A comparative study of cell proliferation markers in breast carcinomas

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Abstract

Aims—To investigate the tumour cell proliferative index obtained by immunostaining of paraffin wax sections of 30 cases of breast carcinoma with monoclonal antibodies MIB1, Ki61 and Ki65, and polyclonal Ki67 antisera to the Ki67 antigen and 19A2 and PC10 antibodies to proliferating cell nuclear antigen and the possible correlation between these indices and that of monoclonal Ki67 antibody in frozen sections of the same tumours.

Methods—All tumour samples had been uniformly fixed and processed and sections were subjected to microwave antigen retrieval before immunostaining in all instances except for monoclonal Ki67 antibody which was used in cryostat sections. Tumour cell proliferative indices were evaluated by two independent examiners, each counting 500 tumour cells with the aid of a cross-hatched grid.

Results—Proliferative indices obtained with MIB1, polyclonal Ki67, Ki61, and Ki65 correlated with those obtained with monoclonal Ki67 in frozen sections. Proliferative indices obtained with monoclonal 19A2 and PC10 showed no correlation with those of monoclonal Ki67 antibody. The staining obtained with MIB1 was the most intense and the easiest to read.

Conclusions—Monoclonal antibodies MIB1, Ki61 and Ki65 and polyclonal Ki67 antisera appear to be suitable substitutes for monoclonal antibody Ki67 in the assessment of tumour cell proliferative index. As these reagents are all immuno-reactive in paraffin wax sections, they overcome the requirement for frozen tissue for immunostaining with monoclonal Ki67.

(Keywords: Proliferation markers, Ki67, breast cancer.)

Assessment of the tumour growth fraction yields important biological information which can be related to the clinical course and therapeutic response in patients with cancer, besides providing greater insights into tumour growth.1-6

While the counting of mitotic figures remains the simplest and most established method of assessing tumour proliferation, it is not a completely reliable or reproducible procedure, and often only reflects the mitotic or M phase of the cell cycle. Prolongation of the M phase of the cell cycle may thus result in a deceptively high rate of proliferation in some tumours such as basal cell carcinoma of the skin. Better established techniques for accurate measurement of the growth rate of tumours are the titrated thymidine labelling or bromodeoxyuridine incorporation techniques,8 but both of these techniques are laborious, difficult to perform and are not appropriate for diagnostic laboratories. The advent of the flow cytometer provides an automated technique which quantifies cellular DNA content and analyses cell cycle distribution,9-12 but its application requires the use of expensive equipment.

Gerdes et al.12 produced the Ki67 monoclonal antibody which recognises a nuclear antigen present only in proliferating cells, permitting an easy immunohistochemical method of assessing tumour cell proliferative fraction. The precise nature and composition of the antigen detected by the Ki67 antibody is largely unknown but it appears to be expressed at all stages of the cell cycle except G0.12-13 Most studies show the Ki67 antigen to increase with progression of the mitotic cycle, rising during the latter half of the S phase and reaching a peak in the G2 and M phases.3 More insights into the biology and structure of the Ki67 antigen have recently been reported14-15 and the gene has been partially cloned and sequenced.16

Ki67 immunostaining has been used as a prognostic indicator in a wide variety of tumours including non-Hodgkin's lymphomas,17-19 gliomas,20 meningiomas,21 pituitary tumours,22 soft tissue tumours,23 bone tumours,24 prostate carcinoma,25 seminoma,27 colorectal carcinoma,28 molar pregnancy,6 hepatocellular carcinoma,29 melanoma,30 and lung carcinoma.31 Several studies have established the relevance of Ki67 analysis in frozen sections as a predictor of behaviour and metastatic potential in breast cancer,32-39 with significant cut off values at 12%40-41 and 16%.40

Proliferating cell nuclear antigen (PCNA) is a 36KDa acidic nuclear protein that has been highly conserved throughout evolution and is essential for DNA synthesis.42 PCNA appears in the nucleus primarily during the synthetic phase of the cell cycle43-49 and functions as an auxiliary protein of DNA polymerase-δ.46 Several monoclonal antibodies have been generated to genetically engineered PCNA and have been used in the demonstration of cell proliferative index in paraffin wax embedded tissues.47 Many papers describing the applications of these monoclonal antibodies to various tumours have been published, claiming a correlation between the proliferative index assessed by immunostaining of PCNA and the

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Accepted for publication 16 January 1995
values obtained with flow cytometric analysis and Ki67 expression.

The monoclonal Ki67 antibody is immunoreactive only in frozen sections and cell preparations. To overcome this handicap, Kreipe et al. have generated two monoclonal antibodies, KiS1 and KiS5, directed against a formalin resistant epitope of the Ki67 antigen. Similarly, Key et al. have succeeded in producing antibodies against bacterially expressed Ki67 fusion proteins which are preserved in formalin fixed, paraffin wax embedded tissues. These latter antibodies are effective in tissues treated by microwave irradiation for retrieval of the Ki67 antigen. Key et al. have also raised a polyclonal rabbit antibody to a synthetic peptide deduced from a 62 base pair region encoding the Ki67 epitope.

In this study we compared the proliferation indices obtained with these new antibodies to cell proliferation markers which survive formalin fixation and embedding in paraffin wax and correlated them with those of Ki67 immunostaining in frozen sections in 30 cases of breast carcinoma.

Methods
Appropriate tissue blocks from 30 cases of infiltrating duct carcinoma of the breast of no specific type were drawn from the files of the Institute of Medical and Veterinary Science, Adelaide. All tissues had been fixed in 10% buffered formalin for one hour before three and a half hours of processing through 95% ethanol, xylene and wax in an automated tissue processor as part of the fixation and processing protocol for oestrogen receptor immunoassay as described previously. Details of the antibodies used in this study are provided in the table. Monoclonal Ki67 antibody was only applied to cryostat sections as described previously. A Streptavidin-biotin peroxidase method was used.

Microwave antigen retrieval was used for all other antibodies which were applied to paraffin wax sections, using a modification of the method previously described. Briefly, deparaffinised, rehydrated tissue sections were immersed in a closed plastic container (Karrell, Milan, Italy) filled with 10 mM citrate buffer, pH 6.0 (2.1 g citric acid in 1 litre and adjusted to pH 6.0 with approximately 13 ml of 2 mM NaOH). The container permitted stacking of 20 slides in 250 ml of solution. The immersed sections were irradiated in a domestic microwave oven with a carousel (NEC Model 702, 650 W) at maximum power setting until the buffer solution boiled. When boiling point was attained, normally in about five minutes, the power was adjusted so that the solution simmered. This simmering was continued for 10 minutes after which the sections were allowed to remain in the hot buffer solution for a further 25 minutes before immunohistochemical staining. All sections were mounted on aminolysilsane treated slides. They were subjected to enzyme digestion before microwave antigen retrieval when immunostaining was performed with MIB1 and KiS1 antibodies. The sections were digested at 37°C with trypsin type II, 0.25 mg/ml, for three minutes before antigen retrieval was performed. Diaminobenzidine in TRIS/HCl buffer, pH 7.4, and 0.03% hydrogen peroxide were used as the chromogen. A light Mayer’s haematoxylin was applied as counterstain.

Consecutive paraffin wax sections were used for immunostaining with each of the antibodies so that similar areas of the tumour could be assessed. Tumour cells with distinct nuclear staining were assessed as positive and only peripheral areas of the tumour were assessed, taking care to avoid areas of haemorrhage and fibrosis. A cross-hatched Whipple grid divided into 100 squares was used with a ×20 objective and only those cells lying within the nine squares in each corner and the central square (that is, 45 squares in total) were counted. The percentage of positively stained cells were determined by counting 500 morphologically malignant cells. Counting was performed independently by two of the authors (SV and CS) and the mean values of the two separate counts were used to calculate the percentage of positive cells or the tumour proliferative index. When the values obtained in the two independent counts differed by more than 5% the counts were repeated. A similar method of counting was adopted for monoclonal Ki67 staining on frozen sections.

Statistical analysis was performed with Stat View 512+ (Brainpower Inc., Calabasas, California, USA), with Spearman’s rank correlation coefficient (r) method used for the analysis of the relation between monoclonal Ki67 proliferative index and those obtained with the other antibodies.

Results
It was easy to count positively stained cells using the method described above and the tumour cell proliferative index appeared to be reproducible by independent counting. The monoclonal antibody MIB1 produced the greatest intensity of staining, whereas both antibodies to PCNA resulted in a wide range of staining intensities within the tumour cell nuclei.
The proliferative index with monoclonal Ki67 antibody ranged from 0.5 to 50% (mean 13.83%, median 10.25%). This was slightly lower than that obtained with MIB1 which ranged from 2.5 to 52.5% (mean 16%, median 13.75%) \( (r=0.1810; p<0.0001) \). Similarly, the correlation coefficient of other proliferative indices compared with monoclonal Ki67 were as follows: KiS1, range 4.0–46.0%, mean 18.53%, median 14.25% \( (r=0.8348; p<0.0001) \); KiS5, range 2.0–72.5%, mean 21.5%, median 15.75% \( (r=0.66945; p<0.0001) \); polyclonal Ki67, range 1.0–58.5%, mean 17.23%, median 17.25% \( (r=0.7339; p<0.0001) \); PC10, range 65.5–100%, mean 86.9%, median 86.25% \( (r=-0.0144; p=0.9399) \); and 19A2, range 44.0–95.5%, mean 75.1%, median 78.5% \( (r=0.18195; p=0.3359) \). These values are depicted in figs 1, 2 and 3.

**Discussion**

By sorting cell suspensions into subpopulations representing distinct phases of the cell cycle, followed by immunolabelling for Ki67 antigen, Gerdes et al. demonstrated that, with the exception of quiescent cells (G0), Ki67 is expressed throughout the cell cycle. The Ki67 molecule is known to be a DNA binding protein that probably has a regulatory function in cell proliferation. The Ki67 antigen is a bimolecular complex of molecular weight 345 and 395 kDa and the gene encoding the antigen has been cloned and sequenced and assigned to chromosome 10 (10q25). A close correlation between the growth fraction of proliferating cells, as measured by autoradiography using tritiated thymidine incorporation, and the number of cells stained by Ki67 has been demonstrated in normal and malignant tissues, a similar correlation has been shown with other techniques such as bromodeoxyuridine incorporation.

Ki67 expression has been studied in a large variety of benign and malignant conditions. In breast carcinoma Ki67 immunostaining of frozen sections has revealed a correlation between Ki67 counts and the mitotic index, tumour diameter, histological grade, lymph node involvement, and vascular invasion. Ki67 counts show an inverse correlation with oestrogen receptor expression.

A recent study of 54 cases of invasive ductal and nine of invasive lobular carcinoma, with a median follow up of 37 months, showed progressively worse disease free and overall survival with increased Ki67 levels. At cut off levels of 12% of nuclear staining for Ki67, survival of low expressers was 95%, whereas that of high expressers approached 60% \( (p<0.05) \). At a cut off of 16% positivity, only 50% survival was seen in the high expressers \( (p<0.01) \). The parameters were independent of age, nodal status or hormonal status. Bouzbar et al. analysed survival data on 124 patients with breast cancer who had been followed for up to 30 months after mastectomy and found that a high level of Ki67 staining \( (>20\%) \) was associated with an increased risk of early recurrence of carcinoma. The major disadvantage of the Ki67 monoclonal antibody is that it is reactive only in frozen tissue or fresh cytological preparations.

Several monoclonal antibodies have been generated to genetically engineered PCNA. The antigen has been shown to function as a co-factor of DNA polymerase-β and appears
to be expressed in the nucleus, primarily during the synthetic phase (S phase) of the cell cycle. Numerous papers have been published describing the use of monoclonal antibodies that recognise PCNA on various tumours, many reports professing a correlation between the proliferative index assessed by immunostaining for PCNA and the values obtained with flow cytometric analysis and Ki67 expression.36-42 We have previously compared the sensitivity and specificity of two commercial PCNA antibodies, namely clones 19A2 and PC10, against that of monoclonal Ki67 antibody73 and found that PCNA is exquisitely sensitive to formaldehyde fixation with marked differences in sensitivity displayed by the two antibodies. Furthermore, although the values obtained with both PCNA antibodies correlated with those of Ki67, they were consistently higher.73 It has since become clear that PCNA may be expressed in non-cycling cells.43,44 Induction of PCNA in non-cycling cells may be mediated by increased messenger RNA (mRNA) stability because of growth factors.45 Moreover, it has been suggested that PCNA may become deregulated from the constraints of the normal cell cycle in malignant cells so that expression may not be tied to G1/S phases.46 PCNA may also have a lengthened half-life and it is possible that in some situations PCNA persists in cells which have come out of their cycling phase, so that total PCNA counts may reflect not only cells in cycle but also those of previous cycles. Their exquisite sensitivity to formaldehyde fixation makes comparisons of PCNA values invalid, particularly with the vast variations in fixation protocols used by different laboratories.

In the present study PCNA counts obtained with both 19A2 and PC10 antibodies did not show a correlation with counts of monoclonal Ki67. By contrast, counts obtained with KiS1, KiS5, and MIB1 antibodies, and polyclonal Ki67 antisera all produced proliferation indices which showed good correlation with those of monoclonal Ki67. However, comparison of monoclonal MIB1 produced the greatest intensity of staining. The antibody was effective only after microwave antigen retrieval and enhanced staining was obtained when the sections were predigested with trypsin before irradiation with microwaves. Cattoretti et al47 reported that the immunohistochemical detection of tumour growth fraction (Ki-67 antigen) in formalin-fixed and routinely processed tissues. J Pathol 1992;168:85-7. Key G, Becker MHG, Key G, Castoretti G. Immunohistochemical detection of tumour growth fraction (Ki-67 antigen) in formalin-fixed and routinely processed tissues. J Pathol 1992;168:85-7. Grogan TM, Lippman SM, Spier CM, Szymon DJ, Kybski JA, Rangel CS, et al. Prognostic significance of the Ki-67 proliferation fraction in prostate cancer. J Urol 1998;159:1657-61. of Ki67 in frozen sections, but few studies have compared the reactivity of both monoclonal antibodies on a large series of tumours. Our experience indicates that the MIB1 monoclonal antibody is a good substitute for the Ki67 monoclonal antibody which has the restriction of being immunoreactive only on frozen sections. With the accumulating evidence that the tumour cell proliferative index is an important prognostic parameter both in breast carcinomas as well as in a wide variety of other tumours, there is an increasing need to be able to perform this assessment in paraffin wax sections and the new generation of monoclonal antibodies, namely MIB1, KiS1, KiS5, and polyclonal Ki67 antisera are potential reagents for the assessment of this biological parameter. It should be noted that some authors have claimed to be able to use monoclonal Ki67 antibody on paraffin wax sections73 but this is not our experience.
Tumour cell proliferation markers


