

MIB-1, Ki67, and PCNA scores and DNA flow cytometry in intermediate grade malignant lymphomas

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Abstract

Aims—To verify the correlation between MIB-1, Ki67, and proliferating cell nuclear antigen (PCNA-PC10) scores and S-phase fraction in intermediate grade non-Hodgkin's lymphomas (Working Formulation F); and their reliability in differently processed tissues.

Methods—Forty one non-Hodgkin's lymphomas were classified as (F) intermediate grade malignant lymphomas according to the Working Formulation; mitotic counts and percentage of large cells were assessed for each case. Sections from formalin fixed, paraffin wax embedded tissues were stained with anti MIB-1 monoclonal antibody, after microwave oven processing, and anti-PCNA (PC10) monoclonal antibody using an avidin-biotin immunoperoxidase (ABC) method. One thousand cells from 10 representative fields were scored. Frozen sections from surgical specimens were stained with Ki67 monoclonal antibody using the ABC method; the fraction of Ki67 positive cells was calculated scoring 1000 cells. Flow cytometry analysis (FCM) was performed on cell suspensions from fresh tissues. Correlations between data were estimated using linear regression.

Results—A linear correlation was found between MIB-1 and Ki67 scores ($r = 0.92$; $p < 0.00001$); between MIB-1 and PCNA scores ($r = 0.79$; $p < 0.00001$); and between MIB-1 score and S-phase fraction ($r = 0.51$; $p = 0.0006$). A linear correlation was also found between Ki67 and PCNA scores ($r = 0.85$; $p < 0.00001$); between Ki67 score and S-phase fraction ($r = 0.6$; $p = 0.0002$); and between PCNA score and S-phase fraction ($r = 0.74$; $p < 0.00001$). A correlation was found between mitotic counts and MIB-1 ($r = 0.56$; $p = 0.0001$), PCNA ($r = 0.51$; $p = 0.0007$), or Ki67 scores ($r = 0.47$; $p = 0.002$); between the percentage of large cells and MIB-1 ($r = 0.49$; $p = 0.0009$), PCNA ($r = 0.6$; $p = 0.00003$), and Ki67 scores ($r = 0.53$; $p = 0.0003$) and S-phase fraction ($r = 0.55$; $p = 0.0002$).

Conclusion—MIB-1, Ki67, and PCNA (PC10) scores and S-phase fraction are highly correlated and equally well represent the proliferative activity of intermediate grade non-Hodgkin's lymphomas in differently processed material. MIB-1 and PCNA stains can be applied even on

small biopsy specimens. MIB-1 produces homogenous staining without background; it also strongly stains mitotic figures. It can be performed on routinely processed tissues, permitting the simultaneous evaluation of the morphology and tumour cell kinetics. The wide standard deviations of the proliferative indices found for intermediate grade NHL suggest that this category probably includes various degrees of malignancy.

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Intermediate grade non-Hodgkin's lymphomas (NHL) (Working Formulation F) are a heterogeneous group of neoplasms with differing histology and prognoses. Cell kinetic studies can provide useful parameters for further characterising this tumour group.

Among the methods used to evaluate the proliferative activity of neoplastic tissue, reactivity with the monoclonal antibody Ki67 is widely accepted. This antibody detects nuclear antigen expressed in all phases of the proliferating cell cycle except G0.¹ In several tumours there is a correlation between Ki67 score and histological grade,²⁻⁷ tumour stage,^{4,5,8,9} and prognosis.^{3,9,10} In NHL the Ki67 score is associated with the degree of malignancy in either the Kiel¹¹⁻¹³ or Working Formulation¹⁴ classification systems.

Proliferating cell nuclear antigen (PCNA/cyclin) is a 36 kilodalton nuclear protein involved in DNA synthesis¹⁵ and related to S-phase¹⁶; its concentration directly correlates with the proliferative state of normal or transformed cells.¹⁷ Recently, a PCNA clone (PC10) was immunohistochemically detected in conventionally fixed and processed tissues.¹⁸ In some neoplasms the PCNA score is associated with histological grade¹⁹⁻²³ and prognosis.^{21,22,24,25} In NHL, in particular, PCNA has been found to correlate with Ki67 score,^{14,26} S-phase fraction,^{27,28} histological grade^{14,28} and prognosis.²⁸

Flow cytometric analysis of the cellular DNA content has shown a correlation between ploidy or aneuploidy, or S-phase fraction, and histological grade, stage, and prognosis in many neoplasms.²⁹ In particular, DNA flow cytometry has been applied to cases of NHL more than to any other type of tumour.³⁰ Associations have been shown between S-phase fraction and histological type of NHL^{31,32} or prognosis.^{33,34} In NHL there is a strong correlation between S-phase fraction and TH3 labelling index,^{33,35} Ki67

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scores,²⁷ PCNA scores^{27 28} and AgNOR counts.³⁶

The new monoclonal antibody MIB-1, prepared against recombinant parts of the Ki67 antigen,³⁷ also reacts in routinely fixed, paraffin wax embedded tissues. To our knowledge, no studies have been performed so far on its expression in intermediate grade malignant lymphoma.

Methods

The study was performed on surgical specimens from 41 patients (29 women, 12 men; mean age 57.8 years) who underwent biopsy for malignant lymphoma. Adjacent tissue fragments were chosen for MIB-1, Ki67, and PCNA stainings and DNA flow cytometry.

Haematoxylin and eosin, periodic acid Schiff (PAS), Giemsa, and reticulin stained paraffin wax sections were examined; the cases were classified according to the Working Formulation³⁸ (by DN and GP). In each case the mean number of mitoses per high power field and the percentage of large cells from 100 consecutive cells in representative areas was determined.³⁹

MIB-1 STAINING AND SCORING

Sections (4 μm) from formalin fixed, paraffin wax embedded tissues on poly-L-lysine coated slides were dewaxed, rehydrated, and brought to water. They were treated with 0.05 trypsin for 10 minutes, placed in a glass box filled with 10 mM, pH 6.0, citrate buffer, and processed in a microwave oven twice at 750 W and then rinsed in phosphate buffered saline (PBS). The sections were stained with MIB-1 monoclonal antibody (Immunotech, Marseille, France) at a 1 in 100 dilution for 2 hours at room temperature, using an immunoperoxidase ABC method⁴⁰ and diaminobenzidine as chromogen; they were then counterstained with haematoxylin and mounted in resin. MIB-1 immunostaining was scored by two pathologists (AP and LC) using a standard light microscope equipped with an ocular reticle (magnification $\times 15$) and a $\times 40$ objective. One thousand cells were counted from 10 different randomly selected areas; the interobserver variation was less than 5%.

Ki67 STAINING AND SCORING

A section of each malignant lymphoma was snap-frozen and stored in liquid nitrogen. Sections (5 μm), fixed in acetone at 4°C for 5–10 minutes, were stained with Ki67 monoclonal antibody (Dakopatts, Glostrup, Denmark) at a 1 in 40 dilution for 90 minutes, using the ABC method⁴⁰ and diaminobenzidine as chromogen. Primary antibody was omitted from negative controls. Sections were counterstained with haematoxylin and mounted in resin. Ki67 immunostaining was scored counting 1000 cells in 10 different randomly selected areas at $\times 400$ magnification.

PCNA STAINING AND SCORING

Immunostaining was performed using the

ABC method.⁴⁰ Sections (4 μm) from formalin fixed, paraffin wax embedded tissues on poly-L-lysine coated slides were dewaxed, rehydrated, and brought to phosphate-buffered solution. Endogenous peroxidase activity was blocked by incubation for 7 minutes in 3% H_2O_2 . Monoclonal antibody against PCNA (PC10) (Dakopatts, Glostrup, Denmark) diluted 1 in 200 in PBS was incubated overnight. Normal mouse serum was substituted for primary antibody as a negative control. The sections were then treated for 30 minutes with a biotin-labelled second layer antibody and avidin-biotin-peroxidase complex (Dakopatts, Glostrup, Denmark) was added. Sections were developed with diaminobenzidine for 10 minutes, counterstained with haematoxylin, and mounted in resin. PCNA (PC10) immunostaining was scored by AP and LC; 1000 tumour cells were counted at $\times 400$ magnification from 10 representative fields (areas in which the reaction was clearly positive): in these fields all the stained nuclei were regarded as positive, regardless of the intensity of the staining, and the fraction of positive cells was determined. The interobserver variation was between 10% and 15%.

DNA FLOW CYTOMETRY

Fresh tissue fragments were processed according to the method of Vindelov *et al* using the Cycle Test Kit (Becton Dickinson, San Jose, California, USA).⁴¹ Briefly, after digestion with trypsin for 10 minutes at room temperature and treatment with RNAase and trypsin inhibitors for 10 minutes at room temperature, cell suspensions were treated with propidium iodide and Spermin for 15 minutes on ice. Ten thousand cells were acquired using a FACScan flow cytometer with a 488 nm argon ion laser, equipped with a doublet discrimination module and the Cell Fit program (Becton Dickinson). Histograms were grouped as diploid and aneuploid.⁴²

In all tumours the DNA index (DI) was calculated by dividing the modal channel number of the peak with higher DNA content by that of the peak with lower DNA content. The peak that determined a DI value of between 1.85 and 2.15 was considered to be in the G_2/M region. Samples with a peak in the G_2/M region and those with the number of cells lower or equal to 15% were regarded as diploid; samples with DI values between 0.80 and 1.20 were regarded as near diploid. The S-phase fraction was calculated using the SOBR (sum of broadened rectangle) model.

Correlations between MIB-1, Ki67, and PCNA scores and S-phase fraction number of mitoses, and percentage of large cells were estimated using Pearson's correlation coefficient (linear regression).

Results

Positivity for MIB-1 monoclonal antibody was confined to the cell nucleus; the sections were homogeneously stained without background; the intensity of the staining was

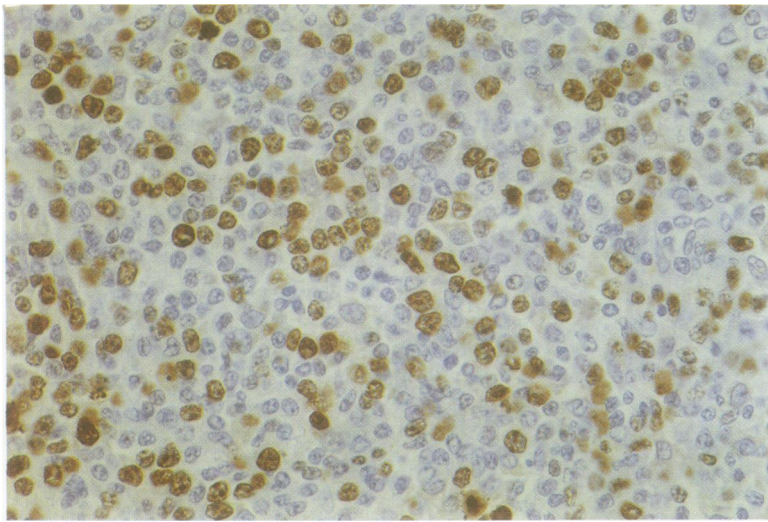


Figure 1 NHL (F): the positive cells are strongly stained with MIB-1 monoclonal antibody; no background is evident (ABC immunoperoxidase).

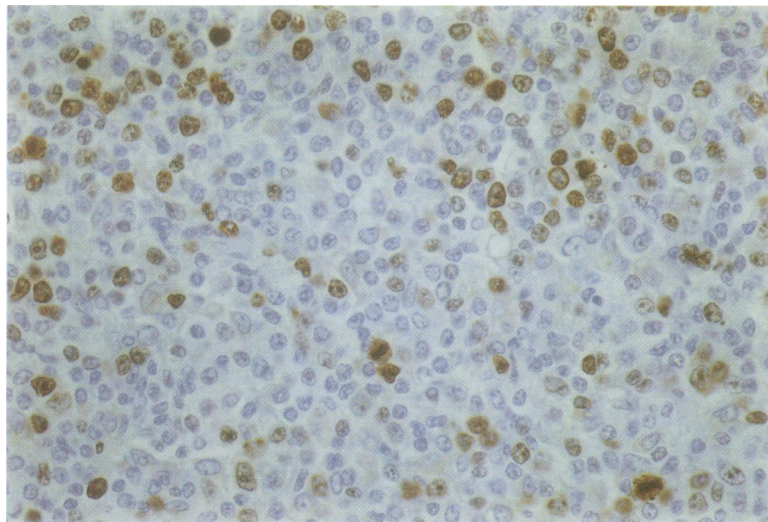


Figure 2 NHL (F): MIB-1 monoclonal antibody strongly stains mitotic figures (ABC immunoperoxidase).

Table 1 MIB-1 Ki67, and PCNA (PC10) scores and S-phase fraction in 41 intermediate grade NHL (F)

	Mean (%)	Median (%)	SD (%)	Range (%)
MIB-1	28.03	28.25	8.95	9.5-44.5
Ki67 score	29.14	28	13.04	7-57
PCNA (PC10) score	23.74	23.25	8.66	8.5-50
S-phase fraction	17.39	14	8.89	4-52

Table 2 Correlation between MIB-1 score, Ki67 score, PCNA (PC10) score, S-phase fraction, mitotic counts and percentage of large cells in 41 intermediate grade NHL (F)

	r	p value
MIB-1 and Ki67 scores	0.92	<0.00001
MIB-1 and PCNA(PC10) scores	0.79	<0.00001
MIB-1 score and S-phase fraction	0.51	0.0006
MIB-1 score and mitotic counts	0.56	0.0001
MIB-1 score and percentage of large cells	0.49	0.0009
Ki67 and PCNA(PC10) scores	0.85	<0.00001
Ki67 score and S-phase fraction	0.6	0.0002
Ki67 score and mitotic counts	0.47	0.002
Ki67 score and percentage of large cells	0.53	0.0003
PCNA(PC10) score and S-phase fraction	0.74	<0.00001
PCNA(PC10) score and mitotic counts	0.51	0.0007
PCNA(PC10) score and percentage of large cells	0.6	0.00003
S-phase fraction and percentage of large cells	0.55	0.0002
S-phase fraction and mitotic counts	0.072	0.65

enhanced by trypsin pretreatment and was generally strong (fig 1). Some gradation in the intensity could be seen from nucleus to nucleus within the same section, but it was always easy to determine whether a nucleus was positive, so that the interobserver variation in scoring was very low. A minimal crumpling of the nuclear membrane, due to the boiling procedure in the microwave oven, was observed, with consequent loss of some fine cytological details. Mitotic figures were strongly stained in all cases (fig 2). The mean MIB-1 score was 28.03%, the median 28.25% (range (SD) 9.5-44.5 (8.95)%).

Ki67 positive staining was found in the nuclei of neoplastic cells in proportions that varied from case to case; the staining was stronger in the nucleoli, but diffuse nuclear staining was also often present. The average Ki67 score for all the cases was 29.14%, the median 28% (range (SD) 7-57 (13.04)%).

PCNA(PC10) staining was mainly confined to the nucleus and showed a diffuse or granular pattern, but with a gradation in the intensity of the staining within the same section. Most of the large cells were positive. In a few cases, especially in very large sections, scattered areas were completely unstained; these areas were excluded from counting. The mean PCNA score was 23.74%, the median 23.25% (range (SD) 8.5-50 (8.66)%).

Of the 41 (F) NHL, 32 (78%) were diploid and nine (22%) aneuploid. Among the latter, eight (19.5%) had a DI below 1.2 (near diploid) and only one (2.44%) had a DI above 1.2. The mean S-phase fraction was 17.39%, the median 14% (range (SD) 4-52 (8.89)%). The results are summarised in table 1.

There was a linear correlation between MIB-1 and Ki67 scores, with a correlation coefficient of 0.92 ($p < 0.00001$) between MIB-1 and PCNA(PC10) scores ($r = 0.79$; $p < 0.00001$), between MIB-1 score and S-phase fraction ($r = 0.51$; $p = 0.0006$), between MIB-1 score and mitotic counts ($r = 0.56$; $p = 0.0001$) and between MIB-1 score and percentage of large cells ($r = 0.49$; $p = 0.0009$). The Ki67 score was directly correlated with PCNA (PC10) score ($r = 0.85$; $p < 0.00001$), S-phase fraction ($r = 0.6$; $p = 0.0002$), mitotic counts ($r = 0.47$; $p = 0.002$) and percentage of large cells ($r = 0.53$; $p = 0.0003$). The PCNA score also correlated with S-phase fraction ($r = 0.74$; $p < 0.00001$), mitotic counts ($r = 0.51$; $p = 0.0007$), and percentage of large cells ($r = 0.6$; $p = 0.00003$). S-phase fraction was related to the percentage of large cells ($r = 0.55$; $p = 0.0002$) but not to the mitotic counts ($r = 0.072$; $p = 0.65$). The results are summarised in table 2.

Discussion

The most significant correlations were found between MIB-1 and Ki67 scores ($r = 0.92$), between MIB-1 and PCNA scores ($r = 0.79$), and between PCNA and Ki67 scores ($r = 0.85$). The linear correlation

between S-phase fraction and PCNA score ($r = 0.74$) was higher than those observed between S-phase fraction and Ki67 ($r = 0.6$) or MIB-1 ($r = 0.51$) scores.

The mean Ki67 score of 29.14% we found in intermediate grade lymphomas is between the values reported for low grade malignant lymphoma (2.5–20%) and high grade malignant lymphoma (50–83%),¹¹ but lower than that (59%) reported in a smaller series of intermediate grade NHL.¹⁴ The associations we found between Ki67 and PCNA scores, S-phase fraction, and mitotic counts were similar to those reported before.^{14 26 27 43} Ki67, however, can be applied only on frozen sections in which it is hard to define the tissue structure and select the most representative fields that are essential for an accurate diagnosis in NHL. Moreover, it needs a consistent amount of tissue and it is not suitable for small specimens. MIB-1 and PCNA immunostainings, which can be performed on formalin fixed, paraffin wax embedded tissues, have no such limitations.

The correlation between PCNA score and S-phase fraction ($r = 0.74$) fits with other reported data in malignant lymphoma²⁷ and gastrointestinal malignant lymphoma²⁸ and is higher than the correlation between S-phase fraction and Ki67 score ($r = 0.6$) or MIB-1 score ($r = 0.51$). Thus the PCNA score is a very reliable indicator of the S-phase in (F) NHL. We did encounter some difficulties in performing PCNA staining in formalin fixed tissues. In some sections, especially when very large, the intensity of the staining varied from area to area and in a few cases the reaction was completely negative in some fields: we believe that such areas must be excluded from scoring. Moreover, the labelling intensity varied from nucleus to nucleus, as previously noted.^{14 44} This can lead to some subjectivity in the scoring process, unless all PCNA reactive nuclei are scored as positive, regardless of the intensity of staining. It could also explain why in our series the interobserver variation for PCNA scores was 10–15%.

Our values for the S-phase fraction agree with those reported before.^{31 32} But flow cytometry also included inflammatory and activated stromal cells, many of which can be highly proliferative and detract from accurate assessment of the S-phase.⁴⁵ Flow cytometry cannot differentiate the highly proliferating cells of the residual germinal centres which are excluded from MIB-1, Ki67, or PCNA scoring. These conditions may account for the lack of correlation between S-phase fraction and mitotic counts in our cases. A consistent amount of fresh material is also required for performing both flow cytometric and histological analyses and inadequate sampling may have accounted for some discrepancies between values.

Because MIB-1 recognises native Ki67 antigen or recombinant fragments of Ki67 molecule,^{37 46} it is not surprising that the linear relation between Ki67 and MIB-1 scores is very high ($r = 0.92$). Like Ki67, MIB-1

score also correlates with the PCNA score and S-phase fraction and with the percentage of large cells and mitotic counts.

In this study we have shown that MIB-1, Ki67, and PCNA immunostaining and DNA flow cytometry are reliable and complementary methods for assessing the proliferative activity of intermediate grade NHL: therefore, it is advisable to perform them all whenever possible.

MIB-1 immunostaining, however, seems to be more flexible and has some advantages over the other procedures. In fact, unlike Ki67, MIB-1 staining can easily be applied to routinely fixed and wax embedded specimens, permitting retrospective analysis. Unlike PCNA, it stains the sections homogeneously with minimal variation in the staining intensity from nucleus to nucleus. This feature reduces the interobserver discrepancy in the scoring process and, together with the absence of the background interference, makes MIB-1 staining particularly suitable for automated image analysis. MIB-1 also strongly stains mitotic figures, making such counts easier and quicker than with Giemsa staining. Finally, unlike flow cytometry, it permits the simultaneous evaluation of the tumour cell kinetics and histology.

In our series of intermediate NHL we found wide standard deviations in the proliferative indices: this suggests that this category probably includes various degrees of malignancy. In fact, by subdividing the cases according to the median values of the proliferative indices, 14/41 (34.14%) had all the indices greater than the medians; 13/41 (31.7%) had all the indices smaller than the medians. Further studies are in progress to ascertain if these two groups of intermediate grade NHL will also have different prognoses.

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