Comparison of extent of disease and morphometric and DNA flow cytometric prognostic factors in invasive ductal breast cancer

ANNE M UYTERLINDE, NELLIE W SCHIPPER, J P A BAAK

From the Pathological Institute, Free University Hospital, Amsterdam, The Netherlands

SUMMARY In 65 patients with primary invasive ductal breast carcinoma the relation between classic prognosticators describing the extent of disease (lymph node metastases and tumour size) and newer promising morphometric and DNA flow cytometric prognostic factors was studied. There was no relation between DNA ploidy, lymph node state, and tumour size. Tumours with a mitotic activity index of more than 10 were predominantly DNA aneuploid (61%) compared with those with a mitotic activity index of less than 10 which showed a DNA aneuploid pattern in 27%. The strongest prognosticator, the morphometric prognostic index (a multivariate combination of mitotic activity index, tumour size, and lymph node state) correlated positively with the DNA index in 63% of the cases ($p = 0.038$). Thus there was a discrepancy between the morphometric and DNA flow cytometric prognostic variables in 37% of the cases. These results indicate that morphometric and flow cytometric analysis may provide additional information on the prognosis in primary breast cancer.

The mortality of breast cancer is 30 per 100 000 women per year in western Europe; the incidence is about 65 per year and still increasing. In spite of many developments in surgery, hormone treatment, and (adjuvant) chemotherapy, prognosis has only slightly improved over the past few decades.^{1,2} Some patients, however, do benefit from adjuvant therapy.³ Indeed, patients with metastases in their lymph nodes have a much less favourable prognosis than patients with no lymph node disease, although the sensitivity and specificity of lymph node state is not very high,^{2,4} which results in an imbalance of treatment. Additional or other prognostic factors, which can help select specific treatments are obviously needed.

Pathologists have developed several grading systems relating to survival over many decades.^{5,6} In spite of its well documented prognostic value, nuclear or histological grading for breast cancer is used much less extensively in therapeutic decision making than clinical staging, principally because grading is not objective and reproducibility is therefore poor.^{7,8} Furthermore, it is difficult to predict the outcome in an individual patient if the grade of tumour falls within the relatively large "grey zone" (moderately

differentiated or grade II). Consequently, the value of grading in an individual patient is restricted, and as a result, a correct prediction of prognosis can be achieved in only 60% of patients.⁸

Many attempts have been made to identify more accurately patients at high risk. Women with tumours in which steroid receptors are demonstrable have a better survival than women with tumours in which such receptors are lacking.⁹ Unfortunately, recent data indicate that this is only true in the short term, thus reducing the predictive prognostic value of these receptors.^{10,11}

Much work has therefore been done during the past few years to develop several objective prognostic variables.¹²⁻¹⁵ Morphometric variables like cellularity index, nuclear area and shape, ratio of longest and shortest axis in primary and secondary tumours, the mitotic activity index (MAI) and the morphometric prognostic index (MPI) have been shown to be accurate prognostic indicators in breast cancer.¹³⁻¹⁵ Moreover, cellular DNA content can be measured on human solid tumours, and according to the findings of many reports, offers additional objective information for prediction of prognosis.^{12,16-18} Using static DNA cytometry, Auer *et al* concluded that DNA ploidy can be used as an independent prognostic variable.¹² Other work has confirmed these results with fluorescence DNA flow cytometry.¹⁶⁻¹⁸

To our knowledge, this is the first study which compares the prognostic value of the lymph node state, tumour size, morphometry, and DNA flow cytometry in relation to each other. Such a comprehensive approach seemed justified because many publications have reported only on individual variables and techniques, and so a comparison of them to determine the quickest and most cost effective method, or the best combination of techniques to give meaningful additional information, is not possible.

Material and methods

Of 78 consecutive breast cancer patients entering our laboratory between 1984 and 1985, 71 had a ductal type of carcinoma.¹⁹ Of this group only 65 patients were included because lymph node state was not known in three patients (old age); DNA histograms of three other tumours were inadequate. None of the patients had received any treatment before operation. Forty three patients were postmenopausal and 22 were premenopausal (age range 31 to 84 years, mean age 58.9 years, table 1). Tumour diameter ranged from 0.5 to 15 cm (median diameter 2.50 cm) and in 28% of cases tumour size was under 2 cm. At least five lymph nodes were found in each patient. Twenty one patients showed axillary lymph node metastases and 44 patients had histologically confirmed unaffected lymph nodes. We therefore considered this group to be representative when compared with other reported groups.^{13 14 18 20}

PREPARATION AND STAINING OF SAMPLES

Sections alternately cut at 50 µm and 4 µm thickness were cut from formalin fixed paraffin embedded tissue blocks that contained the most poorly differentiated areas of the tumour, (sandwich technique). The 4 µm section (stained with haematoxylin and eosin) was used for histological typing and morphometry and also served as a control to be compared with the 50 µm section. Two 50 µm sections were normally sufficient. For smaller tumour samples further sections were required. Single nuclear suspensions were made from 50 µm sections according to a method described elsewhere.²¹ In brief, this method comprises the following: the sections are placed in 10 ml centrifuge tubes and dewaxed in 6 ml xylene for 15 minutes at room temperature. Rehydration is then performed in a sequence of 100%, 96%, 70% and 50% ethanol with centrifugation and decantation of the supernatant after each step. The cells are then washed in 5 ml phosphate buffered saline (PBS, pH 7.4). Three ml of 0.05% protease (Sigma, St Louis, Missouri, USA; P-5255 10 U/mg, type 7) is added and the tubes incubated for 30 minutes at 37°C, with intermittent vortex mixing. The reaction is stopped with 6 ml ice cold PBS and the samples are washed again. Mechanical destruction is performed with a capillary pipette, and the sample is filtered through a 50 µm nylon gauze. Lastly, 2 ml of 0.02% 4',6-diamidino-2-phenyl-indole dihydrochloride (DAPI, Sigma D-1388, 100 mg) is added to the nuclei (residue) and further dispersion into a single

Table 1 Measurements and statistical values of several clinical, morphometrical, and DNA flow cytometrical features

Feature	DNA index = 1.00 (n) (%)		DNA index > 1.00 (n) (%)		Statistical value
Tumour size:					
< 20 mm	10	(56)	8	(44)	$\chi^2 = 0.766$ p = 0.68 (NS)
20–30 mm	14	(48)	15	(52)	
> 30 mm	11	(61)	7	(39)	
Lymph node(s)					
Negative	25	(48)	19	(52)	$\chi^2 = 0.484$ p = 0.49 (NS)
Positive	10	(57)	11	(43)	
Hormonal state					
Premenopausal	13	(59)	9	(41)	Mean DNA index = 1.24 (SD) = 0.33 Mean DNA index = 1.26 (SD) = 0.33
Postmenopausal	22	(51)	21	(49)	
Mean nuclear area (SD)	56.95 18.0		65.60 23.55		t test = -1.64 p = 0.11 (NS)
MAI:					
< 10	22	(73)	8	(27)	$\chi^2 = 8.533$ p = 0.014
10–20	8	(38)	13	(62)	
> 20	5	(36)	9	(64)	
Mean (SD)	10.5 11.2		17.4 10.6		
MPI:					
< 0.6	23	(66)	12	(33)	$\chi^2 = 4.298$ p = 0.038
> 0.6	12	(40)	18	(60)	

nuclei suspension is obtained with a spinal needle (Becton Dickinson, type 20G 3,5, Heidelberg, West Germany).

MORPHOMETRY

In routinely stained 4 µm paraffin sections the most cellular region of a tumour was selected by two investigators. In these areas at least 30 nuclei per tumour were measured at random on a graphic tablet (MOP Videoplan Kontron, Munich, Federal Republic of Germany) at a magnification of 200, as well as mitotic figures counted at a magnification of 400 on a microscope. The MAI is defined as the total number of mitotic figures in 10 adjacent fields in the most cellular region (×40 objective, numerical aperture 0.65, diameter of one field at specimen level 450 µm).

The multivariate MPI is based on tumour size, lymph node state, and MAI^{13 14}:

$$\begin{aligned} \text{MPI} &= 0.3341 (\text{square root of the MAI}) \\ &+ 0.2342 (\text{tumour size in cm}) \\ &- 0.7654 (\text{lymph node state}) \quad (\text{negative} = 2, \\ &\quad \text{positive} = 1) \end{aligned}$$

The reproducibility of the morphometric assessments was tested in 10 random samples (intra-observer and interobserver, table 2). Coefficients of correlation (r) within and between operators in 10 specimens ranged from 0.93 to 0.98 for the mean area, respectively, and 0.98 for both tests in the MAI. The measurements were therefore sufficiently reproducible.

FLOW CYTOMETRY

All samples were analysed within three hours after DAPI staining on a Partec flow cytometer (Partec Instruments, Arlesheim, Switzerland). The coefficient of variation (CV) of the G₁ peak in all the measured samples was between 1.8% and 6%. We defined the CV as the ratio of the half width at 61% (×2 standard deviations) of G₀/G₁ and the value of the G₀/G₁ peak on the abscissa. Formalin fixed mouse thymocytes served as an external standard for instrument setting (optimal CV = 1.5%, with a mean value of 2.1%). Reproducibility of measurements between different blocks of the same tumour as well as between intra- and interobserver measurements were tested in 10 random samples (table 2). No significant

differences were noted (r = 0.98). The DNA index was determined from the histograms: this is the ratio of the second G₀/G₁ to the first G₀/G₁ peak in the DNA histogram. In agreement with others, we made the assumption that the first G₀/G₁ peak represented diploid cells, either from the tumour or other cells present (inflammatory cells or fibroblasts).^{22 21} A diploid (DNA index = 1.0) tumour showed only one G₀/G₁ peak in the expected region (=2c) in the histogram. An aneuploid tumour (DNA index > 1.0) showed an additional peak to the 2c peak. Multiploid tumours showed at least three different G₀/G₁ peaks.

STATISTICAL ANALYSIS

Statistical analysis was carried out using the BMDP package. The difference between means was evaluated by Student's *t* test. The DNA index could not be regarded as a normal continuous distribution because of the over-representation of diploid tumours and was therefore divided into diploid (DNA index = 1.0) or aneuploid (DNA index of > 1.0). Similarly, the MPI was divided into two groups using the previously established cut off point of 0.60.^{5 22} In these studies this cut off point was chosen for comparison with lymph node state so that the number of patients in the low index group was the same as the number of patients with unaffected lymph nodes. Similarly, the number of patients in the high index group equalled the number of patients with affected lymph nodes. Values of *p* < 0.05 (χ² test) were regarded as significant.

Results

Fifty four percent of patients showed a high MAI (> 10) with an average of 13.7 (SD 1.4). Using the cut off value of 0.60, 54% showed an MPI of < 0.60 (low risk) and 46% of > 0.60 (high risk). Measurements of DNA fluorescence showed a diploid tumour pattern in 52%; 48% had an aneuploid tumour peak (table 3). Eighteen (27%) patients with an aneuploid tumour showed a DNA index between 1.50 and 2.0. Two tumours showed an additional aneuploid peak in the DNA index region of 1.55 and 2.1. Only three tumours were tetraploid.

To establish therapeutic relevance the tumour sizes

Table 2 Measurement reproducibility of morphometry and DNA flow cytometry*

Feature	Intraobserver		Interobserver	
	r	α	r	α
Nuclear area (mean)	0.98	1.05	0.93	1.02
MAI	0.98	0.92	0.98	1.09
DNA Index	0.99	1.12	0.99	1.16

*Correlation coefficient (r) and slope of the best linear fit (α) of 10 duplicate measurements.

Table 3 Relative frequency distribution of DNA index of 65 ductal breast tumours

DNA index	Frequency (%)
1.0	52
> 1.0-1.24	7
1.25-1.49	12
1.50-2.00	27
> 2.0	2

Table 4 Relation between DNA index and mitotic activity*

DNA index	MAI		
	0-10	11-20	> 20
Diploid	73	38	36
Aneuploid	27	62	64

* $\chi^2 = 8.533$, $p = 0.014$.

of the 65 tumours were divided into three groups: under 2 cm, over 3 cm, and in between. Statistically, no correlation between ploidy and these categories of tumour size could be found (table 1). Distribution of cellular DNA content in relation to lymph node state is also shown. In agreement with others, we could find no significant correlation.

Table 1 gives an impression of the distributions of the nuclear areas of diploid and aneuploid tumours. In general, DNA aneuploid tumours have a larger nuclear area (65.60 (SD 23.6) μm compared with a nuclear area of 56.95 (SD 18.0) μm for diploid tumours. This difference is just below the level of significance (t test = -1.64 , $p = 0.11$).

There was a significant correlation between MAI and DNA index (tables 1 and 4). Diploid tumours showed a low MAI and aneuploid tumours in general had a high MAI (χ^2 test = 8.533 , $p = 0.0140$). Within the group of aneuploid tumours the correlation between MAI and aneuploidy expressed in different DNA index classes (with cut off points of these classes as shown, figure) was not significant ($CV = 0.0559$).

The strongest morphometric indicator of prognos-

sis, the MPI, was correlated with DNA ploidy as well (χ^2 test = 4.298 , $p = 0.0382$) (table 1 and figure). Most diploid tumours ($n = 23$, 66%) showed an MPI of <0.6 and thus a low risk. Only 12 aneuploid tumours (40%) had an MPI of <0.6 . On the other hand, only 12 patients with diploid tumours (34%) were at high risk (MPI of >0.6) while most cases with an aneuploid pattern ($n = 18$; 60%) were at high risk according to morphometric analysis. There was a discrepancy in 37% of the cases between the predictive value of the MPI and the DNA index.

Discussion

In previous retrospective and prospective studies a multivariate morphometric prognostic index proved to contain the most important prognostic information for patients with invasive ductal breast cancer.^{13 14} Independently, others found that DNA measurements had a prognostic value not related to other classic variables.^{16-18 23} A comprehensive evaluation of clinical, morphometric, and flow cytometric factors is useful, because a combination of several independent more accurate and objective prognostic variables could improve prognosis. The patient group under study showed similar characteristics to those described in other publications concerning clinical features,^{1 6 13} morphometric features,^{13 14} as well as the distribution of cellular DNA content.^{17 23 24}

There was no significant correlation between the classic predictors of prognosis and DNA index (table 1). This agrees with the findings of other publications.^{20 23 24} Theoretically, this could mean that tumour ploidy is a stable feature independent of the stage of disease (characterised by tumour size and nodal state).^{12 25} In practice this could mean that ploidy determinations might be useful even in small lesions with the same predictive value.²⁶ In thymidine labelling experiments the relation between dividing cells and DNA index is stronger with higher index values.²⁷ Of all features investigated in our study, the best correlation was between the MAI and DNA index when subdivided into diploid and aneuploid patterns (table 1). In contrast, no correlation could be found between MAI and aneuploidy (expressed in DNA index) (table 1). This result could be explained by the small number of cases in the various subgroups.

There was only partial agreement (63%) between prognostically favourable (or unfavourable) values of the MPI and DNA index (figure), and this is possibly of clinical importance. This means that in the remaining subsets (MPI of <0.60 and aneuploid; MPI of >0.60 and diploid, in 37% of the cases) the two indices might give additional prognostic information. Can combined morphometric features and DNA measurements replace axillary lymph node dissection? The

Figure Relation between MPI and DNA index. Horizontally dotted line divides the diploid tumours (cytometrically probably favourable) from the aneuploid tumours (prognostically unfavourable). Vertically dotted line divides tumours which are morphometrically probably favourable (MPI of <0.60) from those with a morphometrically unfavourable prediction of prognosis (MPI of >0.60). Note discrepancy in 37% of cases.

relatively strong predictive power of the nodal state and ease of sampling are the main reasons for using presence or absence of locoregional metastases as the criterion for making clinical decisions regarding radiotherapy and adjuvant chemotherapy. Upper limb oedema, however, is one of the risks, especially in extensive axillary dissection. Although the MAI showed an even stronger predictive prognostic value than the nodal state as a single variable, the MAI could not predict lymph node metastasis as has been shown previously.^{13,14} We could not find a correlation between nodal state and any other variable. A combination of features, therefore, including lymph node metastasis is still required to predict outcome.

In conclusion, the combination of the MPI and the DNA index is a promising predictor of prognosis in primary ductal breast cancer. Further evaluation of these tumour characteristics in relation to survival and length of remission is needed. A study on this subject has already been started.

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Requests for reprints to: Professor Dr JPA Baak, Pathological Institute, Free University Hospital, De Boelelaan 1117, 1007 MB Amsterdam, The Netherlands.