Prognostic value of nucleolar morphometric variables in cytological breast cancer specimens

P J van Diest, J Mouriquand, N W Schipper, J P A Baak

Abstract

In a retrospective study on cytological specimens from 86 patients with histologically confirmed invasive breast cancer, the prognostic value of nucleolar morphometric variables was studied and compared with nuclear variables. One hundred nuclei and their nucleoli on each slide were measured with a graphic tablet system at a total magnification of 2800 times using a stratified selection method. The number of nucleoli per 100 nuclei was also noted. Analysis of Kaplan-Meier univariate recurrence free survival curves showed significant differences for eight nuclear features, nine nucleolar features, and three combined nuclear and nucleolar variables. The total number of nucleoli per 100 nuclei was the best single prognostic variable. Multivariate survival analysis (Cox regression model) showed that no other features provided additional prognostic information beyond that given by the total number of nucleoli.

It is concluded that nucleolar morphometric variables assessed in cytological preparations have prognostic value in breast cancer, and the results of this study suggest that their prognostic value may exceed that of nuclear variables.

Methods

Ninety eight patients diagnosed between 1975 and 1985 at the hospital attached to the University of Grenoble, France, were studied. The mean age was 54.5 (range 28-88) years at the time of diagnosis, and the follow up was 56 months on average (range six-120). All patients were submitted to the same diagnostic procedure (physical examination, fine needle aspiration biopsy, mammography) which permitted preoperative diagnosis of breast cancer (with postoperative histological confirmation) in all cases, and underwent the same radiotherapy and surgery. Adjuvant chemotherapy (cyclophosphamide methotrexate 5-fluorouracil) was given to patients with unfavourable factors such as axillary lymph node disease, absence of oestrogen receptors, or grade III cytologic prognostic (unfavourable prognosis) or histology. Most of the remaining patients received adjuvant hormonal treatment, except the patients with grade I cytoprognosis (favourable prognosis). There were no follow up data for two patients, leaving 96 patients.

Imprint slides were made from the resected tumour at the time of surgery, immediately fixed in methanol/acetone (50/50), pH 8.1, and stained according to a hypocromic Papanicolaou staining procedure, which delicately outlines the distribution of chromatin and
visualises nucleoli. One imprint from each patient was considered. Nine slides could not be measured because of low cellularity and one slide because of poor quality, so that 86 cases remained.

On each slide an area with the most atypical cells was selected and marked. In these areas vertical zones of 70 μm in width were scanned using an ocular grid. All clearly outlined, malignant nuclei whose centre of gravity was within these lines were measured, as well as their nucleoli (defined as conspicuous nuclear bodies with an area 1-0 μm² or greater) up to a sample size of 100 cells per slide. A preliminary methodological experiment performed in one of the slides (previously described in detail) to assess experimentally the required sample size, showed that with this sample size highly reproducible results (standard error of the mean <2%) for both nuclear and nucleolar area could be obtained. In these 100 nuclei the total number of nucleoli was also registered.

The reproducibility of all measurements and countings was further tested by duplicate measurements of all variables in 10 slides. Correlation coefficients were, in general, high (mean nucleolar area 0-99, SD 0-80; total number of nucleoli/100 nuclei 0-98), thus confirming the high reproducibility of the measurements. Poorly stained, or fixed, and clotted nuclei were not measured. All measurements were carried out on a graphic tablet linked to a Mop Videoplan (Kontron, Munich, West Germany) at a final magnification of 2800 times (1000 times microscope magnification, projection factor of 2.8). At this magnification, even nucleoli can be accurately measured.

A total of 42 morphometric variables were considered (table) including nuclear and nucleolar features, nucleolar frequency (expressed as the total number of nucleoli per 100 nuclei), and combined nuclear/nucleolar variables such as nucleolar:nuclear area ratio and nucleolar eccentricity.

For statistical analysis, recurrence free survival (defined as the time between date of operation and local recurrence or the first metastasis) was used as a follow up variable. The follow up term was cut off at six years as very few patients had longer follow up.

Univariate survival analysis was performed according to Kaplan-Meier distribution. For this analysis the patients were divided into three groups of roughly the same size on the basis of measurement values. Differences between the curves were analysed using the Mantel-Cox test, with p values below 0.05 as significant. Multivariate analysis to evaluate the additional prognostic value of the features was performed with the Cox regression model.

All analyses were carried out with the BMDP statistical package, using the program’s life tables (P1,1) and survival analysis with covariates (P1,1).

Results

The table shows the results of the univariate Mantel-Cox survival analysis. Eight nuclear variables, nine nucleolar variables, and three

![Figure 1](http://jcp.bmj.com/)

Kaplan-Meier survival curves of patients subdivided according to nuclear area.

![Figure 2](http://jcp.bmj.com/)

Kaplan-Meier survival curves of patients subdivided according to percentage of nuclei without nucleoli.
combined variables gave significant p values. Figs 1–4 show the survival curves of the most significant variables.

Multivariate analysis showed that no variable had additional prognostic value once the total number of nuclei per 100 nuclei was considered.

Discussion

Of the significant nucleolar variables, nucleolar frequency and the standard deviation of the nucleolar area (SDNA) were the best prognostic variables. The strong prognostic value of the SDNA is in agreement with previous studies on nuclei in histological sections of breast cancer,7 lymphomas,7 and ocular melanomas.5 The prognostic value of nucleolar frequency was recently observed in prostatic tumours.3 The previously reported prognostic value of the SD of nucleolar area4 could not be reproduced in this study. This may have been due to differences in fixation and staining techniques or in patient selection.

In multivariate analysis only one feature emerged: the total number of nuclei per 100 nuclei. None of the other variables was selected in multivariate analysis, indicating that the rejected variables contain no additional prognostic information. This variable is the more interesting because its assessment requires no special equipment, and the reproducibility is excellent.

It was very interesting to observe that in this study the nucleolar variables were more powerful from a prognostic point of view than the traditional nuclear variables such as area. Nuclear area is believed to be correlated with differentiation.7,10 It is tempting to speculate on which type of malignant biological behaviour the nucleolar features are dependent. Because of the active role of the nucleolus in cell metabolism17 nucleolar characteristics may be related to increased cell motility and therefore metastatic potential, or they may be related to high proliferative activity as both biological processes require increased cell metabolism. Previous studies on the morphology of mouse mammary tumour cells in vitro have shown that the increase in size and number of nucleoli is not related to cell multiplication but to cell secretion, such as viral production.18 The results of this study therefore suggest that nucleolar frequency may be correlated with metastatic potential. The correlation between nucleolar frequency and number of AgNORs has yet to be clarified.

We conclude that nucleolar morphometric variables assessed in cytological breast cancer specimens give good prognostic information, possibly exceeding that of nuclear variables. Whether these variables offer additional prognostic value to the well established prognostic value of histological morphometric variables and classic variables such as grade, tumour size, and lymph node status should be the subject of further investigation.

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