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QUANTITATIVE HISTOPATHOLOGY
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QUANTITATIVE METHODS IN HISTOPATHOLOGY: EVALUATION OF THEIR PROGNOSTIC POWER IN INFILTRATING DUCTAL BREAST CARCINOMA

Stefano SISTI, Alfredo SANTINELLI, Mirca VALLI, Roberta TABORRO, Bruno MANNELLO, *Gianmario MARIUZZI

Department of Pathology, University of Ancona, Ospedale Nuovo Regionale, I-60020 Torrette di Ancona, Italy. *Department of Pathology, University of Verona, Policlinico Borgo Roma, I-37100 Verona, Italy

ABSTRACT

The value of quantitative histopathologic analysis in prognosis of infiltrating ductal breast carcinoma was assessed. The study was carried out on breast tumours at an early clinical stage, with diameters below 2.5 cm. Ninety cases were studied. At the end of the study there were 45 deceased and 45 surviving patients, the latter with a follow-up of at least 69 months.
Quantitative histopathologic analysis was carried out by simultaneously measuring a number of geometric features in the nuclei and nucleoli of each tumour. In addition, mitotic activity and the percentage of immunohistochemically positive cells for proliferating cell nuclear antigen (PCNA) were recorded in each case. The study showed many statistically significant differences ($p < 0.05$) between the two groups of deceased and surviving patients. However, very few differences were observed when the groups of patients with and without axillary lymph node metastases were compared. Moreover, no significant differences were found between deceased patients with short ($\leq$ 30 months) and long (> 30 months) survival. With multivariate analysis (forward stepwise discriminant analysis) it was possible to produce a canonical discriminant function by which the deceased and surviving patients were correctly classified in 92.2% of the cases. The results stress the usefulness of quantitative methods in the prognostic assessment of breast cancer, as well as the possibility of applying this approach to cytologic material, in order to preoperatively identify patients at high risk for death.

Key words: breast cancer, image analysis, immunohistochemistry, prognosis, quantitative pathology.

INTRODUCTION

Although breast carcinoma is one of the most frequently diagnosed human tumours, and despite the great number of studies related to this topic, considerable controversy still exists about the choice of the most appropriate therapeutic strategies, as well as about the reliability of the clinico-pathologic prognostic criteria presently available. The 5-year-mortality can be estimated as about 40% of the cases (van Diest, 1990).
The most widely accepted therapeutic protocols suggest that chemo- and radiotherapy are reserved for patients with axillary lymph node metastases at the time of surgery. However, 20-35% of the patients without lymph node metastases at the time of surgery die of the disease within 5 years. Another relevant prognostic indicator of breast carcinoma is the tumour size. The current staging systems are based just on these two features, and systemic metastases (TNM staging, based on Tumor size, Nodal status, distant Metastases).
Breast cancer screening has led to the detection of smaller and smaller breast carcinomas and the incidence of patients with axillary lymph node metastases is also decreasing. Because of the improvement in diagnosing early breast carcinoma (at the T_1N_0 or T_2N_0 stage), the prognostic value of the classic criteria of prognosis is becoming limited. Further prognostic information

can be obtained from histopathologic grading of breast carcinomas, which is still performed according to the criteria formulated in 1957 by Bloom and Richardson, but the subjective grading performed by the pathologist is affected by the low diagnostic reproducibility between different observers (Stenkvist et al., 1979; Delides et al., 1982).
To find new prognostic features, we have investigated the quantitative histopathologic analysis in the prognostic assessment of breast carcinomas.

MATERIALS AND METHODS

The histologic slides of all cases of infiltrating ductal breast carcinoma observed between 1977 and 1985 were retrieved from the files of the Department of Pathology of the University of Ancona. All patients had undergone partial or radical mastectomy plus axillary lymph node dissection. Only the cases which met the following criteria were included in the study: a) tumour diameter not exceeding 2.5 cm; b) a minimum of five axillary lymph nodes available for assessing lymph node status; c) no distant metastases at the time of the surgical operation; d) histologic diagnosis of infiltrating ductal carcinoma; e) follow-up of at least six years.
The mean age of the patients was 56.8 years, with a range of 33-76 years; of these, 45 died of the disease during the follow-up period, with a mean survival of 41.9 months and a range of 11-121 months; the remaining 45 patients were alive without disease at the end of the follow-up, the mean duration of which was 106.3 months, with a range of 69-141 months. Axillary lymph node metastases were detected in 48 cases, while the remaining 42 had negative (non metastatic) nodes; by grouping patients according to survival and lymph node status data, the following four categories were obtained: a) 26 deceased patients with positive axillary lymph nodes at the time of the surgical operation; b) 19 deceased patients with negative lymph nodes; c) 22 surviving patients with positive lymph nodes; d) 23 surviving patients with negative lymph nodes. The mean diameter of the tumours was 2.02 cm, with a range of 1-2.5 cm.
Surgical specimens were routinely fixed in 10% formalin and embedded in paraffin. In each case, the most representative specimen of the tumour was selected from the archive material; two 5-μm-thick histologic sections were stained with Haematoxylin-Eosin and with the immunohistochemical method for detecting proliferating cell nuclear antigen (PCNA). The latter stain was applied by using a mouse anti-human PCNA monoclonal antibody (Dako, Glostrup, Denmark), diluted 1:10, incubated overnight at 4 °C and revealed with an avidin-biotin-peroxidase system (LSAB-HrPo Kit, Dako).
All quantitative analyses were carried out on the most actively proliferating tumour area, in which the most prominent cytologic atypias as well as the highest cellularity and mitotic activity were observed; this area was usually found at the tumour periphery. A number of geometric nuclear and nucleolar variables of the neoplastic population were determined on Haematoxylin-Eosin stained sections, using a Kontron-IBAS automatic digital image analyser (Kontron, Munich, Germany), equipped with an RGB camera (Grundig FAC 73, Grundig, Germany) and a Zeiss optical microscope (Zeiss, Oberkochen, Germany). In each case, representative neoplastic fields were randomly selected, in which all clearly defined nuclear profiles were measured, up to a number of 50 elements per case, at 1000x final magnification (objective magnification 100x; ocular magnification 10x). The mean and the standard deviation (S.D.) of the following nuclear and nucleolar variables were determined: nuclear area (μ^2), nuclear perimeter (μ), maximum nuclear diameter (μ), nuclear form factor, nucleolar area (μ^2), ratio of nucleolar area to nuclear area. Nuclear form factor (F.F.) is expressed as:

$$F.F. = P^2 / 4 \pi A \quad (1)$$

where P and A stand for the nuclear perimeter and area, respectively. The percentage of nucleolated nuclei was also determined on the same histologic sections; 500 consecutive nuclei from fields randomly selected in the most representative neoplastic area were evaluated, at 400x microscope magnification (objective magnification 40x; ocular magnification 10x). Furthermore, the volume-corrected mitotic index (M/V index) according to Haapasalo (Haapasalo et al., 1989a, 1989b, 1990) was determined, from the same neoplastic areas and at the same microscope magnification as for the percentage of nucleolated nuclei. Proliferative

activity of the breast tumours was evaluated immunohistochemically on sections stained with the anti-PCNA antiserum; the same neoplastic fields selected for the above measurements were identified on the PCNA-stained slides, and the percentage of PCNA-positive nuclei was established by counting 500 consecutive cells from randomly-selected fields at 400x microscope magnification.
Statistical analysis was carried out using the BMDP package (Berkeley, California, U.S.A.). The statistical significance of the differences observed between deceased and surviving patients, for the group means of any given variable studied, was measured with a t-test (program BMDP3D), adopting the significance level of $p < 0.05$. A multivariate analysis method (forward stepwise discriminant analysis) was also applied to the groups of deceased and surviving patients using the BMDP7M program.

RESULTS

The analysis of the distribution of values of the quantitative variables studied has shown the presence of numerous statistically significant differences between the groups of deceased and surviving patients. Table 1 shows the values of the group means and S.D. for the variables studied, together with the level of significance of the differences observed.
Deceased patients are characterised by higher values of nuclear size (expressed by the mean values of nuclear area, perimeter and diameter) than the survivors. The same trend is observed when considering the S.D. values of the variables studied. The nuclear form factor does not show any significant changes in the two groups, either for the mean or S.D. values. Nucleolar geometric variables (mean and S.D. values of nucleolar area and nucleolar/nuclear ratio, and particularly the percentage of nucleolated cells) are the most discriminant between the two classes (Fig. 1). The variables concerning the degree of cellular proliferation (mitotic activity, evaluated as the M/V index, and the percentage of PCNA-positive nuclei) also showed a

Table 1. List of the quantitative variables studied in respect to survival. For each variable, the mean and the S.D. of the results in the groups of deceased (45 cases) and surviving (45 cases) patients are indicated. Significance level: $p < 0.05$ (n.s.: not significant difference).

Variable	Deceased	Surviving	p
Mean nuclear area	59.36 ± 17.10	49.16 ± 12.10	0.002
S.D. of nuclear area	16.51 ± 5.60	12.23 ± 3.94	<0.001
Mean nuclear perimeter	28.29 ± 4.30	25.96 ± 3.21	0.005
S.D. of nuclear perimeter	4.00 ± 1.00	3.24 ± 0.79	<0.001
Mean nuclear diameter	10.27 ± 1.58	9.32 ± 1.13	0.002
S.D. of nuclear diameter	1.64 ± 0.37	1.34 ± 0.29	<0.001
Mean nuclear form factor	1.11 ± 0.06	1.13 ± 0.10	n.s.
S.D. of nuclear form factor	0.07 ± 0.03	0.08 ± 0.03	n.s.
Mean nucleolar area	4.28 ± 1.55	2.69 ± 1.05	<0.001
S.D. of nucleolar area	1.44 ± 0.69	0.91 ± 0.38	<0.001
Mean nucleolar/nuclear ratio	7.43 ± 1.61	5.56 ± 1.52	<0.001
S.D. of nucleolar/nuclear ratio	2.43 ± 0.69	1.76 ± 0.52	<0.001
% of nucleolated cells	78.04 ± 7.41	55.45 ± 11.28	<0.001
M/V index	29.13 ± 20.17	17.31 ± 14.11	0.002
% of PCNA-positive cells	22.24 ± 16.79	10.77 ± 9.73	<0.001

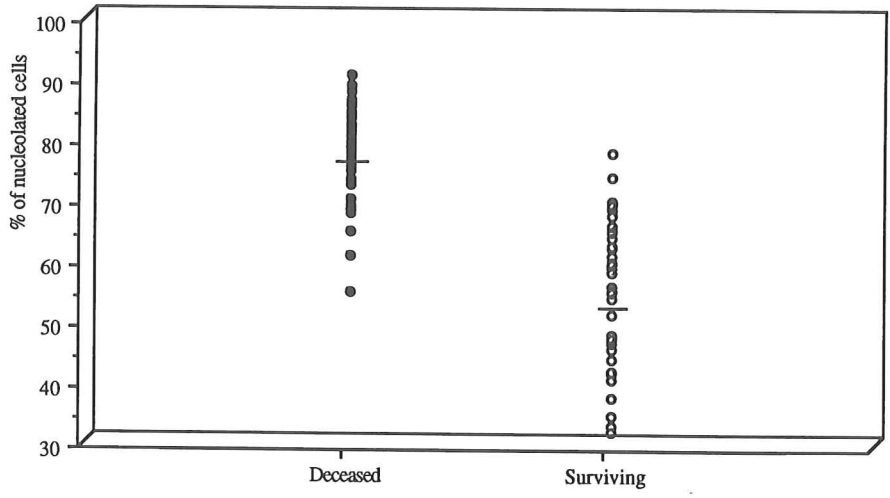

Fig. 1. Scattergram of the distribution of percentage values of nucleolated cells in the groups studied.

Table 2. List of the quantitative variables studied in respect to association with lymph node metastases among deceased patients. For each variable, the mean and the S.D. of the results in the groups of deceased patients with (26 cases) and without (19 cases) lymph node metastases are indicated. Significance level : $p < 0.05$.

Variable	Patients with metastases	Patients without metastases	p
Mean nuclear area	61.73 ± 15.33	56.12 ± 19.20	n.s.
S.D. of nuclear area	15.56 ± 3.90	17.80 ± 7.24	n.s.
Mean nuclear perimeter	28.90 ± 3.96	27.45 ± 4.71	n.s.
S.D. of nuclear perimeter	3.70 ± 0.74	4.41 ± 1.80	0.0186
Mean nuclear diameter	10.46 ± 1.41	10.02 ± 1.80	n.s.
S.D. of nuclear diameter	1.53 ± 0.30	1.78 ± 0.42	0.0225
Mean nuclear form factor	1.11 ± 0.07	1.12 ± 0.03	n.s.
S.D. of nuclear form factor	0.07 ± 0.02	0.09 ± 0.03	0.0251
Mean nucleolar area	4.73 ± 1.19	3.66 ± 1.80	0.0209
S.D. of nucleolar area	1.51 ± 0.66	1.35 ± 0.75	n.s.
Mean nucleolar/nuclear ratio	8.00 ± 1.32	6.64 ± 1.68	0.0039
S.D. of nucleolar/nuclear ratio	2.45 ± 0.65	2.41 ± 0.76	n.s.
% of nucleolated cells	79.31 ± 6.26	76.30 ± 8.61	n.s.
M/V index	34.76 ± 21.45	21.43 ± 15.73	0.0269
% of PCNA-positive cells	26.38 ± 13.87	11.78 ± 12.75	n.s.

Table 3. List of the quantitative variables studied in respect to association with lymph node metastases among surviving patients. For each variable, the mean and the S.D. of the results in the groups of surviving patients with (22 cases) and without (23 cases) lymph node metastases are indicated. Significance level: $p < 0.05$.

Variable	Patients with metastases	Patients without metastases	p
Mean nuclear area	49.71 ± 11.04	48.62 ± 13.25	n.s.
S.D. of nuclear area	12.47 ± 4.17	12.01 ± 3.79	n.s.
Mean nuclear perimeter	26.05 ± 2.98	25.86 ± 3.47	n.s.
S.D. of nuclear perimeter	3.29 ± 0.91	3.20 ± 0.69	n.s.
Mean nuclear diameter	9.42 ± 1.13	9.23 ± 1.14	n.s.
S.D. of nuclear diameter	1.34 ± 0.32	1.34 ± 0.27	n.s.
Mean nuclear form factor	1.12 ± 0.10	1.14 ± 0.10	n.s.
S.D. of nuclear form factor	0.07 ± 0.02	0.08 ± 0.04	n.s.
Mean nucleolar area	2.73 ± 1.08	2.65 ± 1.03	n.s.
S.D. of nucleolar area	0.91 ± 0.37	0.92 ± 0.39	n.s.
Mean nucleolar/nuclear ratio	5.51 ± 1.48	5.61 ± 1.58	n.s.
S.D. of nucleolar/nuclear ratio	1.68 ± 0.43	1.83 ± 0.59	n.s.
% of nucleolated cells	58.31 ± 7.57	52.72 ± 13.56	n.s.
M/V index	14.21 ± 7.57	20.29 ± 18.01	n.s.
% of PCNA-positive cells	9.40 ± 11.28	8.08 ± 5.84	n.s.

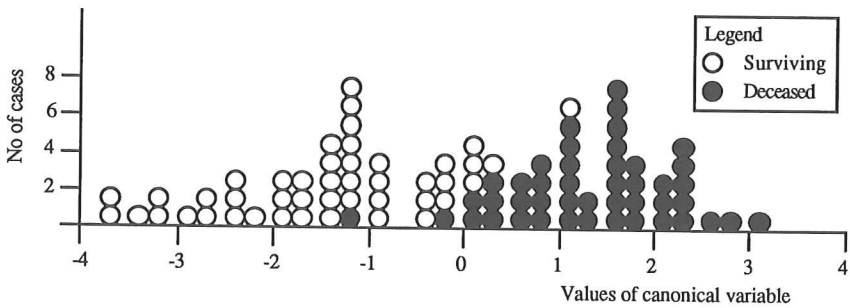

Fig. 2. Distribution of values of the canonical variable calculated in 90 cases of ductal infiltrating breast carcinoma. In the graph the values of the individual cases have been approximated to fit into columns one fourth of unit length wide (canonical variable).

significant increase in values in the group of deceased patients.

When dividing the deceased and surviving patients on the basis of lymph node status, the differences observed are non-significant for the majority of the variables in the group of deceased patients (Table 2), whereas the values of the remaining variables are sometimes higher in the node-negative group and sometimes in the other. Moreover, when considering surviving patients (Table 3), no variable shows statistically significant changes in the groups of node-positive and -negative patients.

In order to evaluate the contribution of the different variables studied in determining the final outcome of the patients, a method of multivariate statistical analysis has been applied (forward

Table 4. List of the quantitative variables studied in respect to short or long survival among deceased patients. For each variable, the mean and S.D. of the results in the groups of deceased patients with short (≤ 30 months) and long (> 30 months) survival are indicated (25 and 20 cases, respectively). Significance level: $p < 0.05$.

Variable	Short Survival	Long Survival	p
Mean nuclear area	57.24 ± 14.24	62.01 ± 20.19	n.s.
S.D. of nuclear area	17.51 ± 6.16	15.25 ± 4.65	n.s.
Mean nuclear perimeter	27.83 ± 3.48	28.86 ± 5.19	n.s.
S.D. of nuclear perimeter	4.31 ± 1.04	3.61 ± 0.82	0.0174
Mean nuclear diameter	10.11 ± 1.40	10.47 ± 1.80	n.s.
S.D. of nuclear diameter	1.72 ± 0.39	1.53 ± 0.33	n.s.
Mean nuclear form factor	1.12 ± 0.04	1.10 ± 0.07	n.s.
S.D. of nuclear form factor	0.08 ± 0.03	0.07 ± 0.03	n.s.
Mean nucleolar area	4.19 ± 1.36	4.37 ± 1.79	n.s.
S.D. of nucleolar area	1.45 ± 0.63	1.42 ± 0.79	n.s.
Mean nucleolar/nuclear ratio	7.66 ± 1.81	7.13 ± 1.32	n.s.
S.D. of nucleolar/nuclear ratio	2.53 ± 0.83	2.32 ± 0.47	n.s.
% of nucleolated cells	76.48 ± 7.13	79.99 ± 7.46	n.s.
M/V index	34.39 ± 23.62	22.55 ± 12.48	0.0493
% of PCNA-positive cells	22.63 ± 17.82	21.76 ± 15.88	n.s.

stepwise discriminant analysis). The canonical discriminant function (C.D.F.) obtained includes the following variables: percentage of nucleolated cells (a), S.D. of nuclear perimeter (b), mean nuclear diameter (c), M/V index (d), combined according to the formula:

$$C.D.F. = 0.11a + 0.55b - 0.33c + 0.017d - 6.47 \quad (2)$$

The canonical variable assumes positive values in patients classified as deceased, while negative values imply a classification among survivors. The solution produced (Fig. 2) correctly classified 43 out of 45 deceased patients (95.6%) and 40 out of 45 survivors (88.9%), thus providing 2 false negative cases (incorrectly predicted as survivors) and 5 false positive cases (incorrectly predicted as deceased), i.e., an accuracy of 92.2%.
By considering only the deceased patients, the possibility of recognising the cases with a short survival has been checked with quantitative methods: the list of values of the variables shows that, in general, there are no statistically significant differences between the two groups (Table 4). Anyway, by graphically examining the values of the canonical variable plotted against survival time in months (Fig. 3), it can be observed that, when arbitrarily introducing a threshold value of 1.50 for the canonical variable, 24 out of 45 (53.3%) deceased patients are identified with feature values beyond the threshold; 16 of these (66.7%, or 35.6% of all deceased patients) have a short (< 30 months) survival.

DISCUSSION

Prognostic assessment of breast carcinoma is a very difficult task for the clinician and the pathologist, because of the difficulty in distinguishing cases with a rapid and aggressive course from those evolving in a slow and indolent manner; the distinction is relevant because of the different therapeutic management required for the two types of tumours. The role of

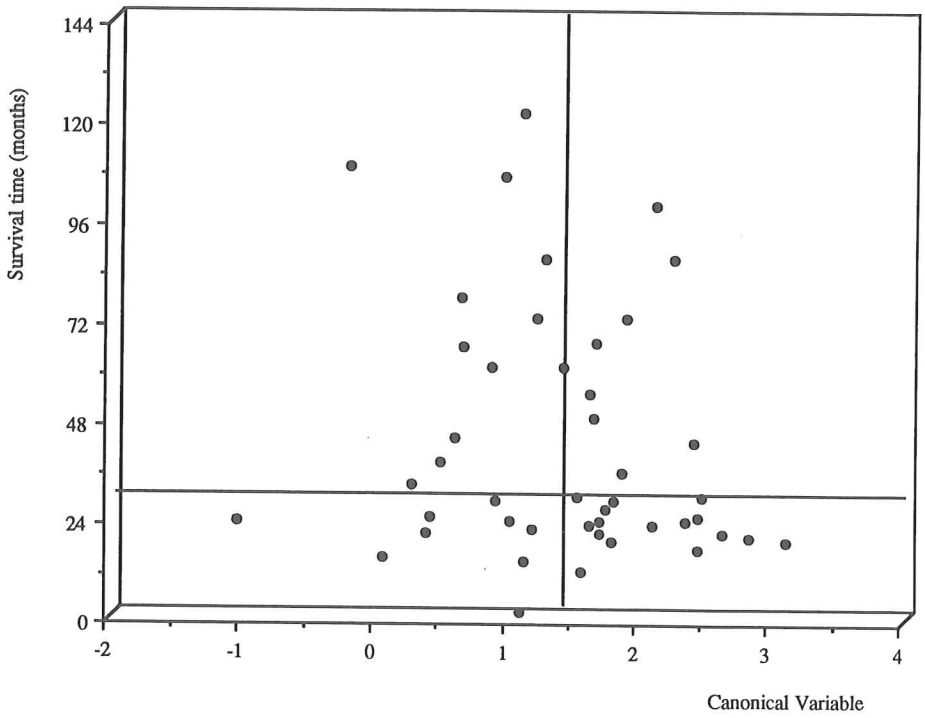

Fig. 3. Scattergram of values of the canonical variable in deceased patients, plotted against survival time. It is possible to identify a relevant proportion of patients with a short survival.

quantitative pathology in this field is twofold, aiming firstly at reducing the subjective component of the classic clinical and pathologic prognostic criteria (TNM staging, histologic grading); moreover, quantitative methods are intended to increase the accuracy of prognosis. Prognosis of any individual case is still based on a few features, such as tumour size, lymph node status and histopathologic grading; as a consequence, a more accurate identification of the biologic characteristics (and therefore of the prognosis) of any newly diagnosed breast tumour can only be achieved by increasing the number of features of the neoplastic population to be simultaneously evaluated with quantifiable, reproducible and thus objective criteria; adopting this approach we were able to improve the prognostic assessment considerably with respect to current methods.

Many quantitative differences in the histopathologic features of deceased and surviving patients have been identified, with particular reference to all nucleolar features: the most discriminant one is the percentage of nucleolated nuclei, but the additional nucleolar features (mean and S.D. values of nucleolar area and of nucleolar/nuclear ratio) also contribute to identifying cases with fatal outcome. These findings are in keeping with literature data, which point to the correlation between the degree of nucleolar changes and the actual malignancy degree of the individual tumours; studies leading to these conclusions have been carried out not only on breast carcinoma (Baak, 1985; Mariuzzi et al., 1989; van Diest, 1990), but also on many other tumours such as lymphomas (van der Valk et al., 1983), prostatic adenocarcinomas (Helpap, 1988), ocular melanomas (Gamel and McLean, 1983) and thyroid nodules (Mariuzzi et al., 1988). As for the biologic significance of these findings, it has been suggested that nucleolar activation, which expresses an increase in cellular metabolism, is correlated to increased cell motility and, as a consequence, to increased metastatic potential (Busch and Smetana, 1970).

The increase in the mean and S.D. values of nuclear geometric variables also appears important, even if to a lesser extent, in identifying patients with bad prognosis. S.D. value changes in deceased patients reflect an increase in phenotypic heterogeneity of the neoplastic population, which is considered an unfavourable prognostic marker.

Many recent papers (Baak et al., 1982; Baak et al., 1985; Russo et al., 1987; le Doussal et al., 1989) have drawn attention to the prognostic significance of mitotic counts in breast carcinoma; this estimate of neoplastic proliferative activity is easily obtained on routine material, can be quickly performed in any laboratory since it does not need complex equipment, and has recently been made more accurate, after the introduction of new quantitation techniques such as the volume-corrected mitotic index (M/V index of Haapasalo et al., cit.) and the number of mitoses per square millimeter of neoplastic tissue (Laroye and Minkin, 1991). In this study M/V index is one of the most discriminant variables between deceased and surviving patients; nevertheless, the values obtained overlap largely in the two groups: in fact, some of the highest values have been observed among survivors, and this is at variance with the results reported in other papers (Baak et al., 1985), according to which mitotic index is by far the best histopathologic prognosticator in breast carcinoma. M/V index shows remarkably different values with respect to another cell proliferation marker, the percentage of PCNA-positive cells. This finding is in keeping with literature data underlining the poor correlation between S-phase fraction (as determined with flow cytometry) and PCNA immunoreactivity (Hall et al., 1990), related to PCNA overexpression in breast carcinoma; this could in turn be related to a deregulation in the control mechanism of the oncoprotein expression (Hall et al., cit.).

Lymph node status has not proven to be prognostically useful in this study; this result can be explained by the relation existing between lymph node status and tumour size (Carter et al., 1989): lymph node status is devoid of prognostic significance in tumours with homogeneous size, as is the case with our material; as already mentioned, we selected relatively small tumours for the study, because of the increasingly rare clinical occurrence of breast tumours diagnosed at a more advanced stage.

The values of each feature studied, although statistically useful in distinguishing the deceased patients from the survivors, overlap largely; thus, the prognostic power of any variable is limited when considered separately from the others; even for the most discriminant variable in our material, the percentage of nucleolated nuclei, the values observed in the two groups overlap in a region including over half of all cases (Fig. 1). It seems therefore necessary that any appropriate prognostic assessment of breast carcinoma should involve the use of multivariate methods, in order to simultaneously take into consideration as many morphologic features of the neoplastic population as possible. The need for a multivariate approach lies in the fact that each morphologic variable seems to be related to a corresponding peculiar change in the neoplastic population, which is presumably determined genetically (Mariuzzi et al., 1992a). The possibility of defining a phenotypic profile for any of the genetically heterogeneous cell clones of a malignant tumour appears very important since any neoplastic cell clone (having its defined genotype) is more or less stable from a genetic point of view: more heterogeneous neoplastic populations possess a greater degree of genetic instability, which is the basis for the emergence of newly-established cell clones with further genetic damage and a selective growth advantage (Poste et al., 1981; Volpe, 1988; Weiss, 1990), whose acquisition constitutes the key feature in malignancy progression of tumours. Therefore, the morphologic assessment of the phenotypic heterogeneity of any tumour is essential in defining its genetic instability and consequently its degree of malignancy, the risk for further progression and, finally, prognosis (Mariuzzi et al., 1992b).

Multivariate analysis of our data produced interesting results, because it selected different features which have all been claimed to be linked with prognosis: the canonical discriminant function includes a feature expressing nucleolar activation (percentage of nucleolated cells), features linked to the degree of phenotypic cell changes as well as to the phenotypic heterogeneity of the tumour (mean nuclear diameter and S.D. of nuclear perimeter) and a feature of altered cell proliferation (mitotic activity, evaluated as M/V index). Moreover, our results suggest a useful application in daily practice: it seems necessary to check whether the same results could be obtained when applying the quantitative approach to cytologic material obtained preoperatively, in order to identify groups of patients at different degrees of risk for death and to allow the most appropriate and effective treatment to be selected for each case.

In conclusion, methods of quantitative histopathologic analysis have led to an improvement in defining breast cancer prognosis; moreover, from a theoretical point of view, the identification and quantitative evaluation of the morphologic changes in the neoplastic population can be considered as a tool for assessing the sequence of genetic alterations which constitute the basic phenomenon of neoplastic transformation and malignancy progression.

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