

EXPRESSION OF THE *BCL2* GENE IN CHRONIC LYMPHOCYTIC LEUKAEMIA PATIENTS

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EKSPRESIJA *BCL2* GENA KOD PACIJENATA SA HRONIČNOM LIMFOCITNOM LEUKEMIJOM

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ABSTRACT

Chronic lymphocytic leukaemia (CLL) manifests as clonal expansion of mature B lymphocytes, whose accumulation is primarily attributed to the dysregulation of apoptosis. Aberrant expression, as well as genetic alterations within various *Bcl2* family members and central regulators of the intrinsic, mitochondria-mediated apoptotic pathway all have been observed in CLL. Here, we report the expression analysis of the anti-apoptotic *Bcl2* gene in a cohort of 58 CLL patients. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis revealed a significant overexpression of *Bcl2* mRNA in CLL samples compared to control samples ($p < 0.001$). Receiver operating characteristic (ROC) analysis showed that the level of *Bcl2* expression exerts a high discriminatory power between patients and healthy subjects ($A = 0.98$, $95\% \text{ CI} = 0.95-1.009$, $p < 0.0001$).

Key-words: Chronic lymphocytic leukaemia, apoptosis, *Bcl2*, expression analysis

SAŽETAK

Hronična limfocitna leukemija (HLL) se manifestuje kao klonaska ekspanzija zrelih B limfocita, čija se akumulacija pripisuje prvenstveno poremećajima procesa apoptoze. U HLL su uočene genetičke promene i aberantna ekspresija različitih članova *Bcl2* genske familije, koji imaju ključnu ulogu u regulaciji unutrašnjeg, mitohondrijskog puta aktivacije apoptoze. U ovom radu je analizirana ekspresija anti-apoptotskog *Bcl2* gena u grupi od 58 pacijenata obolelih od HLL. Metodom kvantitativnog RT-PCRa detektovana je povišena ekspresija *Bcl2* mRNA u HLL uzorcima u odnosu na kontrolne uzorke ($p < 0.001$). "Receiver operating characteristic" (ROC) analiza je pokazala da nivo ekspresije *Bcl2* ima visoku moć diskriminacije između pacijenata i zdravih kontrola ($A = 0.98$, $95\% \text{ CI} = 0.95-1.009$, $p < 0.0001$).

Ključne reči: Hronična limfocitna leukemija, apoptoza, *Bcl2*, analiza ekspresije



ABBREVIATIONS

Bcl2 - B-cell lymphoma 2
CLL - Chronic lymphocytic leukaemia
mRNA - messenger ribonucleic acid

miRNA - micro ribonucleic acid
qRT-PCR - quantitative reverse-transcriptase polymerase chain reaction

INTRODUCTION

Chronic lymphocytic leukaemia (CLL) is the most frequent type of leukaemia in Western countries, affecting predominantly elderly individuals (aged 65 and over). It manifests as monoclonal expansion of small, mature CD5⁺ CD19⁺ CD23⁺ sIgM^{low} B lymphocytes. CLL is characterized by extremely variable clinical presentations, from indolent to rapidly progressive, with different therapy requirements and overall survival (1,2).

Circulating CLL B lymphocytes are arrested in G₀/early G₁ phase of the cell cycle (3), and their gradual accumulation in blood, bone marrow and secondary lymphoid organs is primarily a result of impaired apoptosis.

The *Bcl2* family is a group of evolutionary conserved pro- and anti-apoptotic proteins coded by *Bcl2* family genes, which are involved in the regulation of the intrinsic, mitochondria-mediated pathway of apoptosis. All *Bcl2* family proteins con-



tain at least one of four highly conserved α -helical BH (Bcl2 homology) domains (BH1, BH2, BH3 and BH4). Anti-apoptotic members (Bcl2, Bcl-X_L, Mcl-1, A1, Bcl-W) contain all four BH domains and a C-terminal transmembrane domain. The pro-apoptotic members are divided into “multidomains” (Bax, Bak, Bok), containing BH1-3 and a transmembrane domain, and “BH3-only” (Bim, Bad, Bid, Bik, Bmf, Hrk, Noxa, Puma), containing only the BH3 domain (4). In the absence of apoptotic stimulus, pro-apoptotic members reside in the cytosol, whereas anti-apoptotic are anchored to the mitochondrial membrane and suppress the release of cytochrome c, which is crucial for activation of the caspase cascade and execution of apoptosis (5). Upon receiving a death stimulus, pro-apoptotic proteins translocate from the cytosol to the mitochondrial membrane and interact with anti-apoptotic proteins, ultimately leading to cytochrome c release (6).

BH domains are sites of interaction between Bcl2 family members; this interaction is essential for homo- and heterodimerization, which is one of the main mechanisms of their action. In addition to a prototypical Bcl2/Bax dimer formation, other interactions among Bcl2 family proteins can occur. In addition to dimerization, their activity is modulated through regulation at both transcriptional and post-translational levels (7).

Genetic and epigenetic alterations, as well as aberrant expression of various *Bcl2* family genes, have been observed in CLL (8).

Leukaemic B lymphocytes from the majority of CLL patients (>80%) express high levels of *Bcl2*, a typical anti-apoptotic member of the *Bcl2* family (9). The expression is higher not only in comparison to normal B lymphocytes but also when compared to cells that harbour translocation t(14;18), which places the *Bcl2* gene in close proximity to the immunoglobulin heavy chain enhancer (10). Translocation t(14;18) is common in follicular lymphoma but is a very rare event in CLL. It has been demonstrated that in a high proportion of patients, the *Bcl2* promoter is hypomethylated, leading to increased transcription (11). In addition, overexpression of Bcl2 has been linked to downregulation or loss of miR-15a and miR-16-1. These miRNAs negatively regulate Bcl2 at the post-transcriptional level and are located within the deletion of 13q14, the most frequent genomic aberration in CLL (12,13). Furthermore, Bcl2 overexpression has been reported to associate with -938C>A promoter polymorphism (14); however, this finding was not confirmed by subsequent research (15). The prognostic significance and therapeutic implications of the *Bcl2* gene and protein expression level are still under investigation due to conflicting results obtained in different studies.

The aim of this study was to analyse the expression of the *Bcl2* gene in patients with chronic lymphocytic leukaemia.

PATIENTS AND METHODS

This study enrolled 58 unselected patients from the Clinic for Haematology, Clinical Centre of Serbia (Belgrade,

Serbia), who were diagnosed with typical B cell CLL based on clinical criteria and laboratory features. The study was approved by the medical ethics 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.

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).

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 (PBMCs) of all patients contained >90% of CLL lymphocytes, as confirmed by immunophenotyping. PBMCs 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 the manufacturer's instructions.

Bcl2 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 5'-TCGCCCTGTGGATGACTGA-3' (forward) and 5'-CAGAGACAGCCAGGAGAAATC-3' (reverse). The amplification of Abl using the following primers: forward 5'-TGGAGATAACACTCTAAGCATAACTAAAGGT-3' and reverse 5'-GATGTAGTTGCTTGGGACCCA-3', served as an internal control. The reaction mixture contained 50 ng cDNA, 1 x *Power SYBR*[®] Green PCR Master Mix (Applied Biosystems) and 0.5 pmol (Bcl2) or 2 pmol (Abl) of each gene-specific primer with a final reaction volume of 10 μ l. 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 performed using the comparative ddCt method with the HL-60 cell line as the calibrator.

Statistical analyses were performed using the 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 analysed the expression of the *Bcl2* gene in a cohort of 58 unselected patients with chronic lymphocytic leukaemia.

Using qRT-PCR methodology for expression analysis, we detected significantly higher levels of *Bcl2* mRNA in CLL samples compared to non-leukaemic samples ($p < 0.001$) (Fig. 1).

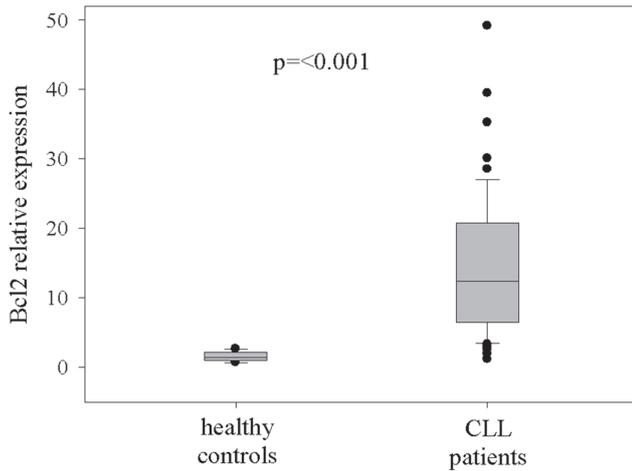


Figure 1
Relative expression of *Bcl2* mRNA in CLL and non-leukaemic samples qRT-PCR analysis showed a significantly higher expression of *Bcl2* in mononuclear cells of CLL patients in comparison to healthy controls ($p < 0.001$; Mann-Whitney Rank Sum Test).

In addition, we observed a wide variability in the expression of *Bcl2* among CLL patients (1.168-49.146 relative units), in contrast to relatively homogeneous expression among healthy controls (0.685-2.629 relative units). We performed receiver operating characteristic (ROC) analysis in order to evaluate the discriminatory power of *Bcl2* expression in CLL. ROC analysis demonstrated that *Bcl2* mRNA expression efficiently distinguished CLL from control samples (**cut-off=2.76 relative units**, $A=0.98$, $95\% \text{ CI}=0.95-1.009$, $p < 0.0001$), exerting an excellent positive predictive value (PPV=1) and a slightly lower negative predictive value (NPV=0.77) (Fig. 2).

Bcl2 expression level did not show any association with either gender or Binet staging. However, a significant negative correlation with the age of patients at diagnosis ($r = -0.4$, $p = 0.004$) was detected.

DISCUSSION

Dysregulation of apoptosis is considered to be a hallmark of chronic lymphocytic leukaemia. In addition to the pro-survival influence of the microenvironment-derived signals, intrinsic defects of different apoptotic pathways have been identified in CLL cells, rendering them resistant to apoptosis. For example, the function of the ATM-p53 pathway is often abrogated by 11q22-q23 and 17p13 deletions, which exert the most adverse prognostic impact among all genomic aberrations detected in CLL. Deletions, as well as mutations and aberrant expression of *p53* and *ATM*, have been associated with progressive disease and shorter overall survival (16,17,18). Impaired function of other apoptotic pathways, namely the PI3K/Akt pathway (19,20,21), NF- κ B pathway (19,22) and Fas/FasL system (23,24), have also been implicated in CLL.

The role of the Bcl2 family of proteins has been extensively studied in CLL because they are key regulators of

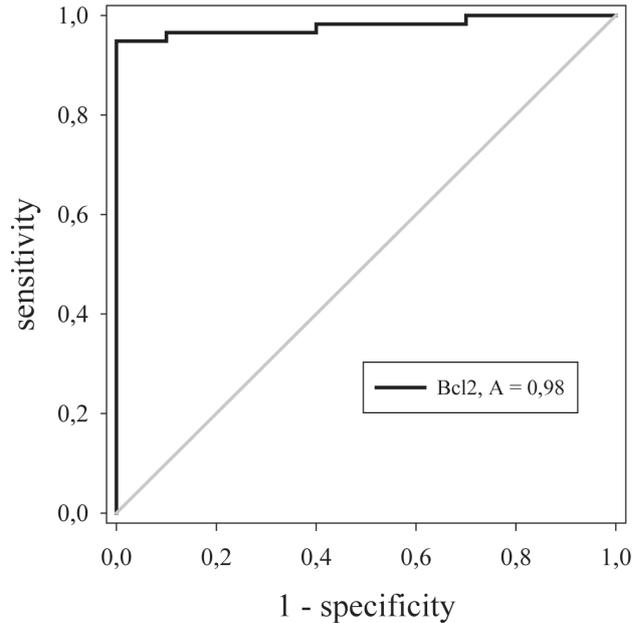


Figure 2
ROC analysis of *Bcl2* expression in CLL and non-leukaemic samples *Bcl2* mRNA expression exerts high discriminatory power between CLL patients and healthy controls. ($A=0.98$, sensitivity=0.95, specificity=1, $95\% \text{ CI}=0.95-1.009$, $p < 0.0001$)
Abbreviations: ROC, receiver operating characteristic; A, area under the ROC curve; CI, confidence interval.

the mitochondrial apoptotic pathway. Genetic alterations and aberrant expression of various pro- and anti-apoptotic members have been observed in different studies. In addition to Bcl2, elevated expression in CLL patients vs. healthy controls of other Bcl2 family proteins and genes has been observed, namely Mcl1, BclX_L and *Bcl2L12* (25,26,27). Moreover, in several studies, an upregulation of pro-apoptotic members was observed as well (28), which may seem paradoxical given the longevity of CLL cells. However, this is thought to represent a mechanism by which cells try to compensate for an excess of anti-apoptotic proteins through elevation of their functional antagonists. It should be noted though, that in other studies, pro-apoptotic Bax, Bak and BclX_s were observed to be underexpressed (29). Moreover, it is generally accepted that relative expression and/or activity levels, rather than the levels of individual proteins, are critical determinants of CLL cells' susceptibility to apoptosis.

Overall, the results of different studies regarding the relationship between the expression of Bcl2 family proteins and genes and the clinical behaviour of CLL are highly discrepant, and no consistent correlation with the disease stage, clinical progression or response to treatment could be established.

In this study, we analysed the expression of *Bcl2* and anti-apoptotic members of the *Bcl2* family in CLL patients and healthy controls.

In concordance with other reports, we observed a significant overexpression of *Bcl2* in CLL samples compared to



non-leukaemic samples. According to the calculated cut-off level, 94.8% of patients in our cohort were high-expressing *Bcl2* cases. Although we detected a substantially wide range of *Bcl2* mRNA expression levels among patients, they overlapped to a very small degree with those of healthy controls; therefore, the ROC analysis showed that the *Bcl2* expression level efficiently discriminates CLL from normal samples.

In our study, no association between *Bcl2* mRNA expression and clinical stage of CLL was detected. However, the results of other studies regarding the association of *Bcl2* protein expression with clinical stage remain controversial. In several cohorts, *Bcl2* expression was higher in advanced stages of the disease (30,31), while in others, such a correlation could not be demonstrated (28,32). The lack of association with clinical stage reopens the question of whether elevated *Bcl2* expression is acquired during leukemogenesis, or if elevated *Bcl2* expression began present in the originating cells of CLL (33). It should be noted that both staging systems currently in use, Rai and Binet, were applied in these studies, which may have led to the inconsistency of the results.

In summary, the findings of the present study further support the hypothesis that *Bcl2* overexpression plays a role in the pathogenesis of CLL. To what extent inter-patient variability in *Bcl2* expression contributes to the clinical heterogeneity of CLL has yet to be elucidated.

Research on the mRNA expression levels of anti-apoptotic *Bcl-2* family members in human cancer cell lines using qRT-PCR techniques and the assessment of the ability of known *Bcl-2* inhibitors to induce cell death within them revealed that the effectiveness of known *Bcl-2* inhibitors depends on the mRNA expression profile of tumor cells. The correlation between the cell-killing properties of known *Bcl-2* inhibitors and the relative mRNA expression levels of anti-apoptotic *Bcl-2* family members has been observed in leukaemia cell lines and has provided critical insights into apoptosis-based anticancer strategies that target *Bcl-2* proteins (34). Moreover, gene expression profiling emphasizes the value of the follow up of molecular markers in CLL patients (especially mRNA expression of *Bcl2* and *NOXA*) in order to facilitate the choice of an effective treatment for individual patients (35). Therefore, the *Bcl2* mRNA expression profile of each patient could lead to personalized treatment. Thus, the methodology used in this study represents a promising tool for individualization and optimization of therapy for CLL patients.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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