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 of 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.

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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.¹⁻⁴ Moreover, oestrogen receptor analysis offers a method for the prediction of response to endocrine therapy.^{5,6} 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^{7,8} 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.⁸ 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.^{7,9} This antigen retrieval method, first described by Shi *et al*,¹⁰ also permits subsequent staining with monoclonal antibody to oestrogen receptor.

In previous studies of breast cancer the percentage of oestrogen receptor positive cells^{6,11-13} and proliferating cells^{12,14-16} varied considerably. Among the possible explanations for the discrepancies are sampling variation, intratumoural 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,^{17,18} 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.¹⁹ 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.

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Methods

Twenty five oestrogen receptor positive breast carcinomas, resulting in five or more slices when cut as described below, were prospectively included in the study. Tissue processing was performed as described in detail by Ladekarl.²⁰ Briefly, each tumour was isolated from the surrounding fatty tissue and cut into 2 mm thick, parallel slices, of which every second was used for routine analyses. The remaining slices (every second) were used for further investigations and cut into bars, 2 mm thick. Specimens were, in general, fixed within 15 minutes of removal for at least 48 hours in 10% formalin at room temperature. However, to investigate the influence of different periods of fixation, tumour bars from nine carcinomas were allowed to fix for two to four hours, four to 24 hours, 24 to 48 hours, and 48 to 166 hours, respectively. In all steps tissue sampling was performed randomly¹⁹—that is, every *n*th of the items (slices or bars) was sampled systematically starting at a random number between one and *n*. After fixation, the tissue was embedded in paraffin wax using standard procedures. From each of the blocks containing systematically sampled tumour bars and from a paraffin wax embedded, “representative” slice selected for routine evaluation, 3 µm thick sections were cut and placed on electrostatic treated slides and air-dried overnight at room temperature.

Immunostaining was performed using oestrogen receptor antibody (Dako, Glostrup, Denmark) and MIB-1 antibody (Immunotech, Marseille, France). Sections were stripped of paraffin in xylene, rehydrated through graded alcohols, and incubated three times for five minutes each in citrate buffer (pH=6.0) in a household microwave oven at 800 W. The slides were then allowed to cool down to room temperature, were washed briefly with Tris buffered saline (TBS) (pH=7.4) and incubated for 20 minutes with 3% hydrogen peroxide in water to block endogenous peroxidase activity. Oestrogen receptor and MIB-1 antibodies were used at 1 in 75 and 1 in 100 dilutions, respectively, for 30 minutes at room temperature. Biotinylated mouse/rabbit antibody (Dako) at a dilution of 1 in 100 was used as the linker molecule. Finally, after washing, avidin-biotin complex (Dako) was applied and aminoethylcarbazole was used for visualisation.

A known oestrogen receptor positive breast carcinoma was included as the positive control and as the negative control, the oestrogen receptor antibody was replaced by TBS. For each MIB-1 staining run, a lymph node with reactive germinal centre cells served as a positive control

and peripheral nerve tissue served as the negative control. The sections were evaluated blindly in a random sequence at high magnification ($\times 1100$, $60\times$ oil immersion lens) using a standard microscope with a projection attachment (Olympus Danmark, Glostrup, Denmark). In each case a median number of 14 fields of vision (range, 8–43) was sampled systematically by moving a fixed distance between fields through the centre of the sectioned bars, along the long axis. The position of the first field of vision was at random from the top of the sectioned bar. An average of 352 tumour cells (range, 97–892) were counted, using a counting frame and an unbiased counting rule.²¹ Highly inflamed and necrotic areas were avoided and only infiltrating tumour cells were evaluated. Tumour cells were considered to be positive for oestrogen receptor and MIB-1 if nucleoplasm or nucleoli were stained regardless of its intensity. The oestrogen receptor and MIB-1 indices were calculated as the percentage of positive tumour cell nuclei. The time spent evaluating each tumour was, on average, 15 minutes.

Finally, the oestrogen receptor and MIB-1 indices were estimated in a “representative” section from each tumour, containing both tumour margin and a central area, selected from the slices used for routine analyses. In these cases a minimum of 10 fields of vision (median 15) were chosen randomly from the whole section, counting an average of 278 tumour cells (range, 136–676) per specimen.

STATISTICAL ANALYSES

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 discontinuous distribution of oestrogen receptor indices, the influence of tumour heterogeneity on these was investigated by Kendal's τ test;²² $2p < 0.05$ 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 ($2p \geq 0.20$).

As illustrated in the figure, the indices estimated in bars sampled systematically from

Mean and coefficient of variation (CV) of the oestrogen receptor and MIB-1 indices obtained at different fixation times ($n=9$)

Index	Fixation time (hours)			
	2–4	4–24	24–48	48–166
MIB-1				
mean (%)	30	35	31	33
CV	0.46	0.37	0.41	0.39
Oestrogen receptor				
mean (%)	76	77	76	75
CV	0.21	0.23	0.27	0.24

Oestrogen receptor and MIB-1 indices obtained in a "representative" section plotted against indices obtained from the entire tumour ($n=25$). ●, MIB-1 index; ○, oestrogen receptor index. MIB-1: slope of correlation line ($=0.93$) and ordinate intersection (-0.001%) did not deviate significantly from unity and zero, respectively. Oestrogen receptor values were not analysed by linear regression because of the very skewed distribution of the data. However, the correlation line is shown for comparison.

the entire tumour were closely correlated with indices obtained in the "representative", routinely processed section ($r=0.88$ and $r=0.79$ 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 unity 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 hour²³) 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 than four hours to more than 48 hours. It has been reported that prolonged exposure to formalin diminishes immunoreactivity of proliferation associated nuclear antigens¹⁷ and oestrogen receptors.¹⁸ 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 content²⁴ and proliferative activity,^{13 14 25} 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.*^{26 27} 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 variation 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 indices 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.

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