The Immunohistochemistry Based Evaluation of Bcl-2 in B non Hodgkin lymphoma & Its Prognostic Significance

El-Esawy, B. H [*1,2]

[1] Pathology Department - Faculty of Medicine - Mansoura University - Egypt
[2]Laboratory & Clinical Biotechnology Department- College of Applied Medical Sciences -Taif University-KSA
Basemelesawy1@yaahoo.com

Abstract: Background: The prognosis for B-Non-Hodgkin's lymphoma (NHL) is known to be determined by multiple differences in tumor cell biology. Bcl-2 is a marker linked to germinal center & thought to have an effect on prognosis of mature B-cell non Hodgkin lymphoma. **Objectives:** The aim of this study was to investigate the value of Bcl-2 expression in B- cell non Hodgkin lymphoma & study of clinico-pathological correlation. **Methods:** Immunohistochemical method was used to detect the expression of Bcl-2 in 132 formalin- fixed, paraffin-embedded tissue samples of human B cell non Hodgkin lymphomas. **Results:** 56 cases of 132 cases of B cell NHL show Bcl-2 expression with higher expression in follicular lymphoma. Also Bcl-2 expressed in low grade follicular lymphoma than in high grade follicular lymphoma. Regarding follicular lymphoma, Bcl-2 is expressed mainly in low grade lymphoma. In correlation with other prognostic parameters, B-cl2 expression is associated with old age, low platelets count, elevated LDH levels & total leukocytic count. **Conclusion:** The over-expression of B-cl2 is a highly characteristic and specific indicator of follicular lymphoma & B-cl2 is a potentially useful diagnostic tool in sub-classification & prognosis of low-grade B-cell lymphomas

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Key Words: Immunohistochemistry, non Hodgkin lymphoma, Bcl-2, Follicular lymphoma, low grade

1. Introduction:

Non Hodgkin's Lymphoma (NHL) is a broad category consisting of several distinct types of lymphoid neoplasm, 85% of which are B cell lymphomas and 15% being T cell lymphoma.(1,2) To establish the therapeutic approach, clinically NHL is categorized into two major subtypes, indolent and aggressive. Indolent group includes the small lymphocytic and the follicular categories, while the aggressive group which responds well against treatment, constitutes the large cell types, the Burkitt's lymphoma and the lymphoblastic lymphomas. (2) The diagnosis and classification of malignant lymphomas are complex and often rely on subtle difference in morphology that are subjected to interpretation so immunohistochemical and genetic studies are often very helpful in establishing correct diagnosis.(3) The prognosis for each of the Non-Hodgkin's lymphoma (NHL) variants is known to be related to the multiple differences in cytogenetics, immune-phenotype, growth fraction, cytokine production found within each of the specific variants.(4) Most of genetic alterations in lymphoma appear to be somatically acquired rather than inherited.(5,6) They result in inappropriate constitutive activation of proto-oncogenes and their transformation into oncogenes that permit an otherwise normal cells to divide independently of normal growth regulatory mechanisms or alternatively inactivation of anti-oncogenes, tumor suppressor gene.(6) Oncogenes typically encode for proteins that are involved in control of cellular proliferation and differentiation.(7) They are classified into four groups: growth factor, growth factor receptor, signal transduction and nuclear transcription factor.(8) There are also proto-oncogenes that do not fit with these categories such as Bcl-2 which is an anti-apoptotic membrane-associated molecule that resides in the nuclear envelope and mitochondria & encodes for cyclin that acts to drive the cell cycle.(9) The growth of the lymphoid cells is regulated by a delicate balance between molecules controlling cell survival and cell death.(10) The Bcl-2 gene product is an anti-apoptotic molecule that modulates the mitochondrial release of cytochrome c, and the interaction of Apoptosis activating factors with caspase 9 and Bax (11,12) Bcl-2 is a marker linked to germinal center B cells. The Bcl-2 gene, located at chromosome 18q21, encodes for a 25kd protein located mainly in the mitochondrial membrane. (12) This Bcl-2 protein is an anti-apoptosis factor that is important in normal B-cell development and differentiation. Bcl-2 over-expression provides a survival advantage for malignant B cells and is thought to play a critical role in resistance to chemotherapy.(13) The curability of many cases of lymphomas depends on the ability of pathologist to determine precisely subtype and cell lineage of lymphoma and hence the importance of proper diagnosis for selection of appropriate therapy in patients with malignant lymphoma.(14) Although many

studies suggest that the Bcl-2 protein expression is related to lymphoma proliferation due to t (14;18) translocation in lymphoma cells that brings *Bcl-2* gene (chromosome 18) into juxtaposition with the enhancer elements of the immnuoglobulin heavy chain locus (IgH) chromosome (14-13) & also over-expression of the Bcl-2 protein in the blood or bone marrow is a feature of follicular lymphoma.(15,16) However analysis of this protein in the different grades of NHL is still lacking. To address the above-mentioned issue, we investigated the expression of the bcl-2 protein in 132 cases of B-NHLs to identify differences in the patterns of expression in different subsets and evaluate the possible significant role of B-cl2 in sub-classification, prognosis & clinic-pathological correlation of B cell NHL.

2. Material and Method:

2.1 Patients

A total of 132 patients with B- NHL were enrolled in this retrospective study. Also, patients refereed from private hospitals were included. The inclusion criteria were histopathologic diagnosis of B-NHL and the availability of detailed clinical sheet details, complete blood count, laboratory investigations, imaging studies and histopathological data and paraffin-embedded tumor tissues for all cases. The diagnosis of NHL was confirmed by tissue biopsy in all cases comprised 38 Follicular lymphomas (FL), 14 Small lymphocytic lymphomas (SLL/CLL), 6 mantle cell lymphomas (MCL), 16 Marginal zone lymphomas, 50 diffuse large B-cell lymphomas (DLBCL) & 8 Burkitt's lymphomas.

2.2 Histopathologic evaluation

Routinely stained hematoxylin and eosin (H&E) slides were reviewed. Also special reticulin stain was applied in some selected cases of lymphomas. Histopathologic sub-typing & grading was performed based on the criteria described in the World Health Organization (WHO 2008), working formulation and Kiel classification.

2.3 Immunohistochemistry

2.3(A): Procedure and antibodies

Serial 3-µm sections were cut from the paraffin block, mounted on positively charged slides and dried overnight in a 60°C oven. Sections were then de-paraffinized in xylene for 24 h and hydrated in a descending grades of alcohol; 100%, 90%, 85% and 70%. Antigen unmasking was performed by heat induced an epitope retrieval method by placing the slides in a plastic Coplin jar filled with citric acid buffer so that the solution covers the slides, then placing the jar in a microwave at 800Watt for 20 min (divided into 4 cycles 5 min each). The Coplin jar was then removed from the oven and allowed to cool for 15 min. Slides were placed in a humidified chamber and rinsed three times in phosphate buffer saline (PBS). Endogenous peroxidase activity was blocked by incubation of the tissue section with 3% hydrogen peroxide in water for 30 min. After washing, the tissue sections were then incubated with the primary monoclonal antibody, ready to use Bcl-2. Two hundred µl of the monoclonal were added for 1 h at room temperature with Bcl-2 (Clone 124, Dako M0887 diluted at 1:40). The streptavidin biotin method was used as a detection kit (LSAB2, Dako, Denmark). Tissue sections were incubated with biotinylated secondary antibody for 40 min, then with streptavidin conjugated enzyme for 30 min during which the 3,3′ diaminobenzidine (DAB) substrate chromogen was freshly prepared then added onto the tissue section for 10 min in the dark. The sections were counterstained in Meyer hematotoxylin, dehydrated, then put in xyelene and cover-slipped by DPX mount media and examined under a light microscope. All cases were stained with CD20 to confirm B cell origin of non Hodgkin lymphomas.(17)

2.3:(B) Quality control of IHC

Appropriate negative controls for the immunostaining, consisting of histological sections of each case processed without the addition of primary antibody were prepared for each antigen, along with a positive control sections prepared with each IHC run then staining results were evaluated.(18)

2.4 Assessment of IHC

Immunohistochemical results were scored semi-quantitatively. For Bcl-2 the positivity cut off was considered when the reactivity of lymphoma cells with antibodies was more than 20%. The pattern of staining was also considered. For Bcl-2, membranous and cytoplasmic patterns were considered positive. The positivity of the immunostaining was detected by percentage of positive cells and intensity of staining. (18)

2.6 Statistical Analysis

The Statistical Package for Social Sciences (SPSS) for windows (version 12) computer program was used for statistical analysis. Comparison between positive cases was calculated by Chi square test. *P* values ≤ 0.05 and ≤ 0.01 were considered significant and highly significant, respectively, in all analyses. T-test to compare mean and SD between two groups.

3. Results

In this study, there were 77 males and 55 females with a male to female ratio 1.4:1.0. As regard Bcl-2 it is expressed in 35 males & 21 females with a ratio of 1.6: 1.0. Ages ranged from 18 to 75 years old with a mean of 48±14.2 years. The results of this study are summarized in tables 1,2,3 & photos 1-6

Table (1): Bcl-2 expression in different subtypes of B cell NHL

Subtypes	N. of cases	Bcl-2 positive N & (%)
Follicular lymphoma (FL)	**38**	**28 (73.6 %)**
Small lymphocytic lymphoma (SLL)	**14**	**4 (28.5 %)**
Mantle cell lymphoma (MCL)	**6**	**2 (33.3 %)**
Marginal zone lymphoma (MZL)	**16**	**10 (62.5 %)**
DLBCL	**50**	**12 (24 %)**
Burkitt's lymphoma	**8**	**-**
Total	**132**	**56 (42.4 %)**

Table (2): The number & percentage of Bcl-2 positive cases of follicular lymphoma

Grading	*Number*	*N & (%) of BCL-2 positive cases*
Grade I	**15**	**13 (86.7 %)**
Grade II	**21**	**14 (66.7 %)**
Grade III	**2**	**1 (50 %)**

Table (3): Bcl-2 expression and prognostic parameters in NHL groups

Parameter	**Bcl-2 positive (56)**			**Bcl-2 negative (76)**		***p***	**Significance**
		N. cases	% cases	N. cases	% cases		
Age (mean)	≥45 yrs <45 yrs	42 14	75% 25%	42 34	55.3% 44.7%	0.020	Significant
Sex (mean)	M F	35 21	62.5% 37.5%	42 34	44.7% 55.3%	0.406	Not sig.
Hb g/dL (mean)	≥10 <10	28 28	50% 50%	38 38	50 50	1.0	Not sig.
Plt $\times 10^9$/L (mean)	≥150 <150	14 42	25% 75%	61 15	80.2% 19.7%	0.000	High sig.
TLC $\times 10^9$/L (mean)	≥8 <8	29 27	51.7% 48.3%	15 61	19.7% 80.2%	0.0001	High sig.
LDH IU/L	≥500 <500	49 7	87.5% 12.5	32 44	42.1% 57.8%	0.0000	High sig.
Stage	I II III IV	2 8 20 26	3.6 14.3% 35.7% 46.4*	7 19 23 27	9.2% 25 30.2% 35.5%	0.056	Not sig.

S, significant; NS, non-significant; Hb, hemoglobin; Plts, platelets; LDH, lactate dehydrogenase. TLC, total leukocytic count; S, significant; NS, non-significant; HS, high –significant.

***As regard Bcl-2 expression in different subtypes of B cell NHL**

Bcl-2 protein was positive in 42.4% (56 of 132) of cases with the most frequent expression in the FL group (*P*= 0.000 High sig.). 73.6% of FL,

62.5% of MZL, 33.3% of MCL, 28.5%% of SLL and 24% of DLBCL.

***Regarding The number & percentage of Bcl-2 positive cases of follicular lymphoma**

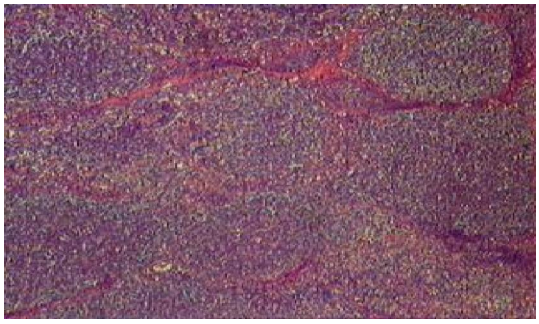
Bcl-2 is highly expressed mainly in low grade FL in a percentage of

86.7% in GI FL, 66.6% in GII FL and 50% GIII FL

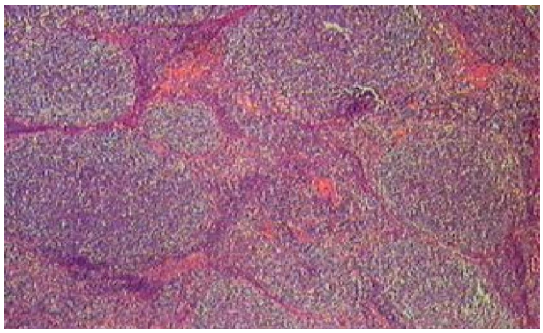
*** Relation of Bcl-2 protein expression to clinical & laboratory prognostic factors**

On comparing Bcl-2 expression with various prognostic parameters, a statistically significant difference was obtained regarding old age & Bcl-2 expression (*p*= 0.020), platelets count in which 75% of Bcl-2 positive cases were thrombocytopenic (*P*= 0.00).Regarding TLC, there was a highly significant value (*P* = 0.001).A higher serum LDH serum level was detected in Bcl-2 positive cases compared to Bcl-2 negative group and the difference was statistically highly significant (*P* = 0.00).

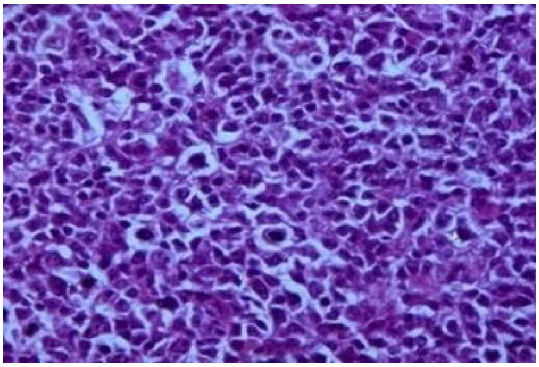
On the other hand, no statistical significance was found as regards sex, hemoglobin level & lymphoma staging.



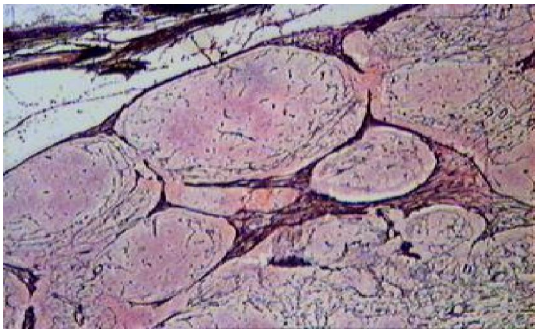
Photomicrograph (1): Follicular lymphoma shows uniform nodularity throughout the lymph node with little variation in size and shape of the follicles. (H & E x 40)



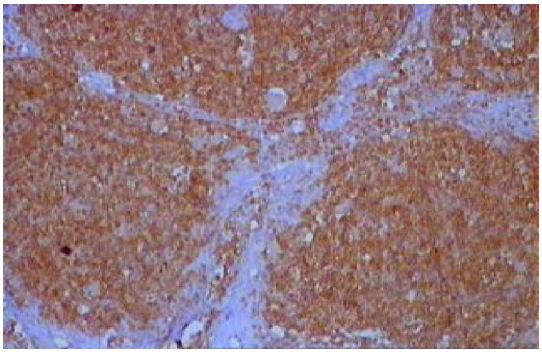
Photomicrograph (2): Follicular lymphoma shows neoplastic follicles that are vague with peripheral fading and coalescence. (H & E x 100)



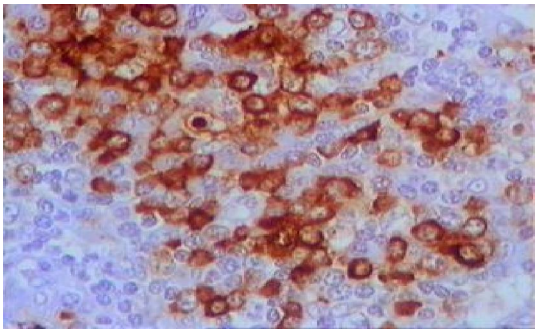
Photomicrograph (3): Follicular lymphoma mixed cell type shows intimate admixture of small centrocytes and large centroblasts with scattered mitosis.(H & E x 400)



Photomicrograph (4): Follicular 1ymphoma shows back to back arrangement of neoplastic follicles by reticulin stain. (x 40)



Photomicrograph (5): Follicular lymphoma shows strong Bcl-2 positivity in the neoplastic follicles. (Immunoperoxidase staining, hematoxylin counter stain x 100)



Photomicrograph (6): Follicular lymphoma shows cytoplasmic & membranous Bcl-2 staining. (Immunoperoxidase staining, hematoxylin counter stain x 400)

4. Discussion:

Several prognostic parameters were used in B-NHL to detect patients' outcome.(19) To detect the value of Bcl-2 in diagnosis and prognosis of such diseases, immunostaining of Bcl-2 was performed for **132** patients with B-NHL. In this study, using a cut off 20%, Bcl-2 protein was positive in 43.75% (42 of 96) of NHL case (73.6 % of FL, 28.5% of SLL/CLL, 33.3% of MCL, 62.5% of MZL and 24% of DLBCL cases). The diagnosis of follicular lymphoma is

confirmed by special reticulin stain in some cases. Navratile *et al.(20)* also found that Bcl-2 is expressed in low grade but not high grade lymphoma which match with our study results. Also in a study by Lai *et al.* (21) performed as a survey of Bcl2 expression in 778 cases of NHL. Of 20 reactive monocytoid B-cell hyperplasia, none were B-cl2 positive, compared with 118 (79%) of 150 marginal zone lymphomas. With respect to the follicular lymphomas of the 110 Grade I lymphomas, 107 (97%) were Bcl-2 positive, 119 (83%) of the 143 Grade II lymphomas were positive, and 71 (74%) of the 96 Grade III lymphomas were positive. In a study performed by Xu *et al*.(22) to evaluate B-cl2 protein expression by immunohistochemistry on paraffin -embedded slices in 35 cases of NHL. They found that the level of Bcl-2 expression in lower grade NHL was higher than that in high grades & The level of Bcl-2 expression in B cell NHL was higher than that in T cell NHL. The variability of relative frequency in different areas all over the world may be due to availability of newer methods to study neoplastic disorders, including immunophenotyping, cytogenetics, and molecular biology. The percentage of positive Bcl-2 in MCL cases was 33.3%. In fact, some MCL cases gave positivity less 10%; yet we considered them negative results according to the previously agreed cut off. Although MCL looks like a slow growing, low-grade tumor under the microscope, it grows fast with biological behavior like a high- grade lymphoma that show low expression of Bcl-2. (22)

Ben-Ezra *et al.*(23) have suggested that Bcl-2 alone is useful in discriminating FL and benign lymphoid aggregates. In spite of the fact that the absence of Bcl-2 was highly specific for benign lymphoid aggregates, the expression of Bcl-2 in some benign and atypical lymphoid aggregates in the study by West *et al.(24*) did not make Bcl-2 a specific marker for the detection of FL however from our present work we can depend on B-cl2 over expression in the neoplastic follicles together with histopathological picture for confirmation of diagnosis of follicular lymphoma. In addition, Llanos *et al.(*25) & Papakonstantinous *et al.(*26) stated that Bcl-2 expression is related to the grade of FL being highest in grade I and lowest in grade III, which matches with our results that show that the expression of Bcl-2 was 86.7% in GI, 66.7% in GII & 50% in GIII follicular lymphoma cases and this may be due to accumulation of more genetic alteration in high grade follicular lymphomas.

From the previous studies we found that BcL-2 is a proto-oncogene located at 18q21 that promotes B-cell survival via inhibition of apoptosis and confers chemotherapy resistance. The oncoprotein, B-cl2 is expressed in various types of non-Hodgkin's lymphoma (NHL) as a result of chromosomal translocation t(14;18)(q32;q21) but Bcl-2 is a highly sensitive marker for follicular lymphoma due to strong positivity. Immune detection of this protein is a useful tool for distinguishing follicular lymphoma. The pattern of Bcl-2 staining in follicular lymphoma is the inverse of the pattern in reactive hyperplasia. The level of B-cl2 expression was closely related with the grade of malignancy and the prognosis of NHL as it is strongly expressed in follicular lymphoma grades I & II & less frequently expressed in follicular lymphoma grade III. So it is a useful marker for understanding the generation and prognosis of NHL.

Comparing Bcl-2 to prognostic parameters in the NHL group in the current study, there was a statistical significance regarding advanced patient age, higher LDH level, elevated total leukocytic count (TLC) and a lower platelet count in Bcl-2 positive NHL cases while in a study performed by Hanan-Yasmine.(27) Bcl-2 expression is significantly associated only with high LDH level & low platelets count but no statistical significance was found as regards age & total leukocytic count.

Hadzi-Pecova *et al.*(28) confirmed that the expression of Bcl-2 protein is significantly present in advanced stage compared with early stages of B-cell lymphoma. Similarly, Hermine *et al.(29)* high Bcl-2 expression was more frequently associated with stages III and IV while in the current study there is no statistical significance between Bci-2 expression & lymphoma staging.

In conclusion, this study confirmed that Bcl-2 was expressed mainly in B- NHL. B-cl2 is expressed more frequently in follicular lymphoma especially low grade follicular lymphoma so this protein should be considered as reliable prognostic marker. This work may help to stratify patients of B- non Hodgkin lymphoma who could benefit from accurate sub-typing, grading & targeted therapy using Bcl-2 immunostaining, though it seems necessary to study B-cl2 expression on a larger number of patients to approve its role in B-NHL.

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