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Evaluation of MIB-1 and p53 Overexpression as Risk Factors in Large Cell Non-Hodgkin Lymphoma in Adults

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ABSTRACT

Background: Improvement of current results of therapy for large cell non-Hodgkin lymphoma patients can be achieved by optimization of initial treatment or application of risk-adapted therapy. The international prognostic index (IPI), introduced to identify high-risk patients, was recently criticized because it was based on clinical risk factors only, ignoring important tumor molecular risk factors and it fails to identify a sector of high-risk patients, who ultimately relapse.

Objective: The aim of this study is to evaluate the value of two tumor biomarkers: MIB-1 and p53 as potential risk factors in diffuse large cell lymphoma. MIB-1 measures tumor cell proliferation, whereas p53 is related to tumor progression and response to chemotherapy.

Patients and Methods: The study was done on 69 adult patients with diffuse large cell NHL (58 B-phenotype and 11 T-phenotype). Clinical risk assessment was determined by the IPI and patients with a score of 3 or more were considered high-risk. Expression of MIB-1 and p53 was determined by immunohistochemistry and nuclear staining was quantitated by image analysis. Immunoexpression was considered high for MIB-1 nuclear count ≥50% and p53 counts ≥20%. Evaluation included both response to chemotherapy (mostly CHOP), as well as 2-year overall survival analysis.

Results: The IPI was the only clinical variable which had a significant impact on survival. Overexpression of both MIB-1 and p53 was associated with poor response to treatment, as well as unfavorable survival. Combined risk factor analysis revealed that only MIB-1 was an independent variable. MIB-1 could also identify some high-risk patients previously categorized in the IPI low-risk group.

Conclusions: MIB-1 is an independent biologic risk factor for large cell NHL. In order to optimize risk assessment of these patients, it is recommended to construct a new prognostic index by adding MIB-1 overexpression to the other clinical factors of standard IPI. This may allow better identification of high-risk patients and help to guide planning of effective initial treatment.

Key Words: *NHL – MIB-1 – p53 – CHOP – Risk factors.*

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INTRODUCTION

Diffuse large cell lymphoma is the most common diagnostic category of non-Hodgkin's lymphoma (NHL), contributing 54.55% of cases in Egyptian patients **[1]** and 41% in Western series **[2]**. It is a heterogeneous group including three main histologic types, namely diffuse large B-cell lymphoma, peripheral T-cell lymphoma and anaplastic large-cell lymphoma **[3]**. Important common features of this group are their clinical aggressiveness and their potential of curability with combination chemotherapy. Thus, it was possible to cure about 41% to 54% of patients by the standard CHOP regimen which was widely used internationally over the past two decades **[2,4]**. In spite the improvement of CHOP regimen by adding Rituximab (R-CHOP regimen), which has led to a marked improvement in survival **[5,6]**, still the therapeutic outcome needs substantial improvement.

Two strategies may be followed to improve the results of treatment of NHL, namely, modify

the protocols of initial (front-line) therapy **[5]**, or apply a risk-adapted therapy in which high-risk patients are identified for more intensive therapy **[2,4]**. The international prognostic index (IPI) was introduced in 1993 for risk stratification of NHL patients **[7]**. This index was based on 5 parameters which reflect the general condition of patients and tumor burden, but did not include biomarkers which directly reflect tumor biologic behavior.

Tumor molecular markers such as MIB-1 **[8]** and p53 **[9]** are potentially valuable risk factors in NHL patients which may influence treatment outcome. Thus, MIB-1 measures tumor cell proliferation which is directly related to tumor proliferation and relapse **[10,11]**, whereas the tumor suppressive gene p53 plays a major role in lymphoma progression **[11]**, chemotherapy response and drug resistance **[12]**.

The aim of the present study is to retrospectively assess the value of MIB-1 and p53 immunoexpression as risk factors in diffuse large-cell NHL. Evaluation is determined by both chemotherapy response, as well as survival analysis. The long-term objective is to use these biomarkers as a guideline for therapy.

PATIENTS AND METHODS

The present retrospective study was based upon 69 Egyptian patients with diffuse large-cell non-Hodgkin's Lymphomas treated at the NCI, Cairo University, during the years 1998 and 1999. The eligibility criteria to include in the study were: Adult patients aged 18 years and above, previously untreated, previous pathologic diagnosis of diffuse large-cell NHL, available paraffin blocks, assessed IPI and compliance of patients to treatment.

The previous pathologic diagnosis was revised and tumors were classified according to the WHO system **[3]**. Our series included 58 patients of large B-cell lymphoma, 8 patients with peripheral T-cell lymphoma and 3 patients with anaplastic large T-cell lymphoma. Clinical records were reviewed to obtain data on international prognostic index category **[7]**, chemotherapy regimen, treatment response and survival data.

Immunohistochemical methods were done on viable areas of tumor sections previously marked on the slides. Standard immuohistochemical methods were adopted **[10]** and tissue sections were routinely microwave-treated to unmask the epitopes of the antigen **[13]**. For lymphoma immunophenotyping, the following primary monoclonal antibodies were used: CD20, CD3 and CD30 (DAKO, USA). For tumor markers, mouse antihuman p53 and mouse antihuman ki-67 (MIB-1) were used (DAKO, USA). The following universals were used, namely, ultravision detection system (Labvision, USA) and LSAB-2 system (DAKO, USA). Diaminobenzidine (DAB) was used as a chromogen since it allows a permanent preparation. For MIB-1 immunostain, the autostainer TFT5030f (DAKO, USA) was employed. The results of immunohistochemistry were interpreted without knowledge of the clinical or pathologic information. Interpretation was limited to areas of strongest reactivity (hot spots). Nuclear immunoexpression was quantitated by image analysis using CAS-200 cell analysis system (Becton and Dickenson, Elmhurst, Illinois, USA). For each tumor section, a total of 1000 tumor cell nuclei were counted and the frequency of stained nuclei determined. For p53, nuclear stain count of 20% (Fig. 4) or more was considered overexpression **[14]**, whereas for MIB-1 nuclear staining of 50% or more was considered over-expression. The latter cutoff value was found optimal after multiple cutoff value survival analysis **[15]**.

The statistical analysis was done using an IBM compatible computer and STATISTICA for MS Windows 98 statistical package. Standard analytical statistical methods were used **[16,17]**. Thus, descriptive statistics were presented as mean and standard deviation, median and percentage. Analytical tests used included unpaired student *t* test (two-sided for comparing two groups) and analysis of variance (F test) for comparing more than two groups. Non-parametric testing was also used to confirm significance. For enumeration data, the chi square test for contingency table analysis and Fisher's exact test for 2x2 tables were used. The overall 2-year survival was estimated by the Kaplan Meier's method **[18]** and comparison of survival curves was done by the log rank test.

A significance level of 0.05 was used throughout all statistical tests in the study.

RESULTS

The present series included 38 males and 31 females, a sex ratio of 1.2. The mean age was 43.1 years (median 44.0 years). Fifty-three patients had nodal disease (76.8%) and 16 patients were primary extra nodal (23.2%). The classification of cases according to the international prognostic index is presented in table (1). For statistical analysis, the low and low-intermediate groups were lumped together as low IPI, whereas, the high-intermediate and high groups were lumped together as high IPI.

Table (1): International prognostic index of patients with large cell NHL, according to Shipp *et al*., 1993 (n=69).

Index	Score	No.	%
Low	0 or 1	28	40.6
Low intermediate	2	18	26.1
High intermediate	3	19	27.5
High	4 or 5	4	5.8
Total		69	100%

The majority of patients (55 cases: 79.7%) were treated by the standard CHOP regimen (Cyclophosphamide, Doxorubicin, Vincristine and Prednisone), whereas, 14 patients (20.3%) received other anthracyclin-based regimens. The response to chemotherapy was as follows: 50 patients had complete response (CR: 72.5%), 14 patients had partial response (PR:20.3%) and 5 patients suffered progressive disease (PD: 7.2%). The 2-year overall survival rate of this series was 34.3%. The mean duration of survival rate was 14.2 months. In table (2), the 2-year overall survival rates of the different clinical prognostic factors are presented, with significantly different survival rate for the high IPI patients (p=0.001, Fig. 1).

The 2-year overall survival rate for the 58 patients with B-phenotype was 62% whereas for peripheral T-phenotype it was 45%. The difference was not statistically significant (p=0.2). It is noteworthy that the peripheral T-phenotype group included only 8 patients, after exclusion of 3 cases of anaplastic large T-cell NHL.

The MIB-1 nuclear expression rate showed a mean value of 48.5%, with a median of 45.0%, a minimal value of 5% and maximal value of 83%. Nuclear staining of 50% and more was considered high expression (Fig. 2) and 33 patients (47.8%) had tumors with high expression. The response to chemotherapy was inversely related to MIB-1 labeling. Thus, in 50 patients with complete response, the mean MIB-1 count was 41.7±15.9, whereas, in 19 patients without complete remission, the mean was 62.2±10.5. The difference was statistically significant (p=0.0001). The relation of MIB-1 expression to the 2-year overall survival rate showed a statistically significant difference in survivals (Fig. 3), with p value of 0.0001.

A total of 23 cases (33.3%) had overexpression of p53 nuclear immunoreactivity. CR rate was inversely related to p53 expression. Thus, 50 patients with CR to chemotherapy had a 26% of p53 overexpression, whereas in 19 without CR (PR: 20.3 and PD: 7.2%), p53 overexpression was 52.6% (p=0.04). The overall survival rate was also related to p53 overexpression, thus the 2-year overall survival rate was 13.5% in p53 positive tumors compared to 60.1% in p53 low expression tumors (p=0.0001, fig. 5).

In order to determine any possible dependence or interaction among the prognostic variables, a multivariate analysis of the variables was done. Since the IPI was the only clinical variable which demonstrated a significant relation with survival, low and high IPI groups were stratified according to MIB-1 and p53 expression (Table 3). This analysis demonstrated that a high MIB-1 rate was an independent variable, since it allowed the subclassification of both low and high IPI into other prognostically different subgroups. Conversely, p53 overexpression proved to be a dependent variable, since the high IPI group could not be subclassified into two prognostically different subgroups according to p53 overexpression.

Table (2): 2-year OS of lymphoma patients with different clinical prognostic factors (n=69).

Prognostic factor	Number	OS%	Survival (*p*-value)
Age (Below 40)	21	39.1	0.39
Age (Above 40)	48	48.7	
Males	38	48.6	0.66
Females	31	41.2	
Low IPI	46	63.4	0.0001
High IPI	23	6.7	
Nodal	53	48.7	0.08
Extranodal	16	35.1	
CHOP	55	42.8	0.42
Others	14	55.3	

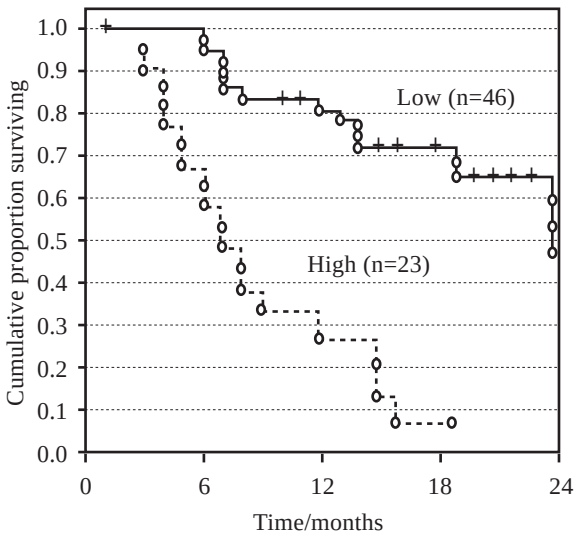

Fig. (1): 2-year overall survival in relation to low (L) and high (H) IPI (n=69, *p*=0.001).

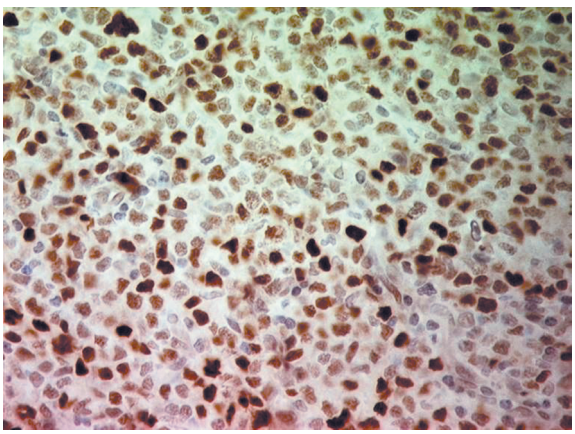

Fig. (2): Overexpression of MIB-1 nuclear immunoreactivity of more than 50%.

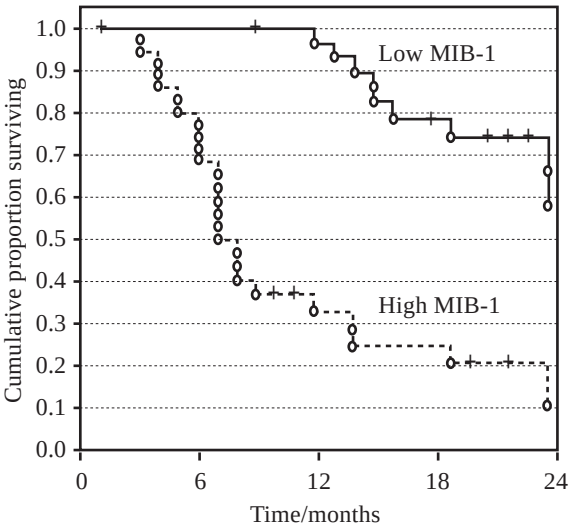

Fig. (3): 2-year overall survival in relation to low and high MIB-1 labeling rate with cutoff value of 50% (n=69, *p*=0.0001).

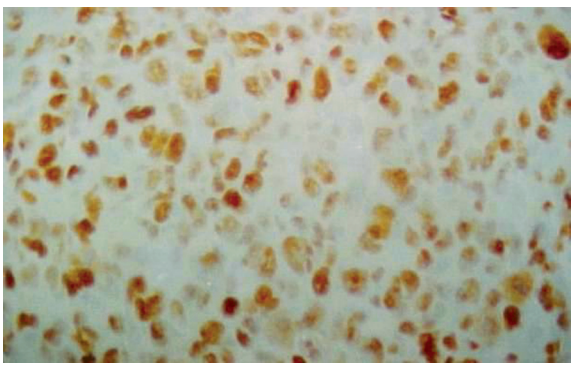

Fig. (4): Overexpression of p53 with nuclear immunoreactivity above 20%.

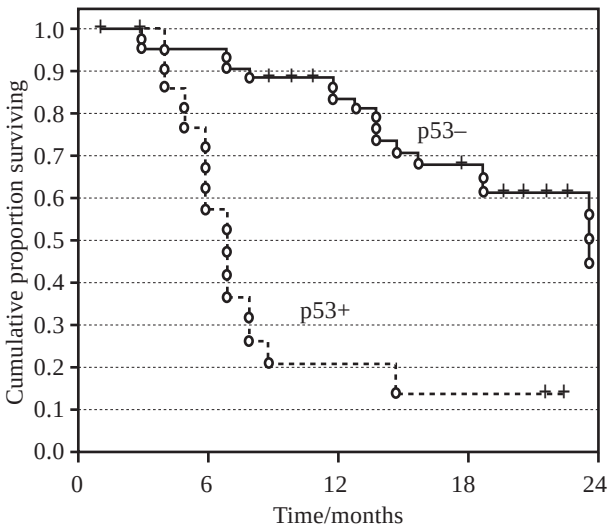

Fig. (5): 2-years overall survival of lymphoma patients in relation to p53 over expression (p53 +) with a cutoff value=20% (n=69, *p*=0.001).

Table (3): 2-year overall survival rate of low and high risk IPI subclassified according to low and high MIB-1 labeling rates, as well as positive and negative p53 (n=69).

IPI	Marker	No.	OS	*p*-value
Low risk (46 cases)	MIB-1 <50	39	70.9	0.002
	MI-1 >50	7	19.5	
	P53 Negative	37	71.50	0.004
	P53 Positive	9	25.5	
High risk (23 cases)	MIB-1 <50	9	18.2	0.0007
	MIB-1 >50	14	0.0	
	P53 Negative	9	13.9	0.14
	P53 Positive	14	0.0	

DISCUSSION

In the present study, the IPI was the only clinical variable which was significantly related to prognosis. This is in agreement with the original report of Ship **[7]** which was later confirmed by other investigators **[5]**. Moreover, immunophenotyping did not show significant relation with survival. Thus, although the 2-year overall survival of peripheral T-cell lymphoma was lower (45%) than that of B-phenotype (62%), the difference was not statistically significant. This result is contrary to other reports which have emphasized the unfavorable prognosis of peripheral T-cell lymphoma **[9]**. The small number of peripheral T-cell lymphoma in our series (only 8 patients) may account for this difference.

The median MIB-1 labeling rate in the present study was 45%. The previously reported values in the literature varied from 15% **[20]** to 50% **[8]**. This variability could be due to variations in the material of study, classification system and immunohistochemical methods. Thus, some reports included all types of NHL **[21]**, whereas other reports were restricted to large cell lymphoma **[22]**. Most reports adopted the Working Formulation classification, but others used the Kiel classification **[23]**. Some reports were based on frozen section studies **[8,24]** but others were based on paraffin sections **[20,22]**. The interpretation of immunohistochemical stain also creates an additional problem. Thus, in view of the heterogeneity of staining of tumor section **[10]** a problem arises whether to count areas of maximal positivity (hot areas) or to count random fields **[25]**. The former approach (which was adopted in our study) yields high counts over 40% **[22]**, whereas, random-field counting yields counts as low as 15% **[20]**. So, a geart discussion is needed to standardize immunohistochemical and scoring methods **[26]**. Moreover, in risk factor assessment analysis, studies must be restricted to one histologic type of lymphoma **[3]** or a group of histologic types of similar biologic behavior **[5]** such as the indolent, aggressive and highly aggressive groups.

Regarding our patients, the response to chemotherapy was inversely related to MIB-1 labeling rate, with fewer tendencies to complete response with high MIB-1 labeling. Also, MIB-1 overexpression was associated with significant reduction in the 2-year overall survival rate. This result is in agreement with other previous reports **[8,24]**. Moreover, in the present report, MIB-1 could identify 7 high risk patients among 46 patients classified as low-risk by the IPI.

Overexpression of p53 was encountered in 33.3% of cases in our study and was associated with poor chemotherapy response. The 2-year survival rate was also significantly lower (13.5%) with p53 overexpression than among those with negative p53 expression (60.1%). This result is in agreement with other investigators **[27,28]** but contradicts with Kramer *et al* **[29]**. The poor survival results associated with p53 overexpression is explained by some basic facts of wild p53 function, which is inhibitory to the cell cycle and is needed for chemotherapy action **[30]**. However, the present inverstigation suggests that p53 is a dependent risk factor.

The management of patients with NHL is a complex endeavor. More than 30 different subtypes are recognized within the WHO classification **[3,30]** and in addition, marked heterogeneity also exists within each subtype. For this reason, the CHOP regimen was only successful in curing less than 50% of patients with large cell NHL **[2,4]**, an achievement generally considered unsatisfactory. Since an adequate initial (front-line) therapy is essential to accomplish maximal curability of cancer patients, two strategies were introduced to improve the results of

treatment of patients with large cell NHL **[1,2,5]**. The first strategy is to improve the present initial treatment protocol by adding new more effective drugs and the second strategy is to apply a risk-adapted therapy in which high-risk patients are identified for more intensive initial therapy. An example of the first strategy is the addition of the monoclonal antibody Rituximab to CHOP chemotherapy (R-CHOP) which has led to a marked improvement in survival to up 70% **[5,6]** in patients with large B-cell lymphoma. At present, R-CHOP is the standard treatment for this disease in developed countries, but its high cost is an obstacle to its general use in developing countries.

The optimal use of risk (or prognostic) factors is essential to apply a successful risk-adapted therapy in patients with NHL. The first system to be used is the Ann Arbor staging system **[31,32]** originally developed for Hodgkin's lymphoma, but has proven to be much less useful in NHL **[33]**. At present, the only use of Ann Arbor staging is to identify a small group (only 10%) of localized disease (stage I and II non-bulky) for combined modality treatment, namely R-CHOP and involved field radiation **[32,34]**. The international prognostic index (IPI), developed in 1993 **[7]**, was based on five clinical risk-factors, namely age >60 years, performance status >2, lactic dehydrogenase above normal, stages III and IV and extra nodal involvement of more than one site. The IPI has been recently subjected to marked criticism since it does not include biologic risk factors of the tumor, besides it gives equal weight to the risk factors in calculating the total risk score **[35,36]**. The main defect of the IPI is its limited ability to identify patients with a very poor outcome in the IPI low risk groups **[35]**. Retrospective analysis with redistribution of the IPI factors into a revised IPI (R-IPI) did not solve this problem **[36]**.

Thus, molecular tumor markers are important potential risk factors for large cell NHL. In the present study, high tumor cell proliferation, determined by MIB-1 over-expression using immunohistochemical methods, was found to be an independent risk factor. Moreover, MIB-1 could identify high-risk patients in the IPI low-risk group. These findings are also supported by others **[10,11]**. Gene expression profiling using DNA microarrays has also been advocated to identify high-risk patients with large cell lymphoma **[35]**. It was possible by this technology to identify two distinct forms of large B-cell lymphoma (LBCL), namely germinal center LBCL with favorable prognosis and activated LBCL with unfavorable outcome **[38]**. In addition, DNA microarray research may allow the development of more targeted therapy in the future based on genetic profile **[35,34]**. Based on findings from gene expression profiling, immunohistochemistry using tissue microarrays, has been used for a limited number of gene products (CD10, bcl6, MUM1 and cyclin D2) as prognostic markers **[39]**.

In conclusion, MIB-1 over-expression is an independent risk-factor in patients with large cell NHL associated with both poor response to treatment and unfavorable survival. It could be added to the other clinical risk factors of the international prognostic index to create a new IPI, which will, hopefully, help in better prediction for patient risk, taking into consideration their relative risk (hazard ratio) in calculating the total risk score.

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