

RELATIVE BIRC3 GENE EXPRESSION IN CHRONIC LYMPHOCYTIC LEUKEMIA**RELATIVNA EKSPRESIJA BIRC3 GENA KOD HRONIČNE LIMFOCITNE LEUKEMIJE**

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Summary

Background: Chronic lymphocytic leukemia (CLL) represents the most frequent type of leukemia in the adult population. It is characterized by highly variable clinical course, predominantly initiated by different genetic alterations responsible for the regulation of cell proliferation and apoptosis. The aim of this study was to evaluate the relative expression of the BIRC3 gene in CLL patients.

Methods: This cross-sectional study included 78 participants: a CLL group (n=50) and a control group of healthy subjects (n=28), both age- and sex-matched. To all participants, CBC (complete blood count), basic biochemical parameters, as well as FISH (fluorescent in situ hybridisation) for determining chromosomal aberrations and PCR analyses for determining the relative expression of the BIRC3 (Baculoviral IAP Repeat Containing 3) gene were performed. All reactions were performed on QIA quant 96 5 plex real-time PCR instrument (Qiagen, Hilden, Germany). Additionally, FISH diagnostics was performed using Abbott Vysis CLL FISH Probe Kit (Abbott Molecular diagnostics, Chicago, IL, USA).

Results: Deletion of the long arm of the 13th chromosome was the most frequent chromosomal aberration in the CLL group. A statistically significant difference was observed in the relative expression of BIRC3, with higher values in the study group (p=0.019). The results of the binary logistic regression model indicated that lactate dehydrogenase and total calcium were statistically significant predictors of relative BIRC3 gene expression (p < 0.05).

Conclusion: Determination of relative BIRC3 gene expression may be a valuable analysis for both the initial diagnosis and the clinical follow-up of CLL patients.

Keywords: baculoviral IAP repeat containing 3, BIRC3, CLL, chronic lymphocytic leukaemia, PCR

Kratak sadržaj

Uvod: Hronična limfocitna leukemija (HLL) predstavlja najčešći tip leukemije kod odraslih. Karakteriše je veoma varijabilan klinički tok, pretežno iniciran različitim genetičkim promenama odgovornim za regulaciju ćelijske proliferacije i apoptoze. Cilj ove studije bio je da se proceni relativna ekspresija gena BIRC3 kod pacijenata sa HLL-om.

Metode: Ova studija preseka obuhvatila je 78 učesnika: HLL grupu (n=50) i kontrolnu grupu zdravih ispitanika (n=28), usklađenih po starosti i polu. Svim učesnicima je urađena kompletna krvna slika (KKS), osnovni biohemijski parametri, FISH (fluorescentna in situ hibridizacija) za određivanje hromozomskih aberacija kao i PCR analiza za određivanje relativne ekspresije gena BIRC3 (Baculoviral IAP Repeat Containing 3). Sve reakcije su izvedene na QIA quant 96 5 plex real-time PCR aparatu (Qiagen, Hilden, Nemačka). FISH analiza je izvršena primenom Abbott Vysis CLL FISH Probe Kit-a (Abbott Molecular diagnostics, čikago, Illinois, SAD).

Rezultati: Delecija dugog kraka 13. hromozoma bila je najčešća hromozomska aberacija u HLL grupi. Statistički značajna razlika je primećena u relativnoj ekspresiji BIRC3, sa višim vrednostima u ispitivanoj grupi (p=0,019). Rezultati binarnog logističkog regresionog modela pokazali su da su laktat dehidrogenaza i ukupni kalcijum statistički značajni prediktori relativne ekspresije gena BIRC3 (p < 0,05).

Zaključak: Određivanje relativne ekspresije gena BIRC3 može biti značajna analiza kako za početnu dijagnozu, tako i za kliničko praćenje pacijenata sa HLL-om.

Ključne reči: BIRC3, HLL, Hronična limfocitna leukemija, PCR

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Introduction

Chronic lymphocytic leukaemia (CLL) is a malignant, lymphoproliferative disease with the highest prevalence in the adult population (4.9 cases per 100,000). In Serbia, about 350 people fall ill with this disease annually (1).

The basic characteristic of CLL is the progressive accumulation of functionally deficient, but apparently mature, small B-lymphocytes of monoclonal origin, which can be reliably demonstrated by immunophenotyping analysis (2). In a significant percentage, the disease can have a stable clinical course; however, frequent relapses are possible, followed by an aggressive form as well as transformation into high-grade diffuse large B-cell lymphoma (Richter's transformation) (3).

The exact etiopathogenesis of CLL has not yet been fully established. Namely, it is considered that a critical event is the appearance of additional changes in the bone marrow, genetic or microenvironmental, that are crucial for the transformation into a malignant cell.

At the basis of CLL, numerous cytogenetic changes occur in almost 80% of cases (4). The most significant are: trisomy of the 12th chromosome, deletion of the 13th chromosome (13q14), deletion of the 11th chromosome (11q) and deletion of the 17th chromosome (17p) (4, 5). Also, gene rearrangements affecting the region encoding immunoglobulin heavy chains have been identified. In the largest percentage, the immunoglobulin heavy chain region is combined with BCL2 (t(14;18)), BCL3 (t(14;19)), and BCL11 (t(2;14)) (6). It is known that at least 45 loci are associated with the tendency to develop CLL (7). As impaired apoptosis is one of the central features of CLL (8), several recent studies pointed out that changes in BIRC3 (Baculoviral IAP Repeat Containing 3) gene expression are associated with the disruption of apoptosis induction, given that the mentioned gene encodes the synthesis of a protein that acts as an inhibitor of apoptosis (9).

All genetic characteristics of tumour CLL cells more closely determine the nature of the cells, enabling a more precise diagnosis and monitoring of the therapeutic effects on the disease itself.

This study aimed to evaluate the relative expression of the BIRC3 gene in CLL patients.

Materials and Methods

Participants and study design

Between November 2024 and June 2025, a total of 78 adult participants were included a group of CLL patients (n=50) and a control group of healthy

subjects (n=28), age- and sex-matched. At the time of examination, all participants in the control group did not have any acute or chronic inflammatory or haematological malignancy. Patients were admitted to the Outpatient Department of Haematology at the University Clinical Centre of Vojvodina (Novi Sad, Serbia). To all participants, blood was drawn to determine CBC (complete blood count), basic biochemical parameters, FISH (fluorescent in situ hybridisation) diagnostics of major chromosomal aberrations, and PCR analyses.

Standard blood parameter tests and FISH diagnostics

CBC analysis was performed on an automated hematology platform, Sysmex XN-1000 (Japan Inc., Kobe, Japan). The following biochemical analyses were performed: lactate dehydrogenase, BUN, creatinine, electrolytes (potassium, sodium and chloride), total and ionised calcium, immunoglobulins (IgG and IgA), beta-2 microglobulin, albumin, and total proteins. They were all analysed on Abbott Alinity c (Abbott Laboratories, Chicago, IL, USA), except ionised calcium, which was performed on an AVL 9180 Electrolyte Analyser (Roche Diagnostics) using standard ion-selective electrode (ISE) methodology.

FISH analyses were performed on an automated Olympus fluorescence microscope BX53 (Olympus Corporation, Tokyo, Japan) using BioView software (BioView Ltd., Rehovot, Israel) to identify and locate specific DNA sequences such as trisomy of the 13th chromosome, deletion of the 11th (long arm, 11q), deletion of the 13th (long arm, 13q14) and deletion of the 17th (short arm, 17p) chromosomes. For all aforementioned chromosomal aberrations, Abbott Vysis CLL FISH Probe Kit was used for in vitro diagnostics (Abbott Molecular Diagnostics, Chicago, IL, USA).

RNA extraction, cDNA synthesis and real-time PCR

To determine relative BIRC3 gene expression, whole-blood specimens were collected from subjects in PaxGene Blood RNA tubes (Becton, Dickinson, Plymouth, UK) and stored at -18 °C for up to 1 week. RNA extraction was carried out with the PaxGene Blood RNA Kit (Qiagen, Hilden, Germany), and cDNA synthesis was performed using the TaqMan® High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), both according to the manufacturer's protocols. The cDNA was amplified, and the resulting PCR product was detected using custom primers for BIRC3 and the β -actin (internal control) gene (Invitrogen, Pleasanton, California, USA), previously reported by

Wang et al., and SYBRTM Green Universal Master Mix (Thermo Fisher Scientific, Nerum, Denmark) (10). All reactions were run on QIA quant 96 5 plex real-time PCR instrument (Qiagen, Hilden, Germany). The temperature profile included denaturation at 95 °C for 3 min, followed by 45 cycles (95 °C for 15s and 60 °C for 1 min). To normalise gene expression levels, the Δ Ct value (the difference between the Ct values of BIRC3 and β -actin) and the $2^{-\Delta\text{Ct}}$ value were calculated for all samples.

Statistical analysis

IBM® SPSS version 25.0 (IBM SPSS Inc., Armonk, NY, USA) was used for data analysis. All collected data were initially summarised using appropriate descriptive statistics. Numerical variables were presented as mean, standard deviation, and minimum and maximum values, while categorical variables were described using frequencies and percentages to provide a clear overview of their distribution within the sample. The normality of numerical variables was assessed using the Shapiro-Wilk test. To compare differences between the two independent groups, either the Student's t-test (for

normally distributed variables) or the Mann–Whitney U test (for non-normally distributed variables) was applied. For paired comparisons involving related samples that did not meet the assumptions of parametric tests, the Wilcoxon signed-rank test was used. The results were considered statistically significant if the p-value was <0.05.

To explore predictors of elevated gene expression, a binary logistic regression model was performed.

Ethical considerations

Informed consent was obtained from all participants. The institutional Ethics Committee approved the study on October 15th, 2024 (Decision No. 00-362).

Results

The mean age in the study group was 65.88 years (SD=11.05) and 64.68 years (SD=14.86) in the control group, indicating a similar age distribution. Table 1 summarises the comparison of major haematological and biochemical parameters

Table 1 Parameters of descriptive statistics.

	Study group N=50				Control group N=28				p-value
	Mean	SD	Min	Max	Mean	SD	Min	Max	
RBC(10^{12} /L)	4.56	0.41	3.56	5.40	4.83	0.48	4.06	5.91	0.009
PLT (10^9 /L)	223.10	76.47	118.00	392.00	266.89	77.62	45.00	428.00	0.006
WBC (10^9 /L)	36.72	32.00	9.45	165.66	6.81	1.56	3.96	10.25	0.000
Lymphocyte count (10^9 /L)	29.88	29.87	3.19	149.95	2.22	0.76	1.28	4.48	0.000
BUN (mmol/L)	6.06	1.72	3.30	13.00	5.37	1.34	3.10	7.90	0.092
Creatinine (μ mol/L)	85.28	29.01	49.00	200.00	74.18	20.06	49.00	129.00	0.094
LDH (U/L)	208.15	63.44	116.00	483.00	200.48	38.39	155.00	311.00	0.775
Na ⁺ (mmol/L)	141.17	1.95	137.00	146.00	140.75	2.03	135.00	144.00	0.374
K ⁺ (mmol/L)	13.36	58.88	3.70	404.00	4.51	0.39	3.60	5.10	0.724
Cl ⁻ (mmol/L)	106.81	2.32	100.00	111.00	102.06	19.82	1.70	109.00	0.070
Total Ca (mmol/L)	2.41	0.12	2.22	3.00	2.34	0.10	2.18	2.51	0.047
Ca ⁺² (mmol/L)	1.28	0.07	1.00	1.420	1.29	0.03	1.22	1.35	0.799
IGG (g/L)	9.74	3.07	3.30	15.70	11.40	1.75	8.30	14.40	0.014
IGA (g/L)	1.49	0.84	0.36	3.69	1.89	1.06	0.70	5.41	0.088
β 2 micro (mg/L)	3.10	1.03	1.55	6.43	2.23	0.64	1.36	4.05	0.000
TP (mg/L)	72.33	5.40	64.00	90.00	72.63	4.23	66.00	80.00	0.500
Albumins (g/L)	48.55	3.33	41.91	57.29	47.41	2.99	41.95	52.00	0.437

Legend: RBC, red blood count; WBC, white blood count; PLT, platelet count; SD, standard deviation; BUN, blood urea nitrogen; LDH, lactate dehydrogenase; Na⁺, sodium; K⁺, potassium; Cl⁻, chloride; Ca⁺², ionised calcium; IGG, immunoglobulin class G; IGA, immunoglobulin class A; β 2 micro, beta2 microglobulin; TP, total proteins.

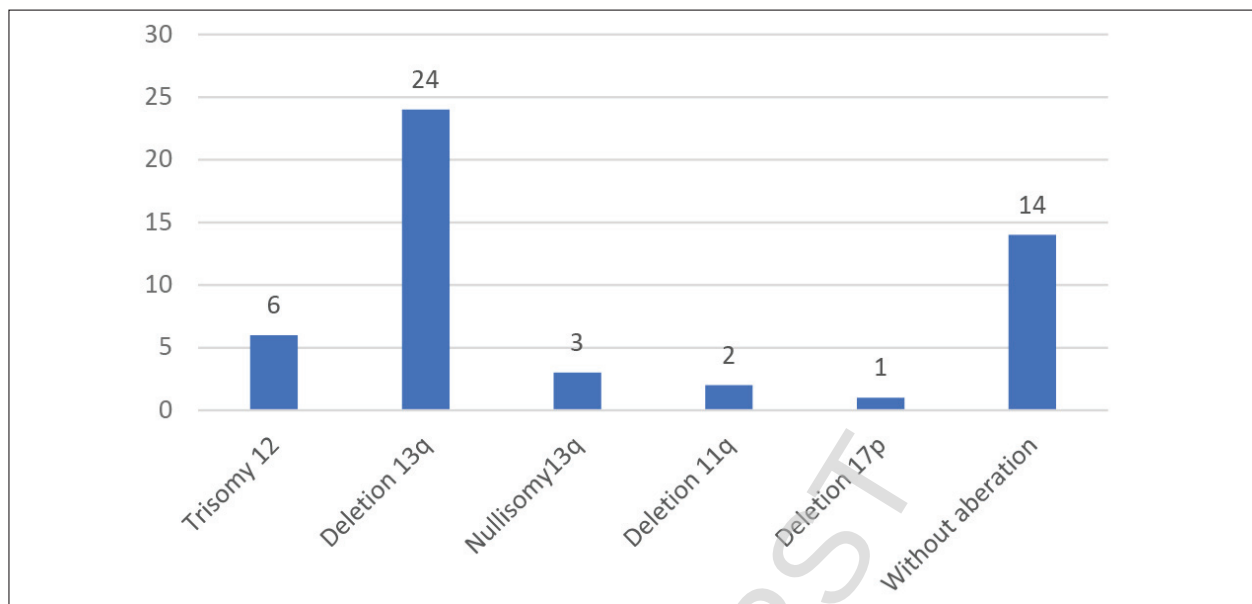


Figure 1 The frequency of chromosomal aberrations in the study group (N=50).

Table II Differences between the study and control group in relative expression of the BIRC3 gene (N=78).

	Study group N=50				Control group N=28				p-value
	Mean	SD	Min	Max	Mean	SD	Min	Max	
Ct BIRC3	16.23	1.38	14.28	21.72	18.00	4.44	10.52	29.97	<0.001
Ct β actin	14.48	6.88	9.13	32.34	14.77	4.59	9.86	26.09	0.258
Δ Ct	1.75	6.32	-15.24	7.29	3.24	6.57	-7.65	11.94	0.019
$2^{-\Delta\Delta\text{Ct}}$	<u>1838.12</u>	<u>7313.44</u>	<u>0.006</u>	<u>38698.77</u>	<u>33.54</u>	<u>62.36</u>	<u>0.00</u>	<u>200.85</u>	<u>0.019</u>
$\Delta\Delta$ Ct	-4.12	6.32	-21.11	1.42	-2.63	6.56	-13.52	6.07	0.019

Legend: Ct BIRC3; Ct β actin; Δ Ct; $2^{-\Delta\Delta\text{Ct}}$; $\Delta\Delta$ Ct; P value

between the study and control groups. Among the analysed variables, the study group had statistically significantly higher white blood cell and lymphocyte counts ($p=0.000$) and lower red blood cell and platelet counts ($p=0.009$ and $p=0.006$, respectively). Significantly higher levels of total calcium and β_2 -microglobulin were observed in the study compared with the control group. Also, the study group had significantly lower total immunoglobulin G (IgG) levels ($p=0.014$).

Figure 1 represents the frequency of chromosomal aberrations characteristic of chronic lymphocytic leukaemia (study group). Deletion of the long arm of chromosome 13 was the most frequent chromosomal aberration in the group. On the other hand, in 14 patients, none of the specific

chromosomal aberrations was observed using FISH.

Table II presents the comparison of relative gene expression levels of BIRC3 between the study and control groups. According to the results, the study group had statistically higher relative expression of the BIRC3 gene, as indicated by a higher $2^{-\Delta\Delta\text{Ct}}$ ($p=0.019$).

To determine the optimal threshold for further classification of gene expression levels, a ROC analysis was performed using the variable $2^{-\Delta\Delta\text{Ct}}$. The analysis yielded an area under the curve (AUC) of 0.662 (95% CI: 0.515–0.810, $p=0.019$), indicating a moderate discriminatory power. The cut-off value of 0.012692 was selected based on

Table III A binary logistic regression model.

N=78	B	S.E.	Wald	df	Sig.	Exp(B)
LDH (U/L)	0.020	0.008	5.868	1	0.015	1.020
$\beta 2$ micro (mg/L)	-2.190	0.630	3.744	1	0.053	0.112
Creatinine (μ mol/L)	0.032	0.022	2.378	1	0.161	1.035
TP (mg/L)	-0.132	0.080	2.676	1	0.102	0.877
Lymphocyte count (10^9 /L)	0.167	0.022	5.909	1	0.056	1.182
Total Ca (mmol/L)	-7.629	4.584	2.770	1	0.006	1998.2
Constant	-3.657	8.660	0.178	1	0.673	0.026

Legend: LDH, lactate dehydrogenase; $\beta 2$ micro, beta2 microglobulin; TP, total proteins; total Ca, total calcium concentration.

the Youden index, which identifies the point on the ROC curve that maximises the difference between sensitivity and $1 - \text{specificity}$. At this threshold, the sensitivity was 86.0%. In comparison, the specificity was approximately 52%, providing a reasonable balance between correctly identifying positive cases and minimising false positives.

To explore predictors of relative gene expression, a binary logistic regression model was performed using a dichotomised outcome based on the optimal cut-off value of $2^{-\Delta Ct} > 0.012692$, determined via ROC analysis. Individuals above this threshold were classified as having higher expression levels.

Model selection was conducted using a backward stepwise method (likelihood ratio). The final model was statistically significant according to the Omnibus Test of Model Coefficients ($\chi^2 = 16.159$, $df = 6$, $p = 0.013$), indicating that the inclusion of predictor variables significantly improved model fit compared to the null model. The Hosmer–Lemeshow goodness-of-fit test was non-significant ($p = 0.102$), suggesting that the model adequately fits the data. The model explained approximately 32.9% of the variance (Nagelkerke R^2) and had a final classification accuracy of 76.2%.

The initial model included eleven potential predictors: LDH, beta-2 microglobulin, red blood count, platelet count, BUN, creatinine, total protein, haemoglobin, leukocyte count, lymphocyte count, and total calcium.

The final model, after excluding non-contributing variables, retained six predictors (Table III). Among these, LDH and total calcium were statistically significant predictors ($p < 0.05$), while beta-2 microglobulin and lymphocyte count approached significance, suggesting a potential trend worth further exploration. The odds ratio for

total calcium suggests that even a small increase in this parameter substantially increases the likelihood of being in the group with a $2^{-\Delta Ct}$ value above the selected cut-off. The negative coefficient for beta-2-microglobulin suggests an inverse association with the outcome. Creatinine and total protein levels were not statistically significant. Still, they remained in the model, likely due to their contribution to overall model fit (Table III).

Discussion

Chronic lymphocytic leukaemia is the most common haematological malignancy in adults. In a significant percentage, it is a disease with a stable clinical course, with patients remaining in the »watch and wait« phase for years (10, 11). Worsening or aggressive forms of the disease may depend on the existence of certain genetic or chromosomal abnormalities (12).

BIRC3 is located on the long arm of the 11th chromosome. It encodes the synthesis of a protein that plays a key role in controlling apoptosis. It inhibits apoptosis by promoting pro-survival nuclear factor κB (NF- κB) signalling by targeting pro-apoptotic proteins for degradation. The BIRC3 gene normally promotes the degradation of MAP3K14, a key kinase in the NF- κB pathway (13). Low BIRC3 levels lead to higher MAP3K14 levels, and increased NF- κB pathway activation causes a more aggressive course of CLL, which is frequently associated with resistance to chemotherapy agents, such as fludarabine (14).

In approximately 80% of CLL cases, the BIRC3 locus is affected by 11q deletions. In addition, BIRC3 can be affected by mutations with an incidence of 3–5% in untreated patients (15). The mutations of the BIRC3 gene were analysed in

most published papers on CLL (15). The laboratory detection of relative BIRC3 gene expression is a relatively novel analysis. It has been reported in only a few published studies on CLL (10, 16). This cross-sectional study aimed to determine whether there is a difference in the relative expression of the BIRC3 gene between CLL patients and the control group. According to our results, increased relative expression of the BIRC3 gene was observed in the CLL group compared to the control group (*Table II*). Balakrishnan and the authors published similar results. (17). They showed that CLL lymphocytes express high levels of several different inhibitors of apoptosis proteins, such as XIAP, cIAP1, and cIAP2, compared to normal lymphocytes (17).

This is a significant result, considering that in the study group of 50 CLL patients, only 2 patients had a verified 11q deletion. Given the important role of the BIRC3 gene in apoptosis, we believe that the initial determination of its relative expression would be of great importance in CLL patients at the time of diagnosis, especially because a dysfunctional apoptotic mechanism is a hallmark feature of chronic lymphocytic leukaemia.

To determine potential predictors of relative gene expression, a binary logistic regression model was performed. The final model retained six predictors, among which the levels of total calcium and LDH were the two most potent predictors of relative BIRC3 gene expression (*Table III*). According to this study's results, total calcium levels were positively associated with relative BIRC3 gene expression. In most cases of CLL, the total calcium level is within the reference range. However, if elevated, it could signal disease progression or transformation into Richter's syndrome (18).

Nevertheless dysregulation of the calcium signalling pathway is a hallmark of CLL, with defects in calcium homeostasis in malignant B cells. This can lead to both anergy and the survival of tumour cells by influencing the apoptotic process (19). An influx of calcium from the blood into a cell triggers apoptosis by overloading intracellular calcium stores and activating several key mechanisms. This also disrupts mitochondrial function, ultimately activating caspase enzymes and initiating the programmed cell death pathway (20). Patients with low BIRC3 mRNA expression have an altered NF- κ B pathway, which is associated with increased dependency of CLL cells on the Bcl-2 rheostat and enhanced sensitivity to Bcl-2 inhibition of apoptosis (12). However, Bcl-2 also inhibits apoptosis indirectly by regulating calcium release from the endoplasmic reticulum and interacting with its calcium release channel

(21). This interaction reduces the release of calcium from the endoplasmic reticulum, thereby decreasing the subsequent influx of extracellular calcium into the cell. Future studies are needed to elucidate the exact pathophysiological relation between the relative expression of the BIRC3 gene and total calcium levels.

On the other hand, LDH levels were shown to be a significant negative predictor of relative BIRC3 gene expression (*Table III*). While a direct link isn't established, a scenario with both low BIRC3 expression and high LDH levels could be significant in certain malignant diseases. LDH represents an easily accessible biomarker in CLL that helps predict disease aggressiveness and treatment outcomes, with higher levels generally indicating a more advanced or active disease state (22). LDH accumulates in the bloodstream due to massive leukemic cell turnover, increased anaerobic glycolysis and/or impaired clearance (23). The primary mechanisms involve high-rate cellular breakdown and altered cellular metabolism in cancerous cells, along with potential secondary factors such as haemolysis (24). LDH and BIRC3 could be linked through processes of inflammation and cancerogenesis, as elevated LDH often indicates cellular damage and high BIRC3 expression promotes cell survival by inhibiting apoptosis, both of which are frequently observed in inflammatory conditions and tumours. Further studies should evaluate the true significance and relationship between BIRC3 gene expression and serum levels of total calcium and LDH.

Conclusion

The CLL group showed significantly higher relative BIRC3 gene expression than the control group. Regarding the role of the BIRC3 gene in the apoptosis process, determining its relative expression could be of great importance in CLL patients at the time of diagnosis and during monitoring of the clinical course.

Acknowledgments. This study was implemented under the auspices of the Provincial Secretariat for Higher Education and Scientific Research, Autonomous Province of Vojvodina, which approved the project »Analysis of BIRC3 Gene Expression in Chronic Lymphocytic Leukaemia Patients«.

Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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Received: April 11, 2026

Accepted: May 12, 2026