Journal of the Egyptian Society of Haematology & Research, Vol. 1, No. 1, March: 65-74, 2005

Detection of BCL-2 Family Proteins in Lymphoid Malignancies: An Immunocytochemical and Immunoelectron Microscopic Study

MAGDA MAHMOUD AHMED ASSEM, M.D.*; NAGLAA MOHAMED KHOLOUSSI, M.D.**; MAHA SALEH MADBOULY, M.D.*; MAHA ABDEL-HAMID ALI EL-TAWEEL, M.D.*; GABALLA HUSSEIN, M.D. and AMIRA KHORSHED, M.D.*

*The Departments of *Clinical Pathology and Medical Oncology, National Cancer Institute, Cairo University and the **Immunogenetic Department, National Research Center.*

ABSTRACT

Background Methods and Purpose: The apoptotic process is under the control of many regulatory genes that have been identified to be involved in inducing apoptosis e.g. p53, Bax and c-myc or inhibiting it e.g. Bcl-2 and Bag. This work aimed at studying the expression of Bcl-2, Bax, Bcl-x and p53 in various lymphoid malignancies using immunohistochemistry inorder to find out whether these oncoprotiens/tumour suppressor gene products have a possible influence on disease progression and outcome as regards to relapse and survival. Additionally, an immune-electron microscopic study was done for proper intracellular localization of Bcl-2.

Patients: The study was carried out prospectively on 60 patients with lymphoid malignancies of which 39 (31 Denovo and 8 cases in relapse) were acute lymphoblastic leukaemia (ALL), and 21 were chronic lymphocytic leukaemia (CLL).

Results: Results revealed a statistically significant higher expression of Bcl-2 and Bax as compared to the control group in ALL (p=0.03 & 0.045 respectively). No correlation was found between Bcl-2 and response to treatment or overall survival in both groups. Bcl-2 expression was significantly higher in stages III and IV as compared to stages I and II in the CLL, thus suggesting a possible role played by Bcl-2 in the poor outcome of advanced disease. Bax showed no correlation to the response of the cells to chemotherapy in both groups of lymphoma studied. Bcl-x showed no statistically significant difference as compared to the control group in both groups. Expression of p53 was significantly higher in the CLL as compared to the control group (p=0.015). There was a significant positive correlation between Bcl-2/Bax ratio and BM lymphocytes i.e. tumour burden (r=0.778, p< 0.01) in the CLL group. In the ALL, the eight relapsed cases showed a statistically significant higher expression of Bcl-2, and Bcl-x (p=0.006, and 0.013) and a statistically lower expression of Bax (p=0.005) when compared to the de novo ones. Immune electron microscopic localization of Bcl-2 and the two-step indirect immune peroxidase method was done. Bcl-2 was visualized in the outer and inner mitochondrial membranes, rough and smooth endoplasmic reticulum and the nuclear envelop.

Conclusion: From this study it can be concluded that: First, Egyptian CLL (chronic lymphocytic leukaemia) patients might be having a different disease biology since they are having more Bcl-2 expression (81% compared to the 73% in the Western CLL), less Bax (48% compared to 96%), more p53 (43% compared to 15%), present at younger age (age range in this study was 37-70y compared to 60-90y in the Western CLL), and present at a more advanced stage (Rai stages III & IV more than stages I & II). Second, Bcl-2/Bax ratio can be used to monitor tumour burden in chronic lymphocytic leukaemia owing to the significant (r=0.778, p<0.01) positive correlation found between this ratio and bone marrow lymphocytes. Third, because Bcl-2 is expressed in 81% of CLL and because all nine cases with positive Bcl-2 expression showed evidence of apoptosis using the electron microscopy, the mechanism of this apoptosis is suggested to be away from the mitochondrial pathway, either through an extrinsic pathway by passing the mitochondrial pathway or through an alternate pathway. Fourth, having a statistically higher expression of the prosurvival Bcl-2, and Bcl-x, and a statistically lower expression of the proapopotic Bax, in relapsed ALL compared to the denovo cases, might explain the cause of relapse as a result of emergence of a resistant clone expressing more prosurvival (Bcl-2 & Bcl-x) and less pro-apoptotic (Bax) markers. Finally, Bcl-2 expression in most of our CLL cases makes it an attractive target for the therapeutic treatment, by using Bcl-2 oligonucleotide antisense or the naturally available Genasense (Bcl-2 antisense).

Key Words: *Bcl-2, Bax - Bcl-x - p53 - ALL & CLL - IHC & immune-electron microscopy.*

INTRODUCTION

The control of cell number and type in multicellular biological system is extremely necessary for maintaining quantitative and functional homeostasis at the cellular level. This control of cell number is maintained through a balance

between cell proliferation and cell death. Each cell possesses its own self-induced destruction which is called programmed cell death **[1]**. Apoptosis- a matter of programmed cell death- is an active, gene-directed, self-inflicted process. It is associated with characteristic morphological & biochemical changes that ends in dissociation of a cell into membrane- bound apoptotic bodies, which are then engulfed by the local phagocytic system **[2]**.

Failure of the cells to undergo apoptosis leads to many diseases e.g. viral infections, autoimmune diseases and cancer. CLL is considered a disease with progressive accumulation of lymphocytes failing to undergo apoptosis. ALL, on the other hand, is a disorder due to uncontrolled increase in proliferation **[3]**. The apoptotic process is under the control of many regulatory genes, some are involved in inducing apoptosis e.g. p53, Bax and c-myc, others are inhibiting apoptosis e.g. Bcl-2 and Bag **[4]**.

This work aimed at studying the expression of Bcl-2, Bax, Bcl-x and p53 in the two distinct groups of lymphoid malignancies, one with increased proliferation and the other with defective apoptosis using immunohistochemistry, in order to find out whether these oncoprotiens/tumour supperssor gene products have a possible influence on disease progression and outcome as regards to relapse and survival. Additionally, immune-electron microscopic study was done for proper intracellular localization of Bcl-2.

PATIENTS AND METHODS

Patients:

The study was carried out prospectively on 60 patients with lymphoid malignancies of which: 39 were acute lymphoblastic leukaemia and 21 were chronic lymphocytic leukaemia. The age range was from 3-80 years in ALL group and 37-70 years in the CLL group. Male: female ratio was 1.5: 1.0 in both groups. Additionally, age- and sex-matched subjects were taken as controls. The patients presented to the National Cancer Institute, Cairo University and subjected to full clinical examination, CBC and BM examination. Immunophenotyping was done for proper lineage affiliation as well as immunohistochemical detection of Bcl-2, Bax, Bcl-x, and p35 using their corresponding monoclonal antibodies. Immune-electron microscopy was done for selected cases (nine CLL and two ALL) with high Bcl-2 expression.

Methods:

Mononuclear cell separation and test for viability were done according to Perper et al. **[5]** and Weir **[6]** respectively.

Immunohistochemistry was done according to Bisgaard and Pluzek **[7]**. The monoclonal antibodies used were as follows:

Bcl-2, Boehringer Mannheeim, Cat. No1624 989.

Bcl-x, Pharmingen BD, Cat. No 66461 A.

Bax, Zymed Laboratories Incorporation, Cat. No 18- 0218.

P53, Dako, Denmark, Cat. No M 7001.

The detection system was Dako Envision+ system, peroxidase, for mouse monoclonal antibodies and DAB as a chromogen, Cat. No 4007.

Interpretation of results:

At least 500 cells were counted for each monoclonal antibody. Negative cells appear homogeneously green while positive cells appear as brown black positivity on the cell. The results are expressed as % of positivity. A cut-off ≥ 5% of the tumour cells with unequivocal positivity in the cytoplasmic membrane and nuclear periphery was used for Bcl-2, Bax, and Bcl-x while a cut-off of ≥ 1% of the lymphoid cells with strong nuclear staining was used for p53 **[8]**.

Bcl-2/ Bax ratio was calculated by dividing the number of cells positive for Bcl-2 by the number of cells positive for Bax.

Immune-electron Microscopy: **[9]**

Immunoelectron Microscopy:

Mononuclear cells were fixed with 4% paraformaldhyde in PBS for 20 min., centrifuged at 200xg for 5 min., washed three times in PBS and stained using two-step indirect immunoperoxidase method. Following two washes, cells were fixed with 2% glutaraldehyde in PBS for 30 min and post fixed with 1% osmium tetroxide in PBS. After 2 washes, cells were stained with 1% uranyl acetone in PBS for 10 min, dehydrated and embedded in Epon 812 according to

routine procedures. Ultra thin sections were prepared and examined in a Joel electron microscope.

Statistical analysis was done according to Ingelfinger et al. **[10]** and Knapp and Miller **[11]**. Survival analysis and curves were done according to Kaplan Miere method. Log rak test was used for comparing survival. Significance level of 0.05 and 0.01was used throughout all statistical tests in the study.

RESULTS

Survival:

Median overall survival for two years of the CLL group was studied among Bcl-2, Bax, Bcl-x and p53 positive cases as compared to negative ones. Bcl-2 positive cases had a significantly shorter survival as compared to Bcl-2 negative ones (p=0.049). Bax, Bcl-x and p53 had no impact on the survival (p=0.46, 0.84, and 0.87 respectively). These data are shown in Fig. (1).

Immunoelectron Microscopy:

Immunoelectron microscopy was carried out for intracellular localization of Bcl-2 protein. Lymphoid cells from an acute lymphoblastic leukaemia case showed lymphoblastoid features characterized by a large nucleus & scarce cytoplasm. In the cytoplasm, abundant mitochondria, smooth & rough endoplasmic reticulum were observed. This is different from the chronic lymphocytic leukaemia case in which cell had sparse intracellular organelles. In both cases, immunoreactivity with anti-bcl-2 antibody was detected prominently in the mitochondria in both outer & inner mitochondrial membrane i.e. in between cisternea, smooth & rough endoplasmic reticulum membrane & in the nuclear membrane. These findings are shown in Figs. (4-6). Some apoptotic changes in the form of increased vesicles, dilated endoplasmic reticulum and condensation of heterochromatin were seen in Fig. (4).

Table (1): Expression of different markers among ALL, CLL and control cases.

Marker	ALL (n=39)	Control (n=10)	*p* value
Bcl-2	23 (59%)	4 (40%)	0.47
Bax	7 (18%)	1 (10%)	0.47
Bcl-x	8 (21%)	2 (20%)	0.67
P53	11 (28%)	0	*0.06
Marker	**CLL (n=21)**	**Control (n=10)**	***p* value**
Bcl-2	17 (81%)	4 (40%)	*0.03
Bax	10 (48%)	1 (10%)	*0.045
Bcl-x	7 (33%)	2 (20%)	0.38
P53	9 (43%)	0	*0.015

* Significant.

Table (2): Expression of different markers in relation to response to treatment among ALL cases and CLL cases respectively.

	CR+PR Mean% ± SD	Resistant Mean% ± SD	*p*-value	CR+PR Mean% ± SD	Resistant Mean% ± SD	*p*-value
Bcl-2	0.112±0.172	0.136±0.225	0.805	0.375±0.227	0.220±0.309	0.265
Bax	0.080±0.164	0.062±0.093	0.820	0.098±0.142	0.034±0.076	0.359
Bcl-x	0.013±0.046	0.096±0.169	0.090	0.111±0.219	0.038±0.085	0.489
P53	0.038±0.064	0.068±0.102	0.442	0.046±0.072	0.066±0.064	0.595
Bcl-2/Bax	5.32±9.29	0.27±0.42	0.377	8.581±11.31	0.059±0.001	0.507

Table (3): Expression of different markers in de novo and relapsed cases among ALL group.

	Denovo n=31	Relapse n=8
Bcl-2	7/31	8/8
Bax	5/31	3/8
Bcl-x	3/31	5/8
P53	12/31	4/8

Table (4): Expression of different markers in relation to disease stage among CLL cases.

	I & II		III & IV		*p*-value
	Mean%	SD	Mean%	SD	
Bcl-2	0.11	0.10	0.38	00.25	0.019*
Bax	0.12	0.08	0.07	00.13	0.450
Bcl-x	0.15	0.22	0.09	00.20	0.534
P53	0.16	0.24	0.04	00.07	0.080
Bcl-2/Bax	1.05	1.53	14.5	17.45	0.121

* Significant.

Table (5): Expression of different markers in Egyptian and Western CLL cases.

	Egyptian CLL	Western CLL
Bcl-2	81%	*73%
Bax	48%	*96%
Bcl-x	33%	?
P53	43%	**15%

* Logdon et al. (1993). ** Cordone et al. (1998).

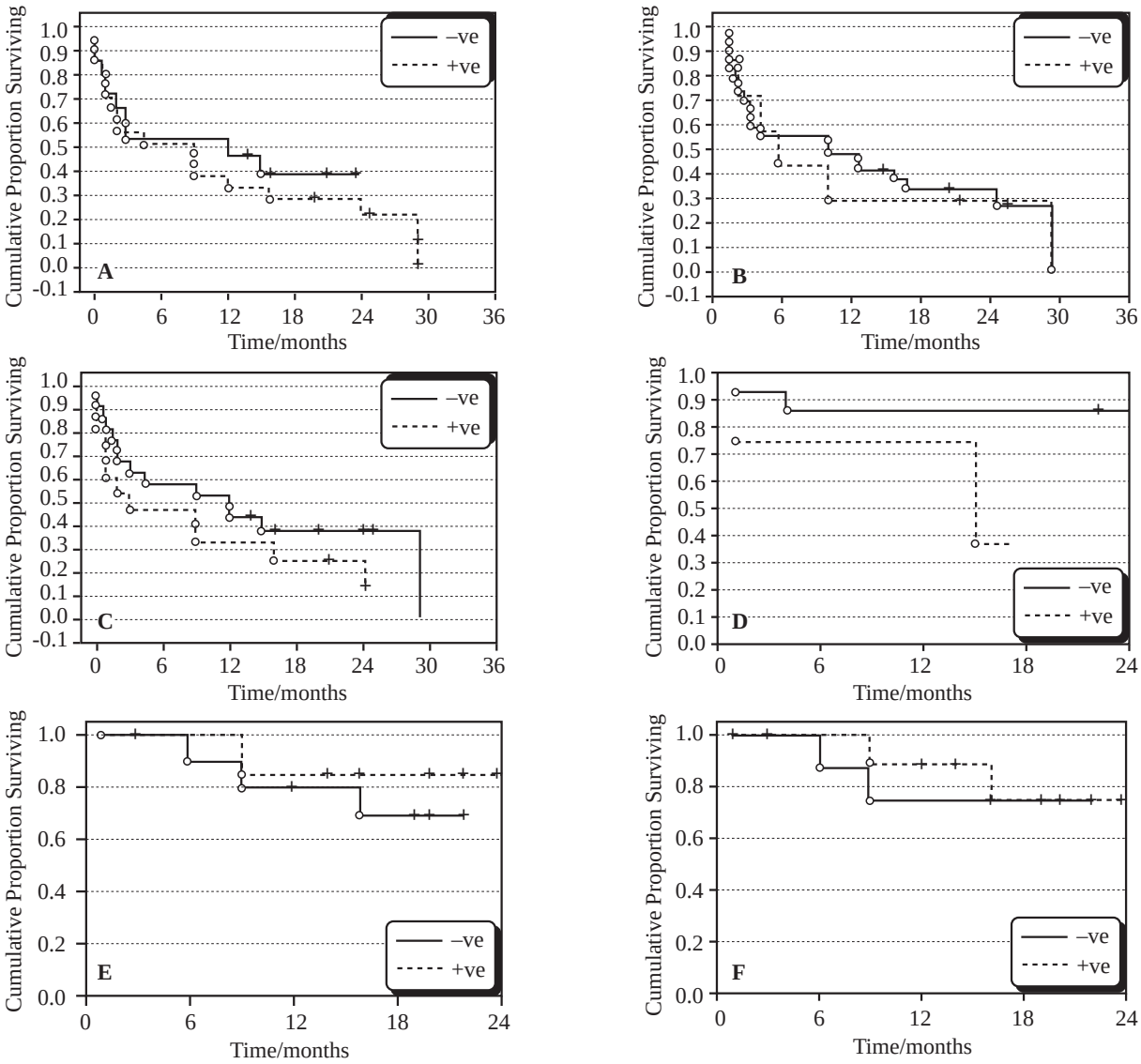

Fig. (1): (A, B, C) Overall survival of the ALL group in relation to Bcl-2, Bax and p53. ºComplete + censored *p* value = 0.54, 0.9 and 0.7 respectively. (D,E,F) Overall survival of the CLL group in relation to Bcl-2, Bax and p53 º Complete + Censored *p* value = 0.049*, 0.46 and 0.87 respectively.

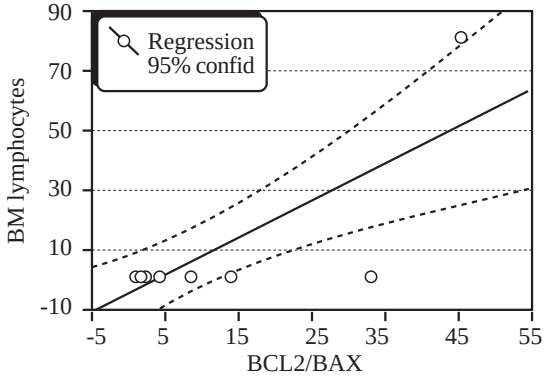

Fig. (2): Correlation between Bcl-2/Bax ratio vs BM lymphocytes in CLL cases Correlation: *r*=0.77800 *p*-value <0.01.

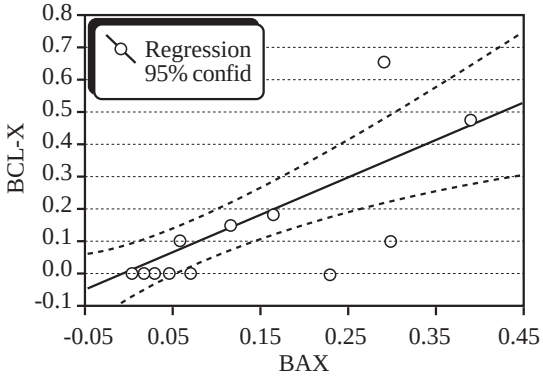

Fig. (3): Correlation between Bax vs. Bcl-x in CLL cases. Correlation: *r*=0.69 *p* value <0.05.

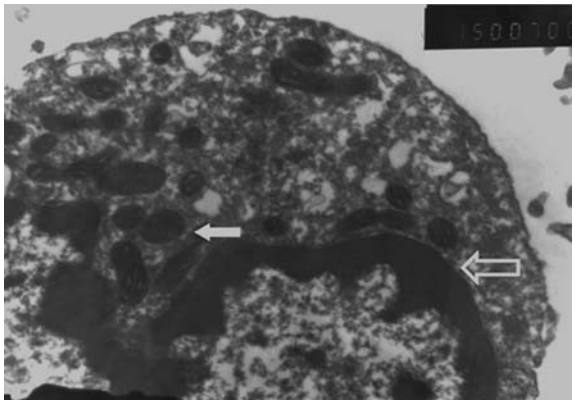

Fig. (4): Immunoelectron micrograph for Bcl-2 in a lymphocyte showing the distribution of Bcl-2 in mitochondria and nuclear membrane (empty arrow: Nuclear membrane, white arrow: Mitochondria). Mild apoptotic changes in the form of condensation of heterochromatin and increased vesicles. X15 000.

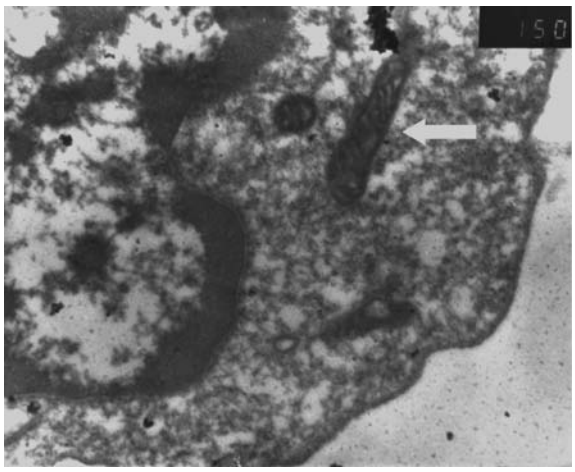

Fig. (5): Immunoelectron micrograph for Bcl-2 in a lymphocyte showing the distribution of Bcl-2 inside the mitochondrial cisternea (white arrow: Mitochondria) X20 000.

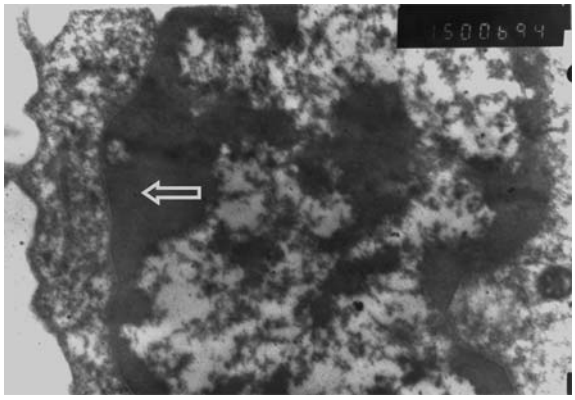

Fig. (6): Immunoelectron micrograph for Bcl-2 in a lymphocyte showing the distribution of Bcl-2 in the nuclear membrane and endoplasmic reticulum (empty arrow: Nuclear membrane) X15 000.

DISCUSSION

Cell division and survival are tightly linked to assure genomic integrity and tissue homeostasis. Failure to apoptose results in propagation of the damaged DNA and the survival of superfluous and potentially harmful cells **[12]**. Apoptosis or programmed cell death is a physiological, active, gene-directed, self-inflicted process by which normal and tumour cells are believed to die **[1]**.

Apoptosis occurs in 3 phases: Initiation, effector phase, and degradation. Susceptibility of a cell to apoptotic signals appears to be regulated, in part, by the relative levels and dimerization between Bcl-2 family members, which act on the effector stage of apoptosis, as well as on the competition for other cellular factors involved in the death pathway **[13]**. Bcl-2 family members can be divided into 3 groups: anti-apoptotic as Bcl-2 and Bcl-xL, pro-apoptotic as Bax and BH3 only proteins as Bid **[14]**. Another protein involved in apoptosis is the tumour suppressor gene, p53, which is involved in cell cycle arrest and induction of apoptosis in genetically damaged cells **[15]**.

In our study, Bcl-2 was expressed in 23/39 (59%) in ALL, in 17/21 (81%) in CLL and in 4/10 (40%) in the control group. A statistically significant higher expression of Bcl-2 as compared to the control group was found in the ALL (p=0.03) but not in the CLL group (p= 0.47). This might be attributed to the relatively small number of CLL subjects studied, increasing the number of CLL cases would bring such borderline difference to the statistical level. Our results are in agreement with Papakonstantinon et al. **[16]** who found a higher expression of Bcl-2 in mature B-cells of CLL/SLL and follicle center cell lymphoma (FCC) than in the immature B-cells of diffuse large B-cell lymphoma.

In our results, Bcl-2 expression of 81% in the CLL group is slightly higher than Logsdon et al. **[17]** who found Bcl-2 in 73% of FCC lymphoma by IHC. However, both Johnston et al. **[18]** and Gaballa et al. **[19]** found Bcl-2 in all cases of B-CLL/SLL by Western Blot (WB) technique.

Bcl-2 was detected in 59% of our ALL cases. This was higher than Gascoyne et al. **[20]** who reported an incidence of 24% using IHC and

lower than both Campana et al. **[21]** who detected Bcl-2 in all their adult ALL cases, Hogarth and Hall **[22]** who detected Bcl-2 in all their cases by WB technique, and Papakonstantinou et al **[16]** who found gene rearrangement in 85% of disseminated lymphoblastic lymphoma by PCR. In the latter study, they found no correlation between gene rearrangement and protein expression as some Bcl-2 translocations may fail to express Bcl-2 protein as a result of mutations in the open reading frame of the translocated Bcl-2 gene leading to absent or diminished Bcl-2 production. On the other hand, Bcl-2 protein expression may be absent in some cases with translocation, suggesting that mechanisms other than translocation can lead to Bcl-2 overexpression. This suggestion was also emphasized by Almasri et al. **[23]** in their study on 28 Jordanian NH lymphoma cases. Accordingly, Bcl-2 protein expression (by either Western Blot or Immunocytochemistry) is far more important than gene rearrangement analysis. Variations in the level of Bcl-2 expression in different studies may be due to different cut-offs used as well as variations among techniques in terms of sensitivity and specificity, its detection signifies its overexpression regardless of the underlying molecular mechanism.

Although Uckum et al. **[24]** found a significant association between high Bcl-2 and low WBC count and Gascoyne et al. **[20]** with high S.LDH, our results showed no significant correlation as regards age, gender, lab. prognostic features (Hb, Platelet count, S.LDH) and immunophenotype. However, both Salomon et al. **[25]**, Srinnivas et al. **[8]**, and Campos et al. **[26]** found similar results.

No correlation was found between Bcl-2 and response to treatment in ALL or CLL groups. This was consistent with that of Logsdon et al. **[17]** and Mekki et al. **[27]** who found no correlation between Bcl-2 and drug resistance or treatment outcome. In contrast to these studies, Baghdassarian et al. **[28]** found that high Bcl-2 expression was linked to poor response to cytotoxic drugs and both Maung et al. **[29]** and Tang et al. **[30]** reported a greater intensity of Bcl-2 staining in cells from ALL patients who failed to achieve complete remission than those who responded to treatment. Other studies concluded that low level of Bcl-2 was related to poor response and shortened survival in childhood ALL **[31]** and myelodysplastic syndrome **[32]**. The discrepancy between the results may be related to the presence of other Bcl-2 family proteins as Bax & Bcl-x modulating the effect of Bcl-2. Variability to the response to treatment could also be attributed to the variability in the drugs used or the presence of drug resistance. Moreover, Alderson et al. **[33]** reported that Bcl-2 expression may be associated with a mutant p53 gene, thereby conferring the resistant phenotype.

In our study, no statistically significant difference was found between Bcl-2 expression and overall survival in the ALL group. Similar results have been reported by many authors **[26,34,35,36]**. In contrast, Gascoyne et al. **[20]** reported a significant difference in overall survival in DL cases. Variations among studies may be due to differences in the uniformity of the patients selected, tumour bulk, or treatment given. A longer follow-up may also reveal more significant results.

Bcl-2 expression has a statistically worse survival in our CLL group. This is not in agreement with both Prowit-MacDonald et al. **[31]** and Lepelley et al. **[32]** as both reported that low Bcl-2 level was associated with shortened survival or Robertson et al. **[37]** who reported that high Bcl-2 expression is associated with adverse outcome.

Bcl-2 expression is significantly higher in stages III and IV as compared to stages I and II in the CLL suggesting a possible role played by Bcl-2 in poor prognosis and advanced disease.

The conformational changes of Bcl-2 and interaction between different family member may contribute to the different levels of Bcl-2 protein expression and the different effects and correlation of Bcl-2 protein with the clinical and laboratory prognostic factors. There is an evidence that post-transcriptional modifications regulate the function of Bcl-2 and possibly other family members **[20]**. This post-transcriptional modification might be the cause of discrepancy between Bcl-2 gene rearrangement and protein expression. Several studies have suggested that phosphorylation of Bcl-2 affects its function, although there have been conflicting reports that this may enhance **[38,39]** or reduce **[40]** its ability to suppress apoptosis. It is also possible

that mutations may alter the function of these proteins by, for example, affecting protein stability **[20]**. Also, Bcl-2 expression may be affected by infection with EBV **[41]** and human CMV **[42]**. IL-5 **[43]** and IL-10 **[44]** have also been reported to induce Bcl-2 expression.

Bax was expressed in 7/39 (18%) of the ALL, 10/21 (48%) of the CLL, and in 1/10 (10%) of the control group. Bax expression was significantly higher than the control group (p= 0.045) in the ALL.

Bax had a range of 0-53% and this was comparable to that of Srinivan et al. **[8]** who reported a range of 1-60%. Bax expression in the CLL was lower than that reported by Logsdon et al. **[17]** who detected an expression of 96% in FCC by IHC.

In the present study, Bax expression in the ALL was lower than Hogarth & Hall **[20]** who detected Bax in all their childhood ALL cases by WB technique. Differences in the level of expression of Bax may be due to the cut-off used, sensitivity of the monoclonal antibody and the sensitivity of the WB technique used to assess the level of Bax.

Bax showed no correlation to the response of the cells to chemotherapy in both groups of lymphoid malignancies studied and this was in agreement with both Hogarth & Hall **[22]** and Srinivas et al. **[8]** on their paediatric ALL cases.

Bcl-x was expressed in 8/39 (21%) of the ALL, 7/21 (33%) of theCLL, and in 2/10 (20%) of the control group. Bcl-x showed no statistically significant difference as compared to the control group both in the CLL and ALL.

Bcl-x used in this study had a common epitope for both Bcl-xl (anti-apoptotic) and Bcl-xs (pro-apoptotic). So, our results were difficult to interpret as due to presence of Bcl-xl or Bcl-xs. However, and in view of the significant positive correlation between Bcl-x and Bax in the homogenous group of CLL, a possible predominance of Bcl-xs (pro-apoptotic) effect could be elucidated in the antibody used (r= +0.58, p< 0.05). Simonian et al. **[13]** found that in FL5.12 lymphoid cell lines, the expression of both Bcl-2 and Bcl-xl has been implicated in the drug resistance of the tumour to therapy and Bcl-xl was better able than Bcl-2 to protect the cells from death induced by certain chemotherapeutic drugs. The study concluded that Bcl-xl expressing tumour cells may be more likely to escape initial destruction by certain chemotherapeutic agents than neoplasms expressing Bcl-2 and thus increasing the likelihood of relapse. In view of the above, and in view of the presence of complete remission in 5 of the relapsed ALL cases expressing Bcl-x i.e. none was resistant to chemotherapy (data not shown) provide a 2nd support of a Bcl-xs effect (pro-apoptotic) of the Bcl-x used in the present work.

P53 was expressed in 11/39 (28%) of the ALL, 9/21 (435%) of the CLL and in none of the control group. Expression of p53 was significantly higher in the CLL as compared to the control group (p=0.015).

The level of p53 expression was variable in the different reports. While it was 43% of our CLL cases, Cordone et al. **[15]** reported a 15% expression in CLL using IHC. Similarly, p53 was expressed in 28% of our ALL as compared to the 38% expression reported by Srinivas et al. **[8]** using ELISA. This variability may be due to different cut offs used and/ or sensitivity of the methods used for p53 detection as ELISA is known to be a more sensitive quantitative technique.

Bcl-2/Bax Ratio:

In the CLL, there was a significant positive correlation between Bcl-2/Bax ratio and BM lymphocytes i.e. tumour burden (r= 0.778, p< 0.01). This finding may correlate with both Thomas et al. **[45]** and Hogarth & Hall **[22]** who reported that increased Bcl-2/Bax ratio correlates with tumour burden and in-vitro resistance to drug-induced apoptosis. However, and in contrast to their results, we could not find any significant correlation between Bcl-2/Bax ratio and in vivo response to therapy.

Expression of the Different Markers in Relapsed Cases:

In the ALL group, the eight relapsed cases showed a statistically significant higher expression Bcl-2, Bax, and Bcl-x as compared to the de novo ones (p= 0.006, 0.005, and 0.013 respectively).

Our finding of higher Bcl-x in relapsed cases (5/8, 63%) than de novo ones (3/31, 10%) is in agreement with Findley et al. **[46]** Additionally,

Datta et al. **[47]** and Minn et al. **[48]** found that Bcl-xl over expression in murine cells and histiocytic lymphoma cells was associated with increased resistance to chemotherapeutic agents in vitro. However, and in contrast to his results, our cases were not drug resistant (five of eight entered complete remission) and the only one with partial remission, showed resistance to chemotherapy and was negative for Bcl-x. This could be attributed to-as mentioned previously-to a Bcl-xs rather than a Bcl-xl effect.

Correlation between Bcl-2 and p53:

Expression of Bcl-2 is down-regulated by the tumour suppressor gene p53. The relative expression and function of these molecules may therefore determine the extent of apoptosis **[49]**. Although, such correlation in our cases was found insignificant, yet it may give an attention to the relationship between Bcl-2 and p53 because only those with high level of Bcl-2 are associated with mutant p53 expression. Several authors reported similar results; Findley et al. **[46]** on paediatric ALL, Pezzela et al. **[50]** on NHL, and Hader et al. **[51]** on cancer breast. These findings could be attributed to the fact that there are p53-dependent and p53-independent apoptotic pathways as well as the heterogenity of the lymphoma group as regards apoptosis. It is worth mentioning that our p53 negative cases were either wild-type or null-type further explaining the insignificant correlation. Also Hussein et al. **[52]** reported that lymphoproliferative lesions had some peculiar clinicopathologic features and that Bcl-2 and p53 proteins are altered in the lymphoproliferative lesions in upper Egyptain cases.

Bcl-2 Localization by Immune Elelctron Microscopy:

Immune electron microscopic localization of Bcl-2 and the two-step indirect immune peroxidase method was done. Bcl-2 was visualized in the outer and inner mitochondrial membranes, rough and smooth endoplasmic reticulum and the nuclear envelop. This coincides with the findings previously reported by Yurihiro et al. **[53]** using the same method and by Lombardi et al. **[54]** using immune gold electron microscopy method on lymphoblastoid cell line.

The pleotropic distribution of Bcl-2 protein argues against the likelihood that mitochondrial function per se is involved in the apoptosis-blocking activity of Bcl-2. Additionally, Bcl-2 may also protect different organelles in the cytoplasm by the same mechanism. Bcl-2 has been suggested to be involved in the antioxidant pathway **[55]** as most of the reactive oxygens are produced in the mitochondria, nuclear envelop and endoplasmic reticulum **[56]**. Luciano et al. **[57]** suggested-through their work on lymphoblastoid cell line using immune gold technique- that Bcl-2 is associated with the nuclear pore and mitochondrial functional complexes; where the inner and outer membranes of these DNA containing organelles come into contact. This location of the Bcl-2 protein suggests that Bcl-2 - at least in part- acts as an adapter protein that targets cytosolic Raf kinase and p23-R-Ras to critical substrates. This is based on the reported data that revealed the association between Bcl-2 and p27 raf-1, **[58]** a serine/ threonine-specific protein kinase and with p23- R- Ras, **[59]** a Gtpase member of the Ras family.

Conclusion:

- Egyptian CLL cases might be having a different disease biology when compared to the Western ones.
- Bcl-2/ Bax ratio had a significant positive correlation with BM lymphocytes in CLL possibly a reflection of tumour burden.
- Because Bcl-2 is expressed in 81% of the CLL cases and because all nine cases with positive Bcl-2 expression showed evidence of apoptosis using EM, the mechanism of such apoptosis is suggested to be away from the mitochondrial pathway i.e. extrinsic or alternate pathways.
- In relapsed ALL, Bcl-2 and Bcl-x were significantly higher while Bax was significantly lower as compared to the de novo ones, possibly explaining the emergence of relapse from a resistant clone expressing the prosurvival markers (Bcl-2 and Bcl-x).
- Bcl-2 expression in most Egyptian CLL cases makes it an attractive target for Bcl-2 oligonucleotide antisense or the naturally available Genasense (Bcl-2 antisense).

REFERENCES

1- Sanders CA and Wride MA. Programmed cell death in development. International Review of Cytology, 1995, 163: 105-173.

2- Kerr JFR, Wyllie AH and Currie AR. Apoptosis, a basic biological phenomenon with wide ranging im-

plication in tissue kinetics. Br. J. Cancer, 1972, 26: 239-257.

3- Vaux DL, Weissman IL and Kim SK. Prevention of programmed cell death in caenorhabditis elegans by human Bcl-2. Science, 1992, 235: 1955-1957.

4- Campana D and Cleveland JL. Regulation of apoptosis in normal haemopoiesis and haematologic disease in: Brenner MK and Hoffbrand AV: Recent advances in Haematology (8), 1st edition, 1996, British Library and library of Congress cataloging ch. 1, p1-20

5- Perper R, Zee TW and Mickelson MM. Purification of lymphocytes and platelets by gradient centrifugation. J. Lab. Clin. Med., 1989, 72: 842.

6- Weir DM. Handbook of Experimental Immunolgy. 3rd edition. Black Well. Oxford. 1987, p15.

7- Bisgaard K and Pluzek KP. Use of polymer conjugate in immunohistochemistry: Comparative study of a traditional staining method to a staining method utilizing polymer conjugates. XXI Intl Cong Intl Pathol and 12th World Cong Acad Environ Pathol. Budapest, Hungary. Oct. 1996, 20-25.

8- Srinivas G, Kusumarumary P, Krishnan MN, Ramachandra KP and Radhakrishna MP. Mutant p53 protein, Bcl-2/ Bax ratio and apoptosis in paediatric acute lymphoblastic leukaemia. J. Cancer Res. Clin. Oncol. 2000, 126: 62-67

9- Catovsky D, (editor), IN The leukaemic cells. Churchill Livingstone Publishing, New York. 1991, pp427.

10- Ingelfinger JA, Mosteller FM, Thibodeau LA and Ware JH. Biostatistics in Clinical Medicine. McCraw-Hill Inc., New York. 1994.

11- Knapp RG and Miller MC. Clinical Epidemiology and Biostatistics. Harvard Publishing Co., Pennsylvania, 1992.

12- Zhong J, Troppmair J and Rapp UR. Independent control of cell survival by Raf-1 and Bcl-2 at the mitochondria. Oncogene. 2001, 20: 4807-4816.

13- Simonian PL, Grillot DA and Nusnez G. Bcl-2 and Bcl-xl can differentially block chemotherapy-induced cell death. Blood, 1997, 90: 1208-1216.

14- Tsujiomoto Y and Shimizu S. Bcl-2 family: life or death switch. FEBS letters. 2000, 466: 6-10.

15- Cordone L, Masi S and Romana Mauro S. p53 expression in B-cell chronic lymphocytic leukaemia: a marker of disease progression and poor prognosis. Blood. 1998, 91: 4342- 4349.

16- Papakonstantinou G, Verbeke C and Hastka J. Bcl-2 expression in Non Hodgkin's Lymphoma is not associated with Bcl-2 gene rearrangements. Br J Haemato. 2001, 1113: 383-390.

17- Logsdon MD, Frigerio S, Collini P and Pilotti S. Apoptosis and the Bcl-2 family- pattern of expression and prognostic value in stage I and II follicular center lymphoma. Int. J. Radiation Oncology Biol. Phys. 1999, 44, 1: 19-29.

18- Johnston JB, Verburg L and Lee K. p53, Bax, and Bcl-2 drug resistance in chronic lymphocytic leukaemia. Leuk. Lymphoma. 1997, 26: 435-449.

19- Gaballa MR, Saleme MS and El-Dansoury AM. Bcl-2 and Bax expression in CLL and their relation with apoptosis, MD thesis in clinical pathology, Ein Shams University. 2000, 97-115.

20- Gascoyne RD, Adomat SA and Krajewski S. Prognostic significance of Bcl-2 protein expression and Bcl-2 gene rearrangement in diffuse aggressive Non-Hodgkin's Lymphoma. Blood. 1997, 90: 244-251.

21- Campana D, Coustan-Smith E and Manabe A. Prolonged survival of B-lineage acute lymphoblastic leukaemia cells is accompanied by over expression of Bcl-2 protein. Blood. 1993, 181: 1025-1031.

22- Hogarth L and Hall A. Increased Bax expression is associated with an increased risk of relapse in childhood acute lymphoblastic leukaemia. Blood. 1999, 93: 2671-2678.

23- Almasri NM, Al-Alami J and Faza M. Bcl-2 gene rearrangement in Jordanian follicular and diffuse large B-cell lymphomas. Saudi Med J. 2005, 26 (2): 251-5.

24- Uckum F, Yang Z and Salter H. Cellular expression of anti-apoptotic Bcl-2 oncoprotein in newly diagnosed childhood acute lymphoblastic leukaemia. Blood. 1997, 89 (10): 3769-3777.

25- Campos L, Sbido O and Sebban C. Expression of Bcl-2 proto-oncogne in adult acute lymphoblastic leukaemia. Leukaemia 1996, 10: 434-438.

26- Salomon GS, Snets LA and Hart A. Bcl-2 family members in childhood acute lymphoblastic leukaemia. Leukaemia, 1999, 13: 1574-1588.

27- Mekki Y, Catallo R and Manel AU. Enhanced expression of p16 is associated with a poor prognosis in childhood acute lymphoblastic leukaemia. Leukaemia. 1999, 13: 181-189.

28- Baghdassarian N, French P and Duhaut P. Role of Bcl-2 and cell cycle regulatory proteins for corticosensitivity assessment in childhood acute lymphoblastic leukaemia. British J of Haematology. 2000, 109: 109-116.

29- Maung ZI, MacLeanFK, Reed MM, et al. The relationship between Bcl-2 expression and response to chemotherapy in acute leukemia. Br. J. Haematology. 1994, 88: 105-109.

30- Tang C, Willingham MC and Reed JC. High levels of p26 Bcl-2 oncoprotein retard taxol-induced apoptosis in human pre-B leukaemia cells. Leukaemia. 1994, 8: 1960-1969.

31- Prowit-Mac Donald A, Ivory K and Wilkinson S. Bcl-2 protein expression in normal human bone marrow precursors and in acute myelogenous leukaemia. Leukaemia. 1995, 9: 1191.

32- Lepelley P, Soenen V and Preudhomme C. Bcl-2 expression in myelodysplastic syndrome and its correlation with hematological features, p53 mutations and prognosis. Leukaemia. 1995, 9: 726.

33- Alderson LM, Castelberg RL, Harsh GR, Louis DN and Hinson JW. Human gliomas with wild-type p53 express Bcl-2. Cancer Res. 1995, 55: 999

34- Hermine O, Haioun C, Lepage E, d'Agay MF, Briere J, Lavignac C, Deibold J, Reyes F and Gaularol P. Prognostic significance of Bcl-2 expression in aggressive Non Hodgkin's Lymphoma. Blood. 1996, 87: 265.

35- Hill ME, Maclennan KA and Cunnningham DC. Prognostic significance of Bcl-2 protein expression and Bcl-2 major breakpoint region rearrangement in diffuse large cell Non-Hodgkin's Lymphoma. Blood. 1996, 88: 1046.

36- Kramer MHH, Herman J, Parker J, Krol ADG, Kluin-Nelemans JC, Haak HL, van Groningen K, VAN Krieken JHJM, de jong D and Kluin PM. Clinical significance of Bcl-2 and p53 protein expression in diffuse large B-cell lymphoma: A population-based study. J. Clin. Oncol. 1996, 14: 2131.

37- Robertson LE, Plunkett W, McConnell K, Keating MJ and McDonell TJ. Bcl-2 expression in chronic lymphocytic leukaemia and its correlation with the induction of apoptosis and clinical outcome. Leukaemia. 1996, 10: 456.

38- Ito T, Deng X, Can B and May WS. Bcl-2 phosphorylation required for anti-apoptotic function. J. Biol. Chem. 1997, 272: 11671.

39- Horiuchi M, Hyashida W and Kanbe T. Angiotensin type 2 receptor dephosphorylates Bcl-2 by activating mitogen activated protein kinase phosphatase-1 and induces apoptosis. J. Biol. Chem. 1997, 272: 19022.

40- Maudrell R, Antonsson B and Magnenant C. Bcl-2 undergoes phosphorylation by C-Jun N-terminal kinase stress- activated protein kinases in the presence of constitutively active GTP -binding protein Rac-1. J. Biol. Chem. 1997, 272: 25238.

41- Henderson S, Rowe M and Gregory C. Induction of Bcl-2 expression by Epstein Barr Virus latent membrane protein 1 protects infected B cell from programmed cell death. Cell. 1991, 65: 1107-1115.

42- Cinatl JJ, Chaudhay D, Rouke K, Koonin E and Dixit V. Persistent human Cytomegalovirus infection induces drug resistance and alteration of programmed cell death in human neuroblastoma cells. Cancer Res. 1997, 58: 367-372.

43- Ochiai K, Kaqami M, Matsumura R and Tomioka H. IL-5 but not INF-γ inhibits eosinophil apoptosis by upregulation of Bcl-2 expression. Clinical Expreimental Immunology. 1997, 137: 225-232.

44- Levy Y and Brouet JC. Interleukin-10 prevents spontaneous death of germinal center B cells by induction of Bcl-2 protein. J. of Clinical Investigation. 1994, 93: 424-428.

45- Thomas A, El Rouby S, Reed JC, et al. Drug induced apoptosis in B cell chronic lymphocytic leukemia. Oncogene. 1996, 12: 1055-1062.

46- Findley HW, Lubing GU, Yeager AM and Zhou M. Expression and regulation of Bcl-2, Bcl-xl and Bax correlate with p53 status and sensitivity to apoptosis in childhood acute lymphoblastic leukaemia. Blood. 1997, 8: 2986-2993.

47- Datta TR and Manome WG. Over expression of Bcl-xl by cytotoxic drug exposure confers resistance to ionizing radiation-produced internucleosomal DNA fragmentation. Cell growth differentiation. 1984, 6: 363.

48- Minn AJ, Rudin CM, Boise LH and Thompson CB. Expression of Bcl-xl can confer a multidrug resistance phenotype. Blood. 1995, 86: 1903.

49- Miyashita T and Reed JC. Tumour suppressor p53 is a direct transcriptional activator of the human Bak gene. Cell. 1995, 80: 293-299.

50- Pezzella F, Morrison H and Jone SM. Immunocytochemical detection of p53 and Bcl-2 proteins in Non Hodgkin's Lymphoma.Histopathology. 1993, 22: 39.

51- Halder S, Negrini M, Monne M. Down-regulation of Bcl-2 by p53 in breast cancer cells. Cancer Res. 1994, 54: 2095.

52- Hussein MR, Al-Sabae TM and Georgis MN. Analysis of the Bcl-2 and p53 Protein Expression in the Lymphoproliferative Lesions in the Upper Egypt. Cancer Biol Ther. 2005, 28: 4 (3).

53- Yurihiro A, Yshinori O, Seiko K, Yuko I and Yoshihide. Multiple subcellular localization of Bcl-2: Detection in nuclear outer membrane, endoplasmic reticulum membrane and mitochondrial membranes. Cancer Res. 1994, 54: 2468-2471.

54- Lombardi L, Frigerio S, Collini P and Piltti S. Immunocytochemical and immunoelectron microscopic analysis of Bcl-2 expression in thyroid oxyphilic tumour. Ultrastructural Pathology. 1997, 21: 33-39.

55- Kane DJ, Sarafian TA, Anton R, Hahn H, Grallla EB, Valllelntine JS, Ord T and Bredesen DE. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. Science. 1993, 262: 1274-1277.

56- Freeman BA and Crapo JD. Biology of disease, free radicals and tissue injury. Lab Invest. 1982, 47: 412-426.

57- Luciano L, Hass R, Busche R, Reale E and Engelhardt WV. Lack of butyrate is associated with induction of Bax and subsequent apoptosis in the proximal colon of guinea pig. Gastroenterology. 1997, 112, (3), 875-881.

58- Wang L, Miura M, Bergeron L, Zhu H and Yuan J. Ich-1, an Ice/ced-3- related gene, encodes both positive and negative regulators of programmed cell death. Cell. 1994, 78, 739-750.

59- Wang HG, Millan JG and Cox AD. R-ras promotes apoptosis caused by growth factor deprivation via Bcl-2 suppressive mechanism. J Cell Biol. 1995, 129: 1103-1114.