Immunohistochemical quantitation of oestrogen receptors and proliferative activity in oestrogen receptor positive breast cancer

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Abstract

Aim—To evaluate the effect of the duration of formalin fixation and of tumour heterogeneity on quantitative estimates of oestrogen receptor content (oestrogen receptor index) and proliferative activity (MIB-1 index) in breast cancer.

Methods—Two monoclonal antibodies, MIB-1 and oestrogen receptor, were applied to formalin fixed, paraffin wax embedded tissue from 25 prospectively collected oestrogen receptor positive breast carcinomas, using a microwave antigen retrieval method. Tumour tissue was allocated systematically to different periods of fixation to ensure minimal intraspecimen variation. The percentages of MIB-1 positive and oestrogen receptor positive nuclei were estimated in fields of vision sampled systematically from the entire specimen and from the whole tumour area of one “representative” cross-section.

Results—No correlation was found between the oestrogen receptor and MIB-1 indices and the duration of formalin fixation. The estimated MIB-1 and oestrogen receptor indices in tissue sampled systematically from the entire tumour were closely correlated with estimates obtained in a “representative” section. The intra- and interobserver correlation on the MIB-1 index was good, although a slight systematical error at the second assessment of the intraobserver study was noted.

Conclusion—Quantitative estimates of oestrogen receptor content and proliferative activity are not significantly influenced by the period of fixation in formalin, varying from less than four hours to more than 48 hours. The MIB-1 and the oestrogen receptor indices obtained in a “representative” section do not deviate significantly from average indices determined in tissue samples from the entire tumour. Finally, the estimation of MIB-1 index is reproducible, justifying its routine use.

(Keywords: Breast cancer, MIB-1, oestrogen receptor, proliferative activity.)

Immunohistochemical analyses of oestrogen receptor status and proliferative activity are of prognostic value in breast cancers. Moreover, oestrogen receptor analysis offers a method for the prediction of response to endocrine therapy. Until recently, these methods have required fresh material. The development of antibodies that can be used on formalin fixed, paraffin wax embedded material for the evaluation of the oestrogen receptor status and proliferative activity permits direct comparison with morphological and morphometrical variables.

The monoclonal antibody MIB-1 reacts with the Ki67 nuclear antigen associated with cell proliferation and found throughout the cell cycle (G1, S, G2, and M phases), but not in resting (G0) cells. A previous study has shown MIB-1 as a robust marker of cell proliferation and that a clear plateau effect is easily discerned. On paraffin wax embedded tissue sections, using a microwave antigen retrieval method, the MIB-1 antibody gives an immunohistochemical staining pattern which is identical with that of the Ki67 antibody in frozen sections. This antigen retrieval method, first described by Shi et al, also permits subsequent staining with monoclonal antibody to oestrogen receptor.

In previous studies of breast cancer the percentage of oestrogen receptor positive cells and proliferating cells varied considerably. Among the possible explanations for the discrepancies are sampling variation, intratumoral heterogeneity, different nature of the tumour samples (fresh, frozen or fixed tissue), variable dilution of antibody, differences in counting methods, and observer variability in the interpretation of the staining. In addition, immunostaining of formalin fixed tissue may be influenced by the duration of fixation, which is often an unknown variable in archival material. In the present study, we evaluated the effect of the duration of formalin fixation on estimates of the percentage of oestrogen receptor positive and MIB-1 positive cells obtained in tissue from 25 prospectively collected, oestrogen receptor positive breast carcinomas. A minimal intraspecimen variation of estimates was ensured by an efficient design of systematic random tissue sampling. This design also enabled the evaluation of the influence of tumour heterogeneity on the variables by comparing estimates obtained from the entire tumour with those obtained in a “representative” tumour section. Finally, the intra- and interobserver reproducibility of estimates of the percentage of MIB-1 positive cells was investigated.
Mean and coefficient of variation (CV) of the oestrogen receptor and MIB-1 indices obtained at different fixation times (n = 9)

<table>
<thead>
<tr>
<th>Index</th>
<th>Fixation time (hours)</th>
<th>2-4</th>
<th>4-24</th>
<th>24-48</th>
<th>48-166</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIB-1</td>
<td>mean (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>35</td>
<td>31</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td>0.46</td>
<td>0.37</td>
<td>0.41</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Oestrogen receptor</td>
<td>mean (%)</td>
<td>76</td>
<td>77</td>
<td>76</td>
<td>75</td>
</tr>
<tr>
<td>CV</td>
<td>0.21</td>
<td>0.23</td>
<td>0.27</td>
<td>0.24</td>
<td></td>
</tr>
</tbody>
</table>

Mean and coefficient of variation of oestrogen receptor and MIB-1 indices were calculated. The correlation between the indices obtained at different fixation times as well as the influence of tumour heterogeneity and observer correlation were investigated by least-square linear regression. However, because of the distribution of oestrogen receptor indices, the influence of tumour heterogeneity on these was investigated by Kendall’s ρ test; 2p<0.05 was considered significant.

Results
The mean tumour diameter was 27 mm (range, 14–50 mm). Eighteen patients were postmenopausal and seven premenopausal. Concerning both antibodies, considerable heterogeneity was observed with respect to the intensity of nuclear staining in adjacent cells within the same region and in different cell groups. The MIB-1 index ranged between 6 and 57%, whereas the oestrogen receptor index ranged between 14 and 95%.

Data for oestrogen receptor and MIB-1 indices obtained in tissue samples fixed in buffered formalin for four different periods are shown in the table. The regression analyses did not show a significant correlation between the mean of estimates and the storage period (2p>0.20).

As illustrated in the figure, the indices estimated in bars sampled systematically from
Quantification of immunohistological prognostic markers in breast cancer

the entire tumour were closely correlated with indices obtained in the "representative", routinely processed section ($r=0.88$ and $\tau=0.79$ for MIB-1 and oestrogen receptor indices, respectively). The interobserver reproducibility of the MIB-1 index was good ($r=0.82$; the slope of the correlation line and the intersection with the ordinate were not significantly different from unit and zero, respectively). The intraobserver correlation (two sets of estimates obtained three months apart) was excellent ($r=0.92$), but the intersection with the ordinate was 9%, indicating a slight systematic error ($2p=0.03$).

Discussion

Formalin fixation may not always be the best choice for preserving tissue antigenicity for immunohistochemical procedures. The process is relatively slow (about 0.8 mm tissue penetration per hour) and if the specimen is large, the central part may be insufficiently fixed. In the present study fixation was performed almost immediately and tissue was cut small to ensure quick and adequate fixation. Using this procedure, we found that oestrogen receptor and MIB-1 indices were not greatly influenced by increasing the period of formalin fixation from less that four hours to more than 48 hours. It has been reported that prolonged exposure to formalin diminishes immunoreactivity of proliferation associated nuclear antigens and oestrogen receptors. Technical differences regarding—for example, specimen thickness, enhancement procedure and the antibody/antigen/epitope detected may explain the conflicting results.

In view of the intratumoral heterogeneity with respect to oestrogen receptor content and proliferative activity, it is obvious that several fields must be analysed. In the present study, the proliferative activity and oestrogen receptor content were determined in fields of vision sampled systematically and randomly from the entire tumour. The use of a clearly defined sampling technique and the evaluation of immunoreactivity in multiple systematically selected fields of vision from the entire tumour provides an effective control against intratumoral heterogeneity and ensures reproducible results. However, the present study indicates that systematic sampling of fields of vision from a single "representative" tumour slice may be sufficient for accurate determination of the oestrogen receptor status and the proliferative activity of the entire specimen.

A commonly used method for quantitation of immunohistochemically determined oestrogen receptors is the HSCORE developed by McCarty et al. This method incorporates both the proportion and intensity of specific, positively staining tumour cells. However, we find it difficult to grade the staining intensity in heterogeneous tumour tissue in an objective and reproducible manner, while the question of whether staining is positive or not is easier to settle. However, even in this case, the systematic differences between the two assessments of the same observer indicates that the threshold, at which a particular cell is termed "positive", is a subjective component inherent in the evaluation technique. Fortunately, this difference is small compared with the large variance between the patients (coefficient of variation, 40%). In view of the very skewed distribution of the oestrogen receptor indices (median oestrogen receptor index = 86%), the prognostic value of the immunohistological quantitation of oestrogen receptor content needs to be examined in a large prospective study with appropriate clinical follow up.

Using immunohistochemical techniques with an antigen retrieval method and microscopic evaluation of systematically selected fields of vision, the following may be concluded: (1) quantitative estimates of the oestrogen receptor content and the MIB-1 percentage are not significantly influenced by the period of fixation in formalin; (2) the MIB-1 and oestrogen receptor indices obtained in one "representative" tumour section closely correlate with indicates determined in several tissue samples from the entire tumour; and (3) estimation of the MIB-1 index is reproducible and may be suitable for routine purposes.

The authors thank Dr F Melgen, Institute of Pathology, Aarhus University, for critical review of the manuscript and the Danish Cancer Society for the gift of antibody to oestrogen receptor. The study was supported by The Danish Cancer Society.

4. Tahan SR, Neuberg DS, Deffenbach A, Yacoub L. Pre-


