

Proliferation Measurement in Breast Cancer by Two Different Methods

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Abstract. *Background:* Different studies show that proliferation measurement in breast cancer may have an independent prognostic value. In the present study, tumor proliferation in breast cancer was analyzed by two radically different methods according to the technique used (immunohistochemistry and flow cytometry), associated costs and necessary equipment. The aim was to evaluate which method discriminates better between tumors with high and low proliferation in relation to all other available clinical and biological parameters. *Materials and Methods:* Two hundred and eighty breast cancers (231 ductal infiltrating, 30 lobular, 19 or less frequent varieties) were studied. The post-surgical staging was as follows: 164 pT1, 87 pT2, 7 pT3, the remaining 22 were multifocal, diffuse tumors. Axillary nodal invasion was found in 99 cases (35.4%). Proliferation was studied by means of flow cytometry (DNA index and S-phase) in fresh tumor tissue and immunohistochemistry (Ki67) in paraffin-embedded tissue. Furthermore, hormone receptor (estrogen receptor, ER; progesterone receptor, PR), c-erb-B2 and p53 expressions were studied using the same method. Finally, histological and nuclear grade, tumor size and axillary nodal invasion were also included as variables of the study. *Results:* A DNA index >1 (aneuploidy) correlated significantly with histological grade 3 ($p=0.01$), nuclear grade 3 ($p<0.0001$), nodal invasion ($p=0.007$), absence of ER ($p=0.006$) and of PR ($p=0.002$), c-erb-B2 expression ($p=0.008$), p53 expression ($p=0.007$) and tumor size ($p=0.01$). An expression of Ki67 in 20% or more of tumor cell nuclei, on the other hand, correlated significantly with histological grade 3 ($p<0.0001$), nuclear grade 3 ($p<0.0001$), absence of ER ($p<0.0001$) and of PR ($p<0.0001$), c-erb-B2 expression ($p<0.0001$), p53 expression ($p<0.0001$) and tumor size ($p=0.0005$), but not with nodal

invasion. Conclusion: Although flow cytometry provides additional data (association with nodal invasion), the study of Ki67 expression emerges from this study as a simple, inexpensive and reliable method to study the proliferation rate of breast cancer.

Cell proliferation is an important prognostic factor in breast cancer (1-6), because it is one of the first events in oncogenic activation. Some authors consider it one of the most relevant biological factors in relation to overall survival (7). The combination of ploidy, S-phase and hormone receptor status accurately predicts the prognosis of this disease (8, 9). Although some authors already consider cell proliferation to be an independent prognostic factor (10, 11), this issue is still controversial.

Different methods allow cell proliferation to be quantified in breast cancer, although none of them has established a routine role in the clinic. The most simple (and inexpensive) of these methods is immunohistochemistry, by which means several proliferation-associated antigens are detected. The most widely used among them is Ki67, especially since the development of the MIB-1 antibody allows for its detection in archival, paraffin-embedded tumor samples (12).

A more sophisticated (and more expensive) method also used in the clinic is flow cytometry, determining the cell fraction in S-phase as a surrogate for the proliferative capacity of the tumor. However, the guidelines on evaluating the S-phase are still not standard worldwide, this being especially true for aneuploid tumors. In fact, in these cancers, two different cell fractions grow together: a diploid and an aneuploid one, the latter usually constituting the minority of tumor cells. Both have extremely different growth patterns and individual cell characteristics, and there is still no consensus about whether the S-phase of these tumors should be studied as a whole, or separately for each of the cell subpopulations (diploid and aneuploid).

In the present investigation, cell proliferation in a series of breast cancers was studied by means of immunohistochemistry

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and flow cytometry, considering the S-phase in aneuploid tumors separately for each of the cell subpopulations, and the results were correlated with all available clinical and biological features of the tumors. The final aim was to elucidate which of the two methods offers the best results for routine use in the clinic.

Materials and Methods

Breast cancers (n=280) operated upon at Fundación Tejerina-Centro de Patología de la Mama, Madrid, Spain, between January 2000 and January 2003, were studied. The histology of the tumors was: 231 ductal infiltrating, 30 lobular and 19 less frequent varieties. After surgical staging, 164 cancers were T1, 87 T2 and 7 T3. The remaining 22 cases were multifocal, diffuse tumors. Of the 280 patients, 99 (35.4%) had invaded axillary nodes. None had received induction chemotherapy or hormone therapy prior to surgery.

All flow-cytometric studies were carried out on fresh tumor tissue. The immunohistochemical studies (hormone receptors, Ki67, c-erb-B2 and p53), were carried out on formalin-fixed, paraffin-embedded matching samples.

Immunohistochemistry. The immunohistochemical technique employed was the standard one at our laboratory. Briefly, 5- μ m paraffin sections were mounted on poly-L-lysine-coated slides for heat-induced epitope retrieval ("HIER" technique) in citrate buffer. We used the same, commercially available streptavidin-biotin-peroxidase kit (Histostain-SP, Zymed, San Francisco, CA, USA) throughout the whole procedure, to ensure uniformity of the results. The antibodies employed were: NCL-CB11 (c-erb-B2), NCL-ER-6F11 (estrogen receptor), NCL-p53-D07 (p53), all from Novocastra Laboratories, Newcastle, UK; prediluted MIB1 (Ki67) and PR-2C5 (progesterone receptor) from Zymed, San Francisco, CA, USA. The incubation time was 1 h at room temperature in a humid chamber for all antibodies, which apart from the prediluted MIB1-Ki67 solution which was used directly as supplied, were employed at the following dilutions: NCL-CB11 (c-erb-B2): 1:40; NCL-ER-6F11 (ER): 1:100; NCL-p53-D07: 1:100. The evaluation of nuclear staining patterns (ER, PR, Ki67 and p53) was straightforward since specimens positive for ER, PR or p53 always showed specific staining in more than 10% of tumor cells. The Ki67 labelling index was expressed as the percentage of reactive tumor cells. The tumors were considered c-erb-B2-positive when more than 10% of cells showed specific membrane staining.

Flow cytometry. The procedure was always carried out on fresh tumor tissue, which was kept in phosphate-buffered saline (PBS) at 4°C for less than 24 h after having been obtained, according to protocols previously described (13). The fresh tissue was first finely minced with a scalpel blade, mixed with 2 ml of DNA-prep Stain reactant and 100 μ l DNA-prep LPR reactant (both from Coulter Corporation, Miami, FL, USA), and incubated for 30 min at 37°C. The resulting mixture was then filtered through a 50- μ m pore filter and was ready for cytometric analysis in a Coulter EPICS XL cytometer (Coulter Corporation).

The analysis of the obtained histograms was carried out with the help of the MultyCycle DNA Cell Cycle Analysis software package (Phoenix Flow Systems, San Diego, CA, USA). The tumors were considered diploid when the DNA-index obtained was 1.0, and

Table I. Number of tumors studied and evaluations performed.

| | Case number | % |
|-----------|-------------|------|
| Ki67 | 277 / 280 | 98.9 |
| DNA-index | 275 / 280 | 98.2 |
| Diploid | 151 | 54.9 |
| Aneuploid | 124 | 45.1 |
| S-phase | 273 / 280 | 97.5 |

aneuploid for any diverging value, including tetraploid tumors, with a DNA-index of 2.0. In aneuploid tumors, the percentage of cells in the S-phase was determined separately for the diploid and aneuploid cell subpopulations. The 75th percentile of distribution was then calculated for both and was 16.4% for the diploid and 12.6% for the aneuploid cell fractions, respectively. In the diploid tumors, the 75th percentile of cells in the S-phase corresponded to 7.2%.

Statistics. All calculations were performed using the GraphPad Prism biomedical statistical package (GraphPad Software, Inc., San Diego, CA, USA). The Spearman's test was used for the comparison of discrete variables and the Pearson's test for the comparison of continuous ones. Values were considered significant when p was <0.05.

Results

The Ki67 labelling index could be successfully evaluated in 277 instances and the DNA-index was determined in 275 out of the 280 studied tumors. Of the 275, 151 (54.9%) were diploid, and the remaining 124 aneuploid (45.1%). The percentage of cells in the S-phase could be determined in 273 instances (97.5% of the whole sample) (Table I, Figure 1).

A significant correlation was found between the proliferation measured by the Ki67 labelling index and flow cytometry. Thus, in the diploid tumors, Ki67 >20% and cells in the S-phase above the 75th percentile were highly correlated ($r=0.42$, $p<0.0001$), whereas in the aneuploid tumors this was only true for the aneuploid cell subpopulation ($r=0.48$, $p<0.0001$). Additionally, a highly significant correlation was found between the percentage of cells expressing Ki67 and the DNA-index ($r=0.24$, $p<0.0001$), indicating that the higher the proliferation, the more the DNA-index shifts towards an aneuploid pattern.

As can be seen in Table II, both the DNA-index and the Ki67 labelling index showed a significant correlation with established prognostic factors in breast cancer, with the only exception, again, of the S-phase of the diploid cell subpopulation of the aneuploid tumors. Significant correlations of both proliferation parameters with a high histological and nuclear grade and an absence of hormone receptors were found. Mutant p53 expression, furthermore, showed a significant correlation with DNA-aneuploidy ($r=0.15$, $p=0.007$), Ki67 ($r=0.33$, $p<0.0001$) and the S-phase of the

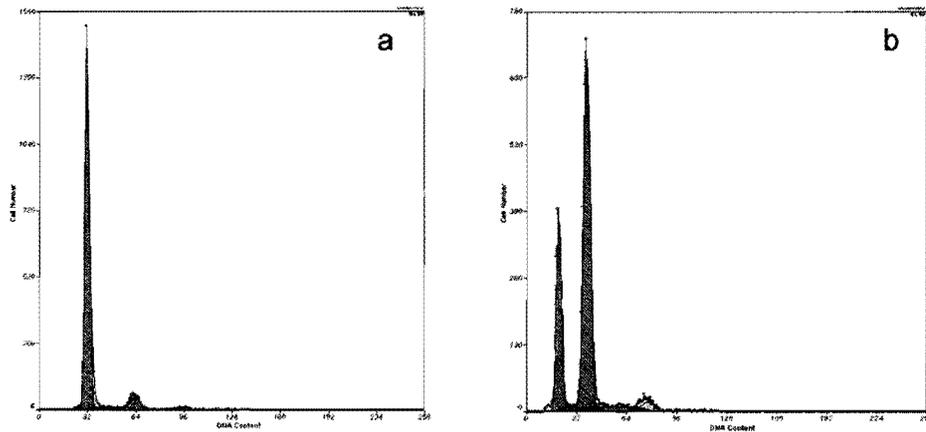


Figure 1. Cell cycle analysis of a diploid (a) and an aneuploid (b) tumor.

Table II. Correlation between the proliferation parameters and other prognostic factors.

| | DNA-index | | Diploid tumor | | Aneuploid tumor | | | | Ki67 | |
|--------------------|-----------|---------|---------------|---------|-----------------|------|-------------------|---------|-------|---------|
| | r | p | r | p | diploid S-phase | | aneuploid S-phase | | r | p |
| | | | | | r | p | r | p | | |
| Histological grade | 0.15 | 0.01 | 0.18 | 0.04 | -0.007 | 0.93 | 0.26 | 0.005 | 0.37 | <0.0001 |
| Nuclear grade | 0.26 | <0.0001 | 0.21 | 0.01 | -0.2 | 0.02 | 0.3 | 0.001 | 0.43 | <0.0001 |
| Nodal invasion | 0.16 | 0.007 | 0.02 | 0.79 | -0.08 | 0.32 | -0.08 | 0.33 | 0.08 | 0.15 |
| ER | -0.15 | 0.006 | -0.37 | <0.0001 | 0.06 | 0.46 | -0.48 | <0.0001 | -0.33 | <0.0001 |
| PR | -0.18 | 0.002 | -0.35 | <0.0001 | 0.11 | 0.22 | -0.37 | 0.0001 | -0.36 | <0.0001 |
| c-erb-B2 | 0.15 | 0.008 | 0.11 | 0.18 | 0.03 | 0.67 | 0.17 | 0.053 | 0.35 | <0.0001 |
| p53 | 0.15 | 0.007 | 0.14 | 0.09 | 0.04 | 0.66 | 0.3 | 0.0009 | 0.33 | <0.0001 |
| Tumor size | 0.15 | 0.01 | 0.28 | 0.0008 | -0.002 | 0.98 | 0.1 | 0.29 | 0.2 | 0.0005 |

ER, estrogen receptor; PR, progesterone receptor.

aneuploid cell fraction of aneuploid tumors ($r=0.3, p=0.0009$). The expression of the c-erb-B2 oncogene showed a significant correlation with Ki67 expression ($r=0.35, p<0.0001$) and DNA-aneuploidy ($r=0.15, p=0.008$), but not with the percentage of cells in the S-phase. Tumor size was significantly related to Ki67 expression ($r=0.2, p=0.0005$) and aneuploidy ($r=0.14, p=0.01$), as well as to the percentage of cells in the S-phase in diploid tumors ($r=0.28, p=0.0008$).

Finally, regarding the most important clinical prognostic factor, axillary nodal invasion, the only associated proliferation parameter was DNA-aneuploidy ($r=0.16, p=0.007$).

Discussion

It is generally assumed that either the proportion of cells in the S-phase or the expression of the Ki67 antigen reflects the same feature of breast tumors, namely their

proliferation rate (7, 13). However, it seems that both methods are not exactly superimposable, but rather complementary to each other. Vielh *et al.* (7) found a significant correlation between Ki67 expression and axillary nodal invasion, on the one hand, and a high S-phase and absence of hormone receptor expression, on the other. The latter finding is coincident with the results of our own study, but we did not obtain the correlation between Ki67 expression and axillary nodal invasion reported by Vielh *et al.* Our study is in closer agreement with that of Gilchrist *et al.* (14), who found a tight relationship between tumor aneuploidy and both axillary metastasis or absence of hormone receptor expression, as additionally corroborated by the study by Pinto *et al.* (11). This group exclusively studied advanced breast cancers and found a significant correlation between DNA-aneuploidy, a high S-phase and absence of hormone receptor expression.

Our results are also in agreement with those reported by Chassevent *et al.* (3), who found DNA-aneuploidy to be directly related to tumor size, axillary nodal invasion, a high histological grade and an absence of hormone receptor expression. These authors reported that patients carrying node-negative tumors with an intermediate or high S-phase had a shorter disease-free survival, whereas for their counterparts with node-positive tumors, both the S-phase and the hormone receptor status had prognostic power. It is interesting to note that these authors only studied the S-phase of the aneuploid cell subpopulation in aneuploid tumors, which seems to corroborate our finding that only the S-phase of aneuploid cells showed a significant correlation with the other clinical and biological features of the tumors studied. In a previous paper (15), we reported that only the S-phase of diploid tumors identified high-risk subgroups, whereas the S-phase of aneuploid tumors showed no prognostic benefit. However, in contrast to the present study, we had determined the S-phase of aneuploid tumors as a mean of the S-phase of both subpopulations, the usual procedure at that time. The present recommendation, in agreement with the guidelines proposed by Kallioniemi *et al.* (16), is to use only the S-phase of the aneuploid cell subpopulation, since the S-phase of the diploid cell fraction of aneuploid tumors seems to be irrelevant for prognostic purposes.

Our results, together with those from the previously cited studies, seem to indicate that Ki67 expression accurately reflects tumor cell proliferation. In addition to proliferation, represented by the S-phase, we can also employ flow cytometry to study DNA-ploidy, which seems to be associated with important prognostic features of tumors, such as axillary nodal invasion. Offersen *et al.* (17) found that Ki67 expression, studied by means of the MIB-1 antibody, was associated with tumor size, tumor grade and absence of hormone receptor expression (in agreement with our results), but had no independent prognostic power.

In conclusion, if the study of tumor cell proliferation is the major end-point, the determination of Ki67 expression by means of immunohistochemistry with the MIB-1 antibody is an inexpensive, reproducible and easy to perform procedure for routine clinical use. However, if more prognostically relevant information than the cell proliferation rate is needed, flow cytometry should be employed.

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