Virchows Archiv B Cell Pathol (1991) 61:133–140

Virchows Archiv B
Cell Pathology
Including Molecular Pathology

Cytogenetics, flow cytometry, cytophotometry and morphometry of 22 cases of primary breast carcinoma

A comparative study

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Received March 14 / Accepted June 13, 1991

Summary. Cytogenetic, flow cytometric, cytophotometric and morphometric analyses were performed on 22 previously untreated, primary solid breast carcinomas. Although the cell nuclei as the primary object of these studies were the same in all the tumors, distinct features were evaluated in each case to determine to what degree the results obtained by these techniques are comparable. From the cytogenetic viewpoint, six tumors had a modal number in the diploid range, seven were in the triploid range, and two in the tetraploid range; seven tumors had no modal number. These data correlate with the flow cytometry and cytophotometry results obtained, with DNA values slightly higher than their respective chromosomal modes. However, no correspondence between chromosomal modes and mean nuclear area was found. Chromosomal markers have been identified that particulary affect chromosomes 1 (p11, q21-qter), 11 and 16, although no common markers existed in all cases. Cytogenetics is the most sensitive technique, but the low yield (22 out of 140 tumors assayed) considerably restricts its value in any prospective breast cancer study.

Key words: Breast carcinoma – Cytogenetics – Cytophotometry – Flow cytometry – Morphometry – Scanning image cytometry

Introduction

As breast cancer is the most common malignant neoplasm in women, several approaches have been made to delve into the biology of the malignantly transformed epithelial cell regarding histological grade, clinical stage and prognosis (Black et al. 1975; Fisher et al. 1983, 1984; Schnitt et al. 1984).

Chromosomal changes are considered to play an important role in malignant transformation, but technical problems and the extensive complicated alterations in most solid malignant tumors have for many years hampered the identification of specific changes in human cancers. Only recently have non-random alterations been reported in a number of solid neoplasms (Sandberg 1990), but information on chromosome abnormalities in untreated, primary solid mammary tumors remains scarce and no specific changes have been found. Nevertheless, several abnormalities in chromosomes no. 1 (Kovacs 1981; Rodgers et al. 1984; Gebhart et al. 1986; Hill et al. 1987; Dutrillaux et al. 1990), no. 11 (Dutrillaux et al. 1990; Ferti-Passantonopoulou and Panani 1987) and no. 16 (Mark 1975; Rodgers et al. 1984) have been identified.

Moreover, numerous studies have been performed in the search for prognostic information and in the classification of tumors based on various fundamental biologic properties such as DNA content and the morphometrical analysis of cells (Baak et al. 1985; Fallenius et al. 1988; Christov et al. 1989; Merkel and McGuire 1990). A comparison of these results with classical chromosome analysis is, however, rare (Petersen and Friedrich 1986; Tribukait et al. 1986; Remvikos et al. 1988).

We report the cytogenetic, flow cytometric and cytophotometric DNA results in 22 human solid breast carcinomas. Karyotype analyses performed using G-banded chromosomes were compared with DNA histograms and morphometric values.

Material and methods

Patients. Samples of 22 mammary carcinomas were obtained at surgery. This group was obtained from a total of 140 primary breast carcinomas which were processed consecutively for cytogenetic analysis. The diagnosis of carcinoma was made in each patient by frozen section. None of the 22 patients had been treated by radiotherapy or adjuvant chemotherapy before surgery. Table 1 summarizes the ages, histological diagnoses and histological grades. A post-surgical clinical staging was performed (PTNM). Any extranodal extension of the carcinoma was recorded in each

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patient (R+) because this has been shown to have prognostic significance (Nobby et al. 1977; Lelle et al. 1987). All patients were treated with adjuvant chemotherapy and some were irradiated after surgery. Survival is indicated in months.

Table 1. Clinical findings

Case	Age	Histology	Grading	PTNM	DFI (months)
1	63	ID	II	pT2N1iiiM1	74
2	38	M	II	pT2NoMo	91
3	50	ID	II	pT2N1Mo	31
4	52	P	II	pT2N1Mo	35
5	41	ID	II	pT2NoMo	69
6	65	ID	I	pT4N1Mo	72
7	54	ID	II	pT2NoMo	39
8	52	ID	II	pT3NoM1	Exitus
9	59	M	III	pT2NoMo	65
10	60	ID	II	pT2N2M1	76
11	40	ID	II	pT2N1Mo	70
12	34	ID	II	pT2N1Mo	71
13	51	ID	II	pT2N1Mo	77
14	62	ID	II	pT2N1iiiMo	70
15	74	ID	III	pT4N1iiiMo	63
16	72	ID	II	pT1NoMo	85
17	43	ID	II	pT2N1Mo	89
18	45	ID	II	pT2N1iiiM1	Exitus
19	55	M	III	pT2N1Mo	97
20	59	M	III	pT2NoMo	84
21	67	ID	II	pT2N1Mo	84
22	75	ID	II	pT2N1iiiMo	74

ID: Ductal infiltrating carcinoma. *M*: Medullar carcinoma. *P*: Papillary carcinoma. *iii*: Capsular invasion=R+. *DFI*: Disease free interval

Histology. Tumors obtained at surgery were histologically diagnosed by frozen section and typified after paraffin-embedding, sectioning and HE staining, according to the WHO classification for breast diseases (Hartmann et al. 1982). Histological grading of the tumors was made in accordance with Bloom and Richardson (1957).

Cytogenetic analysis. The fresh tumor pieces were fine-minced under sterile conditions in RPMI 1640 medium and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 0.2% gentamycin). A cell suspension was obtained after mechanical disaggregation. The tumor cells were resuspended in RPMI 1640 supplemented by 15% FBS, 1% antibiotics and 1% L-Glutamin and cultured for 6–48 h. The culture was exposed to Colcemid (0.2 µg/ml for 2 h at 37° C. This was followed by hypotonic treatment in 0.075 M KCl for 20 min at 37° C. The cells were fixed several times in freshly prepared methanol-acetic acid (3:1) and slides were then air-dried. G-staining bandings were performed according to the Seabright (1971) method. Karyotypes were named according to the ISCN (1985).

Flow cytometry (FCM). This analysis was performed using paraffin-embedded material. A cell suspension was prepared from two 50 µm sections. The sections were dewaxed using two changes of xylene for 10 min at room temperature and then rehydrated in a sequence of 100, 95, 70 and 50% ethanol for 10 min each at room temperature. The tissue was finely minced with a scalpel and resuspended in 2 ml of pepsin (Sigma) at pH 1.5. The tubes were placed in a waterbath at 37° C for 40–60 min with intermittent vortex mixing, and the samples were then filtered through 60 µm nylon gauze. The suspension was centrifuged twice and the pellet resuspended in the staining solution (50 µg/ml propidium iodide) (Sigma). Before analyzing the sample, RNAse (1 mg/ml) was added incubating for 30 min at 37° C. The samples were measured with a Profile (Coulter) Flow Cytometer and analyzed using Multycycle (Autofit version 2.10) software. Tumor populations were considered to be aneuploid if more than one G0/G1 peak was present.

Table 2. Quantitative nuclear determinations

Case	Cytogenetic results				DI (FCM)	DI (CM)	AREA (μ^2)
	Total cells counted	46,XX	Range	Mode			
1	32	–	32–74	36	1.0	1.1	35.7
2	66	6	32–46	41	1.0	1.1	38.4
3	8	–	42–43	42	1.0	0.9	52.3
4	16	–	33–57	46	1.0	1.1	78.3
5	23	14	46–78	48	1.2	1.1	38.6
6	20	–	49	49	1.0	1.2	22.4
7	9	4	46–65	58	1.9	1.8	43.9
8	16	–	39–109	59	1.7	1.6	63.1
9	12	9	46–90	60	1.4	1.3	53.9
10	31	–	39–62	62	1.4	1.4	41.3
11	25	–	41–78	74	1.8	1.8	56.4
12	2	–	75	75	1.8	1.7	45.2
13	21	–	33–110	77	2.0	1.7	51.7
14	25	–	70–82	82	1.9	1.9	39.4
15	12	–	67–89	86	2.0	1.9	45.1
16	21	18	21–70	–	1.2	1.1	58.3
17	26	25	46–60	–	1.9	1.5	31.3
18	10	2	42–69	–	1.7	1.7	48.1
19	7	2	38–72	–	1.6	1.1, 1.7	42.7
20	8	3	42–101	–	1.4	2.1, 3.2	84.6
21	12	4	32–60	–	1.4	1.5, 2.0	57.4
22	30	–	32–85	–	1.4	1.3, 1.7	29.2

The ploidy status of the tumors was expressed by the DNA index (DI), which is the ratio between the mean channel numbers of the tumor Go/G1 peak and the diploid Go/G1 peak. A diploid tumor would thus, by definition, have a DI = 1.0.

DNA cytophotometry (CM). Formalin-fixed, paraffin-embedded material was used for this analysis. Deparaffinized 5 µm sections were stained by the Feulgen method (Deitch 1966), after 3.5 N HCl hydrolysis for 40 min at 37° C. The integrated light absorbance of tumor nuclei was determined by using a Zeiss 01 photomicroscope (Zeiss, Oberkochen, FRG) connected online to a PDP 11/03 computer for scanning and integrating the single cell measurements. The programs used for obtaining and evaluating data were developed by ourselves (Callaghan and Barberá-Guillem 1981). One hundred tumor nuclei were measured in each tumor using a 100× oil-immersion objective, at a 560 nm wavelength. Values were divided by the mean absorbance of 25 lymphocyte nuclei from the same section, used as a control. DI values were considered at the tumor G0/G1 peak(s).

Morphometric methods. Nuclear area was assessed using a MOP Videoplan 2000 (Kontron) semiautomatic image analyzer. At least 100 nuclei were measured in each tumor using microphotographs of Feulgen-stained tissue sections, performed with a Zeiss microscope using a 40× objective, at a 2000× final magnification.

Results

Table 2 shows the cytogenetic results from the 22 tumors studied, indicating the total number of counted cells, the number of cells with normal karyotype and the modal number. Values for the DNA index (DI) obtained by cytophotometry (CM) and flow cytometry (FCM), as well as mean nuclear area are also included. Cases 1–6 have a cytogenetic modal number in the diploid range, cases 7–13 in the triploid range and, cases 14 and 15 in the tetraploid range; cases 16–22 have no chromosomal mode.

From a cytogenetic viewpoint a wide chromosomal range number was observed in most tumors but no mode existed in seven. Together with the anomalous cell population, a second normal population with 46,XX karyotype was found in ten tumors.

Tumors in the diploid range (32–57 chromosomes). Cases 1–6

Six tumors showed chromosomal modes between 36 and 49 chromosomes. All possessed a DI (CM and FCM) close to the diploid value of 1 (from 0.9 to 1.2). Figure 1 shows an example of this correlation between chromosomal and DNA values in the diploid tumor from case 6.

Tumors in the triploid range (58–80 chromosomes). Cases 7–8

Seven tumors had modes between 58 and 77 chromosomes. DI values by CM were also found to be around the triploid value of 1.5 (from 1.3 to 1.8) and by FCM these values were higher (1.4 to 2.0). A predominant diploid population (DI 1.0) was found in all tumors

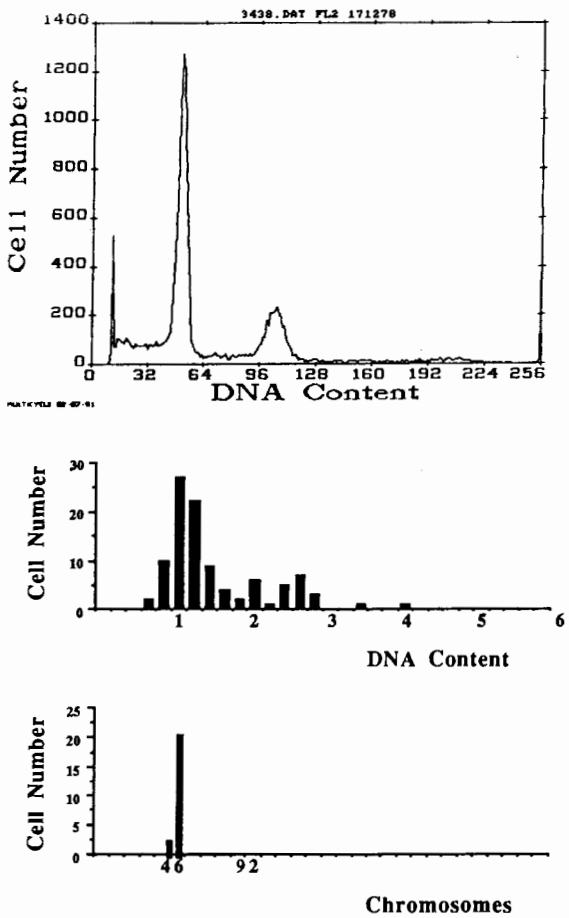

Fig. 1. Example of a breast carcinoma within the diploid range. DNA distribution measured by flow cytometry (*top*) and cytophotometry (*middle*). Chromosome number of the analyzed metaphases is shown as an histogram (bottom)

studied by FCM. Figure 2 shows an example of the correlation between chromosomal and DNA values in the triploid tumor from case 10.

Tumors in the tetraploid range (81–133 chromosomes). Cases 14–15

Two tumors showed chromosomal modes of 82 and 86, respectively. The DI (CM and FCM) was close to the tetraploid value of 2 (from 1.9 to 2.0). A diploid population (DI 1.0) was also found when studied by FCM. Figure 3 shows an example of the correlation between chromosomal and DNA values in the tetraploid tumor from case 14.

Tumors without cytogenetic mode. Cases 16–22

In seven tumors no clear modal number was obtained. Four of these tumors had two different DI values revealed by CM, while FCM values showed a predominant diploid population (DI 1.0) together with an aneuploid population.

As a result of this study it is evident that the tumors

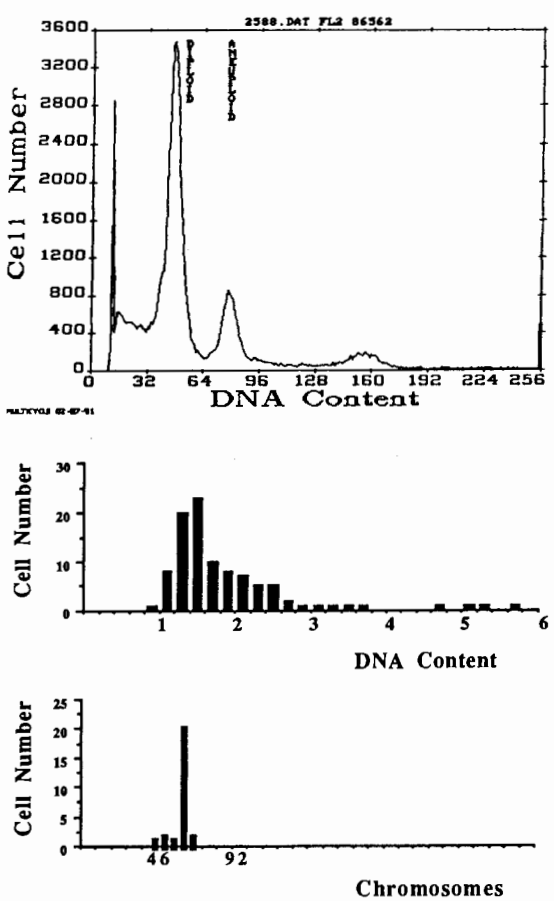

Fig. 2. Example of a breast carcinoma within the triploid range. DNA distribution measured by flow cytometry (*top*) and cytophotometry (*middle*). Chromosome number of the analyzed metaphases is shown as an histogram (bottom)

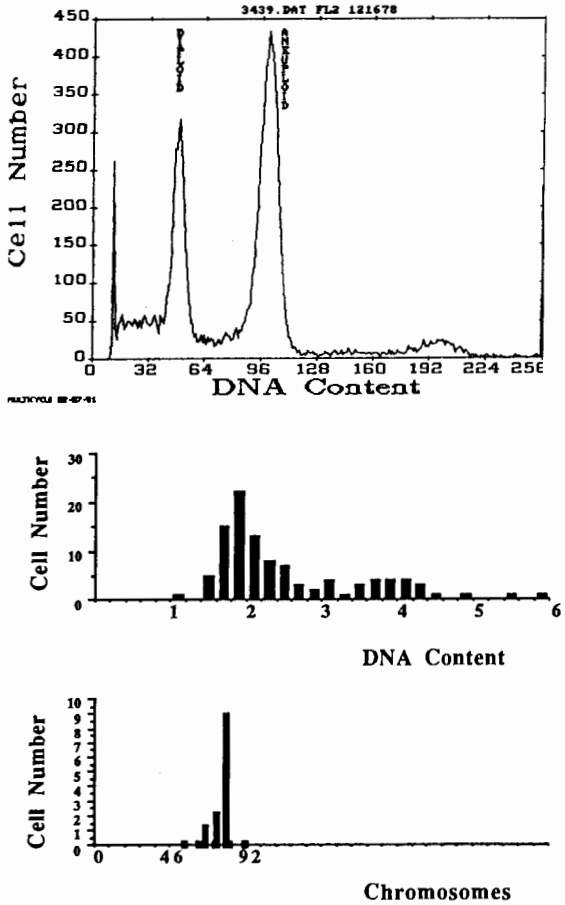

Fig. 3. Example of a breast carcinoma within the tetraploid range. DNA distribution measured by flow cytometry (*top*) and cytophotometry (*middle*). Chromosome number of the analyzed metaphases is shown as an histogram (bottom)

with a defined chromosomal mode show a correlation with the DNA values, although these values were generally somewhat higher than their respective chromosomal modes (Figs. 4A, 4B), especially in the triploid group. Nevertheless, in these tumors the chromosomal mode and the mean nuclear area showed no correlation (Fig. 4C).

Futhermore, an analysis of the chromosomal abnormalities present was performed in 11 tumors in which the chromosomes could be banded. In the following list are compiled the complete karyotypes of the tumors that showed a modal number, as well as the identification of markers where a modal number did not exist.

Karyotypes

Case 2. 41,XX, −3, −4, −4, −7, −9, −10, −10, −12, −13, −13,del(1)(p32), dir ins(1) (q21?), 2q+,del(5) (p13), +del(6)(p23q21), +der(9)t(1;?;9) (q21;?;p24), +der(13)t(7;13)(q11;p11), +del(18)(p11), +der(18) t(18;?) (q21;?), +1 mar.

Case 5. 48,X,+der(x)t(X;15)(p11;q11), +1, −5, −5, −7, −7, +10, +10, −14, −14, −15, −15, −17, −21, −21, −22, −22, +2t(1;21)(p11;q11), +der(1)t(1;14) (p11;q11), +der(?)t(1;?;9)(q21;?;p24), del(4)(p15),6q+, del(6)(q15), 2del(8)(p21), +der(?)t(?;11)(?;q13), del(16) (q21), +der(17)t(1;17) (p22;p25), 18q+, +der(18)t(5;18) (p11;p11), +4 mar.

Case 6. 49,X, −1, −1, +5, −9, +16, +i(1q), +der(?) t(1;?)(p11;?), +del(1)(p32), +der(13) t(13;?)(q11;?), +1 mar.

Case 8. der(?)t(1;?)(p13;?), der(1)t(1;?)(q31;?);, dir dup(1)(q21-q32), i(1q), del(1)(p11), der(6)t(6;11) (q12;q13), der(7)t(7;?)(q11;?), del(11)(p11), i(17q), t(19;?)(q13;?).

Case 10. 62,XX, +3, +5, +6, +7, +8, +9, +12, +13, +14, +19, +20, +20, +del(3) (p23q26), +2der(19)t(1;19)(q21;q13), +1 mar.

Case 11. 74,XX, +1, +2, +3, +3, +3, +4, +4, +5, +5, +5, +6, +7, +8, +10, +10, +10, +12, −13, −13, −14, −14, −15, +18, +20, −21, −21, +dir(1)t(1;13) (p22;q14), +der(1)t(1;21)(p11;q11), +3der(2)t(2;?)(q22;?), 15p+, +2del(16) (p12q22), +2der(19)t(1;19) (q21;q13), +6 mar, +1–3 dmin.

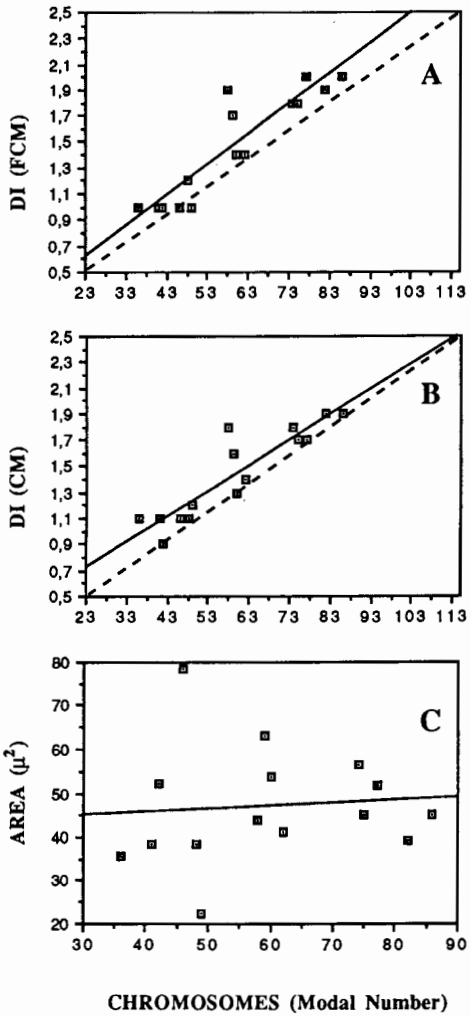

Fig. 4A–C. Relationship between cytogenetic results (*abcissas*) and **A** DI measured by flow cytometry, **B** DI measured by cytophotometry and **C** mean nuclear area. *Solid lines*: linear regression of experimental data. *Dashed line*: theoretically expected correlation

Case 13. i(1q), der(1)t(1;?)(p22;?), t[8;i(1q);8], (q24,q41;p23), der(19) t(19;?) (q13;?).

Case 14. 82,XXX, +t(9;10)(q22;q26), +1, +1, +1, +2, +3, +4, +4, +5, +6, +6, +6, −7, −7, +10, +12, +13, +15, +16, −17, −17, +18, +19, +19, +20,+21, +21, +der(1) t(1;7;13)(p11;p11;p11), +der(2)t(2;14)(q11;p11), +der(2)t(2;?)q11;?), +del(3)(q25),+der(3)del(3)(p21)t(3;12)(q21;q13), +del(6)(q21), +del(9)(q21),+10q+,+13p+,+der(16)t(16;?)(p12;?), +18q+,+6 mar.

Case 20. der(5)t(5;?)(p15;?), dic(10;11)(q22;q13), del(11)(q13), der(11) t(11;?)(p14;?).

Case 21. i(1q), der(5)t(5;?)(p15;?), der(?)t(?;11)(?;q13q23).

Case 22. i(1q), del(1)(q12), der(2)t(2;?)(q11;?).

This cytogenetic study shows a high number of rearrangements, some of unknown origin (Fig. 5A). In these alterations chromosome 1 is preferentially implicated at 1p11 and 1q21-1qter (Fig. 5A–B). Other rearrangements appear in some tumors on chromosomes 11 and 16 (Fig. 5A).

Discussion

Nuclear alterations in tumors, particularly the degree of heterogeneity and nuclear atypicality, are considered in the determination of histological grade. Quantitative methods to give objectivity to such subjective appreciations have been developed and applied to many tumors, including breast carcinoma. Morphometric measurements of nuclear size, as well as techniques that analyze the amount and structural organization of DNA within these nuclei, have been applied to evaluate their implication in the malignancy of these tumors and the relation of these parameters with prognosis (Baak et al. 1985; Fallenius et al. 1988; Christov et al. 1989; Merkel and McGuire 1990).

The most precise technique for evaluating both DNA content and its structural organization within the cell, is cytogenetic analysis. Although the incidence of breast cancer is very high, the number of these studies is relatively small, due to methodological problems (Sandberg 1990; Gibas et al. 1984) and to the difficulties in interpreting karyotypes, most of which show a high number of chromosomes with many rearrangements. This hampers the determination of a chromosomal modal number due to the presence of several cytogenetic populations in these tumors.

However, there is an extensive literature concerning the estimation of DNA values by static as well as flow cytometry, and their correlation with prognosis (Merkel and McGuire 1990). Nonetheless, the comparison between DNA and chromosomal values has been evaluated in a few studies.

In leukemias and lymphomas, the majority show a near-diploid value, and no quantitative differences have been found between the two types of analysis (Barlogie et al. 1977). Also bladder carcinomas show no great distinction between DNA values and chromosomal modal number within the diploid and tetraploid range; however, significant differences have been seen in triploid tumors in which the DNA values are always higher than the chromosomal modes (Wijkstrom et al. 1984; Tribukait et al. 1986). In previous studies on breast carcinomas (Paulete-Vanrell 1970; Remvikos et al. 1988), DNA values were higher than their corresponding chromosomal modes.

In our study a comparison between flow cytometry and cytophotometry measurements of DNA and modal chromosomal number was made, and we found that a correlation exists between them, although DNA values are generally higher than those seen with the chromosomal modes. This difference may be attributed to the high number of chromosomal rearrangements that occur in these tumors with an increase in the proportion of large chromosomes. These results would confirm this hypothesis, advanced previously by others (Paulete-Vanrell 1970; Wijkstrom et al. 1986; Remvikos et al. 1988).

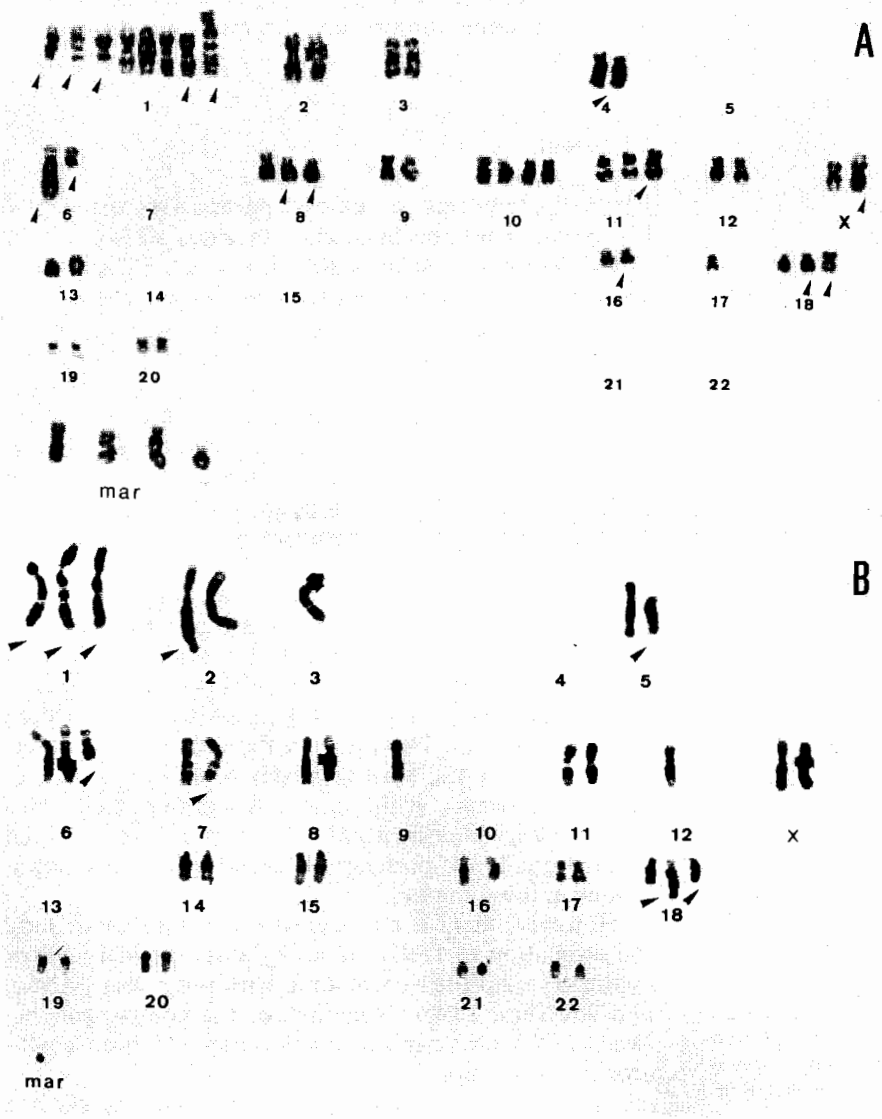

Fig. 5A, B. Karyotypes of **A** case No. 5 and **B** case No. 2. Both show a large number of rearrangements (*indicated by arrows*), some of unknown origin (*mar*), with a preferent implication of chromosome 1

Cytogenetic and flow cytometry analyses show a population of diploid cells together with the aneuploid cell lines. This population does not appear using static cytophotometry, where tumor cells are specifically chosen, and would, therefore, appear to belong to stromal component cells (Rodgers et al. 1984; Sandberg and Turc-Carel 1987) and not to carcinoma cells, as has been described in tissue culture (Wolman et al. 1985).

In some tumors (cases 7, 13, 17, 20 and 22) different DI values were obtained by flow cytometry and cytophotometry. These tumors showed extensive lymphoid infiltration and/or normal mammary gland cells by histological examination. As a result, flow cytometry showed a predominantly diploid population that appeared to overlap the aneuploid one. Therefore, the DI of the minoritary malignant population, as fitted by the DNA analysis program may not represent the actual DI values of these cells. In these tumors static cytophotometry would permit a more objective DI value, as only malignant cells are chosen for this evaluation.

Morphometric measurements of the mean nuclear area showed no clear correlation with the aforementioned parameters. This fact has been addressed by other authors (Uyterlinde et al. 1987) when DNA flow cytometry and morphometry results were compared. Nuclear size, although intrinsically correlated with DNA content, also depends on other factors, such as the metabolic status of the cells, the degree of cellular expression, etc. that could make it an independent prognostic factor in breast cancer.

Finally, an analytical study of DNA organization is given by the cytogenetic study of chromosomal rearrangements and the implication of different chromosomes with their breakpoints. A high number of cytogenetic markers exists in mammary tumors, of which only a restricted number have been identified completely; others have been partially identified or remain unidentified. It is well-known that carcinomas display more complex karyotypes than other tumors, possibly due to the generally advanced stage of the tumor at diagnosis (Sandberg and Turc-Carel 1987).

Chromosome 1 seems to be the most directly impli-

cated in primary breast carcinomas (Kovacs 1981; Rodgers et al. 1984; Dutrillaux et al. 1990; Ferti-Passantonopoulou and Panani 1987), as well as in metastases and cultured cell lines derived from this neoplasm (Satya-Praskash et al. 1981; Bullerdiek et al. 1985). This was apparent in the tumors studied here. Regarding chromosome 1 abnormalities, we found rearrangements principally located at 1p11 and 1q21-1qter, analogous to those found by several authors (Kovacs 1981; Rodgers et al. 1984; Hill et al. 1987). The isochromosome of the long arms in chromosome 1 was found in 5 of 11 of our tumors, as previously described (Kovacs 1981; Rodgers et al. 1984). Furthermore an identical deletion del(1)(p32) was observed in two tumors (cases 2 and 6), and an identical translocation t(1;19)(q21;q13) in two other tumors (cases 20 and 22). Abnormalities of chromosome 1 have been reported in several malignant disorders (Sandberg 1990), and it seems to play an important role in breast cancer progression (Rodgers et al. 1984; Ferti-Passantonopoulou and Panani 1987).

Chromosome 11 had different rearrangements in four cases. The breakpoint q13 is the most frequently seen. Several authors (Dutrillaux et al. 1990; Fertio-Passantonopoulou and Panani 1987) have found this chromosome involved but mainly in the band q22–23.

We have also observed alterations in chromosome 16: different deletions in p12 and q21-q22 in two tumors, and a translocation in another. Other authors found total monosomy of chromosome 16 (Rodgers et al. 1984). As suggested by Hulten et al. (1984), hemizygosity and structural abnormalities in chromosome 16 (segment 16p12-pter) may be of relevance for tumor progression in breast cancer.

Furthermore several other chromosomes were implicated with some frequency in our patients: chromosomes 2, 5, 6 and 13 in different rearrangements, and chromosome 19 always as translocation at the same breakpoint q13.

Although the number of tumors studied is small, no clear-cut correlations between chromosomal abnormalities, DNA pattern and the clinico-pathological staging of the tumor, could be found in the present analysis. The karyotypes in our tumors showed a large number of chromosome rearrangements even in those with a diploid DNA content, where a better prognosis has been described (Merkel and McGuire 1990). Therefore further analysis is necessary for a better understanding of this interesting topic.

Acknowledgements. This work has been supported with grants from Lab. Calderón (Castellón) and IVO (Valencia).

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