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FLOW CYTOMETRIC DNA INDEX AND S-PHASE FRACTION IN BREAST CANCER IN RELATION TO OTHER PROGNOSTIC VARIABLES AND TO CLINICAL OUTCOME

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One frequently used classification of flow cytometric DNA ploidy status (diploid versus nondiploid) was compared with a division into seven ploidy classes based on DNA index (DI) and number of cell populations (hypodiploid, diploid, near-hyperdiploid, hyperdiploid, tetraploid, hypertetraploid, and multiploid). The latter ploidy classification showed a better correlation with prognosis and other prognostic factors (i.e., lymph node involvement, estrogen and progesterone receptor status, and S-phase fraction). The improvement in correlation was mainly due to the identification of near-hyperdiploid cases (DI 1.00–1.14) which could be combined with the diploid cases to form a group with favourable prognosis. In contrast to cases with a small increase in DNA content (near-hyperdiploid), those with a small decrease of DNA content (hypodiploid) manifested a more aggressive disease. In multivariate analysis, S-phase fraction (SPF) was a more important prognostic factor than both the improved or the conventional ploidy classification.

Key words: Breast cancer, flow cytometry, DNA-index, ploidy, S-phase, interphase, proliferation, prognosis.

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The proliferative activity of tumor cells as assessed with the thymidine labeling technique (1–4), and the DNA content of individual tumor cells as determined by static (image) cytometry (5, 6), have been shown to yield significant prognostic information in primary breast cancer. Flow cytometric measurement of nuclear DNA content simultaneously provides information about DNA ploidy and proliferative activity as represented by the fraction of cells in S-phase (SPF). Many investigators have demonstrated independent prognostic value of DNA ploidy (7–10), and of SPF (11–17), while others have failed to confirm such value of these variables (18–20).

Various ploidy classification systems based on flow cytometric DNA analysis have been used in breast cancer. In a frequently used approach, proposed by Hiddemann et al. (21) samples with one cell population are defined as diploid and samples with ≥ 2 cell populations as non-diploid a classification that has been shown to yield prognostic information (17). Diploidy may also be defined on the basis of a DNA index (DI) of around 1.00 (22). Of the non-diploid categories, tetraploid samples can with advantage be combined with diploid samples to form a euploid group; according to this classification system the remaining non-diploid cases constitute an aneuploid group. Tetraploidy is defined on the basis of DI, the fraction of nuclei in the 4C peak and the presence of a peak in the octaploid region, representing G_2-nuclei of the tetraploid cell population. A more detailed subgrouping based on the DNA

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index and the number of cell populations has also been reported (23, 24): hypodiploid, diploid, hyperdiploid, tetraploid, hyper-tetraploid and multiploid tumors. An association between the occurrence of multiple cell populations and the prognosis has been reported (25). Toikkanen et al. (14) have demonstrated that patients with non-diploid breast cancers with a DI below 1.20 have a good prognosis and could therefore be grouped together with strictly diploid cases.

The aim of the present investigation was to evaluate which ploidy classification yields the best prognostic information, by relating the results of DNA analysis to the clinical outcome and to other prognostic factors. Special attention was paid to the hypodiploid cases. In multivariate analysis, the prognostic importance of different DNA ploidy classification systems and of SPF was compared.

Material and Methods

Patients

The series comprised 1 831 breast cancer samples, 1 266 from postmenopausal and 565 from premenopausal patients. The mean age of the patients was 61 ± 14 years (range 18–97). Information about tumor size was available in 1 297 cases (71%): 341 (26%) <20 mm; 859 (66%) 20–49 mm; 97 (7%) ≥50 mm. Histopathologic axillary lymph node status was known for 1 271 (69%) patients; 513 (40%) were node negative; 448 (35%) had 1–3; 225 (18%) had 4–9; and 85 (7%) had 10 or more positive nodes. With a median follow-up time of 34 months, information of recurrence was available for 1 298 patients (71%): 940 (72%) had no sign of recurrence; 322 (25%) developed distant recurrences; while 36 (3%) had loco-regional recurrences only. Of the 1 831 patients, 1 280 (70%) were still alive after a median duration of follow-up of 39 months, 269 (15%) had died of breast cancer, 72 (4%) had died of intercurrent disease, and 210 (11%) had died but no information as to the cause of death was available.

Flow cytometric DNA analysis

Preparation and staining. The samples were prepared for FCM DNA analysis in a one-step procedure previously described (26, 27), with slight modifications as outlined in the following. Briefly, tumor tissue (100–200 mg) was thawed in 100–200 μl of citrate buffer (sucrose 250 mmol/l, trisodium citrate 40 mmol/l, dimethylsulfoxide 5%, pH 7.6) containing chicken and trout red blood cells (CRBC and TRBC, 10^6/ml). To enhance cell elution, the tissue was mechanically disintegrated with two forceps, after which 1–2 ml of a nuclear isolation medium (NIM) containing propidium iodide (PI) was added (50 μg PI/ml, SIGMA P-5264; RNAse 0.1 mg/ml, SIGMA R-5125; Nonidet P 40 0.6% (v/v; SIGMA N-3516) in isotonic buffered saline, GIBCO). The samples were filtered (140 μm) and incubated in the dark for 10 min at room temperature, and then kept at +4°C until required for flow cytometric analysis, which was performed within one hour in an Ortho cytofluorograph 50 H as previously described (28).

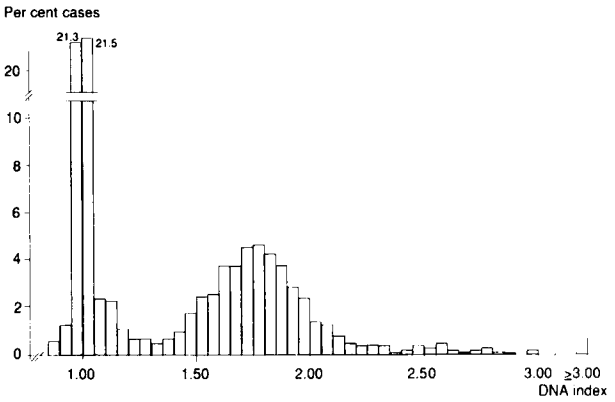

Fig. 1. Distribution of DNA index values (n = 1 786).

Calculation of DNA index (DI). After zero point adjustment of the DNA histogram using the modal values of the CRBC and TRBC G_0/G_1 peaks (29), the mean channel numbers of all G_0/G_1 peaks were adjusted and used for the calculation of the DNA index with TRBC as reference standard. The overall DI distribution (all samples, Fig. 1), is obtained by dividing the DI of the main non-diploid cell population by that of the diploid cell population, hypodiploid samples having a DI below 1.00, and hyperdiploid samples a DI above 1.00. In the present study all hypodiploid samples had a DI below 0.95, and all hyperdiploid samples a DI ≥ 1.05. Diploid samples (one cell population) had a DI, with TRBC as reference standard, between 0.95 and 1.05.

Definition of ploidy status

Diploid/non-diploid. Samples with one G_0/G_1 peak were considered diploid, and those with two or more G_0/G_1 peaks non-diploid (21). The mean coefficient of variation (CV-value) for the diploid G_0/G_1 peak of 603 consecutive breast cancer samples at our laboratory was 3.2 ± 1.0 (30). If a bimodality of the main peak in the 2C region was visible (i.e., the derivative of the smoothed peak function changed from plus to minus or vice versa a second time along the main peak curvature), it was considered to represent a second (near-diploid) cell population. In cases with more than two cell populations, the non-diploid population with the greatest number of nuclei was considered to be the main cell population.

Euploid versus aneuploid. Among the non-diploid samples, tetraploid ones can be combined with diploid ones to form a euploid group, the remaining non-diploid cases being classified as aneuploid. The DI range of the tetraploid G_0/G_1 region was based on the mean quotient of

G_2/G_0G_1 (mean channel numbers) for 100 consecutive diploid breast cancer samples, as measured with FCM, and found to be 1.92–2.04. If the number of nuclei in the tetraploid region (4C) exceeded 20% of the total diploid DNA distribution, the samples were classified as tetraploid, provided a G_2 peak was visible in the octaploid region (22).

Calculation of SPF

The SPF was estimated planimetrically (31) assuming that the S-phase compartment constituted a rectangular distribution between the modal values of the G_0/G_1 and G_2 peaks. In cases of bimodality in the 2C region and where the DI for the non-diploid cell populations was below approximately 1.3, a combined SPF value was calculated. SPF was calculated exclusively in the non-diploid stemline when DI exceeded 1.3, and if the corresponding G_2 peaks were distinctly separated. SPF was calculated in the most prominent non-diploid stemline in cases with two or more non-diploid peaks. Although no correction was made for background debris, SPF was not calculated when background debris predominated in the SPF region(s) of the histogram. SPF was not calculated if the corresponding G_2 peak in the histogram could not be identified, or when the non-diploid stemline was small ($G_0/G_1 < 10\%$ of the total number of observations).

ER and PgR analysis

ER and PgR were measured with two different techniques, ER content with isoelectric focusing in polyacrylamide gels (IF) and enzyme immunoassay (EIA), and PgR content with the dextran coated charcoal method with Scatchard analysis (DCC) and EIA (32–34). In a comparison of previous results obtained with different ER and PgR assays in the same breast cancer samples (33, 34), we found inter-assay agreement to be highly significant for both ER content ($r_s = 0.98$, $n = 127$) and for PgR content ($r_s = 0.88$, $n = 97$), though somewhat higher values were obtained with EIA than with IF or DCC. Thus, the cut-off values adopted for defining receptor positivity had to be adjusted according to the different measuring techniques. Samples with ER and PgR concentration values of $\geqslant 10$ fmol/mg protein, obtained with IF and DCC, were classified as positive, and samples with values below this level as negative (17). The corresponding cut-off level for EIA was 25 fmol/mg protein. The receptor data, covering a period of 10 years and including about 4 000 samples from 15 different hospitals have shown satisfactory stability (35).

Gene analysis

Gene amplification was analyzed with the Southern blot and slot blot techniques, as described previously (36). Filters were sequentially hybridized with probes for the erbB2, int2 and c-myc genes, and with probes for control markers located on the same chromosomal arm as the respective proto-oncogenes (i.e. for erbB2 at 17q-myeloperoxidase, for int2 at 11q-progesterone receptor, and for myc at 8q-mos). The degree of amplification was evaluated with densitometric analysis of slot blot autoradiograms, by comparing the signals from each proto-oncogene and control gene. In the present study, tumors were classified as having a single or an amplified ($\geqslant 2$) gene copy number of the haploid genome.

Analysis of cathepsin D

Cathepsin D was analyzed with a radio immuno assay (CIS, kit instructions). The cathepsin D content was expressed in pmol/mg protein, which was determined according to Lowry et al. (37).

Statistics

The existence of correlations between different factors was checked with Spearman's rank correlation test (the coefficient being denoted as r_s). Life-table analysis with Lee-Desu statistics was used to compare recurrence-free survival (38). Multivariate analyses were performed with Cox's proportional hazards model (39), the covariate being entered in a stepwise fashion, and a p-value of 0.15 being adopted as the limit for the inclusion of a covariate. The RR denotes the relative risk for each covariate ultimately entered into the analysis.

Results

DNA-index and ploidy classes

The number of cell populations was evaluated in 1 821 of the 1 831 samples, the remaining being impossible to evaluate owing to extensive background debris or low cellularity. Regarding the calculation of DNA index, a further 53 samples had to be excluded since the tumor samples had not been frozen for delivery to the laboratory. The DI of the diploid cell population in such samples was higher compared to those arriving properly frozen (1.11 ± 0.10 versus 1.00 ± 0.02).

The distribution of DNA indices (Fig. 1) was bimodal, with peaks at DI = 0.95–1.05 (diploid) and at 1.60–1.90. As shown in Figs 2 and 3 the percentages of recurrence and death were low for diploid cases (one cell population) and for cases with the DI of the non-diploid population between 1.05 and 1.14 (near-hyperdiploid region). Patients with tetraploid samples had a similar low rate of recurrence and death as the diploid cases.

On the basis of the results shown in Figs 2 and 3, seven ploidy classes were defined as listed in Table 1, which also

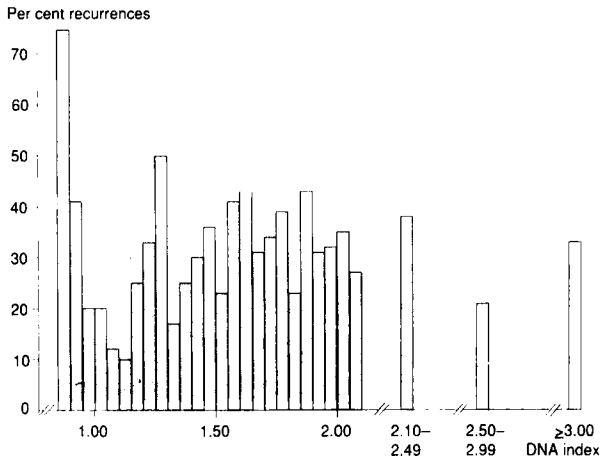

Fig. 2. Percentage of recurrences in relation to DNA index (n = 1 267).

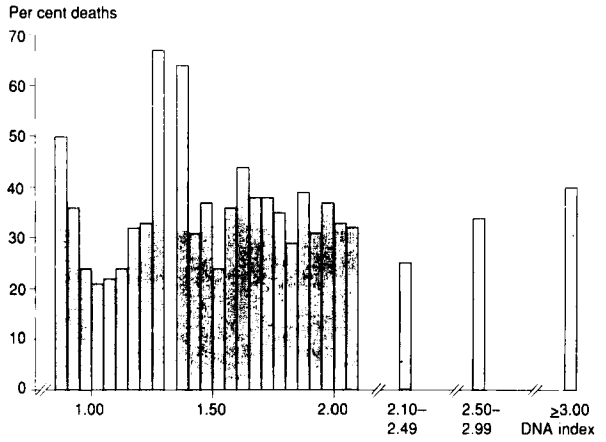

Fig. 3. Percentage of deaths in relation to DNA index (n = 1 786).

shows the distribution by ploidy classes. The median SPF value was lower in diploid and near-hyperdiploid samples than in the other five ploidy classes, as was the rate of recurrences and deaths and the proportion of ER and PgR negative samples (Table 2). The small hypodiploid group (1.8% of the total number of cases) was characterized by aggressive disease manifesting in the lowest percentage of ER and PgR positivity, the largest median tumor size, the highest percentages of recurrence and death, and c-myc amplification (Tables 2 and 3).

Of the seven ploidy classes, those with the most abnormal DNA content (hypertetraploid and multiploid) had the highest proportion of erbB2 amplification and the lowest proportion of int2 amplification (Table 3), which suggests that the presence of erbB2 amplification identifies one category of breast cancer and that of int2 amplification another one. Amplification of c-myc was less frequent among diploid, near-hyperdiploid or tetraploid samples than in the other ploidy classes.

The findings mentioned above suggest that the prognosis in patients with near-hyperdiploid samples may be similar to that in patients with diploid samples. This conclusion derives further support from the life table analysis (Fig. 4), that shows a good prognosis for both these groups of patients. The prognosis was even somewhat better in the near-hyperdiploid group than in the diploid group ($p = 0.076$). In these two groups of patients the recurrence-free survival rate was significantly higher than in the tetraploid cases or in the remaining aneuploid cases (Fig. 4). Patients with hypodiploid samples had a tendency toward poorer prognosis than those with tetraploid, hyperdiploid, hypertetraploid or multiploid samples. The prognosis was no poorer in multiploid cases than in the other non-diploid groups (except near-hyperdiploid).

S-phase fraction

SPF was estimated in 1 674 (91%) of the 1 831 samples. The remaining samples either had extensive debris, contained very small non-diploid cell populations, or were multiploid.

The SPF values showed a significant positive correlation with the number of lymph nodes involved, tumor size, DNA ploidy, cathepsin D content, erbB2 and c-myc amplification and a significant negative correlation with age

Table 1

Characterization of the seven ploidy classes with regard to number of cell populations and DNA-index of the main cell population*

Ploidy class	Number of cell populations	DNA-index of the main cell population	Number of cases (%)
Hypodiploid	2	<0.95	32 (1.8%)
Diploid	1	0.95–1.04	719 (40.4%)
Near-hyperdiploid	2	1.05–1.14	79 (4.4%)
Hyperdiploid	2	1.15–1.91	637 (35.8%)
Tetraploid	2	1.92–2.04	88 (4.9%)
Hyper-tetraploid	2	≥2.05	124 (7.0%)
Multiploid	3–4		101 (5.7%)

* For the calculation of DI see Material and Methods.

Table 2

Relationship between the seven ploidy classes and other prognostic variables in breast cancer

Ploidy class[2]	Percentages						Median value		
	ER +	PgR +	NO	N +	Rec.	Deaths	SPF %	Cath[1]	Size mm
Hypodiploid	50	35	40	60	48	41	8.3	54	30
Diploid	71	61	47	53	20	23	4.4	43	23
Near-hyperdip.	83	60	30	70	11	23	4.8	46	23
Hyperdiploid	60	49	39	61	34	36	13	54	25
Tetraploid	71	66	31	69	32	32	10	70	22
Hyper-tetraploid	53	41	41	59	27	31	12	48	25
Multiploid	66	56	36	64	26	33	13	52	25
Total number in each column	1 736	1 687	509	733	1 267	1 786	1 643	248	1 266

[1] Cathepsin D content expressed in pmol/mg protein.
[2] The totals for each ploidy class are shown in Table 1.

Table 3

Relationship between the seven ploidy classes and oncogene amplification in breast cancer

Ploidy class[1]	Percentages of amplified samples		
	erbB2 (b = 522)	int2 (n = 523)	c-myc (n = 309)
Hypodiploid	0	10	14
Diploid	14	10	6
Near-hyperdiploid	9	15	5
Hyperdiploid	22	11	10
Tetraploid	25	11	6
Hyper-tetraploid	30	9	10
Multiploid	30	5	17

[1] The totals for each ploidy class are shown in Table 1.

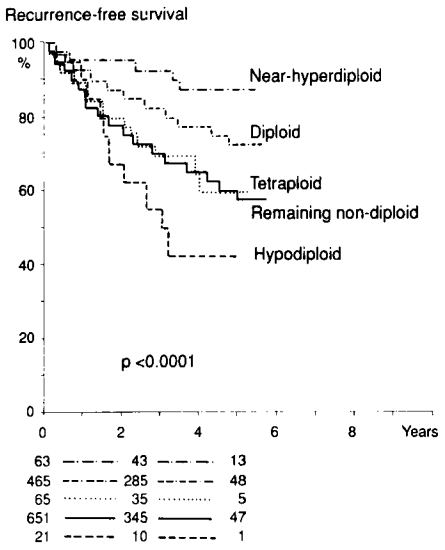

Fig. 4. Recurrence-free survival in relation to five ploidy classes.

Table 4

Correlation between SPF and other prognostic factors (Spearman's rank correlation coefficient, r_s)

Factor	r_s	p-value	number
Lymph node metastases	0.16	<0.001	1 184
Size	0.19	<0.001	1 206
Age	−0.12	<0.001	1 674
Menopause	0.00	0.50	1 786
ER	−0.31	<0.001	1 605
PgR	−0.29	<0.001	1 557
Ploidy status[1]	0.62	<0.001	1 821
Ploidy classes[2]	0.59	<0.001	1 786
erbB2 ampl.	0.27	<0.001	482
c-myc ampl.	0.15	0.004	287
int2 ampl.	0.02	0.30	483
Cathepsin D	0.18	0.003	235

[1] diploid vs. non-diploid
[2] seven ploidy classes

and ER- and PgR content. The SPF values showed no significant overall correlation with menopausal status or int2 amplification (Table 4), however, when diploid and near-hyperdiploid samples were examined separately, a significant positive correlation was found between SPF values and int2 amplification ($r = 0.18$, $p = 0.005$, $n = 212$). For the remaining non-diploid samples, no significant correlation was found.

For the purposes of prognosis, we have previously shown that the most valuable information can be obtained by dividing the series into three groups by SPF values (<7.0%; 7.0–11.9%, ⩾12, (3)). When the same SPF categories were applied on the present data a similar separation with regard to recurrence-free survival was obtained (Fig. 5).

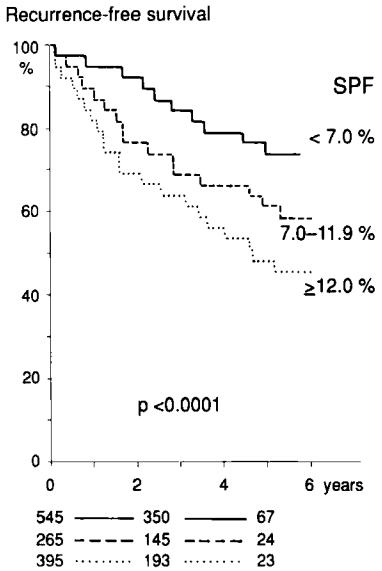

Fig. 5. Recurrence-free survival in relation to S-phase fraction.

Multivariate analysis

In multivariate analysis, lymph node status, tumor size, SPF, PgR and menopausal status were found to be independent prognostic variables vis-à-vis recurrence-free survival, whereas ploidy (both definitions) and ER status were not (Table 5). If SPF was excluded from the multivariate analysis and both classifications for ploidy status were included the modified classification for ploidy status showed the lowest p-values ($p < 0.001$ and $p = 0.020$ respectively).

Discussion

Patients with non-diploid tumors with a DI of up to 1.14 (near-hyperdiploid) had a prognosis similar to those with diploid tumors. These two groups were consequently combined into a low-risk group, the remaining patients with non-diploid tumors being classified as a high-risk group. A better separation was thereby obtained than when the division into diploid (1 cell population) and non-diploid (≥ 2 cell populations) was used. Similar results have previously been obtained by Toikkanen et al. (14) in a study comprising 115 patients with a long clinical follow-up (22 years), where the upper cut-off level for DI in the near-hyperdiploid group was 1.20. In another study, the near-hyperdiploid region was found to be between 1.00 and 1.40 (40). DI was also a better prognostic factor than DNA ploidy status in a study on ovarian carcinoma (41). In agreement with some previous reports (e.g. (42)), but in disagreement with some others (22–24, 43, 44), we did not find a lower recurrence rate among patients with tetraploid samples. This discrepancy may perhaps be explained by

Table 5

Recurrence-free survival, according to uni- and multi-variate analysis (Cox's proportional hazard model, n = 1 064) of prognostic covariates

Covariate	Univariate	Multivariate		
	p-value	p-value	RR[1]	95% confidence interval
Axillary lymph nodes 0, 1–3, 4+	<0.0001	<0.001	2.0	1.8–2.4
Tumor size ≤20 mm vs. >20 mm	<0.0001	0.001	1.6	1.2–2.1
PgR status <10 vs. ≥10 fmol/mg protein	<0.0001	<0.001	1.7	1.3–2.2
ER status <10 vs. ≥10 fmol/mg protein	<0.0001	NS	–	–
S-phase fraction <7.0% vs. 7.0–11.9% vs ≥12%	<0.0001	<0.001	1.4	1.2–1.6
Ploidy status diploid vs. non-diploid	0.0001	NS[2]	–	–
Modified ploidy status diploid + near-hyperdiploid vs. remaining non-diploid	<0.0001	NS[2]	–	–
Menopause pre- vs. post-menopausal	0.0198	0.008	1.4	1.1–1.8

[1] RR = relative risk

[2] When the S-phase fraction was excluded from the multivariate analysis, both ploidy classifications entered the model; modified ploidy status ($p < 0.001$) and ploidy status ($p = 0.020$).

differences in the definition of tetraploidy, or in the composition and/or size of the material. Published results also disagree with regard to prognosis of multiploid tumors. Like some investigators (12, 40, 43), but in contrast to others (23–25), we did not find this prognosis to be poorer than for other non-diploid tumors. One reason for this discrepancy may be the fact that the proportion of multiploid tumors varies from 2.8% to 21% in published studies (12, 24, 25, 42, 44, 45).

In the present study, particular attention was paid to hypodiploid cases, in which the disease was found to be more aggressive than in the other ploidy groups. A similar finding has been indicated by others (23). In this context, it should be borne in mind that, when using paraffin-embedded material, no samples are classifiable as hypodiploid, as by definition the first G_0/G_1 peak appearing in the DNA histogram is considered to be diploid. However, combining near-hyperdiploid cases (DI = 1.00–1.14) with diploid cases to a low-risk group does not seem to have any major effect on the prognostic strength of DNA ploidy when evaluated in a multivariate analysis, as the modified classification was not found to be an independent prognostic factor. As in previous studies by our group (17, 30), the SPF value was found to be an independent prognostic factor and showed also significant correlations with several other prognostic factors.

As indicated above, the modified ploidy classification system was of limited prognostic value—at least when information about SPF was available. Of greater interest is the question why a small decrease of the DNA content (hypodiploid) results in a bad prognosis, whereas a small increase of the DNA content (near-hyperdiploid) results in a comparatively good prognosis. Besides differences in clinical correlations between these two groups, they also differed in the correlations with other prognostic factors such as tumour size, ER, PgR status and SPF.

In an attempt to explain at a molecular level the diverse behavior of different ploidy subtypes of breast cancer, alterations both in proto-oncogenes and tumor suppressor genes must be taken into consideration. Amplification of proto-oncogenes is a frequent finding in breast cancer which, particularly in the case of erbB2, has been found to be associated with aggressive tumor types (36, 46, 47). Gene amplification is cytogenetically visible as homogeneously staining regions (HSRs) and double minutes (DMs) (48). Although amplified DNA may significantly contribute to an abnormal cell DNA content, and an aneuploid DNA pattern as measured with flow cytometry, the origin of the highly aberrant hyperdiploid cells must be sought in other mechanisms such as polysomia. In fact, gene amplification may be a consequence of aneuploidy, caused by replication errors in the instable genome characterizing these cells. Conversely, proto-oncogenes, acting as growth promotors, may have a causative effect on the appearance of aneuploidy. For instance, erbB2 activation is seen in the very initial stages of breast cancer development, being almost universal in the rapidly growing ductal carcinomas in situ of comedo type (49). Increased proliferation may result in a higher rate of mitotic errors such as non-disjunction, polysomia and aneuploidy. Indeed, in the present study we found a lower prevalence of both erbB2 and c-myc amplification in diploid/near-hyperdiploid tumors, than in hyperdiploid or multiclonal tumors, possibly reflecting the less aggressive behavior of the former types. However, int2 amplification did not follow this pattern and has in fact been shown to occur in conjunction with a more differentiated phenotype, albeit also with poor prognosis (50).

The aggressive behavior of hypodiploid tumors is noteworthy in the light of the mechanism described for tumor suppressor gene inactivation (51). These genes, also termed anti-oncogenes, are thought to be inactivated by a two-step process, where the first and phenotypically silent event is usually a point mutation or a small deletion affecting one allele of the gene. The second event, which strikes the remaining allele and completely abolished gene function, can be a larger deletion or a total chromosomal loss. Several tumor suppressor genes may be of importance in breast cancer development, including the p53 gene at chromosome 17p, the retinoblastoma (Rb) gene at 13q, as well as still unknown genes at chromosomes 1, 3p and 11p (52). Loss of one or more chromosomes, as a consequence of tumor suppressor gene inactivation, may result in a hypodiploid DNA pattern, if not balanced by duplication of remaining chromosomes. Conversely, a gain of single chromosomes would not have such a dramatic effect on cellular behavior by analogy with the present finding that near-hyperdiploid tumors are less aggressive.

Our preliminary results (data not shown) indicate loss of an Rb allele and possibly one chromosome 13 in a hypodiploid breast cancer. However, allelic loss of Rb, as well as of regions on 17p and 1p, are also frequently seen in diploid tumors, suggesting that minor deletions not detectable in the quantitative DNA flow cytometry histogram (or by karyotyping) are common (Borg et al., in preparation). The hypodiploid tumors are nonetheless a potentially important subgroup for study in attempting to elucidate the molecular mechanisms underlying breast cancer development and progression.

To sum up, near-hyperdiploid breast cancers have manifested a less aggressive pattern, both with regard to clinical outcome and the relationship with other prognostic factors. This group should consequently be combined with diploid cases in a low-risk group. In contrast, samples with a small decrease in DNA (hypodiploid) manifested several characteristics for a poor prognosis. When information about SPF was available, however, this modified ploidy classification did not reach statistical significance for recurrence-free survival in a multivariate analysis.

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