

The pattern of Ki-67 and bcl-2 expression in lymphoid malignancies

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Abstract

The International Working Formulation divides non-Hodgkin's lymphoma (NHL) into three grades: low, intermediate and high. This grading system implies rate of tumour growth and hence prognosis. Ki-67 antigen is a proliferation-related nuclear antigen and bcl-2 oncogene product is known to inhibit apoptosis. This study aimed to determine the pattern of expression of Ki-67 antigen and bcl-2 oncoprotein in various grades of NHL. Paraffin-embedded tissues from 42 cases of NHL (7 low, 15 intermediate, 20 high grade) were retrieved from the files of the Department of Pathology, University of Malaya. Ki-67 antigen and bcl-2 oncoprotein were detected using immunohistochemistry. The percentage of positively stained neoplastic cells was determined by semi-quantitative estimation and given scores ranging from 0 to 6. Partition chi square test demonstrated the association of Ki-67 antigen expression and histological grade ($p=0.007$). There was no significant difference in Ki-67 antigen expression between intermediate and high grade malignant lymphomas ($p=0.28$), whereas significant difference was demonstrated between low and intermediate/high grade tumours ($p=0.003$). Bcl-2 oncoprotein expression in the neoplastic cells varied widely within the three histological grades. Statistical analysis showed no association between the expression of bcl-2 oncoprotein and histological grade ($p=0.25$). Ki-67 immunostaining is therefore a useful adjunct to histological grading of NHL.

Key words: bcl-2, International Working Formulation, Ki-67, non-Hodgkin's lymphoma.

INTRODUCTION

Non-Hodgkin's lymphoma (NHL) encompasses a wide range of lymphoid malignancy which differ in clinical presentation and behaviour. Between the 1960s and early 1970s, a number of classification systems based on histomorphological features divided NHL into several entities. These included the Rappaport Classification, Kiel's Classification and Luke's Classification which led to much controversy and confusion between pathologists, and between pathologists and clinicians. In 1982, an international panel of haematopathologists proposed a classification system entitled "The Working Formulation for Clinical Usage" (IWF),¹ which divided NHL into three prognostic groups, namely low, intermediate and high grade. With some limitations, this formulation permitted users of the various morphological classifications to convert their respective entities into a common scheme with prognostic implication. The IWF is presently widely used in North America.

Gerdes *et al.* had shown that Ki-67 antigen is a proliferation-related nuclear antigen, expressed

during the G1, S, G2 and M phases of the cell cycle but not in resting (G0) phase cells.² Therefore, Ki-67 expression is being used to determine the growth fraction in neoplasms. Cell proliferation is an important contributing factor to tumour growth, hence, its potential relevance in prognosis.¹

Up-regulation of the bcl-2 oncogene was first detected in association with the t(14;18) chromosomal translocation in follicular lymphoma, originally considered the 'hallmark' of the neoplasm.⁴ It is now known that bcl-2 oncoprotein expression occur in several tumours and non-tumoral cells in the absence of t(14;18) translocation. Bcl-2 oncoprotein inhibits apoptosis, a programmed cell death, and is the counterpart of mitosis? It is widely viewed as a gene-directed activity in which its cellular products result in self destruction. Hence, bcl-2 oncoprotein confers the ability of prolonging the lifespan of cells, which may lead to tumour progression.⁶

This study aimed to correlate the proportion of tumour cells expressing bcl-2 oncoprotein

and growth fraction by Ki-67 antigen expression, with NHL of various prognostic grades by the IWF.

MATERIALS AND METHODS

Cases

42 cases of NHL of the lymph node, reviewed and confirmed histologically as 7 low, 15 intermediate and 20 high grade tumours were retrieved from the Department of Pathology, University of Malaya, Kuala Lumpur. The archival biopsy materials had all been formalin-fixed (10% buffered formalin) and paraffin-embedded. 4µm thick sections were cut and mounted on salinized slides for haematoxylin & eosin and immunohistochemical staining.

Immunohistochemical staining

Sections were pretreated with microwave heat in 0.01M citrate buffer, pH 6.0 for 20 minutes at 100°C with a Biorad microwave processor H2500. Immunostaining was performed with monoclonal antibodies to Ki-67 antigen (clone M722, Dako, Glostrup, Denmark) and bcl-2 oncoprotein (clone 124, Dako, Glostrup, Denmark), using the standard horse-radish peroxidase streptavidin-biotin technique (Biogenex, San Ramon, California) with diaminobenzidine as the chromogen. All reagent dilution and washings were performed in 0.01M phosphate-buffered saline, pH 7.6. Negative control sections were stained with the exclusion of the relevant antibody to rule out non-specific binding of the antibody. Sections of normal tonsils were stained in parallel during every run of the immunostaining process as the external control. Cells exhibiting brown nuclear staining were regarded as Ki-67 positive and those showing brown cytoplasmic staining were regarded as bcl-2 positive.

Assessment of immunoreactivity and scoring system

Neoplastic areas with highest Ki-67 antigen and bcl-2 oncoprotein expression were selected for assessment of immunoreactivity. The staining results were assessed with a semi-quantitative tissue estimation method: where the percentage of positively-stained neoplastic cells was initially estimated by the first author. This was then reassessed together with the second author. The percentage of reactivity was then translated into a scoring system with points ranging from 0 to 6 (Table 1), as represented in Fig. 1. In estimating this percentage of reactivity, small lymphocytes, endothelial cells, nuclear debris, cellular fragments and fibroblasts were excluded. Statistical analysis was performed on the results with Epi Info 6.0. A p-value of less than 0.05 was set as the level of significance for all the statistical findings. Score points which were more than 2 were regarded as indicative of a high percentage of reactivity.

RESULTS

Ki-67 immunostaining

Of the 42 cases stained, both the neoplastic and normal cells in six cases were non-reactive with Ki-67 antibody and hence were excluded from analysis. The percentage of tumour cells expressing Ki-67 antigen for the remaining 36 cases studied was as presented in Table 2. Low grade NHL exhibited a low percentage of Ki-67 reactivity (Score 0-1). In the intermediate and high grade NHL, a wide range of Ki-67 reactivity was observed. Statistical analysis using partition chi square test demonstrated an association between Ki-67 antigen expression and histological grade ($p=0.007$); with an increasing trend of Ki-67 antigen reactivity score as the histological grade increases (Fig. 2a). Further

TABLE 1: Scoring system for immunoreactivity of Ki-67 antigen and bcl-2 oncoprotein

Score	Percentage of reactivity in tumour cells
0	<1
1	1 and <5
2	5 and <25
3	25 and <50
4	50 and <75
5	75 and <95
6	95 and above

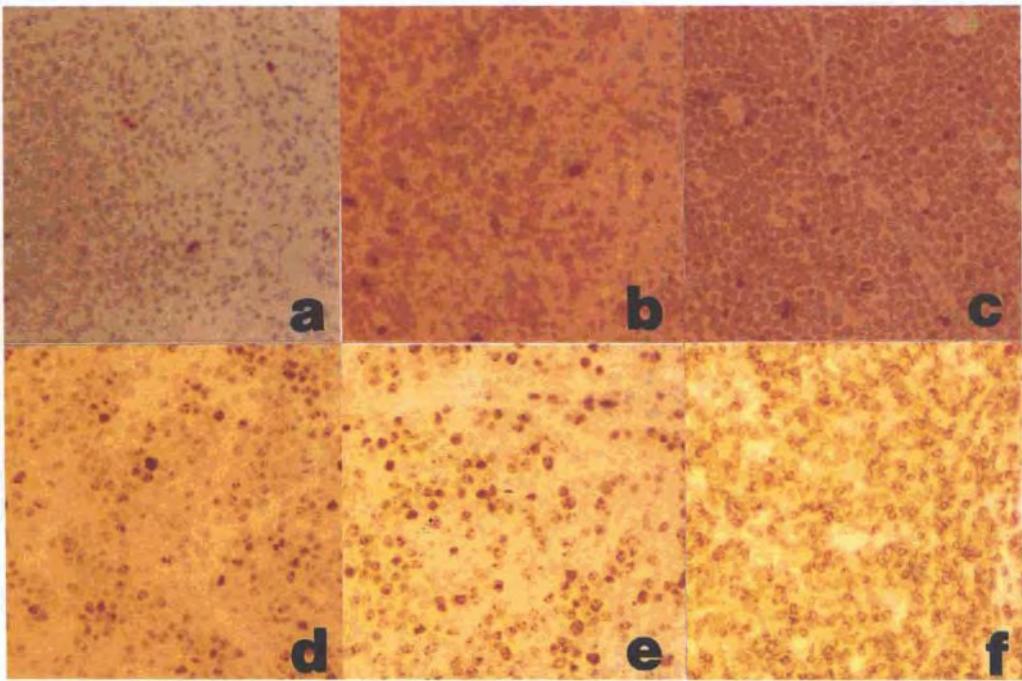


FIG.1: (a) Score point 1, immunostaining for Ki-67 antigen (X25). (b) Score point 2, immunostaining for Ki-67 antigen (X25). (c) Score point 3, immunostaining for bcl-2 oncoprotein (X25). (d) Score point 4, immunostaining for bcl-2 oncoprotein (X25). (e) Score point 5, immunostaining for Ki-67 antigen (X25). (f) Score point 6, immunostaining for bcl-2 oncoprotein (X25).

analysis showed no significant difference in Ki-67 antigen reactivity score between intermediate and high histological grade NHL ($p=0.28$), while it was significant between low and intermediate high histological grade ($p=0.003$) NHL.

Bcl-2 immunostaining

One case showed absence of immunoreactivity for bcl-2 and was excluded from analysis. Of the remaining 41 cases studied, it was observed that cases with a high percentage of immunoreactive

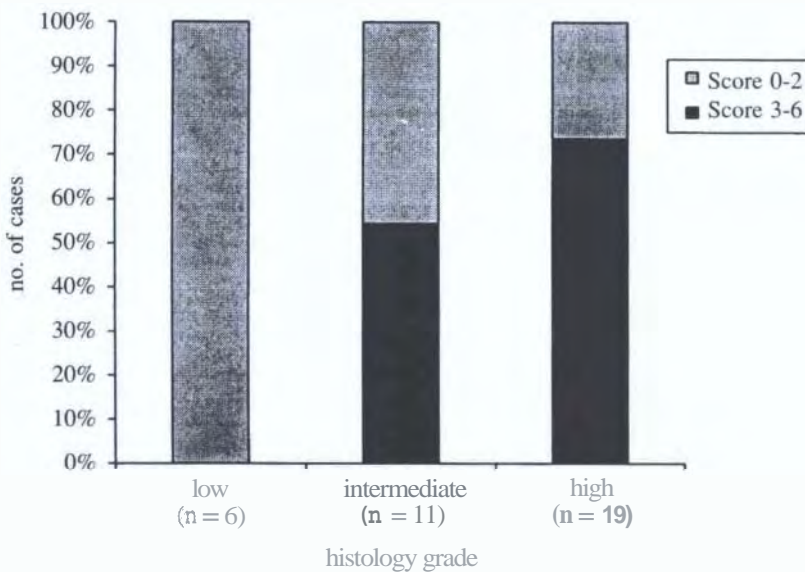


FIG.2(a): Overview of cases stained with Ki-67 antibody expressed in percentages against histological grading.

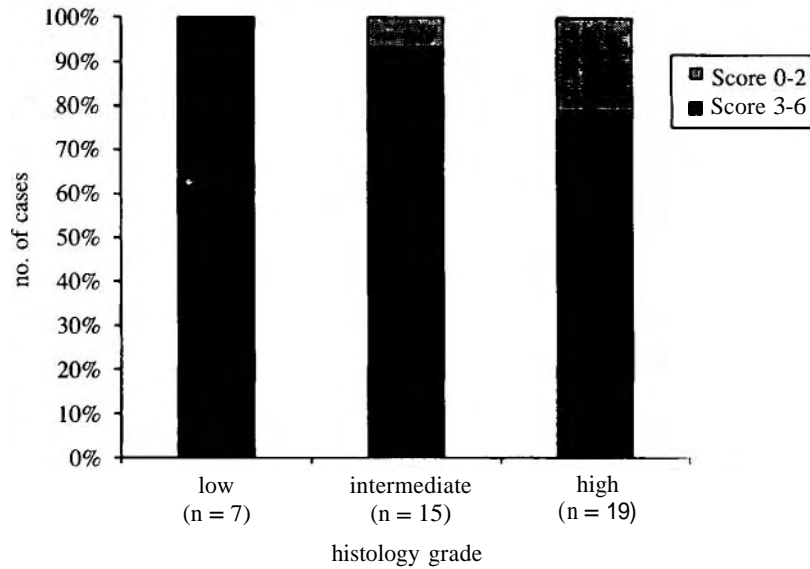


FIG. 2(b): Overview of cases stained with bcl-2 antibody expressed in percentages against histological grading.

neoplastic cells also showed more even and intense staining (Fig. 3a); whereas cases with a lower percentage of positively stained cells showed more variable intensity of staining from cell to cell (Fig. 3b). The results of bcl-2 staining are summarized in Table 2. There is a trend of decreasing reactivity score as the histological grade increases (Fig. 2b). However, there was no significant correlation between bcl-2 oncoprotein expression and histological grading

on statistical analysis ($p=0.25$). There was also no association between Ki-67 antigen and bcl-2 oncoprotein expression.

DISCUSSION

The results of this study showed correlation between proliferative reactivity by Ki-67 antigen expression and histological grading of NHL, which concur with previous studies. Weiss *et al*⁸ demonstrated that the proliferative rate as

TABLE 2: Distribution of Ki-67 antigen and bcl-2 oncoprotein scores in the three grades of non-Hodgkin's lymphoma

<i>Ki-67 antigen (36 cases)</i>							
Grade\Score	No. of cases						
	0	1	2	3	4	5	6
Low (n=6)	2	4	0	0	0	0	0
Intermediate (n=11)	2	0	3	4	0	1	1
High (n=19)	1	1	3	2	2	9	1

<i>bcl-2 oncoprotein (41 cases)</i>							
Grade\Score	No. of cases						
	0	1	2	3	4	5	6
Low (n=7)	0	0	0	0	1	3	3
Intermediate (n=15)	0	1	0	2	3	6	3
High (n=19)	2	0	2	1	4	5	5

represented by Ki-67 expression generally paralleled the **subtypes** of NHL and grades of malignancy in the IWF. Schwartz *et al*⁹ reported **significant** difference in Ki-67 antigen expression in the three grades of NHL.

In our study, there was a wide range of Ki-67 antigen reactivity in intermediate and high grade NHL, from **<1%** to 100% and was not significantly different. Schwartz *et al*⁹ also reported similar observations. The difference in Ki-67 index between low and intermediate grade NHL was more significant (**p<0.00001**) than between intermediate and high grade NHL (**p<0.05**). In a study by Houmand *et al*¹⁰, the authors reported significant agreement between malignancy grade in the IWF (**low/intermediate** versus high grade) and Ki-67 index.

Several studies have analysed the clinical outcome of patients in relation to Ki-67 reactivity scores. Gerdes *et al*¹¹ reported that patients with high Ki-67 indices (**>69%**) had bad prognosis. In a study of diffuse large cell lymphoma, Grogan *et al*¹² concluded that high proliferative reactivity (Ki-67 index **>60%**) correlated with a poor clinical outcome. They suggested the potential role of Ki-67 as a prognostic indicator in predicting survival of patients. A few studies have raised the possibility of using Ki-67 immunostaining to assess the potential therapeutic benefits of anti-proliferative **drugs**.¹³

Unlike Leoncini *et al*¹⁴ who reported decreasing percentage of bcl-2 reactivity of neoplastic cells in NHL as histological grade increases (low **62.5%**, intermediate **5%** and high grade **0%**), our study did not show correlation of bcl-2 oncoprotein expression with histological grading in NHL. All cases were bcl-2 positive of varying density and intensity in our study, whereas most of the other **studies**^{15,16} reported a proportion of bcl-2 negative cases in NHL. The possible reasons for this discrepancy could be that there was no antigen retrieval technique such as microwave heating being employed in the earlier studies, and a different clone of antibody was used (monoclonal antibody to **bcl-2** oncoprotein, clone **100**, supplied by Dr. **D.Y.Mason**). Moreover, **there** are differences in the type of materials used by these studies, as some of the bcl-2 negative cases were **extra-nodal** lymphomas, which may contribute to a different bcl-2 oncoprotein expression from the present study of nodal **lymphomas**.¹⁵

There was no correlation in Ki-67 antigen and bcl-2 oncoprotein expression in this study. However, Schena *et al*¹⁷ found an inverse relationship in Ki-67 antigen and bcl-2

oncoprotein expression in B-Chronic **Lymphocytic** Leukaemia (B-CLL) cells from an *in vitro* study of 183 B-CLL cells. Bcl-2 oncoprotein is down-regulated in cells in proliferation. This renders it useful in distinguishing reactive **lymphoid** hyperplasia from follicular lymphoma. Its presence in normal and various neoplastic cells suggests its role in the pathophysiology of human **tissue**.¹⁸ It may be a useful clinical marker in many malignancies once its role is more completely **understood**.¹⁸

Ki-67 immunostaining is an established technique in **determining** the growth fraction of neoplasms. This study concluded that Ki-67 antigen expression in NHL correlates with histological grading according to the International Working Formulation. It can add to the information provided by conventional histological assessment of NHL. This study found no correlation between bcl-2 oncoprotein expression and histological grading in NHL.

ACKNOWLEDGEMENTS

The authors thank Mr. **Ong Leong Huat** of Department of Social and Preventive Medicine, Faculty of Medicine, University of Malaya for his assistance in statistical analysis. This study was supported by the Malaysian Ministry of Science, Technology and Environment **R&D** grant IRPA 06-02-03-0176 (1996).

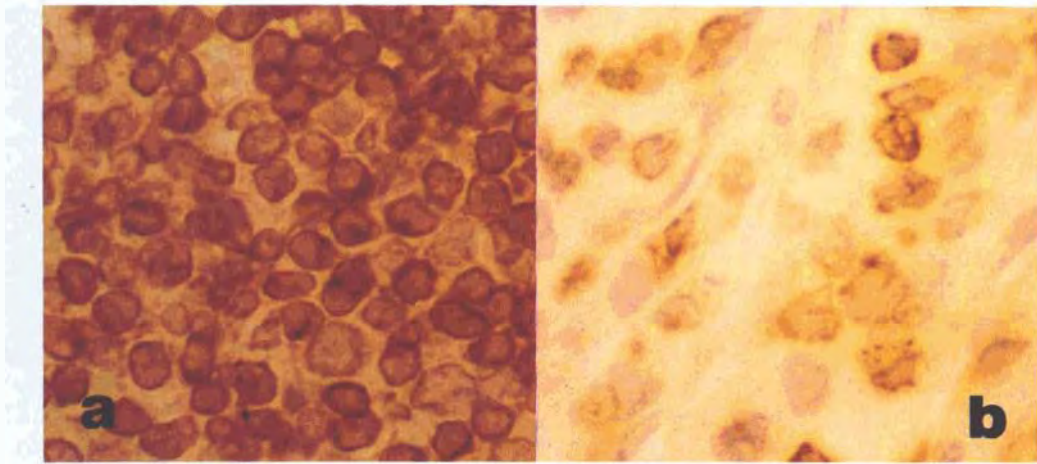


FIG. 3: (a) Low grade NHL: Neoplastic cells showing intense cytoplasmic staining with antibody to bcl-2 oncoprotein (Immunostaining X260). (b) Intermediate grade NHL: Neoplastic cells showing variable staining intensity with antibody to bcl-2 oncoprotein (Immunostaining X260).

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