

The Expression of Bcl-2 and Bax Proteins and Their Clinical Relevance in ALL and CLL Patients

HADIR A. EL-MAHALLAWY, M.D.*; NAYERA H. EL-SHAKANKIRY, M.D.*;
SAAD EL-GUINDY, Ph.D.**; SHERINE EL-MAGHRABY, M.Sc.*; SOHAIR ABD EL-LATIF, M.D. and
INAS EL-ATTAR, Ph.D.***

The Departments of Clinical Pathology, Tumor Biology** and Epidemiology & Biostatistics***,
National Cancer Institute, Cairo University.*

ABSTRACT

The products of the bcl-2 gene prolong survival of lymphohemopoietic cells by inhibition of programmed cell death, whereas, bax has an antagonistic role against the function of bcl-2. In the present study we have examined the expression of bcl-2 and bax proteins in cases of ALL and CLL aiming to establish if measurement of expression of these proteins could be of prognostic relevance. Using quantitative western blotting (WB) and immunocytochemistry (IC) we determined the level of expression of bcl-2 and bax proteins in 42 and 20 newly diagnosed cases of ALL and CLL, respectively. Ten healthy individuals were taken as control for bcl-2 and bax expression by IC and WB. By WB, bcl-2 protein was detectable in 39/42 of ALL cases and all CLL cases studied. Bax protein expression using WB revealed detectable levels in 37/42 ALL cases and all CLL cases studied. The level of bcl-2 and bax expression in CLL patients was significantly higher than in ALL patients $p = 0.003$ and 0.030 , respectively. The level of bcl-2 expression in CLL patients was significantly higher than in ALL patients and control group, $p = 0.006$. Our results confirmed a higher sensitivity of WB in detection of these cell cycle proteins when compared to IC. Higher bcl-2 expression was more evident among ALL patients above 18 years, $p = 0.03$. The level of bax expression was significantly higher in patients with total leukocytic counts (TLC) $\geq 50 \times 10^9/L$, $p = 0.02$. Although no relation was found between bcl-2 and bax expression and an increased probability of relapse yet, overexpression of bax (but not bcl-2) was associated with an unfavorable disease outcome, $p = 0.02$. In cases with strong expression of bcl-2, an over expression of bax as well was detected by WB. This finding reflects the fact that it is the balance rather than the level of either protein product that determines the influence of these cell cycle proteins on prognosis of ALL.

Key Words: Bcl-2 - Bax - ALL - CLL - Western blotting.

INTRODUCTION

Bcl-2 and bax are members of the bcl-2 fam-

ily proteins that play important roles in regulating cell survival and apoptosis. The product of the bcl-2 gene is an anti-apoptotic protein that suppresses apoptosis in response to a wide variety of stimuli [15,36]. Enforced bcl-2 expression delays apoptosis of cell lines deprived of essential survival factors [27]. Conversely, bax is a pro-apoptotic protein that can accelerate death and in some instances sufficient to cause apoptosis [13,34]. The ratio of bcl-2 family pro-apoptotic to anti-apoptotic proteins dictates the susceptibility of cells to a variety of apoptotic stimuli [19,21].

Bcl-2 protein has been consistently found to be upregulated in B-CLL and is thought to be involved in an anti-apoptotic mechanism that facilitates B-cell survival [9]. However, it has been also reported that dysregulation of bax, may in fact be more crucial to the maintenance of this condition [32]. It is stated that the ratio of levels of bax and bcl-2 is more important than the steady state level of each individual protein in the cells [22].

Thus, most cases of CLL express abundant bcl-2 protein that suppresses apoptosis and confers resistance to anticancer drugs [6]. On the other hand, the clinical significance of these apoptotic/anti-apoptotic proteins in acute lymphoblastic leukemia is still unsettled [3,10]. This study was carried out aiming at studying the expression of bcl-2 and bax proteins in newly diagnosed ALL and CLL cases and their relevance to other clinicobiological features and clinical outcome.

PATIENTS AND METHODS

This study included 42 and 20 newly diagnosed ALL & CLL patients, respectively. Ten healthy subjects were taken as controls for bcl-2 and bax expression for IC and WB techniques. The patients attended the outpatient clinic, Clinical Pathology Department, National Cancer Institute, Cairo University in the period from January 1999 to January 2000. They were subjected to clinical examination for the presence of lymphadenopathy, splenomegaly and hepatomegaly. Routine laboratory investigations included a complete blood picture and bone marrow aspiration with cytochemical stains to confirm the diagnosis. Immunophenotyping for ALL lineage classification included a battery of CD3, CD5, CD7, CD4, CD8, HLA-DR, CD10, CD19, CD20, CD22 and surface kappa and lambda monoclonal antibodies. CLL was diagnosed according to standard persistent absolute lymphocytosis $\geq 10 \times 10^9/L$ with small mature, monotonous lymphocytes and $\geq 40\%$ lymphocytes in bone marrow. In suspicious cases, immunophenotyping was performed where a score of ≥ 3 was considered diagnostic of CLL and this included weak expression of SmIg, positive expression of CD23 & CD5, negative FMC7 and weak or negative CD79b [37].

Assessment of bcl-2 and bax by immunoperoxidase: Monoclonal antibodies for bcl-2 and bax were obtained from Boehringer Mannheim, Germany and Oncogene Research Products, USA, respectively. Detection of bcl-2 and bax expression was undergone by the ABC immunoperoxidase staining kit of Novocastra, Oncogene, USA [14]; where 200-500 cells were counted for each protein. Positive staining appeared as distinct brown cytoplasmic precipitate and a cutoff was taken $\geq 10\%$ positive cells after Gascoyne et al. [7].

Semi-quantitation of bcl-2 and bax proteins by western blotting technique:

This technique was carried out for 42 ALL patients from whom enough protein for WB was available. Preparation of whole cell lysates was done according to Hsu and Youl, 1997 [13]; 30 μ g of protein was used. The protein was separated by SDS polyacrylamide gel electrophoresis and transferred by semidry blotting to nitrocellulose membrane [29]. The relative molecular mass of protein was compared to a

Rainbow molecular weight marker (Amersham, Little Chalfont, UK). Addition of bcl-2 and bax monoclonal antibodies was done after the transfer of protein on nitrocellulose filter. Immunodetection of bcl-2 and bax expression was performed using chemiluminescence detection system (Pharmacia biotech-Amersham system life science, England) [13] followed by densitometer quantitation.

Statistical analysis:

Comparison between means was done by non-parametric analysis of variance, namely Kruskal-Wallis test. Comparison between high and low expression of oncoproteins was done by Chi-square test or Fisher's exact if the sample size was too small [5].

RESULTS

Patients' characteristics: Among the 42 ALL patients, 30 were children below 18 years and 12 were adults with an age range from 9 months to 58 years. The male: female ratio was 2.2. Lymphadenopathy, hepatomegaly and splenomegaly were encountered in 31 (73.8%), 23 (54.8%) and 26 (61.9%) of the ALL patients, respectively. Twenty patients (47.6%) presented with a total leukocytic count $\leq 50 \times 10^9/L$ & 22 (52.4%) had a TLC above this figure. Hemoglobin ranged between 3.2 to 11.8 g/dl; being ≤ 10 g/dl in 36 patients (85.7%). Platelet count ranged from 6 to $220 \times 10^9/L$; being $\leq 100 \times 10^9/L$ in 37 (88%) patients. As regards FAB classification, 24 (81%) of the cases were ALL-L2 and 8 were L1 (19%). A B-phenotype was encountered in 28 (66.6%) patients; being a common-B in 15 cases, a pre-B in 10, a pro-B in 2 and a mature B in one case. T-phenotype was found in 14 (33.3%) patients; being an early T in 7 cases, an intermediate T in 5 and a late T in 2 cases. At diagnosis, 9 (21.4%) of the patients had CNS invasion. On follow up, 4 patients had bone marrow relapse and 9 patients died in 6 months period.

Bcl-2 and bax expression in ALL cases: Using immunocytochemistry, bcl-2 was undetectable in 15 cases, of score 1-10% in 22 cases and $> 10\%$ in 5 cases. Fig. (1) is representative of bcl-2 positivity by IC in an ALL patient. By WB technique, bcl-2 was undetectable in 3 cases, 25 cases showed low expression and fourteen cases had high bcl-2 expression as compared with mean ± 2 SD of the control group.

Fig. (2) demonstrates an immunoblot for bcl-2 protein in different ALL patients. By immunocytochemistry, bax protein was undetectable in 11 cases and showed a score of 1-10% in 26 cases; whereas, an expression > 10% was recorded in 5 cases. Fig. (3) demonstrates a bax positive case of ALL by IC. Using WB, bax protein revealed a detectable level in 37/42 (88%) of ALL patients and could not be detected in 5. High expression of bax protein was detected in 11 cases and low expression in 26 cases as compared with mean \pm 2SD of the control group. Fig. (4) is a representative sample of immunoblotting for bax expression in different ALL patients. Results of bcl-2 and bax semi-quantitation by densitometer and their ratio for ALL and CLL patients in comparison to controls are summarized in Table (1). Eleven patients with strong expression of bax by WB were also strongly expressing bcl-2.

The level of bcl-2 and bax expression in ALL cases did not show statistical significant difference when compared to controls. There was marked variation in the level of bcl-2 and bax expression among the positive ALL cases. A statistical significant relation was found between the level of bcl-2 expression and age in ALL patients. Higher bcl-2 expression was detected in adult patients with a *p*-value 0.03. Overexpression of bax (but not bcl-2) was statistically significantly associated with bad prognosis, *p* = 0.02. Correlation of bcl-2 expression with sex, lymphadenopathy, hepatomegaly, splenomegaly, TLC, Hb, platelets, lineage, CNS invasion and relapse failed to reach the level of significance. Comparing the level of bax expression using WB with TLC was statistically significant over expressed in patients with higher TLC *p*-value 0.02. Other studied parameters (Age, sex, lymphadenopathy, hepatosplenomegaly, Hb, platelets, lineage, CNS invasion and relapse) were not significantly different in patients with over expression of bax. Bcl-2/bax ratio in ALL patients was classified according to the mean \pm 2 SD of the controls into high (n=3) and low (n=39) ratio values. However, no significant association could be found with clinicobiologic features of ALL patients. Comparison of different ALL patients' characteristics with bcl-2, bax proteins and their ratio are shown in Table (2).

Patients' characteristics for CLL cases: Their age ranged from 36 to 70 years, with a

male to female ratio of 1.8:1. Clinically, lymphadenopathy, hepatomegaly and splenomegaly were encountered in 17 (85%); 14 (70%) and 11 (55%) of the 20 CLL cases, respectively. The TLC ranged from 12 to 410 x 10⁹/L. Twelve (60%) patients presented with a TLC \geq 100 x 10⁹/L. Absolute lymphocytic count ranged from 11.8 to 385 x 10⁹/L. Lymphocyte percent in bone marrow ranged from 46% to 97%. Hemoglobin level ranged from 4.5 to 14 g/dl, being \leq 10 g/dl in 9 (45%) of the patients. Platelet count ranged from 60 to 435 x 10⁹/L and was \leq 100 x 10⁹/L in 5 (25%) patients.

Expression of bcl-2 and bax proteins in CLL cases: Immunocytochemistry revealed bcl-2 expression with a score < 10% in 15 patients and above 10% in 5 patients. Bax was undetectable by IC in 2 cases and showed a score < 10% in 17 cases. Only one patient had a score above 10%. Western blotting was performed for 6 CLL patients and bcl-2 expression was detected in all cases. The expression was variable among samples ranging from 0.893 to 3.362 with a mean of 2.36 \pm 0.98. Bcl-2 and bax expression in lymphocytes of CLL cases was significantly higher than in lymphoblasts of ALL patients with a *p* value of 0.003 and 0.030, respectively. On comparing the 3 groups together, bcl-2 but not bax expression in lymphocytes of CLL cases was significantly higher than the ALL patients and the control group with a *p*-value 0.006. Bax expression was detected by WB in all CLL cases with a range between 1.94 to 3.43 and a mean of 2.62 \pm 0.67. Although the level of bax expression in CLL cases was higher than the control group, yet it didn't reach the level of statistical significance. Fig. (5) and 6 demonstrate immunoblots for bcl-2 and bax in CLL cases, respectively.

DISCUSSION

Considerable evidence now exists that cytotoxic drugs used in the treatment of malignancies exert their effects through triggering the apoptotic pathways [10]. Although apoptosis involves a complex series of intracellular molecular interactions, cell line studies have indicated that alterations in the expression of bcl-2 and related proteins can have a marked influence on chemosensitivity [24]. High levels of expression of bcl-2 have been shown to correlate with poor treatment outcome in some hematological ma-

Table (1): Bcl-2, bax and bcl-2/bax ratio in ALL and CLL cases compared to the control group by the western blotting technique.

Parameter	ALL N=42 OD	CLL N=6 OD	Control N=10 OD	p value
Bcl-2	1.15±0.69b (0.64-2.95)	2.36±0.98a* (0.99-3.36)	0.88±0.10b (0.80-1.13)	0.007
Bax	1.75±1.03 (0.65-3.38)	2.62±0.67 (1.94-3.43)	1.69±0.55 (0.67-2.19)	0.107
Bcl/bax	0.81±0.53 (0.23-3.54)	0.92±0.36 (0.20-1.30)	0.60±0.30 (0.36-1.27)	0.100

Values are mean ± SD (range).

OD unit is optical density

p-value less than 0.05 is considered significant

Values with different letters are statistically different with the letter a* showing the highest results.

Table (2): Relationship between bcl-2, bax and bcl-2/bax ratio and clinicobiologic features of ALL patients.

Parameter	Bcl-2 % high exp./p-value		Bax % high exp./p-value		Ratio % high exp./p-value	
<i>Age:</i>						
≤ 18 yrs, n=30	23.3	0.03	20.0	0.15	10.0	0.26
> 18 yrs, n=12	58.3		41.7		0.0	
<i>TLC:</i>						
≤ 50x10 ⁹ /l n=20	25.0	0.28	10.0	0.02	5.0	0.61
> 50x10 ⁹ / n=22	40.9		40.9		9.1	
<i>Hb:</i>						
≤ 10gm/dl, n=36	33.3	1.0	25.0	0.65	8.3	0.46
> 10 gm/dl, n=6	33.3		33.3		0.0	
<i>PLT:</i>						
≤ 100x10 ⁹ /L n=37	35.1	0.50	27.0	0.74	8.1	0.51
> 100x10 ⁹ /L n=5	20.0		20.0		0.0	
<i>IP:</i>						
B-, n=28	29.3	0.25	28.6	0.62	7.1	1.0
T-, n=14	21.4		21.4		7.1	
<i>Relapse:</i>						
-, n=38	34.2	0.71	30.0	0.21	8.0	0.56
+, n=4	25.0		0.0		0.0	
<i>CNS invasion:</i>						
-, n=33	39.4	0.11	24.2	0.58	9.0	0.35
+, n=9	11.1		33.3		0.0	
<i>Outcome:</i>						
Alive, n=33	30.3	0.42	18.2	0.02	9.1	0.35
Dead, n=9	44.4		55.6		0.0	



Fig. (1): A representative of bcl-2 positivity in a case of ALL patient by immunocytochemistry technique.

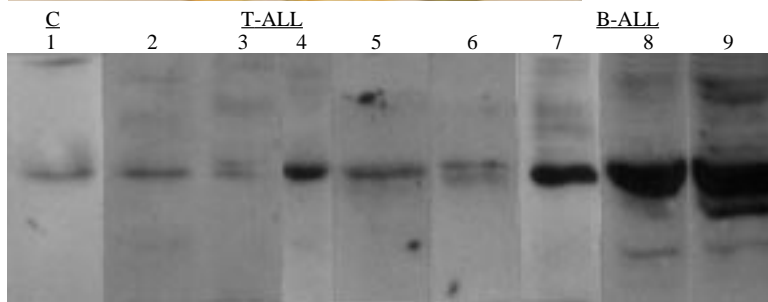


Fig. (2): Western blot analysis of bcl-2 in ALL patients. Lane 1 represents a normal control, lanes 2-6 are T-ALL and lanes 7-9 are B-ALL. In T-ALL cases lanes 2,3 & 5 showed low bcl-2 expression whereas lane 4 showed moderate expression. In B-ALL all the cases showed high expression of bcl-2 protein.

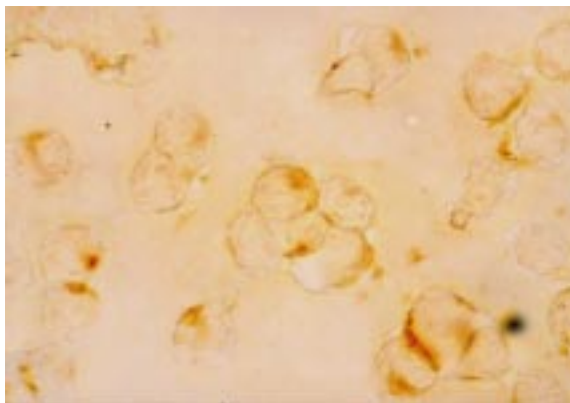


Fig. (3): Demonstrates a bax positive case of ALL patient by immunocytochemistry technique. Note the weak positivity of bax in ALL blasts.

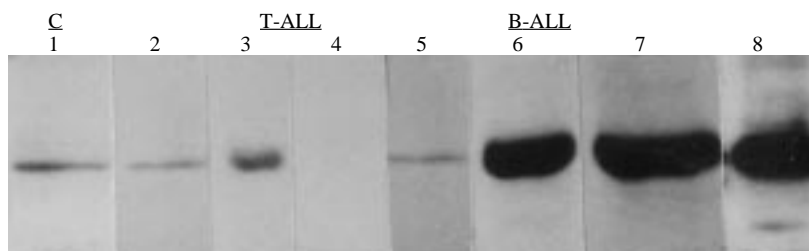


Fig. (4): Western blot analysis of bax in ALL patients. Lane 1 represents a normal control. Lanes 2-5 are T-ALL and lanes 6-8 are B-ALL. In T-ALL cases lanes 2,3 & 5 showed low bcl-2 expression whereas lane 4 showed no expression. In B-ALL all the cases showed high expression of bcl-2 protein.

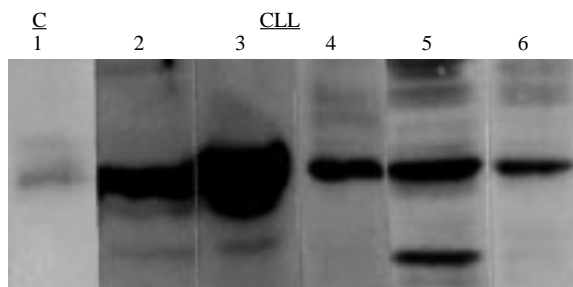


Fig. (5): Western blot analysis of bcl-2 in CLL patients. Lane 1 represents a normal control. Lanes 2,3,4,5 & 6 are example of CLL, showed high expression of bcl-2 protein.

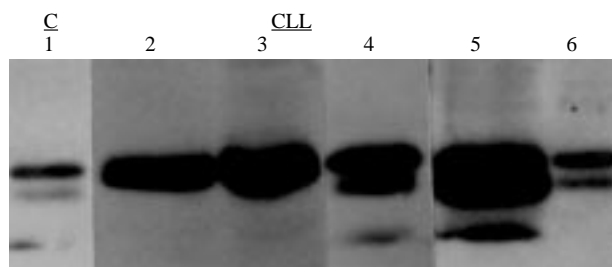


Fig. (6): Western blot analysis of bax in CLL patients. Lane 1 represents a normal control. Lanes 2,3,4,5 & 6 are example of CLL, showed high expression of bax protein.

lignancies, including follicular lymphoma [11], CLL [26] and AML [2].

In this study we aimed at studying bcl-2 and bax expression in newly diagnosed cases of ALL and CLL and its relation with other prognostic factors and treatment outcome. High expression of bcl-2 and bax proteins was demonstrated in lymphocytes of all CLL cases studied by immunoblotting and bcl-2 expression was significantly higher in CLL than in both ALL and the control group.

B-CLL is an example of a human malignancy caused by alterations in the pathways of programmed cell death. In this disease, the anti-apoptotic protein, bcl-2, is over expressed and may lead to the prolonged survival of a malignant CLL clone as opposed to increased cell proliferation [8,16]. Several previous studies reported that higher levels of anti-apoptotic proteins were strongly associated with failure to achieve a complete remission [17] and with a progressive disease [16,22]. On the other hand and despite extensive study of the relationship between the expression of bcl-2 family proteins and the sensitivity of ALL blasts to apoptosis, controversy persists. Several studies have failed to disclose any correlation between bcl-2 expression and outcome of ALL [3,4,33], but these studies have not included measurement of the relative expression of other members of the bcl-2 family. One recent study have shown that, whereas expression of bcl-2 and determination of bcl-2/bax ratio do not appear to have prognostic significance in childhood ALL, high expression of bax at diagnosis is associated with a significant increase in the probability of relapse [12].

In the present study, bcl-2 protein expression by WB revealed detectable level of the protein in 39/42 (92.8%) of ALL cases. Using the densitometer, the level of bcl-2 expression was variable among the samples ranging from 0.64 to 2.95 with a mean of 1.15 ± 0.69 which showed no significant difference when compared to the control group. The level of bax expression was variable among the ALL cases ranging from 0.65 to 3.38 with a mean of 1.75 ± 1.03 , which was also not different than the control group. In agreement with our findings, it was reported that bcl-2 was detectable in 43 ALL cases studied by western blotting [12]. In addition, others reported the presence of

detectable bcl-2 by flow cytometry in all samples tested [3,4].

On the other hand, using immunocytochemistry, bcl-2 and bax expression was detectable in 27/42 and 31/42 of cases, respectively. This confirmed the higher sensitivity of WB as compared to immunocytochemistry in the detection of apoptotic proteins as was previously observed [17]. This might be due to the larger size used in WB (more than 5 million cells). Besides the cell lysis which yielded large amounts of protein, there was the superior resolving power of electrophoresis. In addition, by chemiluminescence, the results were captured on X-ray film and kept as permanent documents.

In the present study, we tried to find a correlation between bcl-2 expression and other prognostic parameters of ALL including age, sex, lymphadenopathy or organomegaly, presenting TLC, immunophenotype, CNS invasion and the occurrence of relapse. A significant association was detected between bcl-2 expression and age. High bcl-2 expression was more evident among patients above 18 years, p -value 0.03. In several other studies, including pediatric [12,28,33] and adult ALL [3] such an association was not evident. However, similar to other reports no significant relation was detected between bcl-2 expression and other clinical and laboratory parameters [3,4].

As the ability of bcl-2 to modulate the apoptotic threshold is affected by the relative expression of bax [1], we attempted to assess the relationship between bcl-2/bax ratio and other prognostic features. A high ratio has been reported to correlate with an inability to achieve a CR in AML [31] and with in-vitro resistance to drug induced apoptosis in CLL [21]. In our study, bcl-2/bax ratio could not predict response to therapy or occurrence of relapse. Similar results were previously reported on hood child ALL cases [12]. However, in the latter study bax expression alone was shown to be associated with an increased probability of relapse ($p = 0.04$).

Our findings documented a statistical association between bax over expression and higher TLC. Moreover, bax overexpression was associated with unfavorable disease outcome for ALL patients. This paradoxical finding is difficult to explain. However, it was found that though bax homodimers promote apoptosis,

still bax and bcl-2 heterodimerize to prevent cell death [35]. Thus, the equilibrium in the formation of bcl-2: bax heterodimers (suppressors of death) and bax: bax homodimers (activators of death) appears to be central in the molecular regulation of apoptosis [35].

Hogarth and Hall [12] reported that bcl-2 was highly expressed in B-ALL rather than T-ALL. In their study, although no significant difference in bax expression was found between B- & T- lineage ALL, the bcl-2/bax ratio was significantly lower in T-ALL compared with B-lineage ALL. Other studies failed to find a significant relation between bcl-2 expression and ALL cell lineage [3,4,30,33]. Although our findings did not show a significant relation between bcl-2 and bax over expression among cases of T & B phenotype, still most cases with high expression were of the B-lineage.

In our study, higher levels of bcl-2 or bax were not associated with poor response to therapy as evidenced by the occurrence of relapse. Similar results were previously reported, as bcl-2 and bax over expression did not have any prognostic significance among several studies [18,33]. Studies which were able to demonstrate a relation between these cell cycle proteins over expression and relapse were done on blast cells during relapse. Prokop and his coworkers [23], stated that both bax and bcl-2/bax ratio were significantly lowered in samples at relapse as compared with samples at initial diagnosis in pediatric ALL (*p*-value 0.003). Thus, to study the effect of apoptotic proteins on the occurrence of relapse, it is essential to study cases during relapse.

In the present study, a significant association between bcl-2 over expression with older age and between bax over expression with higher TLC was found. Thus, higher levels of these proteins expression may be related to unfavorable prognostic factors. This is further supported by the unexpected relationship between high expression of bax and poor prognosis in our ALL cases. In our cases with strong expression of bcl-2, an over expression of bax as well was detected by western blotting. This finding might contribute to the fact that the balance between apoptotic and anti-apoptotic mechanisms rather than the level of each protein that determine the effect of these proteins on the progno-

sis of ALL. Studies using a larger cohort of uniformly treated patients and levels of other apoptotic proteins need to be performed to determine if this is an independent prognostic variable that may be used to aid treatment stratification.

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