SPANDIDOS PUBLICATIONS

Hypoploidy defines patients with poor prognosis in breast cancer

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Abstract. The clinicopathological features currently used in breast cancer prognosis often fail to characterize the clinical heterogeneity of the disease accurately. Our study is aimed to investigate the predictive value of DNA flow cytometry in breast cancer. Previously untreated breast carcinoma samples (584) were snap frozen for flow-cytometry. Tumors were classified into three DNA index (DI) categories: i) tumors showing a DI =0.96-1.15 (diploid and near-diploid); ii) tumors with a DI ≥1.16 (hyperdiploid, tetraploid, multiploid and/or those with more than one diploid population); and iii) tumors with a DI ≤0.95 (hypoploid). The 5- and 10-year cumulative survival rates ± SE for Group I (n=191) were 98±1% and 98±1%. For Group II (n=361) these rates were 77±2% at 5 years and 63±5% at 10 years. In Group III (n=32) the rate at 5 years was 23±8%, with no patients alive at 10 years (p<0.0001). In univariate analysis, tumor size, node status, grade, karyometry, S-phase fraction, MIB-1 index, and estrogen receptors retained prognostic significance; in multivariate analysis, only DI ≤0.95 (hypoploid) was retained as an independent prognostic factor for overall survival. Our data strongly support that DNA hypoploid has a strong, independent prognostic value for predicting the short-term clinical outcome of breast carcinoma patients.

Introduction

Cancer of the breast is one of the most common human neoplasms, accounting for approximately one quarter of all cancers in females (1). Breast carcinomas exhibit a wide range of morphological phenotypes and specific histopathological types have particular prognostic or clinical characteristics (1,2). The clinicopathological features currently used in breast cancer prognosis often fail to characterize the clinical heterogeneity of the disease accurately, particularly with respect to predicting tumor behavior in the individual case (1,2). In recent years, therefore, in several areas, including analytical cytology, immunocytochemistry, and molecular biology, attempts have been made to identify features that could be clinically useful in assessing prognosis (3-7).

The predictive role of DNA flow cytometry in patients with breast carcinoma has been investigated in many studies (8-12). In some it was concluded that DNA ploidy and S-phase fraction (SPF) were useful in predicting clinical outcome, with SPF, particularly, often claimed as having independent prognostic value (9-13). Other studies, however, have shown that flow cytometry data analysis provides no additional prognostic information (14). It is generally accepted that most of the controversy over these results stems from the different methods and criteria used in the various studies (15). Apart from intratumor heterogeneity (15,16), confounding factors include patient selection bias, differences in treatment, insufficient numbers of patients, differences in the type of sample used (fresh versus paraffin embedded), the tissue processing procedures, and the criteria used for interpreting the histograms (14,15,17).

Our aim was to investigate the predictive value of DNA flow cytometry in breast cancer, applying well-recognized software for DNA histogram interpretation. We evaluate the correlation between DNA content (ploidy), S-phase fraction and other established clinicopathological prognostic factors in a series of carcinomas using fresh/frozen material. We analyzed the impact of this information on disease-free survival in the short-term and on overall survival after primary surgical treatment to identify a group of patients who might have a significantly worse prognosis.

Materials and methods

Clinicopathological data. The study involved 584 women with operable breast cancer diagnosed and treated between April 1991 and December 2000 at Clinical University Hospital (Santiago de Compostela, Spain). These patients were treated in our institution according to standard protocols. The mean age of the patients was 59 years (range, 25-85 years). Median follow-up time was 102 months (range, 48-156 months). Informed consent was obtained for this study, which was conducted according to the Spanish law including adherence to the Helsinki Principles of 1975, as revised in 1983.

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Table I. Clinicopathological, karyometric, and immunohistochemical characteristics of a series of primary breast carcinomas in relation to ploidy (univariate analysis).

Groups Ploidy Number of patients	Group I Diploid and near-diploid n=191	Group II Aneuploid[a] n=361	Group III Hypoploid n=32	p value
Age (years)	60.0±12.8	57.7±14.0	58.1±13.4	NS[b]
Hormonal status				NS
Pre-menopausal	38 (19.9%)	110 (30.5%)	9 (28.1%)	
Peri-menopausal	25 (13.1%)	32 (8.9%)	2 (6.3%)	
Menopausal	128 (67%)	219 (60.7%)	21 (65.6%)	
FHBC[c]	26 (13.8%)	65 (18.1%)	5 (15.6%)	NS
Tumor size (cm)	2.7±1.5	3.5±2.1	6.6±5.9	0.000
Histological type				NS
IDC[d]	152 (79.6%)	309 (85.6%)	27 (84.4%)	
DCIS[e]	11 (5.8%)	6 (1.7%)	0	
ILC[f]	15 (7.9%)	30 (8.3%)	5 (8.6%)	
IMC[g]	5 (2.6%)	11 (3%)	0	
Other	8 (4.2%)	5 (1.4%)	0	
Axillary lymph node metastases	69 (37.9%)	196 (56.2%)	25 (78.1%)	0.000
Microscopic grade				0.003
Grade I	45 (29.6%)	59 (19.2%)	3 (11.5%)	
Grade II	89 (58.6%)	170 (55.4%)	15 (57.7%)	
Grade III	18 (11.8%)	78 (25.4%)	8 (30.8%)	
Nuclear area	77 (±13)	114 (±49)	91 (±37)	0.000
Perimeter	61 (±5)	73 (±15)	65 (±12)	0.000
Spherical	65 (±11)	56 (±15)	60 (±12)	0.000
Oval	29 (±7)	30 (±7)	29 (±8)	NS
Cylindrical	5 (±8)	12 (±13)	9 (±7)	0.000
Percentage S-phase (%)	5.5±3.5	8.5±5.9	10.1±6.3	0.000
S-phase fraction				
Low	115 (60.2%)	120 (33.2%)	5 (15.6%)	0.000
Moderate	57 (29.8%)	136 (37.7%)	15 (46.9%)	
High	19 (9.9%)	105 (29.1%)	12 (37.5%)	
MIB-1				
Low	51 (40.2%)	63 (24.6%)	5 (26.3%)	0.008
Moderate	52 (40.9%)	107 (41.8%)	7 (36.8%)	
High	24 (18.9%)	86 (36.6%)	7 (36.8%)	
Estrogen receptors				
Negative	26 (16.0%)	93 (28.2%)	8 (26.7%)	0.012
Positive	136 (84.0%)	237 (71.8%)	22 (73.3%)	
Progesterone receptors				
Negative	55 (35.9%)	124 (40.9%)	11 (55.0%)	0.220
Positive	98 (64.1%)	179 (59.1%)	9 (45.0%)	
P53				
Negative	81 (62.8%)	163 (61.3%)	11 (55.0%)	0.798
Positive	48 (37.2%)	103 (38.7%)	9 (45.0%)	

[a]Aneuploid: hyperdiploid, tetraploid, multiploid and two diploid populations; [b]NS: not significant; [c]FHBC: familial history positive for breast cancer; [d]IDC: invasive ductal carcinoma; [e]DCIS: ductal carcinoma *in situ*; [f]ILC: invasive lobular carcinoma; [g]IMC: invasive medullary carcinoma. Quantitative data are expressed as mean ± SD.

ry tumor, 4-μm histological sections were cut and with hematoxylin and eosin for histopathological examination according to the criteria of the World Health Organization (1). Histological grading was evaluated using the Nottingham modification of the Bloom-Richardson system (18). All relevant clinicopathological characteristics are summarized in Table I. Immunohistochemical analysis on paraffin-embedded material was performed using a universal second antibody kit that used a peroxidase-conjugated labeled-dextran polymer (EnVision®, Peroxidase/DAB; Dako, Glostrup, Denmark), with antibodies for estrogen receptor (clone 6F11, dilution 1:10, microwave oven; Novocastra, Newcastle-upon-Tyne, UK), progesterone receptor (clone PgR 636, dilution 1:50, water bath; Dako), MIB-1 (clone Ki-67, dilution 1:100, water bath; Dako), and p53 (clone DO7, dilution 1:20, water bath; Novocastra). Negative and positive controls were concurrently run for all antibodies with satisfactory results. Cells were considered immunopositive when diffuse or dot-like nuclear staining was observed regardless of the intensity of the staining; only nuclear immunoreactivity was considered specific. The number of positive cells was counted by two different observers independently (EC-U and JC-T). Whenever necessary, a consensus was reached using a double-headed microscope. In accordance with the percentage of positive cells for MIB-1, the proliferation index was considered low (≤17%), moderate (18-34%), and high (≥35%).

Flow cytometry. Flow cytometry analysis was performed on fresh material from specimens obtained at the time of surgery as previously reported (19). Tissue samples were selected by a pathologist and were stored at -80˚C. On average the tissue samples examined measured 0.5x0.4x0.2 cm. The frozen tissue samples were disaggregated in buffer solution (FACSFlow, Becton Dickinson, Franklin Lakes, NJ, USA). The resulting cell suspension was passed through a nylon filter with 30-μm meshes and centrifuged for 10 min at 1500 rpm. The supernatant was aspirated and discarded, and the pellet was resuspended in buffer solution (FACSFlow, Becton Dickinson) 1 ml and passed through 30-μm meshes. The concentration of cells in the resulting suspension was estimated by microscopic examination of a 20-μl sample stained with trypan blue; the bulk of the suspension was brought to a concentration of $1x10^6$ cells/ml, and a 1-ml sample was taken and treated with 100 μl of 1 mg/ml RNA-ase (Sigma, R-5503, St. Louis, MO, USA) and 50 μl of propidium iodide 400 μg/ml (Sigma, P-4170) as a fluorescent marker. After 45 min in the dark at room temperature, the sample was examined by FACSCalibur Flow Cytometer® (Becton Dickinson).

DNA histograms were analyzed by the cytometer software (ModFit LT® for Mac V3.0, Topsham, ME), which automatically suppressed background caused by cell debris and calculated the percentages of G_0/G_1-, G_2M- and S-phase cells in each cell population. Patients with samples whose DNA histogram had a diploid G_0/G_1 peak with a coefficient of variation >5% were excluded from the study (this occurred exclusively at the start of the study, and is attributed to lack of experience for preparing the samples). The tumor samples were classified into three categories in relation to DNA index (DI): Group I diploid (DI =0.96-1.05), and near-diploid (DI =1.06-1.15); Group II hyperdiploid (DI =1.16-1.75), tetraploid (DI =1.76-2.10), multiploid (DI = two or more aneuploid peaks), and two diploid populations; and Group III hypoploid (DI ≤0.95).

Karyometry. Karyometry was carried out at a magnification of x400 using a light microscope with an eyepiece equipped with a micrometer grid. Details of procedures have been described in a previous publication (19). For each sample, the maximum (D_{max}) and minimum (D_{min}) diameters of the nuclei of 100 cells were measured and their cytonuclear areas were calculated as D_{max} x D_{min} x (π/4). On the basis of their mean cytonuclear area, samples were classified as normokaryotic (area <100 μm^2), magnokaryotic (area 100-140 μm^2), or megalokaryotic (area >140 μm^2). The perimeter and shape of these tumor cells were also evaluated.

Database and statistics. All statistical analyses were performed with the Statistical Package for the Social Sciences (version 10; SPSS® Inc., Chicago, IL). Curves for DFS were calculated according to the Kaplan and Meier method, and differences between curves were assessed with the log-rank test for censored data on survival. Cox's regression model was used to evaluate the predictive power of prognostic factors in the multivariate analysis. Continuous variables are expressed as mean ± SD.

Results

Clinicopathological data. The clinicopathological data are summarized in Table I. After histological re-evaluation the study included 584 invasive carcinomas: 488 (88.1%) ductal type, 50 (9%) lobular type, 16 (2.8%) medullary type, and 13 invasive carcinomas of a miscellanous group that included 2 (0.36%) tubular carcinomas, 7 (1.3%) mucinous carcinomas and 4 (0.72%) papillary carcinomas, as well as 17 *in situ* ductal carcinomas.

DNA analysis. Table I shows the correlations between the three groups classified according to the DNA index and the different tumor features. Of the 584 breast tumors analyzed by flow cytometry, 191 were in Group I [107 (18.3%) diploid and 84 (14.4%) near-diploid], 361 in Group II [134 (22.9%) hyperdiploid, 124 tetraploid, 48 (8.2%) multiploid and 55 (9.4%) with two diploid populations], and 32 (5.5%) in Group III (hypoploid) (Fig. 1). A greater size (on average) was found in the group of hypoploid tumors (6.6±5.9 cm) in contrast with Groups I (2.7±1.5 cm) and II (3.5±2.1 cm). In Group III (hypoploid) all 32 cases were invasive carcinomas (84.4% invasive ductal type and 8.6% invasive lobular type), without *in situ* carcinomas in this group. A significantly higher number of patients (78.1%) with axillary lymph node metastases were also found in the hypoploid group ($p<0.0001$).

Overall, more than half of the tumors were histologically classified as grade II; however, the hypoploid group included a higher percentage of grade III tumors, and there were only 3 grade I neoplasms. Regarding karyometric parameters, the nuclear area and the perimeter were higher in the aneuploid group ($p<0.0001$).

In relation to proliferative activity, the fraction (and percentage) of S phase as well as the percentage of immuno-

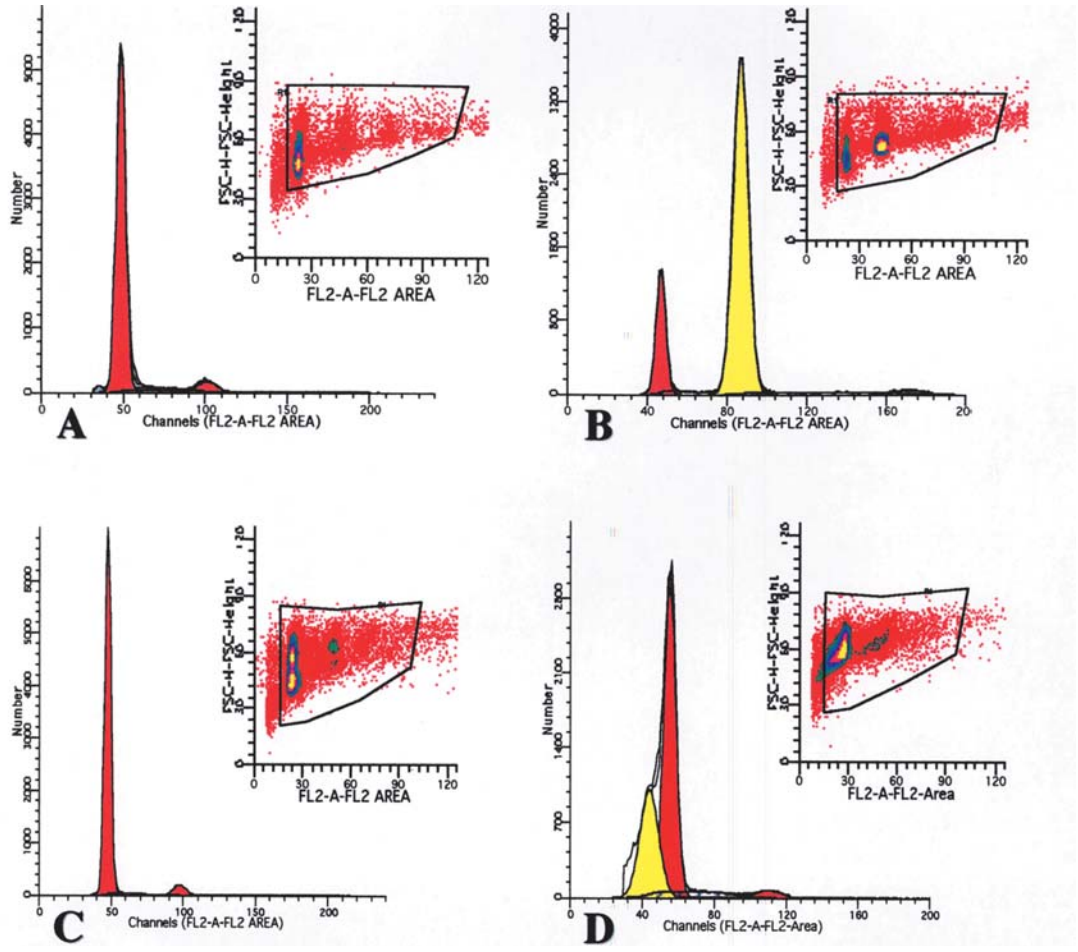

Figure 1. Flow cytometric DNA. A, diploid histogram (Group I); B, tetraploid histogram (Group II); C, two diploid population histogram (Group II); and D, hypoploid histogram (Group III).

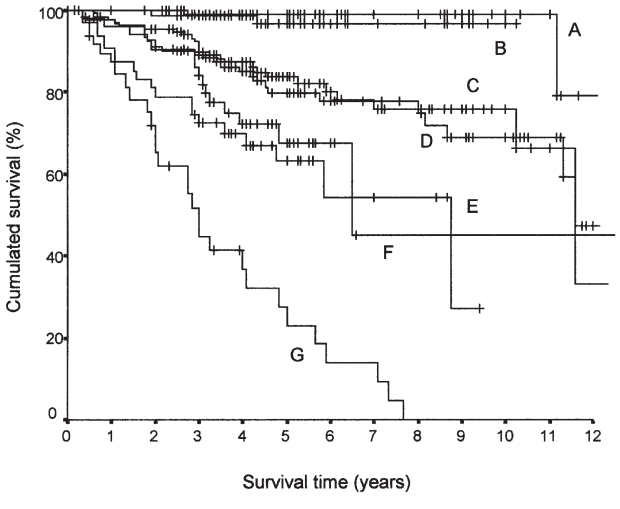

Figure 2. Analysis of disease-free survival according to ploidy (Kaplan and Meier plots). A, diploid; B, near-diploid; C, tetraploid; D, hyperdiploid; E, multiploid; F, two diploid populations; and G, hypoploid.

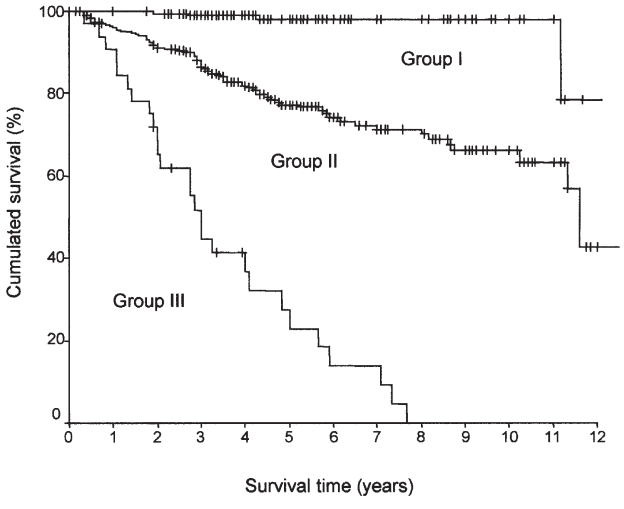

Figure 3. Analysis of disease-free survival according to ploidy (Kaplan and Meier plots). Group I, diploid and near-diploid; Group II, aneuploid; and Group III, hypoploid.

expression of MIB-1 were higher in the hypoploid group ($p<0.0001$). An association between estrogen receptor expression and Group I was also found ($p=0.01$).

Survival analysis. Median follow-up time was 102 months (range, 48-156 months). The 5- and 10-year cumulative survival rate ± SE for Group I (n=191) was 98±1% and 98±1% respectively. For Group II (n=361) the cumulative survival rate was 77±2% at 5 years and 63±5% at 10 years. In Group III (n=32) a cumulative survival rate at 5 years of 23±8% was found with no patients alive at 10 years ($p<0.0001$) (Figs. 2 and 3). In univariate analysis, tumor size, node status, grade,

ry, S-phase fraction, MIB-1 index, and estrogen retained prognostic significance, whereas in the multivariate analysis, only DI ≤0.95 (hypoploid) was retained as having independent prognostic significance in relation with overall survival.

Discussion

Prognosis of breast carcinoma is related to a large variety of clinical and pathological factors (1,2). The overall issue of DNA index, or ploidy, as a marker for breast cancer has been controversial for a number of years. The aims of the present study were to ascertain the prognostic impact of ploidy in breast cancer and its relation to other classic clinicopathological prognosis factors.

The recommendations of the 1993 DNA Cytometry Consensus Conference (14) have appeared to be insufficient to ensure interlaboratory reproducibility. For these reasons Duigou *et al* (17) and others (20,21) established more standardized procedures using frozen tissue samples to minimize fluctuations in measurements when using different cytometers and software. However in a more recent consensus under the auspices of the College of American Pathologists published in 2000, a multidisciplinary group of clinicians, pathologists, and statisticians considered that DNA ploidy analysis is a factor which has still not been studied sufficiently to demonstrate its prognostic value (3). Although the same authors concluded that neither DNA index nor DNA ploidy status achieves independent prognostic significance, they considered that distinguishing hypoploid tumors from near-diploid and hyperploid tumors correlates with different clinical outcomes (3). Michels *et al* (9) and Wenger and Clark (22), however, after reviewing literature restricted to papers involving more than 100 patients and using fresh or frozen samples, demonstrated a relation between DNA ploidy and prognosis in 17 studies, in half of them after multivariate analysis.

The use of fresh/frozen tumor tissue in our study minimized the background, aggregates and debris, made fixation unnecessary, and resulted in sufficient histogram data. We showed that DNA hypoploid has a strong, independent prognostic value for predicting the short-term clinical outcome of breast carcinoma patients. In the study of Fernö *et al* (23), hypoploid aneuploidy was associated with the worst clinical outcome of all types of aneuploidy, even after adjustment of other prognostic factors. In the literature, the incidence of hypoploid tumors ranged from 0 to 7%, with most studies agreeing on a value between 2 and 2.5% (9,22-29). We found 32 (5.5%) cases of hypoploid tumors, which is within the range of previously reported values. In agreement with our results, all other studies (22,25-28) focusing on hypoploid tumors, with the exception of that of Michel *et al* (9), found that these tumors were correlated with a worse prognosis. In the study of Michel *et al* (9) the tumors with one hypoploid peak were found to have a better prognosis than diploid tumors; however, tumors that were both hypoploid and multiploid had a significantly worse prognosis.

In our series, the 584 frozen tissue samples were classified in three categories in relation to DNA index. This classification in three groups was based on a previous evaluation of the relationship between survival and DNA content. We observed that the diploid and near-diploid group showed a very good prognosis in clear contrast to the hypoploid tumor group in which there were no survivors after 10 years. The tetraploid, hyperdiploid, multiploid and two diploid population tumor group had an intermediate outcome. Interestingly, to the best of our knowledge, the category of tumors with two diploid populations had not previously been reported in the literature. The two diploid populations are detected when the sample passes through the cytometer but they are represented in the histogram as a single diploid peak. The consideration of these tumors as aneuploid as opposed to *bona fide* single diploid tumors was supported by survival analysis. In addition, we cannot exclude the possibility that these two diploid population tumors with an intermediate prognosis could include near-diploid or hypoploid cell populations that are not detected by the software of the flow cytometer.

In the present study, DNA hypoploid was shown to be the most important and independently significant prognostic factor in relation to overall survival. The group of hypoploid tumors also correlated with greater tumor size, axillary lymph node metastases, higher histological grade and higher proliferative activity (fraction of S phase and/or percentage of MIB-1 immunoexpression), factors which are all well recognized as associated with a worse prognosis (2-4). A rough correlation was found in our study between fraction of S phase and percentage of MIB-1.

In conclusion, when using generalized guidelines for flow cytometric DNA measurements (3,15,17,21), and classifying tumors in the three DNA index categories that we have designated in this paper, DNA ploidy is a good tool for establishing a prognosis for breast cancer. DNA hypoploid, more specifically, has strong, independent prognostic value for predicting the short-term clinical outcome of breast carcinoma patients. Loss of chromosome 4 and amplification of the cyclin D1 oncogene were defined as characteristic aberrations in hypoploid tumors (30), indicating that histologically indistinguishable ductal invasive breast carcinomas consist of several distinct entities (such as hypoploid tumors) that can be defined by modern molecular techniques (comparative genomic hybridization and fluorescence *in situ* hybridization) (30,31). Our paper strongly supports the usefulness of DNA flow cytometry as a technique that is faster and simpler than other more recently developed molecular techniques in the selection of patients in clinical oncology.

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