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Morphometry of Breast Cancer

I. Comparison of the Primary Tumours and the Axillary Lymph Node Metastases

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SUMMARY

In 234 patients with primary breast cancer, morphometry was performed of three groups of tumour cells: 1. in the primary tumours without lymph node metastases (PRN); 2. in primary tumours with lymphnode metastases (PRP) and; 3. in the axillary lymph node metastatic deposits (LMD). The morphometrical analysis included the cellularity index, the mitotic activity index and seven nuclear features.

The highest mitotic activity indices (mean: 14.1) were found in the metastatic deposits (LMD), the lowest (mean: 12.5) in the lymph node negative primary tumours (PRN), whereas the lymph node positive primary tumours (PRP) fell in between (mean: 13.2). The cellularity index shows a similar, though reciprocal, trend. The largest standard deviations of the nuclear features were found in primary tumours with lymph node metastases (PRP). In contrast, the metastatic (LMD) nuclei had the smallest standard deviations. The nuclei of the latter are therefore more regular in size and shape. The differences found are not sufficiently large to distinguish PRN and PRP with an acceptable accuracy (the efficiency, sensitivity or specificity did not exceed 67% irrespective of the decision threshold used).

Comparison of primary tumours with lymph node metastases and the metastatic deposits, from the same patients, revealed significant correlations of all morphometric features with the exception of the nuclear shape factor and the nuclear axes ratio. Thus, the nuclei of the metastatic cells in general are identical to the nuclei in the primary tumour from which they arise, but in a subset of patients the metastatic tumour cells have much more monomorphous ellipsoid nuclei. Further investigations are needed to see whether this phenomenon indicates a different malignant course.

Introduction

One of the aims of modern pathology is to create and use objective and reproducible methods for diagnosing disease and, in case of cancerous tissue, to assess its malignant potential. Morphometry, defined as the quantitative

description of a structure, is a possibility in achieving this aim²². By using this technique, subjective criteria of the pathologist become more objective and more reproducible parameters are obtained^{3, 4, 6, 10, 18, 20}. Another benefit of morphometry is that tumours are not, in contrast to the subjective grading systems^{7–9}, assigned to one of two or three groups, but instead, are arranged in a continuous spectrum.

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The ultimate goal of such an approach is to achieve a more accurate selection of patients with a higher risk for recurrence, thus reducing over- and undertreatment^{14,16}. In order to achieve this aim, a better understanding of tumour cell malignancy and biological behaviour of the tumour is essential. Alternatively, if complete distinction between primary breast tumours with and without metastases could be obtained with primary tumour characteristics, perhaps the rather mutilating axillary lymph node dissection could be omitted. Several studies have been undertaken in this direction. Zajdela and associates²³, comparing tumours with a large nuclear area and tumours with a small nuclear area, found that the latter had a higher survival rate. Maehle et al.¹⁵, studying histologic grading and the mean nuclear area in the lymph node metastases, concluded that tumours with smaller nuclear areas have a better prognosis. Tosi et al.²¹, investigating primary tumours and their lymph node metastases found larger tumour nuclei in the lymph node metastases, which was slightly at variance with earlier observations³.

Therefore, in the present study three breast cancer cell populations were investigated searching in which these populations differ or resemble for the above mentioned and other morphometric characteristics. The three populations were: 1. tumour cells of primary breast carcinomas without lymph node metastases (PRN); 2. tumour cells of primary breast carcinomas with (PRP) affected lymph nodes and; 3. tumour cells of the axillary lymph nodes metastases (LMD) (Fig. 1).

It will be shown that morphometry of the primary tumours can not accurately predict axillary lymph node metastases. Further, as a group, the lymph node metastatic cells differ morphometrically from the primary tumour cells from which they arise.

Fig. 1. The design of the morphometrical study of the three Fgroups of breast cancer cells.

Material and Methods

Patients

All female patients ($n = 312$), who entered consecutively one of the hospitals affiliated with the Stichting Samenwerking Delftse Ziekenhuizen (SSDZ), Delft, The Netherlands in 1981–1982 and were diagnosed as having a primary carcinoma of the breast without other overt diseases, were included in this study.

The mean age of the patients was 59.9 years (median: 60, range 25–90). The mean tumour size was 2.6 cm (median: 2.1, range 0.4–15). 53% of the patients had positive lymph nodes and 47% had not. All tumours were carefully reviewed by one of us (JPAB) concerning histologic type and grade^{1,9}. The results fell in the range as described in literature^{11,12}, and thus, the material can be considered to be representative.

Morphometry was only performed on primary adenocarcinomas of the "Not Otherwise Specified" (NOS) ductal type ($n = 254$). Adequate – excluding prefrozen – material was available for the study in 234 patients. Due to the detecting procedure, the discovery of micrometastases is responsible for a relatively high (53%) percentage of positive axillary lymph nodes. This also explains why in 77 (62%) of the 125 patients with axillary lymph nodes, it was possible to establish morphometry, because the other 48 patients had too small (i.e. micrometastatic, single cell) deposits, in which not sufficient cancer cells were available for the mitotic counting and the cellularity index measurement.

Morphometrical methods

Details of morphometry, and theoretical background, have been described elsewhere^{2,4,17,22}. For the application of this method to breast cancer reference is made to earlier publications^{3–6}. Briefly, morphometry was performed on standard, 4 μ m thick, H+E sections. The following features were assessed, in the most cellular areas (usually in the periphery) of the tumour:

The mitotic activity index, defined as the number of mitoses in 10 high power fields (magnification 400 X; 40 X objective, n.a. 0.75, field diameter at specimen level 450 μ m).

The cellularity index is the mean of the number of nuclei counted in 5 fields at 1000 X magnification. One field, superimposed on the projection screen, is divided by a grid of lines into 36 squares sized 16 μ m at specimen level. Only nuclei not in contact with any of the margins and with an average diameter of more than 4 μ m at specimen level are counted.

The nuclear measurements are performed at 1000 X magnification on a commercially available graphic tablet (Mop-Videoplan, Kontron, Munchen, BRD) using microphotographs. The nuclear features assessed are perimeter, area, shortest axis, longest axis, diameter, shape factor (defined as: $4\pi \times \text{Area} / \text{Perimeter}^2$), and axes ratio. Twenty-five nuclei of tumour cells were measured and the mean and standard deviation were calculated for each specimen. So, 14 nuclear parameters were assessed. The size independent shape factor is a measure for the regularity of the profile, being 1.0 in perfectly regular circles, and smaller than 1.0 in irregular structures.

Quality control

Previous studies have revealed the reproducibility and consistency of morphometry in primary breast cancers^{3,4,19}. Much less information is known about the reproducibility of morphometry of lymph node metastatic deposits, and therefore special attention was paid to this subject. Quality control studies were carried out on the measuring system as a whole, and not only on the

measurements proper. The quality control studies are summarized in Fig. 2.

First, 5 axillary lymph nodes with metastatic deposits were cut serially in order to investigate intra lymph node variation, and of each gland 5 sections were taken (intersection distance: 50 µm), stained and analysed morphometrically (Fig. 2a). Secondly, in 10 lymph node slides morphometry was performed in 2 different areas of the same section, with the same purpose as the first experiment (Fig. 2b). Another lymph node with metastatic deposits of the same patient was also analysed to evaluate inter lymph node differences (Fig. 2c). These studies revealed that different parts of the same lymph node metastasis, and different metastases in the same patient, have the same morphometric characteristics, as no significant differences could be detected (Student's t-test and F-test $p > 0.90$). Fourthly, intra- and interobserver measuring agreement was evaluated (Fig. 2d). Of the same lymph nodes, areas were independently selected with the above mentioned criteria, and microphotographs of 10 patients were measured independently and "blind" by 2 investigators. Further, measurements of copies of microphotographs

were performed of 10 patients by 2 investigators ($p < 0.005$, $r > 0.90$).

In all studies a very strong agreement was found. As the measurements in the 4 experiments were highly reproducible, it is concluded that morphometry in axillary lymph nodes is a reproducible method as it is in the primary tumour.

Statistics

The statistical method employed were:

1) Student's t-test and the F-test (variance ratio) for comparing the measurements of the quality control studies performed on the lymph node metastases (see above);

2) linear regression analysis for comparing inter- and intra-observer variation and for comparing PRP and LMD morphometrics of the same patients;

3) Wilcoxon's test for determination of statistical differences between the three different groups of cells. Probability values below 0.05 were considered significant. Fig. 3 summarizes the statistical evaluations.

In addition, definitions for efficiency, sensitivity and specificity were adopted from Galen and Gambino¹³, as follows:

$$\text{efficiency} = \frac{TP + TN}{TP + FP + FN + TN} \times 100$$

$$\text{sensitivity} = \frac{TP}{TP + FN} \times 100$$

$$\text{specificity} = \frac{TN}{TN + FP} \times 100$$

Fig. 2. Illustration of the quality control studies performed on morphometry of lymph node metastatic deposits (LMD). a) investigation of serial sections (intra lymph node variation); b) investigation of different areas of the same section (intra lymph node variation); c) investigation of different lymph nodes of the same patient (inter lymph node variation); d) investigation of different photomicrographs and selections by different investigators (intra- and interobserver variation).

Fig. 3. Overview of the different comparisons made and the statistical techniques used.

where:

TP = true positives, i.e. the number of patients with lymph node metastases which are correctly predicted by a test
 TN = true negatives, i.e. the number of patients without lymph node metastases which are correctly predicted by a test
 FP = false positives, i.e. the number of patients without lymph node metastases which are incorrectly predicted by a test
 FN = false negatives, i.e. the number of patients with lymph node metastases which are incorrectly predicted by a test

By using different thresholds of each feature the predictive value of various tests were evaluated, and the optimum of efficiency, sensitivity and specificity were assessed. A Receiver Operating Characteristic (ROC) curve of false positives and false negatives was constructed of the feature with the highest power to discriminate patients with and without axillary lymph node metastases.

Results

Predictive value of morphometry concerning lymph node metastases

Comparison of the morphometry in the two groups of primary tumours (PRP and PRN) showed that more mitoses are found in PRP. There were no other statistically significant differences, although a trend is shown towards more a larger size and a higher standard deviation of the nuclear features of the PRP tumours (Table 1).

Using stepwise regression analysis to predict the lymph node status of a patient, the mitotic activity index again turned out to be the only significantly correlated predictor. The cellularity index and the mean nuclear area showed a similar tendency, but were not significant once that the mitotic activity index was taken into account. In Fig. 4a a histogram of the mitotic activity index of lymph node

Fig. 4. The mitotic activity index of lymph node negative and positive primary tumours. Note the considerable overlap.

Fig. 5. The Receiver Operating Characteristic (ROC) curve shows that there are many false positives and negatives when the mitotic activity index of the primary tumour is used as a predictor of lymph node metastases. No accurate discrimination threshold can be found.

Table 1. Means of – and differences between – morphometry of primary mammary carcinomas without (PRN) and with (PRP) lymph node metastases and of the lymph node metastatic deposits (LMD).

	PRN n = 109	PRP n = 125	P*	PRN n = 109	LMD n = 77	P*	PRP n = 125	LMD n = 77	P*
Cellularity index	12.0	10.6	n.s.	12.0	8.2	<0.001	10.6	8.2	<0.005
Mitotic activity index	12.5	13.2	<0.01	12.5	14.1	<0.001	13.2	14.1	n.s.
Mean nuclear perimeter	25.9	26.1	n.s.	25.9	25.0	n.s.	26.1	25.0	<0.05
S.d. nuclear perimeter	3.1	3.3	0.1 (n.s.)	3.1	2.7	n.s.	3.3	2.7	<0.005
Mean nuclear area	47.1	47.2	n.s.	47.1	46.0	n.s.	47.2	46.0	n.s.
S.d. nuclear area	11.2	12.1	0.1 (n.s.)	11.2	10.2	n.s.	12.1	10.2	0.07 (n.s.)
Mean shortest nuclear axis	6.6	6.6	n.s.	6.6	6.5	n.s.	6.6	6.5	n.s.
S.d. shortest nuclear axis	0.95	1.00	0.1 (n.s.)	0.95	0.92	n.s.	1.00	0.92	0.1 (n.s.)
Mean longest nuclear axis	9.0	9.1	n.s.	9.0	9.0	n.s.	9.1	9.0	n.s.
S.d. longest nuclear axis	1.32	1.34	0.1 (n.s.)	1.32	1.20	n.s.	1.34	1.20	<0.01
Mean nuclear diameter	7.6	7.6	n.s.	7.6	7.5	n.s.	7.6	7.5	n.s.
S.d. nuclear diameter	0.87	0.94	0.1 (n.s.)	0.87	0.83	n.s.	0.94	0.83	<0.05
Mean nuclear shape factor	0.85	0.84	n.s.	0.85	0.90	<0.001	0.84	0.90	<0.001
S.d. nuclear shape factor	0.04	0.05	n.s.	0.04	0.05	<0.001	0.05	0.05	<0.005
Mean nuclear axes ratio	1.37	1.38	n.s.	1.37	1.42	<0.01	1.38	1.42	<0.05
S.d. nuclear axes ratio	0.24	0.23	n.s.	0.24	0.25	<0.05	0.23	0.25	0.1 (n.s.)

* P = Probability of no difference, Wilcoxon's test ($p < 0.05$ was regarded as the level of significance).

S.d. = Standard deviation

negative and lymph node positive primary tumours is shown. The best discriminating threshold (mitotic activity index > 5) did only reach an efficiency of 62% (specificity 68% and sensitivity 54%). There was a considerable overlap, and the ROC-curve (Fig. 5) illustrates the high percentage of false positives and false negatives. Therefore, it is concluded that it is not possible to accurately predict the presence or absence of lymph node metastases by using a single morphometric feature of the primary tumour.

Differences between the investigated groups

The results of morphometry of the three groups (i.e. PRN, PRP and LMD) are summarized in Table 1.

In the lymph node metastatic deposits (LMD) less cells per area (lower cellularity index) were found, there were more mitoses, and the nuclei were more regular, (mean nuclear shape factor = 0.90) and more elongated (mean nuclear axes ratio = 1.42) than in the primary tumour from which they arise (means respectively 0.84 and 1.38). The standard deviations of most nuclear features are significantly smaller in the lymph node metastatic deposits (LMD) than in the primary tumours with lymph node metastatic deposits (PRP). This also reflects a higher regularity of the metastasized cell nuclei (Table 1).

Comparing LMD and Primary tumours without metastases (PRN) the same is true concerning the cellularity index and the mitotic activity index (less cells, more mitoses in the LMD's). Again the cells in LMD are more regular and elongated. No significant differences were found between the standard deviations of nuclear features (Table 1).

Table 2. Results of linear regression analysis: conformity between morphometry of the same patient assessed both in the primary mammary carcinoma (PRP) and in the lymph node metastases (LMD). The lower the probability (P) and the higher the correlation coefficient (r) the more conformity there is between the primary tumours and their metastases. Note the lack of correlation in nuclear shape descriptors.

	P	r
Cellularity index	< 0.05	0.27
Mitotic activity index	< 0.001	0.48
Mean nuclear perimeter	< 0.001	0.55
S.d. nuclear perimeter	< 0.001	0.35
Mean nuclear area	< 0.001	0.59
S.d. nuclear area	< 0.001	0.46
Mean shortest nuclear axis	< 0.005	0.37
S.d. shortest nuclear axis	< 0.01	0.30
Mean longest nuclear axis	< 0.001	0.59
S.d. longest nuclear axis	< 0.001	0.31
Mean nuclear diameter	0.001	0.59
S.d. nuclear diameter	< 0.001	0.37
Mean nuclear shape factor	0.29	0.12
S.d. nuclear shape factor	0.07	0.20
Mean nuclear axes ratio	0.17	0.16
S.d. nuclear axes ratio	0.64	0.05

S.d. = Standard deviation

Comparison of primary tumours and their metastases

In the above described paragraphs, between the PRP and LMD, differences are found using Wilcoxon's test. Because it concerns the same patients, it was also possible to evaluate the *conformity* of the primary tumours and the metastases of each patient (using linear regression analysis) – or, in other words, how much do they resemble?

The results are shown in Table 2. Most morphometric features are significantly correlated. Thus, characteristics found in the primary tumours are also present in the lymph node metastases. This was true for all indices except the nuclear shape factor and the nuclear axes ratio (Figs. 6 and 7). Fig. 6 visualises that the means of the

Fig. 6. The mean nuclear shape factor of the primary tumours with lymph node metastases and their lymph node metastatic deposits.

Fig. 7. The mean nuclear axes ratio of the primary tumours with lymph node metastases and their lymph node metastatic deposits.

shape factors are more centered around 0.90 for the lymph node metastases, whereas in the primary tumours a much larger variation is found, including lower values (mean: 0.84). In Fig. 7 a shift towards more elongated cells in the LMD can be seen. In the primary tumours, the values range from 1.20–1.60 (mean: 1.38), in the LMD's from 1.20 to 1.80 (mean: 1.42). Or, in other words, in a subset of patients with axillary lymph node metastases the nuclei in the LMD are more regular and ellipsoid than in the primary tumour from which they arise.

Discussion

In the present study a comparison was made between PRP, PRN and LMD morphometry. The quality of the measuring procedure being the cornerstone of such a study, special care was paid to this subject. Different studies from other institutes and also from our own laboratory have shown that morphometry of primary breast cancers is a highly reproducible method^{3,5,6,21}. The quality control studies presently described revealed that morphometry of lymph node metastatic deposits is, as in the primary breast tumour, a highly reproducible method. Further, the intra- and inter lymph node variations were so small that axillary lymph node metastatic tumour deposits can be regarded as homogeneous. Therefore, no special criteria for selecting certain areas for morphometric analysis of LMD are necessary.

Earlier observations have shown that larger and more anisokaryotic nuclei are associated, though weakly, with lymph node metastases^{3,19,21}. The same trend was detected by us but the differences were not as significant. This maybe due to the larger number of patients in the present study. Compared with their primary tumours the nuclei in the lymph node metastatic deposits showed significantly smaller standard deviations and differences concerning the nuclear shape. Further, the nuclei in the lymph node metastases are smaller and less polymorphous in appearance. There are several explanations for this phenomenon. The tissue processing of the tissues for each tumour and its metastasis being exactly the same, the presence of the smaller nuclei can be due to a different degree of shrinkage of lymph node metastases, perhaps because of different biochemical properties of the membranes. It also is possible that only special clones of cells metastasize. Finally the spatial distribution can be different in the primary tumours and the lymph nodes, in which the metastases are localized – comparable with the difference between canned and swimming sardines.

The mitotic activity index was significantly higher in the PRP than in the PRN tumours^{3,21}. However, the value of morphometry of the primary tumours to predict the axillary lymph node status of a patient was not very high in the present study (in agreement with earlier studies). The concept of regarding lymph node metastatic tissue as more malignant in its proliferation rate is supported by this finding, because the mitotic activity index is a significant prognostic factor in a number of malignancies and also in breast cancer^{3,6,19}.

Other morphometric criteria are also correlated with prognosis. Zajdela et al. compared patients who died of breast cancer with patients that were free of disease after 5 years²³. As can be expected, using prognosis rather than the lymph node metastases as the dependent variable, the differences in that study were larger than between the currently investigated subsets. Maehle et al.¹⁵, studying lymph nodes with metastatic deposits of breast cancer, found that a greater mean nuclear area was associated with a worse prognosis.

The differences found between the lymph node metastatic deposits and the primary tumours from which they arise, concerning the nuclear shape factor and nuclear axes ratio, may reveal additional information of the malignant potential of a tumour. These findings may contribute to initiate a more subtle treatment of breast cancer patients, better coping with over- and under-treatment. This is currently investigated in a long-term follow-up study.

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