Clinical Significance of ZAP-70 Protein Expression in B-cell Chronic Lymphocytic Leukemia

Short title: ZAP-70 and prognosis in B-CLL

Maria Ilaria Del Principe, Giovanni Del Poeta, Francesco Buccisano, Luca Maurillo, Adriano Venditti, Antonella Zucchetto*, Rita Marini, Pasquale Niscola, Maria Antonietta Irno Consalvo, Carla Mazzone, Licia Ottaviani, Paola Panetta, Antonio Bruno, Riccardo Bomben*, Giovanna Suppo, Massimo Degan*, Paolo de Fabritiis, Maria Cantonetti, Francesco Lo Coco, Domenico Del Principe° and Sergio Amadori

Cattedra di Ematologia, Università Tor Vergata, Roma, Italy
*Clinical and Experimental Hematology Research Unit, Centro di Riferimento Oncologico, Aviano (PN), Italy
°Clinica Pediatrica, Università Tor Vergata, Roma, Italy

Presented in part both at the 45th Annual Meeting of the American Society of Hematology, San Diego, CA, December 6-9, 2003 and at the 46th Annual Meeting of the American Society of Hematology, San Diego, CA, December 4-7, 2004

M.I.D.P and G.D.P. contributed equally to this study

Address correspondence to:

Giovanni Del Poeta, M.D.
Cattedra di Ematologia
Università Tor Vergata
Ospedale S.Eugenio
Via Fiume Giallo, 430 MA
00144 Roma, Italy
Tel.: +390651002509
Fax: +39065915965
e-mail: g.delpoeta@tin.it

Supported in part by MURST, Programmi di Ricerca di Interesse Nazionale, 2003 and by Ministero della Salute (Ricerca Finalizzata I.R.C.C.S. and “Alleanza contro il Cancro”), Rome, Italy
Specific contributions of all Authors

Giovanni Del Poeta, Francesco Lo Coco, Domenico Del Principe and Sergio Amadori provided the original concept and design for the study.

Francesco Buccisano and Maria Irno Consalvo obtained ZAP-70 and CD38 flow cytometry data.

Valter Gattei, Antonella Zucchetto, Riccardo Bomben and Massimo Degan investigated IgVH mutational status and generated part of the ZAP-70 flow cytometry data.

Pasquale Niscola and Antonio Bruno did statistical analyses.

Maria Ilaria Del Principe, Giovanni Del Poeta, and Luca Maurillo drafted the manuscript.

Paola Panetta obtained interphase FISH cytogenetics data.

Rita Marini and Giovanna Suppo obtained soluble CD23 data.

Carla Mazzone, Licia Ottaviani, Adriano Venditti, Maria Cantonetti and Paolo de Fabritiis provided clinical care and recorded clinical data.
Abstract

The clinical course of B-cell chronic lymphocytic leukemia (B-CLL) is variable and novel biological parameters need to be added to the clinical staging systems to predict an indolent or aggressive outcome. We investigated the 70-kD zeta-associated protein (ZAP-70), CD38, soluble CD23 (sCD23) and cytogenetics in 289 B-CLL patients. Both a shorter progression free survival (PFS) and overall survival (OS) were observed in ZAP-70+ (P<0.00001), in CD38+ (P<0.00001) and in sCD23+ patients (P<0.00001 and P=0.013, respectively). ZAP-70+CD38+ or ZAP-70+ patients with an unmutated IgVH status showed both a shorter PFS (P<0.00001) and OS (P=0.00002 and P=0.00004, respectively) as compared to ZAP-70+/CD38− or ZAP-70− patients with mutated IgVH genes. Discordant patients showed an intermediate outcome. Noteworthy, ZAP-70+ patients even if CD38− or mutated showed a shorter PFS, while ZAP-70− patients even if CD38+ or unmutated had a longer PFS. Furthermore, ZAP-70 positivity was associated with a shorter PFS both within normal karyotype (P=0.00006) and within the poor risk cytogenetic subset (P=0.02). The predictive value of ZAP-70 expression was confirmed in multivariate analysis. Thus, ZAP-70 protein determined by flow cytometry improves the prognostic significance of cytogenetics and appears to be a better predictor of outcomes than IgVH gene mutational status. On this line, we recommend and are also interested in conducting a prospective randomized trial of early intervention versus observation for ZAP-70+ patients.
Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world and is characterized by the accumulation of monoclonal CD5+ B cells with the appearance of small mature lymphocytes.1 One the most intriguing features of the disease is its clinical heterogeneity with patients progressing rapidly with early death, whereas others exhibit a more stable non progressive disease lasting many years. Thus, it is more important than ever to develop sensitive stratification parameters to identify patients with poor prognosis.2,3

About 50 to 70 percent of patients with CLL have evidence of somatic hypermutation in the immunoglobulin heavy-chain variable region genes (IgV_H) of the leukemic cells.4 B-CLLs with mutated (M) or unmutated (UM) IgV_H gene configurations may represent the neoplastic counterparts of mature B-cells either in different stages of normal B cell differentiation or, more likely, selected by different, yet unidentified, antigens.5 On average patients with M IgV_H genes have a more indolent clinical course than those with the unmutated phenotype and have a longer survival.6,7

Investigations using DNA microarrays have shown that CLL cells exhibit a characteristic gene-expression profile in which the expression of small subgroup of genes, including those encoding ZAP-70, IM1286077 and C-type lectin, correlates with the mutational status of IgV_H genes.8,9 ZAP-70, a member of the Syk-ZAP-70 protein tyrosine kinase family, is normally expressed in T and natural killer cells and has a critical role in initiation of T-cell signalling.10,11 Recent studies have found that ZAP-70 is associated with enhanced signalling by the cell-surface immunoglobulin receptor in CLL B cells12 and that measurement of ZAP-70 can serve as a surrogate for mutational status of IgV_H.13 Moreover, ZAP-70 protein may be conveniently measured by flow-cytometry, in contrast to the technically demanding IgV_H analysis.14 In a larger series of patients, Rassenti et al15 have shown that an increased expression of ZAP-70 by CLL is a
more significant predictor of need for treatment than the presence of an UM IgV\textsubscript{H} gene. Moreover, the expression of ZAP-70 appears to be constant over time.\textsuperscript{15}

CD38 expression also has been suggested as a surrogate marker for the mutational status in B-CLL.\textsuperscript{6} At present, both parameters are regarded as independent prognostic variables in B-CLL.\textsuperscript{16}

Detection of CD38 cell surface expression can be easily performed by flow cytometry in the general laboratory.

Genetic studies using chromosome analysis or interphase fluorescence in situ hybridization (FISH) have identified recurring abnormalities with prognostic significance. Patients with a normal karyotype or deletion of 13q14 as the sole genetic abnormality have a better prognosis than those with a complex karyotype or deletion 11q23 or 17p13.\textsuperscript{17} In a large study these deletions were associated with a shorter overall survival.\textsuperscript{18}

In this study, ZAP-70, CD38, soluble CD23 (sCD23), mutational status and genomic aberrations in relation to other laboratory parameters and clinical information were examined in a large series of B-CLL patients.

In particular, we aimed to develop a new flow cytometric assay for ZAP-70 protein determination, to assess the prognostic impact of ZAP-70, CD38, sCD23, mutational status and cytogenetic groups, whether combined analysis of ZAP-70 and CD38 or ZAP-70 and IgV\textsubscript{H} mutational status could allow us to identify new prognostic subsets, whether ZAP-70 could predict varied outcome within interphase cytogenetic groups, and, finally, to suggest preliminarily whether ZAP-70 could replace IgV\textsubscript{H} gene mutational analysis for prognosis.
Patients, materials and methods

Patients

Approval for this study was obtained from the institutional review board. Informed consent was provided according to the Declaration of Helsinki. Two hundred eighty-nine consecutive and unselected B-CLL patients were enrolled in this study from 1990 to 2004, all fulfilling the recommended diagnostic criteria with dim surface immunoglobulins and CD5⁺CD23⁺ immunologic pattern. There were 148 men and 141 women with a median age of 65 years (range 37-84) at the time of diagnosis. Fresh or cryopreserved B-CLL cells were available for ZAP-70 and CD38 analysis in 289 patients. The median age of the more than 20% ZAP-70⁺ (median, 65; range 37-84) and less than 20% ZAP70⁺ (median, 65; range 37-83) patients was the same. Fresh or frozen serum samples were obtained for sCD23 from 256 patients. All the samples were collected on a single day for each patient and were evaluated at diagnosis or before disease progression or before any chemotherapeutic approach. We tried to set empirically various cut-off points for each biological variable (ZAP-70, CD38, sCD23) and the selected thresholds were sufficient to predict progression and survival, identifying accurately patients at poor prognosis. Moreover, we applied a discriminant function analysis based on the squared Mahalanobis distances of each case from its group centroids (ZAP-70, CD38, sCD23) to verify the selected cut-points. The percentage correct (observed classification vs predicted classification) was about 90% for all variables. Eighty-seven patients had a low-modified Rai stage, 189 had intermediate stage and 13 had high stage. One hundred-forty nine out of 289 patients received chemotherapy for their disease. Fifty-six were intermittently treated with a combination of chlorambucil at conventional doses and prednisone. The remaining 93 patients received 6 courses of fludarabine monophosphate (Fludara; Schering AG, Berlin, Germany) at 25 mg/m²/day for 5 days each 28 days. A total of 34 patients had died of causes related to B-CLL at the time of analysis.
Cellular immunophenotypic analysis

The following antibody conjugates were used: anti-CD23-PE, anti-CD5-FITC, anti-CD38-PE, anti-CD19-APC, anti-CD45-FITC, anti-CD14-PE, anti-CD95-PE, anti-CD10-FITC (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Peripheral blood mononuclear cells were analyzed for surface expression of CD19/CD5/CD38 and CD19/CD5/CD23 by triple color immunofluorescence, as described elsewhere. 19

ZAP-70 protein determination was performed by flow cytometry. Peripheral blood mononuclear cells were fixed and permeabilized with Fix & Perm kit (Caltag Laboratories, Burlingame, CA, USA). Then the cells were placed into two tubes and incubated the first with 10 μl of mouse IgG1 isotypic control conjugated to Alexa Fluor 488 dye (MG120, Caltag Laboratories) and the second with 10 μl of monoclonal antibody (MoAb) anti-ZAP-70 (clone 1E7.2, isotype IgG1) conjugated to Alexa Fluor 488 dye (MHZAP7020, Caltag Laboratories) for 20 minutes at room temperature (r.t.) in the dark. Alexa Fluor 488 dye is excited at 488 nm and presents a peak emission at 519 nm as FITC. After two washing with PBS, the cells in the first tube were stained with mouse IgG1 PercP, APC, PE isotypic controls and the cells in the second one were incubated with 10 μl of CD19 PerCP, 10 μl of CD5 APC, and 3 μl of CD3 PE plus 3 μl of CD56 PE (Becton Dickinson), for 15 minutes at r.t. Finally the samples were analyzed with a FACS Calibur flow cytometer (Becton Dickinson) with a gate on the fluorescence 2 detector to ensure that at least 5000 T and natural killer cells were analysed in each sample. The ZAP-70 analysis, performed with the CellQuest software, as described elsewhere 13 was shown in Figure 1A. Figure 1B shows the level of expression of ZAP-70 by lymphocytes from four representative patients with CLL according to the mutational status of IgVH genes.
Figure 1. Flow cytometric analysis of ZAP-70 expression and flow cytometric profiles of ZAP-70 protein on 4 B-CLL cases. Panel A shows the method used to quantify the expression of ZAP-70 by B-CLL cells. In the plot 1 forward and side scatter of mononuclear cells is represented with a region (R1) drawn around the lymphocytes. Lymphocytes were R1 gated, and then T cells and natural killer (CD3⁺CD56⁺) cells (the R2 region in the plot 2) and CLL cells (CD5⁺CD3⁻CD56⁻) cells (the R3 region in the plot 3) were selected according to their phenotype. The plot 4 shows the expression of mouse IgG1 isotypic controls after lymphocyte (R1) gating. The plot 5 shows the expression of ZAP-70 after lymphocyte (R1) gating. For the purpose of quantification, markers were placed so that the T cells and natural killer cells (R1 and R2 gated) with a high level of expression of ZAP-70 would appear in the upper right quadrant (plot 6). Mouse IgG1 isotypic antibody conjugated with Alexa Fluor was used as control.
marker for ZAP-70 positivity. Then, B-CLL cells were plotted and the same marker that included T cells and natural killer cells in the upper right quadrant was used to calculate the percentage of CLL cells that were positive for ZAP-70, as shown in the plot 7.

Panel B shows the level of ZAP-70 expression by lymphocytes from four representative patients with CLL according to the mutational status of IgVH status. In each case mouse IgG1 isotypic antibody is used as control for ZAP-70 positivity. The percentage of CLL cells with a high level of ZAP-70 expression is shown in the lower right quadrant of each plot, after the exclusion of T and natural killer cells. FITC denotes Alexa Fluor 488.

**Enzyme-linked immunosorbent assays**

Soluble CD23 (sCD23) immunoenzymometric assay was performed as described elsewhere.\textsuperscript{19} The threshold of positivity was set at sCD23 value of more than 70 U/mL.

**Interphase FISH**

Separate hybridisations were carried out for loci on chromosome 11, 12, 13 and 17. For chromosomes 11 (q23), 13 and 17 commercial probes (ATM-2, Rb-1 and p53, respectively) were used (Vysis, Inc, London, United Kingdom). An alpha satellite DNA probe CEP12, directly labelled with SpectrumGreen, was used to detect aneuploidy of chromosome 12. LSIp53, labelled with SpectrumOrange (Vysis), was used to evaluate chromosome deletion at 17p13.1. We used peripheral blood lymphocytes, which were separated by density gradient centrifugation, treated with hypotonic solution (KCl) and fixed with methanol-acetic acid. Then the slides were aged for 20 min at 80°C on a hot plate and dehydrated for 2 min in 70%, 80% and 100% ethanol and air dried. Gene frames were applied to dried slides in order to mark and separate the hybridisation areas of single probes. Slides were placed on a hot plate at 37°C and 5 μl of each probe buffer solution was applied inside the area of the slides delineated by the frame. After, the slides were sealed with a 22x22 mm gene-frame plastic coverslip and placed in the Vysis Hybrite™ machine.
Co-denaturation was carried out at 68°C for 5 min and hybridisation at 37°C over-night. Then the slides were washed in 0.4xSSC/0.3% NP-40 for 2 min at 71°C, followed by 1 min washing in 2xSSC at room temperature. Finally, the nuclei were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI) and signals were visualized using an Olympus BX51 microscope (Olympus Italia, Milan, Italy). Two hundred interphase cells with well-delineated fluorescent spots were examined.

**IgV\textsubscript{H} mutation analysis**

Total RNA was extracted and reverse-transcribed as previously reported\textsuperscript{20}. The resulting cDNAs, checked for first strand synthesis\textsuperscript{21}, were amplified using a mixture of sense primers annealing either to the V\textsubscript{H}1 through V\textsubscript{H}6 leader sequences or to the 5' end of V\textsubscript{H}1-V\textsubscript{H}6 FR1, as reported\textsuperscript{4,22-24}. These primers were used in conjunction with a mixture of antisense primers complementary to the germ line J\textsubscript{H} regions. The purified amplified products, inserted into the PCR2.1-TOPO vector (Invitrogen, Milan, Italy) were expanded in TOP10 One Shot\textsuperscript{TM} competent cells (Invitrogen) and cloned. Plasmid DNAs were isolated from overnight cultures of randomly selected colonies and sequenced by using an automatic DNA sequencer (ABI PRISM 3100, Applied Biosystem, Foster City, CA, USA). Comparisons between the obtained sequences and those of the various germ line IgV\textsubscript{H} genes were performed with the IgBLAST directory (http://www.ncbi.nlm.nih.gov/igblast) using the Mac Vector 7.1 sequence analysis software (Accelrys, Symantec Co., San Diego, CA). Only when the same V\textsubscript{H}DJ\textsubscript{H} rearrangement was identified in at least 5-10 clones, a given IgV\textsubscript{H} sequence was further analysed. Alignment of the IgV\textsubscript{H} sequences available for each patient often revealed, along with mutations shared by all the transcripts analysed, a number of unique or partially shared mutations.\textsuperscript{22,23} For this reason, all mutational analyses were carried out in each IgV\textsubscript{H} transcript separately, and the percent mutation assigned to a given B-CLL was the mean value of the percent mutations found in each transcript. V\textsubscript{H} gene sequences deviating more than 2% from the corresponding germline gene were defined as mutated.
**Statistical analysis**

All statistical analyses were performed at the end of data collection. Correlations between modified Rai stages or lymphadenopathy/splenomegaly or β2-microglobulin and ZAP-70 percentages were based on the two-tailed Fisher exact test. Associations between ZAP-70 and mutational status or cytogenetic subgroups were analysed by the two-tailed Fisher exact test. The correlations between ZAP-70 or CD38 percentages and response to fludarabine were assessed by the proportional odds ordered logistic regression univariate model for categorical variables. To quantify the degree of association between ZAP-70 and CD38 percentages or sCD23 levels, the Spearman coefficient was calculated. The assessment of response was based on the National Cancer Institute Working Group criteria. Progression-free survival (PFS) and overall survival (OS), measured from diagnosis, were estimated according to the method of Kaplan and Meier and compared between groups by means of log-rank test. Cox proportional hazards regression model was used to assess the independent effect of covariates, treated as dichotomous, on the PFS or OS.
Results

Profiles of ZAP-70, CD38, soluble CD23, IgVH mutational status and FISH

As currently reported in literature, a CLL population was considered ZAP-70 positive when at least 20% of the CD19+ B cells expressed the antigen. The B-CLL cells were ZAP-70+ in 104 patients (36%) and ZAP-70− in 185 patients (64%). In order to confirm the stability of ZAP-70 expression during the disease course, sequential samples from 32 patients (2-3 samples per patient) over time periods ranging from 4 to 36 months were analysed to investigate whether ZAP-70 expression remains constant over time. In 28 out of 32 cases (87.5%) including 15 untreated and 13 treated patients, ZAP-70 expression differences of <10% were detected. Although 4 cases (12.5%) showed a variation greater than 10%, in none of these patients the change of ZAP-70 expression crossed the 20% cut-off.

The proportion of B cells expressing CD38 varied from 0 to 87% and the threshold was set at 30%, as previously reported. Based on this cut-off value, 67 patients (23%) were CD38+ and 222 patients (77%) were CD38−. In order to test the stability of CD38 over time, we further analysed sequential samples from 65 patients (range 3-8 for patient) over periods ranging from 2 to 45 months. In 58 of 65 cases (89%) slight differences (<10%) were detected. A total of 7 (11%) cases showed a variation higher than 10%. However, no patient crossed the used 30% cut-off.

Finally, sCD23 was higher than 70 U/ml in 82 patients (32.2%) and lower than 70 in 172 patients (67.7%). Considering the possibility that also sCD23 levels may be unstable during disease, we analysed samples from 85 patients over periods ranging from 2 to 66 months. In 75 out of 85 cases (88%) we detected ignorable differences (<15%). A total of 10 (12%) cases showed variations higher than 15%, but the selected threshold (70 U/ml) was never crossed.
The Spearman correlation between the percentages of ZAP-70\(^+\) cells and those of CD38\(^-\) cells or the serum levels of sCD23 was \(r = 0.35\) (\(P<0.0001\)) and \(r = 0.32\) (\(P<0.0001\)), indicating moderate direct relationships.

Moreover, in 140 patients analysed both for IgV\(_{H}\) mutations and ZAP-70 expression, 79/85 patients (93\%) with lower ZAP-70 had IgV\(_{H}\) mutations >2\% (\(P<0.00001\)), confirming the close correlation currently described\(^{13-15}\).

Also a relationship between CD38 and IgV\(_{H}\) mutational status was demonstrated (85/101 patients with CD38<30\% showed IgV\(_{H}\) mutations >2\%, \(P<0.00001\)).

Finally, also lower sCD23 was significantly correlated with mutated IgV\(_{H}\) status (66/76 patients with sCD23 <70 U/ml had IgV\(_{H}\) mutations >2\%, \(P=0.0007\)).

One hundred fifty seven patients were analyzed by interphase FISH in order to evaluate deletions in chromosome bands 17p13, 11q23, 13q14 and trisomy of band 12q13. With regard to cytogenetic groups, 85 patients (54\%) had a normal karyotype and 42 (27\%) had 13q- . Thirty patients (19\%) had trisomy 12 (n=16), 11q- (n=11), 17p- (n=3). There was a significant correlation between ZAP-70 >20\% and the poor risk cytogenetic subset, encompassing trisomy 12, 11q- and 17p- (22/30, 73\%, \(P=0.002\)). On the other hand, 13q14 deletion was significantly correlated with CD38 <30\% (34/42, 81\%, \(P=0.01\)), whereas normal karyotype was very frequent in patients with lower sCD23 levels (62/83, 75\%, \(P=0.001\)).

**Clinical course and outcome**

No significant correlation was found between gender and ZAP-70 positivity. On the other hand, there was a trend of association between female gender and low Rai stage (51/87, \(P=0.07\)).

With regard to the clinical course, we found significant associations between higher ZAP-70 and intermediate/high modified Rai stage (\(P<0.00001\), Table 1) or the presence of multiple (3 or more) intrathoracic/abdominal lymphadenopathies (>3 cm in diameter) and/or splenomegaly (\(P<0.00001\), Table 1) or \(\beta_2\)-microglobulin serum levels >2.2 mg/ml (\(P=0.00002\), Table 1).
Table 1. Modified Rai stages or lymphadenopathy/splenomegaly or $\beta_2$-microglobulin and ZAP-70 percentages

<table>
<thead>
<tr>
<th></th>
<th>Low Rai stage (n=87; 30%)</th>
<th>Int/High Rai stage (n=202; 70%)</th>
<th>Lymphadenopathy/Splenomegaly §</th>
<th>$\beta_2$-micro(mg/ml)$§$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Present (n=101;35%)</td>
<td>Absent (n=188;65%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta_2$-micro</td>
<td>&gt;2.2 (n=121;46%)</td>
<td>&lt;2.2 (n=144;54%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ZAP-70*

|                  | > 20% (n=104;36%)         | < 20% (n=185;64%)               |
|                  | 12 (12%)                  | 75 (41%)                        |
|                  | 92 (88%)                  | 110 (59%)                       |
|                  | 66 (63%)                  | 35 (19%)                        |
|                  | 38 (37%)                  | 150 (81%)                       |
|                  | 56 (46%)                  | 65 (54%)                        |
|                  | 31 (22%)                  | 113 (78%)                       |

n indicates the number of samples

§ As determined by the two-tailed Fisher exact test

*For modified Rai stage, P<0.00001; for lymphadenopathy/splenomegaly, P<0.00001; for $\beta_2$-microglobulin, P=0.00002.
Similarly, higher CD38 and sCD23 levels were significantly correlated with intermediate/high modified Rai stages, multiple intrathoracic/abdominal lymphadenopathies and/or splenomegaly or higher than 2.2 mg/ml β2-microglobulin (data not shown).

Lymphocyte doubling time (LDT) less than 12 months was observed in 27 patients: 19/27 (70%) showed higher ZAP-70 expression (P=0.00005).

Moreover, while 80/104 (77%) of the ZAP-70+ patients had received chemotherapy at the time of analysis, only 64/185 (34.6%) ZAP-70− patients had been treated for B-CLL (P<0.00001).

Ninety-three patients (87 cases classified as low or intermediate Rai stage) underwent six monthly courses of fludarabine monophosphate at 25 mg/m² for 5 days as first-line chemotherapy, achieving a global complete remission (CR) rate of 46% (43/93 patients, all belonging to low or intermediate Rai stage). A higher CR rate was found both in ZAP-70− (64% vs 31%, P=0.0037, Table 2) and in CD38− patients (61% vs 19%, P=0.00004, Table 2).

A significant shorter PFS was observed in ZAP-70+ patients (0% vs 59% at 13 years, P<0.00001, Figure 2A), in CD38+ patients (8% vs 42% at 14 years, P<0.00001, Figure 2B) and in sCD23 >70 U/ml cases (0% vs 55% at 13 years, P<0.00001, Figure 2C).
Table 2. Complete, partial or no response to fludarabine and ZAP-70 or CD38 expression

<table>
<thead>
<tr>
<th>Variable *</th>
<th>CR (n= 43)</th>
<th>PR (n= 36)</th>
<th>NR (n= 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;20% (n=104)</td>
<td>31% (13-55)&amp;</td>
<td>43% (23-64)</td>
<td>25% (12-44)</td>
</tr>
<tr>
<td>&lt;20% (n=185)</td>
<td>64% (34-82)</td>
<td>33% (14-57)</td>
<td>2% (0.5-8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable °</th>
<th>CR (n= 43)</th>
<th>PR (n= 36)</th>
<th>NR (n= 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;30%</td>
<td>19% (6-36)</td>
<td>50% (26-72)</td>
<td>31% (11-53)</td>
</tr>
<tr>
<td>&lt;30%</td>
<td>61% (35-83)</td>
<td>33% (13-51)</td>
<td>6% (1-21)</td>
</tr>
</tbody>
</table>

CR indicate complete remission; PR, partial remission; NR, no response or progression; n, number of observations.

*P = 0.0037. P values are determined by the proportional odds ordered logistic regression univariate model.

° P = 0.00004.

& CI 95% throughout.
Figure 2. Progression-free survival (PFS) curves based on ZAP-70, CD38 expression and soluble CD23 (sCD23) levels. (A) Kaplan-Meier plot comparing progression-free survival based on the detection of more than 20% or less than 20% ZAP-70+ B-cells. Less than 20% ZAP-70+ pts experienced a longer PFS (P<0.00001). (B) Equally, B-CLL pts with CD38+ less than 30% showed a significant longer PFS (P<0.00001). (C) sCD23 levels lower than 70 U/ml characterized pts with a longer PFS (P<0.00001).

Likewise, a shorter OS was found in ZAP-70+ patients (16% vs 86% at 18 years, P<0.00001, Figure 3A), in CD38+ patients (0% vs 73% at 18 years, P=0.00001, Figure 3B) and, less significantly, in sCD23+ cases (43% vs 79% at 14 years, P=0.013, Figure 3C). These significant differences in term of PFS and OS were maintained when the analysis was restricted to Rai intermediate risk group (189 patients) with regard to ZAP-70, CD38 and sCD23 (data not shown).
Figure 3. Survival curves according to ZAP-70, CD38 expression and sCD23 levels. (A) Kaplan-Meier plot comparing numbers of ZAP-70+ B-CLL cells with overall survival (OS). The difference between pts with ZAP-70>20% and pts with ZAP-70<20% was highly significant (P<0.000001). (B) CD38 expression lower than 30% identified pts with a longer OS (P=0.00001). Less significantly, a longer OS was found in pts with sCD23 levels lower than 70 U/ml (C) (P=0.013).

Interestingly, the simultaneous positivity or negativity for ZAP-70 and CD38 identified two subsets of patients, the former with a worse prognosis and the latter with a better prognosis with regard to both PFS (8% vs 60% at 12 years, P<0.00001, Figure 4A) and OS (18% vs 94% at 16 years, P=0.00002, Figure 4B). Discordant patients (n=77), ZAP-70\(^{-}\)CD38\(^{-}\) (n=63, 82%) or ZAP-70\(^{+}\)CD38\(^{-}\) (n=14, 18%) showed an intermediate outcome (PFS= 14% at 12 years; OS=41% at 16 years, Figure 4A and 4B). No significant association between ZAP-70/CD38 discordant patients (n=43) and IgV\(_{H}\) mutational status was observed. In detail, 12 of 34 (35%) ZAP-70\(^{-}\)CD38\(^{-}\) had UM IgV\(_{H}\) sequences, while 22 (65%) ZAP-70\(^{+}\)CD38\(^{-}\) patients had M IgV\(_{H}\) genes. On the other hand, 2 of 9 cases (22%) ZAP-70\(^{-}\)CD38\(^{+}\) were UM IgV\(_{H}\) and 7 (78%) ZAP-70\(^{+}\)CD38\(^{-}\) were M IgV\(_{H}\).

Moreover, ZAP-70 expression and IgV\(_{H}\) mutational status showed additive properties: in fact, ZAP-70 positivity and UM IgV\(_{H}\) status identified a subset of patients at worst prognosis with regard to PFS (0% vs 60% at 11 years, P<0.00001, Figure 4C) and OS (20% vs 94% at 13 years, P=0.00004, Figure 4D). Discordant patients (ZAP-70\(^{-}\)M or ZAP-70\(^{-}\)UM, n=28) showed an intermediate outcome (PFS= 13% at 11 years; OS= 62% at 13 years, Figure 4C and 4D).
Figure 4. Progression-free survival (PFS) and overall survival (OS) curves in relation either to combined ZAP-70 and CD38 expression or ZAP-70 and IgVH mutational status. (A,B) PFS and OS were significantly longer within ZAP-70\(^{-}\)CD38\(^{-}\) subgroup (P<0.00001 and P=0.00002, respectively). (C,D) Equally, ZAP-70\(^{\text{Mutated (M)}}\) pts experienced both a longer PFS (P<0.00001) and OS (P=0.00004). Discordant patients (ZAP-70\(^{+}\)CD38\(^{-}\)/ZAP-70\(^{+}\)CD38\(^{+}\) or ZAP-70\(^{+}\) M/ZAP-70\(^{+}\)Unmutated [UM]) showed an intermediate outcome.

Noteworthy, we identified two small subsets of discordant patients ZAP-70\(^{-}\)CD38\(^{+}\) (n=14) and ZAP-70\(^{-}\)UM (n=5) which showed a significant better outcome than that of ZAP-70\(^{+}\)CD38\(^{-}\) and ZAP-70\(^{+}\)M patients with regard to PFS (Figure 5A and 5B).

Interestingly, within the ZAP-70\(^{-}\) subset (185 of 289 patients, 64%) patients with sCD23>70 U/ml levels had a significant shorter PFS, as compared to cases with sCD23 lower than 70 U/ml cut-off (21% vs 76% at 10 years, P=0.0005, Figure 5C).
Figure 5. Progression-free survival (PFS) curves in discordant patients (ZAP-70⁻CD38⁻/ZAP-70⁻CD38⁺ and ZAP-70⁺M/ZAP-70⁻UM) and in ZAP-70⁻ pts by sCD23 levels. (A,B) Interestingly, ZAP-70⁻ either CD38⁺ or UM pts showed a longer PFS in comparison with ZAP-70⁺ either CD38⁻ or M pts (P=0.0009 and P=0.002, respectively). (C) Levels lower than 70 U/ml of sCD23 identified pts with a longer PFS within the ZAP-70⁻ subset (P=0.0005).

With regard to clonal genomic aberrations, a significant shorter PFS was observed in patients with prognostically unfavorable aberrations, such as 17p⁻, 11q⁻ and trisomy 12, which were pooled together and defined as a poor risk cytogenetic subset (n=30), vs normal karyotype patients (n=85, 7% vs 39% at 12 years, P=0.004, Figure 6A). The 13q⁻ patients (n=42) showed an intermediate outcome (22% at 12 years).

To further explore the clinical impact of ZAP-70 expression among different cytogenetic groups, we investigated its expression within the normal karyotype and the poor risk cytogenetic subsets. As a matter of fact, ZAP-70 positivity was significantly associated with a shorter PFS both within normal karyotype (14% vs 67% at 10 years, P=0.00006, Figure 6B) and within the poor risk subset (0% vs 29% at 10 years, P=0.02, Figure 6C).
Figure 6. Progression-free survival (PFS) curves based on cytogenetics and ZAP-70 expression within cytogenetic subsets. (A) B-CLL pts with a normal karyotype showed a longer PFS (P=0.004) as compared to high risk genetic aberrations (11q-, 17p- and +13). (B,C) ZAP-70 protein overexpression distinguished pts at worse prognosis both within normal karyotype (P=0.00006) and within the poor risk B-CLL subset (P=0.02).

Finally, we performed a multivariate Cox regression analysis of PFS and OS, including as covariates, age < or > 60 years, modified Rai stages, $\beta_2$-microglobulin, sCD23, CD38 and ZAP-70. With regard to PFS, ZAP-70, sCD23 and modified Rai stages were confirmed to be independent prognostic factors (Table 3). Concerning OS, only ZAP-70 was an independent prognostic factor, while CD38 showed a trend toward a statistical significance (Table 4).

Table 3. Cox regression analysis of progression free survival

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio</th>
<th>P</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZAP-70</td>
<td>16.05</td>
<td>.0006</td>
<td>5.68-49.23</td>
</tr>
<tr>
<td>sCD23</td>
<td>7.21</td>
<td>.007</td>
<td>2.55-22.35</td>
</tr>
<tr>
<td>Modified Rai stages</td>
<td>7.04</td>
<td>.008</td>
<td>1.98-21.43</td>
</tr>
</tbody>
</table>

Table 4. Cox regression analysis of overall survival

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio</th>
<th>P</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZAP-70</td>
<td>10.96</td>
<td>.0009</td>
<td>3.76-31.85</td>
</tr>
<tr>
<td>CD38</td>
<td>3.29</td>
<td>.06</td>
<td>1.12-10.22</td>
</tr>
</tbody>
</table>
Discussion

ZAP-70 expression, determined by flow cytometry, has already been identified as a significant predictor of disease progression and overall survival in B-CLL.\textsuperscript{13-15,26,27} We used a well-standardized flow cytometric methodology\textsuperscript{13} for ZAP-70 determination, but the employment of the novel fluorochrome Alexa Fluor directly conjugated with the MoAb anti-ZAP-70 clone 1E7.2 allowed us to obtain, as in Rassenti et al. work,\textsuperscript{15} a signal clearer than that currently performed with unconjugated antibodies, such as clone 2F3.2, as shown in Figure 1. Gibbs et al\textsuperscript{28} investigated the measurement of ZAP-70 expression in CLL using two different antibodies and two different staining methods. These Authors demonstrated that ZAP-70 antibody clone 1E7.2 and Fix & Perm kit staining method were the easiest to use and the most sensitive and specific combination. Therefore this combination may provide a standardized flow cytometric method which could be introduced into a routine CLL immunophenotyping panel in a clinical diagnostic laboratory. Moreover, this technologic improvement was important in order to define the optimal cut-off, in term of percent of ZAP-70+ cells, capable to split B-CLL patients into two subsets with different clinical features and outcome. As stability of ZAP-70 expression by the leukemic cell clone over time is an important precondition for a reliable use of this protein as prognostic marker, we analyzed ZAP-70 levels in 32 patients from whom sequential samples were available. ZAP-70 expression levels were stable in the majority of patients and, notably, in none of these patients the change of ZAP-70 expression crossed the 20\% cut-off. Even though data from literature\textsuperscript{15,29} indicate a certain degree of variability in ZAP-70 expression over time, the prognostic prediction made at diagnosis does not change in the majority of cases. Surely, further ZAP-70 time course analyses and standardization of flow cytometry protocols are needed to resolve this issue.

We show here that ZAP-70 expression was significantly correlated with CD38 antigen: both these two markers are more highly expressed in UM IgV\textsubscript{H} CLL and are consistent with a more activated
phenotype. Ongoing antigen-mediated activation of the leukemic cells through the BCR is suggested by the presence of cellular activation markers on the cell surface. Probably, the B-CLL subset expressing ZAP-70 and CD38 may reflect ongoing in vivo stimulation, thereby explaining the more aggressive disease course observed in these patients. Chen et al demonstrated that the expression of ZAP-70 is associated with an increased BCR signalling in CLL cells. In this report, the Authors further suggest that global tyrosine phosphorylation is increased in ZAP-70+ cells. Such increased intracellular signalling could influence the survival or proliferation of CLL cells, leading to a tendency toward disease progression. Moreover, Zupo et al have shown that CD38-expressing B cells appeared to be more responsive to BCR ligation, thereby supporting a potential link between CD38 and ZAP-70 expression.

Our study confirms the significant association between the expression of ZAP-70 in CLL cells and UM IgVH genes in agreement with the results of other studies even though the mechanisms accounting for the relation between ZAP-70 expression and IgVH mutational status are still unknown.

However, the simultaneous information of both ZAP-70 level and IgVH mutational status still provides more useful prognostic information given a certain degree of discordance between these two prognosticators (from 5% to 23%), as underscored in recent studies. We obtained that 20% of the results of the two methods were discordant. Our discordance rate was greater than 5 to 6 percent reported by Crespo et al and Orchard et al, but similar to that reported (23%) by Rassenti et al. Interestingly, we used the same monoclonal antibody (1E7.2) and fluorochrome (Alexa-488 dye) reported by Rassenti et al. and surely identical antibodies may give similar results.

In our study we performed chromosome analysis by FISH in 157 B-CLL patients and a significant correlation between unfavorable cytogenetic aberrations such as trisomy 12, 11q-, 17 p- and ZAP-70 expression was demonstrated.
From a clinical point of view, in our series of consecutive unselected CLL patients there was a large prevalence of low and intermediate Rai stages accounting for about 95% of all patients. It is our opinion that this situation reflects the actual recruitment of patients, as it happens in a first level haematology center such as our Institution; this may at least in part explain both the higher number of females and the lower frequency of ZAP-70 positivity observed in our series.

Higher ZAP-70 expression was significantly correlated with more advanced Rai stages, with large intrathoracic/abdominal lymphadenopathies, splenomegaly and a shorter LDT, all hallmarks of an active and aggressive disease. Moreover, significant differences in treatment histories between ZAP-70+ and ZAP-70− cases were found since a larger number of ZAP-70+ patients received treatment at the time of analysis (P<0.00001).

Besides, the high global CR rate (46%) of fludarabine treated patients was to be attributed to the fact that almost all (87/93) treated patients as progressing belonged to low or intermediate Rai stages. In fact, in literature, considering only this subset of patients, CR percentages are higher than 15-20% range, currently reported in most large series.33,34

Furthermore, complete response to fludarabine as initial therapy was significantly correlated with ZAP-70 percentages (P=0.0008), as well as with CD38 expression (P=0.0001), confirming that these biological markers may be used to predict the chemosensitivity of B-CLL patients.

We demonstrated that ZAP-70 positivity was significantly related to an unfavorable clinical course as regards both to PFS (P<0.00001) and OS (P<0.00001) on a large series of B-CLL patients in accordance with the studies by Crespo et al,13 Orchard et al14 and Rassenti et al.15 These important results were also confirmed by restricting the analysis to patients initially presenting in the Rai intermediate stage which includes clinically heterogeneous cases. Moreover, also higher CD38 expression and sCD23 levels were significantly associated with a shorter PFS and OS in this large cohort of patients, confirming our previous results.19 Interestingly, combined analysis of ZAP-70 and CD38 allowed us to separate our patients into three subgroups: ZAP-70+CD38−
(n=161) presenting good clinical features, ZAP-70\(^{+}\)CD38\(^{-}\) (n=51) with a poor prognosis and discordant ZAP-70/CD38 (n=77) with an intermediate outcome (Figure 4).

Of note, almost all discordant patients (63/77, 82\%) were ZAP-70\(^{+}\) and CD38\(^{-}\) suggesting the superior clinical impact of ZAP-70 over CD38. The phenomenon of discordant ZAP-70/CD38 expression was further explored by IgV\(_H\) mutation analysis in this subset of patients. Interestingly, we found a high proportion of M IgV\(_H\) patients (21/32, 64.5\%) in the ZAP-70\(^{+}\)CD38\(^{-}\) subgroup, indicating that ZAP-70 could be prognostically even more important than Ig-mutational status.

Our results are in disagreement with the observations of Schroers et al.\(^{29}\) who reported a high proportion of UM cases among discordant patients.

Furthermore, the combined analysis of ZAP-70 and IgV\(_H\) mutational status allowed us to identify a discordant group ZAP-70/IgV\(_H\) presenting an intermediate outcome, as described in Figure 4. Again, almost all discordant patients were ZAP-70\(^{+}\) and M IgV\(_H\) suggesting that ZAP-70 may be a better predictor of outcomes than Ig-mutation status.\(^{15,35}\)

Interestingly, in our study we described a novel clinical preliminary observation that the two small discordant subsets ZAP-70\(^{+}\)CD38\(^{-}\) (n=14) and ZAP-70\(^{-}\) UM IgV\(_H\) (n=5) presented a much more favorable outcome than that of ZAP-70\(^{-}\)CD38\(^{-}\) or ZAP-70\(^{+}\) M IgV\(_H\) patients, as reported in Figure 5. This result suggests that the prognostic impact both of IgV\(_H\) mutational status and CD38 can be dependent mainly by the ZAP-70 contemporary positivity, even if it should be confirmed both on larger numbers of patients and in the setting of a prospective controlled trial. Concerning that, Chen et al.\(^{12}\) observed that the enhancement of IgM signalling with an increased tyrosine phosphorylation and increased calcium flux, responsible of an aggressive clinical behaviour, was more tightly higher in UM or M ZAP-70\(^{+}\) CLL as compared to UM ZAP-70\(^{-}\) CLL.

Another interesting point of discussion is how to evaluate the disease progression and to establish the time to treatment within ZAP-70 negative patients who represent a large subgroup (185 of 289 patients) with variable outcome also in our experience. Interestingly, sCD23 levels varied within ZAP-70 negative patients allowing us to distinguish different prognostic subsets with regard to
PFS. Therefore, this biological marker reveals prognostic capabilities and has to be added to the other well known factors (ZAP-70, CD38, mutational status, clinical stage) in order to monitor the clinical outcome of B-CLL patients.

Moreover, our study confirmed the prognostic significance of chromosome analysis by FISH in B-CLL demonstrating a longer PFS in normal karyotype and 13q deletion in comparison with high risk genomic aberrations such as trisomy 12, 11q- and 17p-, as previously reported. Up today, there are not large studies on the combined prognostic significance of ZAP-70 and cytogenetics in B-CLL. In our study, ZAP-70 protein confirmed a predictive power distinguishing patients who have early PFS or short OS within normal karyotype and poor risk cytogenetic subsets, where progression was heterogeneous.

The independent prognostic effect of ZAP-70 expression on the clinical outcome of B-CLL patients was finally corroborated by the multivariate analysis with regard both to PFS and OS (Table 3 and 4).

In conclusion, our study demonstrates that the increased expression of ZAP-70 by CLL cells is a more significant predictor of disease progression than the presence of CD38 and sCD23. Moreover, ZAP-70 was able to predict a different outcome within interphase cytogenetic groups. In addition, combined analysis of ZAP-70 and CD38 or ZAP-70 and IgV H status allowed us to identify discordant subsets of patients in which the presence of ZAP-70 alone was sufficient to define a poor prognosis. These results suggest a superior prognostic role of ZAP-70 over CD38 and IgV H mutational status. Importantly, this parameter can be determined easily and rapidly by flow cytometry, even though further studies are required to develop a standardized flow cytometry protocol. Finally, because ZAP-70 expression appears to be stable over time, it should be used at the time of diagnosis to identify patients at increased risk for early disease progression. Noteworthy, the large prevalence of the intermediate Rai stage found in our series (189/289, 65%) reinforces the importance of ZAP-70 determination; since patients belonging to this stage may have either an indolent course or a rapid aggressive outcome, information on ZAP-70 expression
levels may be of great help for clinicians to select which patients are eligible or not for treatment. Clinical trials of effective treatment stratified by more reliable prognostic markers, such as ZAP-70, are surely now warranted.\textsuperscript{38}
References


Clinical significance of ZAP-70 protein expression in B-cell chronic lymphocytic leukemia

Maria Ilaria Del Principe, Giovanni Del Poeta, Francesco Buccisano, Luca Maurillo, Adriano Venditti, Antonella Zucchetti, Rita Marin, Pasquale Niscola, Maria Antonietta Iro Consalvo, Carla Mazzone, Licia Ottaviani, Paola Panetta, Antonio Bruno, Riccardo Bomben, Giovanna Suppo, Massimo Degan, Valter Gattei, Paolo de Fabritiis, Maria Cantonetti, Francesco Lo Coco, Domenico Del Principe and Sergio Amadori