

Clinical significance of bax/bcl-2 ratio in chronic lymphocytic leukemia

Maria Iliaria Del Principe,¹ Michele Dal Bo,² Tamara Bittolo,² Francesco Buccisano,¹ Francesca Maria Rossi,² Antonella Zucchetto,² Davide Rossi,³ Riccardo Bomben,² Luca Maurillo,¹ Mariagiovanna Cefalo,¹ Giovanna De Santis,¹ Adriano Venditti,¹ Gianluca Gaidano,³ Sergio Amadori,¹ Paolo de Fabritiis,¹ Valter Gattei,² and Giovanni Del Poeta¹

¹Ematologia, Dipartimento di Biomedicina e Prevenzione, S. Eugenio Hospital and Università Tor Vergata, Roma; ²Unità Clinica e Sperimentale Onco-Ematologica, Centro di Riferimento Oncologico, I.R.C.C.S., Aviano; and ³Ematologia, Università del Piemonte Orientale, Novara, Italy



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ABSTRACT

In chronic lymphocytic leukemia the balance between the pro-apoptotic and anti-apoptotic members of the bcl-2 family is involved in the pathogenesis, chemorefractoriness and clinical outcome. Moreover, the recently proposed anti-bcl-2 molecules, such as ABT-199, have emphasized the potential role of bcl-2 family proteins in the context of target therapies. We investigated bax/bcl-2 ratio by flow cytometry in 502 patients and identified a cut off of 1.50 to correlate bax/bcl-2 ratio with well-established clinical and biological prognosticators. Bax/bcl-2 was 1.50 or over in 263 patients (52%) with chronic lymphocytic leukemia. Higher bax/bcl-2 was associated with low Rai stage, lymphocyte doubling time over 12 months, beta₂ microglobulin less than 2.2 mg/dL, soluble CD23 less than 70 U/mL and a low risk cytogenetic profile ($P < 0.0001$). On the other hand, lower bax/bcl-2 was correlated with unmutated *IGHV* ($P < 0.0001$), mutated *NOTCH1* ($P < 0.0001$) and mutated *TP53* ($P = 0.00007$). Significant shorter progression-free survival and overall survival were observed in patients with lower bax/bcl-2 ($P < 0.0001$). Moreover, within *IGHV* unmutated (168 patients) and *TP53* mutated (37 patients) subgroups, higher bax/bcl-2 identified cases with significant longer PFS ($P = 0.00002$ and $P = 0.039$). In multivariate analysis of progression-free survival and overall survival, bax/bcl-2 was an independent prognostic factor ($P = 0.0002$ and $P = 0.002$). In conclusion, we defined the prognostic power of bax/bcl-2 ratio, as determined by a flow cytometric approach, and highlighted a correlation with chemoresistance and outcome in chronic lymphocytic leukemia. Finally, the recently proposed new therapies employing bcl-2 inhibitors prompted the potential use of bax/bcl-2 ratio to identify patients putatively resistant to these molecules.

Introduction

Chronic lymphocytic leukemia (CLL) is a clinically heterogeneous disease characterized by the accumulation of CD5⁺CD23⁺ B-cell lymphocytes.¹ The genetic and molecular mechanisms involved in the development of the disease are not well known, but recent observations suggest that the modulation of survival signals interfering with apoptosis may be a pivotal tool in the pathogenesis of CLL.² Bcl-2 (B-cell lymphoma-2) family of antiapoptotic (bcl-2, bcl-xl, bcl-w and mcl-1) and proapoptotic (bax, bak and bok) proteins are critical regulators of apoptosis in CLL.³ In particular, bcl-2 has emerged as the most important protein in predicting

Correspondence:

g.delpoeta@tin.it
del.principe@med.uniroma2.it

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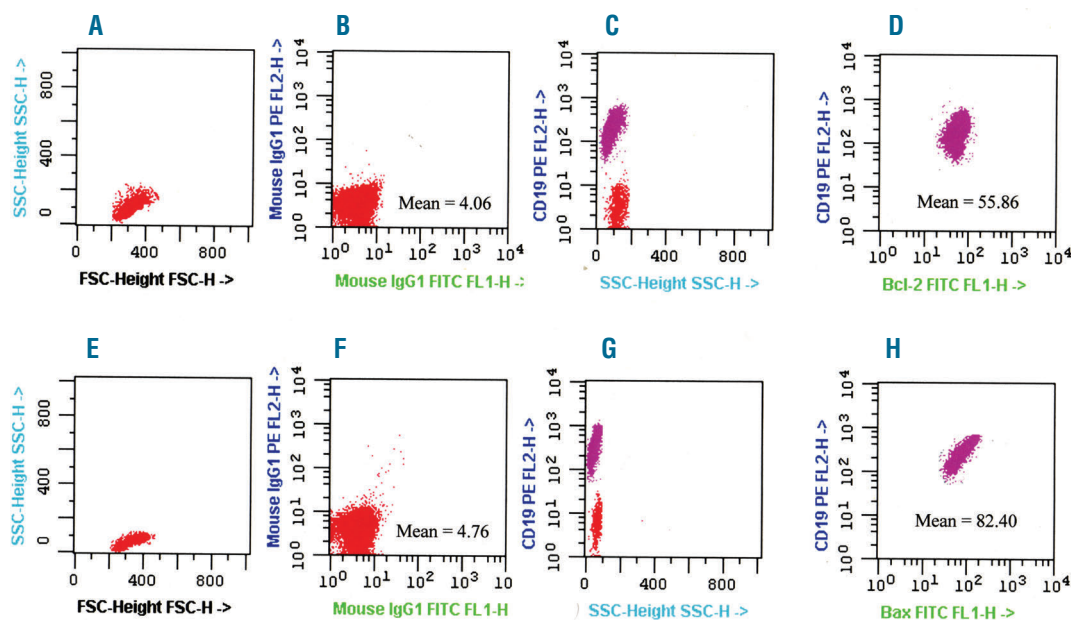


Figure 1. Characteristics of bcl-2 and bax expressions. (A-E) Forward and side scatter of representative gated lymphocytes are shown. (B-F) Mouse IgG1 isotypic antibodies are used as controls for bcl-2 and bax positivities with their mean fluorescence intensities (MFI). (C-G) B-CLL (CD19⁺) cells (violet) were selected on the gated lymphocytes according to their phenotype. (D-H) Flow cytometric dot plots of bcl-2 and bax (violet) on CD19⁺ cells on peripheral blood sample of one representative CLL case with reported MFI values.

survival in CLL between 11 proteins that are implicated in the control of apoptosis, proliferation and differentiation.⁴ High levels of bcl-2 protein are found in CLL cells even in the absence of classical t(14;18) translocation that is detected frequently in follicular lymphomas.⁵ In this context, Cimmino *et al.*⁶ demonstrated that the loss of microRNAs (miR), miR-15a and miR-16-1, is involved in regulation of the bcl-2 family expression levels. Moreover, bcl-2 family members have also been linked to resistance of chemotherapy in CLL. In particular, Pepper *et al.*⁷ demonstrated that high levels of bcl-2 and low levels of bax protein expression are linked to chemoresistance in CLL cells treated with chlorambucil and a remarkable elevation of bax protein was found in CLL cells undergoing apoptosis. Several reports further confirmed that in CLL the balance between the pro- and anti-apoptotic members of the Bcl-2 family determines the chemotherapy sensitivity and survival.^{8,9} Finally, the recent introduction in the clinical use of novel potent oral pro-apoptotic BH3 peptidomimetics such as ABT-199^{10,11} and ABT-737¹² has further emphasized the importance of bcl-2 family proteins in the context of CLL therapy. In the present study, we addressed the clinical impact of bax/bcl-2 ratio, evaluated by a flow cytometric approach, as independent prognosticator of progression-free survival (PFS) and overall survival (OS) in CLL. In this context, we also correlated the bax/bcl-2 ratio with the other well-established clinical-biological prognosticators in CLL.

Methods

Study design and patients

This is a retrospective study that includes peripheral blood (PB) from 502 CLL patients recruited in a single center during the period 1989-2014. Approval of this study was obtained from the Institutional Review Board of the Centro di Riferimento

Oncologico (IRCSS) of Aviano (PN), Italy. Informed consent was provided in accordance with the Declaration of Helsinki. Patients were 279 males and 223 women with a median age of 66 years (range 33-89) at the time of diagnosis. Using the modified Rai staging system, 170 patients had a low stage, 318 an intermediate stage, and 14 a high stage. Median follow up was 7.9 years with 103 deaths and 399 censored patients. Two hundred and ninety-one patients out of 502 (58%) received chemotherapy for their disease. Eighty-seven patients were treated with a combination of chlorambucil plus prednisone and rituximab was added in 11 of them. One hundred and sixty patients received six courses of fludarabine monophosphate (Fludara; Genzyme, Modena, Italy) at 25 mg/m² intravenously or 30-40 mg/m² orally for five days every 28 days followed by four courses of rituximab (Mabthera; Roche, Basilea, Switzerland) at 375 mg/m² for one day every week.¹³ Twenty-seven patients were treated with six courses of fludarabine, cyclophosphamide and rituximab.¹⁴ Finally, 17 patients aged over 60 years received bendamustine at 70 mg/m² for two days and rituximab for one day at conventional dosages.¹⁵

Cellular immunophenotypic analysis

All flow cytometric analyses were performed on a FACSCalibur flow cytometer (BD, Biosciences, CA, USA). The instrument was aligned and calibrated daily with the use of a 4-color mixture of CaliBRITE beads (BD, Biosciences) with FACSComp Software (BD, Biosciences), according to the manufacturer's instructions. Triple-color immunofluorescence analysis of surface antigens was performed by using combinations of phycoerythrin (PE), fluorescein isothiocyanate (FITC), and allophycocyanin (APC) monoclonal antibodies (MoAbs). The following conjugated MoAbs were used: anti-CD23 PE, anti-CD5 FITC, anti-CD5 APC, anti-CD38 PE, anti-CD19 PE, anti-CD19 APC, anti-CD49d PE and anti-CD45 FITC (BD, Biosciences). The peripheral blood (PB) mononuclear cells were analyzed for surface expression of CD19/CD5/CD38, CD19/CD5/CD23 and CD19/CD5/CD49d in order to confirm CLL diagnosis.

Bcl-2 and bax expression

Bcl-2 and bax oncoproteins were evaluated by flow cytometry using the following two MoAbs: 1) Anti-human bcl-2 clone 124 FITC-conjugated MoAb (Dako, Glostrup, Denmark) is an immunoglobulin G1 (IgG1) that reacts specifically with bcl-2 oncoprotein associated with mitochondria, smooth endoplasmic reticulum, and perinuclear membrane;^{16,17} 2) anti-bax clone Ab-2 (Oncogene, Cambridge, MA, USA) is an IgG1 that recognizes bax, normally localized to the cytoplasm, but translocating rapidly to the mitochondria after the induction of an apoptotic signal.¹⁸

All samples were tested at diagnosis or before any therapeutic approach. For multiparameter analysis of bcl-2 and bax combined with lymphoid antigens, mononuclear cells were initially incubated with CD19 PE and CD5 APC MoAbs for 30 min at 4°C. Subsequently, the cells, after washing twice in PBS, were fixed and permeabilized with Fix & Perm Cell Permeabilization kit® (Fix&Perm, Caltag Laboratories, Carlsbad, CA, USA). All samples were then incubated at 4°C for 30 min with either 10 µL anti-bcl-2 FITC or 20 µL unconjugated anti-bax. For bax, cells were further incubated at 4°C for 30 min with 100 µL FITC-

Table 1. Distribution of prognostic factors in CLL according to bax/bcl-2 values.

Parameter	bax/bcl-2, no		P [†]	n [‡]	10-year OS, %	P*	10-year PFS, %	P*
	≥1.50	<1.50						
Age								
<60 years	83	85	0.25	502	93	<0.0001	36	0.51
>60 years	183	151			77		35	
B2M								
<2.2 g/L	185	92	<0.0001	502	92	<0.0001	52	<0.0001
>2.2 g/L	81	144			69		15	
Mod-Rai								
I	121	49	<0.0001	502	92	0.00001	59	<0.0001
II-III	145	187			79		24	
sCD23								
<70 UI/ml	193	101	<0.0001	447	92	<0.0001	52	<0.0001
>70 UI/ml	44	109			60		6	
CD38								
<30%	222	151	<0.0001	502	87	<0.0001	44	<0.0001
>30%	44	85			70		12	
FISH								
Normal/del13q	221	120	<0.0001	489	90	<0.0001	46	<0.0001
Int/Poor (+12, del11q, del17p)	38	110			64		9	
IGHV								
M	221	106	<0.0001	495	92	<0.0001	47	<0.0001
UM	38	130			61		13	
TP53								
M	8	29	0.00007	502	87	<0.0001	5	0.0001
WT	258	207			30		38	
NOTCH-1								
M	9	49	<0.0001	502	85	<0.0001	0	<0.0001
WT	257	187			60		40	

[†]Fisher exact tests were performed to evaluate the association between bax/bcl-2 values (below or above the established threshold set at ≥1.50 of positive CLL cells) and other prognostic factors. [‡]Values refer to the number of cases analyzed for a given feature. *P values were calculated by the log rank test.

Table 2. Multivariate Cox regression analysis of progression-free survival.

Parameter	N=489 patients		N=435 patients	
	HR	P	HR	P
Bax/bcl-2	0.52	0.000004	0.57	0.0003
NOTCH1	1.32	0.09	1.26	0.19
sCD23	NT	NT	1.57	0.006
B2M	1.91	0.000002	1.65	0.0011
Mod-Rai	2.08	0.000008	1.59	0.0007
CD38	1.79	0.00003	1.65	0.0009
IGHV	0.73	0.022	0.77	0.11
FISH cytogenetics	1.37	0.023	1.34	0.05
TP53	2.32	0.00002	2.24	0.0001

HR: hazard ratio; NT: not tested.

conjugated F(ab)2 fragment of goat antimouse immunoglobulin (dilution 1:50; Dako, Glostrup; Denmark). Negative controls were performed by incubating cells with non-specific isotype IgG1 antibodies (Figure 1B and F). The results were performed by gating lymphoid cells on a SSC versus FSC plot (Figure 1A and E) and examining bcl-2 and bax as mean fluorescence intensity (MFI) on CD19 positive/CD5 positive cells (Figure 1C, D, G and H).¹⁹ Bcl-2 and bax were then evaluated as relative mean fluorescence intensities (rMFIs), calculated as the ratio between bcl-2 or bax MoAbs MFI and non-specific MoAb MFI on B cells. Finally, the results were expressed as an index (bax/bcl-2) obtained by dividing rMFI bax and rMFI bcl-2. Estimation of bax/bcl-2 ratio yielding the best separation of 2 subgroups with different OS and/or PFS probabilities was made by applying various methods including Youden's index, median value and receiver-operating characteristic (ROC) analysis. Finally, the threshold was set at the bax/bcl-2 median value equal or higher than 1.50 (range 0.27-6.10) confirmed also by ROC analysis (*Online Supplementary Figure S1*). Two small subsets of patients were studied using 20 µl of two directly conjugated anti-bax MoAbs (clone 2D2), one conjugated with FITC (Santa Cruz Biotechnology, Dallas, TX, USA) and the other with APC (Abcore, Ramona, CA, USA).

Soluble CD23 and B2-microglobulin detection

Soluble CD23 (sCD23) immunoenzymometric assay and β₂-microglobulin (β₂M) determinations were performed as described elsewhere.²⁰ The threshold of positivity was set at 70 U/mL for sCD23 and 2.2 g/L for β₂M on the basis of normal laboratory references values.

Interphase FISH

Separate hybridations were carried out for loci on chromosome 11, 12, 13 and 17. For chromosome 11, 13, 12 and 17 specific probes and procedures were used, as reported previously.²¹

IGHV mutation analysis

Total RNAs were extracted and reverse-transcribed. The resulting cDNAs, checked for first strand synthesis,²² were amplified using a mixture of sense primers annealing to either V_H1 through V_H6 leader sequences or 5'ends of V_H1-V_H6 FR1. Purified amplifications were cloned and a minimum of 10-20 colonies for each case were sequenced; CLL specific IGHV transcripts, identified by sharing the same CDR3 in multiple clones were analyzed for percent mutation, as previously described. V_H gene sequences deviating

more than 2% from the corresponding germline gene were defined as mutated.²³

NOTCH1 mutational status and TP53 mutational status

The presence of NOTCH1 mutations was investigated with amplification refractory mutation system (ARMS) PCR for c.7544-7545delCT and by Sanger sequencing of NOTCH1 exon 34.²⁴ Mutation analysis of TP53 exons 2 to 11 was carried out by DNA direct sequencing on an ABI Prism 3130 automated DNA sequence analyzer (Applied Biosystems, Foster City, CA, USA) according to the International Agency for Research on Cancer (IARC) guidelines (www.p53-iarc.fr) and analyzed with the Sequencing Analysis v.5.2 software (Applied Biosystems), as previously reported.²⁵ Mutations were confirmed on both strands on independent amplimers and validated by the IARC TP53 Mutation Database R15, as previously reported.

Statistical analysis

Correlations between bax/bcl-2 ratio and the other biological and clinical variables were based on the two-tailed Fisher exact test.

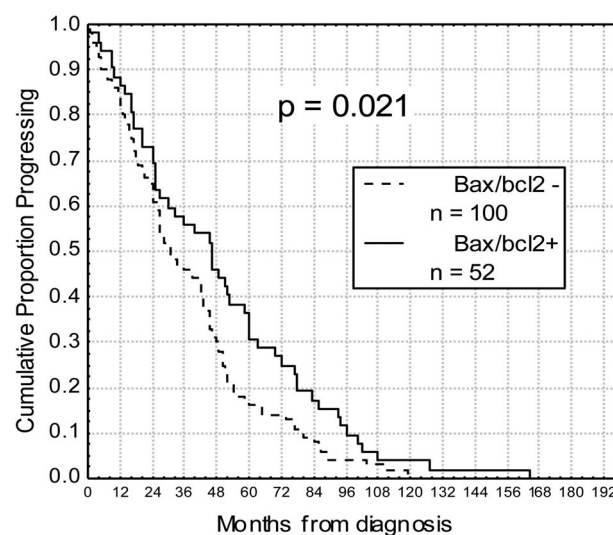


Figure 2. Duration of response after fludarabine and rituximab in relation to bax/bcl-2 values. Progression-free survival after treatment was significantly longer within chronic lymphocytic leukemia subgroup showing bax/bcl-2 ≥ 1.50 (bax/bcl2⁺; 46% vs. 30% at 4 years; $P=0.021$).

Table 3. Multivariate Cox regression analyses of overall survival.

Parameter	N=489 patients		N=435 patients	
	HR	P	HR	P
Bax/bcl-2	0.41	0.0022	0.46	0.011
Age > or < 60 years	3.91	0.000001	3.51	0.00002
B2-M	2.50	0.001	2.11	0.022
TP53	2.74	0.0001	2.71	0.0004
CD38	1.13	0.58	1.03	0.91
NOTCH1	1.83	0.02	1.76	0.045
Mod-Rai	1.81	0.03	1.64	0.057
IGHV	0.37	0.0001	0.41	0.001
FISH cytogenetics	1.85	0.010	1.88	0.016
sCD23	NT	NT	1.37	0.27

HR: hazard ratio; NT: not tested.

The clinical assessment of CLL patients was based both on the National Cancer Institute Working Group criteria²⁶ and on the International Workshop on Chronic Lymphocytic Leukemia criteria after 2008.²⁷ PFS and OS, measured from diagnosis, were estimated according to the Kaplan-Meier method and compared between groups by means of the log rank test. Cox proportional hazards regression models were used to assess the independent effect of co-variables, treated as dichotomous, both on the PFS and OS.

Results

Expression of bax/bcl-2 and association with other prognostic factors

Bax and bcl-2 presented variable pattern of mean fluorescence intensity. Median rMFIs were 12.39 (range 3.85-103.0) for bcl-2 and 17.44 (range 2.20-100.0) for bax, respectively. Median bax/bcl-2 ratio (bax/bcl-2) was 1.50 (range 0.26-6.10). The median bax/bcl-2 ratio was chosen as cut off to discriminate subgroups of cases, being very similar to the best cut off defined by ROC analysis (*Online Supplementary Figure S1*). Using this cut off, 263 patients (263 of 502, 52%) were bax/bcl-2 positive (≥ 1.50). An inverse correlation was observed between bax/bcl-2 levels and PB B-cell count (Spearman correlation, $r=0.26$, $P<0.0001$). Higher bax/bcl-2 was significantly associated with low Rai stage, lymphocyte doubling time (LDT) over 12 months, $\beta 2M$ less than 2.2 mg/dL and soluble CD23 less than 70 U/mL ($P<0.0001$) (Table 1). Moreover, lower bax/bcl-2 was significantly associated both with CD38 more than 30% ($P<0.0001$) (Table 1), and CD49d more than 30% ($P=0.010$) (Table 1). Four hundred and eighty-nine patients were analyzed by interphase FISH to evaluate deletions in chromosome bands 17p13, 11q23, 13q14, and trisomy of band 12q13. There was a significant correlation between higher bax/bcl-2 and low risk (normal karyotype or del13q) cytogenetics ($P<0.0001$) (Table 1). Conversely, lower bax/bcl-2 was significantly represented within the intermediate/high risk (trisomy 12, del11q and del17p) classes ($P<0.0001$) (Table 1). Significant associations were also found between lower bax/bcl-2 and *IGHV* unmutated status ($P<0.0001$) or *NOTCH1* mutations ($P<0.0001$) or *TP53* mutations ($P=0.00007$) (Table 1). Moreover, we analyzed a validation cohort of 233 CLL patients recruited in another Institution (Policlinico Tor Vergata) and analyzed in another laboratory located at University Tor Vergata obtaining similar results with regard to bax/bcl-2 ratio and all the above mentioned variables (*Online Supplementary Table S1 and Figure S2*). Interestingly, 54 of these patients were the same as our series with similar results regarding bax/bcl-2 ratio. We repeated these assays in 20 thawed samples using an anti-bax MoAb APC (Abcore, Ramona, CA, USA) and an anti-bcl-2 MoAb APC (Abcore, Ramona, CA, USA). Then we compared these bax/bcl-2 ratios with the bax/bcl-2 ratios previously obtained on the same patients using fresh cells and an anti-bax unconjugated MoAb. We obtained similar results in 20 of 20 cases (*Online Supplementary Table S2*) using the same cut off (≥ 1.50). Interestingly, significant correlations between bax/bcl-2 ratio and some other prognostic factors (*Online Supplementary Table S3*) were confirmed in 52 patients who were recently studied using an anti-bax FITC MoAb (clone 2D2, Santa Cruz Biotechnology, Dallas, TX, USA) and anti-bcl-2 FITC MoAb (clone 124). In this subset, median rMFI for bax

FITC was 18.32 (range 4.0-38.9) and the same value of bax/bcl-2 ratio (≥ 1.50) was used as cut off. Interestingly, we confirmed the stability over time of bax/bcl-2 ratio in 46 of our untreated patients in which this index was repeated three times along the course of the disease (*Online Supplementary Table S4*). It is worthy of note that, in some patients treated with fludarabine, we observed that bax/bcl-2 ratio increased progressively from one day post treatment to one month post treatment and this increase was higher in responsive patients (*data not shown*).

Relevance of bax/bcl-2 ratio as biological prognostic marker

With regard to clinical behavior, a significant correlation was found between bax/bcl-2 and treatment. In particular, 197 of 236 (83%) with lower bax/bcl-2 received

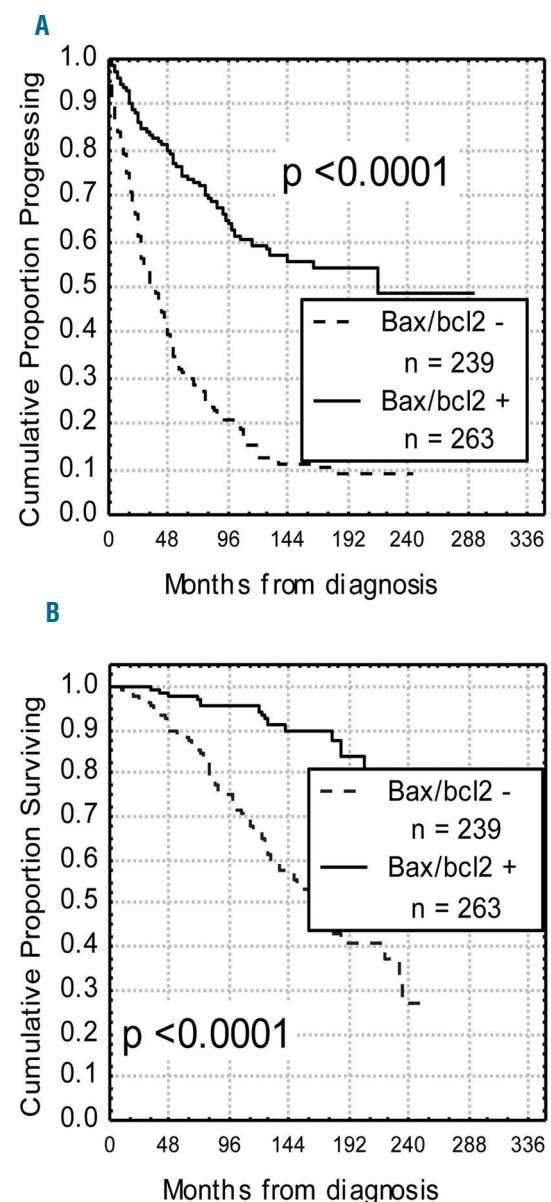


Figure 3. Progression-free survival (PFS) and overall survival (OS) curves based on bax/bcl-2 values. Kaplan-Meier plot comparing PFS (A) and OS (B) based on the detection of bax/bcl-2 ratio ≥ 1.50 (bax/bcl2⁺) or < 1.50 (bax/bcl2⁻). Bax/bcl2⁺ patients experienced both a longer PFS and OS ($P<0.0001$).

chemotherapy, while only 90 (33%) of 263 patients with bax/bcl-2 1.50 or over were treated ($P<0.0001$). Moreover, 82 (35%) bax/bcl-2 negative patients received two or more treatment regimens versus 25 (9%) bax/bcl-2 positive patients ($P<0.0001$). Interestingly, patients with lower bax/bcl-2 went more rapidly to treatment (27 months vs. 36 months; $P=0.019$). It is worthy of note that, in a series of 160 patients treated in induction with fludarabine plus rituximab, all resistant cases (8 patients) were bax/bcl-2 negative, while among patients who achieved a response (152 patients), those bax/bcl-2 positive showed a significant longer response duration compared to bax/bcl-2 negative cases (46% vs. 30% at 4 years; $P=0.021$) (Figure 2). With regard to clinical outcome, significant shorter PFS and OS were observed in patients with lower bax/bcl-2 (10% vs. 52% at 16 years, $P<0.0001$ and 46% vs. 79% at 16 years, $P<0.0001$, respectively) (Figure 3A and B). Similar results were obtained when analyzing the impact on PFS and OS of *IGHV* gene mutations as well as of *TP53* and *NOTCH1* mutations (Table 1). A better refinement in the prognostic assessment of PFS and OS was obtained by combining bax/bcl-2 values with those of *TP53* and *NOTCH1* (Figure 4A and B, and D and E). For all these variables, bax/bcl-2 expression had true additive properties. In particular, positive or negative bax/bcl-2 combined both with wild-type or mutated *TP53* or with wild-type or mutated *NOTCH1* identified 2 subsets of patients, the former with the best prognosis and the latter with the worst prognosis with regard to both PFS (65% vs. 10% at 8 years, $P<0.0001$ and 66% vs. 4% at 8 years, $P<0.0001$) and OS (96% vs. 33% at 10 years, $P<0.0001$ and 95% vs. 52% at 10 years, $P<0.0001$). In all combinations, discordant

patients showed intermediate outcomes (Figure 4A and B, and D and E). Similar behavior was observed by combining bax/bcl-2 and *IGHV* mutational status in additional bivariate analyses, the shortest PFS and OS time intervals being found for bax/bcl-2 negative/UM *IGHV* patients (7% vs. 63% at 10 years and 52% vs. 97% at 10 years; $P<0.0001$) (Figure 4C and F). To further explore the prognostic impact of bax/bcl-2, we investigated its expression within *TP53* mutated ($n=37$) and *IGHV* unmutated ($n=168$) subgroups that notoriously have the worst prognosis. As a matter of fact, higher bax/bcl-2 identified patients with a significant longer PFS (50% vs. 10% and 43% vs. 10% at 7 years; $P=0.039$ and $P=0.00002$, respectively) (Figure 5A and B), so confirming its very high prognostic impact.

Multivariate analysis

The clinical impact of bax/bcl-2 as independent prognosticator both for PFS and OS was also checked by multivariate Cox proportional hazards analysis applied to models including the prognosticators proven to be significant in univariate analysis (Table 1). In a cohort of 489 patients, in which all the above mentioned prognostic factors were available at the same time, bax/bcl-2 was confirmed to be an independent prognosticator with regard to PFS along with mod-Rai, $\beta 2M$, FISH, *TP53* and *IGHV* (Table 2). Similarly, multivariate analysis selected age, $\beta 2M$, bax/bcl-2, FISH, *NOTCH1*, *TP53* and *IGHV* as significant independent prognosticators for OS (Table 3). Equally, within a subset of 435 patients, in which sCD23 was included, bax/bcl-2 was confirmed again as an independent prognosticator both with regard to PFS and OS (Tables 2 and 3).

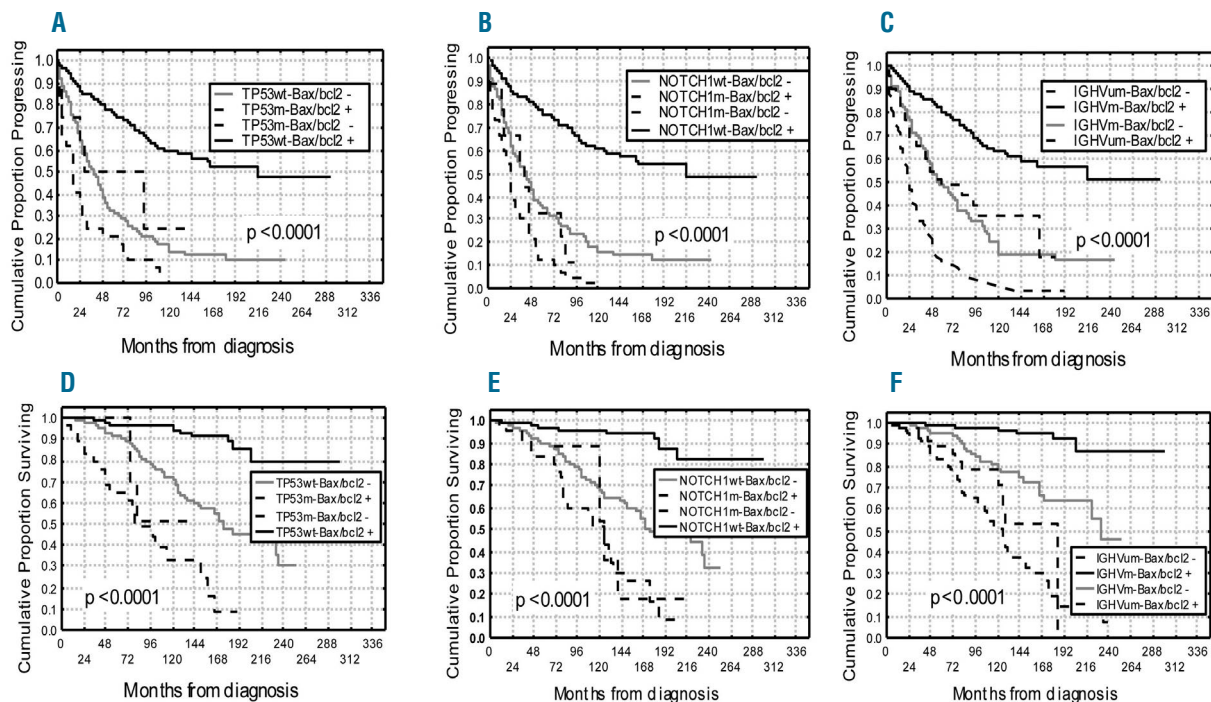


Figure 4. Progression-free survival (PFS) and overall survival (OS) curves in relation to combined bax/bcl-2 ratio and *TP53* or bax/bcl-2 and *NOTCH1* or bax/bcl-2 and *IGHV* status. PFS and OS were significantly longer within the bax/bcl-2+ (≥ 1.50) *TP53* wild-type (wt) subgroup (A-D), or within bax/bcl-2' *NOTCH1* wt patients (B-E) or within bax/bcl-2' *IGHV* mutated (m) cases (C-F). Discordant patients showed an intermediate outcome.

Discussion

In CLL malignant clonal B cells accumulate because of dysregulated production and inappropriately prolonged survival due to impairment of apoptosis.² In fact, the alteration of some apoptotic mechanisms such as the balance between the pro- (bax) and anti-apoptotic members (bcl-2) of the bcl-2 family, is recognized to be an important factor not only in the pathogenesis but also in the development of chemorefractoriness in CLL.⁷ Thus, measures of bax/bcl-2 could provide information on the intrinsic chemosensitivity of CLL influencing response to treatment and clinical outcome.

In the present study, we addressed the clinical impact of bax/bcl-2 ratio, evaluated with a flow cytometric approach, as independent prognostic factor in a large homogeneous series of 502 patients affected by CLL. We had previously demonstrated that the quantification of bcl-2 and bax, measuring fluorescence intensity by flow cytometry, is technically adequate and useful for a correct evaluation of clinical and prognostic correlations both in acute myeloid leukemia¹⁷ and in CLL.¹⁹ In the present study, we used a well-standardized and simple flow cytometric method for bcl-2 and bax determinations. In particular the methodological feasibility and reproducibility of this flow cytometric approach was confirmed by the finding that superimposable results of bax/bcl-2 ratios were obtained in a series of CLL cases for which bax/bcl-2 measures from fresh PB samples were repeated in two different laboratories (*Online Supplementary Table S1*), or when two directly conjugated anti-bax MoAbs were employed (*Online Supplementary Tables S2 and S3*).

The robustness of this flow cytometry-based method was also highlighted by the fact that in a series of 46 untreated patients in which the bax/bcl-2 measures were performed three times along the course of the disease, but always before any treatment, the bax/bcl-2 ratio was seen to be stable over time (*Online Supplementary Table S4*). This stability of the ratio underlines a reliable use of bax/bcl-2 ratio as prognostic marker, although further analysis is required to clarify this important issue. However, to the best of our knowledge, this is the largest study in CLL patients on clinical significance of bax/bcl-2 index by flow cytometry. Clinically, by using a cut off of 1.50, representing the median value of bax/bcl-2 ratio of our case cohort, and further confirmed as best cut off by ROC analysis, we found significant correlations of bax/bcl-2 ratio both with tumor burden indicators (PB B lymphocytes, Rai stages, β 2M, sCD23 and LDT) and with other biological prognosticators (CD38, CD49d, *IGHV* mutations, cytogenetic abnormalities, *NOTCH1* and *TP53*). Specifically, we observed that a higher bax/bcl-2 ratio showed a strict correlation with some favorable clinical features such as low PB B cells and low/intermediate Rai stages. Moreover, low levels of sCD23 and β 2M, known markers of disease activity in CLL, were correlated with higher apoptosis levels,²⁸ although previously published studies did not find any significant association between some apoptosis regulation proteins such as bcl-2, mcl-1 and Rai stages, while their correlation with CD23 or β 2M was not investigated.²⁹⁻³² Most of these studies involved a small number of patients and methods other than flow cytometry, such as Western blotting, immunoblotting and PCR.³³ However, Kitada *et al.*³² demonstrated that high bcl-2/bax ratio was

associated with elevated white blood cell count, similar to that found in our series. Moreover, we noted also that LDT was significantly longer in bax/bcl-2 positive patients, thus suggesting a significant correlation between this ratio and low proliferation.

From a biological point of view, we found a significant relationship between this index and some well-known biological prognosticators. First, we observed a significant lower bax/bcl-2 ratio in CD38 and CD49d positive patients. It is well known that CD38 and CD49d overexpression are adverse prognostic markers in CLL^{19,34,35} and it has been reported that interactions involving CD38 and CD49d prevent spontaneous and drug-induced apoptosis of normal and neoplastic B cells.³⁶ Interestingly, co-culture of CLL cells with human vascular endothelial cells determined a simultaneous overexpression of CD38, CD49d

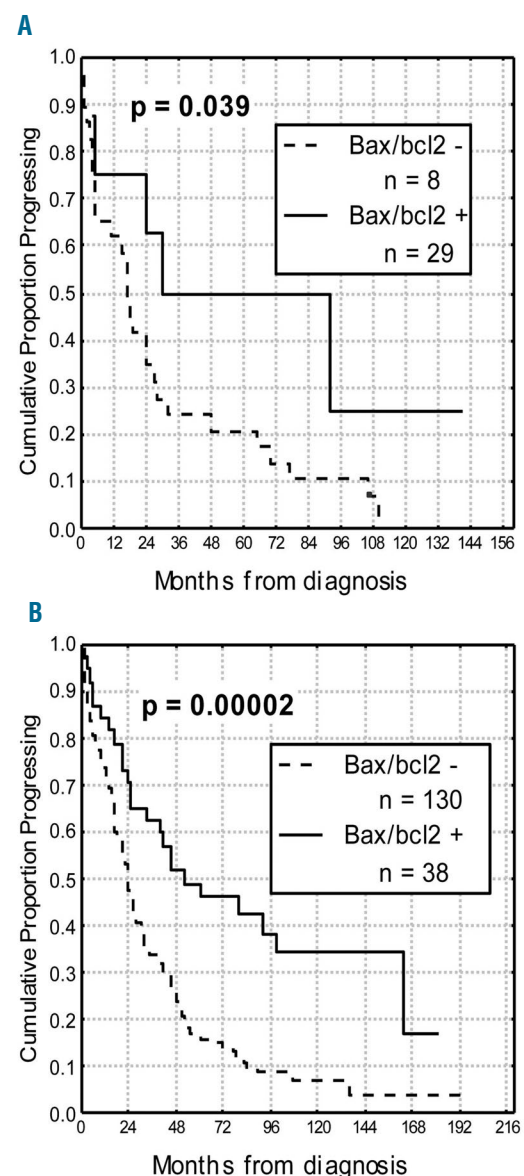


Figure 5. Progression-free survival (PFS) curves based on bax/bcl-2 within IGHV unmutated subgroup and TP53 mutated subgroup. Bax/bcl-2⁺ (≥ 1.50) patients showed a significant longer PFS both within TP53 mutated subgroup (A) and within IGHV unmutated subset (B).

and bcl-2 with an enhanced tumor cell survival targeting NF- κ B activation.³⁷ Recently, it has been observed that CD49d was nearly universally expressed in trisomy 12 CLL patients.³⁶ In our study, we demonstrated a significant correlation between lower bax/bcl-2 ratio and trisomy 12 (Table 1), so suggesting that this ratio may represent a further mechanism of chemoresistance in this subset. A strict association was also observed between bax/bcl-2 negative patients and other high risk (del11q and del17p) classes (Table 1), thus confirming the clinical impact of the apoptotic mechanisms in these poor risk cytogenetic subsets. Conversely, higher bax/bcl-2 was significantly represented in low risk (normal or del13q) cytogenetic groups (Table 1).

In addition, we found that a lower bax/bcl-2 ratio was associated with *NOTCH1* mutations (Table 1), unfavorable prognosticator in CLL,³⁸⁻⁴⁰ in keeping with previous studies showing that *NOTCH1* mutations activate NF κ B pathway resulting in upregulations of target genes such as bcl-2 in T-acute lymphoblastic leukemia.^{41,42}

Significant associations were also found between lower bax/bcl-2 ratio and *UM-IGHV* or *TP53* mutations ($P < 0.0001$) (Table 1), thus confirming that biological mechanisms related to defective apoptosis play a pivotal role both in the chemoresistance and in the unfavorable clinical course of these patient subgroups.

Results of this study also confirmed a correlation between bax/bcl-2 levels and chemoresistance in CLL, in keeping with previous studies.^{7,33,43,44} In particular, significant differences in treatment histories were found between bax/bcl-2 positive and negative patients. In fact, bax/bcl-2 negative patients underwent treatment more frequently and rapidly than bax/bcl-2 positive patients. Moreover, they presented a shorter response duration (Figure 2) and received, significantly, more chemotherapy regimens than patients with higher levels of bax/bcl-2.

Clinically, we demonstrated that the bax/bcl-2 ratio had an impact for PFS and OS in our large series of CLL patients, together with CD38, *IGHV* mutations, cytogenetic abnormalities, *NOTCH1* and *TP53* (Table 1). Moreover, bax/bcl-2 ratio was confirmed to be an independent prognostic factor with regard both to PFS and OS in multivariate analysis, considering 489 patients in which all the most important recognized prognostic factors were included (Tables 2 and 3).

Furthermore, bax/bcl-2 ratio demonstrated significant additive prognostic properties by the fact that positive or negative bax/bcl-2 combined both with wild-type or mutated *TP53* or with wild-type or mutated *NOTCH1* identified 2 subsets of patients, the former with the best prognosis and the latter with the worst prognosis with regard to both PFS and OS (Figure 4). The superior prognostic capacity of bax/bcl-2 with respect both to *IGHV* mutation status and *TP53* mutations^{25,45,46} was corroborated by the observation that higher bax/bcl-2 ratio identified patients with a significant longer PFS within *IGHV* unmu-

tated and *TP53* mutated subgroups, notoriously with the worst prognosis (Figure 5).

The capacity of bax/bcl-2 index to identify subsets of patients with a different apoptotic profile that reflects prognosis could be of interest in the light of the recently introduced bcl-2 inhibitors such as ABT-199, a 2nd-generation BH3 mimetic, highly selective for bcl-2 with demonstrated high rates of activity in relapsed/refractory CLL patients.^{5,10} In this regard, the bax/bcl-2 ratio, determined with the flow cytometric approach, as proposed in the present study, could represent a proper predictive biomarker to identify CLL patients who could benefit from the use of ABT-199 or who, on the contrary, could be more effectively treated with conventional chemotherapy. Experiments of apoptosis testing ABT-199 on CLL cells in order to use bax/bcl-2 ratio for identifying the sensitivity degree of patients and thus monitoring the anti-bcl-2 therapy are in progress at our Institution (*data not shown*). In this context, antibodies able to detect bax conformational changes could be useful to characterize apoptosis levels.⁴⁷

In conclusion, in the present study, we defined the prognostic power of bax/bcl-2 ratio, as determined by a flow cytometric approach, easily transferable to routine laboratory practice. Moreover, we highlighted the correlation of these two mitochondrial apoptotic proteins with chemoresistance and clinical outcome in CLL. Finally, the recently proposed new therapies employing bcl-2 inhibitors, such as ABT-199, prompted the potential use of bax/bcl-2 ratio to identify CLL cases putatively resistant to these molecules.

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