

FLOWCYTOMETRIC EVIDENCE OF DNA PLOIDY IN HUMAN BREAST CANCER

Dorina Munteanu¹, Mihaela Zlei¹, Octăvița Ailiesei², Camelia Chifu³,
C. Diaconu³, E. Carasevici⁴

1. Laboratory of Immunology and Genetics "Sf. Spiridon" Hospital Iași; 2. Department of the Biochemistry, Genetics and Microbiology, "Al. I. Cuza" University of Iași; 3. The III-rd Surgical Clinic of "Sf. Spiridon" Hospital, Iași; 4. Department of Immunology, "Gr. T. Popa" University of Medicine and Pharmacy, Iași

Abstract. Breast cancer is the most common form of malignancy that affect women. Tumor proliferation can be monitored by measuring DNA synthesis using flow cytometry which provides rapid and precise analysis of large numbers of cells. The aim of the present study was to evaluate DNA ploidy in breast cancer and its relationship to other classical clinicopathological parameters (age, tumor size, histological type, grade of cellular differentiation, lymph node status). This study was initiated in September of 2003 and continued until April of 2004. A number of 30 patients diagnosed with mammary carcinoma and subjected to surgery at the III-rd Surgical Clinic of the "Sf. Spiridon" Hospital were taken into consideration. None of the patients were subjected to chemo- or radio-therapy prior to surgery. Cell cycle analyses were performed with fresh tumour sample and normal tissue surrounding the tumour. Data were analysed by Modfit (Verity Software House, USA). A number of 12 cases (40%) were cytometrically diploid (DNA index, DI = 1.00), whereas 18 (60%) were nondiploid tumors. In these latter cases, 16 (53.33%) were simple aneuploid and 2 (6.67%) were tetraploid. When aneuploid and tetraploid cases were analyzed as one group, a statistical correlation was evidenced between DNA ploidy and tumor size ($p=0.01$). Our study indicated that DNA ploidy could be an important factor for estimating the degree of genomic instability which may be reflected by the aggressiveness of the tumor.

Key words: ploidy, flowcytometry, breast cancer

Rezumat. Cancerul de sân este cea mai comună formă de malignitate care afectează femeile. Proliferarea tumorală poate fi monitorizată prin măsurarea sintezei de ADN folosind flow-citometria care furnizează o analiză rapidă și precisă a unui număr mare de celule. Scopul studiului prezent a fost de a evalua ploidia ADN în cancerul de sân și relația sa cu alți parametri clinicopatologici (vârsta, dimensiunea tumorii, tipul histologic, gradul de diferențiere celulară, *status*-ul ganglionar). Acest studiu a fost inițiat în septembrie 2003 și a continuat până în aprilie 2004. 30 paciente diagnosticate cu carcinom mamar și operate în Clinica a III-a Chirurgie, Spitalul „Sf. Spiridon”, Iași au fost luate în studiu. Pacientele nu au beneficiat de chimio- sau radio-terapie anterior tratamentului chirurgical. Analiza ciclului celular a fost realizată pe probe de țesut tumoral și țesut normal peritumoral iar datele au fost procesate prin intermediul programului Modfit (Verity Software House, USA). 12 cazuri (40%) au fost diploide (DI = 1.00), în timp ce 18 (60%) au fost tumori nondiploide. Dintre tumorile non-diploide 16 (53,33%) au fost simple aneuploide și 2 (6,67%) au fost tetraploide. Cazurile caracterizate de aneuploidie și tetraploidie au fost grupate și analizate împreună. Aplicarea unor metode statistice a evidențiat o corelație între ploidia ADN și dimensiunea

tumorii ($p=0,01$). Rezultatele studiului nostru indică faptul că ploidia ADN ar putea fi un factor important pentru aprecierea gradului de instabilitate genetică, care s-ar putea reflecta în potențialul agresiv tumoral.

Cuvinte cheie: ploidie, flow citometrie, cancer de sân

INTRODUCTION

A malignant tumor is a disorder characterized by abnormal cellular proliferation due to abnormal changes in the control mechanism of the cell cycle and dynamic cellular characteristics. Many factors are involved in these abnormal cellular changes and may influence progress of the cell cycle and cell development (1).

Therefore, the assessment of factors such as cell cycle and DNA ploidy may be eventually useful to determine the response to treatment and prognosis (2, 3).

Breast cancer is the most common form of malignancy affecting women. The incidence of breast cancer tends to increase. In Iasi, in only one year, the incidence of breast cancer increased from 47,17 ‰ (192 new cases in 2002) to 64,73‰ (267 new cases in 2003).

Breast tumors, as other solid tumors undergo multiple genetic changes as they progress to advanced stages and these changes are thought to be responsible for many of the clinico-pathologic features of neoplastic cells. However, women with similar prognostic features can vary significantly in their outcome (1).

Measurement of ploidy by flow cytometry has been investigated as prognostic factors in breast cancer (3). In this regard, numerous investigators have compared flow cytometric DNA content with age, tumor size, histo-

morphologic classification, histological grade, clinical stage, nodal status, estrogen receptor status, and a variety of other features. DNA aneuploidy (DNA index, $DI \neq 1.00$) has been associated with a more aggressive behavior of the disease, thus providing prognostic information on overall tumor progression.

MATERIALS AND METHODS

Patients

This study was initiated in September of 2003 and continued until April of 2004. A number of 30 patients of the III-rd Surgical Clinic of the "Sf. Spiridon" diagnosed with mammary carcinoma and subjected to surgery at Hospital were taken into consideration. None of the patients were subjected to chemo- or radio-therapy prior to surgery. All clinical parameters were obtained from hospital tumor registries. These parameters included: age of patient, tumor size, histological type, histological grade and nodal status. Local treatment consisted of modified radical mastectomy.

Sample preparation, staining and flow cytometric DNA measurement

The tumor samples were harvested in sterile media and incubated with 0.4% collagenase, at 37°C over night. 1×10^6 isolated cell were incubated with a non-ionic detergent (Beckman-Coulter) and stained with propidium iodide. After incubation at room temperature for at least 30 minutes, the cell

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suspensions were analyzed using a FacsCalibur flow-cytometer (Becton Dickinson).

Data analysis

Flow cytometry acquisition of data was performed using the CellQuest software (Becton Dickinson). At least 2×10^4 cellular events were acquired for each histogram. The red DNA fluorescence signal was analyzed as area versus peak signal, in order to eliminate doublets and aggregates. To localize the diploid peak position around channel 200 on the red DNA fluorescence histogram an external standard was used (normal tissue surrounding of tumor) (coefficient of variation, $CV < 2$). Histograms exhibiting one G_0/G_1 cell population positioned in the expected diploid range were classified as DNA diploid histograms. Tumors displaying an additional G_0/G_1 peak with more than 10 % of events were classified as DNA aneuploid. DNA aneuploid histograms with a DNA index ranging from 1.9 to 2.1 and more than 15 % of events presents in additional peak were classified as DNA tetraploid - according to the DNA Cytometry Consensus Conference on breast carcinomas (3). The DNA index determination and the cell cycle distribution analysis were carried out using the ModFit (Verity Software House, USA).

Descriptive statistics were generated, including means, ranges, standard deviations (SD), frequencies and percentages. Association between ploidy and clinico-pathological parameters was evaluated by the chi-square method.

RESULTS AND DISCUSSION

In the present study, the median age of patients was 57 years (ranged 42-78 years). All breast cancers were primary tumors, among them 2 (6,67%) well-differentiated (G1-low grade), 17 (56,67%) moderately differentiated (G2-intermediate grade) and 11 (36,66%) poorly differentiated (G3-high grade).

As histological types, a number of 25 cases (80%) were ductal carcinomas and 5(20%) lobular carcinomas. From the point of view of the size of tumour, 5 (20%) tumors were classified as ≤ 2 cm and 25 (80%) as > 2 cm.

The grade of cellular differentiation was evaluated according to the criteria of Scarff and Bloom and their modified criteria: 3 cases (10%) were N0 and 27 (90%) were N1 (4,5).

All tumors were successfully analyzed by flow-cytometry. Twelve cases (40 %) were cytometrically diploid ($DI = 1.00$), whereas eighteen (60%) were nondiploid tumors. In these latter cases sixteen (53.33 %) were simple aneuploid and two (6.67 %) were tetraploid. DNA aneuploid and tetraploid cases were grouped for statistical analysis. The mean DNA index for aneuploid tumors was 1.40 ± 0.25 SD and median was 1.39.

The coefficient of variation (CV) of the histogram peak corresponding to G_1/G_0 cells was also calculated for the diploid and aneuploid tumors. The CV (in %) mean value was 4.67 ± 2.08 SD for diploid tumors. In the case of aneuploid tumors, the mean CV relative to the diploid peak was 5.08 ± 1.84 SD, and 5.69 ± 3.4 SD for the aneuploid peak.

Over the past decade, flow cytometric studies have shown that analysis of DNA, ploidy and cell cycles in malignant tumors provides valuable information for the diagnosis, therapy and prognosis of cancer patients (2). It is worth noting that the aneuploidy incidence we observed is comparable to that reported in the literature for other similar studies. For example, Kute et al., and Michels et al., reported an incidence of aneuploid tumors of 54%, respectively 50% (6,7).

A correlation was evidenced between DNA ploidy and tumor size ($p = 0.01$). $RR = 3.57$; $CI\ 95\% = 1.90-6.70$ (table 1). A significant relationship between increasing tumor size and DNA aneuploidy was also reported previously (8, 9). These studies showed that small tumors ($\leq 2\text{cm-T1}$) were predominately diploid or near-diploid, while larger tumors ($> 2\text{ cm-T2, T3, T4}$) were predominately aneuploid. Other study did not evidence this correlation (10, 11).

Table 1. Clinicopathologic Characteristics of the Study Population

Variable	Patients n (%)	DNA diploid cases (%)	DNA aneuploid cases (%)	p
Tumor size				
≤2cm	5 (20)	5 (100)	0 (0)	0.01
>2cm	25 (80)	7 (28)	18 (72)	
Histological type				
ductal	25 (80)	9 (36)	16 (64)	NS
lobular	5 (20)	3 (60)	2 (40)	
Histologic grade				
G1	2 (6.67)	2 (100)	0 (0)	NS
G2	17 (56.67)	7 (41.18)	10 (58.82)	
G3	11 (36.66)	3 (27.27)	8 (72.73)	
Nodal status				
pN0	3 (10)	2 (66.67)	1 (33.33)	NS
pN1/2	27 (90)	10 (37.04)	17 (62.96)	

NS, not significant

More than half of all incident breast cancer has not apparently spread to the lymph nodes. These patients have an excellent prognosis, but 20% to 30 % will recur later in their lives. Most investigators agree that patients with N0 breast cancer whose tumors are large and poorly differentiated have the worst prognosis. The current trend in oncology is to treat all N0 patients whose tumors are greater than 1 cm

with some form of adjuvant therapy, even though the absolute benefits are small (6). Duigou et al found a strong correlation between DNA ploidy and positive lymph nodes ($p=0.007$) (9). However, in our study, no such correlation was demonstrated, although in 17 N1/2 cases aneuploidy was found.

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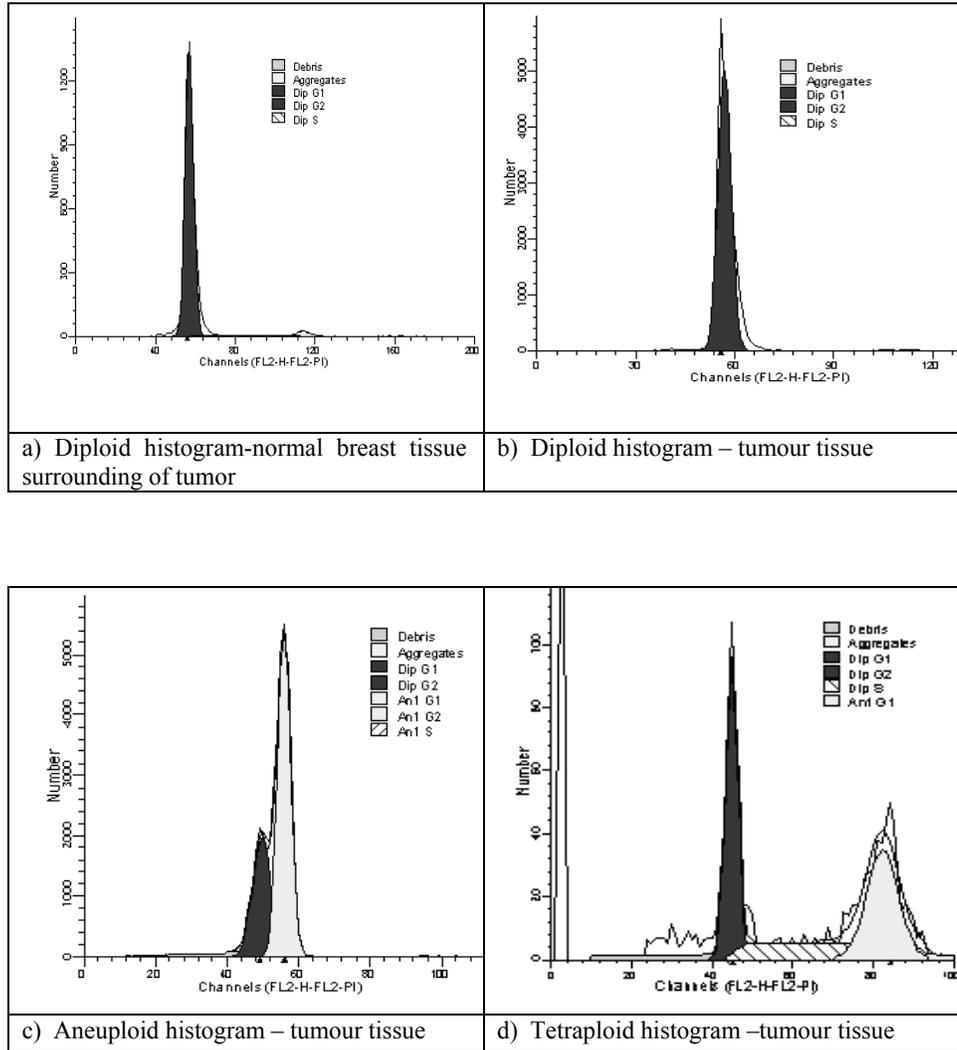


Fig. 1. Flow cytometric analysis of DNA

DNA tetraploid histograms have been reported to be associated with low to intermediate as well as a high risk of relapse (12,13). Michels et al., 2004 reported that tetraploid tumors had approximately the same prognosis as aneuploid tumors, whereas hypoploid tumors had a slightly better outcome than even diploid tumors (7).

Discrepant results are partially explained by differences in the technical steps involved in the implementation of clinical DNA cytometry. Flow-cytometry technique on fresh or frozen material requires compliance to a number of different technical steps. These include type of dissociation, type of DNA binding dye, instrument settings, conditions of data acquisition, external standard, histogram analysis (9).

Numerous published studies have been concerning the impact of FCM in the prognosis of breast carcinomas. As Wenger and Clark reported, a review of the literature was performed that was restricted to studies involving more than 100 patients, using fresh or frozen material (14). This review showed that a majority of 17 out of 23 studies evidenced a relationship between DNA ploidy and prognosis, 8 of them after multivariate analysis.

Figure 1 illustrates flow cytometric histograms with overlapping population: diploid (normal breast tissue surrounding of tumor -a), diploid (tumor tissue-b), aneuploid (tumor tissue-c) and tetraploid (tumor tissue- d).

CONCLUSIONS

The results of DNA study in breast carcinoma may vary according to the criteria used for histogram interpretation. A statistical correlation was evidenced between DNA ploidy and tumor size ($p=0.01$).

The results of our study indicate that DNA ploidy might be an important factor for estimating the degree of genomic instability which may be reflected by the aggressiveness of the tumor.

Further information will be provided by analyzing a larger number of cases in order to confirm the value of DNA ploidy as a prognostic factor in the breast cancer.

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