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ZAP-70 and CD38 expression are independent prognostic factors in patients with B-cell chronic lymphocytic leukaemia and combined analysis improves their predictive value

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Abstract: Recently identified biological risk factors in B-cell chronic lymphocytic leukemia (B-CLL) include ZAP-70 and CD38 expression. The present study was conducted to clarify whether a combined analysis could improve predictive impact of these two parameters. We examined the expression of ZAP-70 and CD38 by flow cytometry method in 217 newly diagnosed, consecutive, unselected and well characterized B-CLL patients in relation to laboratory parameters and clinical outcome. We confirmed that both ZAP-70 as well as CD38 were independent of prognostic factors. There was a significant correlation between the percentage of leukemic cells positive for ZAP-70 and the percentage of CD38+CD19+ cells (R=0.629; p=0.000001). Combined analysis of ZAP-70 and CD38 showed concordant results in 158/217 patients (72.8%), while in 59 patients the results were discordant (27.2%). A mean treatment free survival (TFS) was the longest in ZAP-70-CD38- patients (45.6 months, comparing to 13.6 months in ZAP-70+CD38+ group). Also a mean overall survival was the longest in ZAP-70-CD38- patients (224.7 months compared to 77.9 months in ZAP-70+CD38+ patients).

Key words: B-cell chronic lymphocytic leukaemia - Prognostic factors - ZAP-70 - CD38

Introduction

Over the last few years, technological advances have led to identify important biological and genetic parameters related to the clinical course of B-cell chronic lymphocytic leukaemia (B-CLL). The immunoglobulin variable heavy chain (IgV_H) gene was determined as one of the most powerful prognostic factors, where B-CLL cases with unmutated IgV_H genes are characterized by an unfavorable clinical outcome, whereas the prognosis for patients with germline status is unfavourable [1,2]. Recently two other prognostic parameters were also identified in patients with B-CLL: intracellular ZAP-70 expression [3,4] and cell-surface expression of CD38 on leukemic cells [1,5]. In contrast to technically demanding and expensive method of IgV_H mutations analysis, both markers can be conveniently measured using the flow cytometry method. ZAP-70 (zeta-associated protein) was shown to be the most promising surrogate marker for IgV_H mutation status with a high predictive value. CD38 expression was also initially reported by Damle *et al.* to be correlated with IgV_H mutations [1], however subsequent studies showed only some degree of correlation [6,7]. Irrespective of the extent of concordance with rearrangement of the IgV_H genes, CD38 expression was proved by many investigators to be a reliable predictor of the outcome in B-CLL [8,9]. In the literature, there are only few studies [10,11], including our preliminary data [12] reporting clinical significance of combined expression of ZAP-70 and CD38 in predicting the outcome of B-CLL patients. In order to clarify further prognostic impact of combined analysis of ZAP-70 and CD38 we examined the expression of both factors in peripheral blood samples obtained from 217 newly diagnosed, consecutive, unselected

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Table 1. Cox regression analysis of prognostic factors for B-CLL.

Independent predictors	Hazard ratio	P value	95% confidence interval
Hb level	0.766512	0.035383	(0.598331 ; 0.981967)
ZAP-70 expression	1.033586	0.032345	(1.002785 ; 1.065334)
CD38 expression	1.036427	0.002653	(1.012523 ; 1.060894)

Table 2. Comparison of laboratory parameters in groups of patients with B-CLL according to ZAP-70 and CD38 expression. p^1-between ZAP-70-CD38- group and ZAP-70-CD38+group (not significant); p^2-between ZAP-70-CD38- group and ZAP-70+CD38-group; p^3-between ZAP-70-CD38-group ZAP-70+CD38+ group (only significant p values shown).

Parameter	ZAP-70-CD38- (n=105)	ZAP-70-CD38+ (n=25)	ZAP-70+CD38- (n=34)	ZAP-70+CD38+ (n=53)	p (U Mann–Whitney test)
WBC (G/L)	58.23±63.66	76.68±85.02	66.97±59.91	93.24±90.92	p^3=0.02
LYMPH (G/L)	49.62±59.85	59.16±66.64	58.12±57.22	77.26±70.68	p^3=0.009;
Hb (g/dL)	13.35±1.96	12.16±2.86	12.53±2.40	12.41±2.43	p^3=0.01
PLT (G/L)	199.5±74.14	173.00±67.03	164.59±69.86	150.46±71.76	p^2=0.03 p^3=0.0002
B_2M (mg/L)	3.03±1.68	4.93±4.59	4.07±.25	5.17±4.04	p^3=0.00008
LDH (U/L)	288.24±129.61	389.29±248.99	322.13±126.19	373.02±192.45	p^3=0.005

and well characterized B-CLL patients in relation to laboratory parameters and clinical outcome.

Materials and methods

Patients. Two hundred seventeen newly diagnosed and previously untreated, consecutive, unselected B-CLL patients were enrolled into this study from 1987 to 2006. Diagnosis of B-CLL was made on the basis of a clinical examination as well as morphological and immunological criteria described by NCI-WG (National Cancer Institute-Working Group) [13]. There were 78 women and 139 men (M : F ratio - 1:1.78 with a median age of 64 years (range 34 - 86) at the time of diagnosis. Patients were staged according to the Rai classification. Fifty patients were diagnosed at stage 0 and fifty five at stage 1, sixty at stage 2, and twenty-five and twenty seven at stages 3 and 4, respectively. For statistical analyses modified Rai stages were used. Fifty patients had low-modified Rai stage, 115 had intermediate stage and 52 had high stage. All peripheral blood samples were collected in heparinized tubes and immediately processed. Mononuclear cells were isolated by density gradient centrifugation on Gradisol L (Aqua Medica, Poland). Interphase cells were removed and washed twice in phosphate-buffered saline (PBS) and then resuspended at 1×10^6 cells for future staining.

Cell-surface antigen expression. Peripheral blood mononuclear cells were stained with the following antibody conjugates: negative control IgG1 FITC/IgG_{2a} PE/IgG_{2a} TC, anti-CD45 FITC/ anti-CD14 PE (Caltag Laboratories, USA), anti-CD19 PE, anti-CD19 FITC, anti-CD3 PE (BD PharMingen, USA), anti-CD19 TC (PE-Cy5), anti-CD5 TC (PE-Cy5), anti-CD3 FITC/ anti-CD19 PE, anti-CD8 FITC/ anti-CD4 PE, anti-CD5FITC/anti-CD19PE (Caltag Laboratories, USA. 5 µl of each MoAb was added to the appropriate tubes and incubated for 20 min at RT. Finally, the cells were washed and analyzed by flow cytometry.

Flow cytometry analysis. Samples were analysed by flow cytometry using a Becton Dickinson FACSCalibur instrument equipped with a 488-nm argon laser. Five data parameters were assessed: linear forward and side scatter (FSC, SSC), FL-1(FITC), FL-2(PE) and FL-3 (CyChrome). An acquisition gate was established on the basis of FSC and SSC that included mononuclear cells and excluded dead cells and debris. For each analysis 10.000 events were acquired and analysed using CellQuest software. Each sample was run with an appropriate isotype control and this was used to define the negatively stained cells.

ZAP-70 staining using Zenon™ Alexa Fluor 488 Mouse IgG2a Labeling Kit. All samples were stained for ZAP-70 protein expression. We used the method described earlier [14]. Anti-ZAP-70 antibody, clone 2F3.2 (mouse monoclonal IgG_{2a}), was purchased from Biomol Research Laboratories. Zenon™ Alexa Fluor 488 Mouse IgG_{2a} Labeling Kit was obtained from Molecular Probes. CyChrome conjugated monoclonal antibody (MoAb) anti-CD5, CyChrome conjugated MoAb anti-CD19 and PE conjugated MoAb anti-CD19 were obtained from Caltag Laboratories.

100 µl of peripheral blood mononuclear cells (1×10^6cells/mL) were stained with MoAbs against the cell-surface markers CD19 PE and CD5 CyChrome. The cells were then fixed in a 1% paraformaldehyde solution in PBS for 15 minutes at room temperature (RT) and permeabilized with 70% ethanol for at least 1 hour at -20°C. Thereafter, anti-ZAP-70 antibody labelled by the Zenon mouse IgG labelling reagents was added to the sample tubes. The samples were incubated for 30 minutes at RT, washed and immediately analysed by flow cytometry.

Zenon Complex Formation. For ZAP-70 staining 1 µg cells of anti-ZAP-70 antibody per 10^6 cells was evaluated. Anti-ZAP-70 antibody was prepared in 20 µl of PBS. Next, the Zenon mouse IgG labelling reagent, which contained a fluorophore-labelled Fab fragment, was added to the antibody solution (5 µl of the Zenon labelling reagents were used for each 1 µg of IgG). The mixture was incubated for 5 minutes at RT. Then 5 µl of the Zenon blocking reagent was added to the reaction mixture. The solution was incubated for additional 5 minutes and applied to the sample.

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Statistical analysis. The statistical significance of the flow cytometric results was analysed by means of the Mann-Whitney U test. Spearman rank test was used to assess the correlation between the variables. Analysis of the effect of potential prognostic factors was undertaken using a Cox regression. Treatment free survival time and overall survival time measured from diagnosis were estimated according to the method of Kaplan-Meier and compared between groups by means of log-rank test. Differences were considered as statistically significant when the p-value was <0.05. We used Statistica 5.0 PL software for all statistical procedures.

Results

Cox proportional hazards regression analysis

Using Cox proportional hazards regression analysis, we compared ZAP-70 and CD38 expression with other prognostic factors, namely age >60 years, clinical stage, white blood cell, lymphocyte and platelet count, hemoglobin level, serum concentration of LDH and β_2-microglobulin (β_2M). Univariate analysis of the overall survival identified 6 significant factors: Rai stage of the disease, platelet count, hemoglobin level and LDH concentration, as well as ZAP-70 and CD38 expression, which subsequently were included in multivariate Cox regression analysis. Only hemoglobin concentration, ZAP-70 and CD38 expression remained significant and were confirmed to be independent prognostic factors (Table 1).

Correlation between ZAP-70 and CD38 expression

Spearman rank-correlation test revealed significant correlation between the percentage of leukemic cells with ZAP-70 expression and the percentage of CD38+CD19+ cells in the whole patients group (r=0.629; p=0.000001) (Fig. 1).

To evaluate further correlation between ZAP-70 and CD38 expression the patients were divided into four groups. Entire correlation between the expression of studied parameters was noted in 158/217 patients (72.8%). Among them there were 105 ZAP-70-/CD38- patients (48.4%) and 53 ZAP-70+CD38+ patients (24.4%). Among the remaining 59 patients, 25 were ZAP-70-CD38+ (11.5%) and 34 ZAP-70+CD38- (15.7%). Table 2 presents the comparison of clinical and laboratory parameters with four groups of patients mentioned above. The most significant differences were revealed among the patients characterized by positive ZAP-70 and CD38 expression as well as lack of expression in the two studied proteins. The differences referred to all evaluated laboratory parameters. The only significant difference among the remaining groups concerned a higher number of PLT count in the group of ZAP-70-CD38- patients in comparison with ZAP-70+CD38- ones (Table 2).

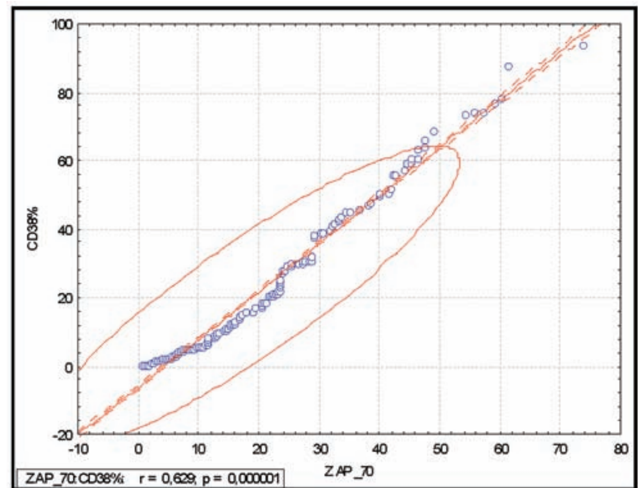

Fig. 1. Correlation between intracelullar expression of ZAP-70 and expression of CD38 on leukaemic cells (Spearman rank-correlation test).

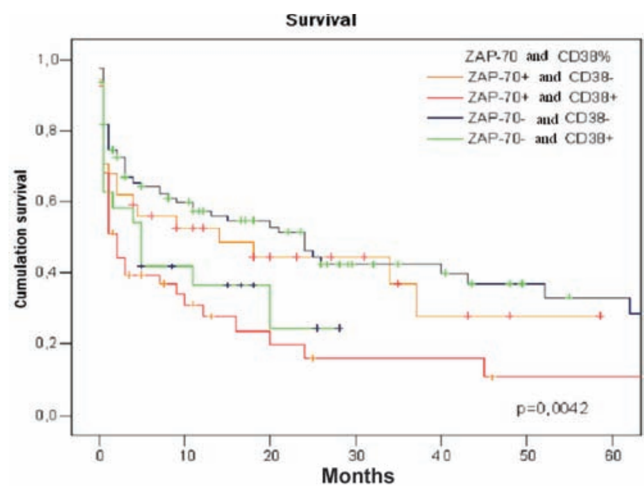

Fig. 2. Kaplan-Meier's curves presenting TFS in groups of B-CLL patients with phenotype: ZAP-70-CD38-; ZAP-70-CD38+; ZAP-70+CD38-; ZAP-70+CD38+.

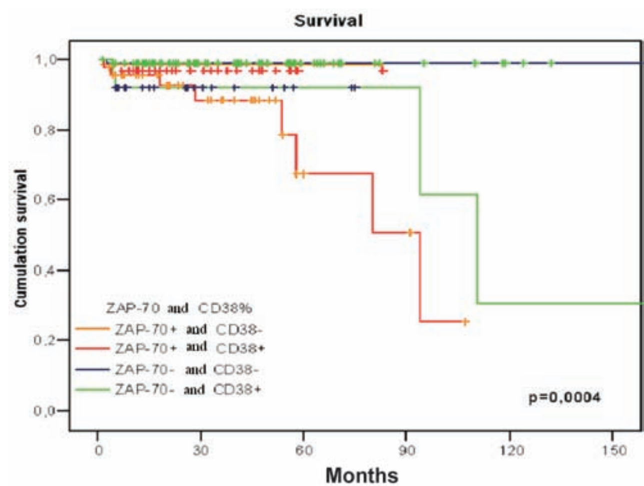

Fig. 3. Kaplan-Meier's curves presenting OS in groups of B-CLL patients with phenotype: ZAP-70-CD38-, ZAP-70-CD38+, ZAP-70+CD38- and ZAP-70+CD38+.

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Table 3. Comparison of TFS in groups of patients with B-CLL with phenotype: ZAP-70-CD38-; ZAP-70-CD38+; ZAP-70+CD38-; ZAP-70+CD38+.

	ZAP-70- CD38-	ZAP-70- CD38+	ZAP-70+ CD38-	ZAP-70+ CD38+
No. of treated patients	57	16	20	42
No.r of untreated patients	48	9	14	11
TFS (mean; months)	45.6	10.8	24.2	13.6
TFS (median; months)	24	5	14	2

Table 4. Comparison of OS in groups of patients with B-PBL with phenotype: ZAP-70-CD38-; ZAP-70-CD38+; ZAP-70+CD38-; ZAP-70+CD38+.

	ZAP-70-CD38-	ZAP-70-/CD38+	ZAP-70+CD38-	ZAP-70+/CD38+
No. of patients who died	2	4	3	10
No. of living patients	103	21	31	43
OS (average, months)	224,7	94,7	80,4	77,9
OS (median; months)	-	94	-	94

Treatment-free survival (TFS) and overall survival (OS) in relation to combined ZAP-70 and CD38 expression

In order to define the impact of simultaneous ZAP-70 and CD38 expression on TFS, the analysis of Kaplan-Meier's curves was done in the following groups of patients: ZAP-70+CD38+; ZAP-70-CD38-; ZAP-70-CD38+ and ZAP-70+CD38-. It was found that TFS curves for groups ZAP-70-CD38- and ZAP-70+CD38+, as well as groups ZAP-70+CD38- and ZAP-70+CD38+ showed essential differences (p=0.0004, p=0.0499, log-rank test). A significant difference in TFS between groups ZAP-70+CD38- and ZAP-70+CD38+ suggests that in the group of patients with positive ZAP-70 expression, defining CD38 expression enables also to distinguish patients with better and worse prognosis (Fig. 2). With the data presented in Table 3 one may come to conclusion that the longest TFS time is observed in patients with neither ZAP-70 nor CD38 expression.

The results of the TFS analysis show the best prognosis in patients with lack of ZAP-70 and CD38 expression, the group with the worst prognosis is the one with ZAP-70+CD38+ phenotype. It seems that the presence of positive CD38 expression on leukaemic cells is the most important parameter determining the shortening of TFS time.

The log-rank test showed significant difference in the OS curves for groups ZAP-70-CD38- and ZAP-70+CD38+, as well as for ZAP-70-CD38- and ZAP-70-CD38+ (p=0.0001, p=0.0023). A significant difference in OS between groups ZAP-70-CD38- and ZAP-70-CD38+ suggests that additional defining of CD38 expression enables to distinguish patients with better and worse prognosis in ZAP-70- group (Fig. 3). Analysis of the data from Table 4 revealed that overall survival time was longer in ZAP-70-CD38- group in comparison with another three groups.

Additional information can be obtained by evaluating the survival curves in ZAP-70+CD38+ and ZAP-70-CD38- groups depending on LDH activity, concentration of β_2M and hemoglobin. Because of lack of decrease in favorable prognosis groups, only TFS but not OS was defined. As for groups: ZAP+CD38+/LDH >350U/l; ZAP+CD38+/β_2M >3.0 mg/L and ZAP+ CD38+/Hb <12 g/dL the mean TFS was: 51.76; 48.9; and 58.13 months and it was shorter in comparison with ZAP+CD38+ patients (77.9 months).

Discussion

The aim of the presented study was to establish whether combined analysis of ZAP-70 and CD38 expression could improve predictive impact of both parameters. First, we confirmed their value as independent prognostic factors using multivariate Cox regression analysis. The importance of ZAP-70 as an independent prognosis parameter has been shown in numerous earlier studies [10,15,16], in the case of CD38, this issue has been the matter of many controversies. Hamblin *et al.* in a multifactoral analysis, conducted in 2002, underlined that both IgV$_H$ mutational status and CD38 expression were independent prognostic factors [17], but according to Oscier *et al.* [18] the expression of CD38 cannot play the role of an independent prognostic parameter, but gives an additional predictive information in patients with defined IgV$_H$ mutational status. Other research concerning the

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value of CD38 as an independent prognosis parameter, also revealed divergent results [8,15,16,19-22]. The majority of recent studies conducted on large groups of patients confirms the predictive significance of CD38 expression.

For the clinical practice it is important to establish whether combined evaluation of the two independent prognostic factors, ZAP-70 and CD38 contribute to the increase in their predictive value. According to Orchard *et al.* CD38 expression has no influence on the increase in the prognostic significance of ZAP-70 and IgV_H mutational status [15]. Similar conclusions were drawn by Del Principe *et al.* They stated that it was adequate just to evaluate ZAP-70 expression [16]. However, the results of our studies are in favor of simultaneous evaluation of both parameters that was presented in the earlier publication concerning the discussed issue [12]. The analysis showed the concordance in the results regarding the expression of the two studied antigens in 72.7% of patients. In the group with divergent results, 15.6% of patients were ZAP-70+CD38- and 11.5% were characterized by the phenotype of ZAP-70-CD38+. In the studies of Del Principe *et al.* the vast majority of patients from the group with divergent expression of both factors was characterized by the phenotype of ZAP-70+CD38- (82%) which, according to the authors indicated a major importance of ZAP-70 expression [16]. The results of our studies do not confirm such observations as the percentage of ZAP-70+CD38- and ZAP-70-CD38+ patients was approximately the same. The most significant differences in laboratory parameters were found between ZAP-70-CD38- and ZAP-70+CD38+ groups, whilst the differences between the two groups with divergent expression of studied antigens were not statistically significant. Corresponding observations deal with the evaluation of survival curves as well. Simultaneous evaluation of both antigen expression enables to distinguish subgroups with the best and the worst prognosis, which was revealed in the earlier studies of other authors [10,11]. Schroers et al, on the basis of combined evaluation of ZAP-70 and CD38 expression displayed three groups of patients: the good prognosis group (event-free survival, EFS 130 months; phenotype ZAP-70-CD38-); the poor prognosis group (EFS 30 months; phenotype ZAP-70+CD38+) and the intermediate prognosis group (EFS 43 months; phenotype ZAP-70+CD38+ and ZAP-70-CD38-) [10]. Similar data on the treatment free survival were presented by Del Giaudice et al, who also distinguished three groups with different prognosis on the basis of ZAP-70 and CD38 evaluation [11]. Furthermore, combined analysis used in our study enabled to separate subgroups in ZAP-70+ as well as in ZAP-70- patients, differing sometimes in OS and TFS time. Analogically, evaluation of ZAP-70 expression helps to distinguish additional subgroups among patients with positive or negative CD38 expression. These interdependences were acknowledged as significant for the prognosis by Durig *et al.* [4] and Bosch *et al.* [23]. The fact that the analysis of survival curves presented in our studies revealed that shortening of TFS depends more on CD38 expression, but ZAP-70 expression has a major impact on shortening of OS is in favor of the importance of combined evaluation of ZAP-70 and CD38 expression. Simultaneous evaluation of ZAP-70 and CD38 expression approves to enroll patients into an appropriate prognostic group in the case of threshold values of one of the evaluated parameters. The results of our study revealed also that analysis of ZAP-70 and CD38 expression together with chosen laboratory parameters with prognostic importance (concentration of hemoglobin and β_2M, serum activity of LDH), enabled to distinguish patients with a shorter TFS time than in ZAP+CD38+ group. These observations may be of great clinical value, since qualification of patients in early stages of B-CLL for the therapy on the basis of the presence of unfavourable prognostic parameters is more often considered. Our results suggest that standardized evaluation of ZAP-70 and CD38 expression ought to be included in the panel of routine laboratory tests studies at B-CLL diagnosis. However, whilst taking therapeutic decisions, it would be advisable to elaborate point system which would include both ZAP-70 and CD38 expression, as well as other laboratory parameters with determined prognostic value.

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