ASSOCIATION OF BAX EXPRESSION AND BCL2/BAX RATIO WITH CLINICAL AND MOLECULAR PROGNOSTIC MARKERS IN CHRONIC LYMPHOCYTIC LEUKEMIA

UDRUŽENOST EKSPRESIJE BAX GENA I BCL2/BAX ODNOSA SA KLINIČKIM I MOLEKULARNIM PROGNOSTIČKIM MARKERIMA U HRONIČNOJ LIMFOCITNOJ LEUKEMIJI

Ksenija Vucicevic¹, Vladimir Jakovljevic¹, Natasa Colovic²,³, Natasa Tosić⁴, Tatjana Kostić⁴, Irena Glumac⁴, Sonja Pavlović⁴, Teodora Karan-Djurasevic⁴, Milica Colovic³

¹Department of Physiology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
²Hematology Clinic, Clinical Center of Serbia, Belgrade, Serbia
³Medical Faculty, University of Belgrade, Belgrade, Serbia
⁴Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia

Summary

Background: In chronic lymphocytic leukemia (CLL), in vivo apoptotic resistance of malignant B lymphocytes results, in part, from the intrinsic defects of their apoptotic machinery. These include genetic alterations and aberrant expression of many apoptosis regulators, among which the Bcl2 family members play a central role.

Aim: The aim of this study was to investigate the association of pro-apoptotic Bax gene expression and Bcl2/Bax ratio with the clinical features of CLL patients as well as with molecular prognostic markers, namely the mutational status of rearranged immunoglobulin heavy variable (IGHV) genes and lipoprotein lipase (LPL) gene expression.

Methods: We analyzed the expression of Bax mRNA and Bcl2/Bax mRNA ratio in the peripheral blood mononuclear cells of 58 unselected CLL patients and 10 healthy controls by the quantitative reverse-transcriptase polymerase chain reaction.

Results: We detected significant Bax gene overexpression in CLL samples compared to non-leukemic samples (p=0.003), as well as an elevated Bcl2/Bax ratio (p=<0.001). Regarding the association with prognostic markers, the Bcl2/Bax ratio showed a negative correlation to lymphocyte doubling time (r=-0.307; p=0.0451), while high-level Bax expression showed a positive correlation with lymphocyte doubling time (r=0.25; p=0.015).

Relevant clinical features associated with high Bcl2/Bax ratio were shorter lymphocyte doubling time (p=0.0451) and higher levels of lactate dehydrogenase (LDH) (p=0.015). On the other hand, high Bax expression was associated with lower levels of LDH (p=0.0451).

Cytogenetic analysis revealed a higher frequency of trisomy 12 in patients with high Bcl2/Bax ratio compared to those with low ratio (p=0.0451).

Conclusion: The study suggests that the Bcl2/Bax ratio and Bax expression may be useful biomarkers for the prognosis of CLL patients, providing new insights into the molecular mechanisms underlying the disease.

Kratak sadržaj

Uvod: Rezistencija na apoptozu koja karakteriše maligne B limfocite in vivo u hroničnoj limfocitnoj leukemiji (HLL) delimično je uzrokovana unutrašnjim poremećajima apopotske mašinere u ovim ĉelijama. Ti poremećaji su rezultat genetičkih promena i aberantne ekspresije regulatora procesa apopitoze, među kojima ključnu ulogu imaju članovi Bcl2 familije.

Cilj: Cilj ove studije je bio da se ispita udruženost nivoa ekspresije proapoptotskog Bax gena, kao i Bcl2/Bax odnosa, sa kliničkim karakteristikama bolesnika sa HLL kao i molekularnim prognostičkim markerima, i to mutacionim statutom rearanjiranih gena za teške lance immunoglobulina (IGHV) i ekspresijom gena za lipoproteinski lipazu (LPL).

Metode: Analizirana je ekspresija Bax iRNK i Bcl2/Bax iRNK odnos u mononuklearnim ĉelijama periferne krvi 58 bolesnika sa HLL i 10 zdravih kontrola metodom reverzne transkripcije i lanĉane reakcije polymeraze u realnom vremenu (qRT-PCR).

Rezultati: Detektovana je povišena ekspresija Bax gena u HLL uzorcima u odnosu na kontrolne uzorke (p=0,003), kao i povišen Bcl2/Bax odnos (p=0,001). Kada je u pitanju udruženost sa prognostičkim markerima, Bcl2/Bax odnos je ispoljio negativnu korelaciju sa vremenom udvo-

Abbreviations: CLL, chronic lymphocytic leukemia; Bcl2, B-cell lymphoma 2; Bax, Bcl2-associated X; IGHV, immunoglobulin heavy chain variable region genes; LPL, lipoprotein lipase; mRNA, messenger ribonucleic acid; miR, micro ribonucleic acid; PBMC, peripheral blood mononuclear cells; LDT, lymphocyte doubling time; LDH, lactate dehydrogenase; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; ROC, receiver operating characteristic; A, area under the ROC curve; CI, confidence interval.
was associated with LPL-positive status (p=0.035). Both the expression of Bax and Bcl2/Bax ratio were higher in patients with unmutated vs. mutated IGHV rearrangements, but this difference did not reach statistical significance.

**Conclusions:** Our results suggest that dysregulated expression of Bcl2 and Bax, which leads to a high Bcl2/Bax ratio in leukemic cells, contributes to the pathogenesis and clinical course of CLL.

**Keywords:** apoptosis, Bax, Bcl2/Bax ratio, chronic lymphocytic leukemia, expression analysis

**Introduction**

Chronic lymphocytic leukemia (CLL) is the most frequent type of leukemia in Europe and North America, affecting predominantly elderly individuals aged approximately 65–70 years at diagnosis. It is characterized by monoclonal expansion of circulating small, mature CD5+/CD19+ CD23+ slgMlow B lymphocytes. The most striking feature of CLL is its extremely variable clinical presentation, with diverse therapy requirements and overall survival. In some patients the disease can follow an indolent course for years without developing any symptoms, while in others rapid progression and need of treatment occur soon after diagnosis (1). This fact has led to an extensive search for new cellular and molecular prognostic markers which could predict the clinical course of CLL.

Circulating CLL B lymphocytes are arrested in G0/early G1 phase of the cell cycle (5) and their gradual accumulation in blood, bone marrow and secondary lymphoid organs is being attributed primarily to defective apoptosis. Although a growing body of evidence suggests that CLL clone turnover is more dynamic than previously assumed, and that CLL cells proliferate and die at considerable rates (6, 7), impairment of apoptosis remains one of the hallmarks of CLL. The fact that CLL cells undergo spontaneous apoptosis when placed in culture points to the role of microenvironment-derived signals in their survival in vivo (8). On the other hand, marked inter-patient variability in the rate of apoptosis of CLL cells ex vivo implicates the existence of inherent differences in their apoptotic potential (9–11). Indeed, genetic alterations and aberrant expression of many apoptotic regulators involved in both intrinsic (mitochondrial) and extrinsic (death receptor) apoptotic pathways have been described in CLL.

Bcl2 family of proteins plays a central role in the regulation of the mitochondria-mediated pathway of apoptosis, and it is generally accepted that their deregulated function is involved in the pathogenesis and/or progression of CLL. Bcl2 family consists of pro-apoptotic (Bax, Bak, Bok, Bim, Bad, Bid, Bik, Bmf, Hrk, Noxa, Puma), and anti-apoptotic proteins (Bcl2, Bcl-XL, Mcl-1, A1, Bcl-W) (12). Given the functional antagonism between the pro- and anti-apoptotic Bcl2 family members, it is believed that the ratio of their activity levels is a critical determinant of the cells’ susceptibility to apoptosis, rather than the levels of individual proteins.

Leukemic cells of the majority of CLL patients overexpress the anti-apoptotic Bcl2 gene even though translocation (14;18), which juxtaposes Bcl2 to the immunoglobulin heavy chain enhancer, is a very rare event in CLL (13, 14). It has been suggested that the main mechanism underlying Bcl2 upregulation is hypomethylation of its promoter region, detected in a large proportion of patients (15). In addition, miR-15a and miR-16-1, which negatively regulate Bcl2 at the posttranscriptional level, are frequently downregulated or lost by the deletion of 13q14, the most common genomic aberration in CLL (16, 17). Abnormal expression of other Bcl2 family members has also been observed in CLL, namely Mcl1, BclXL, Bag-1, Bax, Bak, Bad (18–20), as well as Bcl2L12 and Bfl-1 (21, 22). However, the results of different studies regarding the relationship between the expression of Bcl2 family genes and proteins and the disease stage, clinical progression and response to treatment are highly discrepant.

In our previously published paper, we reported a significant overexpression of Bcl2 in a cohort of CLL patients compared to non-leukemic controls, and association of high Bcl2 mRNA levels with adverse prognostic parameters: progressive CLL, high serum β2-microglobulin, shorter lymphocyte doubling time (LDT) and high lipoprotein lipase gene (LPL) expression (21). In the study presented here, we broadened our previous research by analyzing the expression of the pro-apoptotic Bax gene in the same cohort of patients. The aim was to evaluate the association of Bax mRNA levels, as well as Bcl2/Bax ratio, with clinical and molecular prognostic markers in CLL, namely the mutational status of rearranged immunoglobulin heavy chain variable region genes (IGHV) and lipoprotein lipase gene expression. IGHV mutational status is the most powerful and the most stable molecular marker in CLL. Unmutated IGHV rearrangements represent an adverse prognostic factor and are
associated with shorter time to progression and overall survival (23–25). Lipoprotein lipase (LPL) is a novel molecular marker whose high-level expression is associated with unfavourable prognostic parameters in CLL, and which has been proposed as a surrogate marker for the IGHV mutational status (26–28).

Materials and Methods

This study enrolled a total of 58 unselected patients from the Hematology Clinic, Clinical Center of Serbia (Belgrade, Serbia), diagnosed as typical B cell CLL based on the clinical criteria and laboratory features. The study was approved by the medical ethic committee of the institution.

The patient group consisted of 45 men and 13 women (male/female ratio = 3.5), with a median age of 63.5 years (range 39–86) at the time of diagnosis. Median white blood cell count was $55 \times 10^9/L$ (range 13.5–413), and median lymphocyte count was $42 \times 10^9/L$ (range 4.1–371). The distribution of clinical Binet stages was as follows: 22 patients (42.3%) stage A, 7 patients (13.5%) stage B and 23 patients (44.2%) stage C (the staging information was unavailable for 6 patients). Lymphocyte doubling time (LDT) was determined in 43 out of 58 patients; LDT ranged from 1 to 84 months, with a median of 12 months.

Among 52 patients for whom we possessed follow-up information, progressive disease was observed in 40 patients (76.9%), whereas non-progressive disease was observed in 12 patients (23.1%). Patients were considered to have progressive disease based on at least one of the following criteria: lymphocyte doubling time of less than 1 year, progression to a more advanced Binet stage, development of systemic symptoms or Richter syndrome, or a downward trend of hemoglobin/platelet count to below the normal range.

Serum markers β2-microglobulin and lactate dehydrogenase were determined in 32/58 and 37/58 patients, respectively. The levels of β2-microglobulin ranged 0.21–13.5 mg/L, with a median of 3.86 mg/L. Twenty-five (67.6%) patients had normal levels of LDH, while in the remaining 12 patients (32.4%) LDH was elevated.

CD38 expression, lipoprotein lipase expression and IGHV mutational status were determined as reported in Karan-Djurasevic et al. (21). CD38 expression was assessed in 38 out of 58 CLL samples and, applying the cut-off level of 30% of CD38 positive cells, 14 patients (36.8%) were classified as CD38-positive, and 24 patients (63.2%) as CD38-negative. Regarding IGHV mutational status, 29 of our patients (50%) belonged to the mutated CLL subset (M-CLL), while the other 29 patients (50%) belonged to the unmutated CLL subset (U-CLL).

In all patients who received treatment (40/58) no therapy had been administered for at least 6 months prior to blood sampling. The control group consisted of 10 healthy individuals, 3 men and 7 women, with a median age of 53 years (range 44–84).

Peripheral blood mononuclear cells (PBMC) of all patients contained >90% of CLL lymphocytes, as confirmed by immunophenotyping. PBMC were isolated by Ficoll density-gradient centrifugation and total RNA was extracted using TRI reagent (Sigma-Aldrich). The isolated RNA was reverse-transcribed using RevertAid M-MuLV Reverse Transcriptase (Fermentas) and random hexamer primers, according to manufacturer’s instructions.

Bax mRNA expression was analysed by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) using SYBR Green chemistry in a 7500 Real Time PCR system (Applied Biosystems). The specific primers used for qRT-PCR amplification were: forward 5'-TGGCAAGCTGACATTTTCTGAC-3' and reverse 5'-TCACCCACACCCTGTCTT-3'. The amplification of Abl using the following primers: forward 5'-TGGAGATAACCTACTAAGCATAAAG-GT-3' and reverse 5'-GACGATGTTGCTTTGGAC-CA-3', served as internal control. The reaction mixture contained 50 ng cDNK, 1 × Power SYBR® Green PCR Master Mix (Applied Biosystems) and 0.5 pmol (Bax) or 2 pmol (Abl) of each gene-specific primer, in a final reaction volume of 10 μL. The cycling conditions were as follows: denaturation of the template at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Each qRT-PCR reaction was performed in duplicate, in order to evaluate reproducibility of the results. Quantification of target gene expression was made by a comparative ddCt method, using HL-60 cell line as the calibrator.

Statistical analysis

Statistical analyses were performed using Fisher’s exact test, Mann-Whitney rank-sum test, Spearman rank order correlation and receiver operating characteristic (ROC) analysis. All statistical tests were carried out using Sigma Stat 3.5 and SigmaPlot 11.0 software (Systat Software Inc.). Statistical significance was defined as p<0.05.

Results

In this study, we analyzed the expression of Bax gene and Bcl2/Bax ratio in a cohort of 58 unselected patients with chronic lymphocytic leukemia.

Bax expression

qRT-PCR expression analysis of Bax revealed significantly higher levels of Bax mRNA in CLL samples in comparison to non-leukemic samples (p=0.003;
Mann-Whitney rank sum test). However, the patient-to-patient variability and the increase of expression level in CLL vs. healthy controls was less prominent than in the case of Bcl2 (Figure 1).

The expression of Bax did not show association with gender and Binet staging. Although we observed a tendency towards a negative correlation between Bax expression and the age at diagnosis, statistical significance was not reached ($r=-0.26; p=0.0683; \text{Spearman rank order correlation}$). Bax mRNA level was not associated with the course of the disease (progressive vs. non-progressive) and LDT. In addition, neither correlation to the levels of serum markers β2-microglobulin and LDH, nor association with CD38 status were detected.

Bax was expressed at higher levels in U-CLL vs. M-CLL, but the difference in Bax expression between these two groups of patients did not reach statistical significance ($p=0.056; \text{Mann-Whitney rank sum test}$).

In order to investigate the relationship between LPL expression and the expression of Bcl2, Bax and Bcl2/Bax ratio, we used median LPL mRNA expression as a cut-off level to define LPL status. According to this cut-off level, 29 patients (50%) were LPL-positive and 29 patients (50%) LPL-negative. In addition, LPL-positive status showed strong association with unmutated IGHV genes ($p<0.001; \text{Fisher’s exact test}$), with only 6 discrepant cases (10.3%).

Having divided our cohort into two groups based on LPL status, we then used median expression of Bcl2 and Bax as a cut-off level to discriminate between high and low expressing cases. By applying this approach, we found that high levels of both Bcl2 and Bax expression were associated with an LPL-positive status ($p=0.008$ and $p=0.035$, respectively; Fisher’s exact test).

\[ Bcl2/Bax \text{ ratio} \]

In our cohort of patients, Bcl2 and Bax expression levels were positively correlated ($r=0.6; p=0.00; \text{Spearman rank order correlation}$). The ratio of Bcl2 and Bax mRNA expression ($Bcl2/Bax$ ratio) was significantly higher in CLL samples in comparison to healthy controls ($p<0.001; \text{Mann-Whitney rank sum test}$) (Figure 2).

Bcl2/Bax ratio was not found to be significantly associated with either gender, Binet stage or the course of the disease. Similar to Bax expression, the observed trend toward negative correlation to the age at diagnosis was not statistically significant ($r=-0.265; p=0.0627; \text{Spearman rank order correlation}$). On the other hand, there was a significant negative correlation between Bcl2/Bax ratio and LDT ($r=-0.307; p=0.0451; \text{Spearman rank order correlation}$). No association with the levels of β2-microglobulin, LDH and CD38 status was observed.

Bcl2/Bax ratio was higher in U-CLL vs. M-CLL and LPL-positive vs. LPL-negative groups of patients, but the association with either IGHV mutational status or LPL status was not statistically significant.

We performed receiver operating characteristic (ROC) analysis in order to evaluate the discriminatory power of Bcl2 and Bax mRNA expression in CLL. ROC analysis demonstrated that both Bcl2 expression and Bcl2/Bax ratio efficiently distinguished CLL from non-leukemic samples ($A=0.98, 95\% \text{ CI}=0.95–1.009,$)

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\text{Figure 1 Relative expression of Bcl2 and Bax mRNA in CLL and non-leukemic samples. qRT-PCR analysis showed a significantly higher expression of both Bcl2 and Bax in mononuclear cells of CLL patients (Bcl2 median 12.374; Bcl2 range 1.168–49.146; Bax median 2.346; Bax range 0.670–10.642) in comparison to healthy controls (Bcl2 median 1.411; Bcl2 range 0.685–2.629; Bax median 1.395; Bax range 0.972–2.243).}
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\text{Figure 2 Bcl2/Bax ratio in CLL and non-leukemic samples. qRT-PCR analysis showed a significantly higher Bcl2/Bax mRNA ratio in mononuclear cells of CLL patients in comparison to healthy controls.}
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Discussion

Chronic lymphocytic leukemia is considered to be a paradigmatic example of malignancy caused by dysregulation of apoptosis. However, the unique mechanism preventing CLL cells from undergoing apoptosis in vivo is still elusive, as is the significance of their apoptotic resistance for the clinical course of the disease.

Impairment of apoptosis results from the combination of microenvironmental survival signals and inherent genetic and epigenetic alterations of apoptotic machinery in CLL cells, both of which exert high discriminatory power between CLL patients and healthy controls. Bcl2 mRNA expression and Bcl2/Bax mRNA ratio exert very high discriminatory power between CLL patients and healthy controls.

Bcl2: A=0.98, sensitivity=0.95, specificity=1.00, 95% CI=0.95–1.009, p<0.0001

Bcl2/Bax ratio: A=0.96, sensitivity=0.90, specificity=1.00, 95% CI=0.9230–1.005, p<0.0001

Bax: A=0.80, sensitivity=0.79, specificity=0.90, 95% CI=0.6920–0.9097, p=0.002514

The level of Bax expression we measured was significantly increased in CLL samples, but it was less homogeneous and more overlapping with that of healthy controls than in the case of Bcl2. Given its pro-apoptotic role, the overexpression of Bax in CLL cells may seem paradoxical but, interestingly, an overexpression of both pro- and anti-apoptotic proteins has been observed in CLL (43). Moreover, their relative expression levels were positively correlated (44, 45). This is considered to be a compensatory mechanism used by cells in an attempt to regain equilibrium between pro- and anti-apoptotic proteins, which is crucial for apoptosis regulation.

Although in some studies higher expression of Bax mRNA and protein was detected in non-progressive vs. progressive CLL (46, 47), in our cohort no association with the course of the disease, Binet stage or LD) was observed. Regarding the association of Bax with the molecular prognostic markers, we observed higher Bax mRNA levels in U-CLL vs. M-CLL patients but, as was the case with Bcl2, without reaching statistical significance. On the other hand, high expression of both Bcl2 and Bax was associated with an LPL-positive status which, in turn, was an excellent predictor of unmutated IGHV genes. Several studies that investigated differential gene expression in U-CLL vs. M-CLL did not detect a significant association between Bcl2 and Bax expression and the IGHV mutational status (48, 49). However, our results show high Bcl2 and Bax expression in the group of patients defined by LPL positivity and unmutated IGHV rearrangements. In a study of Pallasch et al. (50), it was demonstrated that the LPL inhibitor orlistat has a cytotoxic effect on primary CLL cells through specific and concentration-dependent induction of apoptosis. In addition, the authors found that BcR stimulation significantly increases LPL expression in CLL cells. Thus, it would be important to elucidate the mecha-
nisms by which intracellular pathways of lipid metabolism, apoptotic and BcR signalling are interconnected in CLL.

Analysis of the Bcl2/Bax mRNA ratio revealed that it was higher in CLL samples in comparison to control samples and that it efficiently discriminated patients from healthy controls. However, the Bcl2/Bax ratio was not significantly related to either clinical characteristics of CLL (with the exception of LDT), or molecular prognostic markers, although it was slightly higher in LPL-positive vs. LPL-negative and U-CLL vs. M-CLL groups of patients. According to the results of some previous studies, an elevated Bc2/Bax ratio, measured at both the mRNA (46) and protein level (47), is associated with adverse prognostic parameters in CLL and is more relevant for the survival of CLL cells than the expression levels of Bc2 and Bax individually. In other studies, including ours, however, this association was not observed (43). The explanation for these contradictory results may lie in the fact that other members of the Bcl2 family modulate the function of Bc2 and Bax and, hence, their relative expression and/or activity levels also affect the CLL cells susceptibility to apoptosis. For example, the anti-apoptotic protein Mc1 forms heterodimers with Bax, so elevated expression of Bc2 and Mc1 has an additive or synergistic negative effect on the Bax’s pro-apoptotic function. Saxena et al. (51) suggested that, when it comes to response to apoptosis-inducing chemotherapeutics, a negative effect of high Bcl2/Bax ratio may be overcome by low Mc1 expression.

In summary, the results of this study further emphasize the complexity of the role that the dysregulated expression of Bc2 family members plays in prolonged survival of CLL cells and phenotype of the disease. Considerable inter-patient variability in their expression may contribute to the heterogeneity of CLL, but the association with clinical characteristics and molecular prognostic markers remains controversial. Further studies are needed to clarify to what extent the pattern of expression and/or activity of Bc2 family genes and proteins influences the clinical behaviour of CLL.

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Conflict of interest statement
The authors stated that they have no conflicts of interest regarding the publication of this article.


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