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Flow Cytometry *vs.* Ki67 Labelling Index in Breast Cancer: A Prospective Evaluation of 181 Cases

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Abstract. *Backaround: Excessive proliferation is one of the first steps in oncogenic activation and one of the most important biological features defining the aggressiveness of. tumors. Quantifying the proportion of tumor cells in S-phase by means of flow cytometry has shown, in the past, to be useful for defining high-risk subgroups in breast cancer. Several antigens closely associated with proliferation are also detectable by means of immunohistochernistry, offering in theory an easy to perform and cheap alternative to flow cytometry for measuring proliferation. To test this hypothesis, we compared both methods prospectively in a series of breast cancers. Materials and Methods: We studied the proliferation rate of 181 breast cancers (152 ductal infiltrating, 17 lobular infiltrating, 12 other histological varieties), operated upon at our institution, by means of flow cytometry and the Ki67 labelling index, using the MIB1 antibody. Ploidy (expressed as DNA content or DNA-index), S-phase fraction and the Ki67 labelling index were the variables of the study. The S-phase fraction was considered separately for diploid and aneuploid tumors, following the 1992 Maine Consensus guidelines and was judged abnormally elevated if higher than the 75th percentile for each group. The Ki67 labelling index was expressed as percent positive tumor cells, positive cells being those showing specific nuclear staining. Results: DNA-ploidy and the Ki67 labelling index could be evaluated in all tumors. Of the total, 96 (53%) were diploid and 85 (47%) aneuploid. S-phase fraction could be measured in 172 out of the 181 tumors (95%). The 75th percentile cut-offs for diploid and aneuploid tumors were 9.9% and 15.8%, respectively. We found a significant correlation beween rising DNA content and increasing Ki67 index ($r = 0.18$; $p = 0.022$), as well as between the percentage of cells in S-phase of the whole tumor population and Ki67 ($r = 0.22$; $p = 0.0055$). A Ki67 cut-off of 50% or higher identified most aneuploid tumors, or a small group of diploid ones with a high S-phase fraction (specificity = 96.7%; positive predictive value 92.5%), however at the price of a very low sensitivity (62.6%). This was due to the presence of many aneuploid tumors with a low S-phase fraction. Conclusion: The Ki67 labelling index and S-phase fraction are significantly correlated. However, flow cytometry provides additional indirect information on tumor aggressiveness associated with DNA-ploidy. Further studies are needed to determine whether Ki67 alone is sufficient as a routinely applicable method.*

Although proliferation is an important feature defining the aggressiveness of tumors, it has not been generally accepted as an independent prognostic factor in breast cancer. This may be partly due to the fact that there are different methods of measuring proliferation and it has still not been defined which of them is the ideal one in practice. Standard histology, especially when applying exhaustive evaluation scales such as the Elston score (1), flow cytometry and immunhistochemistry are among the most widely tested tools for measuring the proliferation rate of breast cancers. Measuring the proportion of cells in S-phase by means of flow cytometry has shown in the past to be useful for defining high-risk subgroups in breast cancer (2) and, in general, for defining prognosis. However, this method, although having passed the test of time, has some drawbacks which prevent it from being generally adopted in the clinic: it requires costly equipment to be used by highly-trained personnel and, furthermore, S-phase measurements are not obtainable in a number of tumors, ranging from around 5% for fresh specimens, to up to 25-40% in the case of paraffin-embedded ones (2-5). Immunohistochemistry, on the other hand, is now a routine procedure in virtually every pathological department around the world, being cheap and easy to perform. Its drawbacks are the usual ones attributed to this technique, mainly the subjectiveness of the evaluation and the differences in reactivity between the various antibodies usually available for detecting any given antigen, together with other sources of interobserver variability, such as the different reactivity of the detection kits or methods (epitope unmasking techniques, etc) used. In spite of these criticisms, a number of studies have been performed on the expression of proliferation-associated markers, such as PCNA, Ki67, AGNOR's and

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others, comparing them to standard techniques of proliferation measurement such as BrdU uptake or radioactive thymidine labelling index (6,7). It seems from most of them that Ki67 is the one immunohistochemical marker that most closely reflects the proliferative rate of the studied cells, although the correlation with the standard, aforementioned techniques, is less than complete (7,8). Ki67, finally, was originally only detectable on fresh-frozen tumor tissue, which posed an additional difficulty for its standard use in the clinic. This has been overcome by the development of the MIB-1 antibody, which effectively recognizes Ki67-expression on archival, paraffin-embedded tissue. In a comparative study between several antibodies detecting proliferation markers, furthermore, it yielded the best results among them all (9).

In the present study we have compared flow cytometry and the Ki67 labelling index in a series of breast cancers, in order to establish if they deliver the same message concerning proliferation and, if so, which one to choose for further routine use in our clinical practice.

Materials and Methods

We studied 181 breast cancers consecutively operated upon at Fundación Tejerina-Centro de Patologia de la Mama, Madrid, Spain, between January 2000 and April 2001. Of them, the vast majority were infiltrating ductal carcinomas (152), followed by infiltrating lobular carcinomas (17) and other, less frequent variants (12). The proliferation rate of the tumors was assessed by two different methods: immunohistochemistry for the detection of the Ki67 antigen on slides from formalin-fixed, paraffin-embedded samples and flow cytometry, which was carried out mostly on fresh tumor specimens (145 cases), as well as on a smaller proportion of archival, paraffin-embedded tumors (35 cases).

Immunohistochemistry. The immunohistochemical procedure was the standard one normally employed at our laboratory, which has been extensively described elsewhere (10). It involves the use of the MIB1 antibody and, after completion, allows for the quantitation of specifically-stained nuclei and the establishment of the proportion of reactive tumor cells, which is expressed as per cent positive. Briefly, the procedure was carried out on 5-μm sections mounted on poly-L-lysine-coated slides. The immunohistochemical kit was always the same, to ensure uniformity of results (Histostain-SP, Zymed, San Francisco, Ca., U.S.A). The antibody used, as has been said, was prediluted MIB1 (Ki67) from Zymed (San Francisco, CA, U.S.A.). We employed the heat-induced antigen retrieval (HIER) technique developed at Zymed laboratories, as described on the data sheet accompanying their antibodies. The slides were de-paraffinized in three xylene baths, 5 minutes each and then rehydrated in phosphate-buffered saline (PBS) for 10 minutes after passages through graded ethanols (100%, 96%, 70%), 3 minutes each. Afterwards, the preparations were pretreated for antigen retrieval (see above), incubated with blocking serum (component 1A of the kit) for 10 minutes and subsequently with the prediluted antibody for 1 hour at room temperature. The slides were then washed in PBS three times for 3 minutes, after which the second, biotinylated bridge antibody was applied (component 1B of the kit) for 10 minutes. Following three washes in PBS 3 minutes each, the slides were incubated with the streptavidin-peroxidase complex (component 1C of the kit) for 10 minutes, washed again In PBS and stained with Diamino-benzidine. They were then counterstained with hematoxylin for 30 seconds and mounted.

Flow cytometry. Fresh tumors were finely minced with a scalpel blade, mixed with 2 ml of DNA-prep Stain reactant and 100 μl DNA-prep LPR reactant (both from Coulter Corporation, Miami, FL, U.S.A.), and incubated for 30 minutes, at 37 °C. The resulting mixture was then filtered through a 50 μm pore filter and was ready for cytometric analysis. Preparation of paraffin-embedded samples was more laborious: 4 to 5 slides with a thickness of 50 μm were deparaffinized in xylene and rehydrated in graded ethanols and finally PBS, then finely minced with a scalpel blade and incubated with 2 ml 0.5% pepsin for 30 minutes at 37 °C. The mixture was then filtered through a 50 μm pore filter and afterwards centrifugued at 2,000 rpm for 10 minutes. The supernatant was then resuspended in 1 ml DNA-prep Stain and 50 μl DNA-prep LPR (see above), incubated for 30 minutes at 37 °C, and was ready for analysis in a Coulter EPICS XL cytometer (Coulter Corporation, Miami, FL, U.S.A.). 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, U.S.A.). Tumors were considered diploid when the DNA-index obtained was 1.0 and aneuploid for any diverging value, including tetraploid tumors, with a DNA-index of 2.0.

Statistics. All calculations were performed using the GraphPad Prism biomedical statistical package (GraphPad Software, Inc., San Diego, CA, U.S.A.). Values were considered significant, when $p < 0.05$.

Results

DNA-ploidy and the K67 labelling index were evaluated in all 181 tumors. Following this, 96 (53%) of them were found to be diploid, with a DNA index of 1.0 and 85 (47%) aneuploid, with a DNA-index >1.0. S-phase fraction could be calculated in 172 out of the 181 tumors (roughly 95%), which is well within the expected limits, especially considering that 36 samples came from archival paraffin blocks. Following the recommendations of the 1992 Maine Consensus Meeting (11), we calculated the percentage of cells in S-phase independently for both subpopulations of diploid and aneuploid tumors. The 75th percentile for each of them was 9.9% and 15.8%, respectively, and only tumors showing values above these levels were considered "high S- Phase". There was a significant correlation beween rising DNA content and increasing Ki67 index ($r = 0.18$; $p = 0.022$), as well as between the percentage of cells in S-phase of the whole tumor population and Ki67 ($r = 0.22$; $p = 0.0055$). However, when one observes the corresponding linear regression graph (Figure 1), it is obvious that a direct correlation indeed exists between the plotted results, but the scatter of values is unacceptably high. Our working hypothesis was that this was attributable to the presence of "odd men" in both subgroups of diploid and aneuploid tumors. In fact, when we eliminated from the subgroup of diploid tumors those with a high proportion of cells in S-phase (*i.e.*, "bad diploid tumors) and, conversely, from the subgroup of aneuploid tumors those with an unusually low percentage of cells in S-phase (*i.e.* "good" aneuploid tumors), the correlation and its corresponding p value improved remarkably ($r = 0.3794$, $p = 0.0005$). However this, at first glance excellent, result was obtained at the price of purging from the whole series more than 50% of the tumors, which makes it useless for practical purposes.

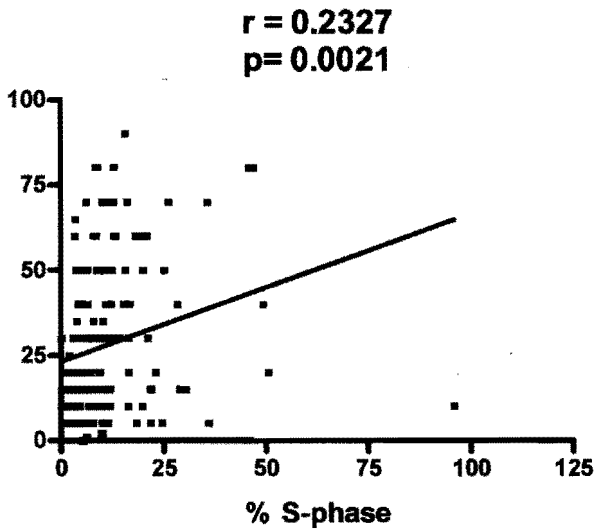

Figure 1. *Correlation between the S-phase fraction of breast cancers and their Ki67 labelling index. N=181.*

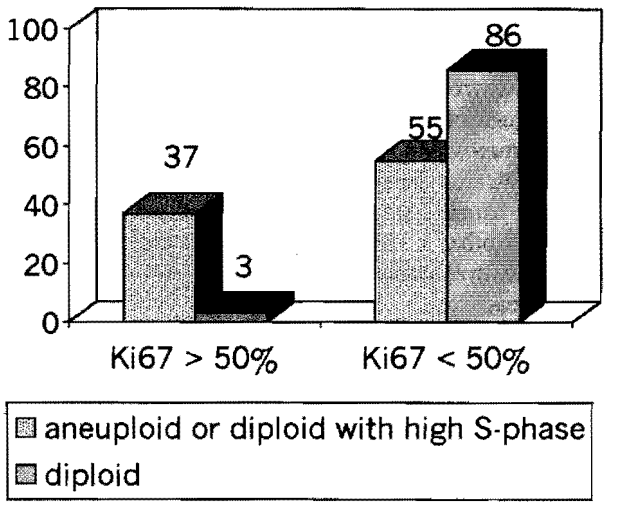

Figure 2. *Relationship between ploidy and proliferation measured by flow cytometry vs. proliferation measured by means of the Ki67 labelling index*

As a further step, we tried to establish a Ki67 cut-off which might have clinical applications. By means of stepwise regression analysis, this was found to lie at 50% or more of reactive tumor cells. As can be seen from Figure 2, very few diploid tumors (10 out of 66) showed a Ki67 labelling index of 50% or higher. All other tumors with a Ki67 index equal to or higher than 50% (40 cases) were aneuploid. Of the 10 diploid tumors with unexpectedly high Ki67 scores, furthermore, 7 had a proportion of cells in S-phase above the 75th percentile, which, according to our previous paper on the subject, automatically put them in a bad prognostic category (2). Therefore, a Ki67 index of 50% or above yields a false-positive rate regarding bad prognosis of just 3.1%, (specificity = 96.7%; positive predictive value 92.5%), which seems acceptable for clinical purposes. Unfortunately, the false-negative rate, on the other hand, does not match this excellent result. In fact, as can be seen again from Figure 2, 55 out of 85 aneuploid tumors had a Ki67 index below 50%, for a negative predictive value of just 60.1% (Figure 3). When we analyzed these cases in search of an explanation for this unexpected result, we found that most of them (40 out of 50 in which the S-phase fraction could be evaluated, *i.e.*, 80%) showed S-phase values below the 75th percentile. Therefore, it seems clear that the Ki67 labelling index just reflects the proportion of cells in S-phase (or of replicating cells in general), whereas DNA-aneuploidy seems to reflect something else in addition, closely associated with a bad prognosis.

Figure 3. *Prognostic model for ploidy and proliferation rate of breast cancers using the data provided by flow cytometry and the Ki67 labelling index.*

	Ki67 ≥ 50%	Ki67 < 50%	
Aneuploid or diploid with high S-phase	37	55	Sensitivity = 62.6%
Diploid	3	86	Specificity = 96.7%
	PPV = 92.5	NPV = 60.1 %	

PPV = positive predictive value
PV = negative predictive value

Discussion

Flow cytometry and the Ki67 labelling index for measuring the proliferation rate of breast cancers have been compared before by Vielh *et al.* (12). They found a correlation of a very similar range to the one obtained by us (r = 0.3, p< 0.05), but in their case only restricted to the subgroup of aneuploid tumors. This may be explicable by the smaller size of their series, the higher percentage of aneuploid tumors found by them and the lower proportion of cases in which a useful S-phase reading could be obtained. However, given the other correlations they found between both methods and other indicators of higher proliferation and tumor aggressiveness (nuclear grade, mitotic index, node invasion), we are certain that a higher case number would have elicited results very similar, if not identical to ours. In any case, what the results by Vielh *et al.*, as well as our own ones, clearly show is that K67 and S-phase tend to indicate the same feature in breast tumors (*i.e.*, proliferation), but are not exactly superimposable. This had already been studied before by Silvestrini *et al.* (7), who showed that nucleic acid precursor uptake was the feature most exactly reflecting the proliferation rate of tumor

cells, followed by S-phase, with the Ki67 index in last place. On the other hand, both S-phase and Ki67 have been independently reported to be clinically useful for identifying high-risk subgroups in breast cancer (2, 13-15), so that the relevant question in practice seems to be which one to choose for that purpose. In this respect, Ki67-labelling, in spite of being simple and cheap to perform and easily reproducible, has been harmed by the appearance of other antibodies, identifying other proliferation-associated antigens, such as PCNA. However, almost every time they have been compared between each other, Ki67 has emerged as the one most closely associated with the effective proliferation rate of the studied tumors (9). A different question is whether, by using flow cytometry, we are able to determine not just the proliferation rate, but also indirectly other features of the tumors, by measuring their DNA-content. Our results seem to support this hypothesis. In fact, it is plausible that an aberrant DNA-content might reflect the presence of mechanisms associated with the development of a more aggressive tumor phenotype, such as the amplification of genes associated with invasion and metastasis, the inhibition of apoptosis, *etc.* A very recent report seems to indicate that this is indeed the case. In it, Rennstam *et al.* (16) show that chromosomal rearrangements and oncogene amplification (which would translate into an abnormal DNA-content by flow cytometry) precede aneuploidization in breast cancer. However, DNA-ploidy has not been identified up to this date as an independent predictor of prognosis in breast cancer. In one of the few reports where it has been found to have prognostic significance, moreover, the close association with histologic grade also disclosed by the statistical analysis offers a much cheaper alternative for obtaining the same message (17).

From our results, finally, it can be concluded that tumors with a Ki67 labelling index of 50% or above are highly proliferative or aneuploid (*i.e.*, carry a bad prognosis), whereas those with lower values merit further study, if the knowledge about their DNA ploidy is considered important, since aneuploid tumors with a low S-phase fraction may also show low Ki67 indexes.

If proliferation alone is considered the only significant parameter of prognosis, either S-phase or Ki67 reflect this feature, although not in exactly the same way. It should be disclosed in further prospective, large comparative studies which of them most closely predicts the outcome of the patients, since at the present moment we are at a loss if we have to decide between any one of them for their routine use in clinical practice.

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