The clinical significance of ZAP-70 and CD38 expression in B-cell chronic lymphocytic leukaemia

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Background: B-cell chronic lymphocytic leukaemia (B-CLL) is a disease with a highly variable clinical course; some patients never need treatment, while others require intensive treatment early after diagnosis. Recently, some new prognostic factors, such as IgVH mutational status, ZAP-70 and the expression of CD38 in leukaemic cells were introduced to identify attenuated versus progressive types of CLL bearing the potential to facilitate risk-adapted treatment strategies.

Patients and methods: To evaluate the clinical value of ZAP-70 and CD38 as predictors of disease progression we assessed the expression of these markers by the flow cytometry method in 156 B-CLL patients.

Results and conclusions: Both ZAP-70 and CD38 expression were shown to predict the clinical course of the disease, while ZAP-70 expression appeared to be more predictive than CD38 expression and more relevant in defining the cases of B-CLL responsive or refractory to first line chemotherapy. A simultaneous evaluation of ZAP-70 and CD38 expression allowed distinguishing the patients groups with the most favourable prognosis as well as those with the worst. Taken together we recommend assessing both ZAP-70 and CD38 protein expression for the definition of prognostic subgroups in patients with B-CLL

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

introduction

B-CLL is the most frequent type of adult leukaemia in western countries. It is a heterogeneous disease with a highly variable clinical course and prognosis [1, 2]. Some patients show an indolent disease and never require treatment, while in others the clinical course is aggressive requiring intensive treatment shortly after diagnosis. The staging systems developed by Rai et al. [3] and Binet et al. [4] have been recognised as standard methods of assessing the survival and the treatment requirements in B-CLL patients. However, these systems cannot identify stable or progressive forms of the disease, especially in the early stages of B-CLL, which include most of the patients at diagnosis. Thus, there is a need to seek out other prognostic factors in the early stage of the disease to identify stable or progressive forms of CLL that might facilitate risk-adapted treatment strategies [5, 6].

The presence or absence of somatic mutations in the immunoglobulin heavy-chain variable region (IgVH) of B-CLL cells has been described as one of the most powerful prognostic factors distinguishing two disease subsets [7, 8]. The cases with mutated IgVH genes exhibit a favourable clinical course and they may never require treatment [9]; while patients with unmutated IgVH genes are characterised by a reduced survival and responsiveness to chemotherapy [10, 11]. However, IgVH sequencing is difficult to perform in a routine diagnostic laboratory. Finding a surrogate for IgVH mutational status would seem to be an important priority.

Damle et al. suggested that CD38 might identify IgVH mutation having a prognostic significance in B-CLL [11]. The prognostic value of CD38 expression was also confirmed by other authors [12]. However, subsequent studies failed to confirm CD38 as an independent prognostic factor in multivariate analysis [9, 10]. Moreover, Chevallier et al. [13] found that CD38 expression by the leukaemic cells might change during the course of the disease.

Recently the role of ZAP-70 (zeta associated protein) as a surrogate marker for IgVH mutation to help identify patients with a more aggressive clinical course was reported [14–16]. ZAP-70 is a member of the Syk-ZAP-70 protein tyrosine kinase family. It is expressed by normal T cells and natural killer cells and it plays a critical role in the development and differentiation of these cell types [15]. There is no ZAP-70 expression on normal B lymphocytes, but it was detected on B-CLL cells. It was suggested that the expression of ZAP-70 could not only predict IgVH mutational status, but it could also serve as a prognostic
factor in B-CLL and its expression would be stable during the course of the disease [15].

Here, we present results from a single centre retrospective study of 156 CLL patients estimating the clinical value of ZAP-70 and CD38 expression as B-CLL prognostic factors. We analysed ZAP-70 and CD38 expression on CD19+/CD5- leukemic cells using flow cytometry and correlated the results with the clinical outcome and risk factors like the stage of the disease, the lymphocyte count, the lactate dehydrogenase (LDH) and B2-microglobulin level in the series of our patients.

patients, materials and methods

patients and samples

Between September 1996 and July 2004, peripheral blood samples were obtained from 156 newly diagnosed, previously untreated patients with B-CLL. The median age was 65 years (ranging from 34 to 84 years), including 60 female and 96 male patients. Patients were staged at the time of diagnosis according to the Rai classification. Thirty-seven patients were diagnosed at stage 0 and thirty-eight at stage 1, forty at stage 2, and twenty-one and twenty at stages 3 and 4, respectively. Diagnosis of B-CLL was made on the basis of a clinical examination as well as morphological and immunological criteria. Seventy patients whose disease was stable did not receive chemotherapy and 86 patients whose disease was progressive were treated. Response to the therapy was evaluated in 66 patients in which the following regimens were used: purine nucleoside analogues (37 patients), CHOP (cyclophosphamide, vincristin, doxorubicine, prednisone) and COP (cyclophosphamide, vincristin, prednisone) (14 patients), chlorambucil (11 patients), alemtuzumab (4 patients). All peripheral blood samples were collected in heparinized tubes and immediately processed. Mononuclear cells were isolated by density gradient centrifugation on Gradiol L (Aqua Medica, Poland). Interphase cells were removed and washed twice in phosphate-buffered saline (PBS) and then resuspended at 1×10^6 cells/ml for future staining.

reagents

Anti-ZAP-70 antibody, clone 2F3.2 (mouse monoclonal IgG2a), was purchased from Biomol Research Laboratories. Zenon™ Alexa Fluor® 488 Mouse IgG2a Labeling Kit was obtained from Molecular Probes. Fluorescein isothiocyanate (FITC)-labelled anti-CD38 and the corresponding negative controls were obtained from PharMingen. CyChrome conjugated monoclonal antibody (MoAb) anti-CD5, CyChrome conjugated MoAb anti-CD19 and PE conjugated MoAb anti-CD19 were obtained from Caltag Laboratories.

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 [17]. Additionally in 20 patients we used two methods to identify ZAP-70 expression: indirect labelling ZAP-70 procedure using Zenon™ Alexa Fluor® 488 Mouse IgG2a Labeling Kit and direct labelling procedure with PE conjugated MoAb anti-ZAP-70 from Caltag Laboratories. The results obtained by these two different techniques were concordant.

100 μl of peripheral blood mononuclear cells (1×10^6 cells/ml) were stained with MoAbs against the cell-surface markers CD19 PE and CD5 CyChrome. The cells were then fixed in 1% paraformaldehyde solution in PBS for 15 minutes at room temperature (RT) and permeabilised 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 ml 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 an additional 5 minutes and applied to the sample.

cell-surface antigens expression

Peripheral blood mononuclear cells were stained with the following MoAbs: CD38 FITC, CD19CyChrome or IgG1 isotypic control. 5 μl of each MoAb was added to the appropriate tubes and incubated for 20 min at RT. Finally, the cells were washed and analysed 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 based on 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 (Figures 1 and 2).

statistical analysis

The statistical significance of the flowcytometric results was deduced by means of the Mann-Whitney U test. Spearman rank test was used to assess the correlation between the variables. Differences were considered as statistically significant when the P value was 50.05. Survival and time to progression were estimated according to the method of Kaplan-Meier. We used Statistica 5.0 PL software for all statistical procedures.

results

ZAP-70 expression in B-CLL cells

Patients were considered positive for ZAP-70 when the expression was found in 20% or more leukaemic cells. In 57 of 156 B-CLL patients (36.5%) ZAP-70 expression was found in more than 20% of leukaemic cells (32.99±11.94%) and 99 patients (63.5%) tested negative for ZAP-70 expression (8.15±4.83%) (P = 0.00001).

A significant difference was noted for the two groups in terms of Rai staging (P = 0.01): of the 57 patients who expressed ZAP-70, 28 (49.1%) were in Rai stages 3–4, whereas, of the 99 patients who did not express ZAP-70, only 13 (13%) were in Rai stages 3–4. Fifteen CLL-related deaths occurred during the observation period and 12 patients of this group were ZAP-70+ (80%). In contrast only three out of 99 patients deceased in the ZAP-70− group (3%). There was a significant difference (P = 0.009) in the mean ZAP-70 expression between patients who died and patients who are still alive (37.82% versus 16.10%, respectively). ZAP-70 expression was associated with most of the known parameters for poor prognosis in B-CLL. There was a significant difference between the two groups in white blood
Figure 1. The dot plots show representative data from one B-CLL patient, illustrating the analysis method for identification of CD19+/CD5+ leukaemic cells and CD3+ T-cells expressing ZAP-70 following three- or two-colour staining. ZAP-70 antibody was labelled using Zenon technology. An acquisition gate was established based on FSC and SSC that included mononuclear cells. A region R1 was drawn around the lymphocytes (A). Next, the R1 gated events were analysed for CD19+/CD5+ PE or CD3 PE staining and positive cells (CD19+/CD5+ or CD3+) were selected (region R2) (B, C). We used dot plots of CD19 PE versus CD5 CyChrome (B) or SSC versus CD3 PE (C). The final dot plots: CD19 PE versus ZAP-70 (D) and CD3 PE versus ZAP-70 (E) were established by combined gating of events using R1 and R2. The numbers in the upper right quadrant on the dot plots represent the percentage of CD19+/CD5+/ZAP-70+ and CD3+/ZAP-70+ cells. The dot plot F shows ZAP-70 expression after lymphocyte gating (R1). Lower right quadrant shows ZAP-70 expression by CD19+ lymphocytes (generally T and NK cells), which provided a useful positive control for antibody activity.

Figure 2. The dot plots show representative data from one B-CLL patient, illustrating the analysis method for identification of CD19+/CD38+ leukaemic cells following three-colour staining. An acquisition gate was established based on FSC and SSC that included mononuclear cells. A region R1 was drawn around the lymphocytes (A). Next, the R1 gated events were analysed for CD19+CyChrome staining and positive cells (CD19+) were selected (region R2) (B). We used dot plots of CD19 CyChrome versus SSC (B). The final dot plot: C (C) CD38 FITC versus CD19 CyChrome was established by combined gating of events using R1 and R2. The numbers in the upper right quadrant on the dot plot C represent the percentage of CD19+/CD38+ cells.
cell (WBC) count, lymphocytosis, LDH serum activity, haemoglobin level, and platelet count (Table 1). The percentage of leukaemic cells expressing ZAP-70 correlated with the percentage of CD38+/CD19+ cells (P = 0.0001) (Figure 3). ZAP-70 expression correlated significantly with the stage of disease. In patients with stages 3–4 according Rai the percentage of leukaemic cells with the expression of ZAP-70 was higher than in patients with lower stages (P = 0.001).

CD38 expression
Patients were considered CD38 positive when the expression was found in at least 20% of B CD19+ cells. This threshold value was previously used by Ibrahim et al. [18] and Deaglio et al. [19]. However other authors used 30% [9, 11, 12] or even 7% [10] of CD38+ cells as the cutoff point and this issue still remains controversial. In our study, we compared two most often used values, 20% and 30%, and similarly to the comparison made previously by Domingo-Domenech et al. [20]. We did not find any significant differences in estimated parameters between these two cut-offs (data not shown).

Table 1. Comparison of clinical and laboratory data between ZAP-70 positive and ZAP-70-negative patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>ZAP-70 positive patients (n=57)</th>
<th>ZAP-70 negative patients (n=99)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (G/L)</td>
<td>96.10±81.61</td>
<td>60.83±58.14</td>
<td>0.018*</td>
</tr>
<tr>
<td>Lymphocyte count (G/L)</td>
<td>80.63±73.76</td>
<td>50.94±42.100</td>
<td>0.007*</td>
</tr>
<tr>
<td>Hb (mg/dl)</td>
<td>12.46±2.22</td>
<td>13.01±2.08</td>
<td>0.050*</td>
</tr>
<tr>
<td>PLT (G/L)</td>
<td>142.37±71.37</td>
<td>192.98±67.27</td>
<td>0.0001*</td>
</tr>
<tr>
<td>β2-microglobulin (IU/L)</td>
<td>4.33±3.87</td>
<td>3.37±2.74</td>
<td>0.071</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>347.18±181.98</td>
<td>298.43±170.73</td>
<td>0.01*</td>
</tr>
<tr>
<td>% CD19+/CD38+ cells</td>
<td>26.58±19.16</td>
<td>13.72±12.89</td>
<td>0.0001*</td>
</tr>
</tbody>
</table>

Data are mean ± standard deviation, significant P-values are marked by asterisk (*).

The B-CLL population was considered ZAP-70 positive when at least 20% of CD38+/CD38− cells were positive for the protein, as assessed by flow cytometry. The P-value was calculated using the Mann-Whitney U-test.

In 52 patients (33.3%) CD38 expression was found in more than 20% of leukaemic cells (40.46 ± 16.96%), and 104 patients (66.7%) tested negative for CD38 expression (7.40 ± 3.53%) (P = 0.00001). CD38 positive patients as well as ZAP-70+ patients were in the more advanced Rai stage of the disease (P = 0.013). Of the 52 CD38 positive patients, 23 (44.2%) were in stages 3–4 according to Rai. Whereas, of the 104 patients who did not show 38 expression, 18 (17.3%) were in stages 3–4 according to Rai. In addition, ten (19.2%) of the CD38+ patients died (CLL-related deaths) during the observation period, whereas among the 104 patients negative for 38 expression there were only five (4.8%) CLL-related deaths. There was no significant difference in CD38 expression between patients who died and patients who survived. As shown in Table 2, in the CD38 positive group we noted significantly higher lymphocytosis, WBC counts and LDH levels than in CD38 negative patients. In CD38 positive patients, the percentage of leukaemic cells expressing ZAP-70 protein was significantly higher than in CD38 negative ones. Likewise, the Spearman correlation between the percentage of B CD19+ cells expressing CD38 and clinical and laboratory data showed a strong and direct correlation with the percentage of leukaemic cells expressing ZAP-70 (Figure 3). In addition, the percentage of CD38+ B cells correlated slightly with the lymphocyte count (R = 0.164; P = 0.05).

correlation between ZAP-70 and CD38 expression
For further statistical analysis, B-CLL patients were divided into four groups. In 118 patients (75.6%) there was a complete concordance of ZAP-70 and CD38 expression: 35 patients (22.4%) were positive for ZAP-70 and CD38 and 83 patients (53.2%) showed a ZAP-70+/CD38− phenotype. 22 patients (14.1%) were characterized by ZAP-70−/CD38+ phenotype and 16 patients (10.3%) showed a ZAP-70−/CD38− phenotype. Figure 4 shows a comparison of the clinical and laboratory data of the four groups. The combination of ZAP-70 and CD38 increased the prognostic power of both of

Table 2. Comparison of clinical and laboratory data between CD38 positive and CD38 negative patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>CD38 positive patients (n=52)</th>
<th>CD38 negative patients (n=104)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (G/L)</td>
<td>103.02±97.64</td>
<td>59.47±54.09</td>
<td>0.007*</td>
</tr>
<tr>
<td>Lymphocytosis (G/L)</td>
<td>84.05±76.56</td>
<td>50.97±30.72</td>
<td>0.004*</td>
</tr>
<tr>
<td>Hb (mg/dl)</td>
<td>12.47±2.19</td>
<td>12.97±2.11</td>
<td>0.142</td>
</tr>
<tr>
<td>PLT (G/L)</td>
<td>152.09±68.85</td>
<td>185.21±72.57</td>
<td>0.077</td>
</tr>
<tr>
<td>β2-microglobulin (IU/L)</td>
<td>4.61±4.37</td>
<td>3.32±2.43</td>
<td>0.083</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>369.33±223.38</td>
<td>289.66±139.47</td>
<td>0.040*</td>
</tr>
<tr>
<td>% CD19+/CD38− cells</td>
<td>29.50±16.26</td>
<td>12.09±10.26</td>
<td>0.0001*</td>
</tr>
</tbody>
</table>

Data are mean ± standard deviation, significant P-values are marked by asterisk (*).

The B-CLL population was considered CD38 positive when at least 20% of CD19+ cells were positive for the CD38, as assessed by flow cytometry. The P value was calculated using the Mann-Whitney U-test.
the factors. The most significant differences in WBC count, lymphocytosis, PLT count and LDH serum activity were observed between patients with ZAP-70+/CD38+ and ZAP-70+/CD38- phenotype. Additionally, we found significant differences in the haemoglobin and β2-microglobulin level between the both groups (Figure 4) and no significant differences between ZAP-70+/CD38- or ZAP-70+/CD38+ patients. Patients with ZAP-70+/CD38+ phenotype had a shorter mean event-free survival than all other patients; however, this difference was not statistically significant (Figure 4).

ZAP-70 and CD38 expression in treated and untreated patients

Comparing the subgroups of patients with regard to their clinical characteristics, we found significant differences in time to progression as indicated by the event-free interval.

**Figure 4.** Comparison of clinical and laboratory data between CD38+/ZAP-70+, CD38-/ZAP-70-, CD38+/ZAP-70- and CD38-/ZAP-70+: (A) haemoglobin level; (B) β2-microglobulin level, (C) lymphocyte count, (D) lactate dehydrogenase level, (E) platelet count, (F) white blood cell count, (G) age of the patients, (H) event free survival (EFS). Significant P-values (P < 0.05) are marked by an asterisk (*).
There was a significant difference in median event free survival (EFS) between ZAP-70 negative and ZAP-70 positive patients groups (10 ± 20.8 vs. 4 ± 15.1 months, respectively; \( P = 0.001 \)) (Figure 5A).

There was a significant difference in the median event free survival (EFS) between CD38 negative and CD38 positive patients groups (10 ± 20.9 versus 3 ± 13.4 months, respectively; \( P = 0.03 \)) (Figure 5B).

In the group of patients requiring anticancer therapy, there were more patients positive for ZAP-70 and CD38 expression than in untreated patient group (Table 3). We also found a significantly higher percentage of leukaemic cells expressing ZAP-70 in patients requiring therapy during the follow-up period than in patients who did not (\( P = 0.032 \)). CD38 expression was also higher in patients requiring therapy, although this difference was not statistically significant. In the group of untreated patients the number of ZAP-70 negative patients was higher. On the contrary, the percentage of CD38 negative patients was higher in both untreated as well as treated patient group (Table 3).

Among the B-CLL patients who received anticancer treatment (Table 4) 37 patients were treated with purine nucleoside analogues (PNA) and haematological remission was achieved in 23 of them. Complete response to PNA was significantly correlated with ZAP-70 percentages (\( P < 0.008 \)) demonstrating that this factor might be used to predict the chemosensitivity of B-CLL patients. We found no significant differences in the CD38 percentages. Likewise, there were no significant differences in the groups treated with CHOP, COP, chlorambucil or alemtuzumab (Table 4).

**discussion**

The objective of the present study was to assess the clinical significance of ZAP-70 and CD38 expression on leukaemic cells of B-CLL patients. We used flow cytometry assay to evaluate both markers expression. Flow cytometry was demonstrated earlier to be a reliable method for ZAP-70 estimation with results correlating with those obtained by the RT-PCR method [15, 17].

In our study both ZAP-70 and CD38 expression was shown to predict the clinical course of the disease. EFS was significantly longer in ZAP-70 negative as well as in CD38 negative patient groups. However, more significant differences were found between ZAP-70 positive and ZAP-70 negative patients in B-CLL poor prognosis parameters (stage of the disease, laboratory parameters at diagnosis: WBC count, lymphocytosis, PLT count, Hgb level and LDH serum level) than between CD38 positive and negative patients (WBC count, lymphocytosis and LDH activity).

ZAP-70 was proven by many authors to be a sensitive and specific surrogate marker for IgVH mutational status, though the mechanisms accounting for the relation between both factors remain unknown [21, 23]. Moreover according to Rassenti et al. [24] the prognostic value of ZAP-70 expression is even more significant than IgVH mutational status.

The clinical significance of CD38 expression as well as its value as a surrogate marker for IgVH gene mutational status still remains more controversial. A high CD38 expression was found to correlate with IgVH gene mutations as well as an unfavourable clinical course of B-CLL [11, 18], but this relation was not confirmed in further studies by Hamblin [22] and Krober [10]. According to Oscier et al. [9] CD38 expression cannot be used as an independent prognostic factor, but rather gives additional information in patients with an established mutational status. Domingo-Domenech et al. [20] found a link between CD38 expression and clinical and biological data, but in multivariate analysis it lost statistical significance. In multiple regression analysis by Crespo et al. [15] only ZAP-70

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**Table 3.** The percentage of ZAP+ versus ZAP− and CD38+ versus CD38− patients in the group requiring treatment and in the untreated group

<table>
<thead>
<tr>
<th>Patients requiring treatment (n=86)</th>
<th>ZAP-70 positive patients (46 (53.5%))</th>
<th>ZAP-70 negative patients (40 (44.5%))</th>
<th>CD38 positive patients (35 (40.7%))</th>
<th>CD38 negative patients (51 (59.3%))</th>
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</thead>
<tbody>
<tr>
<td>Untreated patients (n=70)</td>
<td>ZAP-70 positive patients (11 (15.7%))</td>
<td>CD38 positive patients (17 (24.3%))</td>
<td>CD38 negative patients (53 (75.7%))</td>
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</tbody>
</table>
expression, not CD38 expression, maintained a correlation with IgVH mutational status.

Results of our study demonstrated an association between ZAP-70 expression and the treatment requirement as well as the response to anticancer therapy. In patients who required chemotherapy ZAP-70 expression was significantly higher than in still untreated patients ($P = 0.032$). Furthermore in all patients responding to first-line chemotherapy with purine analogues ZAP-70 expression was significantly lower than in non-responding patients ($P < 0.008$). There were no significant differences in CD38 expression. Among the four patients receiving MabCampath as a first line therapy complete haematological remission was achieved in only one, a ZAP-70 negative patient, while the other three patients were ZAP-70 positive and failed to respond to the treatment. In our study the expression of ZAP-70 appeared to be more relevant than CD38 expression in defining the cases of B-CLL responding and not responding to first line chemotherapy, though it was demonstrated before that CD38$^+$ CLL patients respond poorly to chemotherapy. Del Poeta et al. [12] found a significant correlation between higher CD38 percentages and the lack of a complete response to six courses of fludarabine monophosphate as a first line approach. According to Damle et al. [11] there are significant differences, not only in survival, but also in chemotherapy requirements depending on CD38 expression. Domingo-Domenech et al. [20] found that CD38$^+$ patients required treatment more frequently than CD38$^-$ patients. According to Westner et al. [16] CD38 expression distinguished patients who required treatment early versus late, albeit less well than IgVH mutation status or ZAP-70 expression. Our observations are in agreement with the earlier results of Durig et al. [14], which found that ZAP-70 positive patients required more intensive chemotherapy over longer periods of time than ZAP-70 negative patients.

For evaluation of clinical significance ZAP-70 and CD38 co-expression of B-CLL cells we analysed four groups: patients positive or negative both for ZAP-70 and CD38, and patients with discordant results: ZAP-70$^+$/CD38$^-$ and ZAP-70$^-$/CD38$^+$. Among several clinical and laboratory parameters significant differences were observed only between groups that were negative or positive for both ZAP-70 and CD38 in WBC and PLT count, peripheral blood lymphocytosis and LDH activity (Figure 4). This observation suggests taking into account both parameters when undertaking therapeutic decisions in CLL patients. There are still controversies in this issue. Orchard et al. [21] noted that CD38 had weak prognostic value in univariate analysis but did not improve the predictive power of either ZAP-70 or IgVH mutational status in multivariate analysis. However, according to Durig et al. the combination of ZAP-70 and CD38 may increase the prognostic power of either of the two factors [14], and Schroers et al. showed recently in a large series of patients, that combined analysis of ZAP-70 and CD38 expression provided complementary prognostic information [25].

In conclusion, both ZAP-70 and CD38 expression were shown to predict the clinical course of the disease. ZAP-70 expression appeared however to be more predictive than CD38 expression and more relevant in defining the cases of B-CLL responding and non-responding to first line chemotherapy. Our results suggest that a simultaneous estimation of ZAP-70 and CD38 expression could distinguish the patient groups with the most favourable, as well as the least prognostic. Both markers are evaluated by flow cytometry, which is a routine technique in most laboratories. Performing this combined analysis could serve for either precise definition of prognostic subgroups or might be an option to confirm the prognosis if the value of one predictive factor is borderline.

**references**


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**Table 4.** Expression of ZAP-70 and CD38 antigens in patients responding and refractory to different therapeutic regimens

<table>
<thead>
<tr>
<th></th>
<th>ZAP-70 (%)</th>
<th>CD38 (%)</th>
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<tbody>
<tr>
<td>Purine nucleoside analogues</td>
<td>Remission (n=25) 13.78$\pm$13.65 &amp; 18.59$\pm$18.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Progression (n=12) 25.22$\pm$10.21 &amp; 30.56$\pm$20.60</td>
<td></td>
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<tr>
<td>MabCampath</td>
<td>Remission (n=1) 4.62 &amp; n.t.</td>
<td></td>
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<tr>
<td></td>
<td>Progression (n=3) 30.95$\pm$6.25 &amp; 21.04$\pm$18.34</td>
<td></td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>Remission (n=6) 8.41$\pm$6.89 &amp; 16.29$\pm$8.61</td>
<td></td>
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<tr>
<td></td>
<td>Progression (n=5) 26.39$\pm$20.01 &amp; 22.08$\pm$17.13</td>
<td></td>
</tr>
<tr>
<td>CHOP, COP</td>
<td>Remission (n=2) 4.02$\pm$3.67 &amp; 3.59$\pm$4.22</td>
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</tr>
<tr>
<td></td>
<td>Progression (n=12) 32.19$\pm$22.99 &amp; 26.09$\pm$23.69</td>
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