

## The Prognostic Value of International Prognostic Index and MIB-1 Immunostaining of Peripheral Lymphoid Tissues and Bone Marrow in Patients with High-Grade Non-Hodgkin's Lymphoma

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### ABSTRACT

**Background:** Cell kinetic data are important indicator of the aggressiveness of tumour and clinical response. The Ki-67 antigen plays a pivotal role in maintaining cell proliferation and the expression of this antigen was found to be a valuable indicator for aggressive disease in a variety of neoplastic disorders. **Aim of the study:** This study aimed to assess the prognostic significance of the expression of Ki-67 antigen in peripheral lymphoid tissues and bone marrow, using the monoclonal antibody MIB-1 that is applicable in formaline-fixed paraffin embedded samples in cases with high-grade non-Hodgkin's lymphomas. **Material and methods:** The MIB-1 immunostaining was performed on 96 samples from 48 patients with high-grade non-Hodgkin's lymphomas. The study was performed on tissue sections, nodal or extranodal, as well as on BM smears or BM paraffin embedded sections of same patients. Ki-67 index was determined using image analyzer. **Results:** Forty-five out of the studied 48 cases (93.8%) were positive with a median labelling index of 20.425% (Range, 0-58%). We were able to detect bone marrow involvement by detecting MIB-1 positive cells in BM samples of 29 patients who were not morphologically diagnosed to have BM infiltration. There was a strong correlation between BM positivity for Ki-67 and Ki-67 labelling index ( $p < 0.001$ ). Twenty-eight (58.3%) out of the studied 48 cases achieved complete remission (CR). The median duration of CR was 35 months (range, 8-42 months) and the overall survival at 48 months was 35.4% (median 22 months, 95% CI, 13-31 months). The median Ki-67 index (20.425%) was chosen as a cut-off level for statistical analysis of the variables that influence clinical outcome. The probability of inducing CR was associated with low and low intermediate International Prognostic Index (IPI) whereas a low growth fraction was associated, although not significant, with a trend toward a higher probability of inducing a CR. In univariate analysis, high MIB-1 labelling index and increased IPI were associated with shorter duration of complete remission and overall survival time. Multivariate analysis showed that an extended overall survival time was associated with low MIB-1 label-

ling index and with the known clinical variables included within the IPI. Patients with high intermediate or high IPI who expressed high Ki-67 labelling index had a worse overall survival compared with patients with low or low intermediate IPI and low Ki-67 index ( $p < 0.001$ ). BM infiltration as detected by MIB-1 was correlated in univariate analysis ( $p = 0.04$ ), although not confirmed in multivariate analysis, with poor survival. **Conclusion:** In conclusion, the MIB-1 monoclonal antibody immunostaining appears to be a simple and reproducible method of determining tumour proliferative index and provides useful prognostic information in patients with high-grade NHL. We recommend using apoptotic markers in addition for proper assessment of the impact of detecting MIB-1 positive cells in BM on disease outcome.

**Key Words:** I.P.I - Non-Hodgkin's lymphoma - MIB-1.

### INTRODUCTION

Non-Hodgkin's lymphomas (NHL) represent a spectrum of haematological malignancies characterized by a highly diverse clinical behaviour in terms of clinical presentation, histology, immunophenotype, genetic alternation, prognosis and response to therapy.

Although the International Prognostic Factor Project was developed to provide a model system for predicting the outcome of patients with aggressive NHL depending on some clinical parameters (age, stage, performance status, number of extranodal sites and serum lactic dehydrogenase level) [12], an accumulating information's has shown that cell proliferation activity may contribute to a more precise identification of patients at different risk [19].

The identification of proliferating cells in

tissue sections has previously required the assessment of number of mitosis in histological sections or the use of the costly and time consuming quantitation of S-phase cell fraction utilizing active incorporation of tritiated thymidine [4], or by quantifying nuclear DNA content of individual cells by flow cytometry [5]. In all of these studies a significant correlation was found between S-phase index and overall survival.

The Ki-67 antigen plays a pivotal role in maintaining cell proliferation and is expressed in all phases of cell cycle except G<sub>0</sub> and has been extensively used as a marker of cell proliferation in a variety of neoplastic [7] and non-neoplastic disorders [1].

The monoclonal antibody anti-Ki-67 was the only available till the early 90s and it had the inherited drawback that it could be applied only to sections from fresh-frozen tissues. Recently, using recombinant parts of Ki-67 protein as an immunogen, an equivalent monoclonal antibody, MIB-1, has been developed [7] and can be utilized as a routine stain on paraffin-embedded sections from fixed tissues through microwave process [3] providing an interest in the prognostic validation of MIB-1 in different human tumors, including NHL.

The purpose of this study was to determine whether MIB-1 labelling index could add to the information provided by International Prognostic Index in patients with high-grade NHL and to define the impact of detecting bone marrow infiltration on disease outcome by applying the MIB-1 immunostaining technique to bone marrow sample of the same patients.

## PATIENTS AND METHODS

### *Patients:*

Ninety-six samples from 48 previously untreated patients with non-Hodgkin's lymphoma presented to the National Cancer Institute, Cairo University in the period between August 1997 and September 2001 were studied.

Samples were lymph nodes in 33 cases, extranodal tissue in 15 cases and bone marrow aspirate or biopsy in 48 cases. All cases were staged according to Ann Arbor Staging System [2] and pathologically classified according to the Revised European American Lymphomas (REAL) classification [12].

All patients had received an anthracycline containing regimen which included, CHOP (Cyclophosphamide 750 mg/m<sup>2</sup>, Vincristine 1.4 mg/m<sup>2</sup>, Doxorubicin 50 mg/m<sup>2</sup> IV on dl and oral Prednisone 60 mg/m<sup>2</sup> for 5 days) (30 cases), CHOP followed by involved field irradiation (9 cases), or leukaemia-like regimen incorporating weekly Doxorubicine, Vincristine and Prednisone, central nervous prophylaxis and maintenance therapy for patients with Lymphoblastic and Burkitt's lymphomas (9 cases).

Complete re-staging was scheduled at the end of the treatment program and periodic follow up examinations were performed every 3 to 4 months for the first 2 years after completion of therapy and every 6 months thereafter.

### *Clinical variables:*

Age, clinical stage, performance status, serum LDH level and the number of extranodal sites of the disease were used as criteria as determined by the International Prognostic index [1]. Complete remission (CR) was defined as the resolution of clinical and radiological evidence of disease for a minimum of 4 weeks. Other degrees of response were considered to represent the failure of treatment.

Nodal organ was assigned to those cases with a clinical presentation in lymph nodes, waldeyer's ring, spleen or bone marrow by the criteria of Kramer et al. [16]. Extranodal organ was defined as presentation in other sites with or without local lymph node involvement.

### *Immunohistochemistry:*

Five-µm thick paraffin sections from the samples were mounted onto poly-L-lysine coated slides (Sigma, Milan, Italy) and dried overnight at 37°C. Subsequently, sections were de-waxed in xylene, rehydrated according to histopathological standards. Slides were immersed in a jar filled with sufficient antigen retrieval solution (BioGenex Cat. No. HK 090-5K) at a dilution of 1:4 with deionized water. The slides were processed for two cycles of 5 minutes at maximum power (1000w) in a microwave oven (Goldstar, USA). Sections were never allowed to dry. The sections were incubated with the monoclonal antibody MIB-1 (BioGenex ready-to-use) at room temperature overnight in a humid air and successively with a biotinylated rabbit anti-mouse IgG serum. Then treated with an avidin-biotin system (No-

vostain super ABC kit, Novocastra Laboratories, Newcastle, UK) for 30 minutes followed by rinsing with buffer for 10 minutes. The antigen-antibody complex was visualized using DAB (3,3'-diaminobenzidine tetrahydrochloride) and then counterstained with haematoxylin. A tissue with known positive immunostaining for MIB-1 or Ki-67 was used as positive control; negative controls were obtained by omission of the primary monoclonal antibody. The CAS-200 Image analyzer system was used for Ki-67 quantitation. A cell was considered positive when it exhibits a weak, moderate or strong nuclear and/or nucleolar staining. At least 1000 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.

#### Statistical analysis:

SPSS version 10.0 was used for data analysis. Chi-square and Fisher exact test were used for testing proportions independence. Disease-free survival and OS were studied by the Kaplan Meier method and the survival curves were compared by the log-rank test [15]. Cox backward proportional hazard model was performed for multivariate analysis of the factors that might be of independence significance in influencing the overall survival. Factors included in the maximum model were IPI (Low and Low intermediate or high intermediate and high), MIB-1 (< 20.425% or  $\geq$  20.425%) and bone marrow MIB-1 (negative or positive). All *p* values were always two tailed and values of 0.05 or less were considered statistically significant.

## RESULTS

Immunoreactivity to MIB-1 was always nuclear (Fig. 1) and was found to be expressed in 45/48 (93.8%) of the studied tissue samples with a mean index of  $23.4 \pm 18.9$  and a median value of 20.425% (range, 0%-58%) while 3 cases were completely negative for Ki-67.

Twenty-nine cases expressed positive immunostaining to MIB-1 in bone marrow sections in absence of BM infiltration by morphology (Fig. 2). The positive Ki-67 BM cases, which were not morphologically diagnosed to have BM infiltration, included 24 cases with aggressive and 5 cases with highly aggressive lymphomas.

There was a strong correlation between BM positivity for Ki-67 and Ki-67 labelling index. The positive BM for Ki-67 had a median index of 30% (range, 1.7%-57.92%) while negative BM cases had a median of 1.76% (range, 0%-24.62%)  $p < 0.001$ .

#### Clinical outcome:

The clinicopathological characteristics of the 28 (63.9%) male and 20 (36.1%) females included in the study are shown in Table (1). Their age ranged between 3 and 71 years (median 42 years).

Table (1): Clinicopathological features of the studied 48 NHL cases.

Total	Number / %
<i>Age (years):</i>	
$\leq 60$	41 (85.4)
$> 60$	7 (14.6)
<i>Sex:</i>	
Male	28 (58.3)
Female	20 (41.7)
<i>PS (ECOG):</i>	
0-1	15 (31.2)
$\geq 2$	33 (68.8)
<i>Stage:</i>	
I-II	15 (31.2)
III-IV	33 (68.8)
<i>Extranodal disease:</i>	
0-1	33 (68.8)
$\geq 2$	15 (31.2)
<i>LDH:</i>	
Normal	18 (37.5)
High	30 (62.5)
<i>IPI:</i>	
Low	12 (25)
Low intermediate	10 (20.8)
High intermediate	21 (43.8)
High	5 (10.4)
<i>Pathology:</i>	
- Aggressive	39 (81.3)
Diffuse large	32 (66.7)
Peripheral T-cell	7 (14.6)
- Highly aggressive	9 (18.7)
Burkitt's lymphoma	7 (14.6)
Lymphoblastic lymphoma	2 (4.1)
<i>Ki-67 labelling index:</i>	
$< 20.425\%$	24 (50)
$\geq 20.425\%$	24 (50)
<i>BM Ki-67:</i>	
Negative	19 (39.6)
Positive	29 (60.4)

Twenty-eight patients (58.3%) achieved complete remission (CR). The median duration of CR was 35 months (range, 8-42 months) and the overall survival at 48 months was 35.4% (median 22 months, 95% CI, 13-31 months). The median Ki-67 index (20.425%) was chosen as a cut-off level for statistical analysis of the variables that influence clinical outcome.

Analysis of probability of inducing CR showed that, poor performance status > 1 ( $p = 0.04$ ), extranodal disease > 1 site ( $p = 0.003$ ) and increased IPI ( $p = 0.01$ ) were significantly associated with decrease probability of achieving CR Table (2).

Analysis of the factors which affect the duration of CR showed that, advanced clinical stage ( $p = 0.05$ ), high serum-LDH level ( $p = 0.003$ ), increased IPI ( $p = 0.002$ ) and MIB-1 labelling index greater than 20.425% ( $p = 0.01$ ) were significantly associated with shorter duration of CR (Table 3; Figs. 3&4).

The investigation of overall survival showed that, advanced clinical stage ( $p = 0.01$ ), extra-

nodal disease > 1 site ( $p = 0.002$ ), poor performance status > 1 ( $p = 0.007$ ), high serum-LDH level ( $p = 0.006$ ), increased IPI ( $p < 0.001$ ), MIB-1 labelling index  $\geq 20.425\%$  ( $p = 0.01$ ) and BM positivity for MIB-1 ( $p = 0.04$ ) were significantly associated with shorter overall survival time (Table 4; Figs. 5,6&8).

Nine out of the 12 patients with low or low intermediate IPI who expressed low Ki-67 labelling index < 20.425% and achieved CR are alive disease-free at 42 months (median survival, 48 months; range 12-48 months). Whereas all of the 6 out of the 14 patients with high intermediate or high IPI and high Ki-67 labelling index  $\geq 20.425\%$  who achieved CR, had relapsed (median survival, 8 months; range 3-29 months). The difference was statistically highly significant ( $p < 0.001$ ) (Fig. 7).

In Cox's regression model, the parameters associated with a prolonged overall survival were MIB-1 index (< 20.425%) and the clinical variables included within the International Prognostic Index (Table 5).

Table (2): Effect of various variables on achieving complete remission (CR).

	CR (58.3%)		NO CR (42.7%)		p-value
	Number	%	Number	%	
<i>Age (years):</i>					
≤ 60	25	61	16	39	0.42
> 60	3	42.9	4	57.1	
<i>Stage:</i>					
I-II	11	73.3	4	26.7	0.16
III-IV	17	51.5	16	48.5	
<i>PS (ECOG):</i>					
0-1	12	80	3	20	0.04
≥ 2	16	48.5	17	51.5	
<i>Extranodal:</i>					
0-1	24	72.7	9	27.3	0.003
≥ 2	4	26.7	11	73.3	
<i>LDH:</i>					
High	16	53.3	14	47.7	0.36
Normal	12	66.7	6	33.3	
<i>IPI:</i>					
Low & low intermediate	17	77.3	5	22.7	0.01
High & high intermediate	11	42.3	15	57.7	
<i>Ki-67 labelling index:</i>					
< 20.425%	15	62.5	9	37.5	0.5
≥ 20.425%	13	54.2	11	45.8	
<i>BM Ki-67:</i>					
Negative	13	68.4	6	31.6	0.25
Positive	15	51.7	14	46.3	

Table (3): Factors affecting duration of CR.

	Duration of CR (months) Mean±SE*	p-value
<i>Age (years):</i>		
≤ 60	31±3	0.97
> 60	28±8	
<i>PS(ECOG):</i>		
0-1	35±3	0.17
≥ 2	27±3	
<i>Stage:</i>		
I-II	37±2	0.05
III-IV	27±3	
<i>Extranodal disease:</i>		
0-1	32±3	0.5
≥ 2	26±7	
<i>LDH:</i>		
Normal	38±1	0.003
High	25±3	
<i>IPI:</i>		
Low & low intermediate	37±2	0.002
High intermediate & high	22±4	
<i>Ki-67 labelling index:</i>		
< 20.425%	37±2	0.01
≥ 20.425%	23±3	
<i>BM Ki-67:</i>		
Negative	34±3	0.3
Positive	27±3	

\* Because median time to disease progression was not attained in many factors, survival was estimated by mean ± standard error.

Table (4): Univariate-survival analysis.

	Median survival (months)	p-value
<i>Age (years):</i>		
≤ 60	25	0.5
> 60	13	
<i>PS(ECOG):</i>		
0-1	48	0.007
≥ 2	16	
<i>Stage:</i>		
I-II	48	0.01
III-IV	16	
<i>Extranodal disease:</i>		
0-1	29	0.002
≥ 2	12	
<i>LDH:</i>		
Normal	48	0.006
High	16	
<i>IPI:</i>		
Low & low intermediate	48	< 0.001
High intermediate & high	13	
<i>Ki-67 labelling index:</i>		
< 20.425%	48	0.01
≥ 20.425%	16	
<i>BM Ki-67:</i>		
Negative	48	0.04
Positive	16	

Table (5): Multivariate analysis of factors that influence overall survival by Cox's regression model.

	RL	RR	95% CI	p-value
IPI	Low & low intermediate	0.2031	0.0854-0.4831	< 0.001
Ki-67 labelling index	< 20.425	0.4529	0.2077-0.9879	0.04
BM Ki-67	Negative	1.409	0.6224-3.1919	0.41

Abbreviation: RR: Risk ratio in disease-specific mortality. RL: Reference level.

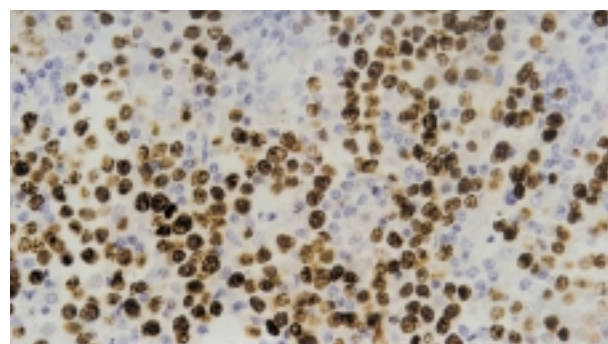


Fig. (1): NHL, diffuse large cell showing diffuse strong nuclear Ki-67 immunostaining (ABC immunoperoxidase-DAB chromogen, x 400).

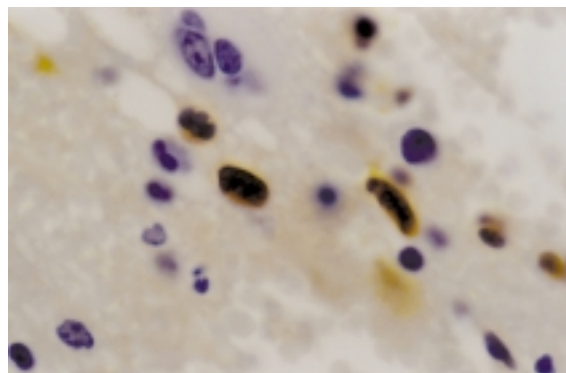


Fig. (2): Oil immersion magnification of BM aspirate paraffin block showing 2 positive cells (x 1000).

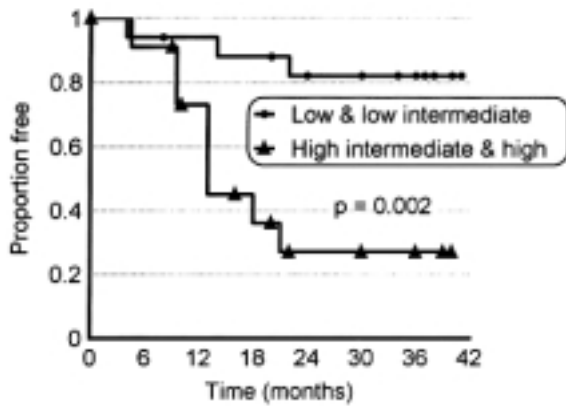


Fig. (3): Time to disease progression in 28 cases with high grade NHL according to IPI (International prognostic index).

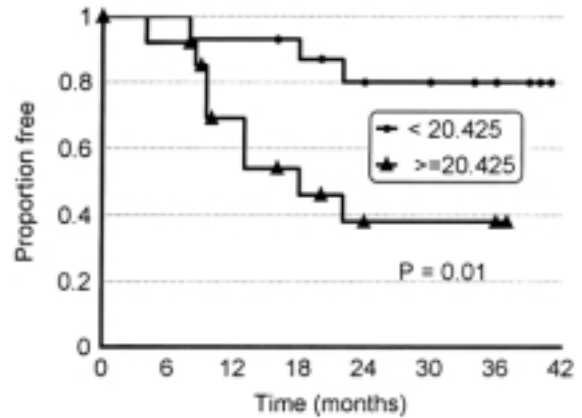


Fig. (4): Time to disease progression in 28 cases with high grade NHL according to Ki-67 labelling index.

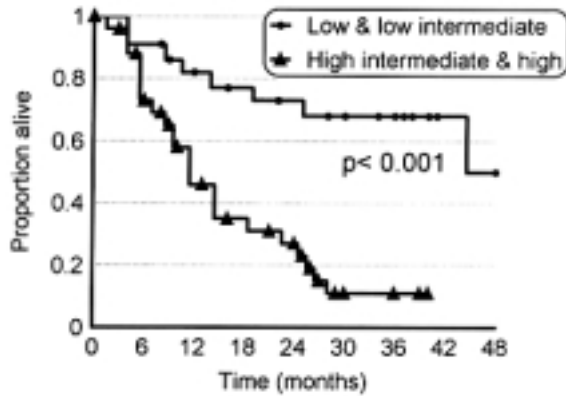


Fig. (5): Overall survival in 48 cases with high grade NHL according to IPI (International prognostic index).

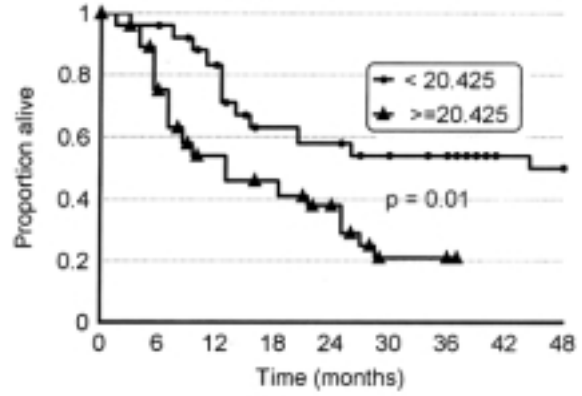


Fig. (6): Overall survival in 48 cases with high grade NHL according to Ki-67 labelling index.

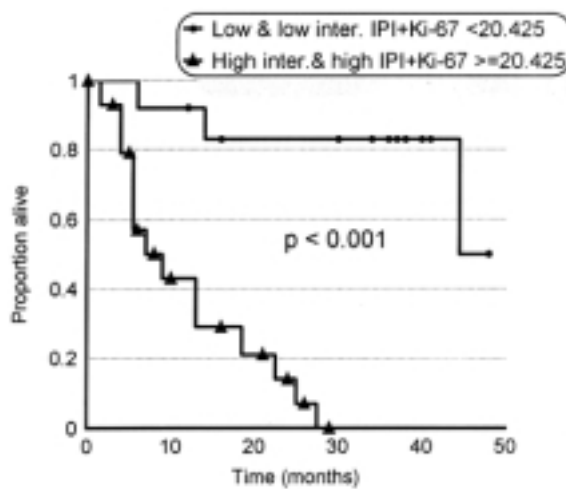


Fig. (7): Overall survival of patients with low and low intermediate IPI, low Ki-67 index compared with patients with high intermediate and high IPI, high Ki-67 index.

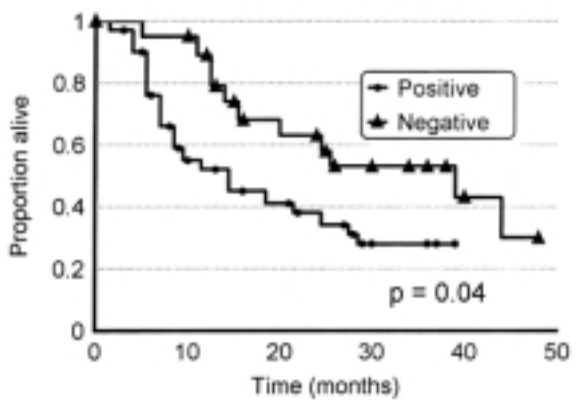


Fig. (8): Overall survival in 48 cases with high grade NHL according to Ki-67 (bone marrow).

## DISCUSSION

MIB-1 labelling index was estimated using the CAS-200 image analyzer in the tissue samples of 48 NHL cases. The MIB-1 index had a mean of  $23.4 \pm 18.9$  and a median of 20.425% with a range of 0-58%. Three cases (7.2%) were completely negative for Ki-67 immunostaining. This was explained by Lopez et al. [17], who stated that among the cycling cells antigen's expression is transiently minimal during GI phase, thus not all the Ki-67 negative cells are non-cycling cells. It is therefore; in accurate to identify the percentage of Ki-67 positive cells as the growth fraction as is commonly practised [6].

The median value of Ki-67 labelling index in the studied group is close to those of Hall et al. [11] and Sanchez et al. [21], but lower than that reported by Gerdes et al. [8] whose cut-off point was 26% and Houmand et al. [13] who used 45% Ki-67 positivity as the cut-off point in their studies.

This variation in results may be due to sampling errors and counting of non-neoplastic cells as there is a variable and often large, number of reactive proliferating cells other than the neoplastic population present in non-Hodgkin's lymphomas [10]. Careful morphological examination is required to establish a diagnosis of malignant lymphoma rather than the quantitation of nuclear Ki-67 immunoreactivity in isolation.

The question whether to study areas of maximum Ki-67 positivity or random field in typical tumour area is a subject of some debate [17]. In our study, random number table for selection of fields, counting a sufficient large number of cells and careful examination of hematoxylin and eosin stained serial sections were used to avoid false high results [1].

The relationship between the cell proliferation marker and clinical outcome has not been conclusively defined. In fact, a high Ki-67 index was correlated with a poor outcome with low-grade lymphoma [11], whereas for high-grade lymphoma, inconsistent data have been reported. In the study of Hall et al. [11], a very high index was correlated with a good survival, whereas Grogan et al. [9] reported contrasting findings, which were confirmed by Miller et al. [18], Mochen et al. [19] and Sanchez et al. [21].

High MIB-1 index ( $\geq 20.425\%$ ) was in this series associated with statistically significant shorter duration of CR ( $p = 0.01$ ) and overall survival ( $p = 0.01$ ) in univariate analysis. Moreover, multivariate analysis showed that MIB-1 labelling index provided predictive information on survival independent of other clinical variables included in IPI.

In our study, all the patients with high intermediate or high IPI who expressed high Ki-67 labelling index have been relapsed with poor overall survival as compared with the group of patients with low or low intermediate IPI and low Ki-67 index ( $p < 0.001$ ). These results, if confirmed in a larger study, suggest that Ki-67 labelling index should be added to the prognostic factors included within the IPI in patients with high-grade lymphoma as it could identify a sub-group of patients with a very poor prognosis when treated with conventional chemotherapy.

Indeed very little is known about Ki-67 reactivity in non-neoplastic conditions. Van Bockstaele et al. [22] could not detect any convincing Ki-67 positivity in the nuclei of normal BM cells. They evaluated the Ki-67 reactivity in samples of normal human BM in order to have an inherent control for proliferation. Although the BM is the most obvious natural example of proliferating cells yet they reported that cycling cells in normal human BM do not express detectable amount of Ki-67 nuclear antigen.

In this study normal BM was considered as the negative control during the immunostaining of BM sections with MIB-1. Because no Ki-67 positive cells were observed in normal BM samples used as negative control. BM smears or aspirate were considered positive when at least 1% of lymphoid cells showed a nuclear staining with MIB-1 monoclonal antibody. BM positivity for MIB-1 detected as nuclear brown staining of cells, was strongly correlated ( $p = 0.001$ ) with the median Ki-67 labelling index. Based on the fact that normal BM cells growing under-steady state conditions, showed no Ki-67 positivity in nuclei of normal BM samples, we were able to detect BM infiltration by detecting Ki-67 positivity in BM samples of patients who were not morphologically diagnosed to have BM involvement.

BM infiltration as detected by MIB-1 monoclonal antibody in BM samples was associated

with a trend, although not significant, toward decreased probability of achieving CR and a short duration of disease free survival. With statistically significant ( $p = 0.04$ ) shortened overall survival time in univariate but not in multivariate analysis.

Careful consideration is required in the assessment of the impact of the detection of BM infiltration by MIB-1 monoclonal antibody on disease outcome as during the study performed by Qiao et al. [20], they discovered in cancer colon and acute promyelocytic leukaemia (M3), a sub-population of apoptotic cells. This sub-population strongly expressed PCNA and Ki-67, suggesting that their specificity as proliferation markers may need re-assessment. Thus, including apoptotic markers in addition to exclude this sub-population would be appropriate.

In conclusion, Ki-67 immunostaining using the monoclonal antibody MIB-1 represents a simple, quantifiable and reproducible method that enables the histopathologists to define the tumour proliferation index. These data also prove that Ki-67 labelling index in lymphoid tissues is determinant factor in BM involvement. To our knowledge this is the first report to clarify this positive correlation. Furthermore, it would be prudent to include MIB-1 labelling index for predicting DFS and OS in addition to the clinical parameters that have already been recognized in patients with high-grade lymphoma.

Based on the fact that normal BM cells growing under-steady state conditions, showed no Ki-67 positivity in nuclei of normal BM samples, we were able to detect MB involvement by detecting MIB-1 positive cells in BM samples of patients who were not morphologically diagnosed to have BM infiltration. However, not all cases could be shifted to stage IV, because of the false positivity, which could be expressed by apoptotic sub-population of cells. We recommend using apoptotic markers in addition to exclude this subcategory in a larger study.

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