Prognostic comparative study of S-phase fraction and Ki-67 index in breast carcinoma

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Abstract

Aims—To investigate the prognostic value of recently proposed flow cytometric S-phase fraction (SPF) variables (average SPF and SPF tertiles) compared with conventional SPF, and to compare the one with the best predictive value with the immunohistochemical Ki-67 index in breast carcinoma.

Methods—A short term follow up study (median, 39.6 months) of a large series of patients (n = 306) was conducted. DNA ploidy was analysed on fresh/frozen tumour samples by flow cytometry, and the SPF was calculated from the DNA histogram using an algorithm. The Ki-67 index was assessed on paraffin wax embedded material by immunohistochemistry (cut off point, 10%). The two methods were compared by means of K statistics, and the prognostic significance of both in relation to disease free survival (DFS) and overall survival (OS) was determined.

Results—SPF and Ki-67 analysis was performed on 234 (76.5%) and 295 (96.4%) tumours, respectively. The two assessments were simultaneously available in 230 cases. All SPF variables analysed in the whole series significantly correlated with disease evolution, with the conventional median SPF (cut off point, 6.1%) showing the highest predictive value in relation to both DFS (p = 0.0001) and OS (p = 0.0003). SPF tertiles and median SPF evaluated according to DNA ploidy status had no prognostic significance. The Ki-67 index showed a trend in relation to DFS (p = 0.086) that did not reach significance, and no correlation with OS was found (p = 0.264). The comparative analysis of SPF and Ki-67 revealed some agreement between the two methods (agreement, 69.13%; K statistic, 0.3844; p < 0.001), especially in the subgroup of diploid tumours.

Conclusions—Flow cytometric SPF is a better prognosticator than the Ki-67 index, but only SPF variables applied in the whole series show potential clinical usefulness.

Keywords: breast carcinoma; DNA flow cytometry; immunohistochemistry; S-phase fraction; Ki-67; prognosis

It is well recognised that the proliferative activity of neoplastic cells influences the clinical course of certain types of human malignancy. However, methodological issues, such as the choice of the best method for the assessment of proliferation and the standardisation of criteria for interpretation of results, have limited its clinical application.

In a previous study, we showed that the S-phase fraction (SPF) is an independent marker of disease outcome in breast carcinoma. For prognostic purposes, some investigators adopt the median value as the cut off point, whereas others use two thresholds for defining a three group classification system. Therefore, we investigated which SPF variable has the greatest predictive value and would provide useful prognostic information for the clinician. In addition, we sought to determine the best cell proliferation method for predicting disease outcome, comparing distinct markers in the same series of patients. We tested Ki-67, a nuclear antigen present in all active phases of the cell cycle (G1, S, G2, and mitosis (M)), which is a valuable indicator of tumour proliferation and prognosis in patients with breast cancer. The immunohistochemical Ki-67 index has the technical advantage, in relation to flow cytometry, of allowing the morphological evaluation of proliferating cell populations.

Our study was designed to investigate the following three areas in a series of 306 patients with breast cancer, namely: (1) to elicit the SPF category with the best prognostic strength, by applying SPF variables with distinct cut off points; (2) to compare SPF with immunohistochemical Ki-67 results; and (3) to correlate both indices with disease outcome (disease free survival (DFS) and overall survival (OS)).

Materials and methods

The study group consisted of 306 women with primary operable invasive breast cancer (stage I/II of the disease), diagnosed and treated at the Instituto Português de Oncologia, Lisbon between October 1990 and December 1996. The eligibility criteria for patients included the lack of treatment before surgery, the availability of frozen samples for flow cytometry, and accurate follow up information. The mean age of the patients was 58.5 years (range, 23–88). The histological type and tumour staging of breast carcinomas were evaluated according to the TNM-UICC system. The series comprised 273 invasive ductal carcinomas (89.2%) and 33 carcinomas of other histological types (10.8%). One hundred and twenty tumours (39.2%) were classified as pT1 (< 2 cm), 163 (53.3%) as pT2 (2–5 cm), and 23 (7.5%) as pT3 (> 5 cm). One hundred and seventy three patients (56.5%) had no axillary lymph node positivity (pN0), whereas 133 (43.5%) had...
nodal involvement (pN1). Two hundred and seventy eight patients (90.9%) underwent modified radical mastectomy and axillary lymphadenectomy, and 28 (9.1%) were submitted to conservative surgery (tumorectomy or quadrantectomy) and axillary lymph node dissection as primary surgical treatment. Heterogeneous adjuvant therapeutic regimens were given to the patients: 84 received chemotherapy; 46 hormonotherapy; 33 chemo-therapy, hormonotherapy, and radiotherapy; 43 chemotherapy and hormonotherapy; 84 chemotherapy and radiotherapy; and nine hormonotherapy and radiotherapy, whereas seven patients received no adjuvant treatment. Information on DFS and OS was obtained from clinical chart review or consultation of the epidemiological registry service at our institution (ROR-Sul). DFS and follow up period were defined as the time that elapsed between primary surgical resection and the first recurrence, locally or at a distance, and the last clinical observation or death, respectively. The median follow up was 39.6 months (range, 3–84). At the end of follow up time, 254 patients (83%) were alive without evidence of disease, 17 (5.6%) were alive with disease, and 34 (11.1%) had died of their disease. One patient who died from an unrelated cause was censored from the survival analysis study.

**DNA FLOW CYTOMETRY STUDY**

Flow cytometric analysis was performed on representative fresh/frozen samples obtained at the time of surgery, as described previously. Briefly, the tissue samples were mechanically disaggregated in cold phosphate buffered saline (PBS) using scalpel blades, and the cell suspension obtained was rinsed twice in PBS and checked by counting in a Bürker haemocytoscope, and the cell disaggregated in cold phosphate bu-

**IMMUNOHISTOCHEMICAL STAINING**

Ki-67 immunostaining was performed on 2–3 µm thick sections cut from formalin fixed, paraffin wax embedded tissue using the streptavidin–biotin complex peroxidase technique. First, the sections were attached to gelatine coated slides and dried overnight at 37°C. Dewaxing in xylene and washes in 100% ethanol were followed by two pretreatment procedures: endogenous peroxidase was blocked by 0.6% hydrogen peroxide in methanol for 10 minutes, and antigen retrieval was carried out using a pressure cooker and citrate buffer, pH 6.0 for one minute. After washing in water, the sections were rinsed in Tris buffered saline (TBS), pH 7.4–7.6, and incubated for 30 minutes at room temperature with primary monoclonal anti-Ki-67 antibody (anti-Ki-67/B11 clone; Zymed Laboratories, San Francisco, California, USA) at a 1/50 dilution. The sections were then washed in TBS
and incubated with a secondary biotinylated goat antimouse/antirabbit serum (K492; Dako) at a 1/100 dilution for 30 minutes. The sections were rinsed again in TBS, and the StreptABC complex (K492; Dako) at a 1/100 dilution was applied for 30 minutes. After washing in TBS, diaminobenzidine tetrahydrochloride (D-5637; Sigma, St Louis, Missouri, USA) was used as chromogen for eight minutes. The sections were then washed in water and finally counterstained with Mayer’s haematoxylin. As negative control, staining was performed without primary antibody, and human normal appendix tissue was used as positive control.

**Staining assessment**

The entire slide was scanned for immunostaining evaluation by two observers using a two headed light microscope. All malignant cells with nuclear staining were considered to be positive (fig 2). When Ki-67 immunoreactivity was distributed diffusely, randomly chosen tumour cells were assessed in several high power fields; whenever there was focal/heterogeneous staining, the scoring was carried out in the area with the highest number of positive nuclei. The Ki-67 index was expressed semiquantitatively only in the invasive component of the tumour in at least 200 neoplastic cell nuclei. A cut off point of 10% was used to distinguish between the categories of low and high proliferative tumours.

**Statistical analysis**

The $\kappa$ statistic was used to compare flow cytometric SPF and immunohistochemical Ki-67 results. $\kappa$ Values between 0.21 and 0.40 suggested a reasonably better agreement and values between 0.00 and 0.20 suggested a slightly better agreement than would be expected by chance alone. Analysis of survival data was performed using the Kaplan-Meier method, with differences between survival curves being evaluated by the log rank test. Probabilities of $p < 0.05$ were regarded as significant.

**Results**

Table 1 illustrates the correlation between the SPF categories and the disease outcome as assessed by DFS and OS. Only the SPF variables analysed in the whole series showed a significant correlation with the evolution of the disease, with the “conventional median SPF” having the greatest predictive strength in relation to both DFS ($p = 0.0001$) and OS.
Therefore this SPF category was used for comparison with Ki-67 results. Neither SPF tertiles nor median SPF evaluated by DNA ploidy status (DNA diploid vs DNA aneuploid) showed significance in relation to disease outcome.

SPF analysis was feasible in 234 cases (76.5%), half of which were considered as slowly proliferative tumours and the other half as highly proliferative. The Ki-67 index was obtained in 295 cases (96.4%), including 159 low and 136 high proliferative tumours. In the remaining 11 cases, Ki-67 was not determined because of the lack of representative pathological material in the paraffin wax blocks.

The concomitant assessment of both cell proliferation parameters was available in 230 cases.

Table 1  Correlation between SPF variables and disease outcome in breast carcinoma

<table>
<thead>
<tr>
<th>Variables</th>
<th>Disease free survival</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>No. recurrences</td>
</tr>
<tr>
<td>% Average SPF (whole series)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5.9</td>
<td>119</td>
<td>8</td>
</tr>
<tr>
<td>≥5.9</td>
<td>120</td>
<td>29</td>
</tr>
<tr>
<td>% SPF tertiles (whole series)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;4.5</td>
<td>78</td>
<td>4</td>
</tr>
<tr>
<td>4.5–9.2</td>
<td>78</td>
<td>12</td>
</tr>
<tr>
<td>≥9.2</td>
<td>78</td>
<td>19</td>
</tr>
<tr>
<td>% SPF tertiles (diploid tumours)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>3–4.7</td>
<td>50</td>
<td>3</td>
</tr>
<tr>
<td>≥4.7</td>
<td>42</td>
<td>5</td>
</tr>
<tr>
<td>% SPF tertiles (aneuploid tumours)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>34</td>
<td>7</td>
</tr>
<tr>
<td>10–13</td>
<td>35</td>
<td>8</td>
</tr>
<tr>
<td>≥13</td>
<td>36</td>
<td>9</td>
</tr>
<tr>
<td>% Median SPF (whole series)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3.8</td>
<td>117</td>
<td>7</td>
</tr>
<tr>
<td>3.8–6.1</td>
<td>66</td>
<td>4</td>
</tr>
<tr>
<td>≥6.1</td>
<td>63</td>
<td>6</td>
</tr>
<tr>
<td>% Median SPF (diploid tumours)</td>
<td></td>
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<tr>
<td>&lt;3.8</td>
<td>117</td>
<td>7</td>
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<tr>
<td>3.8–6.1</td>
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<tr>
<td>≥6.1</td>
<td>63</td>
<td>6</td>
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<tr>
<td>% Median SPF (aneuploid tumours)</td>
<td></td>
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<tr>
<td>&lt;3.8</td>
<td>117</td>
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<td>3.8–6.1</td>
<td>66</td>
<td>4</td>
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<tr>
<td>≥6.1</td>
<td>63</td>
<td>6</td>
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</table>

SPF, S-phase fraction.

Table 2  Comparison between flow cytometric SPF and immunohistochemical Ki-67 index methods

<table>
<thead>
<tr>
<th>Variable</th>
<th>SPF</th>
<th>n</th>
<th>Low</th>
<th>High</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67 index</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Low</td>
<td>134</td>
<td>88</td>
<td>46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>96</td>
<td>25</td>
<td>71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agreement: 69.13%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>χ: 0.3844</td>
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</tbody>
</table>

SPF cut off point, 6.1%; Ki-67 cut off point, 10%. SPF, S-phase fraction.

Table 3  Relation between SPF and Ki-67 proliferation indices in DNA diploid breast carcinomas

<table>
<thead>
<tr>
<th>Variable</th>
<th>SPF</th>
<th>n</th>
<th>Low</th>
<th>High</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67 index</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.005</td>
</tr>
<tr>
<td>Low</td>
<td>93</td>
<td>84</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>32</td>
<td>23</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agreement: 74.40%</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>χ: 0.2154</td>
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</table>

SPF cut off point, 6.1%; Ki-67 cut off point, 10%. SPF, S-phase fraction.

Figure 3  (A) Probability of disease free survival and (B) overall survival according to S-phase fraction (SPF) (cut off point, 6.1%) in breast carcinoma (n = 234). Low SPF groups have a more favourable outcome (p < 0.001).
Cell proliferation in breast carcinoma

S-phase cells in breast cancer. is the most reproducible method for estimating some studies have shown that the average SPF variables evaluated according to DNA ploidy status showed no predictive significance, of prognostic strength (table 1). In contrast, the median SPF being the best indicator in terms both DFS and OS, with the conventional SPF) through their correlation showed that all SPF variables applied in the conventional SPF) determination is related to (B) overall survival (OS) according to Ki-67 index weak differences between survival curves in relation to DFS (not significant; p = 0.086) were verified. No differences in relation to OS were found (p = 0.264).

Discussion
The technical issues of validity and reproducibility related to prognostically useful methods to measure tumour cell proliferation are a matter of controversy. Although some authors have demonstrated a highly reproducible way of estimating the mitotic index in breast carcinoma, it has been difficult to reach general consensus on standardised conditions for SPF assessment, as well as on the cut off values to be used for prediction purposes. In our study, we evaluated the prognostic value of three SPF variables (average SPF, SPF tertiles, and conventional SPF) through their correlation with disease outcome. The use of the SPF tertiles classification, which applies two thresholds dependent on the aneuploid group, whereas others found a significant correlation between both methods, irrespective of the DNA ploidy status. In contrast, Jansen et al failed to demonstrate such a correlation. The comparative study revealed the existence of two groups of tumours exhibiting apparently contradictory results (table 2): one group comprised tumours with a low Ki-67 index and high SPF (n = 46), and the other comprised tumours with a high Ki-67 index and low SPF (n = 25). The discordant data found in this last group could be explained by the fact that the two methods evaluate different cell cycle compartments of proliferating cell populations: Ki-67 stained cells in the G1 phase being responsible for the higher percentage of cycling elements in these tumours. The other group included a few tumour samples that contained numerous mitotic figures but lacked Ki-67 immunostaining, a surprising finding because Ki-67 staining commonly identifies G2/M phases. Several
reasons have been advocated for the discrepancy; namely, a very low amount of Ki-67 antigen undetectable by the antibody used, or the occurrence of a mutated protein. The alteration of protein expression in nutritionally deprived cells has also been suggested together with the inability of the Ki-67 antibody to identify S-phase arrested tumour cells.

The main finding of our study is that flow cytometric SPF is the most useful cell proliferation method in predicting the short term prognosis of patients with breast cancer, with the conventional median SPF category being the best indicator of disease outcome compared with other SPF variables and the Ki-67 index. Gasparini et al compared SPF with other immunohistochemical indicators of cell proliferation, such as Ki-67 and proliferating cell nuclear antigen (PCNA), in a consecutive series of 195 patients with breast cancer, and also concluded that SPF is the best cell kinetics marker to assess disease prognosis. Similarly, Dettmar et al determined SPF and MIB-1 indices in their retrospective study of 90 node negative breast carcinomas, and showed by multivariate analysis that SPF has the highest prognostic value. However, it has to be taken into account that, despite promising results, SPF could not be assessed in 23.5% of our cases, owing to technical drawbacks. Furthermore, the high intratumour heterogeneity of breast carcinoma might affect SPF determination, which is a crucial problem when applying this parameter to the individual patient. To improve the accuracy of the method, some authors have recommended the separate analysis of multiple samples from the same specimen.

In conclusion, the comparative study of SPF and the Ki-67 index in breast carcinoma showed that: (1) the two methods show reasonable agreement; (2) Ki-67 appears to have limited prognostic usefulness; (3) flow cytometric SPF is a better prognosticator than the Ki-67 index; and (4) only the SPF variables assessed in the whole series constitute reliable proliferative indicators for estimating the disease outcome.

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