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Morten Ladekarl · Vibeke Jensen

Quantitative histopathology in lymph node-negative breast cancer. Prognostic significance of mitotic counts

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Abstract Reliable prognostic factors are needed to improve the stratification of patients with lymph node-negative breast cancer to different therapy modalities. We investigated the prognostic value of quantitative histopathology in a retrospective study of 98 "low-risk" breast cancer patients (T1+2N0M0) with a median follow-up of 9 years. An interactive video system and stereological and morphometric techniques were used to obtain estimates of four nuclear features (mean volume, mean profile area, volume fraction, and profile density), and two mitotic counts [mitotic profile frequency (MF) and mitotic profile density (MD)]. All measurements were performed in fields of vision sampled systematically from the whole tumour area of a routine histological section. Histological grade, histological type, and oestrogen receptor (ER) status was reassessed, whereas tumour diameter and age at diagnosis were recorded from the files. We found that all quantitative histopathological variables and ER status were correlated with histological grade. Single-factor prognostic analyses showed a highly significant value of MF ($2p=0.001$) and a marginally significant value of MD ($2p=0.09$), whereas no other variable approached statistical significance ($2p\geq0.25$). In a multivariate Cox analysis, MF was the only parameter of significant independent prognostic value ($2p=0.03$). Thus, the prognostic value of nuclear features found in previous studies could not be reproduced, whereas the marked value of mitotic counts for prediction of the outcome in patients with breast cancer was confirmed. Mitotic counts are easily obtained and may be of clinical value for identification of high-risk cases among patients with lymph node-negative breast cancer.

M. Ladekarl[1] (✉)
Stereological Research Laboratory, University Institute of Pathology, Second University Clinic of Internal Medicine,
Institute of Experimental Clinical Research, University of Aarhus, Denmark

V. Jensen
University Institute of Pathology, Aarhus Amtssygehus, Denmark

Mailing address:
[1] Stereological Research Laboratory, Bartholin Building, University of Aarhus, DK-8000 Aarhus C, Denmark

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Introduction

Clinical trials have shown that patients with lymph node-negative breast cancer survive significantly longer if they receive adjuvant treatment [12, 26]. It is, however, desirable to treat only patients at the greatest risk of recurrence, because the drugs given may induce serious side effects. Reliable parameters for the identification of high-risk cases among node-negative patients are thus urgently needed.

The diameter of the primary tumour is a useful prognostic variable in node-negative breast cancer [28, 29], although its value may be reduced in patients with very small tumours [37]. Among factors of possible additional predictive value are some obtained by immunohistochemistry [21, 30, 31], flow-cytometry [7], and morphological evaluation [28, 29, 37]. A number of these factors require expensive equipment and highly skilled personnel, however, while others may be hampered by poor reproducibility.

Quantitative evaluation of histopathological features may present a useful way of obtaining reproducible variables of prognostic value by a low-technology approach. In previous studies of quantitative histopathology in breast cancer patients, morphometric estimates of mitotic indices have been especially promising [1, 5, 8, 10], but stereological estimates of the mean size of nuclei have also been found to be of prognostic value [23, 24]. The present study addresses the ability of quantitative histopathology to predict the outcome in patients with lymph node-negative breast cancer. A multivariate statistical technique is applied to analyse the independent significance of quantitative histopathological factors relative to that of conventional clinicopathological variables.

Materials and methods

Patients

A consecutive series of 118 women with "low-risk" breast cancer was studied. In the present context, low-risk means absence of tumour in the axillary lymph nodes, tumour diameter no more than 50 mm, and no histological evidence of invasion to skin or deep fascia, corresponding to stage T1+2N0M0 [34]. The patients had their disease diagnosed from 1980 through 1984 at the University Institutes of Pathology, Aarhus, Denmark. The median age at treatment was 59 years (range 30–86 years) and the median tumour diameter was 20 mm. Tumour diameters were assessed clinically by palpation in half the cases. Oestrogen receptor (ER) status was determined using a monoclonal antibody in microwave-treated, deparaffinized sections (for details, see [20]). Tumours were considered ER positive if more than 10% of cancer cells were stained. At least two lymph nodes (median six lymph nodes) were removed at the primary treatment, which consisted of mastectomy followed by axillary dissection, according to the protocol of the Danish Breast Cancer Cooperative Group [2]. No adjuvant treatment was given. Three patients who did receive anticancer drugs were excluded from the study, as were 15 patients for whom only frozen sections were available and two for whom the quality of the histological material was too poor. The survival status of the remaining 98 patients was extracted from the Central Personnel Registry, and the cause of death was established from the death certificate or autopsy report in each case. Patients were excluded on the date of death if they had died of any other disease than carcinoma of the breast. The median follow-up was 8.7 years (range 0.4–11.9 years).

Histological specimens

In each case, one haematoxylin-eosin (HE)-stained section was cut at 4 μm from each of the original, paraffin embedded tissue blocks taken from the primary specimen. Tumour specimens were, in general, fixed immediately after removal. The tumours were typed according to the criteria of the World Health Organization [43]: 83 carcinomas were of ductal type, 11 of lobular type, 3 of medullar and 1 of mucinous type. Histological grading of ductal carcinomas was performed by two independent observers according to the method published by Bloom and Richardson [6]. In cases of disagreement, the specimens were reviewed by both together to obtain a consensus grade.

For quantitative histopathology, a colour video microscope connected to an AMIGA computer was employed. The software package GRID (Olympus Danmark, Copenhagen, Denmark) and a motorized scanning object stage made automatic sampling of fields of vision possible: at low magnification, the circumference of the whole tumour area of the histological section was indicated by the observer using a computer mouse. Within this area, the software randomly selected the first field of vision and the subsequent fields were sampled systematically with respect to the first, the scanning stage moving in equidistant steps of a size proportional to the demarcated area. Fields of vision showing inflammation, necrosis or nuclear pyknosis were excluded, and only invasive cancer was assessed. Mitotic profiles were counted using a ×40 oil immersion lens (N.A.=1.3) at a final magnification of ×890, whereas measurements of nuclei were performed at ×2225 using a ×100 oil immersion lens (N.A.=1.4).

Using a projected counting frame (Fig. 1), the mitotic profile density, MD, i.e. the number of mitotic profiles per square millimeter of tumour, could be estimated:

$$MD = \frac{Q(mit)}{n_f \cdot A}$$

where Q(mit) is the total number of mitotic profiles counted in n_f fields of vision, and A is the area of the counting frame. In the same fields, a small counting frame (of area A_1) was employed for counting nuclear profiles. Knowing the total count of nuclear profiles, $Q_1(nuc)$, the mitotic profile frequency, MF, i.e. the number of mitotic profiles per 1000 nuclear profiles, could be estimated:

$$MF = \frac{Q(mit) \cdot A_1}{Q_1(nuc) \cdot A} \cdot 10^3$$

The mitotic counts were assessed by *screening* an estimated mean number of 1800 nuclear profiles (range 700–5700) in, on average, 14 fields of vision (range; 5–27). This procedure took about 10 min per tumour, while the actual counting of, on average, 56 nuclear profiles (range 23–177) took an additional 5 min.

Four variables of nuclear features were obtained in the same fields of vision using the test system illustrated in Fig. 1 and previously described formulas (see Table 1 in [22]): the mean volume of nuclei, $\bar{v}_V(nuc)$, the mean profile area of nuclei, $\bar{a}_H(nuc)$, the fraction of tumour volume occupied by nuclei, $V_V(nuc/tis)$, and the number of nuclear profiles per square millimeter of tumour, ND. A mean of 75 nuclear measurements (range 41–183) were performed per tumour; a procedure that took less than 15 min.

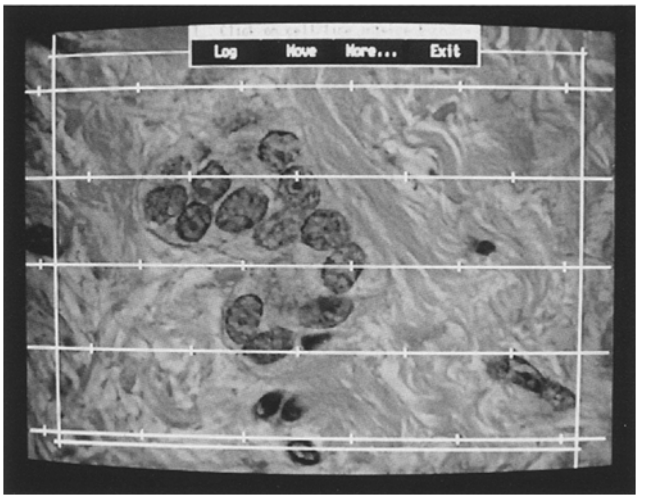

Fig. 1 Video image of a breast carcinoma projected onto a test-system. The numbers of nuclear and mitotic profiles per tumour area are estimated using counting frames and an unbiased counting rule [14]: profiles in focus, completely or partly inside the frame, are counted provided they do not touch or intersect the lower or left frame edges or their extensions. For the estimation of $\bar{v}_V(nuc)$, the length of the test-line is measured from nuclear border to nuclear border each time a nuclear profile is hit by a point (for details, see [15]). Original magnification ×2225

Statistics

Differences between group means were tested by a Student's *t* test, except for mitotic indices; as these were discontinuously distributed they were tested by Kendal's τ [33]. All continuous variables were logarithmically transformed due to their right-skewed distribution. A least-square linear regression analysis was used for investigating the correlation between continuous parameters, whereas the correlation and the distribution of discontinuous and categorical data was tested by Kendal's τ or by Fisher's exact test. In all tests, $2p \leq 0.05$ was considered the level of significance.

Using the BMDP statistical package (BMDP-Statistical Software, Calif., USA), the prognostic value of individual factors was analysed by log-rank tests and the cancer-specific survival illustrated in Kaplan-Meier plots. In these analyses, continuous variables were tested using 25% percentiles as cut-off points. The relative prognostic value of parameters was investigated in multivariate Cox analyses using standard stepwise, backward elimination. The assumptions for making these analyses were tested as follows:

Table 1 Single-factor survival analyses of patients with lymph node-negative breast carcinoma

Variable	Cut-off points	No. of cases[b]	2p-value
Tumour diameter	20 and 30 (mm)	52/32/14	0.28
Age at diagnosis	49 and 69 (years)	27/47/24	0.61
Histological type	Ductal and non-ductal	83/15	0.89
Histological grade[a]	I, II, III	22/34/27	0.41
ER status	Positive and negative	63/35	0.58
MD	0.00 and 0.30 (mm^{-2})	28/46/24	0.09
MF	0.00 and 2.84 (10^{-3})	28/47/23	0.001
$\bar{v}_V$(nuc)	215 and 418 (μm^3)	24/50/24	0.60
$\bar{a}_H$(nuc)	33.8 and 48.4 (μm^2)	24/51/23	0.25
ND	1810 and 3450 (mm^{-2})	24/50/24	0.66
V_V(nuc/tis)	0.071 and 0.131	24/50/24	0.68

[a] Ductal type carcinomas only
[b] Number of patients initially at risk in each group as defined by the cut-off points

Table 2 First and final step of the multivariate survival analysis of patients with lymph node-negative breast carcinoma (β regression coefficient estimated by maximizing the likelihood function in the Cox analysis, and *SE* standard error of β)

Variable[a]	First step			Final step		
	β	β/SE	2p-value	β	β/SE	2p-value
Tumour diameter	0.023	1.0	0.31	–	–	0.28[b]
Histological type/grade	–0.48	–0.8	0.42	–	–	0.42[b]
$\bar{v}_V$(nuc)	–0.003	–1.6	0.11	–	–	0.12[b]
ND	0.0004	0.1	0.93	–	–	0.93[b]
MF	0.27	2.2	0.03	0.19	2.1	0.03

[a] All parameters entered on a continuous scale, except histological grade/type which was scored 1 for ductal grade I and non-ductal carcinomas, and 2 for ductal carcinomas grades II and III
[b] 2p-value obtained at exclusion from the analysis

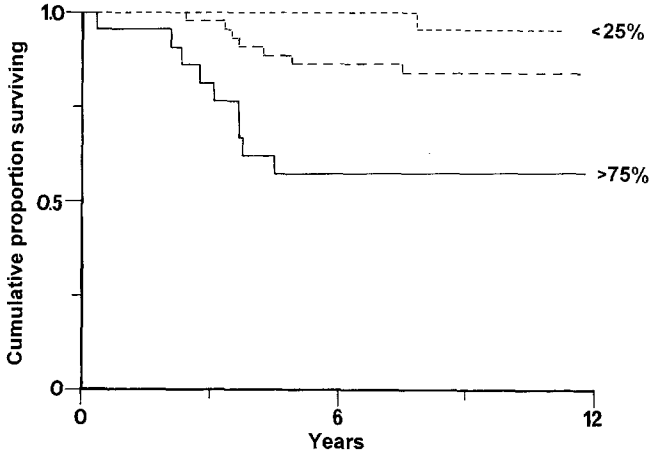

Fig. 2 Survival plot of 98 lymph node-negative breast cancer patients according to the mitotic profile frequency (2p=0.001). The 25% percentiles are used as cut-off points

the scoring of categorical variables was checked by log-minus-log plots, whereas the scoring of continuous variables was investigated by the "local smoothing technique" [17]. Moreover, the existence of time trends influencing the prognostic variables was tested by introducing an additional "time-dependent" covariate in the model by multiplying each variable by log(time). The tests showed no statistically significant departure of the scoring of prognostic variables from the assumption of proportional hazard rates, and no time trends that could influence the parameters were detected.

Results

Several parameters correlated with the histological grade of ductal carcinomas: all quantitative histopathological variables increased significantly with increasing malignancy grade (2p≤0.04), and a positive ER status was seen more frequently in low-grade tumours (τ=0.30, 2p=0.003). A significant correlation was found between tumour diameter and age at diagnosis (r=0.28, 2p=0.005) whereas ductal tumour type was associated with younger age (2p=0.04). The mean $\bar{a}_H$(nuc) was smaller in ER-positive than in ER-negative tumours (2p=0.02). Finally, a number of significant correlations were found among the quantitative histopathological variables – strongest between MD and MF (τ=0.85), $\bar{v}_V$(nuc) and $\bar{a}_H$(nuc) (r=0.75), and V_V(nuc/tis) and ND (r=0.74).

At the end of follow-up, 62 patients were alive, 17 had died of breast cancer, and 19 had died of other diseases. The overall cancer-specific 5- and 10-year survival rates were 83% and 81%, respectively. The results of single factor prognostic analyses are shown in Table 1 and the survival function according to MF is illustrated in Fig. 2. Using the 25% percentiles as cut-off points, MF was of high statistical significance, whereas only marginal prognostic value could be attributed to MD. The median value (0.14 mm^{-2}) seemed to be a more appropriate cut-off point for MD, however, resulting in a 2p-value of 0.004. In the multivariate Cox analysis, MF was the only parameter of prognostic significance (Table 2).

If MF was replaced by MD all variables were rejected, which means that MD was not a significant independent prognostic factor.

Discussion

In this study, none of the traditional prognostic parameters was found to have any statistical significance. Apart from the relatively small sample size, several explanations may be given for this result. Clearly, clinical assessments of tumour diameters, as performed in half the cases in the present study, may be too inaccurate. Measured with greater accuracy, this variable is of well-established and important prognostic significance [28, 29]. The missing prognostic value of ER status is in agreement with some [29, 42], but in conflict with other reports [13], whereas the association between ER status and histological grade has been recognized previously [27, 42]. Most surprisingly, no prognostic value of histological grade was seen, although the morphological evaluation was performed by observers experienced in breast pathology. This finding might be explained by the probably great observer-variability of this parameter [9, 36, 39] which, in smaller studies, may reduce the prognostic value to statistically insignificant levels. The results underscore the need for improving reproducibility and accuracy of malignancy grading by replacing subjective scoring with objective measurements.

The strong prognostic value of quantitative assessments of the mitotic activity is well recognized [1, 3, 5, 8, 10, 23, 24]. Confusingly, however, mitotic counts have been reported in a number of ways [25], most often as counts per field of vision or per tumour area. These figures are greatly influenced by variations in cellularity, which in itself may be a variable of independent prognostic significance [3, 24]. In the present study, we related the mitotic profile number to the number of nuclear profiles, resulting in an estimate of the mitotic profile frequency. Since this variable is virtually independent of variations in cellularity, it should be preferred to estimates of the number of mitotic profiles per unit area or per field of vision [32]. Moreover, in the multivariate analyses, we found the mitotic profile frequency, but not the mitotic profile density, to be of independent prognostic value.

The estimation of the mitotic profile frequency need not take much longer than the estimation of the mitotic profile density if a large sampling frame is employed for counting mitotic profiles and a small frame is used for sampling nuclear profiles. In contrast to most authors, we obtained the variables in fields of vision sampled systematically from the *whole* tumour sectional area. This method of sampling is efficient [16] and, most probably, less liable to observer-dependent bias than the usually employed sampling performed inside a subjectively selected, "worst" area of the section. Although from a biological point of view, sampling in such selected areas may seem attractive [41], it has been shown that the prognostic strength is the same if mitotic counts are obtained in the whole tumour area or inside the "worst" tumour compartment [19]. In the current study, a computerized video-microscope was employed for obtaining quantitative histopathological variables, mainly to increase the speed of sampling. However, the actual counting of mitotic and nuclear profiles may easily be performed using a routine microscope with a square grid in the eyepiece [25].

Several issues have been recognized that could hamper the clinical value of mitotic counts. Thus, tissue should be fixed immediately, because delays in fixation may lead to reductions in the number of mitoses observed [35]. Also strict definitions of mitotic figures should be employed to ensure reproducibility among laboratories [11]. Variations in the thickness of histological sections might influence mitotic counts, but could be diminished by using embedding in methacrylate rather than paraffin [18]. Finally, the scarceness of mitotic figures in many breast tumours is likely to produce large statistical errors of those indices which are based on few observations. Consequently, from a statistical point of view, immunohistochemical detection of proliferating cells may be more attractive, because positively stained cells occur much more frequently than do mitotic figures [41]. The prognostic value of such proliferation markers has recently been demonstrated in breast cancer patients [21, 30, 31], but confirmation in larger prospective studies is still lacking.

In contrast to previous studies of breast cancers [23, 24], estimates of the mean size of nuclei were of no prognostic significance. The mean nuclear volume is a genuine three-dimensional parameter which, using an unbiased stereological technique, seems suitable for routine use [38]. Thus, the negative result for this variable is disappointing, and, moreover, the reason for it is obscure. However, the mean nuclear volume and other quantitative histopathological variables may be influenced by variations in the processing of tissue [4, 22], an uncontrollable factor in retrospective studies. Prospective investigations are needed to assess the impact of this kind of bias on the prognostic value of the parameters. The same may apply to estimates of the mean area of nuclear profiles and of their density, which were of prognostic value in some studies of breast cancer patients [4, 10, 23, 24, 40], but not in the present one. In the multivariate analysis, we did not include mean nuclear profile areas, because the mean nuclear volume reflects nuclear size more realistically. Similarly, the nuclear volume fraction was not included, because of its close correlation with the nuclear profile density.

Previous studies have shown the prognostic significance of variables revealed by, for example, immunohistochemistry, flow-cytometry and tumour morphology in lymph node-negative breast cancer patients. Objective malignancy grading by quantitative histopathology represents an alternative, reproducible, low-technology approach. In the present study and in agreement with others, mitotic counts were of great prognostic significance. This easily obtained variable could be of future value in

the clinical management of patients with lymph node-negative breast cancer.

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