Improved Grading of Breast Adenocarcinomas Based on Genomic Instability

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ABSTRACT

Numerous investigations have shown that in primary breast adenocarcinomas DNA aneuploidy in contrast to DNA diploidy indicates high malignancy potential. On the basis of the study of 104 breast carcinomas, we describe a subtype of aneuploidy, which demonstrates a low degree of malignancy. In image cytometric DNA histograms, this subtype possessed a low percentage (≤8.8%) of nonmodal DNA values as measured by the stemline scatter index (SSI), which is defined as sum of the percentage of cells in the S-phase region, the G2 exceeding rate and the coefficient of variation of the tumor stemline. The cut point of SSI = 8.8% (P = 0.03) enabled us to also subdivide diploid and tetraploid tumors into clinically low and high malignant variants. One possible reason for aneuploidy is impaired distribution of chromosomes at mitosis caused by numerical or structural centrosome aberrations. Cyclins A and E seem to be involved in centrosome duplication. Real-time quantitative PCR measurements of cyclin A and E transcript levels and immunohistochemical determination of cyclin A protein expression showed statistically significantly increased values in the tumors with a high SSI (>8.8%), compared with those with a low SSI. A pilot study demonstrated centrosomal aberrations in an average of 9.6% of the measured cells in four aneuploid carcinomas with high SSI values and in an average of 2.5% of the cells in three aneuploid and three diploid tumors with low SSI. Our data indicate that the SSI, most likely reflecting the degree of genomic instability, allows additional classifying of the known aneuploid, diploid, and tetraploid categories of primary breast adenocarcinomas into low and high malignant subtypes.

INTRODUCTION

Cyclin E, associated with cyclin-dependent kinase 2 during G1-S-phase transition of the cell cycle, and cyclin A, interacting with cyclin-dependent kinase 2 during S phase, are found to be overexpressed in different types of cancer, e.g., colon, lung and endometrium (1–4). In human breast tumors and breast cancer cell lines, cyclin E expression is increased at both the protein and mRNA levels (5, 6) and is furthermore encountered to a high extent in undifferentiated species with poor prognosis (7). Cyclin A protein expression is reported as being up-regulated in breast carcinomas and correlates with an unfavorable clinical course (8, 9).

Particularly in the case of cyclin E, a connection with oncogenesis is suggested. Although cyclin E and its associated kinase are under cell cycle control in normal tissue, in tumor cells, it remains in an active complex throughout the cell cycle (10). The constitutive expression of cyclin E may lead to aberrant regulation of S-phase progression (11). This could be a cause of karyotypic instability in addition to others such as the overexpression of centrosome-associated kinase STK15/BTAK or the malfunction of mitotic checkpoint genes (12, 13). In some authors’ opinion, karyotypic instability is directly proportional to the degree of aneuploidy. Thus, the further the distance of a cell’s stemline karyotype from either diploidy or tetraploidy, the greater the instability of its genome (14).

Closely involved in the process of generating aneuploidy seems to be disturbed regulation of the centrosome duplication. The centrosome as the microtubule assembling and organizing cellular compartment is responsible for correct chromosome segregation (15). During G1-S-phase transition of the cell cycle, centrosome duplication initiates, and cyclins A and E appear to partake in this process. Blocking of either cyclin-dependent kinase 2 or of either cyclin will lead to an abolition of centrosome duplication (16–18). Throughout G2-M-phase passage, replicated centrosomes separate and constitute the poles of the mitotic spindle (15, 18). When centrosome duplication and DNA replication are no longer synchronized, e.g., because of cyclin E overexpression, this may lead to interference with centrosome duplication, formation of multipolar mitotic spindles, and impaired distribution of chromosomes at cell division and, thus, to aneuploidy (19–21). Numerical and qualitative deviations of centrosomes, as well as aneuploidy, are common in human cancers (22, 23).

Aneuploid tumors prove to be more malignant than their diploid counterparts (24, 25), and in general, they are regarded as being genomically unstable (gu; Ref. 13). A type of genomic instability, described as chromosomal instability, was first characterized by Lengauer et al. (26). A tumor cell population with chromosomal instability attribute indicates, by definition, high clonal heterogeneity (27).

In the histograms obtained by us from image cytometry showing the nuclear DNA content of 154 primary breast tumors, we could clearly distinguish two subtypes of aneuploid species (see “Results”), as did previously Baladetorp et al. (28), using other criteria. Our measurements resulted from the detection of a genomically stable (gs) aneuploid type, essentially demonstrating clonal homogeneity and a gu one, demonstrating clonal heterogeneity (Fig. 1, A and B). As stated above, the cell cycle genes cyclin E and cyclin A seem to be closely involved in generation of karyotypic instability because they are crucial for G1-S-phase transition and centrosome duplication. In this study, we have measured the mRNA levels of cyclin A and cyclin E in primary breast tumors of different DNA ploidy. We additionally determined cyclin A protein expression in these tumors. Chosen by random from those samples with evaluable mRNA levels, we investigated centrosomal aberrations in 10 breast carcinomas of different nuclear DNA content.

MATERIALS AND METHODS

Patient Samples. A total of 154 consecutive biopsies of breast adenocarcinomas was collected at Cell and Molecular Analysis, Karolinska Hospital, during 1998–1999. There was no radio- or chemotherapy before surgery. From each sample, imprints were taken for DNA ploidy measurements, and 4-μm histological sections were prepared for histopathological assessment and immunohistochemistry. Furthermore, total RNA was extracted from the same tumor sample for real-time quantitative PCR (qPCR) determinations. Additionally, we investigated four benign lesions (3 fibroadenomas and 1 phyllodes adenoma), and for the real-time qPCR, we also included three normal breast tissue samples.

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DNA Cytometry. Image cytometry was performed on imprints taken from the biopsies and Feulgen stained to measure the nuclear DNA content of the tumor cells. The staining, internal standardization, and tumor cell selection were based on previously described methods (29). All DNA values were determined in relation to a corresponding staining control, which obtained the value 2c, denoting the diploid DNA content. Our specimens could be divided determined in relation to a corresponding staining control, which obtained the value 2c, denoting the diploid DNA content. Our specimens could be divided
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Immunohistochemistry. Directly after surgery, a part of each tissue sample was fixed in 4% phosphate-buffered formalin and subsequently paraffin embedded. From each specimen, 10 contiguous sections (4 μm) were prepared for immunohistochemistry. All slides were deparaffinized with xylene, rehydrated, and microwave treated at 500 W for 2 min (1/2'H11003). The slides were then incubated for 15 min at room temperature, we inactivated DNase I in a 2.5 mM (final concentration) EDTA solution, followed by heating at 70°C for 10 min. We reverse transcribed 2 μg of total RNA by following the manufacturer's instructions. The final reaction volume of 2 μl contained 1X first strand buffer (50 mM Tris-HCl, 75 mM KCl, and 3 mM MgCl2), 100 ng random hexamers, 0.1 μM DTT, 10 mM deoxynucleotide triphosphate mix (Applied Biosystems or ABI, Warrington, United Kingdom), 200 units of Superscript II RNase H Reverse Transcriptase and diethyl pyrocarbonate-treated water (all other reagents from Invitrogen-Life Technologies, Inc.).

Real-Time qPCR. Real-time qPCR enables the measurement of product accumulation during the exponential phase of PCR when all reagents are still abundant and limiting conditions do not interfere with precise quantitation. The instrument’s optic device detects an increase in fluorescent emission that occurs if Taq polymerase performs a 5′-3′-nucleotidolytic cleavage of the dual-labeled fluorogenic probe, which has to be hybridized to the template. The threshold cycle (Ct) is the fractional cycle number during PCR at which the amount of amplified target in each sample reaches a fixed threshold above background noise (31, 32). The logarithm of initial template copy number results from the measured Ct value in consequence of their negative linear correlation (33).

A validation experiment (34) confirmed comparable amplification efficiencies of the cyclin A, cyclin E, and 18S rRNA amplicons. This is the prerequisite for a relative quantitation without using standard curves. Relative quantitation of cyclin A and E mRNA levels was carried out using the comparative Ct method; 18S rRNA served as an endogenous external control, and the interrun standard was cDNA prepared from MDA-MB-231 cells. All PCR reactions were performed in triplicates on a 7700 Sequence Detector (ABI). From the mean Ct values of the respective triplicates, the mean Ct value of the 18S rRNA amplicon was subtracted to give a ΔCt value for each sample and for each of the two cyclins. The mean Ct value of the interrun standard was then subtracted from the ΔCt values to yield ΔΔCt values for each sample and for each of the two cyclins. The relative amount of target mRNA was calculated as two to the power of -ΔΔCt (34).

We designed primers and probes for the target genes cyclin A and cyclin E using Primer Express 1.0 (ABI). The probes were synthesized by ABI and the primers by Life Technologies, Inc. The amplicon of 18S rRNA was designed and synthesized by ABI. The sequences of primers and probes (all in 5′-3′ direction) were as follows: cyclin A—forward primer, GAGAGC-GAGACGGGTGGTCGA; probe, AGGCTCGGCTTCACCATCATTGTGGA; reverse primer, GAGATTCAGCTGGCTTCTTCTG; cyclin E—forward primer, GAAGACTGGTCTGCTCCTTCTG; probe, CCCACACTGGTCTCTGCGG; reverse primer, TGGTATTTGCTCCAGGCT; 18S rRNA—forward primer, CGGCTACCACATCCAAGAAAAA; probe, TGTCGACCAGACCTT

Fig. 1. Aneuploid (A), diploid (D), and tetraploid (T) DNA histograms. Nuclear DNA content of the cells, given on the horizontal axis, is normalized to the nuclear DNA content of leukocytes (2c denotes diploid DNA content). A, characteristic profile of the genomically stable A subtype with insignificant scattering; B, characteristic profile of the genomically unstable A subtype with significant scattering; C, DNA histogram of a genomically stable B tumor; D, typical profile of a genomically unstable B tumor; E, histogram of a genomically stable T tumor; and F, typical profile of a genomically unstable T tumor.
GCTCC C, reverse primer, GCTGGAATTACCGGGCT. To exclude amplification from genomic DNA, we situate one primer of each amplicon across an exon/exon junction (31) such that up to 6 bases of the 3'-end of the primer corresponded to one of the exons, and the remaining nucleotides corresponded to the adjoining one.

Each PCR comprised 1× TaqMan Buffer A, 6 mM MgCl₂, 200 μM dATP, dCTP, dGTP, and dTTP, and 0.625 units of AmpliTaq Gold DNA polymerase (all reagents ABI) in a final volume of 25 μl. We used the probes for cyclin A and cyclin E at a concentration of 100 nM and the probe for 18S rRNA at a concentration of 75 nM. Cyclin A and cyclin E primers had concentrations of 200 nM, the forward primer of 18S rRNA had a concentration of 50 nM, and the reverse primer a concentration of 40 nM. Each reaction contained 25 ng of cDNA. Thermal cycling conditions were as follows: 10 min at 95°C, followed by 40 cycles of 95°C, 15 s and 62°C, 1 min. Triplicate nontemplate controls were included for the endogenous control and all targets.

Statistical Analysis. The Excel statistics package (Office 98) was used to perform the majority of the statistical calculations, e.g., the unpaired two-tailed Student’s t test and the Pearson correlation coefficient r. Spearman’s rank order correlation was carried out with SPSS software and the logistic regression analysis with the R statistical package.7

RESULTS

Image cytometric measurements of 154 consecutive biopsies of breast adenocarcinomas resulted in 70 aneuploid (A), 61 diploid (D), and 23 tetraploid (T) tumors, as defined by the modal DNA content of cells from each individual cancer (29). The subjective classification of DNA histograms of all three ploidy categories indicated subtypes that differed clearly in their percentage of cells with nonmodal DNA content values (Fig. 1).

To define an objective measure, which allowed the identification of these subtypes, we added up (a) the percentage of cells with DNA content values in the S-phase region (S phase); (b) the percentage of cells with DNA content values exceeding twice the modal value plus 1c (G₂ exceeding rate or G₂ Exc); and (c) the coefficient of variation (CV) of the respective tumor stemline. The sum of these three DNA histogram variables was called the “stemsline scatter index” (SSI), which is a measure of the clonal heterogeneity of the constituent tumor cells. We calculated each of these three values by the Ahrens image program. Logistic regression analysis on all SSI values of the 154 tumor samples showed S phase (z = 3.5, P = 5.3 × 10⁻⁴; Fig. 2) as contributing most to the discriminative strength of the SSI, followed by CV and G₂ Exc (z = 3.0, P = 0.003 and z = 2.1, P = 0.03, respectively; Fig. 2). None of the three summands was found as being sufficiently selective on its own. Using logistic regression, we could also determine the cut point of SSI = 8.8% (P = 0.03) for discrimination of all tumors showing significantly scattered DNA histograms (SSI > 8.8%) from those with insignificantly scattered ones (SSI ≤ 8.8%), independent of their cytophotometrically assessed modal DNA content values. Breast cancers with an SSI > 8.8% were termed gu, and those with an SSI ≤ 8.8% were termed gs. The cut point and the discriminative strength of the three summands of SSI have been validated on a data set of 890 breast cancer cases.8

To evaluate whether the mRNAs for cyclin A and E are differentially expressed in the two subtypes of each of the A, D, and T ploidy categories, we performed real-time qPCR on the 154 biopsies from primary breast carcinomas studied above on four biopsies from benign tumors and three biopsies from normal breast tissue. Of the 154 biopsies, 104 gave evaluable results. In the case of the remaining tumors, either the Cₚ value of 18S rRNA exceeded 23 (a sign of RNA degradation), or the Cₚ values of the cyclins were higher than 36, both of which render unreliable results (35). As a measure of intra-assay reproducibility, the CVs of triplicate reactions for cyclins A and E for each sample were on an average 2.2 and 1.9%, respectively. Interassay reproducibility, as demonstrated by the interrun standard CV, was 4.7% for cyclin A and 4.9% for cyclin E.

The two aneuploid lesion subtypes could not only be characterized according to DNA histogram and SSI value but also demonstrated statistically highly significant differences in cyclin A and E expression levels between them, according to the Student’s t test (Table 1). The transcript level of cyclin A was found to be four times higher in Ags cases than in Dgs cases. Measuring the amount of cyclin E transcripts, we observed a 3-fold difference between the two types of aneuploid carcinomas (Fig. 3). On the other hand, the expression of these cyclins did not significantly vary in Dgs versus Dgs tumors (P = 0.5, cyclin A; P = 0.9, cyclin E). The Dgu and Dgu lesions also produced comparable expression levels for both cyclins, whereas transcript levels differed significantly in Dgs versus Dgu breast cancers (Table 1, Fig. 3). In the case of T lesions (Tgs and Tgu), cyclin A mRNA, but not cyclin E mRNA, gave a significant difference between the ploidy subtypes (Table 1). The mRNA expression of the two cyclins was ~10–15 times lower in normal breast tissue and benign lesions than in Dgs tumors (data not shown). The best linear correlation between the mRNA levels of cyclin A and E were obtained from Dgs carcinomas (r = 0.76, P = 1 × 10⁻⁴; Fig. 4), followed by Dgs lesions (r = 0.755, P = 2 × 10⁻⁴; Fig. 4). In Dgs tumors, the coefficient of correlation was 0.44, with P = 0.009, and in Dgu lesions, we did not measure any linear relationship between the mRNA expression of these cyclins (r = 0.05, P = 0.9). At the protein level, as determined by immunohistochemistry for cyclin A, we found significant differences in the same tumor categories that differed significantly from each other at the mRNA level (Table 1). There was a good linear correlation between protein and mRNA expression of cyclin A in

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7 Internet address: http://www.r-project.org.
these two cyclins. A E mRNA values, whereas 31% showed low mRNA levels for each of
ses and/or disease-related death) appeared to have high cyclin A and
2% of the patients died and 4% had distant metastases.

In a pilot study, we examined three fibroadenomas, three Dgs and three
Ags breast carcinomas and four Agu breast tumors for potential centro-
some defects. We chose these samples at random from the 104 breast
carcinomas that yielded evaluable real-time qPCR data. The tumor sec-
tions were immunostained with an antibody against γ-tubulin, a well-
characterized marker of the centrosome (36) localized within the centri-
oles and the pericentriolar region (37). The aberrations observed included
single centrosomes of increased size (diameters larger than twice that of

Table 1 Results of the $t$ test between the different ploidy categories

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>n</th>
<th>Cyclin A mRNA, $P$</th>
<th>Cyclin E mRNA, $P$</th>
<th>Tested categories</th>
<th>n</th>
<th>Cyclin A protein, $P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dgs$^a$</td>
<td>24</td>
<td>$5.0 \times 10^{-4}$</td>
<td>0.01</td>
<td>Dgs/Agu</td>
<td>23</td>
<td>$4 \times 10^{-3}$</td>
</tr>
<tr>
<td>Dpg$^a$</td>
<td>12</td>
<td>0.04</td>
<td>0.02</td>
<td>Dgs/Dgu</td>
<td>9</td>
<td>0.02</td>
</tr>
<tr>
<td>A$^a$</td>
<td>19</td>
<td>0.001</td>
<td>0.007</td>
<td>Ags/Agu</td>
<td>17</td>
<td>$2 \times 10^{-4}$</td>
</tr>
<tr>
<td>Agu</td>
<td>34</td>
<td>0.5</td>
<td>0.9</td>
<td>Ags/Dgs</td>
<td>29</td>
<td>0.6</td>
</tr>
<tr>
<td>T$^a$</td>
<td>6</td>
<td>0.04</td>
<td>0.1</td>
<td>Tgs/Tgu</td>
<td>6</td>
<td>0.05</td>
</tr>
<tr>
<td>Tgu</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Type I tumors with a diploid (D) stemline.

$^b$ Genomically stable (gs) tumor.

$^c$ Genomically unstable (gu) tumor.

$^d$ Type IV tumors with an aneuploid (A) stemline.

$^e$ Type II tumors with a tetraploid (T) stemline.

Agu lesions ($r = 0.85$, $P = 1.0 \times 10^{-4}$; Fig. 5), whereas this was not
observed in Dgu and Tgu lesions ($r = 0.16$ and 0.1, respectively), nor in
the stable ploidy subtypes (Ags, Dgs, and Tgs; $r = 0.02$, 0.25, and 0.05,
respectively).

We also examined the clinicopathological data of this cohort of 104
women to observe how this related to the grouping of patients into those
having a tumor containing cells with unstable genomes and an
SSI $> 8.8%$ and those having a tumor containing cells with stable
genomes and an SSI $\leq 8.8%$. The two groups did not differ significantly
from each other concerning the clinical stage, nor was there any differ-
ence in the percentage of positive lymph nodes between them. The
majority of the tumors were ductal (Table 2). Our follow-up data showed
76% of the patients who developed distant metastases and/or died as
possessing low differentiated carcinomas. During the follow-up period
of 2–3 years (average follow-up interval was 2 years and 5 months) in
the group with an SSI $> 8.8%$, 25.6% of the patients died of breast cancer
and 18.6% evolved distant metastases. In the group with an SSI $\leq 8.8%$,
2% of the patients died and 4% had distant metastases.

Fifty-four percent of all patients with progression (distant metastases
and/or disease-related death) appeared to have high cyclin A and
E mRNA values, whereas 31% showed low mRNA levels for each of
these two cyclins. A $\chi^2$ test proved this difference to be significant
($P = 0.03$). The median cyclin A and E mRNA expression levels
served as cutoff values and were 0.46 and 0.28, respectively. Cyclin
A protein expression was high (>5% positive cells, general directions
of the Department of Oncology-Pathology, Karolinska Hospital) in
62% of all patients with progression; in the remaining 38%, it was low
(5%). This difference was not significant ($P = 0.32$).

In addition, we did not observe any relation between cyclin A and
E mRNA overexpression and tumor size ($r = 0.02$, $P = 0.8$ and
$r = -0.2$, $P = 0.1$, respectively), suggesting that overexpression of
these cyclins may be an early event in cancer development.

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Fig. 3. Cyclin A and E mRNA expression in breast carcinomas of different ploidy
categories. Box plots describe the relative cyclin A and E mRNA expression levels, the
values of which are logarithmic. The cyclin mRNA expression level for each tumor is
normalized to its respective 18S rRNA expression level. The algorithm for relative
quantitation results in unitless values. The detection range of the instrument in our hands
is five orders of magnitude. The ploidy categories are as follows: genomically stable
aneuploid (Ags); genomically unstable aneuploid (Agu); genomically stable diploid
(Dgs); and genomically unstable diploid (Dgu).

Fig. 4. Linear correlation between the amount of cyclin A and E mRNAs in genomi-
cally stable diploid (Dgs) carcinomas and in genomically stable aneuploid (Ags) carci-
nomas. Pearson’s correlation between cyclin A and E mRNA levels normalized to 18S
rRNA levels, measured in each of the Dgs and Ags tumors ($r = 0.76$, $P = 1 \times 10^{-4}$;
$r = 0.755$, $P = 2 \times 10^{-4}$, respectively).

Fig. 5. Linear correlation between the levels of cyclin A protein and cyclin A mRNA
in genomically unstable aneuploid (Agu) tumors. Pearson’s correlation between the
percentage of cells with positive nuclear staining for cyclin A protein and relative cyclin
A mRNA expression in Agu tumors ($r = 0.85$, $P = 1.0 \times 10^{-5}$).
Table 2: Patient age and breast tumor characteristics

<table>
<thead>
<tr>
<th>Age (yrs)</th>
<th>63.8 (36–91)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological type</td>
<td>Ductal 87 (83.6%)</td>
</tr>
<tr>
<td>Lobular 8 (7.7%)</td>
<td></td>
</tr>
<tr>
<td>Others 9 (8.7%)</td>
<td></td>
</tr>
<tr>
<td>Tumor stagea</td>
<td>I: 34 (32.7%)</td>
</tr>
<tr>
<td>II: 56 (53.8%)</td>
<td></td>
</tr>
<tr>
<td>III: 11 (10.6%)</td>
<td></td>
</tr>
<tr>
<td>Not determined 3 (2.9%)</td>
<td></td>
</tr>
<tr>
<td>Tumor sizea</td>
<td>T1: 45 (43.3%)</td>
</tr>
<tr>
<td>T2: 54 (51.9%)</td>
<td></td>
</tr>
<tr>
<td>T3: 2 (1.9%)</td>
<td></td>
</tr>
<tr>
<td>Not determined 3 (2.9%)</td>
<td></td>
</tr>
<tr>
<td>Nodal statusa</td>
<td>N0: 76 (73.1%)</td>
</tr>
<tr>
<td>N1: 28 (26.9%)</td>
<td></td>
</tr>
<tr>
<td>Grading</td>
<td>Elston grade 1a: 10 (9.6%)</td>
</tr>
<tr>
<td>Elston grade 2: 31 (29.8%)</td>
<td></td>
</tr>
<tr>
<td>Elston grade 3: 54 (51.9%)</td>
<td></td>
</tr>
<tr>
<td>Not determined 9 (8.7%)</td>
<td></td>
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</tbody>
</table>


DISCUSSION

Image cytometrical measurement of diploid, tetraploid, and aneuploid primary breast adenocarcinomas resulted in two different subtypes of DNA histograms for each of the three ploidy categories. The histograms were clearly dissimilar in their percentage of cells with nonmodal DNA content values (Fig. 1), manifesting the clonal heterogeneity that is characteristic of cancer (27). By calculating the SSI as an objective measure of the nonmodal part of DNA content values or scattering of DNA histograms and then using a cut point of SSI values above this cut point, it appears to be of minor importance for clinical malignancy grade where the tumor’s stemline is situated but very critical how pronounced the scattering of the tumor’s DNA histogram proves to be as indicator of genomic instability. As described above, stable aneuploid and diploid cancer types show similarities in cyclin E and A expression and also in the degree of centrosome aberration. However, the representation of such tumors in the breast cancer population does deviate. In a data set derived from 890 consecutive primary breast adenocarcinomas, we measured 15% of the aneuploid cases as having SSI values ≤ 8.8%; among the diploid cancers, 62% had a SSI ≤ 8.8% (manuscript in preparation).8

We found a linear correlation between the mRNA levels of the cyclins and the extent of centrosomal aberrations. This is consistent with the proposal that cyclins A and E are involved in centrosome duplication (16, 18). However, this correlation has to be verified with a larger set of samples. There was no relation between the overexpression of cyclin A and E mRNAs and tumor size, which may indicate that disturbance of the expression of these cyclins is an early event in carcinogenesis. Aneuploidy and chromosomal instability are also early events in tumor development, as reviewed in Lengauer et al. (27), and furthermore, centrosome aberrations are detectable even in

Table 3: Centrosome defects in breast tumors of different ploidy categories

<table>
<thead>
<tr>
<th>Cyclin A mRNA</th>
<th>Cyclin E mRNA</th>
<th>Ploidy</th>
<th>Centrosome defects (%)</th>
<th>Aberrant mitotic spindles</th>
<th>Mean centrosome defects (%)</th>
<th>SD centrosome defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.003</td>
<td>0.008</td>
<td>D</td>
<td>0.4</td>
<td>0.4</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>0.001</td>
<td>0.003</td>
<td>D</td>
<td>0.5</td>
<td>–</td>
<td>–</td>
<td></td>
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<tr>
<td>0.001</td>
<td>0.006</td>
<td>D</td>
<td>0.2</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>0.19</td>
<td>0.05</td>
<td>D</td>
<td>1.4</td>
<td>2.6</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>0.06</td>
<td>0.02</td>
<td>Dgs</td>
<td>2.6</td>
<td>–</td>
<td>–</td>
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</tr>
<tr>
<td>0.05</td>
<td>0.53</td>
<td>Dgs</td>
<td>3.7</td>
<td>–</td>
<td>–</td>
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</tr>
<tr>
<td>0.13</td>
<td>0.04</td>
<td>Ags</td>
<td>1.8</td>
<td>2.4</td>
<td>0.6</td>
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<tr>
<td>0.09</td>
<td>0.03</td>
<td>Ags</td>
<td>2.9</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>0.12</td>
<td>0.11</td>
<td>Ags</td>
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<td>Agu</td>
<td>9.9</td>
<td>+</td>
<td>9.6</td>
<td>2.2</td>
</tr>
<tr>
<td>6.96</td>
<td>5.42</td>
<td>Agu</td>
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<td>+</td>
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</tr>
<tr>
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<td>Ags</td>
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<td>–</td>
<td>–</td>
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</tr>
<tr>
<td>8.51</td>
<td>1.34</td>
<td>Ags</td>
<td>7.3</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

a Cyclin A mRNA expression relative to the 18S rRNA expression in the same tumor (unitless).
b Cyclin E mRNA expression relative to the 18S rRNA expression in the same tumor (unitless).
c Centrosome defects: centrosomes with increased size and centrosome clusters were counted and divided by the number of investigated cells.
d Fibroadenoma, benign lesion with a diploid (D) stemline.
e Type I tumors with a diploid (D) stemline.
\( ^{f} \) Genomically stable (gs) tumors.
\( ^{g} \) Type IV tumors with an aneuploid (A) stemline.
\( ^{h} \) Genomically unstable (gu) tumors.

8.8% determined the stable aneuploid subtype of breast carcinomas, aberrant mitotic spindles could be observed, whereas this was not seen in any of the other cases (Table 3). Our results showed a positive linear correlation between the extent of centrosomal aberrations and the mRNA levels of both cyclin E and cyclin A (\( r = 0.83, P = 0.01 \)).

The two groups of aneuploid tumors. In the gus aneuploid subtype, the centrosome aberrations is diagnostic of genomic instability, then the measurement of these indicators should lead to different results in the two groups of aneuploid tumors. In the gu aneuploid subtype, the amounts of cyclin A and E transcripts were significantly higher than those in the gs aneuploid lesions. Furthermore, expression levels of these two mRNAs were not found to be significantly different in gs aneuploid and diploid tumors. The same was true for these expression levels in gu aneuploid and diploid lesions (Table 1, Fig. 3). Cyclin A protein expression was significantly up-regulated in all unstable ploidy subtypes, compared with the gs subtypes (Table 1).

We also investigated the presence of centrosomal aberrations in some of the ploidy subtypes and measured by far the most defects, in an average of 9.6% of cells, in gu aneuploid tumors. In accordance with the cyclin expression measurements, stable aneuploid and diploid cancers demonstrated comparable and much lower ranges of centrosomal aberrations. On average, 2.4% and 2.6% of the tumor cells, respectively, possessed centrosomal abnormalities (Table 3). Our results suggest greater affinity between tumors with comparable SSI values than between those of the same ploidy category.

A follow-up period of 2–3 years revealed a much better prognosis for patients with tumors having an SSI ≤ 8.8% than for patients with tumors having SSI values above this cut point. It appears to be of minor importance for clinical malignancy grade where the tumor’s stemline is situated but very critical how pronounced the scattering of the tumor’s DNA histogram proves to be as indicator of genomic instability. As described above, stable aneuploid and diploid cancer types show similarities in cyclin E and A expression and also in the degree of centrosome aberration. However, the representation of such tumors in the breast cancer population does deviate. In a data set derived from 890 consecutive primary breast adenocarcinomas, we measured 15% of the aneuploid cases as having SSI values ≤ 8.8%; among the diploid cancers, 62% had a SSI ≤ 8.8% (manuscript in preparation).8

We found a linear correlation between the mRNA levels of the cyclins and the extent of centrosomal aberrations. This is consistent with the proposal that cyclins A and E are involved in centrosome duplication (16, 18). However, this correlation has to be verified with a larger set of samples. There was no relation between the overexpression of cyclin A and E mRNAs and tumor size, which may indicate that disturbance of the expression of these cyclins is an early event in carcinogenesis. Aneuploidy and chromosomal instability are also early events in tumor development, as reviewed in Lengauer et al. (27), and furthermore, centrosome aberrations are detectable even in

Table 3: Centrosome defects in breast tumors of different ploidy categories

<table>
<thead>
<tr>
<th>Cyclin A mRNA</th>
<th>Cyclin E mRNA</th>
<th>Ploidy</th>
<th>Centrosome defects (%)</th>
<th>Aberrant mitotic spindles</th>
<th>Mean centrosome defects (%)</th>
<th>SD centrosome defects</th>
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<td>Ags</td>
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Aneuploidy with Low Degree of Malignancy

noninvasive breast cancers (23). This coincidence might emphasize connections between the above mentioned cellular biological events. Both Duesberg et al. (14) and Lingle et al. (23) have postulated the existence of stable aneuploid tumors, as characterized by fluorescence in situ hybridization. Duesberg et al. (14) found in cell lines a direct relation between genomic instability and the degree of aneuploidy that predicts relatively stable aneuploidy, with its stemline closely situated to the diploid or tetraploid stemline. These aneuploid cases were supposed not to involve chromosomes encoding mitosis-regulating genes. In contrast, we measured stable aneuploidy far from the diploid or tetraploid stemline peak. Aneuploid breast tumors with, e.g., a modal DNA content value of 3.2c, showed SSI values in the range of gs diploid and tetraploid tumors and proved to have a good prognosis. On the other hand, we detected gs diploid and tetraploid cancers with SSI values in the range of unstable aneuploid ones and an unfavorable prognosis. Lingle et al. (23) also investigated primary breast tumors and, according to their centrosome observations, could distinguish between stable and unstable aneuploid lesions. The unstable specimens exhibited significantly increased centrosome size and number in comparison to stable ones. This is consistent with our measurements where one criterion, in addition to others, to distinguish between gs and unstable aneuploid cancers has been the presence of differing extents of centrosome abnormalities.

We did not confirm yet whether the gs subtypes of the different ploidy categories remain stable with time. However, we have evidence from a 9-year follow-up study showing that the stable subtypes maintain a significantly better prognosis than the unstable ones (manuscript in preparation). Furthermore, there are studies concerning stable DNA ploidies of breast adenocarcinomas, which have been studied during the history of the disease. Modal DNA content values have been found to be stable in the primary tumor and its corresponding lymph node and distant metastases, occurring many years later (42, 43).

Until now, the occurrence of stable and unstable subtypes of ploidy categories has only been observed in breast cancer. It would be interesting to investigate the existence of this phenomenon in other malignancies.

In conclusion, we here describe a new classification of DNA ploidy profiles, which appears to be more relevant for estimation of the clinical malignancy potential of breast carcinomas than the traditional one. Aneuploid cancers are generally considered to be more dangerous and aggressive than their diploid counterparts. However, ~15% of aneuploid tumors have a malignancy potential comparable with that found in gs diploid cancers. The findings reported herein may provide support for approaches aimed at improving the prognostic value of ploidy analysis in breast adenocarcinomas.

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Improved Grading of Breast Adenocarcinomas Based on Genomic Instability

Ulrike Kronenwett, Sören Huwendiek, Carin Östring, et al.

Cancer Res 2004;64:904-909.

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