicating subsequent chromosome losses as a cause for hypodiploidy. These findings were confirmed by FISH, which showed *Cyclin D1* amplification in cells monosomic or disomic for chromosome 11. Similarly, a structural aberration of chromosome 8 (*MYC* duplication) was present in another tumor before loss of the other homologue of chromosome 8. An isochromosome formation could also explain findings revealed by FISH, but CGH from the same case did not show 8p loss (Table 2), which suggests that *MYC* duplication is due to subregional aberration, not isochromosome formation. In this study DOP-PCR CGH allowed comparison of two DNA FCM-defined subclones. These results, together with supporting evidence acquired by FISH, directly suggest that unbalanced structural chromosomal aberrations (gains and losses) occur earlier during breast carcinogenesis than aneuploidization. Both types of DNA alterations probably occur very early during carcinogenesis, as already the preinvasive breast carcinomas are often aneuploid^{1,5} and show chromosomal aberrations,^{16,17,25} loss of heterozygosity,²⁶ and oncogene amplifications.^{27,28}

Previously, allelotyping and FCM studies have hypothesized that the most common type of aneuploidy, the near tetraploidy (DNA index, 1.7 to 1.9), is a consequence of endoreduplication of a hypodiploid clone.^{1,8,9} These endoreduplicated cell clones would then gradually replace the original hypodiploid clone.^{1,8,9} Our results support the hypothesis of a common pathogenetic origin, because many gains typical of diploid and hyperdiploid cancers (eg, gains of 1q, 8q, and 16p) were common also in hypodiploid tumors. The reason why hypodiploid stemlines persist in 1 to 2% of breast tumors remains unknown.³ It is possible that the persisting hypodiploid clones have accumulated certain critical genetic changes and acquired a growth advantage, which is only marginally improved by polyploidization. Cells persisting in a hypodiploid state may be more susceptible to loss of tumor suppressor gene activity, because unmasking of a somatic recessive mutation would require loss of only one wild-type allele. Loss of several wild-type alleles is required for total tumor suppressor gene inactivation in tetraploid cells. A candidate for such a locus is chromosome 4, which was frequently lost in hypodiploid (53%) but rarely in unselected breast cancers (11%). Thus, chromosome 4 may harbor one or more critical tumor

suppressor genes that may contribute to the maintenance of the hypodiploid state.

Hypodiploid tumors showed evidence for high-level gene amplification for only one locus, chromosome band 11q13. Nine of fifteen tumors (60%) showed this aberration by CGH, which led us to study this locus more specifically with FISH. Using a PAC probe we were able to demonstrate that the gain of 11q13 was due to amplification of the well characterized *Cyclin D1* oncogene.^{29,30} Thus, the overall prevalence of *Cyclin D1* amplification in hypodiploid tumors is approximately three to four times higher than in unselected breast tumors, where large studies have reported 15 to 20% occurrence.³⁰⁻³² *Cyclin D1* amplification is therefore a characteristic feature of hypodiploid breast cancers, similar to ERB-B2 amplification in intraductal comedo-type tumors.²⁸ The *Cyclin D1* oncogene amplification is known to be associated with positive hormone receptor status,³⁰⁻³² and evidence for this was obtained also in the hypodiploid tumors. Seven of ten *Cyclin D1*-amplified tumors were hormone receptor positive, compared with two of five tumors with no *Cyclin D1* amplification. *Cyclin D1* amplification has also been associated with aggressive clinical outcome,^{31,32} similar to the hypodiploid DNA content.³ It is tempting to speculate that the poor prognosis of hypodiploid breast cancers may be explained in most cases by *Cyclin D1* amplification. Using the information obtained from the present study, it is now possible to test more specifically the association of hypodiploidy and *Cyclin D1* amplification with FCM and FISH.

In conclusion, the combination of CGH and FISH with DNA FCM turned out to be a powerful combination in characterizing genetic aberrations in hypodiploid cancers and defining the possible genetic evolution pathways. CGH revealed a partly distinct pattern of genetic aberrations in hypodiploid breast carcinomas compared with unselected ones. Loss of chromosome 4 and amplification of the *Cyclin D1* oncogene were defined as characteristic aberrations without any previous knowledge of involvement of these aberrations in hypodiploid tumors. Thus, the present CGH and FISH study indicates that histologically indistinguishable ductal invasive breast cancers consist of several distinct entities (such as the hypodiploid tumors) that can be defined and characterized by modern molecular techniques. In the future, this molecular subclassification of breast cancers may benefit clinicians in selecting optimal therapy individually for each patient.

Acknowledgments

We thank Mrs. Minna Ahlstedt-Soini, Maria Johansson, and Mrs. Ghita Fallenius for excellent technical assistance.

References

1. Devilee P, Cornelisse C: Somatic genetic changes in human breast cancer. *Biochim Biophys Acta* 1994, 1198:113-130

2. Bieche I, Lidereau R: Genetic alterations in breast cancer. *Genes Chromosomes Cancer* 1995, 14:227-251
3. Fernö M, Baldetorp B, Borg Å, Olsson H, Sigurdsson H, Killander D: Flow cytometric DNA index and S-phase fraction in breast cancer in relation to other prognostic variables and to clinical outcome. *Acta Oncol* 1992, 31:157-165
4. Kallioniemi OP, Blanco G, Alavaikko M, Hietanen T, Mattila J, Lauslahti K, Koivula T: Tumour DNA ploidy as an independent prognostic factor in breast cancer. *Br J Cancer* 1987, 56:637-642
5. Beerman H, Kluin PM, Hermans J, van de Velde CJ, Cornelisse CJ: Prognostic significance of DNA-ploidy in a series of 690 primary breast cancer patients. *Int J Cancer* 1990, 15:34-39
6. Coulson PB, Thornthwaite JT, Woolley TW, Sugarbaker EV, Seckinger D: Prognostic indicators including DNA histogram type, receptor content, and staging related to human breast cancer patient survival. *Cancer Res* 1984, 44:4187-4196
7. Remvikos Y, Gerbault-Seureau M, Vielh P, Zafrani B, Magdelenat H, Dutrillaux B: Relevance of DNA ploidy as a measure of genetic deviation: a comparison of flow cytometry and cytogenetics in 25 cases of human breast cancer. *Cytometry* 1988, 9:612-618
8. Dutrillaux B, Gerbault-Seureau M, Remvikos Y, Zafrani B, Prieur M: Breast cancer genetic evolution. I. Data from cytogenetics and DNA content. *Breast Cancer Res Treat* 1991, 19:245-255
9. Cornelisse CJ, Kuipers-Dijkshoorn N, van Vliet M, Hermans J, Devilee P: Fractional allelic imbalance in human breast cancer increases with tetraploidization and chromosome loss. *Int J Cancer* 1992, 50:544-548
10. Ewers SB, Långström E, Baldetorp B, Killander D: Flow-cytometric DNA analysis in primary breast carcinomas and clinicopathological correlations. *Cytometry* 1984, 5:408-419
11. Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D: Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science*, 1992, 258:818-821
12. Kallioniemi A, Kallioniemi OP, Piper J, Tanner M, Stokke T, Chen L, Smith HS, Pinkel D, Gray JW, Waldman FM: Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. *Proc Natl Acad Sci USA* 1994, 91:2156-2160
13. Isola JJ, Kallioniemi OP, Chu LW, Fuqua SA, Hilsenbeck SG, Osborne CK, Waldman FM: Genetic aberrations detected by comparative genomic hybridization predict outcome in node-negative breast cancer. *Am J Pathol* 1995, 147:905-911
14. Ried T, Just K, Holtgreve-Grez H, du Manoir S, Speicher M, Schröck E, Latnam C, Biegen H, Zetterberg A, Cremer T, Auer G: Comparative genomic hybridization of formalin-fixed paraffin-embedded breast tumors reveals different patterns of chromosomal gains and losses in fibroadenomas and diploid and aneuploid breast carcinomas. *Cancer Res* 1995, 55:5415-5423
15. Tirkkonen M, Tanner M, Karhu R, Kallioniemi A, Isola J, Kallioniemi OP: Molecular cytogenetics of primary breast cancer by CGH. *Genes Chromosomes & Cancer* 1998, 21:177-184
16. Kuukasjärvi T, Tanner M, Pennanen S, Karhu R, Kallioniemi OP, Isola J: Genetic aberrations in intraductal breast cancers by comparative genomic hybridization. *Am J Pathol* 1997, 150:1465-1471
17. James A, Mitchell ELD, Menasce L, Varley JM: Comparative genomic hybridization of ductal carcinoma in situ of the breast: identification of regions of DNA amplification and deletion in common with invasive breast cancer. *Oncogene* 1997, 14:1059-1065
18. Kuukasjärvi T, Karhu R, Tanner M, Kähkönen M, Nupponen N, Pennanen S, Schaffer A, Kallioniemi A, Kallioniemi O, Isola J: Clonal heterogeneity and clonal evolution underlying development of asynchronous metastasis in human breast cancer. *Cancer Res* 1997, 57:1597-1604
19. Baldetorp B, Fernö M, Fallenius A, Fallenius-Vecchi G, Idvall I, Olsson H, Sigurdsson H, Åkerman M, Killander D: Image cytometric DNA analysis in human breast cancer analysis may add prognostic information in diploid cases with low S-phase fraction by flow cytometry. *Cytometry* 1992, 13:577-585
20. Kallioniemi OP, Kallioniemi A, Piper J, Isola J, Waldman FM, Gray JW, Pinkel D: Optimizing comparative genomic hybridization for analysis of DNA sequence copy number changes in solid tumors. *Genes Chromosomes & Cancer* 1994, 10:231-243
21. Karhu R, Kähkönen M, Kuukasjärvi T, Pennanen S, Tirkkonen M, Kallioniemi O: Quality control of CGH: impact of metaphase chromo-

- somes and the dynamic range of hybridization. *Cytometry* 1997, 28:198–205
22. Kuukasjärvi T, Tanner M, Pennanen S, Karhu R, Visakorpi T, Isola J: Optimizing DOP-PCR for universal amplification of small DNA samples in comparative genomic hybridization. *Genes Chromosomes & Cancer* 1997, 18:94–101
23. Ioannou PA, Amemiya CT, Garnes J, Kroisel PM, Shizuya H, Chen C, Batzer MA, de Jong PJ: A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. *Nature Genet* 1994, 6:84–89
24. Tanner MM, Tirkkonen M, Kallioniemi A, Collins C, Stokke T, Karhu R, Kowbel D, Shadravan F, Hintz M, Kuo WL, Waldman FM, Isola JJ, Gray JW, Kallioniemi O-P: Increased copy number at 20q13 in breast cancer: defining the critical region and exclusion of candidate genes. *Cancer Res* 1994, 54:4257–4260
25. Nielsen KV, Blichert-Toft M, Andersen J: Chromosome analysis of *in situ* breast cancer. *Acta Oncol* 1989, 28:919–922
26. Fujii H, Szumel R, Marsh C, Zhou W, Gabrielson E: Genetic progression, histological grade, and allelic loss in ductal carcinoma *in situ* of the breast. *Cancer Res* 1996, 56:5260–5265
27. Murphy DS, Hoare SF, Going JJ, Mallon EEA, George WD, Kaye SB, Brown R, Black DM, Keith WN: Characterization of extensive genetic alterations in ductal carcinoma *in situ* by fluorescence *in situ* hybridization and molecular analysis. *J Natl Cancer Inst* 1995, 87:1694–1704
28. Van de Vijver M, Peterse JL, Mooi WJ, Wisman P, Lomans J, Dalesio O, Nusse R: Neu-protein overexpression in breast cancer: association with comedo-type ductal carcinoma *in situ* and limited prognostic value in stage II breast cancer. *N Engl J Med* 1988, 319:1239–1245
29. Schuurin E: The involvement of the chromosome 11q13 region in human malignancies: cyclin D1 and EMS1 are two new candidate oncogenes. *Gene* 1995, 159:83–96
30. Dickson C, Fantl V, Gillett C, Brookes S, Bartek J, Smith R, Fisher C, Barnes D, Peters G: Amplification of chromosome band 11q13 and a role for cyclin D1 in human breast cancer. *Cancer Lett* 1995, 90: 43–50
31. Borg Å, Sigurdsson H, Clark G, Fernö M, Fuqua S, Olsson H, Killander D, McGuire WL: Association of INT2/HST1 coamplification in primary breast cancer with hormone-dependent phenotype and poor prognosis. *Br J Cancer* 1991, 63:136–142
32. Berns EM, Foekens JA, vanStaveren IL, vanPutten WL, de Koning HY, Portengen H, Klijn JG: Oncogene amplification and prognosis in breast cancer: relationship with systemic treatment. *Gene* 1995, 159: 11–18