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Prognostic significance of static cytophotometric DNA analysis in invasive ductal breast cancer.

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SUMMARY

To evaluate the role of image cytometry in prognostic assessment of breast cancer, static cytophotometric DNA analysis was performed on smears obtained from cell suspensions from 60 paraffin-embedded ductal breast carcinomas. The prognostic significance of DNA histogram parameters was tested along with other clinical and pathologically determined factors (age, menstrual status, nodal status, season at diagnosis, tumor location, tumor size, histologic grade, clinical stage and morphometric prognostic index), and a multivariate analysis was performed to determine which of the significant prognostic factors provide independent prognostic information, and to identify the parameter of greatest prognostic value.

Nodal status, location (retroareolar-no retroareolar), tumor size, clinical stage, morphometric prognostic index, DNA-index and S-phase fraction were significantly correlated with prognosis in univariate analysis. In multivariate analysis, only nodal status, DNA index and location appeared as independent prognosticators. This finding suggests that static cytophotometric DNA index analysis on paraffin embedded material provides additional prognostic information in invasive ductal breast carcinoma.

Key words: Breast cancer. DNA content. Static cytometry. Paraffin embedded tissue.

INTRODUCTION

Most of the studies about prognostic factors in mammary cancer show a probable, but not settled, correlation between DNA ploidy and prognosis, and have been performed by flow cytometry (1,2). This method, whose main advantage over static cytometry is speediness, has the disadvantage of not allowing morphologic identification of elements to measure, what may restrict reliability on results, specially when tumor samples with high proportions of inflammatory and stromal cells are studied.

Static cytometry can be used as an interactive technique that allows cell analysis under visual control. Thus, malignant cells may be preferentially selected, giving analysis greater specificity. Furthermore, it requires fewer cells than flow cytometry. Although interactive measurement is time-consuming, new systems have appeared recently that shorten time of study.

Since a good correlation between results in fresh and formol fixed paraffin-embedded material by flow and static cytometry has been demonstrated (3-5), paraffin blocks can be used for cytometry. The advantage of using archival blocks for DNA analysis is that a long follow-up is available and DNA-ploidy pattern can be analyzed in that context.

The current study analyzes DNA histogram parameters obtained by static cytometry performed on smears obtained from cell suspensions of paraffin-embedded tissues of patients with invasive ductal breast carcinoma. The prognostic significance of DNA ploidy data was correlated with various other histopathologics and clinical parameters. A multivariate analysis was performed to identify independent prognostic factors, as well as the most powerful prognosticator.

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Tabla I. Univariate survival analysis

Variable Name	Group Index	Category Name	Frequency Category	Total	Mantel-Cox	P Breslow	Tarone	P Prentice
Age				56*	0.7212	0.7251	0.7282	0.7214
	1	30-35	1					
	2	36-40	3					
	3	41-45	5					
	4	46-50	10					
	5	51-55	11					
	6	56-60	4					
	7	61-65	10					
	8	66-70	5					
	9	71-75	5					
	10	76-80	2					
Age				56	0.8161	0.7953	0.7750	0.7712
Van der Linden 9	1	35-48	17					
Adami 45, Rosai 46	2	49-65	27					
	3	>65	12					
AGE				56	0.6217	0.6356	0.6482	0.6524
Adami 45	1	<=53						
	2	>53						
Age				56	0.6217	0.6356	0.6482	0.6524
Bloom 47	1	other	39					
	2	50-60	17					
Location				52	0.0246	0.0213	0.0187	0.0184
Haagensen48	1	LIQ	3					
	2	LEQ	9					
	3	UIQ	2					
	4	UEQ	24					
	5	Retroareolar	14					
Location				56	0.5281	0.5178	0.5071	0.5061
Joensuu 18	1	Left	21					
	2	Right	35					
Location				38	0.2783	0.2785	0.2792	0.2790
	1	Internal	5					
	2	External	33					
Location				56	0.0000	0.0000	0.0000	0.0000
	1	other	48					
	2	Retroareolar	8					
Season				56	0.1334	0.1431	0.1542	0.1569
	1	Spring	16					
	2	Summer	15					
	3	Autonm	5					
	4	Winter	20					
Menopause				56	0.1663	0.1641	0.1628	0.1610
	1	Post	34					
	2	Pre	22					

* Maximum number of cases is 56 because diffuse tumors (4) were excluded from analysis.

MATERIAL AND METHODS

This study concentrated on invasive ductal carcinoma NOS (not otherwise specified) subtype, because it is the most common type of mammary cancer, and to get a group of lesions as homogeneous as possible, avoiding the possible prognostic impact of histologic type.

Minimum follow-up time was five years (mean follow-up time 81 months), and disease-free-interval (DFS) was used as follow-up parameter. Patients who died during follow-up period because of concurrent diseases were excluded.

Only patients without distant metastases nor locally advanced disease at diagnosis were selected. Likewise, patients with other neoplastic or non-neoplastic pathologies associated with the tumor were excluded. To avoid the prognostic effect of different therapeutic modalities, only patients who had been treated with modified radical mastectomy, with or without adjuvant chemotherapy, were included.

Of 97 invasive ductal breast carcinomas registered at the Pathology Department II of San Carlos Hospital

Tabla I: Cont.

Variable Name	Group Index	Category Name	Frequency Category	Total	Mantel-Cox	P Breslow	Tarone	P Prentice
Menopause for Age (45,55)				25	0.0208	0.0185	0.0166	0.0166
Goldenberg 49	1	Post	8					
Cutler 50	2	Pre	17					
Tumor Size				56	0.0109	0.0131	0.0158	0.0154
Ewers 12	1	1.2-2	15					
	2	2.1-4	28					
	3	> 4	13					
Tumor Size				56	0.0069	0.0070	0.0074	0.0073
Joensuu 51	1	<=2.00	15					
	2	>2.00	41					
Clinical Stage				56	0.0002	0.0002	0.0002	0.0002
	1	I	12					
	2	II	13					
	3	III	31					
Nodal Status				56	0.0000	0.0000	0.0000	0.0000
	1	Unnafected	26					
	2	Affected	30					
Nodal Status				56	0.0000	0.0000	0.0000	0.0000
Russo 52	1	=0	26					
	2	1-3	11					
	3	>=4	19					
Histologic Grade (Scarff-Bloom-Richardson)				33	0.1558	0.1555	0.1557	0.1505
	1	I	9					
	2	II	24					
Histologic Grade				56	0.1300	0.1288	0.1283	0.1263
Black 53	1	I	9					
	2	II	23					
	3	III	24					
Histologic Grade				56	0.2213	0.2186	0.2164	0.2131
Black 53	1	I	9					
	2	II-III	47					
Histologic Grade				33	0.1558	0.1555	0.1557	0.1505
Black 53	1	I	9					
	2	III	24					
MPI		MorphometricPrognostic Index	56	0.0008	0.0010	0.0012	0.0012	
	1	<=0.60	24					
	2	>0.60	32					

(Madrid), sixty were selected according to previous criteria.

Age at diagnosis, menstrual status, season at diagnosis, tumor location (left or right breast, upper or lower quadrants, internal or external quadrants, retroareolar location), tumor size, nodal status, clinical stage, histological grade, tumoral proliferative activity (mitosis number per 10 high power field) (6), morphometric prognostic index (6) and DNA histogram parameters were analyzed in every patient.

Apart from classical DNA histogram parameters: DNA index (DI) and S-phase fraction (SPF), Böcking (7) algorithms for DNA cytophometric diagnosis and grading of malignancy were also evaluated: 5c exceeding rate (5cER), 2c deviation index (2cDI), malignancy index (MI), and malignancy grade (MG). 5cER is defined as the percentage of aneuploid cells having a DNA content of more than 5c. 2cDI is defined as the sum of the squares of the differences between the DNA values of single cells and the 2c value, divided by the number of measured cells. It represents, therefore, the mean square deviation from the diploid value. MI es the product of 2cDI and 5cER. The scale of MI stretches from 0 to 3758 (the highest observed MI). The MG is computed from the MI as follows: MG=3xlog (MI+1)/log 3758=0.84xlog (MI+1). The scale of MG stretches from 0.0 to 3.0.

Sampling procedure

New sections of each specimen were performed at different levels. Histological grade was independently established by two observers, according to Scarff-Bloom-

Tabla II: Univariate survival analysis of DNA data

Variable Name	Group Index	Category Name	Frequency Category	Total	Mantel-Cox	P Breslow	Tarone	P Prentice
DI	DNA Index			38	0.0087	0.0089	0.0091	0.0091
Kallionemi 10	1	Diploid	9					
	2	Aneuploid	21					
	3	Tetraploid	2					
	4	Hypertetraploid	6					
G0G1				43	0.5107	0.5506	0.5913	0.5782
	1	<=74.16	29					
	2	>74.16	21					
SPF	S-phase fraction			43	0.0209	0.0214	0.0222	0.0229
	1	<=20	29					
	2	>20	14					
G2M				43	0.0538	0.0550	0.0567	0.0547
	1	<=5.43	22					
	2	>5.43	21					
Thisto 2				43	0.5899	0.5781	0.5666	0.5622
	1	1-5	31					
	2	= 6	12					
Idesviac	Deviation Index			43	0.4218	0.4138	0.4066	0.4037
	1	<=3.35	22					
	2	>3.35	21					
P5CER	5c Exceeding Rate			43	0.5062	0.5369	0.5702	0.5777
	1	<=8.98	22					
	2	>8.98	21					
MI	Malignancy Index			43	0.4856	0.4585	0.4337	0.4215
	1	<=25.08	22					
	2	>25.08	21					
MG	Malignancy Grade			43	0.4856	0.4585	0.4337	0.4215
	1	<=1.19	22					
	2	>1.19	21					

Richardson system (8). Cases in which disagreement existed were jointly reviewed.

The most representative tumor area -usually peripheral areas with high mitotic activity - was selected for DNA analysis, avoiding areas showing necrosis or inflammation. From this area, two five-microns sections (one for hematoxylin-eosin control and one to join the bank that was going to be stained with Feulgen) and ten 50-microns sections were cut. From the last ones, nuclear suspensions were prepared using a modified Hedley technique (9). Sections were deparaffinized in xylene, rehydrated and refixed in 10% neutral formalin solution. They were cut in smaller fragments to facilitate pepsin action, and incubated in a 2 ml solution of 0.5% pepsin (pepsin SIGMA p-7012 0.5 gr, NaCl 0.9 gr, and destilled water 100 ml) at 37°C.

After incubation, tissue fragments were resuspended in 5 ml cold PBS and syringed through the tip of a Pasteur pipette. The suspension was filtered through a 60 microns mesh and centrifugated for 15 min (2000 r.p.m.). The aliquot was then smeared upon a glass slide, and dried in a desiccator for a week. After acid hydrolysis in 5N HCl for 60 min. at 25°C, the nuclei were stained according to Feulgen technique.

DNA-Feulgen analysis

The Feulgen stained tumor cell nuclei were analized with Microm DNA-MIP System. The system uses as quantitative DNA index the parameter IOD (Integrated Optical Density), which integrate in a unique value Nuclear Surface Area and Optical Nuclear Density (IOD=SnxOD).

System calibration was performed with a 450 nuclei control specimen (simultaneously stained with each bank of problem specimens) made up of three nucleated

erytrocytes populations corresponding to three animal species of known DNA content (External Patterns for densitometric calibration of DNA-MIP System from Microm Spain SA, Ref. A3-178). For diploid internal control value 50 small lymphocytes on the same specimen were measured. A minimum of 100 and a maximum of 300 nuclei were measured in every specimen, depending on specimen cellularity. More than 175 nuclei were measured in 80% of the cases. Time of measurement ranged between 10 min. and 1 hour and 30 min., depending on nuclei disintegration.

Statistical methods

Statistical analysis was performed with BMDP computer program (BMDP Statistical Software, Los Angeles, CA) in a Convex C3210 computer. Cumulative survival was estimated with the product limit method, and comparison of cumulative survival between groups was performed with four tests: Mantel-Cox, Breslow, Tarone-Ware and Peto-Prentice (1L subprogram). The relative importance of risk factors was assessed with Cox's proportional hazard model (2L subprogram). 1D and 2D subprograms were used for statistical data description, and correlation matrix between variables was established with the 4M subprogram.

RESULTS

The prognostic significance of classical parameters is shown in Table I. Natural subgroups were established, as well as those that were previously established by other investigators. Location of the tumor (comparing retroareolar with no retroareolar tumors), menstrual status (in 45-55 years interval), tumor size, nodal status, clincal stage, and MPI were significantly correlated with prognosis (p values < 0.05).

Table 2 shows prognostic significance of DNA data. Technical problems (incorrect fixation of the original tumor, low cellularity, bad nuclei disintegration, or too weak Feulgen stain) prevented measurement of seventeen of the specimens. DNA index subgroups were established according to modified Kallioniemi (10) classification: diploid (DI 0.75-1.25), aneuploid (DI 1.25-1.8), tetraploid (DI 1.8-2.2), hypertetraploid (DI > 2.2). Coefficients of variations were next to 10%. Diffuse tumors were excluded from analysis because they were too scanty to constitute an independent subgroup.

We detected 19.6% diploid tumors, 49% aneuploid tumors, 17.6% tetraploid tumors and 13.7% hypertetraploid tumors. 23.5% of the cases showed multiclonality. These cases were considered as an independent group in the assessment of DNA ploidy prognostic significance. and they were excluded of SPF analysis, because it was not possible for us to evaluate SPF when there was more than one neoplastic population.

According with other authors (11), S-phase fraction was considered as the parameter that represents the percentage of cells that are synthesizing DNA. The mean value was 15%. There was a significant correlation between S-phase fraction and DNA ploidy (diploid tumors had the lowest value of SPF, followed by aneuploid tumors, tetraploid tumors and hypertetraploid tumors), S-phase fraction and 2cDI, S-phase fraction and 5cER, S-phase fraction and IM and S-phase fraction and MG. There was also a surprising correlation between S-phase fraction and location, retroareolar tumors having significantly higher S-phase fraction values than the no retroareolar ones. Retroareolar tumors had significantly highest values of DNA index too. A cut point of 20% established the most powerful subgroups.

For 2cDI, 5cER, MI and MG median was used as cut point, since we couldn't find reference points in the literature.

In univariate analysis DNA index (evaluated according previous groups) and S-phase fraction has statistically significant prognostic value, while 2cDI, 5cER, MI, and MG failed to provide prognostic information.

The results of Cox's multivariate analysis are summarized in Table 3. The most important independent prognostic factor was the presence of axillary nodal metastases (p 0'000), followed by DNA index (p 0'017) and location (retroareolar - no retroareolar) (p 0'05).

Table III: Multivariate Survival analysis

Step	Enter/Remove	Variable Name	Improvement CHI-SQ	P Value	Global CHI-SQ	P Value
0						
1	E	Nganga	14.793	0.000	23.188	0.000
2	E	Inddna 2	5.701	0.017	29.521	0.000
3	E	Locali	3.457	0.063	32.037	0.000

DISCUSSION

Since breast cancer is an heterogeneous disease, with different behaviour in patients within the same clinical stage, many attempts have been made to find prognostic indicators which allow patients classification in prognostic groups.

During the last decade, nuclear DNA content has emerged as a new prognostic indicator in breast cancer. Most of the studies on respect have been performed by flow cytometry (2,10, 12-21). Static cytometry studies, in which interactive selection of cells by an experienced pathologist is possible, are fewer, and most of them have been performed on fresh material (1,22,23). The present study has been realized on nuclei smears obtained from 50 microns sections of paraffin wax embedded tissue blocks. This method lacks the problem of DNA image analysis of thin (5 microns) sections, in which most of the nuclei are not completely contained within the section (24). This technique, developed by Hedley and coworkers (25,26), has been perfectioned by Delgado and coworkers, and automated by Babiak and Poppema (27), and several studies have demonstrated a good correlation between results obtained on fresh and paraffin embedded material (3,4).

We obtained a percentage of 79% aneuploid tumors (including tetraploid and hypertetraploid ones). This value is descretly higher than the values obtained by most of authors by flow cytometry (17,28-30), what we attribute to greater sensibility of static cytometry in detection of aneuploid peaks (31,32). Coefficients of variation (CV) were next to 10%, which coincides with those obtained by other investigators on deparaffined specimens of breast carcinoma (33).

Using Kallioniemi (10) histogram classification, DNA index appears as a strong prognosticator in invasive ductal breast carcinomas in univariate analysis, as an independent prognostic factor in multivariate analysis, and as the second most powerful factor after nodal status. Our results agree with those of Opfermann (34), Koss (1), Longin (23) and Arnëlov (22), whose studies were realized by image cytometry on fresh material, and with those of Auer (35), whose study, performed on paraffin embedded material by citodensitometry, was the first retrospective analysis about prognostic value of DNA analysis in breast carcinoma. We have not used Auer's histogram classification (group I, diploid tumors: group II, markedly aneuploid tumors, and group III and IV, tetraploid tumors or tumors with a combination of diploid and tetraploid patterns), because it forced us to exclude from the study a great number of cases (hypertetraploid and mosaicisms other than diploid-tetraploid patterns of Auer). Analizing paraffin-embedded material by flow cytometry, DNA index appeared as a significant prognostic factor in the series of Stanton (36), Joensuu (37), and Witzig (38). This author (38) suggests that sonication of samples better quality histograms by reducing debris and nuclear clumpling. We have tried to improve nuclei disintegration by sonicating nuclear suspensions for ten minutes, and increasing time and temperature of pepsinization, and the results obtained were bad (smears results in clots of nuclear material weakly stained).

When we analized DNA index as a continuous variable, it was not statistically significant as prognostic factor. In this way, Uyterlinde (15) finds a weak relation between DI and prognosis.

There is not general agreement on which parameter of tumoral kinetics defines the percentage of cells that are synthesizing DNA; S-phase fraction for some authors and combined S-phase fraction and G2-M fraction for others. According with Sharma (11) who demonstrated that G2-M phase fraction loses all prognostic effect when S-phase fraction is eliminated, we have considered S-phase fraction alone. In our study, we obtained S-phase fraction values sensibly greater than the ones obtained for most authors (mean value 15% on respect 11% of most authors), what may be due to most studies on mammary cancer have been made by flow cytometry, in which is not possible selecting neoplasic cells from non neoplasic ones. That means that non neoplasic cells, with a low S-phase fraction, contribute to resuce S-phase fraction on respect total (in diploid histograms in which neoplasic cells occupy the same numeric channel than non neoplasic ones). In out series, S-phase fraction was lower than mean value in diploid tumors (9%), and higher in tetraploid (19'4%) and hypertetraploid ones (22.02%). Aneuploid tumors had values very close to the mean (14.2%), owing to they were the most numerous group in our series. According with most authors (20,39-43), S-phase fraction was, in our study, a significant prognostic factor (p value 0.04). Cut-off level was established in 20% (higher than cut-off level established in most of flow cytometry studies, owing to the former reasons) the prognosis being better in patients with S-phase fraction lower than 20%. Retroareolar tumors had a significantly higher S-phase fraction than no retroareolar ones, besides significantly higher values of DI. This suggests a peculiar biology of retroareolar tumors, although longer studies are necessary to confirm this aspect.

5c Exceeding rate, 2c Deviation Index, Malignancy Index and Malignancy Grade are a group of algorithms defined by Böcking (7) to process data obtained from DNA content by image analysis. The aim of Böcking was to get a system of objective discrimination between benign and malignant lesions, besides a system of objective gradation of tumor malignancy in a continuous scale of tumor grades.

In univariate analysis we have established median value of each parameter as cut-off level, and we have failed to demonstrate any prognostic significance in none of them. We are, then, discreetly sceptical about Böcking algorithms, although it would be necessary to

analyze a greater number of cases to evaluate them accurately. The fact that they are parameters scantly divulged in literature (we have found just one study (44) which alludes to 5c Exceeding rate and Malignant Grade of Böcking), without reference values of cut-off points, may have influenced negatively our results.

The classical parameters location (as retroareolar - no retroareolar), tumor size, nodal status, clincal stage and MPI appeared as prognostic indicator in univariate analysis. Season of the year at diagnosis, patient age, menstrual status and histological grade were not significantly correlated with prognosis (Table I). Although location classification in quadrants seemed to be statistically significant as prognostic factor, results are not valuable because of the few total cases located in internal quadrants in our series.

Multivariate analysis results are summarized in Table III. The most important independent factor was the presence of axillary nodal metastases, followed by DNA index (according Kallioniemi classification), and tumor location (as retroareolar - no retroareolar). Our findings coincide with those obtained by Joensuu (18), Merkel (20), Sharma (11), Kallionemi (10) and Lewis (17), in whose studies DNA index appeared as an independent prognosticator that add new information to that obtained from classical histopathologic and clinical parameters. DNA index lost prognostic significance in multivariate analysis in the series of Stanton (35), Noguchi (21) and Clark (16).

Our results confirm nuclear DNA content as an objective marker of tumor aggressiveness that can significantly enhance our prognostic capabilities, and support the role of image analysis DNA content on paraffin-embbeded material to assess the prognosis of invasive ductal breast carcinoma.

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RESUMEN

Con el fin de evaluar el papel de la citometría de imagen en el establecimiento del pronóstico del carcinoma de mama, hemos realizado un análisis de DNA por citofotometría estática sobre frotis obtenidos a partir de suspensiones celulares de 60 casos de carcinoma ductal invasor incluídos en parafina. La significación pronóstica de los parámetros extraídos del histograma de DNA ha sido contrastada con la proporcionada por otros factores clínicos y patológicos (edad, status menstrual, status ganglionar, estación del año en el momento del diagnóstico, localización del tumor, tamaño tumoral, grado histológico, estadío clínico e índice pronóstico morfométrico multiparamétrico). Además, se ha realizado un análisis multivariante con el fin de determinar cuales de los factores pronósticos significativos proporcionan información pronóstica independiente y cual es el parámetro de mayor valor pronóstico.

En el análisis univariante, el status ganglionar, la localización (como retroareolar - no retroareolar), el tamaño del tumor, el estadío clínico, el Indice Pronóstico Morfométrico (MPI), el Indice de DNA y la fracción de células en fase S (SPF), aparecían significativamente relacionados con el pronóstico ($p < 0.05$). En el análisis multivariante sólo el status ganglionar, el Indice de DNA y la localización, por ese orden, se comportaron como factores pronósticos independientes, por lo que podemos concluir que el análisis del Indice de DNA por citofotometría estática proporciona información pronóstica adicional en el establecimiento del pronóstico del carcinoma ductal invasor de mama.

Palabras clave: Cancer de mama. Ploidía de ADN. Citometría estática. Tejido incluído en parafina.

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