MIB-1 and S-phase cell fraction predict survival in non-Hodgkin's lymphomas

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Abstract. The monoclonal antibody anti-Ki67 is used to detect proliferating cells, but its main limitation is the requirement of fresh-frozen material. On a series of patients with non-Hodgkin's lymphoma, we used a Ki67 equivalent monoclonal antibody, the recently proposed MIB-1, on formalin-fixed histopathological material using microwave antigen retrieval. MIB-1 expression was analysed in relation to other proliferation indices, such as autoradiographic ^3H -thymidine labelling index ($^3\text{HTL1}$) and flow cytometric S-phase cell fraction (FCM-S) and to pathological status. Moreover, the prognostic relevance of the cell kinetic indices was defined in uni- and multivariate analyses including histology and tumour stage. The relationship between MIB-1 index and the other proliferation indices was statistically significant even though the correlation coefficient was around 0.6. The MIB-1 index was also related to the REAL (Revised European American Lymphoma) classification, but not to the Ann Arbor stage classification. Univariate analysis showed that the MIB-1 index was a significant predictor of 6-year survival in the overall series and in distinctly analysed low-grade and high-grade lymphoma subgroups. With regard to S-phase indices, $^3\text{HTLI}$ was a powerful prognosticator in patients with high-grade histologies and FCM-S in patients with low-grade histologies. Multivariate analyses revealed that MIB-1 index, $^3\text{HTLI}$ and FCM-S retained their prognostic significance independent of histology. In conclusion, the MIB-1 antibody provides prognostic information in non-Hodgkin's lymphomas and has the main advantage that it can be used in formalin-fixed, paraffin-embedded specimens.

The prognostic definition and the choice of therapy in non-Hodgkin's lymphomas (NHL) are based on clinical staging and on histological classifications, which distinguish NHL with an indolent behaviour from those with an aggressive one. However, within the two subgroups there are entities with considerably different biological and clinical outcomes (Urba *et al.* 1990, Armitage 1993, Hiddemann & Unterhalt 1994).

During the last 20 years, accumulating information has shown that cell proliferation activity may contribute to a more precise identification of patients at different risk. Some studies have addressed quantification of the S-phase cell fraction by utilizing active incorporation of ^3H -thymidine, iodo- or bromodeoxyuridine (Kawauchi *et al.* 1989, Silvestrini *et al.* 1989, Brons *et al.* 1992, Costa *et al.* 1992, Silvestrini *et al.* 1993, Witzig *et al.* 1995), or

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by quantifying nuclear DNA content of individual cells by flow cytometry (Slymen *et al.* 1990, Costa *et al.* 1992, Duque *et al.* 1993, Joensuu *et al.* 1994). In all these studies a significant correlation was found between the S-phase index and overall survival, with the worst prognosis for patients with rapidly proliferating NHL.

Alternatively, among approaches proposed to define the entire cell proliferating fraction, the detection of the proliferation-associated antigen Ki67 has been extensively used (Gerdes *et al.* 1984, Gerdes *et al.* 1987, Grogan *et al.* 1988, Hall *et al.* 1988, Veneroni *et al.* 1988, Medeiros *et al.* 1989, Leoncini *et al.* 1993, Sabattini *et al.* 1993, Miller *et al.* 1994, Czader *et al.* 1995). A prognostic relevance of this proliferation index has been observed for some morphological subgroups (Gerdes *et al.* 1987, Grogan *et al.* 1988, Hall *et al.* 1988, Medeiros *et al.* 1989, Miller *et al.* 1994). The monoclonal antibody anti-Ki67 was the only one available until the early 90s, and it had the inherent drawback that it could be applied only to sections from fresh-frozen tissues and to archival material if adequately fixed. Recently, using recombinant parts of the Ki67 protein as an immunogen, an equivalent monoclonal antibody, MIB-1, has been developed (Key *et al.* 1992). This new antibody can be utilized as a routine stain on paraffin-embedded sections from fixed tissues through a microwave process (Cattorretti *et al.* 1992). Thus there is new interest in the prognostic validation of MIB-1 in different human tumours, including NHL.

In the present series of patients with NHL, the relationship between MIB-1 and other proliferation indices and pathological factors was analysed, and we defined the independent prognostic value of MIB-1 in uni- and multivariate analyses including histology and pathological stage.

MATERIALS AND METHODS

Case studies

Biopsies were obtained from 103 untreated adult patients with NHL admitted to the National Cancer Institute of Milan (INTM) from May 1979 to December 1989. Cases were selected for the study on the basis of MIB-1 information in formalin-fixed samples, and the availability of other cell-proliferation markers. The median age of the patients was 55 years (range 17–81); 52 were men and 51 were women. All patients were pathologically staged and classified according to the Ann Arbor Staging System (Carbone *et al.* 1971): 14 patients had stage I, 18 stage II, 22 stage III and 49 stage IV disease at presentation.

Histological specimens were retrospectively reviewed and classified by the pathologist (RG) according to the updated Kiel classification, Working Formulation and REAL classification (Harris *et al.* 1994). For prognostic purposes we divided the whole series into two subgroups: low-grade (62 cases) and high-grade (41 cases) lymphomas (Table 1).

Forty-six patients had received an anthracycline-containing regimen with or without irradiation (RT). Forty-nine patients had received a polychemotherapy regimen without anthracyclines, either associated or not associated with RT, and the remaining eight patients received RT alone. Complete restaging was scheduled at the end of the treatment programme, and periodic follow-up examinations were performed every 3 to 4 months for the first 2 years after completion of therapy and every 6 to 12 months thereafter.

Cell proliferation markers

For all 103 cases information on MIB-1 index and ³H-thymidine labelling index (³HTLI) was available, whereas flow-cytometric S-phase (FCM-S) data were limited to 78 cases.

Table 1. Histological subtypes according to REAL classification

Type of lymphoma	Number of patients
Low-grade	10
B-cell chronic lymphocytic leukaemia	
Lymphoplasmacytoid lymphoma	12
Follicular centre lymphoma, grade I	21
Follicular centre lymphoma, grade II	11
Mantle cell lymphoma	8
High-grade	
Follicular centre lymphoma, grade III	4
Follicular centre lymphoma, diffuse, small cell	4
Peripheral T-cell lymphoma, unspecified	5
Diffuse large B-cell lymphoma	23
Burkitt's lymphoma	2
Precursor lymphoblastic lymphoma	3

MIB-1

Two- μm thick paraffin sections from the samples used for diagnosis were mounted onto poly-L-lysine-coated slides (Sigma, Milan, Italy) and dried overnight at 37°C. Subsequently, sections were dewaxed in xylene, rehydrated according to histopathological standards, and immersed in 10 mM citrate (in distilled water, pH 6.0). The slides were processed for two cycles of 8 and 5 min at maximum power (700 W) in a household microwave oven (Quelle Versand, Furth, Germany). The sections were incubated with the monoclonal antibody MIB-1 (Immunotech, Marseille, France; dilution 1:100) for 1 h at room temperature and successively with a rabbit anti-mouse IgG serum (Dako, Glostrup, Denmark) and then treated with an avidin–biotin system. The antigen–antibody complex was visualized using alkaline phosphatase and then counterstained with haematoxylin. A tissue with known positive immunostaining for MIB-1 or Ki67 was used as positive control; negative controls were obtained by omission of the primary monoclonal antibody. A cell was considered positive when it exhibited a weak, moderate or strong nuclear and/or nucleolar staining. At least 3000 cells from the most representative areas of each tumour were scored, and the MIB-1 index was evaluated as the ratio between positive cells and total tumour cells.

³HTLI

Cell suspensions were obtained by mechanical disaggregation of the fresh lymph node material as previously described (Costa *et al.* 1981). Cells were incubated with ³HTLI (148 kBq; specific activity, 925 GBq mmol⁻¹, Radiochemical Centre, Amersham, UK) for 1 h at 37°C. Samples were processed by autoradiography according to the stripping film (Kodak AR10, Kodak, London) technique. The ³HTLI was determined on the entire cell population by scoring 3000–10000 cells from different cytopins for each tumour.

FCM-S

From the same samples used for diagnosis, single-cell suspensions were obtained from 50- μm paraffin-embedded sections according to methodology previously described (Costa *et al.* 1992). Adjacent 5- μm sections were used for the microscopic control of tumour tissue adequacy. DNA was stained with propidium iodide. Flow cytometry was performed with a FACScan flow cytometer (Becton Dickinson, San José, CA), and a total of 30000 nuclei was acquired for each tumour. The median coefficient of variation of the G_{0/1} peak was 4.9%.

with a range of 2.4% to 8%. FCM-S was computed by a planimetric method (RFIT, Becton Dickinson). According to this model, the S phase was calculated from a rectangle, whose height is the average height of mid S phase. For aneuploid tumours, the S-phase fraction of the aneuploid population alone was calculated (i.e., (number of aneuploid S-phase cells/total number of aneuploid cells) $\times$ 100).

Statistical analysis

The concordance between MIB-1 immunoreactivity and the other cell proliferation markers was evaluated by Spearman's rank correlation coefficient (r_s). Survival curves were estimated starting from the date of diagnosis to the date of death (any cause) or last contact, by the product-limit method of Kaplan–Meier. The role of each of the putative prognostic variables (univariate analysis) and their joint effect (multivariate analysis) were evaluated by resorting to a Weibull regression model. In this model, each regression coefficient (β) is recognizable as the log of the hazard ratio (HR), and is constant with time. For each variable, the unadjusted HR and its 95% confidence limits (CL) were estimated using the putative best prognosis as reference category. To investigate the joint prognostic relevance of the variables, the adjusted HR was estimated by resorting to a multiple regression model containing all variables. The parsimonious regression model was obtained by means of a backward elimination procedure. The cell proliferation markers, histology and stage, were used as categorical variables. Cut-off values for MIB-1, 3 HTLI, and FCM-S were tested in 1% steps through the experimental-value distribution to seek the optimal categorization (Sigurdsson *et al.* 1990).

RESULTS

MIB-1 and other cell proliferation markers

In this series of 103 formalin-fixed samples, immunoreactivity to MIB-1 was always nuclear. A strong correlation was observed between MIB-1 index and the REAL classification. In particular, the median MIB-1 index of low-grade lymphomas was 16.7% (range, 4% to 78.7%) and that of high-grade lymphomas 40.9% (range, 6.2% to 97%) ($P=0.0001$).

A statistically significant correlation was observed on the overall series between MIB-1 index and 3 HTLI ($P=0.0001$) or FCM-S ($P=0.0001$), but the correlation coefficients (r_s) were only 0.6 and 0.57, respectively. Moreover, a breakdown analysis showed quite similar correlation coefficients between MIB-1 index and 3 HTLI in low-grade ($r=0.55$) and high-grade ($r_s=0.63$) lymphomas, and a correlation coefficient between MIB-1 index and FCM-S lower in low-grade ($r_s=0.34$) than in high-grade ($r_s=0.59$) lymphomas (Figure 1a,b).

No association was found between the median values of the three cell proliferation indices and stage according to the Ann Arbor classification, except for a lower median 3 HTLI in stage I lymphomas.

Clinical outcome

An initial analysis of the relation between cell proliferation and patient's clinical status at 6 years to follow-up (Table 2) showed similar median values of the three proliferation indices for disease-free, alive with disease, or dead for progression in patients with low-grade lymphomas. Conversely, in patients with high-grade disease, the median value of MIB-1 index, 3 HTLI and FCM-S was approximately two times higher in patients dead for disease progression than for those alive and disease-free.

In seeking the best cut-off to discriminate good and poor prognosis, a wide range of values significantly predictive of 6-year survival was observed for MIB-1 index in the overall series

Figure 1. Relationship between growth fraction, expressed by the MIB-1 index, and the S-phase cell fraction expressed as $^3\text{HTLI}$ (a) or FCM-S (b) in individual lymphomas ($\circ$, low-grade NHL; $\bullet$, high-grade NHL).

(Figure 2) and in the distinctly analysed high-grade lymphomas, and a narrower range was observed in low-grade lymphomas. With regard to $^3\text{HTLI}$, a wide range of prognostic cutoff values was found on the overall series. However, only the value of 4% was a significant discriminant of prognosis within high-grade lymphomas, and no value reached a statistically significant relevance within low-grade tumours. Again, a wide range of significant cut-off values of FCM-S was observed on the overall series; only 12% was a prognostic indicator within low-grade lymphomas, and no cut-off value was discriminant in high-grade lymphomas.

Cumulative survival percentages at 6 years for patients with low- or high-grade lymphomas as a function of the three cell proliferation markers are shown in Figure 3. By using cut-off values specific for each histology, within patients with low-grade tumours a low MIB-1 value was associated with an 86% survival rate, compared to only 52% for patients with high MIB-1 tumours ($P=0.01$). The hazard of death was about four times higher for the latter than for the former subgroup. Similarly, within patients with high-grade lymphomas, a low MIB-1 value was associated with a 67% survival rate, compared to only 15% for patients with

Table 2. Median value of cell proliferation indices in relation to prognosis

Patient's status (6 years)	MIB-1 (%)		$^3\text{HTLI}$ (%)		FCM-S (%)	
	Low-grade*	High-grade*	Low-grade*	High-grade*	Low-grade*	High-grade*
Alive disease-free	16.9 (4.0–43.8)	29.0 (6.2–85.3)	2.3 (0.1–11.6)	7.4 (0.6–28.6)	9.3 (3.4–17.0)	11.4 (3.9–41.3)
Alive with disease	13.8 (6.6–77.4)	45.2 (7.8–91.6)	2.8 (0.01–23.8)	23.1 (1.5–35.5)	10.0 (2.9–22.3)	19.7 (13.1–41.4)
Dead for progression	20.9 (6.3–78.7)	58.3 (19.9–97.0)	2.7 (0.5–16.7)	14.0 (0.01–40.0)	12.2 (4.7–33.7)	27.2 (9.7–60.7)

*Range given in parentheses.

high MIB-1 tumours ($P=0.001$), with a hazard of death 11 times higher in the latter than in the former subgroup.

In the present series, $^3\text{HTLI}$ was not a prognostic indicator in low-grade tumours but it was a powerful prognosticator of survival in high-grade lymphomas. In fact, patients with high $^3\text{HTLI}$ tumours had a 29% survival rate, compared to 70% for patients with low $^3\text{HTLI}$ tumours ($P=0.008$), and a hazard of death 10 times higher in the latter than in the former subgroup (Figure 3).

In the limited series of patients (78 cases) with available information, FCM-S showed a prognostic power similar to MIB-1 in low-grade tumours and lesser than that of the other two proliferation indices in high-grade lymphomas (Figure 3).

Figure 2. Cut-off values of MIB-1 index for predicting overall survival plotted against the relative P value (—) and HR (---). Statistical significance is indicated by the line drawn at the χ^2 value corresponding to the $P=0.05$ level for 1 degree of freedom.

The relative prognostic relevance of MIB-1 index and 3 HTLI from histology and Ann Arbor stage was defined by multivariate regression analysis on the overall series (Table 3). Using a backward method for regression, some variables were removed from the model, leaving MIB-1 and 3 HTLI in the final step as the only independent prognostic factors.

To verify the independent prognostic relevance of FCM-S, a similar multivariate analysis was performed on the series of 78 cases for which this cell kinetics information was available

Figure 3. Probability of 6-year survival in low-grade and high-grade tumours as a function of MIB-1, 3 HTLI and FCM-S.

Table 3. Multiple regression analysis for survival: overall series

Variable	Hazard ratio (95% CL)	P*
<i>Initial model</i>		
Stage		
III–IV v. I–II	3.2 (1.4–7.6)	0.006
Histology		
High-grade v. low-grade	2.4 (0.9–6.5)	0.08
MIB-1 index		
High v. low	4.0 (1.5–10.8)	0.006
³ HTLI		
High v. low	2.6 (1.0–6.7)	0.05
<i>Final model</i>		
MIB-1 index	6.0 (2.1–17.7)	0.001
High v. low		
³ HTLI		
High v. low	3.4 (1.4–8.5)	0.008

*Computed by Weibull model.

(Table 4). The final model left MIB-1 index, histology and FCM-S as independent prognostic factors.

DISCUSSION

Many reports have documented a strong correlation between Ki67 staining and histological grade of lymphomas, but a relationship between the cell proliferation marker with clinical

Table 4. Multiple regression analysis for survival: 78 cases with evaluable FCM-S

Variable	Hazard ratio (95% CL)	P*
<i>Initial model</i>		
Stage		
III–IV v. I–II	1.2 (0.4–3.3)	0.07
Histology		
High-grade v. low-grade	3.1 (1.2–17.5)	0.02
MIB-1 index		
High v. low	5.7 (1.8–17.5)	0.003
FCM-S		
High v. low	4.0 (1.3–11.8)	0.01
<i>Final model</i>		
MIB-1 index	7.0 (2.2–22.0)	0.0009
High v. low		
Histology		
High-grade v. low-grade	3.0 (1.2–7.6)	0.02
FCM-S		
High v. low	4.0 (1.3–12.0)	0.01

*Computed by Weibull model.

outcome has not been conclusively defined. In fact, a high Ki67 index was correlated with a poor outcome within low-grade NHL (Hall *et al.* 1988), whereas for high-grade lymphomas inconsistent data have been reported. In the study of Hall *et al.* (1988), a very high Ki67 index was correlated with a good survival, whereas Grogan *et al.* (1988) reported contrasting findings, which were confirmed by Miller *et al.* (1994) in a series of patients with advanced stages of intermediate- and high-grade histologies. MIB-1 is a new antibody prepared against recombinant Ki67 antigen, which also detects the antigen in formalin-fixed, paraffin-embedded tissues (Cattoretti *et al.* 1992, Key *et al.* 1992). For this reason, its prognostic relevance would be of particular interest for retrospective analyses. In a few recent papers, the prognostic significance of MIB-1 immunostaining has been observed in some solid human tumours (Kerns *et al.* 1994, Skalova *et al.* 1994, Nakamura *et al.* 1995), but to our knowledge, the current study is the first addressed to evaluate the prognostic relevance of MIB-1 in lymph node NHL.

In NHL, a correlation has been demonstrated between the S-phase cell fraction (expressed as FCM-S, ³HTLI or BrdUrd LI) and the Ki67 index (Veneroni *et al.* 1988, Kawauchi *et al.* 1989, Brons *et al.* 1992). Moreover, a relationship has been observed between FCM-S and Ki67 or MIB-1 index in a series of intermediate-grade lymphomas (Pich *et al.* 1994). In the present series, we observed a relationship between the fraction of DNA-synthesizing cells, expressed as ³HTLI or FCM-S, and the growth fraction, expressed by the MIB-1 index, even though with a different correlation coefficient as a function of histology and biological variables.

The prognostic relevance of the S-phase cell fraction has been already reported by others (Duque *et al.* 1993) and by us (Costa *et al.* 1992) in several studies including all histologies or only subgroups of NHL patients, such as low-grade lymphomas (Witzig *et al.* 1995) or diffuse large-cell lymphomas (Slymen *et al.* 1990). In the present study, in univariate analysis ³HTLI was a significant indicator of survival only in high-grade NHLs, FCM-S reached a statistical significance as indicator of survival only in low-grade lymphomas, and MIB-1 was a powerful prognosticator in both histology subsets. Moreover, multivariate analysis showed that MIB-1 index, ³HTLI and FCM-S provided predictive information on survival independent of histology and stage. That is, the different cell proliferation indices give additive contributions to prognosis of patients with NHL.

Unfortunately, the number of cases investigated did not allow analysis of the known interaction between therapy, histology and stage. Moreover, when adjusted for the type of treatment, including or not anthracyclines, MIB-1 index and ³HTLI maintained their prognostic power. These data are consistent with those reported in homogeneous series of patients with diffuse large-cell lymphomas (Grogan *et al.* 1988, Silvestrini *et al.* 1993, Miller *et al.* 1994).

In conclusion, immunostaining with MIB-1 represents a simple and quantitative approach, applicable to formalin-fixed, paraffin-embedded NHL specimens. However, the present data should be validated on larger and consecutive series of homogeneously treated patients.

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