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Which proliferation markers for routine immunohistology? A comparison of five antibodies

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Abstract

***Aims*—To determine the best of five antibodies for immunohistochemical assessment of growth fraction in formalin fixed, paraffin wax embedded tissues.**

***Methods*—Sections from 100 recent, and 17 ten year old or over wax embedded blocks of normal and malignant tissues were immunostained with monoclonal Ki67, polyclonal Ki67, PC10, MIB1, and JC1. The antibodies were evaluated for specificity of nuclear versus cytoplasmic staining, cleanliness of background, and compared with the expected pattern of staining in normal tissues, defined immunohistochemically by monoclonal Ki67 antibody in frozen tissues or by tritiated thymidine uptake.**

***Results*—No marker was ideal, but best results were obtained with MIB1 and polyclonal Ki67, followed by JC1, PC10, and monoclonal Ki67.**

***Conclusions*—For routine use, MIB1 or polyclonal Ki67 are the best proliferation markers in conventional histological preparations. The other markers tested cannot be recommended.**

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Measurement of cell proliferation may provide useful information concerning tumour prognosis and aid diagnosis, especially for low grade non-Hodgkin's lymphomas.[1–6] Methods directed at different parts of the cell cycle are available. Each has advantages and disadvantages.[6] Mitotic counts (M phase) give diagnostic and prognostic information for some tumours, such as smooth muscle tumours,[7] and are used as an adjunct to tumour grading—for example, in breast carcinoma.[8] Mitoses counting is simple to perform, but errors can occur[9] because of delays in fixation,[10] section thickness, the size of the microscope field,[11] and difficulties in the recognition of mitoses. DNA synthesis (S phase) can be assayed by measuring labelled DNA precursor incorporation, such as tritiated thymidine, or a nucleotide analogue, such as bromodeoxyuridine (BrDU). These methods permit direct estimation of DNA synthesis and are therefore the standards against which other methods must be assessed. Tritiated thymidine in tissue sections can be revealed with photographic emulsion, and BrDU can be revealed immunohistochemically, enabling the morphology of proliferating cells to be identified. Alternatively, scintography or flow cytometry can give an objective measure of large numbers of cells labelled with these reagents. However, both methods require in vivo administration or in vitro[12] incubation of tissue with associated ethical and logistic problems, preventing widespread clinical use. Many routine laboratories will also not want to use radionucleotides. Flow cytometry can provide a quantitative measurement of the DNA content of large numbers of cells from fresh, fixed, or paraffin wax embedded tissue.[13] All nucleated cells contain DNA throughout the cell cycle, so flow cytometry measures ploidy rather than proliferation fraction. Statistical correction for distortions due to aneuploidy must be used to isolate the S phase of tumours.[14] Flow cytometry requires dissociation of nuclei, permitting contamination of tumour samples by reactive cells and preventing morphological analysis. Silver staining of proteins associated with nucleolar organiser regions (AgNORs) of interphase chromosomes may be used on conventional histological sections for assessment of proliferation rates; correlation with other indices of cellular proliferation has been shown.[15] The value of AgNOR counting to discriminate between benign and malignant lesions, and the relevance of AgNOR counts to prognosis is not clear.[16 17]

Several antibodies to cell cycle related antigens are available. Monoclonal Ki67 is the prototypic antibody,[18] recognising a nuclear protein[19] expressed in proliferating cells in G1, S, G2, and M but not G0.[20] Ki67 labelling correlates well with cell proliferation as assessed by tritiated thymidine uptake,[20] and with histological grade and prognosis in non-Hodgkin's lymphomas.[2] A major limitation of the use of monoclonal Ki67 is the requirement for fresh or frozen tissue because fixation abolishes staining in most but not all laboratories.[21] Newer antibodies, polyclonal Ki67 and MIB1, raised against peptides from recombinant fragments of the gene for Ki67 antigen, are effective in conventional sections following microwave irradiation.[22 23] JC1 is a monoclonal antibody which has been reported to identify a nuclear protein associated with cell proliferation.[24] Proliferating cell nuclear antigen (PCNA) is a nuclear protein associated with DNA polymerase δ[25 26] which is present throughout the cell cycle in proliferating cells. The monoclonal antibody PC10 recognises PCNA and is effective in formalin fixed, paraffin wax embedded tissues.[27] It is therefore a potentially useful proliferation marker,

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but has been shown to stain non-proliferating cells in some circumstances.[27 28] PC10 is sensitive to the length of tissue fixation before processing[28] and section handling.[29]

Because most diagnostic laboratories use immunohistochemistry, we evaluated five immunohistochemical markers of proliferation (three to Ki67, one to PCNA, and one to JC1), specifically from the viewpoint of a diagnostic pathologist. Our aim was to find the marker that best matched the proliferation profile of normal and abnormal tissues as defined by established methods.

Methods

Paraffin wax blocks of recently processed formalin fixed normal tissues and tumours were retrieved from the files of the Histopathology Department, Whittington Hospital, together with archival tissue processed at least 10 years previously. The tissue had been fixed in neutral buffered 10% formol-saline and fixation periods ranged from 12 hours to several days. Tissues stained included one archival and five recent samples of each of the following: normal stomach, small intestine, colon, liver, skin, reactive lymph node, kidney, and testis, five (recent samples only) of thyroid and fetal thymus; one archival and five recent samples of each of the following: cutaneous basal cell carcinoma, carcinoma of stomach, colon, breast, endometrium, kidney, prostate, bladder; and five (recent samples only) of squamous cell carcinoma of the cervix. One archival and five recent leiomyosarcomas of the uterus were also stained. For each carcinoma category, a similar grade of tumour was used.

To assess large numbers of samples under very similar conditions, multiblocks were prepared as described before.[30] Use of the multiblock technique for individual tumour prognosis has been criticised on the grounds of sampling error[31] but these objections do not apply to our study. Multiblocks are ideal for the comparison of different antibodies because sequential sections are stained and intertumoural comparisons of proliferation rates were not attempted. The primary and secondary antibodies used in this study are presented in table 1.

In accordance with standard practice and the manufacturer's suggestions, we determined the optimum antibody concentrations before performing the study.

Preliminary experiments with the antibodies on untreated sections obtained moderate results with PC10 and poor results with the others. Trypsin abolished staining with all five antibodies. Therefore, we used microwave postfixation as many antibodies show enhanced staining after microwave irradiation of conventionally processed sections.[32]

Sections (4 μm) were cut on to organosilane coated[33] slides and dried at 38°C to prevent loss of PCNA that can occur with drying at 60°C.[29] Slides were dewaxed and endogenous peroxidase blocked with hydrogen peroxide in methanol. Microwave postfixation was performed using a domestic oven (Kenwood KM 2001T) at 700 W, delivered to slides immersed in 0·01M citrate buffer, pH 6·0, as two five minute doses separated by a two minute break, enabling refill of buffer if required. The slides were permitted to cool to room temperature before removal. Sections were incubated with primary layer antibodies at 4°C overnight. Biotinylated rabbit or swine second layer antibodies (table 1) were applied for 60 minutes at room temperature. Bound antibody was visualised with avidin-peroxidase conjugate (Dako K355, High Wycombe, UK) and diaminobenzidine (Sigma D5905, St Louis, Missouri, USA). Sections were lightly counterstained with Mayer's haematoxylin. Negative control sections were included. The slides were labelled with a code so that an independent assessment by two pathologists would be unprejudiced. Semiquantative scoring was used as in previous studies of proliferation antigens.[3 28]

Sections were scored for:

(1) Intracellular distribution of antibody, scoring 1 for restriction to the nucleus, 0 for the presence of cytoplasmic positivity. Cells in mitosis and the basal layers of the epidermis were not included because cytoplasmic staining is expected in mitotic cells and in the basal layers of the epidermis when frozen tissue is stained by monoclonal Ki67.[18 24]

(2) Degree of background staining, scoring 2 for clean nuclear staining, 1 for cytoplasmic staining not preventing interpretation of proliferation, 0 for uninterpretable.

(3) Expected pattern of staining was scored with reference to the distribution of proliferating cells determined by monoclonal Ki67 in frozen tissue or tritiated thymidine uptake[18]; antibodies scoring 3 for faithful reproduction of pattern, 2 for excess positive cells, 1 if most cells stained.

With the multiblock technique, if one tissue sample is lost from a multiblock section it is often lost throughout sequential sections. We excluded three of 117 tissue sample sections from the analysis because of section loss.

Table 1 First and second layer antibodies

Antibody	*Suggested concentration*	*Working concentration*	*Second layer*
Monoclonal Ki67	Neat	Neat	RAM
Polyclonal Ki67 (Dako; A047)	1 in 90	1 in 90	SAR
PC10 (Dako; M879)	1 in 50 to 1 in 100	1 in 100	RAM
MIB1 (Immunotech; 0505)	1 in 50 to 1 in 100	1 in 100	RAM
JC1	Neat	Neat	RAM

RAM = biotinylated rabbit anti-mouse; SAR = biotinylated swine anti-rabbit.

Results

The major difference between antibodies was in degree of cytoplasmic staining, the cleanliness of the background, and in the restriction of specificity (table 2).

It is not immediately apparent why there was a discrepancy between the degree of nuclear versus cytoplasmic staining and the cleanliness of background staining. When scoring distribution of the stain, however,

Table 2 Comparison of staining performance by each antibody expressed as percentage of maximum (100%) possible score

Index	Antibody				
	Monoclonal Ki67	*Polyclonal Ki67*	*PC10*	*MIB1*	*JC1*
Nuclear *v* cytoplasmic staining*	6·5	92·6	38·9	93·5	62·0
Background*	13·2	93·0	70·0	93·8	75·8
Expected pattern**	20·2	88·5	38·5	95·2	56·7

*Scoring for 114 of 117 tissue sample sections (three lost).
**Expected pattern for 52 of 53 tissue sample sections of non-neoplastic tissue for which data on expected distribution were known (one lost).

cytoplasmic positivity gained no points; in scoring background, if the nuclei were rendered unassessable the score was 0, but if the cytoplasmic staining did not prevent interpretation, one point was given. The best antibodies gave sufficiently little cytoplasmic staining that the discrepancy between the two scoring methods was negligible.

Monoclonal Ki67 stained nuclei and cytoplasm in almost all cells in the sections, often preventing any meaningful assessment of proliferation status.

PC10 and JC1 were more specific with less cytoplasmic staining but the antibodies still stained most cell nuclei. However, intensity of staining varied such that—for example, nuclei in follicle centres in lymph nodes stained more strongly than in the mantle zones, but differences between follicle centre cells could not be visualised. Polyclonal Ki67 and MIB1 compared well with proliferation patterns in normal tissues, as defined by DNA precursor uptake studies or immunohistochemistry using monoclonal Ki67 in frozen tissue.[18] Both were largely confined to nuclei, with the most common pattern being a granular nuclear stain with highlighting of nucleoli and, occasionally, the nuclear membrane. Both Ki67 and MIB1 stained renal tubular cytoplasm, and one renal cell carcinoma with a very granular, proximal convoluted tubule-like appearance. Staining of tubular cytoplasm was largely responsible for the less than 100% score obtained by these antibodies on assessment of background. This non-specific staining of renal tubules is not restricted to proliferation markers (personal observations, Rose DSC). Of the well differentiated tumours, the basal cell carcinomas and papillary transitional cell carcinomas often showed basal layer staining, but no staining of the more mature upper epithelial layers. In dividing cells mitotic figures stained strongly, with weak diffuse cytoplasmic positivity. Polyclonal Ki67 scored worse than MIB1 for adherence to the expected pattern because of lack of staining of early testicular germ cells. If the sections of testicular tissue are excluded from the analysis, the expected pattern score for polyclonal Ki67 is 93·5%, very similar to MIB1.

If normal testis is to be examined MIB1 is preferable. In all tissues examined MIB1 gave a more intense positive stain, affording greater positive versus negative contrast and a more confident categorisation of nuclei, often at lower magnification. The effect is difficult to quantify, and so on objective analysis according to the parameters we measured, the two antibodies must rank equally.

We found no difference in the staining performance of the antibodies in archival as opposed to recent tissues (table 3).

Table 3 Comparison of summed results (all antibodies, all tissues) from archival (>10 years old) and recent samples expressed as percentage of maximum (100%) possible score

Index	*Archival*	*Recent*
Nuclear v cytoplasmic staining	63·8	57·8
Background	70·7	68·2
Expected	57·5	60·6

Discussion

Over the past 10 years, there has been an explosion of interest in the measurement of the proliferation fraction of tumours and normal tissues. There are two practical applications of interest to a diagnostic pathologist.

The first relates to tumour prognosis. Staging and grading systems are commonly used in reporting carcinomas of breast, colon, bladder, prostate, and melanomas. Biological markers of prognosis are not often used, largely because of the requirement for fresh tissue. Hormone receptor status in breast carcinoma is the exception[34] and now a reliable immunohistochemical marker for formalin fixed tissue is available.[35]

The proliferation fraction, whatever the measurement method, has been correlated with prognosis for many tumours,[1] especially breast[4 5] and lymphoid neoplasms.[2] However, several authors have urged caution because different methods measure different indices, not all directly related to the proliferation fraction.[6 36] In this study we used three antibodies directed against the Ki67 antigen, one to the JC1 antigen, and one to PCNA. The latter is expressed continuously in proliferating cells and shows good correlation with BrDU and Ki67 determined proliferation in normal cells and non-Hodgkin's lymphomas.[27] In cultured HeLa cells, however, the correlation between the PCNA measured proliferation fraction and the S phase fraction as assessed by flow cytometry is poor.[27] In rapidly growing tumours this may be because of the long $t_{1/2}$ of PCNA[37] relative to the rate of cell turnover. PCNA staining may also be seen in non-proliferating (Ki67 negative) hepatocytes adjacent to tumours, and in hepatitis,[28] possibly because of the enhancement of PCNA expression by growth factors released from the tumour cells or the inflammatory infiltrate. Similar excess staining is seen in biopsy specimens of normal and inflamed gastric mucosa.[38] PC10 staining in these circumstances may not indicate proliferation even though it may be technically faultless in showing the presence of PCNA. Conversely, Ki67 expression is regarded as a good index of the proliferation fraction as long as the antibody and staining method have been validated.

The second practical application concerns diagnosis. New techniques are often welcomed in the hope that they will contribute to decision making in differential diagnosis. Deficiencies often emerge, particularly in the grey areas between reactive hyperplasia, dysplasia, and malignancy. Proliferation markers are no different. However, preliminary investigations have shown the value of staining for Ki67 antigen in one difficult differential diagnosis: reactive hyperplasia of lymph nodes versus follicular lymphoma. MIB1 staining of reactive lymph nodes reveals a high proliferation fraction in the follicle centres with prominent zonation. Centroblasts in the dark zone stain more strongly than centrocytes in the light zone. Proliferation centres in follicular lymphoma lack zonation and stain much less strongly than in reactive follicle centres. In combination with immunostaining for the product of the antiaptosis gene, *bcl-2*, this is a useful technique for the distinction between the two conditions (personal communication, Isaacson PG).

One possible criticism of our study is that the target tissues did not have a uniform fixation period. We deliberately selected a wide range of normal and abnormal tissues from archival samples to reflect routine diagnostic practice in which specimens are not usually received fresh and are inevitably exposed to variable rates and duration of fixation. Many of the larger specimens had at least 36 hours of fixation and tissues from a Friday evening operation may not enter the processor cycle until 80 hours later. This may cause problems with some antibodies. PCNA, as assessed by PC10, is very fixation dependent. Positivity in proliferating cells is reduced after 48 hours fixation and is almost abolished at 72 hours.[27]

Antigen retrieval from fixed material using protease treatment is an established method. Recently, there has been increasing interest in the use of microwave heating as an alternative. It often gives equivalent or superior results.[39] Protease digestion has to be carefully controlled to prevent artefactual changes in antigen exposure and similar problems could be anticipated for microwave treatment. In an analysis of 256 antibodies, including PC10 and MIB1, Cattoretti *et al*[32] found no adverse effects on staining specificity following microwave irradiation. However, divergence from the normal pattern of antibody binding can be seen as in a study of microwaved sections of lymph nodes from cases of Hodgkin's disease stained with CD15 and CD30.[40] We followed a commonly used protocol,[32 41] placing the tissue in the microwave oven for two closely separated five minute exposures, partially because it was effective, and partially to impose a simple pretreatment protocol. Other investigators, using multiples of seven minute exposures, have shown that MIB1 staining of overfixed material is enhanced if the duration of exposure is increased from seven to 14 minutes.[42] Surprisingly, even prolonged irradiation does not have an adverse effect on staining[42] so if overfixed tissues are to be examined, prolonged irradiation may be preferred. The main requirement for good antigen retrieval is application of wet heat, which can be provided satisfactorily by a range of methods.[32 39]

The differences in performance of the antibodies tested is not surprising. The three antibodies to Ki67 were raised against different epitopes which probably vary in accessibility and resistance to denaturation. PC10 will underperform in a study (or in diagnostic practice) using overfixed tissues; this problem, in conjunction with deregulated expression, makes PC10 a poor choice for routine use.

In conclusion, reliable antibodies for the measurement of the proliferation fraction in current or old conventionally processed tissues are now available. The most suitable for routine work is either MIB1 or polyclonal Ki67.

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