Research article

Prognostic value of immunohistochemical expression of ZAP-70 and CD38 in chronic lymphocytic leukaemia detected on bone marrow and lymph node biopsies

Valoarea prognostică a expresiei imunohistochimice a ZAP-70 si CD38 în leucemia limfocitară cronică detectată pe biopsii osteomedulare şi limfoganglionare

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Abstract

Chronic lymphocytic leukemia (CLL) has a heterogeneous clinical course. Among useful markers in identifiyng patients with poor outcome are unmutated IgVH, ZAP-70 and CD38 expression. Both ZAP-70 and CD38 were shown to be capable of identifying aggressive CLL.

We analysed data from 35 patients diagnosed with CLL based on morphological and immunophenotypical criteria. In all cases peripheral blood immunophenotyping was performed as initial diagnostic test. Immunohistochemical expression of ZAP-70 and CD38 was evaluated on 21 cases of lymph node biopsies and 14 cases of bone marrow biopsies, performed at the time of diagnosis. In addition in-situ hybridization for EBER-1 was evaluated.

The median age of patients was 60 years and we noted a slight male predominance. The immunophenotypic criteria (C23+, CD5+, CD20+, CD10-, CD3-, cyclinD1-) for B-cell CLL were achieved in all 35 patients. We found that CLL cases showing expression of both markers (ZAP-70+CD38+ patients) are characterised by an unfavourable clinical course as compared with cases that did not show expression of markers (ZAP-70-CD38- patients). Our data showed significant differences in terms of overall survival at 5 years between the two groups. We also found statistically significant differences between patients ZAP-70-CD38- and patients with one or both positive markers (ZAP-70+ and/or CD38+).

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Prognostic information given by ZAP-70 and CD38 could be used in guiding treatment decisions and they probably should be recommended to all patients with B-CLL in trying to obtain a more clear profile of the disease at the time of diagnosis.

Rezumat

Leucemia limfocitară cronică (LLC) are o evoluție clinică heterogenă. Printre markerii utili în identificarea pacienților cu prognostic nefavorabil se numără statusul nemutat al IgVH, expresia ZAP-70 și expresia CD38. Atât ZAP-70 cât și CD38 evaluați separat s-au dovedit a fi eficienți în identificarea pacienților cu evoluție agresivă a bolii.

În studiul nostru am analizat 35 de pacienți diagnosticați cu LLC din punct de vedere al expresiei anticorpilor ZAP-70 și CD38. Diagnosticul inițial s-a bazat pe imunofenotiparea din sângele periferic. Expresia imunohisto-chimică a ZAP-70 și CD38 a fost evaluată pe 21 de biopsii limfoganglionare și 14 biopsii osteomedulare, efectuate la momentul diagnosticului. Deasemenea am evaluat și prezența infecției cu virus Ebstein-Barr prin hibridizare in situ pentru EBER-1.

Vârsta medie a pacienților a fost de 60 ani cu o ușoară predominanță a sexului masculin. Criteriile imunofeno-tipice pentru LLC (C23$^+$, CD5$^+$, CD20$^+$, CD10$^-$, CD3$^-$, cyclinD1$^-$) au fost întrunite la toate cele 35 de cazuri. În urma evaluării expresiei imunohistochimice a ZAP-70 și CD38 am observat că pacienții cu expresia ambilor mark-eri (pacienți ZAP-70$^+$CD38$^+$) au prezentat o evoluție clinică nefavorabilă în comparație cu pacienții la care cei doi markeri au fost negativi (ZAP-70$^-$CD38$^-$). Datele noastre au arătat diferențe semnificative în ceea ce privește supraviețuirea generală la 5 ani între cele două grupuri. Am constatat diferențe semnificative statistic și între pa-cienții ZAP-70$^-$CD38$^-$ și pacienții cu unul dintre markeri pozitivi (ZAP-70$^+$ și / sau CD38$^+$).

Pozitivitatea pentru ZAP-70 și CD38 la pacienții cu LLC poate fi utilizată ca și surogat pentru statusul nemutat în orientarea deciziilor terapeutice.

Cuvinte cheie: *leucemie limfocitară cronică, ZAP-70, CD38, EBER*

Introduction

Chronic lymphocytic leukemia (CLL) is a disorder with a variable clinical course. Some patients remain stable for a long period of time, while others have an aggressive clinical course, despite chemo-immunotherapy. Clinical staging systems do not have the ability to distinguish between patients with a rapidly progressive disease and patients with a relatively stable disease (1). Consequently a number of prognostic markers have been introduced in an attempt to identify the patients with poor clinical outcome in an early stage of CLL (2,3). Among these markers the immunoglobulin gene mutation status is probably the most significant, based on the presence or absence of somatic mutations in the expressed immunoglobulin heavy chain variable region

(IgV$_H$) (4). Patients with unmutated IgV$_H$ status often have progressive disease, reduced survival and poor response to chemotherapy, while patients with somatic mutations of IgV$_H$ have an indolent disease.

Determination of IgV$_H$ mutation is based on DNA sequencing which is not always available for routine clinical use. Therefore, several surrogate markers (5) that correlate with IgV$_H$ mutational status have been identified. Recent evidence has suggested that ZAP-70 and CD38 expression in B cells of CLL is associated with the mutational status of the IgV$_H$ gene. Several studies have confirmed that ZAP-70 and CD38 positivity is correlated with an unmutated IgV$_H$ gene status whereas their negativity is associated with a mutated IgV$_H$ gene status (4,6).

ZAP-70 is an intracellular tyrosine kinase, normally expressed in T cells and natural killer cells (7) and it is involved in T-cell receptor signaling (8,9). It has also been found to be expressed in some subsets of activated B cells and in normal B cells precursors (10,11). Expression of ZAP-70 in CLL B cells is associated with unmutated IgV_H (12-15) and contributes to a more aggressive clinical course, probably by enhancing B cell receptor signaling (16,17).

CD38 is a membrane glycoprotein found in B cells, monocytes, natural killer cells and acts as a signaling molecule in CLL B cells (18). CD38 positivity can identify unmutated CLL clones. It has also been found that CD38 expression in CLL cells is higher in lymph nodes and bone marrow, tested by flow cytometry (19).

Evaluation of the prognostic value of ZAP-70 and CD38 has been performed using different methods including reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, flow-cytometry and immunohistochemistry (17,20).

In this study we analysed the expression of ZAP-70 and CD38 detected by immunohistochemistry, because it is a sensitive, easy and inexpensive technique that can be performed on routinely prepared histological samples, primarily formalin-fixed-paraffin-embedded tissue sections.

Material and methods

Thirty-five patients diagnosed with CLL in our institution between 2004 and 2007 were included in this retrospective study. The initial workup for CLL diagnosis included routine peripheral smear morphological examination and lymphocyte immunophenotyping by peripheral blood flow cytometry. The antibody panels that were used for flow-cytometry included CD19, CD20, CD23, CD5, CD79, CD3, CD4, CD8, CD7. Due to technical constraints, ZAP-70 and CD38 were not routinely used as initial flow-cytometry diagnostic markers in our cases.

Lymph node and bone marrow biopsies were fixed in 10% neutral buffered formalin for 24 hours. Bone marrow biopsies were decalcified in ethylenediaminetetraacetic acid (EDTA) disodium salt acid buffer (Osteodec) for 3 hours and conventionally embedded in paraffin. All cases were defined as classical CLL using morphological and immunohistochemical criteria (CD20+, CD5+, CD23+ CD10-, CD3-, cyclinD1-). The diagnosis was re-evaluated in an Epidemiological study for Non-Hodgkin Lymphoma performed by the International Non-Hodgkin Lymphoma Classification Project.

At presentation, age, sex, stage according to Rai classification and serum levels of lactate dehydrogenase (LDH) were evaluated; the patients were followed-up until the end of 2013.

21 lymph node biopsies and 14 bone marrow biopsies performed at time of diagnosis were selected. We retrospectively evaluated the ZAP-70 and CD38 immunohistochemical expression on these biopsies.

Mouse liquid monoclonal antibody to ZAP-70 (clone L453R Novocastra, dilution 1:50) and mouse lyophilized monoclonal antibody to CD38 (clone SPC32 Novocastra, dilution 1:50) were used. T-cell component was assessed with CD3. CLL cells were scored for nuclear and/or cytoplasmic ZAP-70 staining as well as membrane and cytoplasmic CD38 staining. For ZAP-70 staining we used a three-tier scale: negative (score 0), weakly positive (score +1) and positive (score +2). CD38 was considered positive if more than 20% of cells presented membrane and cytoplasmic staining (plasma cells were used as control). In-situ hybridization for EBER-1was assessed in all cases.

OS was defined as the time from diagnosis until death or last follow-up. The Kaplan-Meier product-limit method to estimate survival curves and the log rank test to evaluate the differences

between them have been used. Stepwise multivariate Cox regression analysis was used to determine associations of ZAP-70 and CD38 positivity with overall survival. We considered the limit of significance for all tests as $p<0.05$. All statistical analyses were performed using the statistical package STATA 13.

The authors state that there were no ethical aspects or competing interests involved.

Results

The median age at diagnosis was 60 years (patients' ages ranged from 34 to 77 years). We noticed a slight male predominance: there were 23 men (65.71%) and 12 women (34.29%). At the time of diagnosis 19 patients were staged according to Rai classification: 1 (5%) patient stage 0, 6 (31%) patients stage II, 3 (15%) stage III and 9 (47%) stage IV. After a median follow-up of 60 months, a total of 19 patients (54.28 %) have died at the time of analysis.

ZAP-70 expression appeared mostly as nuclear staining in neoplastic cells and was considered positive (score +1 and +2) in 19 cases (54.28%) and negative in 16 cases (45.71%). From the positive cases 14 were lymph node biopsies and 5 were bone marrow biopsies. ZAP70 expression was found among patients with advanced Rai stage (6 cases with stage IV).

The CLL cells were considered positive for CD38 if ≥20% of cells showed membrane and cytoplasmic staining. Based on this cutoff value, 16 patients (45,71%) were

CD38 positive and 19 patients (54.28%) were CD38 negative. Among positive cases 13 were lymph node biopsies and 3 were bone marrow biopsies.

The relation between ZAP-70 and CD38 expression was analysed: 14 of 35 patients (40 %) were ZAP-70$^+$CD38$^+$, 14 patients (40%) were ZAP-70$^-$CD38$^-$ and 7 patients (20%) were positive for either CD38 or ZAP-70.

Genderwise we found that males had a better survival compared to females: 65.22% versus 50% 5-year survival, but the difference was not statistically significant (log rank p=0.16).

The linear regression analysis between the percentage of ZAP-70 and CD38 showed a positive correlation (Pearson correlation coefficient r=0.6, p=0.0001). Association between the two variables was also found using the contingency table and Pearson's chi-squared test (p= 0.001) (Figure 1).

CD38 \ ZAP-70	-	+	Total
-	14	5	19
+	2	14	16
Total	16	19	35

Contingency table

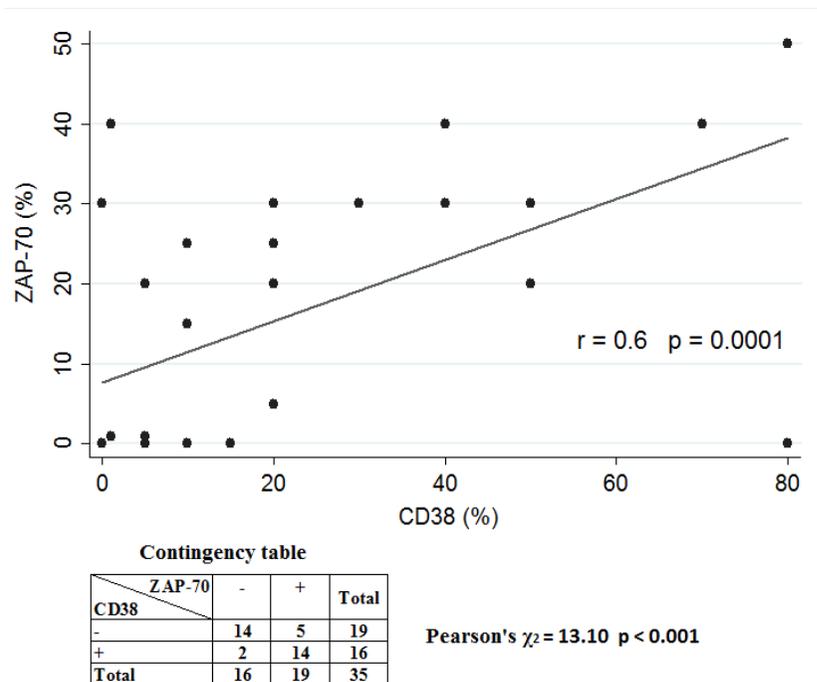Pearson's χ_2 = 13.10 p < 0.001

Figure 1. Correlation of ZAP-70 and CD38 percentages. Contingency table of the association between ZAP-70 and CD38 expressions.

Kaplan-Meier plots were used to analyze 5-year observed survival rates for patients with ZAP-70 and CD38 detected by immunohisto-chemistry. A 75% OS rate was obtained for ZAP-70⁻ patients, compared to a 47.37% value obtained for ZAP-70⁺ patients (log rank p=0.0611) (Figure 2a,b). When the same parameter was evaluated according to the expression of CD38, a 73.68% value was obtained for CD38⁻ patients versus 43.75% for CD38⁺ patients (log rank p=0.11) (Figure 3a,b).

When we compared the patients with both negative markers (ZAP-70⁻CD38⁻) to those with both positive markers (ZAP-70⁺CD38⁺) we found statistically significant differences between the two groups: 85.71% against 50% 5-year survival rate (log rank p=0.04) (Figure 4).

Cases with only one positive marker had significantly lower OS (28.57%) when compared to double negative cases (85.71%, p=0.001) or double positive cases (50%).

Using Cox proportional hazards regression analysis we compared patients with ZAP-70⁻CD38⁻ to patients with at least one positive marker (ZAP-70⁺ and/or CD38⁺). Overall predicted survival at 5 years was 85% for patients
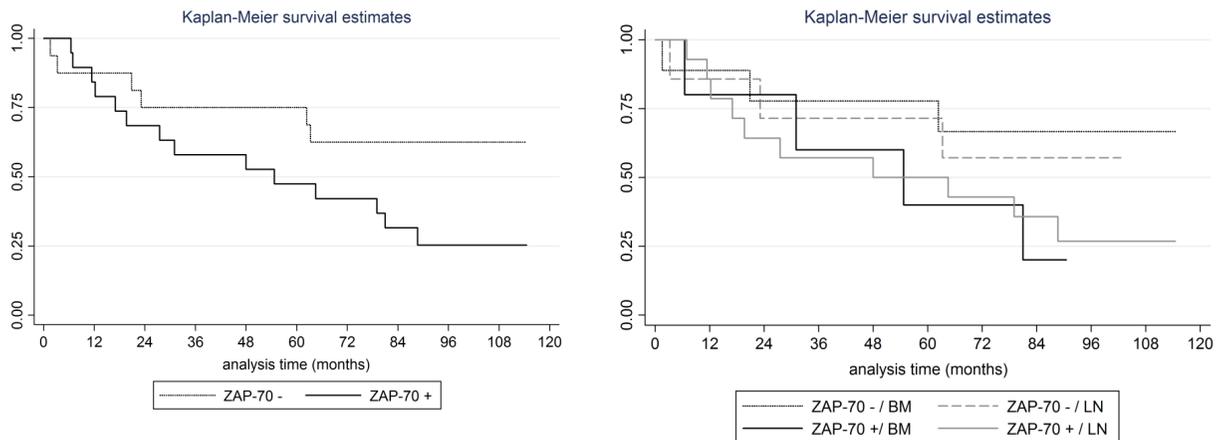

Figure 2. Kaplan–Meier plots estimate overall survival in a) 16 ZAP-70 negative and 19 ZAP-70 positive CLL patients b) on lymph nodes and bone marrow biopsies. (log rank p = 0.0611)
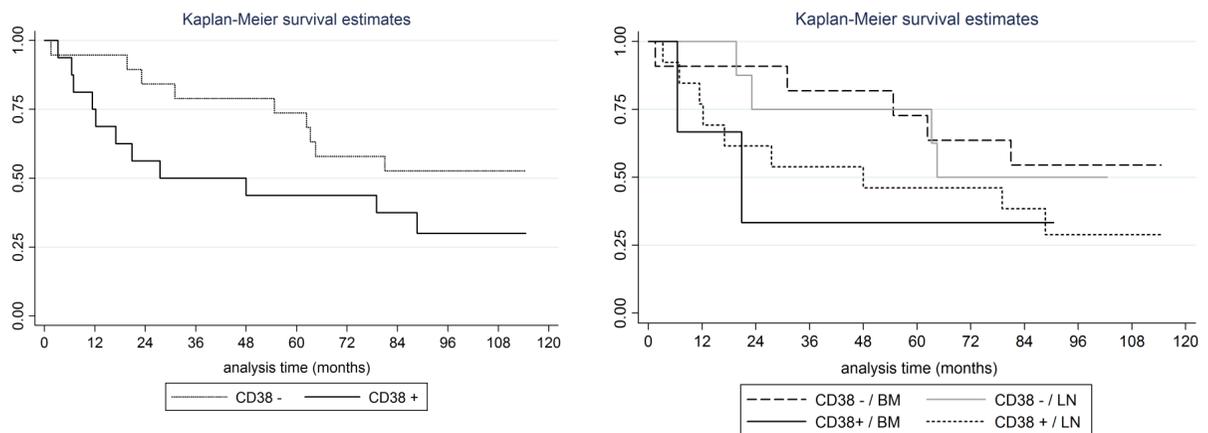

Figure 3. Kaplan–Meier plots estimate overall survival in a) 19 CD38 negative and 16 CD38 positive CLL patients b) on lymph nodes and bone marrow biopsies (log rank p = 0.11).
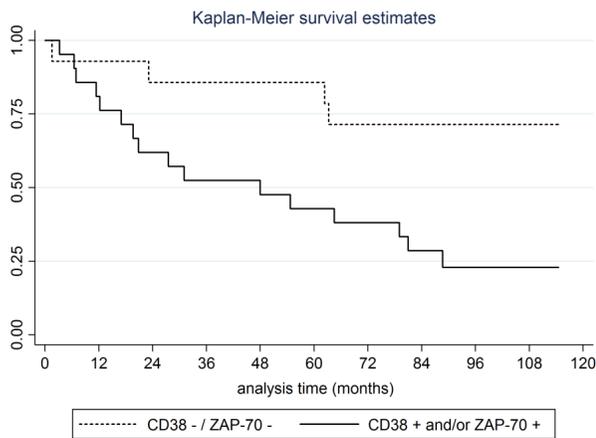
Figure 4. Kaplan–Meier plots estimate overall survival in CD38⁻ZAP-70⁻ patients and CD38⁺ZAP-70⁺ patients (log rank p= 0.04).
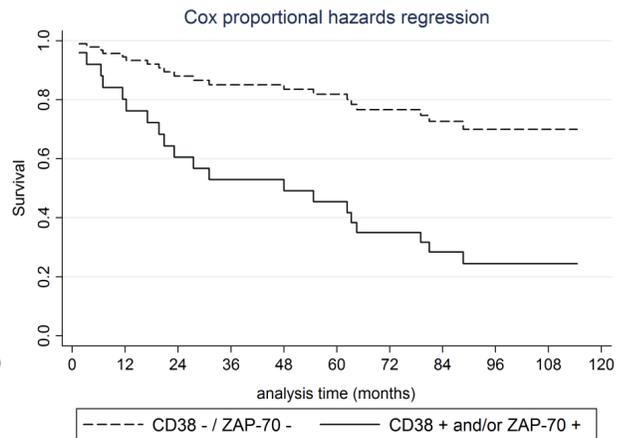

Figure 5. Cox proportional hazards regression in CD38⁻ZAP-70⁻ patients and CD38⁺ and/or ZAP-70⁺ patients (p=0.015).

with both negative markers compared with 42% for patients with ZAP-70⁺ and/or CD38⁺ (Figure 5). The statistical analysis showed that if at least one marker is positive the hazard ratio (HR) is increased four times compared with both markers being negative: HR=3.94 (1.30-11.89 95% confidence interval) (p=0,015).

Using in-situ hybridization for EBER-1 we found two positive cases with no statistical significance.

Due to the reduced number of cases for which Rai clinical staging was available, we tried to group lower stages (Rai 0-II) and higher stages (Rai III-IV) to see if there is any correlation with both markers. We found that for lower stages the overall survival at 5 years is better than for higher stages with both markers being positive (p=0.0036).

Discussions

This retrospective study was performed to evaluate the potential of ZAP-70 and CD38 expression, detected by bone marrow and lymph node immunohistochemistry, as prognostic indicators in CLL. Our study confirmed the prog-

nostic value of ZAP-70 and CD38 protein expression in CLL cells, detected here by immunohistochemistry in lymph node and bone marrow biopsies. Both markers, evaluated by immunohistochemistry, were able to predict the clinical outcome of our patients.

We found that CLL cases showing expression of both markers (ZAP-70⁺CD38⁺ patients) are characterised by an unfavourable clinical course when compared with ZAP-70⁻CD38⁻ patients. Our data showed significant differences in terms of OS at 5 years between the two groups. We also found statistical correlations between ZAP-70⁺CD38⁺patients and patients with either ZAP-70 or CD38 positivity.

Previous studies have identified both ZAP-70 and CD38 expression, as determined by flow cytometry, to be independent predictors of progression and OS in CLL (21). Based on those findings, in the present study we assessed immunohistochemical expression of both markers. The 20% cutoff value for CD38 was used in our study by reference to previously published studies concerning hematological malignancies evaluated by flow cytometry (22). ZAP-70 expression on CLL cells has been analysed by

immunohistochemistry in several studies and concordance between immunophenotypical and immunohistochemical evaluation of ZAP-70 was reported (23,24). The problems with the standardization of ZAP-70 assessment by flow cytometry (type of monoclonal antibody, intracellular staining (25)) and the fact that retrospective studies are impeding, make detection by immunohistochemistry more affordable.

The concordance rate between ZAP-70 and CD38 assessed by immunohistochemistry was similar to that reported in studies where their expression was tested by flow cytometry. Both markers significantly correlated with poor clinical outcome in terms of OS. Their concordance with OS was found both on lymph nodes and bone marrow biopsies.

The discordant cases (with only one positive marker) were compared with ZAP-70⁻CD38⁻ cases and the overall survival was much worse for discordant cases: 28.57% (discordant cases) versus 85.71% (negative for both markers) at 5 years (p=0.001). The discordant subgroup was also compared with ZAP-70⁺CD38⁺ subgroup and we noticed again a worse prognosis for discordant cases in term of survival which is not in accordance with literature data (in studies using flow cytometry analysis). We interpreted this „odd behavior" as being the consequence of the reduced number of cases.

Epstein-Barr virus (EBV) markers are detected infrequently in CLL cells and their presence is associated with an accelerated clinical course (26,27). Few cases of CLL with transformation to diffuse large B cell lymphoma or Richter syndrome have been associated with EBV infection (28). The two cases positive for EBER-1 were also found to be positive for ZAP-70; in addition one of them was positive for CD38. At the time of analysis one of the patients had deceased (the case with ZAP70⁺CD38⁻).

To our knowledge, the current study represents the largest series of CLL patients tested for both ZAP-70 and CD38 expression by immunohistochemistry on lymph node and bone marrow biopsies. In this study, the difference of OS according to ZAP-70 and CD38 expression was significant (p=0.04).

We tested ZAP-70 and CD38 using a different technical perspective on both lymph node and bone marrow biopsies and we found a strong prognostic value in terms of survival (results comparable to flow cytometry data in the literature). Although the method needs better standardization our results suggest that routinely formalin-fixed, paraffin-embedded (and decalcified) biopsies can represent a reliable material for assessing both ZAP-70 and CD38 as prognostic markers in CLL cases.

Acknowledgments

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