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ORIGINAL RESEARCH REPORT

Prognosis biomarkers evaluation in chronic lymphocytic leukemia

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Abstract
Objective/Background: From clinical and biological points of view, chronic lymphocytic leukemia (CLL) is a heterogeneous disease characterized by a progressive accumulation of lymphocytes in the peripheral blood, bone marrow, and lymphoid organs. New prognostic markers in CLL may be useful to clinicians for predicting outcome and in clinical decision-making. The aim of this study was to evaluate the potential prognostic value of the apoptotic/survival-controlling proteins and protein tyrosine kinase ZAP-70 gene expression in CLL patients and control individuals, correlating such findings with patients' clinical data.
Methods: Fifty-three patients diagnosed with CLL attending the hematology service of a clinical hospital, and 24 healthy individuals with no history of leukemia (Control group) were enrolled in this study. Analyses of apoptotic/survival-controlling proteins were performed by western blot and ZAP-70 gene expression was evaluated by real-time polymerase chain reaction.
Results: Significant differences were observed for the p-p38, Mcl-1 long, and Mcl-1 short proteins when patients were compared with CLL and controls. A positive correlation between the results for Mcl-1 short and Mcl-1 long and lymphocyte count was observed, corroborating the hypothesis of an imbalance between proteins of cell survival pathways/apoptosis in CLL.

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Conclusion: ZAP-70 gene expression was not detected as a discriminant biomarker in these CLL patients. An imbalance between apoptosis-related proteins was observed in the present study, corroborating the hypothesis of increased survival of lymphocytes in CLL patients.

Introduction

Chronic lymphocytic leukemia (CLL) is known as an indolent disease, with slow and progressive accumulation of lymphocytes, affecting more elderly individuals, and with a shortened biological expectation of survival. However, this concept is no longer easily applied to all patients affected by this disease. Nowadays, CLL is seen as a heterogeneous disease with a variable clinical course [1]. The most important recent advance in the understanding of CLL pathogenesis was the identification of new prognostic factors in addition to clinical staging. Among them are the subgroup of cytogenetic abnormalities, the mutational status of the immunoglobulin, expression of ZAP-70 and CD38, and evaluation of proteins involved in apoptotic mechanisms.

CLL has been linked to an imbalance between proliferation of blood cells and their ability to undergo apoptosis [2]. Many chemotherapeutic agents kill target cells through protein activation of the bcl-2 family mitochondria-dependent apoptotic pathway. This family of cytoplasmic proteins is characterized by the presence of members that suppress apoptosis (e.g., Mcl-1, Bcl-2, Bcl-xL) or promote apoptosis (e.g., Bax, Bak, Bad, Bid, Bim, and Puma) [3]. The increased survival of B lymphocytes in CLL *in vivo* is considered primarily a result of inappropriate expression of proteins of the Bcl-2 family, particularly the increase of those that suppresses apoptosis and decrease of those that promote apoptosis. The imbalance between antiapoptotic and proapoptotic proteins seems to be one of the mechanisms of apoptosis resistance in CLL, and thus a key factor that determines the longevity of CLL B cells [4,5].

During B lymphocyte apoptosis, activation of B cell receptor (BCR) culminates in the activation of Cy2 phospholipase which, in turn, results in the release of intracellular calcium and activation of protein kinase C, essential for activation of mitogen-activated protein kinases, such as extracellular signal-regulated kinase (ERK), c-jun kinase, and p38, as well as transcription of nuclear factor κB and nuclear factor of activated T cells [6]. In CLL, B lymphocyte CD40 stimulation provokes activation of nuclear factor κB, ERK, and complex PI3K/Akt, also involved in apoptosis, leading to a reduction of both spontaneous and induced chemotherapeutic agent apoptosis. By contrast, proapoptotic protein p38 appears to have reduced phosphorylation, contributing to increased resistance to apoptosis, an important factor in the pathophysiology of CLL [7,8].

The zeta chain-associated protein (ZAP-70) is a 70-kDa protein associated with the T-cell receptor. ZAP-70 is a tyrosine kinase essential to initiate the signaling pathway promoted by activation of the T-cell receptor. Although not found in normal B-lymphocytes, ZAP-70 is highly expressed in most CLL cells in which the variable region of immunoglobulin heavy chain (IgVH) is not mutated. CLL lymphocytes with IgVH mutation rarely show expression of this protein [9]. The presence of ZAP-70 expression in patients without somatic damage in the IgVH gene is related to worse prognosis and shorter survival [10].

It should be noted that substantial changes already occur in the apoptotic process when the CLL is diagnosed, but most patients are asymptomatic at diagnosis and are therefore classified as Binet A. However, as the progression of the disease varies from one individual to another, it is extremely important to search for new biomarkers with potential prognostic implications, which would be important for the adoption of more individualized therapeutic measures. In this context, we evaluated the apoptotic/survival-controlling proteins and the protein tyrosine kinase ZAP-70 gene expression in CLL patients and in control individuals with no history of any hematology disorders. Prognostic evaluation of this kind of biomarker is still lacking in our CLL patient population.

Material and methods

Patients

Patients were selected by hematologists from the Hematology Unit of Clinical Hospital, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil. Clinical data on patients were obtained from their medical records. A total of 53 patients with confirmed chronic lymphocytic leukemia (CLL) rated by the Binet criteria [2] were included in the study: 38 with low risk, nine with moderate risk and six with high risk. Twenty-four clinically healthy individuals with normal blood counts and no history of blood disorders comprised the control group. The institutional Ethics Committee of Federal University of Minas Gerais approved this study, and informed consent was obtained from all participants. This study was carried out in accordance with the Declaration of Helsinki [11].

Blood samples

Whole blood samples were obtained by venipuncture using heparin and EDTA vacuum systems tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Samples were processed immediately after collection and stored at −80 °C until further analysis.

Western blot for cell survival proteins

Mononuclear cells from whole blood were washed with phosphate-buffered saline (PBS) and whole cell extracts were prepared as described [12—14]. The protein content

of the lysate was determined by Bradford assay (Bio-Rad, Hercules, CA, USA). The total protein extracts (~40 μg) were separated by electrophoresis on 10% SDS-PAGE and electrotransferred to nitrocellulose membranes, as described [13]. Subsequently, membranes were blocked with PBS-0.1% Tween containing 5% skim milk powder, washed with PBS-0.1% Tween, and incubated with the antibody of interest overnight at 4 °C (anti: p38, p-ERK, Bcl-xL and Mcl-1; Cell Signaling Technology, Massachusetts, USA and Santa Cruz Biotechnology, Santa Cruz, California, USA). After further washing with PBS-0.1% Tween and incubating for 1 hour at room temperature with the secondary antibody linked to peroxidase, the respective membranes were incubated in ECL-Plus developing solution (Amersham, Chalfont St Giles, Buckinghamshire, UK), exposed to X-ray film (Hyper film ECL; Amersham), developed, and fixed (Kodak, Eastman Kodak Company, Rochester, NY, USA), according to the manufacturer's instructions. Densitometry analyses were performed using ImageJ software (Image Analysis Processing in Java rings; NIH, Bethesda, MD, USA) and values in each sample were normalized to the β-actin values and expressed as arbitrary units (AU).

RNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis were performed using the PureLink Whole Blood RNA Purification Kit and the High-Capacity cDNA Transcription Kit, respectively (Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions.

ZAP-70 gene expression analysis

The cDNA was subjected to amplification by quantitative real-time polymerase chain reaction (qPCR) to quantify the relative expression of ZAP-70 using the Taqman system (Life Technologies) according to the protocol described by the manufacturer. PCR was performed in an Applied Biosystems Step One Real Time PCR System. Each sample was assayed in duplicate and quantified by the $\Delta\Delta C_T$ method. β-actin was used as an endogenous control and a sample from a healthy individual as a reference.

Statistical analysis

Statistical analysis was performed using analysis of variance, followed by the Tukey's multiple comparison test using the Sigma Stat version 2.03 software (SigmaPlot (Systat Software, San Jose, CA)). The Spearman's test was used for investigating the possible correlation between studying variables. Significance levels were estimated by applying the chi-square test. GraphPad Prism version 3.0 software (GraphPad Software, La Jolla California USA) was used to plot the graph. Differences were considered significant when $p < .05$. Outliers were calculated using the GraphPad QuickCalcs Web site (http://www.graphpad.com/quickcalcs/Grubbs1.cfm).

Results

Significant differences for leukocytes, hemoglobin levels, lymphocytes, and platelet counts were observed when patients were compared with control groups ($p < .05$; Table 1). When patients were analyzed according to Binet staging, significant differences for platelet count (B + C group vs. Control group, $p < .05$), lymphocyte count (A and B + C vs. Control group, $p < .05$), leukocytes (A vs. Control group, $p < .05$), and hemoglobin levels (A vs. Control group $p < .05$) were observed.

Data on ZAP-70 mRNA gene expression are presented in Fig. 1. No significant differences were observed when comparing patients and control participants. Three CLL patients belonging to Groups Binet A, B, and C presented very high levels of expression of ZAP-70 (73.5, 92.5, and 47.5, respectively). Coincidentally, these three patients had changes in blood count as a significant increase in the overall count of leukocytes and absolute lymphocyte and thrombocytopenia, suggesting poor prognosis. However, there was no worsening in clinical status of these patients until this moment, despite the high levels of ZAP-70.

The proteins associated with cell survival were analyzed in 21 CLL patients, seven of them with low risk (Binet A), 12 with moderate risk (Binet B), and two with high risk (Binet C). The values of p-p38 protein were significantly reduced in patients (0.0195 [0.000—0.665]; $p = .015$) with CLL compared with the Control group (0.856 [0.412—1.010]; Fig. 2A). By contrast, the values for both Mcl-1 long (Mcl-1L; 0.266 [0.0704—1.128]; $p = .008$) and Mcl-1 short (Mcl-1S; 0.798 ± 0.601; $p = .019$) proteins (Fig. 2B) were significantly higher in the CLL group than in the Control group (0.0539 [0.0135—0.135] and 0.337 ± 0.443, respectively), whereas the values of ERK (0.0774 [0.0260—0.229]; Fig. 2C) and Bcl-xL (0.749 [0.0514—1.003]; Fig. 2D) proteins did not

Table 1 Clinical and laboratory (blood analysis) of patients with chronic lymphocytic leukemia and controls.

Variables	Control	Patients	*p*
M/F	14/10	31/22	.81
Age (median)	63.75 (26—88)	65.44 (27—87)	.394
Leukocytes (10^9/L)	5.3 (4.3—5.8)	16.6 (4.2—3.7)	.009
Hemoglobin (g/L)	140.3 ± 15.6	127.3 ± 18.7	.012
Lymphocytes (10^9/L)	1.9 (1.5—2.1)	9.7 (2.8—3.1)	<0.001
Platelets (10^9/L)	194 (150—236)	156.5 (121.3—203.5)	.011
Binet A	na	38	
Binet B	na	9	
Binet C	na	6	

Note. Data are presented as mean ± standard deviation or median (interquartile range). F = female; M = male; na = not applicable.

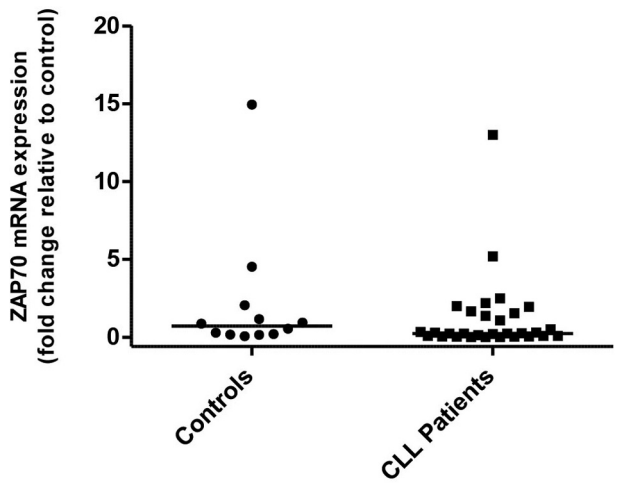

Fig. 1 ZAP-70 mRNA expression in controls and chronic lymphocytic leukemia patients.

differ between groups. No significant difference was observed in the expression of proapoptotic proteins and staging of the disease. A positive correlation between the results of Mcl-1S and the value of absolute lymphocyte count (R^2 = .562; p < .05) was observed. Likewise, there was a positive correlation between the results of Mcl-1L and the absolute value of lymphocyte count (R^2 = .719, p < .05). Fig. 3 shows a representative profile of some controls and some CLL patients for the p-p38, p-ERK, Mcl-1L, Mcl-1S, and Bcl-xL proteins by western blot. β-actin was used as an endogenous control in the different channels and to standardize densitometric analysis.

Discussion

CLL is a disease characterized by the accumulation of monoclonal small B lymphocytes in the blood, bone marrow, and lymphoid tissue [15]. Lymphocytosis was also observed in our study and was significantly higher in both Groups A and B + C, compared with the Control group. Because lymphocytosis leads to leukocytosis, the highest overall leukocyte counts were observed in Group B + C. Even in Group A, leukocytosis was significantly higher than in the Control group.

Patient immunophenotyping agreed with the expected profile for CLL (data not shown). The markers CD19, CD20, and CD22 appeared positive as expected for leukemic cells. CD20 is a low density and CD22 appeared weak or negative. These cells also presented at the cell surface light-chain *kappa* and *lambda* immunoglobulin and antigen for heavy chain IgM and IgD immunoglobulin, and CD5-like T cells [16]. The negative CD38 and low expression of ZAP-70 are indicative of a better prognosis for CLL patients. It was observed that the majority of patients had immunophenotyping results with negative CD38 and low expression of ZAP-70.

According to the findings for proteins related to cell survival/apoptosis, it was observed that the profile of patients (significant differences in p-p38, Mcl-1S, and Mcl-1L compared with the Control group) corroborates other studies already published. Mcl-1 is an antiapoptotic protein of the Bcl-2 family and is associated with malignant cell survival, worse prognosis, and chemoresistance in the CLL [17]. Bae et al. [18] have also demonstrated that increased levels of Mcl-1 are associated with protection of cells against chemotherapeutic agents. The Mcl-1S protein is encoded by an Mcl-1 messenger RNA that is missing exon 2. Thus, Mcl-1S has an alteration in the C-terminus as compared with full-length protein Mcl-1L. Normally, Mcl-1S does not interact with the proapoptotic protein, but dimerizes with Mcl-1L to form a precipitate. Excess Mcl-1S can favor apoptosis. However, Le Gouill et al. [19] showed that the effect of dimerization of Mcl-1S and Mcl-1L can result in antiapoptotic effects in patients with multiple myeloma. In accordance with this study, our data indicate an increase in Mcl-1 consistent with an antiapoptotic role of this protein in CLL, which may affect the treatment response to chemotherapy agents.

To verify the relationship between our findings and the clinical outcome several months after blood collection, we examined the medical records of CLL patients who had changes related to the prognostic markers evaluated in this study. No aggravation of the clinical condition for any of the patients was observed during this time interval. In summary, our findings related to low levels of p-p38 and high levels of both Mcl-1S and L in some CLL patients allow us to suggest that the prognosis for the respective individuals may not be favorable in the near future. However, monitoring for a long time and an increased number of asymptomatic Binet stage A CCL patients must be assayed and followed-up to establish a definitive conclusion.

Awan et al. [20] reported that Mcl-1 was an independent prognostic factor in the group of patients evaluated with CLL, with no relationship to other prognostic factors known as ZAP-70, CD38, and IgVH. Corroborating the data from this study, our study also showed no relationship between prognostic factors in CLL patients. By contrast, significant correlations between both Mcl-1S and Mcl-1L and the absolute number of lymphocyte count were found. The p38 is a protein of MAPK family and has proapoptotic role in B cells and is phosphorylated and thus activated in normal B cells but not in CLL patients. Activation of p38 is required for induction of apoptosis by various anticancer agents and may be an important point to be considered for new drug development; it has high efficiency and low toxicity [21—23]. The decrease in p-p38 relative to the controls observed in the present study confirms the apoptosis resistance reported in the studies, which is expected in patients with CLL.

Data obtained in this study clearly show an imbalance between proteins of the apoptosis pathways, antiapoptotic proteins prevailing over proapoptotic ones, which explains, at least in part, the increased survival of lymphocytes in CLL patients. This statement may be evidenced by increased expression of the Mcl-1 protein (known to increase survival of leukocytes), and decreased levels of phosphorylation of p38 protein (associated with apoptosis of leukocytes) in CLL patients.

Finally, clinical criteria are still used for the prognostication of CLL patients. It is widely known that those still classified as Binet stage A may present a heterogeneous clinical course that may or may not require treatment.

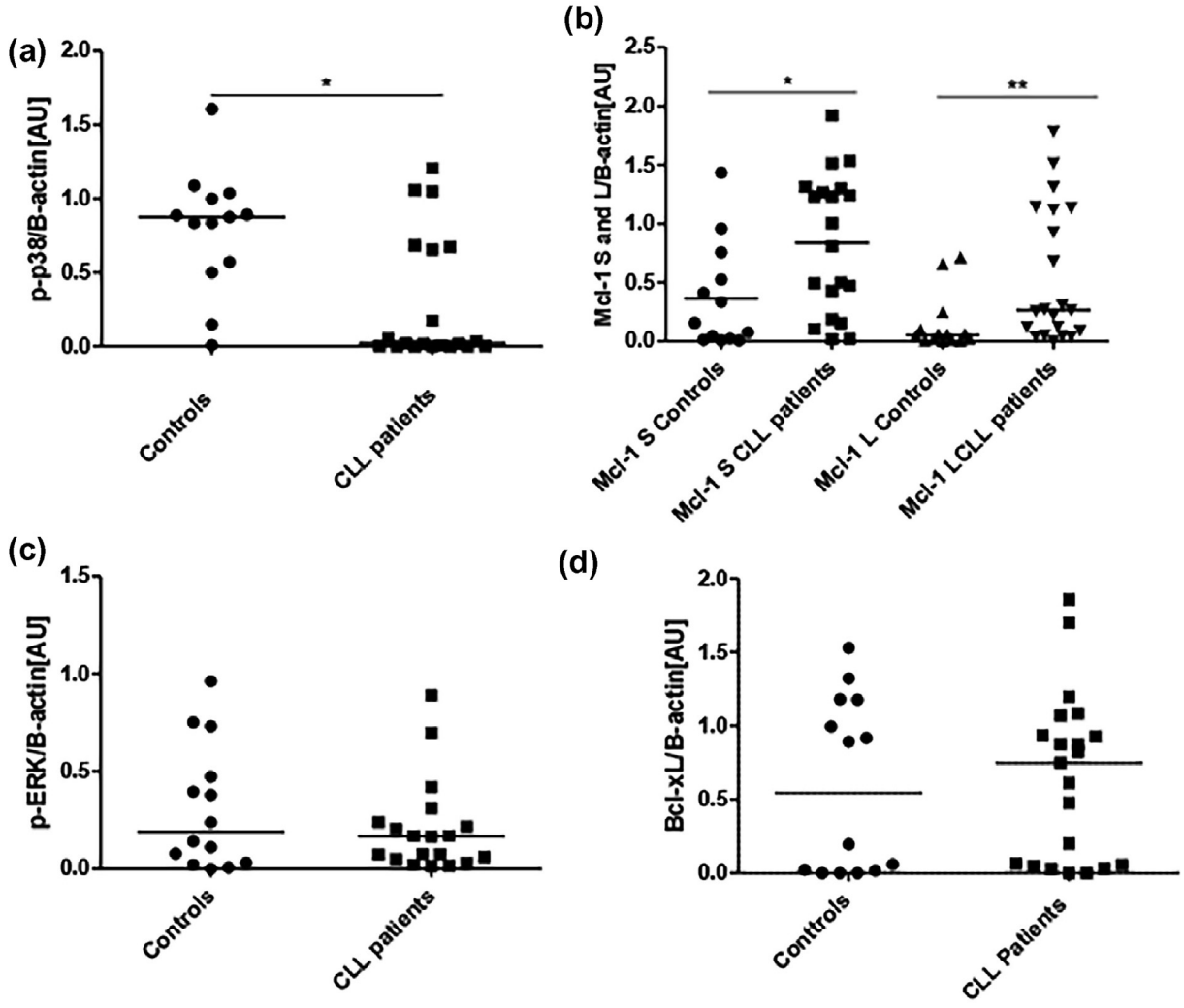

Fig. 2 (A) Levels of p-p38 apoptosis/survival-controlling protein in controls and patients with chronic lymphocytic leukemia (CLL). Densitometry analysis was performed using ImageJ software (NIH, Bethesda, MD, USA) and the values were normalized to the values of β-actin in the same sample and expressed in arbitrary units (AU), $*p < 0.05$. (B) Levels of Mcl-1 short and long apoptosis/survival-controlling proteins in controls and patients with CLL. Densitometry analysis was performed using ImageJ software and the values were normalized to the values of β-actin in the same sample and expressed in AU. $*$, $**p < 0.05$. (C) Levels of p-ERK apoptosis/survival-controlling protein in controls and patients with CLL. Densitometry analysis was performed using ImageJ software and the values were normalized to the values of β-actin in the same sample and expressed in AU. (D) Levels of Bcl-xL apoptosis/survival-controlling protein in controls and patients with CLL. Densitometry analysis was performed using ImageJ software and the values were normalized to the values of β-actin in the same sample and expressed in AU.

Prospective studies using new biomarkers to predict disease progression targeting this group of CLL patients are promising [24]. Our data, extracted from a very limited number of samples, showed different profiles for some new biomarkers linked to the risk of disease progression, which reinforces their crucial role in early-stage CLL patients. Moreover, standardization and validation of these new prognostic biomarkers are mandatory for clinical application and better patient management, particularly for those in Binet stage A.

Most of the studied CLL patients were asymptomatic and classified as Binet stage A indicative of a better prognosis *a priori*. However, regardless of the disease stage, either early or late, alterations in apoptotic mechanisms have been observed in all Binet stages. Regarding the proteins related to cell survival/apoptosis, decreased p-p38 in patients relative to controls corroborates the resistance to apoptosis in CLL patients, whereas increasing Mcl-1 is also consistent with the antiapoptotic role of this protein in this disease. Thus, an imbalance between proteins of the cell survival/apoptosis pathways was observed in the present study, with a lower protein activation related to cell death and higher protein expression associated with increased cell survival in CLL patients, which may explain, at least in part, the increased survival of lymphocytes in these patients.

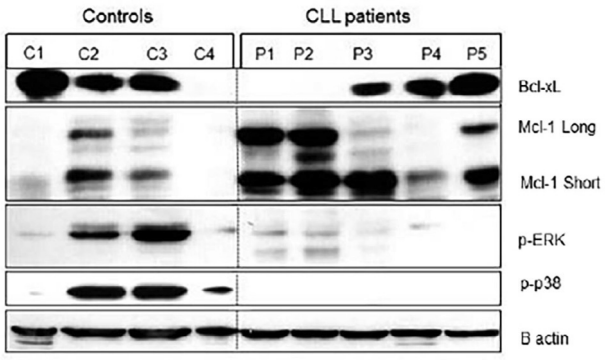

Fig. 3 Apoptosis/survival protein expression/activation profile by western blot in controls and in patients with chronic lymphocytic leukemia. *Note*. C = controls; P = patients.

Conflict of interest

The authors declare that there are no conflicts of interest in this study.

Acknowledgments

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