

De Novo Nodal Diffuse Large B-Cell Lymphoma: Identification of Biologic Prognostic Factors

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ABSTRACT

Background: Diffuse large B-cell Lymphoma (DLBCL) represents the most frequent type of non-Hodgkin lymphoma (NHL). Although combination chemotherapy has improved the outcome, long-term cure is now possible for approximately 50% of all patients, making the search for parameters identifying patients at high risk particularly needed. The presence of bcl-2 gene rearrangement in de novo DLBCL suggests a possible follicle center cell origin and perhaps a distinct clinical behavior. This study investigated the frequency and prognostic significance of t(14;18) translocation and bcl-2 protein overexpression in a cohort of patients with de novo nodal DLBCL who were uniformly evaluated and treated.

Material and Methods: A total of 40 patients with de novo nodal DLBCL treated at National Cancer Institute (NCI), Cairo University were investigated. Formalin-fixed, paraffin-embedded sections were analyzed for: 1) bcl-2 gene rearrangement including major break point region (mbr) and minor cluster region (mcr) by polymerase chain reaction (PCR), and 2) bcl-2 protein expression by immunohistochemistry using Dako 124 clone. Results were correlated with the clinical features and subsequent clinical course.

Results: Bcl-2 gene rearrangement was detected in 8 cases (20%), 2 cases at mbr, and 6 cases at mcr. Bcl-2 protein (>10%) was expressed in 24 cases (60%), irrespective of the presence of t(14;18) translocation. The t(14;18), and bcl-2 protein overexpression were more frequently associated with failure to achieve a complete response to therapy ($p=0.008$, and 0.04 , respectively). DLBCL patients with t(14;18), and bcl-2 protein expression had a significantly reduced 5-year disease free survival ($p=0.04$, and 0.01 , respectively).

Conclusion: The t(14;18) translocation, and bcl-2 protein expression define a group of DLBCL patients with a poor prognosis, and could be used to tailor treatment, and to identify candidates for therapeutic approaches. Geographic differences in t(14;18) may be related to the difference in distribution of bcl-2 breakpoints.

Key Words: DLBCL - Prognosis - t(14;18) - bcl-2.

INTRODUCTION

Diffuse large B-cell lymphoma is a generic term for a clinically and biologically heterogeneous group of tumors, comprising 30-40% of newly diagnosed NHL in Western countries [1]. In Egypt it constitutes 49% of NHL cases at National Cancer Institute Pathology Registry [2]. Approximately half of all patients can be cured by conventional chemotherapy regimens. The remainder have tumors that are either refractory to currently available treatment or have a relapse after a period of remission, and most of these patients will die of the disease. It is possible that alternative therapeutic strategies such as the use of antibody and chemotherapy combinations or more intensive primary therapy with stem cell rescue may be effective in some of these patients. It is therefore important to be able to identify these patients at presentation to allow effective trials to be designed.

The formulation of an International Prognostic Index (IPI) has provided a widely accepted set of criteria to predict the evolution of aggressive lymphomas [3,4]. IPI takes into account factors that are mostly linked to the patient's characteristics or to the disease extension and growth including age, lactate dehydrogenase level, performance status, clinical stage, and number of extranodal sites. However, one limitation of this prediction strategy is that IPI does not encompass molecular abnormalities of tumor cells, which may play a critical role in determining profoundly different clinical outcomes in patients within the same group defined by IPI. Also patients defined as IPI high risk include elderly patients, or patients with poor performance status who may be unable to tolerate a more aggressive treatment.

Gene expression studies using cDNA microarrays have identified molecularly distinct subgroups in DLBCL. However, because this technology is still expensive and not generally available, a more widely available method is needed in routine clinical practice [5].

The B-cell leukemia-lymphoma 2 (*bcl-2*) gene was initially discovered by virtue of its association with the t(14;18) chromosomal translocation in which the *bcl-2* gene at 18q21 is juxtaposed with the Ig heavy chain locus at 14q32, resulting in deregulation of transcription of *bcl-2* protein [6]. The cytogenetic abnormality t(14;18) is found in a variable number (10-40%) of DLBCL [7]. It has been suggested that there are differences in the frequency of the t(14;18) which might point to a real underlying biologic behavior according to geographic factors [8]. Also previous studies of its clinical significance have been hampered by patient selection, non-uniform treatment strategies, variable methodology, detection only of mbr, and the inclusion of patients with antecedent follicular lymphoma. Thus the purpose of this study was to determine the frequency and clinical utility of *bcl-2* gene rearrangement (both major breakpoint region, and minor cluster region) and *bcl-2* protein expression in a carefully selected cohort of Egyptian patients with de novo nodal DLBCL to improve the risk stratification of patients at presentation.

MATERIAL AND METHODS

This study included 40 cases of nodal diffuse large B-cell lymphoma diagnosed between 1998 and 2003 at National Cancer Institute, Cairo University. Eligibility criteria were adequate histologic material for analysis, available clinical and follow-up data, no prior or concomitant history of follicular lymphoma, and no follicular structures were seen morphologically or by immunostaining for follicular dendritic cells.

In all cases the diagnosis was confirmed by histopathologic review and by positivity of tumor cells for B-cell marker CD20. Classification was done according to the World Health Organization classification [1].

Immunohistochemistry:

Five micron paraffin sections were dewaxed in xylene and alcohol and then microwaved for 10min in antigen retrieval solution (0.01M

citrate buffer, pH 6.0). After cooling, they were immunostained with streptavidin-biotin peroxidase detection system (Dako, Denmark) using diaminobenzidine as a chromogen. Antibodies were used against *bcl-2* (clone 124, Dako), CD20 (clone L26, Dako), and CD21 (clone 1F8, Dako).

Bcl-2 was considered positive only when there was cytoplasmic staining in >10% of neoplastic cells [9]. Normal human lymph node served as a positive control. For negative controls, the primary antibody was omitted and replaced with normal serum.

t(14;18) (bcl-2) Gene Rearrangements:

DNA Extraction: Three 25µm paraffin sections were deparaffinized in xylene, and the fragments were recovered by centrifugation. They were washed in absolute ethanol. The pellet was incubated overnight at 40°C with 200µg/ml proteinase K, 50mM Tris-HCL pH 8.3, 0.5% Tween 20, 1mM EDTA. The proteinase K was inactivated by incubation for 10min at 95°C. Centrifugation was carried out and the supernatant was transferred into a new tube. Phenol-chloroform extraction and ethanol precipitation was done [10]. DNA concentration and purity was estimated by agarose gel electrophoresis and spectrophotometry. The quality of DNA samples was further checked by PCR amplification of a target DNA within a β-globin gene fragment.

Polymerase Chain Reaction: PCR detection for the mbr-JH and mcr-JH rearrangements of the t(14;18) translocation was performed in 50µL of final volume using 1-1.5µg of genomic DNA, 1µM of each oligonucleotide primer (Table 1), 25µL PCR master mix (200µM dNTPs, 10mM Tris-HCL pH 8.8, 50mM KCL, 2mM MgCL2, 0.01% gelatin, IU Taq polymerase of Qiagen Operon). Nested PCR reactions were performed as described by Gribben et al. [11]. The original amplified product was reamplified by a second PCR reaction using a second set of oligonucleotide primers internal to the oligonucleotides used for the initial amplification.

The first PCR reaction consisted of 25 cycles that included 1min at 94°C, 1min at 55°C for mbr or 58°C for mcr, and 1 min at 72°C, followed by 10min at 72°C for tailing. The second round of PCR was performed on 5µL

of the first product and consisted of 30 cycles that included 1min at 94°C, 1min at 58°C, and 1min at 72°C, followed by 10min at 72°C for tailing (Robocycler gradient 96, Stratagene). PCR products were analyzed by 2% agarose gel electrophoresis. Specific PCR products give bands at 150-200bp for mbr-JH and at 200-300bp for mcr-JH. Each experiment included the clinical samples, a positive control (known positive case of follicular NHL), and a negative control DNA. These control reactions detect PCR product contamination, avoid false negativity caused by suboptimal PCR efficiency, and standardize the variation in PCR efficiency.

Statistics:

Complete response to therapy was defined as complete disappearance of all measurable tumor for at least 3 month. Partial remission required at least 50% reduction in the product of the maximal tumor diameter and its perpendicular, lasting at least 1 month. Therapeutic response lasting less than 1 month was defined as progressive disease. Disease free survival was calculated as the interval between diagnosis and relapse, progression, or treatment-related death. Follow-up was censored at other causes of death and at end of follow-up if the patient was relapse/progression free.

The relationship between the clinico-pathologic data and t(14;18), and bcl-2 protein expression was analyzed using chi-square and Fisher exact test whenever appropriate. Survival curves were calculated by the method of Kaplan and Meier. Statistical comparison between curves was made by the log-rank test. All directional *p* values were two-tailed and a *p* value of < 0.05 was considered significant [12]. Stat-View 5 software was used for the data analysis.

RESULTS

The mean age of the 24 men and 16 women was 50 years (range, 19-74 years, SD± 14). According to Ann Arbor staging system 19 cases (47.5%) were of early clinical stage (stages I or II). Systemic manifestations of fever, sweat and weight loss were present in 15 cases (37.5%). International Prognostic Index scores were low (0-2) in 20 cases (50%), and high (3-5) in 20 cases (50%).

During follow-up (range 3-60 month; median 15 month), 27 cases (67.5%) achieved a complete response to therapy. Relapse/progression was encountered in 10 cases (25%), and death in 8 cases (20%).

Eight cases (20%) were found to bear bcl-2 rearrangement. This included 2 cases (5%) with mbr, and 6 cases (15%) with mcr. The positive bands were detected at 150-200bp for mbr and between 200-300bp for mcr (Figs. 1,2). The relationship between clinical factors and the presence of bcl-2 gene rearrangement was shown in table (2). Bcl-2 rearrangement did not correlate significantly with age, sex, B-symptoms, stage, or IPI (*p*>0.05).

The differences in complete response to therapy and the 5-year disease free survival were statistically significant demonstrating a worse outcome for t(14;18) positive cases (*p*= 0.008, and 0.04, respectively). These data are shown in table (2), and Fig. (3).

Positivity for bcl-2 (> 10%) was detected in 24 (60%) cases (Fig. 4). The clinical features of the bcl-2 positive and bcl-2 negative cases were compared in table (3). Bcl-2 immunoreactivity was found not to be related to age, sex, B-symptoms, stage, or IPI (*p*>0.05).

Bcl-2 expression proved to be a statistically significant prognostic factor. Thirteen (54.2%) of the bcl-2 positive cases, achieved complete response to therapy, whereas 14 (87.5%) of the bcl-2 negative cases showed complete response, *p*=0.04. Also bcl-2 immunopositivity was associated with a shorter disease free survival, *p*=0.01 (Fig. 5).

As shown in table (4), no correlation was found between t(14;18) translocation and bcl-2 protein expression, *p*=0.44.

Table (1): The sequences of oligonucleotide primers for nested PCR.

Primers	Nucleotide sequences 5' → 3'
Mbr external	CAGCCTTGAAACAATGATGG
Mbr internal	ATGGTGGTTTGACCTTTAG
Mcr external	CGTGCTGGTACCACTCCTG
Mcr internal	CCTGGCTTCCTTCCCTCTG
JH external	ACCTGAGGAGACGGTGACC
JH internal	ACCAGGGTCCCTTGCCCCA

Table (2): Clinicopathologic parameters in relation to t(14;18).

	t(14;18) Positive n = 8		t(14;18) Negative n = 32		p value
	No.	%	No.	%	
Age (years):					
< 50	1	12.5	14	43.8	0.22
≥ 50	7	87.5	18	56.2	
Sex:					
Male	5	62.5	19	59.4	>0.99
Female	3	37.5	13	40.6	
B-symptoms:					
Present	3	37.5	12	37.5	>0.99
Absent	5	62.5	20	62.5	
Ann Arbor stage:					
I-II	3	37.5	16	50	0.70
III-IV	5	62.5	16	50	
IPI:					
Low (0-2)	3	37.5	17	53.1	0.69
High (3-5)	5	62.5	15	46.9	
Complete response to therapy:					
Yes	2	25	25	78.1	0.008
No	6	75	7	21.9	

Table (3): Clinicopathologic parameters in relation to bcl-2 expression.

	Bcl-2 Positive n = 24		Bcl-2 Negative n = 16		p value
	No.	%	No.	%	
Age (years):					
< 50	9	37.5	6	37.5	> 0.99
≥ 50	15	62.5	10	62.5	
Sex:					
Male	15	62.5	9	56.3	0.75
Female	9	37.5	7	43.7	
B-symptoms:					
Present	9	37.5	6	37.5	> 0.99
Absent	15	62.5	10	62.5	
Ann Arbor stage:					
I-II	11	45.8	8	50	> 0.99
III-IV	13	54.2	8	50	
IPI:					
Low (0-2)	12	50	8	50	> 0.99
High (3-5)	12	50	8	50	
Complete response to therapy:					
Yes	13	54.2	14	87.5	0.04
No	11	45.8	2	12.5	

Table (4): Relationship between t(14;18) and bcl-2 protein expression.

	t(14;18) Positive n = 8		t(14;18) Negative n = 32		p value
	No.	%	No.	%	
Bcl-2 positive	6	75	18	56.2	0.44
Bcl-2 negative	2	25	14	43.8	

Table (5): Bcl-2 rearrangement in DLBCL from different geographical regions.

	Number of patients	Bcl-2 rearrangement (%)		
		Mbr	Mcr	Total
United States:				
Jacobson et al. [15]	45	7 (16)	2 (4)	9 (20)
Gascoyne et al. [16]	102	11 (11)	3 (3)	14 (14)
Pooled data	147	18 (12)	5 (3)	23 (15)
Europe:				
Lee et al. [17]	10	1 (10)	0 (0)	1 (10)
Volpe et al. [18]	70	12 (17)	1 (1)	13 (18)
Pooled data	80	13 (16)	1 (1)	14 (17)
South America:				
Noriega et al. [19]	23	2 (9)	1 (4)	3 (13)
Jordan:				
Almasri et al. [20]	23	6 (26)	2 (9)	8 (35)
Present study	40	2 (5)	6 (15)	8 (20)

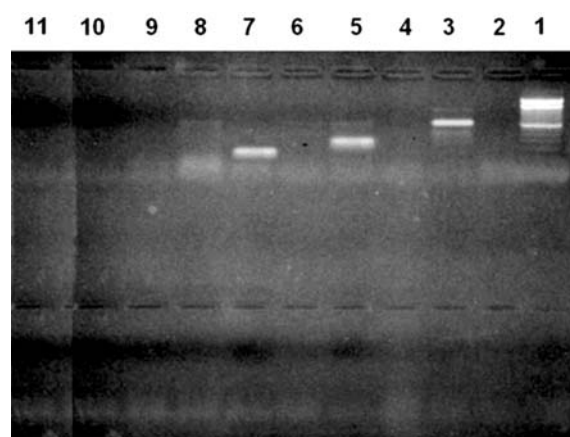


Fig. (1): Ethidium bromide-stained/UV light-illuminated gels of nested PCR amplified products at mbr. Lane 1 represents ladder marker; lane 2 negative control; lane 3 positive control; and lanes 5 & 7 represent the positive cases.

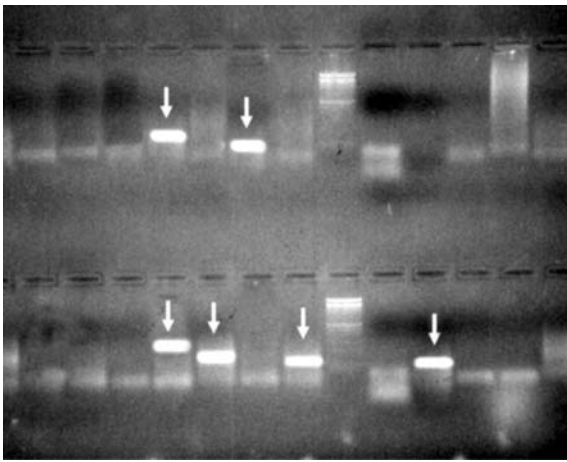


Fig. (2): Ethidium bromide-stained/UV light-illuminated gels of nested PCR amplified products at mcr. The arrows point to the positive cases.

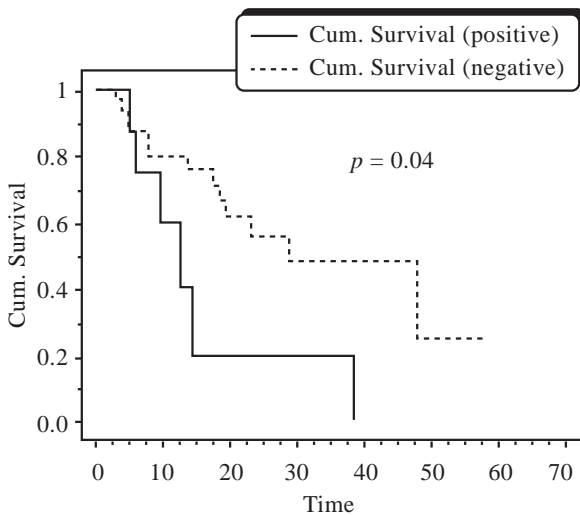


Fig. (3): Disease free survival of 40 patients with DLBCL according to t(14;18) translocation.

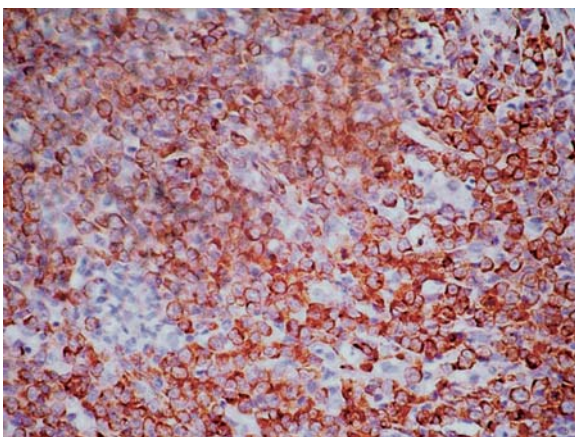


Fig. (4): DLBCL strongly positive for bcl-2 (x 400).

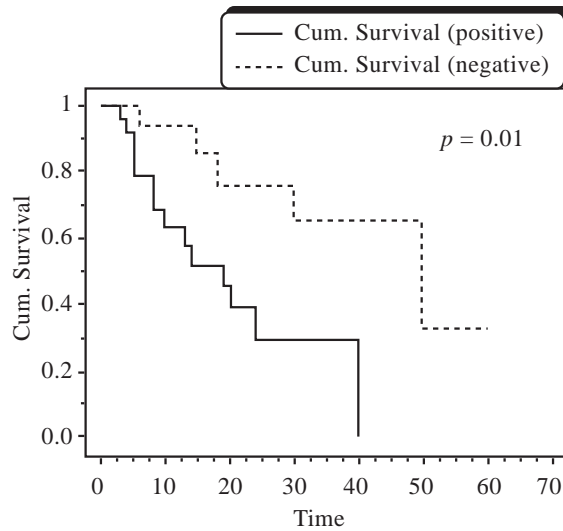


Fig. (5): Disease free survival of 40 patients with DLBCL according to bcl-2 expression.

DISCUSSION

In the present study, bcl-2 gene rearrangement and bcl-2 protein expression were investigated in a carefully selected cohort of patients with Egyptian nodal DLBCL. This work has focused attention on clinically de novo DLBCL by excluding all cases with either a past history of lymphoma, or in which the pathologic specimen contained areas of follicular architecture seen morphologically or by immunostaining for follicular dendritic cells. Also the patient group was treated with a standardized manner at a single institution.

The fact that primary DLBCL with the t(14;18) has gene expression profiles similar to normal germinal center B cells suggests that the t(14;18) plays an important role in the pathogenesis of this subset of DLBCL, just as it does in follicular lymphoma. The t(14;18) occurs as a rare random event in naive B cells, probably during the D/J rearrangement of the immunoglobulin heavy chain gene. Such a B-cell may encounter the appropriate antigen in the context of dendritic and T-cells in the paracortical region of a lymph node. The stimulated B-cell then migrates to and proliferates in a germinal center. Overexpression of bcl-2 protein protects the cell and its progeny from apoptosis even when the affinity of the antibody is not very high. Repeated cycles of proliferation from subsequent antigenic encounters expand this clone and result in additional genetic abnormalities. Different secondary genetic alteration lead to

the development of a primary DLBCL or a follicular lymphoma. Further genetic changes occurring during the course of follicular lymphoma will give rise to a secondary DLBCL in some cases [13].

The chromosomal breakpoint on *bcl-2* have been shown to cluster at two main regions, the major breakpoint region in the 3' untranslated region of the third exon of the *bcl-2* gene, and the minor cluster region in a DNA stretch approximately 30 kb downstream of the *bcl-2* locus. On chromosome 14, breakpoints are predominantly found within the joining region (JH) of the IgH gene [14]. In this study using nested PCR technique i.e. two amplifications have been used with oligonucleotide primers including variable sequences of the *bcl-2* on one side and sequences of the IgH on the other side, 20% of cases were found positive bearing *bcl-2* rearrangement.

The reported frequency of t(14;18) in DLBCL documented by cytogenetics and/or molecular genetics is highly variable in different studies [7]. Not surprisingly, those studies that include patients with transformed follicular lymphoma report higher frequencies of *bcl-2* gene rearrangement. Also the inclusion of extranodal tumors, the use of various probes (*mbr*, *mcr*, or both), different molecular techniques (southern blot analysis, PCR, or fluorescence in situ hybridization), and differences in fixation were responsible for this heterogeneity. The majority of previous studies that have used PCR to evaluate *bcl-2* rearrangements have analyzed only *mbr* breakpoints. Hill et al. [7] reviewed the literature and reported a cumulative frequency of 204 of 1030 (19.8%), with a range of 10% to 40%. This included their own published work from the British National Lymphoma Investigation Study using only *mbr* primers.

The frequencies of *bcl-2* gene rearrangement from different geographic areas in which *mbr* and *mcr* were evaluated, and the data of the present study are shown in table (5). These findings support the preferential breakage of *bcl-2* in Egyptian tumors outside *mbr*. This explains the low frequency (7%) of *bcl-2* rearrangement in DLBCL in the Egyptian series of Fahmy [21] by using only *mbr* primers.

Bcl-2 rearrangement in this study did not correlate significantly with age, sex, B-

symptoms, stage, or IPI ($p>0.05$). Similarly the IPI factors were evenly distributed between t(14;18) positive versus negative groups in the series of Gascoyne et al. [16], with the exception that t(14;18) positive cases tended to be younger. Also Barrans et al. [22] found no association between the presence of t(14;18), and IPI. In contrast, Kramer et al. [23] stated that *bcl-2* rearrangements were associated with disseminated disease since none of 40 patients with stage I disease showed a rearrangement.

A significant difference in complete response to therapy ($p=0.008$) and disease free survival ($p=0.04$) was demonstrated in this work based on the presence of *bcl-2* rearrangement. These differences cannot be explained simply by differences in the clinical features of these patients because there was no association between the presence of the translocation and the IPI. Prior studies have reached varying conclusions regarding the prognostic significance of *bcl-2* rearrangement in DLBCL. Decreased survival in patients having rearrangement was reported by Barrans et al. and Tang et al. [22,24]. The data in the study of Hill et al. [7] did not exclude the possibility that *mbr* rearrangement may have some influence on relapse rate, in so far as actuarially approximately 85% of the *mbr* positive patients have relapsed by 6 years, although this was not significantly different from the relapse rate of patients without an *mbr* rearrangement. They suggested that this failure to detect a significant difference could be due to the small number of patients with an *mbr* rearrangement, and a much larger study could resolve this issue. In contrast, the majority of published series have shown no effect [16,23,25-28], and the poor outcome or resistance to treatment has been attributed by some authors to the expression of *bcl-2* protein, rather than the presence of the translocation [7,16,23,26]. Other studies suggested that patients with *bcl-2* gene rearrangement had better survival [29,30].

Human *bcl-2* is a 239 amino acid 25 kDa protein that localizes primarily to mitochondrial, nuclear, and cytoplasmic membranes. The *bcl-2* protein is expressed in the progenitor and long-lived cells in tissues characterized by apoptotic cell death. It can prevent programmed cell death without promoting proliferation [31]. High level of *bcl-2* protein is believed to play an important role in lymphomagenesis and in the development of drug resistance.

The frequency of bcl-2 expression in this series (60%) was in the mid-range of that in prior studies which report figures from 24% to 79% [32,33,34]. The variation is at least in part likely to represent differences in technique, but the most recent series (including this one), using similar antibodies and methods point to a true figure of 50% to 60%.

In this work, no statistical significant correlation was found between bcl-2 expression and patient age, sex, B-symptoms, stage, or IPI ($p>0.05$). In accordance, some investigators found no association between IPI, or components of IPI, and bcl-2 expression [16,35,36]. Others reported that bcl-2 positive cases were associated with an elevated serum LDH [16], stage III-IV disease [37,38,39], or high IPI [38,39].

Some reports of the prognostic significance of bcl-2 protein expression in DLBCL failed to show any impact on survival [24,28,40]. This was explained by Hill et al. [7] by smaller number of cases or the differing patient populations, treatments, and follow up time. Other groups [7,36,37] using similar techniques and thresholds of bcl-2 immunopositivity have reported that bcl-2 positive cases have a worse disease free or cause specific survival, although none were able to demonstrate a significant difference in overall survival.

Barrans et al. [35] revealed that bcl-2 was associated with decreased 2-year overall survival. In their series using IPI alone they identified 8% of patients as high risk. Expression of bcl-2 in the intermediate IPI group identified a further 28% of patients with an overall survival comparable to the high IPI group. In the series of Biasoli et al. [41], bcl-2 expression was independent poor prognostic factor and identified a subgroup of patients within the high risk IPI category who had a dismal outcome.

Drug resistance related to bcl-2 function has been proposed as the underlying basis for worse clinical behavior in bcl-2 positive lymphoma. Evidence has accumulated that many and perhaps all agents of cancer chemotherapy affect tumor cell killing in vitro as well as in vivo by inducing apoptosis [42]. Tumors that are intrinsically resistant to chemotherapy are unable to activate the apoptotic machinery and may therefore be fundamentally resistant to chemotherapeutic cell death. Many cancer cells circumvent

the normal apoptotic mechanisms to prevent their self destruction. The bcl-2 protein, because of its antiapoptotic effects is considered to be an important multidrug resistance agent.

In keeping with previous published reports [7,13,16,23], our data demonstrate that cases with a molecular rearrangement may fail to express the protein. This may be explained by mutations in the open reading frame of the translocated bcl-2 gene leading to absent or diminished production of bcl-2 protein, or genetic events developing during tumor progression may abrogate the need for bcl-2. Also the expression of bcl-2 protein in cases lacking t(14,18) was detected [7,16], and suggests that mechanisms other than translocation can lead to increased levels of bcl-2 such as activation of the nuclear factor-kB pathway or an increased copy number of the bcl-2 gene [43,44]. So the presence of bcl-2 gene rearrangement is not synonymous with overexpression of bcl-2 protein and vice versa.

In conclusion, assessment of t(14;18) translocation, and bcl-2 protein expression can be introduced as part of the routine investigation in patients with DLBCL for risk-adjusted therapy, and to identify patients who may require different treatment. The comparison of our results of bcl-2 gene rearrangement with data of the literature suggests that some of the epidemiologic difference may be related to the distribution of bcl-2 breakpoints.

REFERENCES

- 1- Jaffe E, Harris N, Stein H, Vardiman J. Pathology and genetics of tumors of the hematopoietic and lymphoid tissues. WHO classification of tumors. Lyon-France, IARC Press. 2001.
- 2- Mokhtar N, Khalid H. Diffuse large B-cell lymphoma. In: Lymphoma Text Book. First edition. Published by the National Cancer Institute, Cairo. 2002, pp. 123.
- 3- The International Non-Hodgkin's Lymphoma Prognostic Factor Project. A predictive model for aggressive non-Hodgkin's lymphoma. N Engl J Med. 1993, 329: 987-994.
- 4- Armitage J, Weisenburger D. New approach to classifying non-Hodgkin's lymphoma: Clinical features of the major histologic subtypes. Non-Hodgkin's Lymphoma Classification Project. J Clin Oncol. 1998, 18: 2780-2795.
- 5- Hans C, Weisenburger D, Greiner T, Gascoyne R, Delabie J, Ott G. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using tissue microarray. Blood. 2004, 103: 275-282.

- 6- Bakhshi A, Jensen J, Goldman P, Wright J, Mc Bride O, Epstein A, et al. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: Clustering around JH on chromosome 14 and near a transcriptional unit on 18 Cell. 1985, 41: 889-906.
- 7- Hill M, MacLennan K, Cunningham D, Hudson B, Burke M, Clarke P, et al. Prognostic significance of bcl-2 expression and bcl-2 major breakpoint region rearrangement in diffuse large cell non-Hodgkin's lymphoma: A British National Lymphoma Investigation Study. Blood. 1996, 88: 1046-1051.
- 8- Biagi J, Seymour J. Insights into the molecular pathogenesis of follicular lymphoma arising from analysis of geographic variation Blood. 2002, 99: 4265-4275.
- 9- Mc Cluggage W, Catherwood M, Alexander H, Mc Bride H, Smith M, Morris T. Immunohistochemical expression of CD10 and t(14;18) chromosomal translocation may be indicators of follicle centre cell origin in nodal diffuse large B-cell lymphoma Histopathology. 2002, 41: 414-420.
- 10- Sambrook J, Fritsch E, Maniatis T. Molecular cloning. A laboratory manual, second edition. New York: Cold Spring Harbor Laboratory Press. 1989, pp. E 2-E 10.
- 11- Gribben J, Freedman A, Woo S, Blake K, Shu R, Freeman G, et al. All advanced stage non-Hodgkin's lymphomas with a polymerase chain reaction amplifiable breakpoint of bcl-2 have residual cells containing the bcl-2 rearrangement at evaluation and after treatment Blood. 1991, 78: 3275-80.
- 12- Peto R, Pike M, Armitage P, Breslow N, Cox D, Howard S, et al. Design and analysis of randomized clinical trials requiring prolonged observations of each patient. Analysis and examples. Br J Cancer. 1977, 35: 1-39.
- 13- Huang J, Sanger W, Greiner T, Staudt L, Weisenburger D, Pickering D, et al. The t(14;18) defines a unique subset of diffuse large B-cell lymphoma with a germinal center B-cell gene expression profile. Blood. 2002, 99: 2285-90.
- 14- Takacs I, Radvanyi G, Szegedi G, Matolocsy A, Semsei I. Detection of t(14;18) chromosome translocation in follicular lymphoma by polymerase chain reaction. Orv Hetil. 1997, 138: 1129-32.
- 15- Jacobson J, Wilkes B, Kwiatkowski D, Medeiros J, Aisenberg A, Harris N. Bcl-2 rearrangements in de novo diffuse large cell lymphoma. Association with distinctive clinical features Cancer. 1993, 72: 231-236.
- 16- Gascoyne R, Adomat S, Krajewski S, Krajewski M, Horsman D, Ticher A, et al. Prognostic significance of bcl-2 protein expression and bcl-2 gene rearrangement in diffuse aggressive non-Hodgkin's lymphoma. Blood. 1997, 90: 244-51.
- 17- Lee K, Goepel J, Winfield D, Hancock B, Goyns M. Investigation of bcl-2 gene rearrangement in a United Kingdom series of low and high grade non-Hodgkin's lymphomas. Leuk Lymphoma. 1993, 11: 91-8.
- 18- Volpe G, Vitolo U, Carbone A, Pastore C, Bertini M, Botto B, et al. Molecular heterogeneity of B-lineage diffuse large cell lymphoma. Genes Chromosomes Cancer. 1996, 16: 21-30.
- 19- Noriega M, DeBrasi C, Narbaitz M, Slavutsky I. Incidence of bcl-2 gene rearrangement in Argentinean non-Hodgkin lymphoma patients. Increased frequency of breakpoints outside of mbr and mcr. Blood Cells Molecules and Diseases. 2004, 32: 232-39.
- 20- Almasri N, Al-Alami J, Faza M. Bcl-2 gene rearrangement in Jordanian follicular and diffuse large B-cell lymphomas. Saudi Med J. 2005, 26: 251-55.
- 21- Fahmy A. Frequency of bcl-2 oncogene rearrangement in non-Hodgkin's lymphoma among Egyptians: A clinicopathologic, cytogenetic, and molecular study. MD Thesis. National Cancer Institute, Cairo University, 1998.
- 22- Barrans S, Evans P, O'Connor S, Kendall S, Owen R, Haynes A, et al. The t(14;18) is associated with germinal center derived diffuse large B-cell lymphoma. Clinical Cancer Research. 2003, 9: 2133-39.
- 23- Kramer M, Hermans J, Wijburg E, Philippo K, Geelen E, van Krieken J, et al. Clinical relevance of bcl-2, bcl-6, and myc rearrangements in diffuse large B-cell lymphoma. Blood, 1998; 92: 3152-62.
- 24- Tang S, Visser L, Hepperle B, Hanson J, Poppema S. Clinical significance of bcl-2 mbr gene rearrangement and protein expression in diffuse large cell non-Hodgkin's lymphoma: An analysis of 83 cases. J Clin Oncol. 1994, 12: 149-54.
- 25- Romaguera J, Pugh W, Luthra R, Goodacre A, Cabanillas F. The clinical relevance of t(14;18)/bcl-2 rearrangement and DEL 6q in diffuse large cell lymphoma and immunoblastic lymphoma. Ann Oncol. 1993, 4: 51-4.
- 26- Martika M, Comeau T, Foyle A, Anderson D, Greer W. Prognostic significance of t(14;18) and bcl-2 gene expression in follicular small cleaved cell lymphoma and diffuse large cell lymphoma. Clin Investig Med. 1997, 20: 364-70.
- 27- Iqbal J, Sanger W, Horsman D, Rosenwald A, Pickering D, Dave B, et al. Bcl-2 translocation defines a unique tumor subset within the germinal center B-cell like diffuse large B-cell lymphoma. Am J Pathol. 2004, 165: 159-66.
- 28- Hirose Y, Masaki Y, Karasawa H, Shimoyama K, Fukushima T, Kawabata H, et al. Incidence of diffuse large B-cell lymphoma of germinal center B-cell origin in whole diffuse large B-cell lymphoma. Tissue fluorescence in situ hybridization using t(14;18) compared with immunohistochemistry. Int J Hematol. 2005, 81: 48-57.
- 29- Vitolo U, Gaidano G, Botto B, Volpe G, Audisio E, Bertini M, et al. Rearrangements of bcl-6, bcl-2, c-myc, and 6q deletion in diffuse large cell lymphoma: Clinical relevance in 71 patients. Ann Oncol. 1998, 9: 55-61.

- 30- Kawasaki C, Ohshim K, Suzumiya J, Kanda M, Tsuchiya T, Tamura K, et al. Rearrangement of bcl-6, bcl-2, and c-myc in diffuse large B-cell lymphomas. *Leuk lymphoma*. 2001, 42: 1099-1106.
- 31- Korsmeyer S. Bcl-2 initiates a new category of oncogenes: Regulators of cell death *Blood*. 1992, 80: 879-86.
- 32- Fang J, Finn W, Hussong J, Goolsby C, Cubbon A, Variakojis D. CD10 antigen expression correlates with the t(14;18) (q32;q21) major breakpoint region in diffuse large B-cell lymphoma. *Mod Pathol*. 1999, 12: 295-300.
- 33- King B, Chen C, Locker J, Kant J, Okuyama K, Falini B, et al. Immunophenotypic and genotypic markers of follicular center cell neoplasia in diffuse large B-cell lymphoma. *Mod Pathol*. 2000, 13: 1219-31.
- 34- Xu Y, Mckenna R, Molberg K, Kroft S. Clinicopathologic analysis of CD10+ and CD10- diffuse large B-cell lymphoma. Identification of a high-risk subset with co-expression of CD10 and bcl-2. *Am J Clin Pathol*. 2001, 116: 183-90.
- 35- Barrans S, Carter I, Owen R, Davies F, Patmore R, Haynes A, et al. Germinal center phenotype and bcl-2 expression combined with the International Prognostic Index improves patient risk stratification in diffuse large B-cell lymphoma *Blood*. 2002, 99: 1136-43.
- 36- Jerkeman M, Anderson H, Dictor M, Kvaloy S, Akerman M, Cavallin-Stahl E. Assessment of biological prognostic factors provides clinically relevant information in patients with diffuse large B-cell lymphoma- a Nordic Lymphoma Group Study. *Ann Hematol*. 2004, 83: 414-19.
- 37- Hermine O, Haioun C, Lepage E, d'Agay M-F, Briere J, Lavnignac C, et al. Prognostic significance of bcl-2 protein expression in aggressive non-Hodgkin's lymphoma *Blood*. 1996, 87: 265-72.
- 38- Clomo L, Lopez-Guillermo A, Perales M, Rives S, Martinez A, Bosch F, et al. Clinical impact of the differentiation profile assessed by immunophenotyping in patients with diffuse large B-cell lymphoma. *Blood*. 2003, 101: 78-84.
- 39- Zinzani P, Dirnhofer S, Sabattini E, Alinari L, Piccaluga P, Stefoni V. Identification of outcome predictors in diffuse large B-cell lymphoma *Hematologica*. 2005, 90: 341-47.
- 40- Piris M, Pezella F, Martinez-Montero J, Orradre J, Villaendas R, Sanchez-Beato M. p53 and bcl-2 expression in high grade B-cell lymphomas. Correlation with survival time. *Br J Cancer*. 1994, 69: 337-341.
- 41- Biasoli I, Morais J, Scheliga A, Milito C, Romano S, Land M, et al. CD10 and bcl-2 expression combined with the International Prognostic Index can identify subgroups of patients with diffuse large-cell lymphoma with very good or very poor prognoses *Histopathology*. 2005, 96: 328-333.
- 42- Hannun Y. Apoptosis and the dilemma of cancer chemotherapy. *Blood*. 1997, 89: 1845-53
- 43- Bureau F, Vanderplasschen A, Jaspar F, Minner F, Pastoret P, Merville M, et al. Constitutive nuclear factor-B activity preserves homeostasis of quiescent mature lymphocytes and granulocytes by controlling the expression of distinct bcl-2 family proteins. *Blood*. 2002, 99: 3683-91.
- 44- Monni O, Joensuu H, Franssila K, Klefstrom J, Alitalo K, Knuutila S. Bcl-2 overexpression associated with chromosomal amplification in diffuse large B-cell lymphoma. *Blood*. 1997, 90: 1168-74.