# DNA ploidy and chromosomal imbalances in invasive ductal breast cancer. A comparative study of DNA image cytometry and comparative genomic hybridization (CGH)

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Chromosomal imbalances were analyzed in 62 breast cancers with different DNA ploidy by CGH. The results of DNA image cytometry and CGH are consistent with peridiploid and aneuploid cases. The peritetraploid tumors harbored a high number of chromosomal imbalances, as a hint for an unfavorable prognosis. The quantitative analysis of imbalances highlighted the role of different physical constituents of the chromosome, and of chromosomal losses in different DNA ploidy groups. The peritetraploid and aneuploid tumors differed from the peridiploid tumors in losses at 8p and 18q. The peritetraploid cancers exhibited more gains at 8q, the aneuploid tumors more losses at 17p than their peridiploid counterparts. The aneuploid cases differed from the peritetraploid tumors in a higher number of losses at 11q and 14q. Combinations of imbalances provide further insights into the genetic background of DNA ploidy. Hypotheses for the progression from peridiploid to nondiploid breast cancers are given.

Figures on http://www.esacp.org/acp/2000/20-2_3/friedrich.htm.

Keywords: Breast cancer, chromosomal imbalances, DNA ploidy, CGH, tumor progression

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## 1. Introduction

The development and progression of tumors are associated with an accumulation of aberrations in the genome of the tumor cells. If such genetic aberrations exceed a certain degree, the deviation from the normal genome is detectable by DNA cytometry.

In the last 35 years, a large amount of data has been gathered that reveals correlations between the findings of DNA cytometry and clinicopathological features, and the prognosis of the breast cancer as well [1–5,7,9, 10,18,20]. DNA aneuploidy is always regarded as indicating an unfavorable course of the disease, whereas the prognosis of a peridiploid tumor is more favorable. DNA peritetraploidy remains doubtful for prognosis in breast cancer.

DNA cytometry is based on the comparison of analysis cells of unknown DNA content with reference cells possessing the known DNA content of a normal diploid cell nucleus. Thus DNA cytometry provides information on the net amount of DNA and features describing the distribution of DNA amount in a tumor cell population.

The behavior of a malignant tumor is influenced by complex changes in the genome. Such complex genetic aberrations were shown by cytogenetic investigations. For invasive breast cancers numerical aberrations at chromosomes 7, 8, 21, X, and structural aberrations involving chromosomes 1, 7, 4, 12, 16, 17 as well as complex translocations [6,23,30] and homogeneously staining regions [35] were described. The applications of conventional cytogenetic banding techniques to solid tumors are limited by the difficulties of preparation of metaphases. The techniques of fluorescence *in situ* hybridization (FISH) and, in particu-

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lar, comparative genomic hybridization (CGH) [17,21] circumvent the disadvantages of banding methods and have led to a rise in studies on genetic aberrations in solid tumors.

As with DNA cytometry, CGH compares an unknown (tumor) genome with a known (normal) genome. Nevertheless, the information obtained with CGH differs from that obtained using DNA cytometry. CGH detects copy number changes in their position in the genome by the use of metaphases with a normal karyotype as targets for the cohybridization of the differently labeled normal (or reference) and tumor (or test) DNA.

Different subsets of breast cancers studied by CGH showed recurrent imbalances at chromosomes 1q, 8q, 16 and 17 [14–16,19,24,25,29,32,33]. Hermsen et al. [14] and Ried et al. [29] included some aspects of DNA ploidy in their CGH analyses. Nevertheless, a detailed analysis of the relationship between CGH and DNA cytometry is still missing for the methodology as well as for biological background.

Thus the aims of the present study are the comparison of the CGH and DNA image cytometry and the detection of non-randomly distributed chromosomal imbalances in breast cancers with different DNA ploidy. The findings may provide new insights for the interpretation of findings from DNA cytometry and with regard to the process of breast cancer progression.

## 2. Material and methods

The study included 62 invasive ductal breast cancers. In Table 1 some clinicopathological variables and the DNA ploidy are summarized.

The DNA cytometry was performed on Feulgen stained imprints or fine needle aspirations from fresh tumor specimens. At least 250 tumor cells and 15 internal reference cells (lymphocytes) were measured per case. Image analysis was done by means of an image cytometry workstation, described in Table 2. The software for the DNA cytometry includes correction procedures for glare and diffraction [12].

The DNA histograms were classified according to the recommendations of the 1997 ESACP consensus report [13]. Only cases with a stemline comprising at least 75% of the analysis cells in their $G_{0/1}$ fraction were studied.

For the CGH procedure, the DNA was isolated from frozen tissue stored in liquid nitrogen or from routinely processed, paraffin embedded tissue specimens by means of a standard phenol extraction protocol. Each tissue specimen was checked using HE stained sections on at least 80% of tumor cell nuclei in the specimen for DNA extraction. The normal DNA was obtained from a karyotypically normal female donor. The labeling was performed by nick translation with biotin-16-dUTP for the tumor DNA and with digoxigenin-11-dUTP for the normal

Table 1
Clinicopathological variables and the DNA ploidy of the studied breast cancers

| Variables | Number |
|---|---|
| Tumor size | |
| pT1 | 30 |
| pT2 | 29 |
| pT3 | 0 |
| pT4 | 3 |
| Lymph node stage | |
| pN0 | 29 |
| pN1–3 | 32 |
| pNx | 1 |
| Grade of malignancy according to Bloom-Richardson | |
| G1 | 14 |
| G2 | 24 |
| G3 | 24 |
| DNA-ploidy | |
| Peridiploid | 23 |
| Peritetraploid | 18 |
| Aneuploid | 21 |

Table 2
Technical equipment of the image cytometry workstation for DNA cytometry

| Component | Specification | Source |
|---|---|---|
| Microscope | Axioplan | Zeiss (FRG) |
| Objective | Plan-neofluar $\times 63/1.25$ | Zeiss (FRG) |
| Condensor | Condensor 0.9 | Zeiss (FRG) |
| Filter | "green" filter $570 \pm 10$ nm | Zeiss (FRG) |
| XY scanning stage | with MCU 26 | Zeiss (FRG) |
| TV camera | XC77CE<br>Pixel size: $11 \times 11$ $\mu$m, 0.03 $\mu m^2$ in the objective plane | Sony (Japan) |
| Frame grabber | MFG | Imaging Technology (USA) |
| Image analysis basic software | Optimas® | Optimas (USA) |

DNA (Böhringer Mannheim, Germany). One microgram each of the labeled tumor and normal DNA and 25 $\mu$g Cot-1-DNA (Böhringer Mannheim, Germany) were cohybridized on metaphases from a karyotypically normal female donor for three days at 37°C. Hybridization was verified using fluorescein isothiocyanate avidin (FITC) for the tumor DNA (Vector Laboratories, Burlington, CA, USA) and anti-digoxigenin conjugated with tetramethylrhodamine isothiocyanate (TRITC) (Böhringer Mannheim, Germany) for the normal DNA. Slides were counterstained with 4′6-diamidino-2-phenylindol (DAPI) for identification of chromosomes. For control purposes, each hybridization procedure includes at least one cohybridization of normal female with normal male DNA as well as one hybridization of a case with known aberrations, usually the breast cancer cell line SKBR3 or in some cases DNA from autopsy material of a case with a cytogenetically detected aberration.

The acquisition of the FITC-, TRITC- and DAPI images was performed by a cytometry workstation for fluorescence image acquisition (described in the Table 3). At least 10 metaphases per case were evaluated with a custom-made CGH analysis program (IBSB, FRG) [26,31] based on AMBA digital image analysis software. Besides calculating the mean ratio profile, the program also comprises a component for assessing chromosomal gains and losses based on a $t$-test (according by Student) and a further component for the comparison of chromosomal imbalances in tumor subgroups based on a chi-square test. To exclude random effects arising from the multitude of possible aberrations, the integrated chi-square test was performed twice with a randomly selected learn and test set. The significance threshold for these conditions was $p \leqslant 5\%$. The search for combinations of chromosomal imbalances was conducted using multiple chi-square tests.

Table 3
Technical equipment of the image cytometry workstation for acquisition of fluorescence images

| Component | Specification | Source |
|---|---|---|
| Microscope | Axioplan 2 | Zeiss (FRG) |
| Objective | Plan-apochromat $\times 100/1.40$ | Zeiss (FRG) |
| Filter | Double band pass filter set 23 for the FITC- and TRITC image (DBP 485/20, 546/12 FT500/560 DBP 515–530, 580–630); filter set 02 for DAPI (G365, FT 395, LP420) | Zeiss (FRG) |
| XY scanning stage | with MCU 26 | Zeiss (FRG) |
| TV camera | 950C<br>Pixel size: $13 \times 13$ $\mu$m, 0.017 $\mu$m$^2$ in the objective plane | Sony (Japan) |
| Frame grabber | MFG | Imaging Technology (USA) |
| Image analysis basic software | Optimas® | Optimas Inc. (USA) |

The analysis of the number of chromosomal imbalances was performed at different levels:

- averaged number of copy alterations (ANCA) [28]: all imbalances regardless of the chromosome constituent involved and the length of the aberration,
- averaged number of copy alterations of whole chromosomes (ANWC): only imbalances of whole chromosomes,
- averaged number of copy alterations of chromosomal arms (ANWA): only imbalances of whole chromosome arms, but not of whole chromosomes,
- averaged number of copy alterations of chromosomal bands (ANCB): all imbalances with at least one border outside of the centromere or telomere of the chromosome,
- imbalances in correlation to the normal diploid karyotype (PERNK): the size of imbalances expressed in percent of the normal karyotype (estimated on a scaled human gene map [22]).

An additional "plus" $(+)$ marks gains, a "minus" $(-)$ indicates losses in the levels set out above.

Statistical analysis of the number of imbalances was conducted using the $t$-test (according to Student). The Bonferroni principle was applied for defining significance thresholds at $p < 5\%$ (with the exception of the difference between gains and losses for PERNK).

## 3. Results

Figures on http://www.esacp.org/acp/2000/20-2_3/friedrich.htm.

### *3.1. Number of chromosomal imbalances*

Table 4 summarizes the mean values and the standard deviations of all analyzed categories of the number of chromosomal imbalances. The lowest number of copy alterations in all categories except gains in

Table 4
Mean values and standard deviations of the number of chromosomal imbalances

| Category | Peridiploid | | Peritetraploid | | Aneuploid | |
|---|---|---|---|---|---|---|
| | Mean | Standard deviation | Mean | Standard deviation | Mean | Standard deviation |
| ANCA | 13.70 | 4.88 | 17.72 | 4.28 | 17.67 | 6.26 |
| ANCA+ | 6.35 | 4.09 | 7.22 | 2.65 | 6.00 | 4.05 |
| ANCA− | 7.35 | 3.78 | 10.50 | 2.87 | 11.67 | 4.85 |
| ANWC | 1.48 | 1.16 | 2.78 | 1.66 | 3.24 | 2.21 |
| ANWC+ | 0.61 | 1.08 | 0.78 | 0.81 | 0.90 | 1.14 |
| ANWC− | 0.87 | 0.76 | 2.00 | 1.19 | 2.34 | 1.80 |
| ANWA | 4.26 | 1.66 | 6.89 | 2.61 | 5.48 | 2.25 |
| ANWA+ | 2.09 | 1.20 | 2.89 | 1.71 | 2.19 | 1.81 |
| ANWA− | 2.17 | 1.56 | 4.00 | 1.83 | 3.29 | 1.82 |
| ANCB | 7.83 | 3.87 | 7.94 | 3.39 | 8.90 | 4.18 |
| ANCB+ | 3.57 | 2.89 | 3.50 | 2.15 | 3.00 | 2.55 |
| ANCB− | 4.26 | 2.73 | 4.44 | 2.23 | 5.90 | 3.21 |
| PERNK | 22.29% | 8.92 | 33.64% | 8.35 | 32.05% | 15.25 |
| PERNK+ | 9.79% | 6.55 | 12.87% | 4.95 | 11.18% | 6.98 |
| PERNK− | 12.48% | 8.20 | 21.44% | 7.92 | 22.89% | 10.21 |
| Difference between gain and loss | −2.69% | 11.85 | −8.57% | 9.45 | −11.71% | 11.05 |

all chromosomal imbalances (ANCA+) and gains in chromosomal bands (ANCB+) were demonstrated for peridiploid tumors. The highest values were detected in the aneuploid group for ANCA−, all categories of ANWC, ANCB and ANCB−, imbalances in correlation to normal karyotype losses (PERNK−) and the difference between gains and losses. The highest values for ANCA and ANCA+, ANWA and ANWA+ and the sum and gains of imbalances in correlation to the normal karyotype were found in peritetraploid breast cancers.

All in all, the difference between peridiploid and peritetraploid or aneuploid tumors was larger than the difference between peritetraploid and aneuploid tumors.

A lower number of chromosomal imbalances (ANCA) were revealed in peridiploid than in peritetraploid and aneuploid tumors, but without statistically significant differences. Significant differences were detected for the number of losses of chromosomal material (ANCA−) between peridiploid tumors and peritetraploid or aneuploid tumors ($p = 0.003$, $p = 0.002$, respectively) (see also Fig. 1). Taking account of imbalances in whole chromosomes (ANWC) only, significant differences were evident between peridiploid cases and peritetraploid or aneuploid breast cancers ($p = 0.003$, $p < 0.001$, respectively). The same is true for the losses for whole chromosomes (ANWC−) ($p < 0.001$ for the difference between peridiploid and peritetraploid as well as for the difference between peridiploid and aneuploid tumors) (see also Fig. 2). Significantly more imbalances at chromosomal arms (ANWA) were detected in peritetraploid than in peridiploid cases ($p < 0.001$ for ANWA as well as for ANWA−). No significant difference was apparent in the analysis of imbalances of chromosomal bands (ANCB). The sum of all imbalances in correlation to the normal karyotype (PERNK) was significantly higher in peritetraploid tumors than in peridiploid tumors ($p < 0.001$). Peritetraploid and aneuploid tumors showed more losses of chromosomal material in correlation to the normal karyotype (PERNK−) compared with the peridiploid tumors ($p < 0.001$). Averaged differences between gains and losses increased from peridiploid through peritetraploid to aneuploid cases ($p = 0.047$ for peridiploid vs. peritetraploid, $p = 0.006$ for peridiploid vs. aneuploid, $p = 0.345$ for peritetraploid vs. aneuploid) (see also Fig. 3). An overview of the statistical analysis is given in Table 5.

### *3.2. Imbalances in specific chromosomal regions*

The most frequent gains for all cases were detected at chromosomal regions 1q42–qter (36 out of 62), 8q24

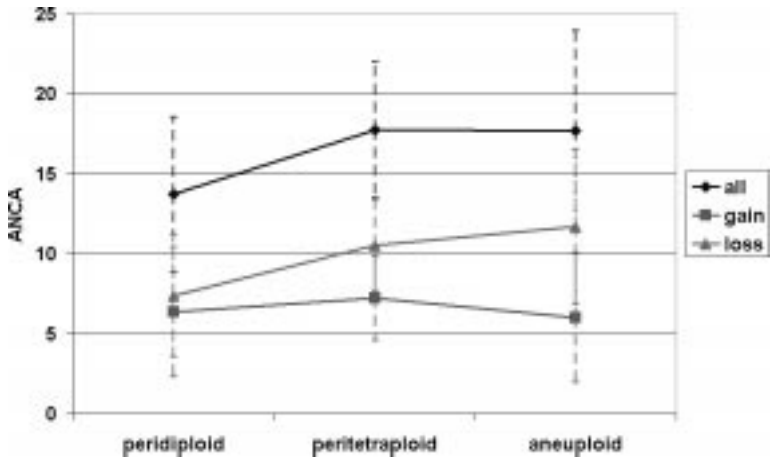


Fig. 1. Averaged number of copy alterations (ANCA). The vertical lines represent the standard deviations.

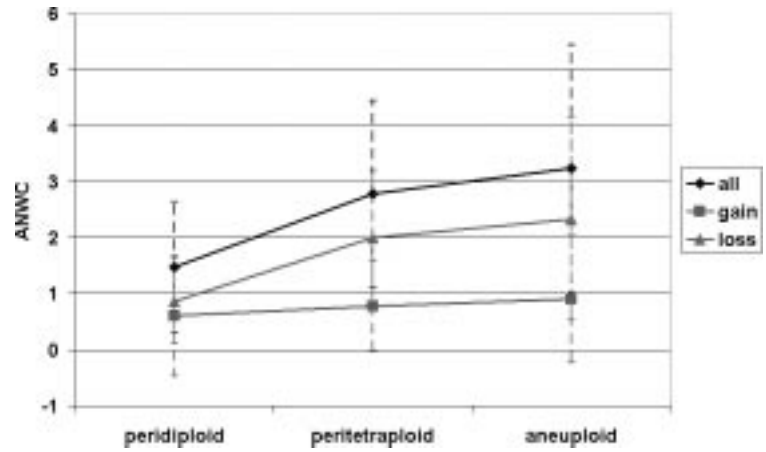


Fig. 2. Averaged number of copy alterations of whole chromosomes (ANWC). The vertical lines represent the standard deviations.

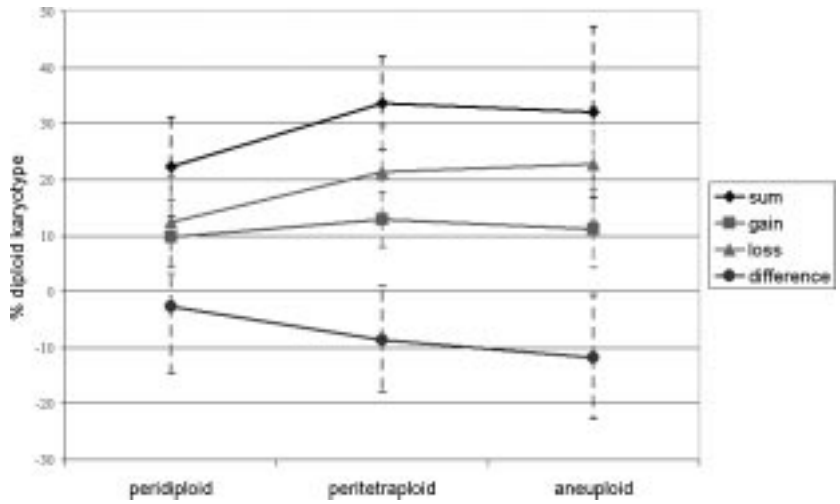


Fig. 3. Chromosomal imbalances in correlation to the normal karyotype (PERNK). The vertical lines represent the standard deviations.

Table 5
Results of the statistical analysis of the number of imbalances by the $t$-test (by Student)

| Category | Peridiploid vs. peritetraploid $p$-value | Peridiploid vs. aneuploid $p$-value | Peritetraploid vs. aneuploid $p$-value |
|---|---|---|---|
| ANCA | 0.004 | 0.01 | 0.9 |
| ANCA+ | 0.4 | 0.8 | 0.3 |
| ANCA− | **0.003** | **0.002** | 0.4 |
| ANWC | **0.003** | **0.0009** | 0.5 |
| ANWC+ | 0.3 | 0.2 | 0.3 |
| ANWC− | **0.0003** | **0.0005** | 0.5 |
| ANWA | **0.0002** | 0.05 | 0.08 |
| ANWA+ | 0.1 | 0.8 | 0.2 |
| ANWA− | **0.0005** | 0.02 | 0.1 |
| ANCB | 0.5 | 0.2 | 0.2 |
| ANCB+ | 0.5 | 0.2 | 0.2 |
| ANCB− | 0.4 | 0.04 | 0.06 |
| PERNK | **0.00008** | **0.006** | 0.3 |
| PERNK+ | 0.05 | 0.2 | 0.2 |
| PERNK− | **0.0006** | **0.0003** | 0.3 |
| Difference between gains and losses* | **0.05** | **0.006** | 0.3 |

Significant differences are marked by bold types. * Analysis without Bonferroni correction.

(26 out of 62) and 20q (26 out of 62). The regions with the most frequent losses in all cases were 9pter–p23 (36 out of 62), 13q31–qter (34 out of 62) and 8p23 (28 out of 62).

Peridiploid tumors showed the highest number of gains at 1cen–q25 (13 out of 23), 1q32–q42 (12 out of 23), 20q (10 out of 23), 15q26 (9 out of 23) and 11q13 (9 out of 23). The regions with the most frequent losses were 9p (11 out of 23), 11q22 (11 out of 23), 13q21–q32 (10 out of 23) and 16cen–q22 (10 out of 23) (see also Fig. 4). The most frequent gains for peritetraploid tumors were mapped at 1q41–ter (13 out of 18), 8q21 (11 out of 18) and 17cen–q21 (9 out of 18). These tumors exhibited the most frequent losses at 9pter–p21 (14 out of 18), 13q31–qter (11 out of 18) and 16q23–q24 (11 out of 18) (see also Fig. 5 on the ACP server). Gains at 1q (11 out of 21), 8q21–qter (8 out of 21), 16p (8 out of 21) and 20q (8 out of 21) and losses at 8pter–p21 (13 out of 21), 13q21–qter (12 out of 21) and 18q21 (12 out of 21) were the most frequent imbalances in the aneuploid tumor group (see also Fig. 6).

Differences between peridiploid and peritetraploid breast cancers were found at 8q12–q21 and 8q23–q24.1, with more gains in the peritetraploid group. Peritetraploid tumors showed more losses than peridiploid tumors at 8p22–p23 and 18q12–q21 (Fig. 7). A combination of losses at 9p and region 18cen–q12 was seen in 8 peritetraploid breast cancers but in none of the peridiploid tumors ($p = 0.0005$).

A higher number of losses at 8p22–p23, 17p13 and 18q12–q21 was found in aneuploid than in peridiploid tumors (Fig. 8). Furthermore, losses at the p-arm of chromosome 8 (8pter–p21) were significantly coupled with losses at the q-arm of chromosome 13 (13q14 and 13q21–qter) or 18q21. None of the peridiploid tumors featured such linkage, but 9 of the aneuploid cases did ($p = 0.0004$).

Aneuploid tumors differed from peritetraploid tumors in losses at 11q23 and 14q23–q24 (Fig. 9). The search for differences in combined chromosomal aberrations between these ploidy groups revealed a loss of 17p together with a gain of 17cen–q21 in 6 peritetraploid tumors, but in none of the aneuploid cancers. The difference was not statistically significant applying the Bonferroni correction ($p = 0.005$).

## 4. Discussion

The aim of the study was the quantitative analysis of chromosomal imbalances including such nonrandomly distributed in breast cancers with different DNA ploidy.

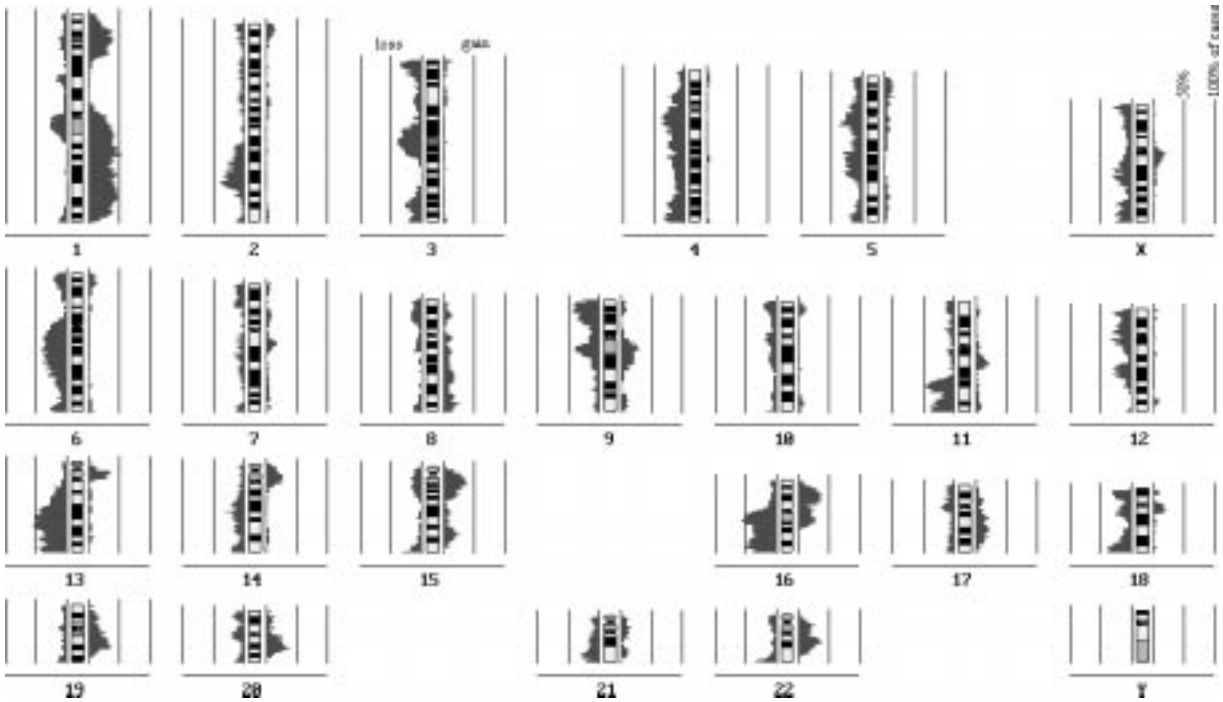


Fig. 4. Histogram of the CGH result of 23 peridiploid breast cancers. The gray areas besides the chromosomes represent the percentage of cases with imbalances in the chromosomal region. Losses are reflected on the left side, gains on the right side of the chromosome ideograms. Significance level of 95% for gains or losses in the Student's *t*-test.

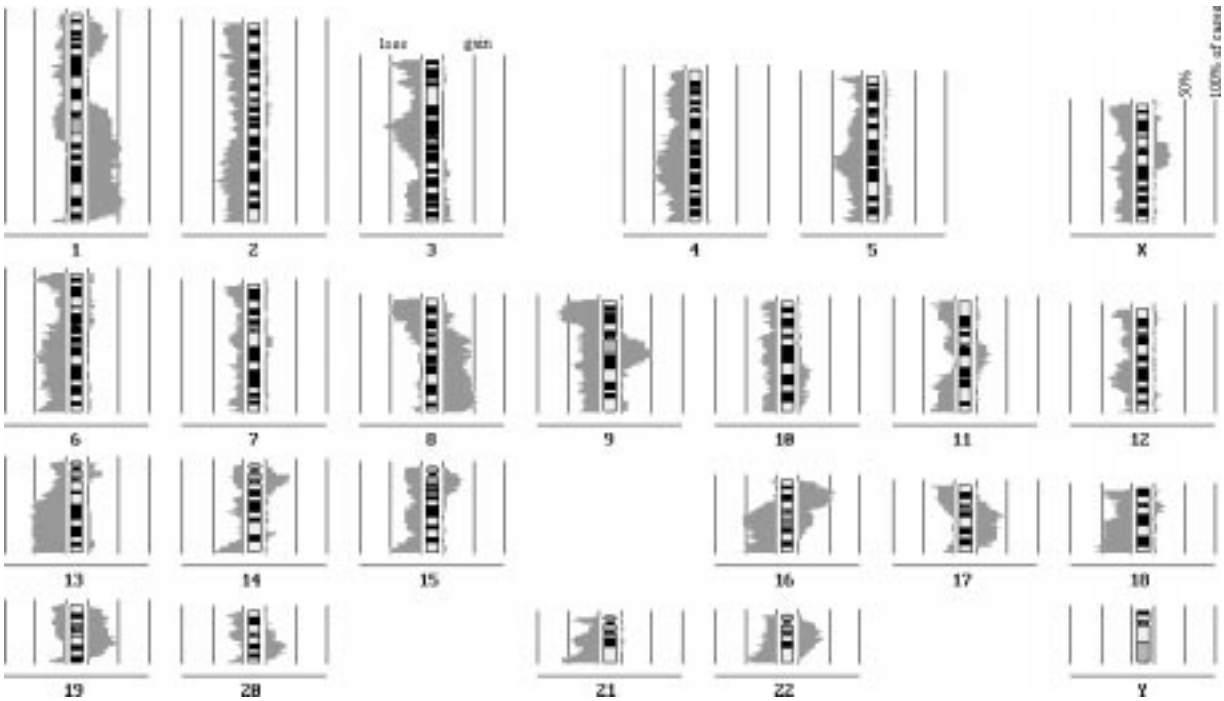


Fig. 5. Histogram of the CGH result of 18 peritetraploid breast cancers shown by gray areas besides the chromosome ideograms. Significance level of 95% for gains or losses in the Student's *t*-test.

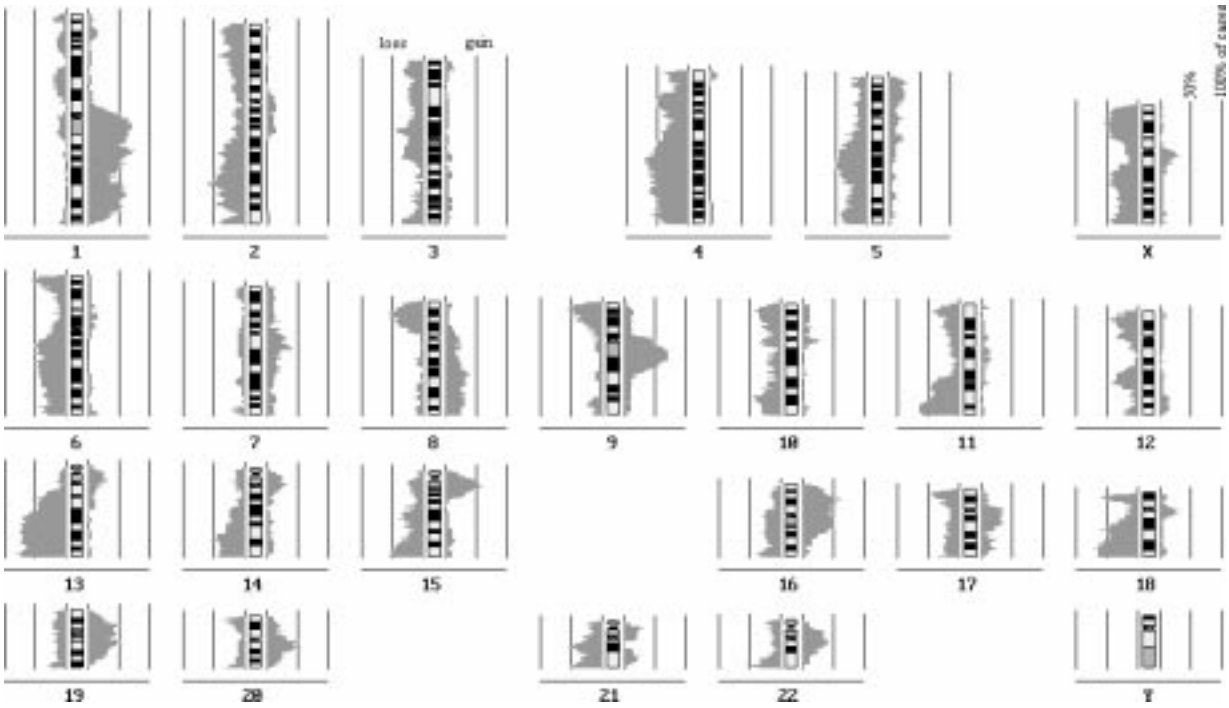


Fig. 6. Histogram of the CGH result of 21 aneuploid breast cancers shown by gray areas besides the chromosome ideograms. Significance level of 95% for gains or losses in the Student's $t$-test.

Peridiploid breast cancers are generally regarded as tumors at an early stage of tumor progression, with aneuploid cancers seen as indicating tumors of the most progressed kind. The place of peritetraploid tumors in the process of tumor progression is not yet clear.

One of the most important findings was evidence of chromosomal imbalances in all peridiploid tumors, showing the impact of numerical chromosomal aberrations in breast cancers at an early stage of tumor evolution when prognosis is still favorable.

In agreement with the results of DNA cytometry, the lowest average number of copy alterations (ANCA) and the lowest sum of imbalances as well as of the difference between chromosomal gains and losses in correlation to the normal karyotype (PERNK) were detected in peridiploid tumors, with peritetraploid and aneuploid breast cancers yielding higher values. Standard deviations were comparatively high for all three categories. This indicates heterogeneity of the peridiploid group, which includes tumors with a low number of chromosomal imbalances as well as tumors with a high number of imbalances but a balanced ratio between chromosomal gains and losses. Although cases were carefully selected, and fine needle aspirates as well as imprints were prepared for DNA cytometry, the high level of standard deviation may also result from intratumoral heterogeneity.

Further studies including follow-up data should show whether breast cancers with a high number of imbalances, either with or without a balanced ratio between chromosomal gains and losses, represent peridiploid cases showing an unfavorable prognosis.

Nevertheless, the majority of peridiploid breast cancers are tumors with a lower degree of imbalances in the genome than nondiploid tumors.

This is supported by the fact that peridiploid breast cancers did not show any additionally non-randomly distributed chromosomal imbalances compared with the nondiploid breast cancers.

There was no statistically significant difference in the number of imbalances between aneuploid and peritetraploid breast cancers. In particular, the difference between chromosomal gains and losses in peritetraploid cancers is expected to be similar to the value for peridiploid tumors. But there was a significantly higher value for peritetraploid than for peridiploid tumors. Besides the problems of intratumoral heterogeneity mentioned above, part of the reason may lie in the evaluation of CGH findings. Assessment of chromosomal gain or loss was conducted using a $t$-test (according to Student), which analyses whether the devi-

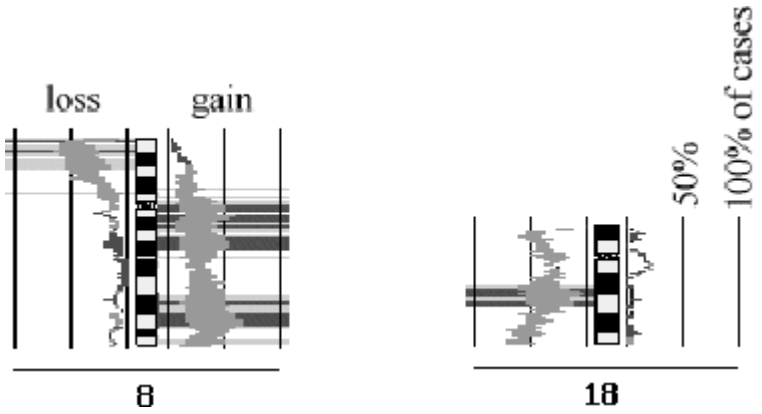


Fig. 7. Differences of chromosomal imbalances between peridiploid and peritetraploid tumors expressed as histogram. The percentage of cases with imbalances is displayed by the colored areas (dark gray for the peridiploid tumors; light gray for the peritetraploid tumors) on both sides of the ideogram chromosomes. The statistical analysis is based on an Chi-square-test integrated in CGH software. Significant differences are displayed by light gray bars for a significance level $p \leqslant 5\%$ and by dark gray bars for a significance level $p \leqslant 1\%$. The modification of the statistical analysis for this study is described in the part Material and methods. The peritetraploid tumors showed more gains on 8q12–q21 and 8q23–q24.1 and more losses on 8p22–p23 and 18q12–q21 than the peridiploid tumors.

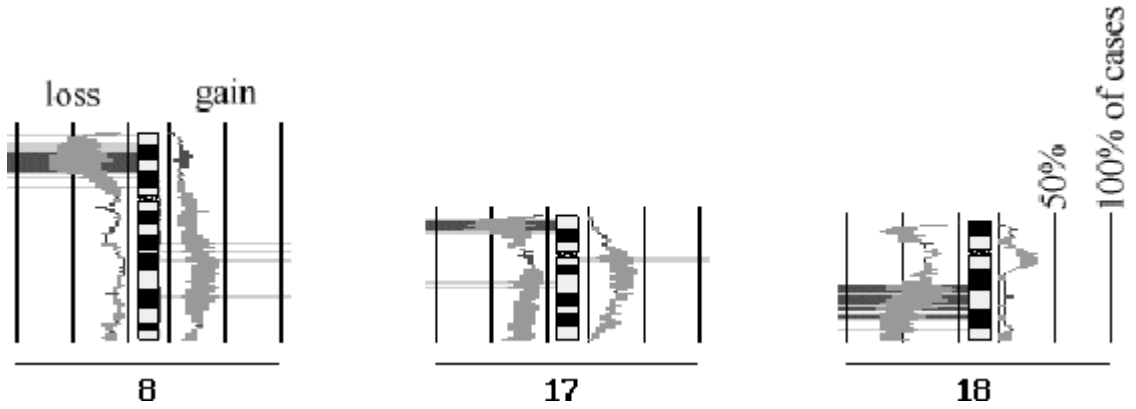


Fig. 8. Differences of chromosomal imbalances between peridiploid (dark gray) and aneuploid tumors (light gray). The aneuploid tumors exhibited more losses on 8p21–p22, 17p13 and 18q12–q21 than the peridiploid cases.

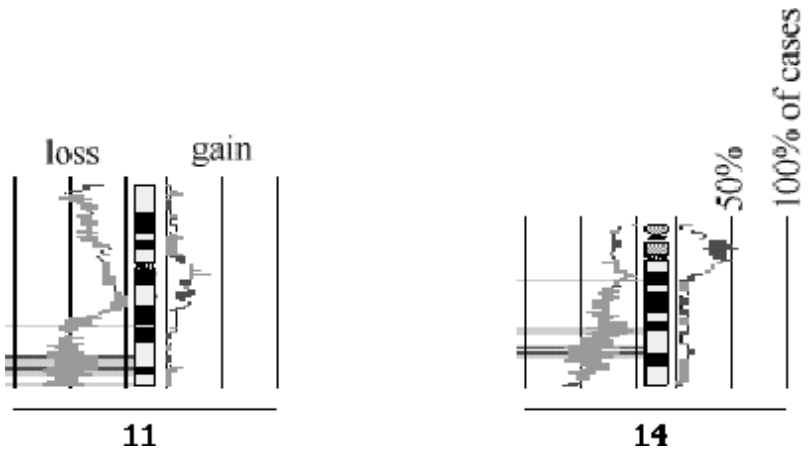


Fig. 9. Differences of chromosomal imbalances between peritetraploid (dark gray) and aneuploid tumors (light gray). The analysis reveals more losses on 11q23 and 14q23–q24 in the aneuploid tumors than in the peritetraploid tumors.

ation in the ratio profile is significantly different from the normal profile ($p < 5\%$). It seems possible that this difference is rendered significant through gain or loss of one of the theoretically existing four copies of chromosomes. This assumption can only be proven by conventional cytogenetics or by fluorescence in situ hybridization on interphase nuclei.

Further similarities between peritetraploid and aneuploid tumors were detected in the localization of chromosomal imbalances. Both tumor groups differed from the peridiploid tumors in losses at 8p22–p23, harboring the PRLTS (PDGFR $\beta$-like tumor suppressor gene) gene (and other still unknown tumor suppressor genes), and 18q12–q21, harboring the genes DPC4, DCC and bcl-2. With the exception of the bcl-2 gene locus, a crucial difference between peridiploid and nondiploid breast cancers are losses of chromosomal regions harboring tumor suppressor gene loci. The more frequent losses of the bcl-2 gene locus in aneuploid and peritetraploid tumors compared with peridiploid tumors may cause the known loss of normal expression of the bcl-2 protein in more progressed breast cancers.

Confirmation of this hypothesis necessitates a comparative study of FISH on interphase nuclei and bcl-2 immunohistochemistry. Peritetraploid and aneuploid tumors showed additional differences in their chromosomal imbalances compared with their peridiploid counterparts. Peritetraploid tumors exhibited more gains at 8q12–q21 and 8q23–q24.1, including the gene locus of c-myc, whereas aneuploid tumors displayed more losses than peridiploid tumors at 17p13, harboring the p53 gene locus.

These results provoke the question whether there is continuous progress from peridiploid through peritetraploid to aneuploid tumors (hypothesis 1), or whether peritetraploid and aneuploid tumors are separate final stages in tumor progression (hypothesis 2). A further hypothesis (hypothesis 3) would assume a development from peridiploid through aneuploid to peritetraploid tumors.

Hypothesis 1 is supported by a continuous increase in losses of chromosomal material from peridiploid through peritetraploid to aneuploid tumors (for ANCA$-$, ANWC$-$, ANCB$-$, PERNK$-$) and by the detection of differences in imbalances in the same chromosomal regions in peritetraploid and aneuploid tumors as against peridiploid tumors. The fact that there are also differing chromosomal imbalances between peritetraploid (gain at 8q12–q21 and 8q23–q24.1) and aneuploid tumors (loss at 17p13) compared with peridiploid tumors appears to rule out any continuous progression from peritetraploid to aneuploid breast cancers. However, a proportion of aneuploid breast cancers exhibited gains at 8q and some peritetraploid tumors were characterized by a loss at 17p (see also Figs 5 and 6). In both groups, the differences compared with the peridiploid cases were not statistically significant. A further argument in favor of continuous progression was provided by the comparison of chromosomal imbalances in peritetraploid and aneuploid tumors. Aneuploid breast cancers exhibited more losses their peritetraploid counterparts at 11q23, harboring the Ataxia Teleangiektasia gene locus (AT) and at 14q23–q24, whereas the latter did not display additional imbalances in individual chromosomal regions as against aneuploid tumors.

On the other hand, peritetraploid tumors have a number of special features, indicating that peritetraploidy could conceivably be a second final stage in the progression of breast cancer tumors (hypothesis 2). Peritetraploid tumors exhibited the highest number of gains in chromosomal material (ANCA$+$, ANWA$+$, PERNK$+$), though without any statistically significant difference relative to the other two ploidy groups. Furthermore, there is a coupling of loss at 17p (with the gene locus of p53) and gains at 17cen–q21 (with the c-erbB2 gene) in 6 out of 18 peritetraploid cases, but in none of the aneuploid tumors.

The path of progression from peridiploid to nondiploid breast cancers still remains unclear. Mechanisms for all three hypotheses mentioned above could be imagined.

The process of progression towards aneuploid tumors is characterized by losses of chromosomal material, especially by the loss of regions harboring tumor suppressor gene loci. Development from peridiploid to aneuploid breast cancers merely through loss of chromosomal material contradicts the findings of DNA cytometry, which yields hyperdiploid DNA indices for all aneuploid tumors investigated. An endoduplication step of peridiploid tumor cells is therefore necessary (supporting hypothesis 1). In case of a pure endoduplication, the resulting peritetraploid tumor cells should not differ from peridiploid cells in the number and localization of chromosomal imbalances. But peritetraploid tumors share more features with aneuploid than with peridiploid tumors (supporting hypothesis 2). There are two possible explanations for this. Firstly, the endoduplication step may be immediately followed by further changes in the genome, possibly caused by endoduplication itself. The first subsequent

step may include the losses of 8p and 18q. The next steps should lead to the differences between peritetraploid and aneuploid tumors mentioned above, corresponding with a combination of hypothesis 1 and 2. These steps may differ within the group of peritetraploid and that of aneuploid breast cancers. A hint for such a difference in development among peritetraploid tumors is the subgroup combining a 17p loss and a gain at 17cen–q21.

The second explanation could be the development of aneuploid tumors from peridiploid ones by randomly distributed chromosomal gains and losses, possibly caused by an initial loss of p53 function, including a disturbed duplication of centrosomes with missegregation of chromosomes. The loss of the p53 function results usually from a point mutation of one allele and loss of the second (wild type) allele. As shown, one feature of aneuploid breast cancers is a loss at 17p, including the p53 gene locus. Peritetraploid tumor cells may develop by endoduplication of peridiploid or aneuploid tumors with additional losses in the genome. In this case, the similarity between the peritetraploid and aneuploid indicates that peritetraploid tumors are derived from aneuploid tumors (hypothesis 3).

The study of Dutrillaux et al. [8] using cytogenetic banding techniques and DNA flow cytometry describes a sequence of chromosomal changes during the evolution of breast cancer. This sequence includes losses of chromosomal material followed by endoduplication steps. Endoduplications leading via an aneuploid stage with losses of chromosomal material to the near tetraploid stemline as an endpoint of breast cancer evolution support the assumption that peritetraploid breast cancers are progressed tumors with an unfavorable course of the disease (hypothesis 3). This model embraces the phenomenon of hypodiploid breast cancers, which are very rarely found in practice.

Our results only allow for the hypotheses mentioned above concerning the progression of breast cancer from peridiploid to the various nondiploid tumors. The development of a tumor progression model for breast cancer based on DNA ploidy requires studies with animal models, possibly in combination with cell culture studies. But the results of the study of Dutrillaux et al. [8] and of this study show peritetraploid breast cancers at a stage of tumor progression comparable with that of their aneuploid counterparts. A possible model of progression from peridiploid to peritetraploid and aneuploid breast cancer is shown in Fig. 10.

Finally, some relationships between DNA ploidy, special aspects in the number of chromosomal imbalances and imbalances in specific chromosomal regions should be discussed.

The losses at 11q23, harboring the Ataxia Teleangiektasia gene locus, which occurs more frequently in aneuploid than in peritetraploid tumors, may lead to a higher fragility of the chromosomes in aneuploid tumors. A higher fragility of chromosomes may be mirrored by a higher number of imbalances in chromosomal bands. There was a continuous increase in the

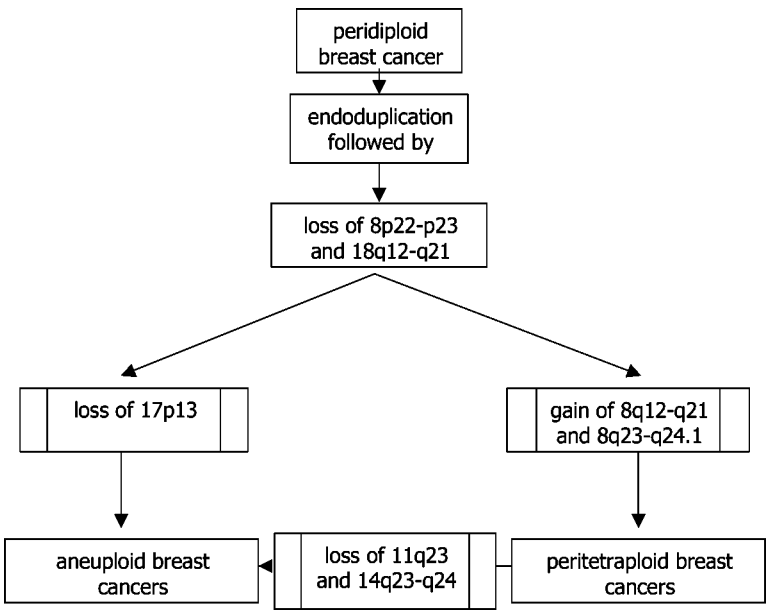


Fig. 10. A possible model of progression from peridiploid to peritetraploid and aneuploid breast cancers.

number of all imbalances and in losses of chromosomal bands from peridiploid through peritetraploid to aneuploid tumors, though not attaining statistical significance. On the contrary, the gains decreased from peridiploid through peritetraploid to aneuploid tumors.

Since not only aneuploid tumors harbored a 11q23 loss, further statistical analysis ($t$-test according to Student, $p < 5\%$) reveals significantly more losses of chromosomal bands (ANCB−) in cases with a loss at 11q23 than in cases without a loss of this chromosomal region. The number of all imbalances of chromosomal bands (ANCB) was higher in tumors with an 11q23 loss compared to cases without this loss, but again not reaching statistical significance.

Imbalances damaging the integrity of the chromosome itself are likely to indicate a higher disorder in the genome. Furthermore, an aberration starting or ending in a coding region of DNA includes not only the loss or gain of chromosomal material but also the possibility of translocation of other chromosomal material. A translocation may lead to the activation of oncogenes. The inactivation of tumor suppressor genes in this way seems not to be a frequent event, but is also imaginable. Such translocations can be assumed to provoke more aggressive behavior in a tumor. The absence of significant differences in the ANCB category between aneuploid and peridiploid or peritetraploid tumors may be caused by the heterogeneity of all ploidy groups as mentioned above. A further reason may lie in the CGH method, which is only able to detect unbalanced aberrations. The identification of balanced aberrations requires other cytogenetic methods involving metaphases of the tumor cells.

Imbalances in large chromosomal constituents such as the whole chromosome or whole chromosomal arms can contribute more to deviations from normal DNA amount than aberrations in small chromosomal subregions comprising a few bands. Thus, significant differences between peridiploid and peritetraploid or aneuploid tumors as regards the number of imbalances in whole chromosomes and whole chromosomal arms (ANWC, ANWA) seem to be logical. The high number of imbalances in whole chromosomes may be caused by defects in centrosomes, a mechanism known to further the development of aneuploidy, leading to a missegregation of whole chromosomes [11,27,34]. Notably in the case of aneuploid breast cancers, the disturbance of centrosomes may be caused by a loss of p53 function in the regulation of centrosome duplication [11,34].

Summarizing all findings of the study, it can be concluded, that

1. All breast cancers examined, including DNA peridiploid tumors, harbor chromosomal imbalances. Thus, a clear distinction needs to be made between cytometrically assessed DNA ploidy and chromosomal ploidy analyzed using cytogenetics, CGH or FISH.
2. There is conformity between the results of DNA image cytometry and CGH as regards peridiploid and aneuploid breast cancers. CGH findings on peritetraploid tumors show a high number of chromosomal imbalances, comparable with aneuploid tumors. This, too, can be interpreted as indicating an advanced stage of disease in peritetraploid breast cancers.
3. A detailed quantitative analysis of chromosomal imbalances has yielded further insights into changes in the genome during tumor evolution. Such an analysis shows the role of imbalances in whole chromosomes, whole chromosome arms and of losses of chromosomal material in breast cancers with different DNA ploidy.
4. Specific patterns of chromosomal imbalances may allow further insights into the genetic background of changes in the phenotype of breast cancers.

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