Prognostic value of flow-cytometric DNA analysis in breast cancer

V. Petrozza[1,2], F. Verna[2], L. Lenti[2], F. Carpino[2]

[1]Department of General Surgery "Paride Stefanini"
[2]Department of Experimental Medicine and Pathology and University of Rome "La Sapienza"

Background. Prognosis of breast cancer is mostly dependent on the biological aggressiveness of the neoplasia. New parameters, including DNA content and cellular cycle of neoplastic cells, are now routinely used to assess malignancy. In this work we have studied the clinical value of flow-cytometric DNA ploidy analysis. Particular attention has been addressed towards establishing if these values give additional information to that of histological examination.
Methods. Patients who underwent surgical treatment for primary breast cancer have been selected. At present, all of them are still living, no one has long-distance metastases and all are free of tumor relapses.
Thin sections were tested, after staining, by a Coulter Epics Profile flow cytometer. DNA-index (DI) was assumed to be 1.0 if only a single G0/1 peak was evident. When a second peak was clearly identified (more than 10%), this was considered as an indicator of clonal abnormality of the DNA content. The slides were also examined to define the stage of the neoplasia. Grading was made by evaluation of tubular growth, nuclear polymorphy and number of mitosis. Cell cycles, DI and coefficent of variation G0-G1 peak were compared.
Results. A significant relationship among histological stage, S-phase fraction (SPF) and ploidy was observed.
In accord to the survival rate, nodule size and SPF determine 3 classes of infiltrating ductal carcinoma with negative axillary lymph nodes. Our twenty cases show a correlation between DNA ploidy and histological stage. In fact there are no aneuploid tumors in stage I group, all cases of stage III and most of stage II are aneuploid. We did not find a correlation with survival.
Conclusions. The new screening methods and the improvement of the available methodologies have permitted an earlier diagnosis of breast cancer. Unfortunately, according to our data, flow-cytometric information does not seem to offer predictive elements about tumor clinical outcome.

Introduction

The most important factor influencing prognosis in breast cancer is its biological aggressiveness, generally expressed by parameters such as tumor size, number of metastatized lymph nodes, histological grade and receptors status.
In recent years, the proliferative rate of tumors has been widely investigated in an attempt to define additional prognostic factors. New factors have thus been proposed to evaluate the DNA content and cellular cycle of neoplastic cells (1-5).
Tumor cell nuclear DNA content has been recognized as "one of the best prognostic indexes" in a wide range of human cancers. Barlogie suggested that the presence of an abnormal DNA stemline (DNA aneuploidy) should be regarded as "the single most reliable marker of neoplasia". Also the clinical value of flow-cytometric DNA ploidy analysis has recently been examined on paraffine-embedded archival tumor samples and established in carcinomas of some organs including breast, lung and prostate (2-4).
Many studies have showed correlations between cellular DNA content, measured by flow-cytometry and other pretreatment factors (1,2,5). In addition, the relationship between the results of flow-cytometric DNA analysis, with particular attention to S-phase fraction, and clinical course, has been examined.
Patients whose tumors have a low proportion of cells in S-phase do appear to have a better prognosis. Recent reports also show a relapse free survival and survival advantage for patients with diploid tumors.
In general, available data suggest that aneuploidy is an indicator of greater tumor aggressi-

Corrispondenza a: Dipartimento di Medicina Sperimentale e Patologia Università di Roma "La Sapienza" V.le Regina Elena 324 - 00161 Roma.

veness. In fact, tumors exhibiting DNA values within the limit of normal tissue (DNA euploidy), were found to be correlated with a favorable prognosis. In contrast, tumors with increased and abnormal DNA values (DNA aneuploidy) were found indicative of poor prognosis although this finding is not universal and may be confined to subgroups defined by other prognostic factors (1,3,5,7).

In order to evaluate if those data give additional information to histological data, we performed the present study on paraffin-embedded tissues, thus allowing a retrospective evaluation of the relationship between the results of DNA flow-cytometry performed today and the clinical outcome in patients followed up for a minimun of eight years.

Twenty patients who underwent surgical treatment for primary breast cancer eight years ago have been selected. At present, all of them are still living, no one has long-distance metastases and all are free of tumor relapses.

Materials and methods

Preparation of single cell suspensions from paraffin-embedded material.

Single nuclear suspension was prepared from 50 μm sections cut from formalin-fixed paraffin-embedded tissue from primary breast cancer. Usually a 50 μm section is adequate but for small samples two or three sections are required. The sections were placed in 10 ml glass centrifuge tubes and dewaxed with xylene and then rehydrated in a sequence of 3 ml of 100, 95, 70 and 50% ethanol. The tissue, after washing in distilled water, was resuspended in 1 ml of 0.5% pepsin (Sigma), 0.9% NaCl adjusted to pH 1.5 with 2N HCl and placed in a waterbath at 37°C for 30 min. The cells were stained with 0.13 mg/mL propidium iodide (Sigma) and 7.5 Kunitz units/mL ribonuclease I-A (Sigma) for 15 minutes then analyzed within 1 hour from staining.

Flow Cytometry

The stained samples were tested with a Coulter Epics Profile flow cytometer provided with an argon ion laser of 15 mW and a 488 μm wavelength. A total number of nuclei ranging from 20,000 to 65,000 was scanned for setting up each histogram, which was considered interpretable if the coefficent of variation (CV) was less than 8%. The mean CV was 5%, DNA-ploidy status was expressed as DNA-index (DI) assumed to be 1.0 if only a single G0/1 peak was evident. When a second peak was clearly identified (more than 10%), this was considered as an indicator of clonal abnormality of DNA content and the tumor was defined as aneuploid. The degree of DNA aneuploidy expressed as DNA Index was calculated by dividing the mean fluorescence channel number of the higher (aneuploid) G0/1 peak by the mean channel number of the lower (diploid) G0/1 peak. For DNA diploid and aneuploid were calculated by a computer modeling program.

Histological grade

The slides prepared for the first histological diagno-

Table I. Histological stage and grading of 20 cases of infiltrating ductal carcinoma

Case number	Size reac.	Desmopl. Infiltration	Inflamm.	Necrosis	Stage Growth	Tubular Polymorph.	Nuclear	Mitosis
BC1	2	++	++	absent	IIA	+++	+	+
BC2	1	++	+	absent	I	+	+	+
BC3	1	+	+	+	I	+	++	+
BC4	2	+++	+	absent	IIA	+	+	+
BC5	1	+++	absent	absent	IIB	++	++	+
BC6	2	++	absent	++	IIA	+	++	+
BC7	1.2	+++	++	+	I	++	++	+
BC8	2.1	+++	absent	+	IIB	++	++	+
BC9	1.5	+	absent	intraduct.	I	++	+	+
BC10	2	++	absent	absent	IIA	++	++	+
BC11	4	+	+	+	IIA	+++	+++	+
BC12	2.5	+	+	++	IIB	+++	+++	++
BC13	1.5	absent	absent	absent	IIA			
BC14	1	+	absent	absent	I	++	++	+
BC15	2	+	++	+	IIA	+	++	+
BC16	2.5	+	+	absent	IIIB	+++	+++	++
BC17	2	+++	+	absent	IIA	+++	+++	++
BC18	2	+	+	absent	IIB	++	+++	++
BC19	2.2	+	+	absent	IIIA	+++	+++	+
BC20	1	++	+	Absent	I	+	+	+

sis were re-examined in order to define the neoplasia's stage. Staging was made according to TNM. The evaluation considered only histomorphological data and all neoplasias were classified as pMx according to the long distance metastases.
Grading was made by using Bloom & Richardson's score (8) with the evaluation of tubular growth, nuclear polymorphy and number of mitosis in ten microscope fields at 10X magnification. Scoring obtained from each parameter allows to make a subdivision into three classes.
We have considered other morphological paramethers (desmoplastic reaction, inflammatory infiltration, necrosis) which can give further prognostic elements (4).

Results

Histological stage and grade of our 20 cases of infiltrating ductal carcinoma are reported in Table I, together with other morphological parameters that were divided into a three-grade scale: low (+), medium (++), severe (+++).
Diploid tumors were present in 12 patients (DI=1); aneuploid tumors were present in 8 patients (DI>1).
As shown in Table II, we compared cell cycles (percentage of G0-G1, S and G2-M), DI and coefficent of variation of the G0-G1 peak obtained from 20 different tumors (12 diploid and 8 aneuploid).
Fig.1 shows an example of a DNA histogram from one of 13 diploid tumors (top) and an example of a DNA histogram of the 8 tumors where G2-M of the diploid population overlaps into the S-phase of the aneuploid population (bottom).
As reported in Table III, there was a significant correlation among histological stage, SPF and ploidy.

Figure 1: (Top) Example of a case of breast cancer presenting a diploid DNA content. Only one peak is present. (Bottom) Example of a case of breast cancer presenting an aneuploid DNA content. A second diploid peak is present showing an abnormal content of DNA in the cells.

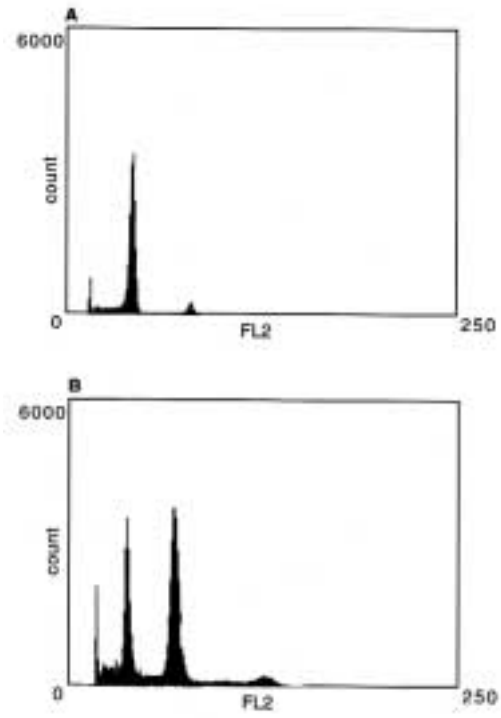

Table II. DNA content of cell cycles

Case number	D	S	T	DI
BC1*	25.5	4.9	44.2	1.9
BC2	68.2	2.4	11.7	1.0
BC3	73.3	2.1	7	1.0
BC4	34.4	40.4	3.2	1.0
BC5	31.1	36.5	11.2	1.0
BC6	44.7	25.5	3.1	1.0
BC7	56.4	23.1	3.2	1.0
BC8*	49.9	2.4	22.2	1.9
BC9	67.5	2.5	6.4	1.0
BC10*	50.3	1.9	11.3	1.4
BC11	72.6	2.3	7.8	1.0
BC12	63.8	2.7	2.9	1.0
BC13*	20.9	10.6	40	2.0
BC14	76.8	1.6	3.4	1.0
BC15*	72.7	2.3	6.3	1.3
BC16*	51.1	4.5	20	2.1
BC17*	31.3	36	14.7	1.8
BC18	16.8	47.5	20.6	1.0
BC19*	50.9	7	22	1.4
BC20	68	1.5	3	1.0

* = Aneuploid DNA content

D = Diploid (G0-G1) S = S phase T = Tetraploid (G2-M) DI = DNA index

Table III. Correlation between histological stage, S-Phase Fraction and ploidy in our twenty cases

Case number	Stage	D	S	T	DI
BC2	I	68.2	2.4	11.7	1.0
BC3	I	73.3	2.1	7	1.0
BC7	I	56.4	23.1	3.2	1.0
BC9	I	66.5	2.5	6.4	1.0
BC14	I	76.8	1.6	3.4	1.0
BC20	I	68	1.5	3	1.0
BC1*	IIA	25.5	4.9	44.2	1.9
BC4	IIA	34.4	40.4	3.2	1.0
BC6	IIA	44.7	25.5	3.1	1.0
BC10*	IIA	50.3	1.9	11.3	1.4
BC11	IIA	72.6	2.3	7.8	1.0
BC13*	IIA	20.9	10.6	40	2.0
BC15*	IIA	72.7	2.3	6.3	1.3
BC17*	IIA	31.3	36	14.7	1.8
BC5	IIB	31.1	36.5	11.2	1.0
BC8*	IIB	49.9	2.4	22.2	1.9
BC12	IIB	63.8	2.7	2.9	1.0
BC18	IIB	16.8	47.5	20.6	1.0
BC19*	III	50.9	7	22	1.4
BC16*	III	51.1	4.5	20	2.1

*= Aneuploid DNA content

D = Diploid (G0-G1) S = S phase T = Tetraploid (G2-M) DI = DNA index

We did not find cases of aneuploidy in stage I tumors, while all cases at stage III are aneuploid. As for stage II cases, four out of eleven are aneuploid (36.36%) and, among these, three of seven cases of stage IIA (42.85%) and one of stage IIB (25%).

Discussion

The new screening methods and the improvement of the available methodologies have permitted an earlier diagnosis of breast cancer, that can be made on a small nodule, with no lymph nodes interested and no long-distance metastases.
On the other side, the increase of therapeutic choices requires to select a group of patients to be subjected to specific treatment. The research of new prognostic factors to obtain a more accurate prognostic evaluation has became imperative (2,10,11).
Now there is a wide agreement regarding the correlation among DNA ploidy, S-phase fraction (SPF) and histological grade. Nevertheless various studies give less importance to these predictive factors because DNA ploidy does not correlate with clinical course and SPF looses its prognostic influence when included in a multi-variable analysis.
In accord to survival rate, nodule size and SPF indicate 3 classes of infiltrating ductal carcinoma with negative axillary lymph nodes:
a) size < 1cm (free survival at 5yrs 96%);
b) size > 1cm and SPF < 10% (free survival at 5yrs 78%);
c) size > 1cm and SPF > 10% (free survival at 5yrs 52%).

The absence of correlation between flow-cytometric parameters and axillary lymph nodes status emerged in numerous studies showing that DNA ploidy and SPF are factors expressing carcinoma development index without a correlation with its metastatic power (3,12,13).
These data seem to be confirmed by studies made on patients with long-distance metastases, who show no correlation among flow-cytometric parameters and treament response, treatment response length, survival and metastases distribution (6-14).
In our study, the 20 cases of breast cancer show a correlation between DNA ploidy and histological stage. In fact, as shown in Table 3 there are no aneuploid tumors in stage I group, all cases of stage III and most of stage II are aneuploid. We do not find a correlation with survival, since all patients were still living after a follow-up period of eight years.
It is therefore important to underline that all patients who underwent post-surgical treatments have a probably modified survival. These treatments could be more useful in some cases and less useful in others.
From the analysis of the obtained data the impossibility of a clear correlation between histological grade and flow-cytometric analysis emerges as pointed out by the presence of tumors in the same stage but with different ploidy and SPF (15-17).
The difference of values may suggest a different tumor clinical history leading to a late diagnosis related to the less sensitive patients (18,19).
An example is offered by the comparison between BC11 and BC4 which have different TNM characteristics and flow-cytometric aspects. A lower pa-

tient sensitivity allowed the BC11 tumor to grow faster in spite of its lower biological aggressivenes. On the contrary the BC4 tumor seems to present a greater biological aggressiveness but its diagnosis was made earlier with a small nodule.
On the other side BC11 and BC6 tumors which have the same stage (IIA) and same histological diagnosis (T2N0) present different diploidy (44.7 vs 72.6), SPF (25.5 vs 2.3) and tetraploidy (3.1 vs 7.8) values although they have a DI less than 2.
According to our data, flow-cytometric information does not seem to offer predictive elements about tumor clinical outcome. This is explainable from the great number of variables which concur to its determination and to the lack of their relative importance.
According to the data reported in the literature and according to our experience in the flow-cytometric examination made immediately after the first diagnosis, the presence of aneuploid tumors is higher than that of diploid ones (70% vs 30%). In these analyses, correlation between histological and flow-cytometric data is possible (20,21). Survival curves of aneuploid tumors showed a high mortality rate during the first post-surgical period followed by a period of lower mortality rate with a course similar to that of diploid tumors. Patients living after five years from surgical treatment present a high percentage of diploid tumors because of its lower mortality rate (30% vs 70%). At present the flow-cytometric data do not permit to establish a precise clinical outcome of aneuploid tumors.
The importance of this new method and its usefulness as a device to evaluate therapy effects and ability to give further data about tumor sensibility to the treatment should be investigated.

Acknowledgement

The authors are grateful to the Foundation for the Advancement of Research in Molecular Medicine (FARMM onlus) for the scientific support.

References

1. Fallenius AG, Auer GU, Carstensen JM. Prognostic significance of DNA measurement in 409 consecutive breast cancer patients. Cancer 1988; 62: 331- 41.
2. Herman CJ. Cytometric DNA analysis in the management of cancer. Clinical and laboratory considerations. Cancer 1992; 69:1553-6.
3. Fox MS. On the diagnosis and treatment of breast cancer. JAMA 1979; 241(5):489-94.
4. Bacus SS, Goldshmidt R, Chin D, et al. Biological grading of breast cancer using antibodies to proliferating cells and other markers. Am J Pathol 1989; 135:783-92.
5. Doky Y, Shiozaki H, Tahara H et al. Prognostic value of DNA ploidy in squamous cell carcinoma of esophagus. Analyzed with improved flow-cytomeric measurement. Cancer 1993; 72: 1813-8.
6. De Lena M, Romero A, Rabinovich M, et al. Metastatic pattern and DNA ploidy in stage IV breast cancer at initial diagnosis. Relation to response and survival. Am J Clin Oncol 1993; 16(3): 245-9.
7. Hellmen E, Bergstrom R, Holmberg L, et al. Prognostic factors in canine mammary tumors: a multivariate study of 202 consecutive cases.Vet Pathol 1993; 30:20-7.
8. Bloom HJG, Richardson WW. Histological grading and prognosis in breast cancer: a study of 1409 cases of which 359 have been followed for 15 years. Br J Cancer 1957; 9: 359-77.
9. Kallionemi OP, hietanen T, Mattila J, et al. Aneuploid DNA content and high SPF of tumor cells are related to poor diagnosis in patients with primary breast cancer. Eur J Cancer Clin Oncol 1987; 27:227-82.
10. Vindelov LL, Christensen IBJ, Jensen et al. Limits of detection of nuclear DNA abnormalities by flow-cytometric DNA analysis: results obtained by a set of methods for sample-storage, staining and internal standardization. Cytometry 1983; 3:332-9.
11. Batsakis JG, Sneige N, El-Naggar AK. Flow cytometric (DNA content and SPF) analysis of brest cancer. Cancer 1993; 71:2151-3.
12. Askensten UG, Von Rosen AK, Nilsson RS et al. Intratumoral variations in DNA distribution patterns in mammary adenocaricnomas. Cytometry 1989; 10: 326-33.
13. Dutrillaux B, Gerbault-Serureau M, Remvikos Y et al. Breast cancer genetic evolution. Data from cytogenetics and DNA content. Breast Cancer Res Treat 1991; 19:245-55.
14. Hay ID, Ryan JJ, Grant CS, et al. Prognostic significance of nondiploid DNA determined by flow cytometry in sporadic and familiar medullary thyroid carcinoma. Surgery 1990; 108:97-980.
15. Haak HR, Cornelisse CJ, Goslings BM et al. Nuclear DNA content of medullary thyroid carcinoma in a large family with MEN-2A syndrome. J Endocrinol Invest 1991;14: 261-24.
16. Blanco G, Holly K, Heikkinen M, Kallionemi OP et al. Prognostic factors in recurrent breast cancer: relationship to the site of recurrence, disease-free interval, female sex steroid receptors, ploidy and histological malignancy grading. Br J Cancer 1990; 62: 142-6.
17. Clark GM, Dressler IG, Owens MA et al. Prediction of relapse or survival in patients with node-negative breast cancer by DNA flow cytometry. N Engl J Med 1989; 320: 627-33.
18. Hitchcock A, Ellis IO, Robertson JF et al. An observation of DNA ploidy, histological grade and immunoreactivity for tumor-related antigens in primary and metastatic breast carcinoma. J Pathol 1989; 159: 129-34.
19. Frierson HF. Ploidy analysis and S-phase fraction determination by flow cytometry of invasive adenocarcinomas of the breast. Am J Sur Pathol 1991; 15: 358-67.
20. O'Reilly SM, Camplejohn RS, Barnes DM et al. Node-negative breast cancer: prognostic subgroups defined by tumor size and flow cytometry. J Clin Oncol 1990; 8: 2040-6.
21. Del Bino G, Silvestrini R, Zucconi MR et al. DNA ploidy of human breast cancer. Ann Cell Pathol 1989; 1: 215-23.