ZAP-70 Protein Expression and CD38 Positivity in B-cell Chronic Lymphocytic Leukemia

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Abstract: ***Background:*** The clinical course, disease progression, and survival of B-cell chronic lymphocytic leukemia (B-CLL) have been correlated with immunoglobulin heavy-chain variable region mutation status. The biologic parameters 70-kDa zeta-associated protein (ZAP-70) and CD38 expression are easier and faster surrogate markers for mutational status. ***Objective:*** To assess retrospectively ZAP-70 expression in B-CLL cells using flow cytometry and examine its relationship with CD38 expression and the median time from diagnosis to initial therapy. ***Methods:*** Ninety-four unselected patients who had their follow-up in the outpatient clinic from 2004 to 2005 were reviewed for immunophenotyping ZAP-70 and CD38 expression. Direct immunolabeling with clone 2E3.2, isotype IgG2a, enabled easy quantification of ZAP-70 by flow cytometry in association with CD38 expression; in addition, the mean fluorescence intensity ratio (MFIR) of CD19+CD5+ B-CLL cells compared to an isotype control monoclonal antibody was determined. ***Results:*** ZAP-70 expression levels in B-CLL cells varied widely (0.3–99%). The median time to therapy was significantly shorter for the 54 patients with 20% or more ZAP-70+ cells (30 months) than for the 40 patients with less than 20% ZAP-70+ cells (median time to treatment not reached). The optimal MFIR for classifying patients as ZAP-70+ was 2. Thirty-two patients had a threshold of ZAP-70+CD38+ greater than 30%, with a median time from diagnosis to treatment of 19 months. Regardless of CD38 expression level, CD38 and ZAP-70 expressions were significantly associated. The median interval from diagnosis to initial therapy was 16.2 months for ZAP-70+CD38+ patients, 60 months for ZAP-70+CD38- or ZAP-70-CD38+ patients, and had not yet been reached for ZAP-70-CD38- patients. ***Conclusion:*** The association of ZAP-70+CD19+CD5+ B-CLL cells and percentage of CD38+CD19+CD5+ B-CLL cells evaluated by flow cytometry provide reliable methods that could be introduced into a routine diagnostic B-CLL panel to predict outcome.

Introduction

The clinical course of chronic B-cell lymphocytic leukemia (B-CLL) is heterogeneous; some patients progress rapidly and others have stable nonprogressive disease lasting many years. It is now thought that B-CLL may result from the malignant transformation of either a pregerminal or postgerminal center B-lineage cell as defined by immunoglobulin heavy-chain variable region (IgV_H) gene mutational status.[1,2] The presence or absence of IgV_H gene mutations can be used to help predict the clinical course[1] of B-CLL: those patients without mutations (~30%) have relatively more aggressive disease with shorter survival times (79–119 months) than those with mutations.[3-5] However, because sequencing rearranged immunoglobulin genes is not easily amenable to routine clinical laboratory practices, several surrogate markers have been studied.

CD38 is a receptor membrane that induces proliferation and increased survival of CLL cells.[6] CD38 expression, as determined by flow cytometry, has been shown to have prognostic significance for B-CLL patients and proposed as a surrogate marker for IgV_H gene mutational status.[7-9] A confounding issue, however, is that CD38 expression by leukemia clones can change during the course of the disease and is not a clear independent prognostic factor according to multivariate analyses.[4,5]

Gene microarray analyses have identified a small number of genes, including those encoding the 70-kDa zeta-associated protein (ZAP-70), IM1286077, and C-type lectin, that predict IgV_H gene mutational status in B-CLL with high accuracy.[10,11] ZAP-70, a member of the Syk/ZAP-70 tyrosine kinase family, is normally expressed in T cells and natural killer (NK) cells and plays a critical role in the initiation of T-cell signaling.[12,13] ZAP-70 is associated with enhanced signaling by the cell-surface immunoglobulin receptor of B-CLL cells.[11] ZAP-70 expression detected by flow cytometry was correlated with IgV_H mutational status, disease progression, and survival.[14-16]

Previously, ZAP-70 expression was assessed by flow cytometry with two main monoclonal antibodies: 2F3.2 clone, isotype IgG2a, and 1E7.2 clone, isotype IgG1.[14,15] Each clone binds to specific ZAP-70 epitopes and can be conjugated with different fluorochromes (Alexa Fluor 488, fluorescein [FITC], or phycoerythrin [PE]).[17] Antibody affinity to different ZAP-70 epitopes and differences in fluorochrome-conjugated chemistry are the source of variability. The use of fluorochromes directly conjugated with the monoclonal antibody anti–ZAP-70 allows researchers to obtain a signal clearer[18,19] than that currently obtained with unconjugated antibodies.[14,15] The ratio of mean fluorescence intensity (MFI) of ZAP-70–stained CD19+CD5+ cells compared with the same population treated with an isotype control monoclonal antibody is an alternative method.[11] We assessed ZAP-70 expression in B-CLL cells by flow cytometry using monoclonal antibody anti–ZAP-70, clone 2E3.2, conjugated directly with FITC as fluorochrome according to Tinhofer and associates.[19] ZAP-70 levels were then calculated as percentages, and the ratio of MFI CD19+CD5+ cells compared to an isotype control monoclonal antibody, and their relationships with CD38 expression and time to treatment, were analyzed.

Methods

Patients and Samples

Between January 2004 and May 2005, 94 consecutive B-CLL patients followed at the outpatient clinic of our institution were eligible for this retrospective study. All patients had either viable frozen or fresh peripheral blood samples, collected at the time of the first consultation or B-CLL diagnosis, and clinical follow-up.

The diagnosis of B-CLL required persistent lymphocytosis of more than 5×10^9/L, and, according to the scoring system by Matutes and colleagues, the tumor cells were typically CD19+CD5+CD23+ and surface immunoglobulin weakly expressed.[20]

The population consisted of 57 men and 37 women whose median age at diagnosis was 60 years. The median duration of follow-up from diagnosis was 54 months (range, 4–308 months). At the time of diagnosis, 82 patients were at Binet stage A and 12 patients were Binet stage B.[21] Progression was defined as a change to a more advanced clinical stage or the need for treatment according to National Cancer Institute Working Group criteria.[22] The time to progression and overall survival were calculated from the time of diagnosis. A total of 42 patients required treatment.

Flow Cytometry

Mononuclear cells were separated from fresh peripheral blood or before freezing by Ficoll-Hypaque density-gradient centrifugation. Immunophenotypes were determined using a FACScalibur (Becton Dickinson) flow cytometer.

Determination of ZAP-70 Expression

The following antibody conjugates were used: anti–CD19-PerCP-Cy5.5 (BD Biosciences), anti–CD3-PE (BD Biosciences), anti–CD56-PE (BD Biosciences), and anti–CD5-APC (BD Biosciences). Mononuclear cells (500,000) were suspended in 200 µL of phosphate buffered saline (PBS) and incubated with 5 µL CD19-PerCP-Cy5.5, 10 µL CD3-PE, 10 µL CD56-PE, and 2.5 µL CD5-APC for 15 minutes at room temperature. After two washings with PBS, cells were fixed and permeabilized with Fix & Perm kit (Caltag), according to the manufacturer's instructions. Externally labeled cells were suspended in 100 µL of medium A for 15 minutes and washed with PBS, suspended in 100 µL of medium B for 15 minutes and washed with PBS, then resuspended in 100 µL of PBS. The cells were finally incubated with 1.5 µL monoclonal antibody anti–ZAP-70-FITC (clone 2F3.2, isotype IgG2a; Upstate) for 20 minutes at room temperature. Cells were then washed twice with PBS before flow cytometry analysis using 4-color gating. Immunoglobulin isotype–matched negative controls were used in all cases to distinguish positive and negative cells.

Each patient's gated lymphocytes were further gated to select CD3+CD56+ cells (T and NK cells), used as a positive internal control for ZAP-70 expression, and CD19+CD5+ cells (B-CLL cells). Biparametric dot plots of cells labeled with ZAP-70 (FITC), CD3 and CD56 (both PE), CD19 (PerCP-Cy5.5), and CD5 (APC) were individually drawn for T cells, NK cells, and B-CLL cells. ZAP-70 expression on CD19+CD5+ B-CLL cells was expressed as a percentage.

In order to increase the sensitivity of ZAP-70 expression, we calculated the mean fluorescence intensity ratio (MFIR).[11] This is the ratio of the median fluorescence intensity of gated CD19+CD5+ cells stained with monoclonal antibody anti–ZAP-70-FITC versus CD19+CD5+ cells stained with the IgG2a isotype control monoclonal antibody conjugated with FITC.

CD38 Expression

CD38 positivity was assessed using a four-color technique. The cells were labeled with the following conjugated antibodies: CD20-FITC, CD19-PerCP-Cy5.5, CD79-PE, and CD38-APC (BD Biosciences). Immunoglobulin isotype–matched controls were used in all runs to separate positive and negative cells. For each sample the dot plot was generated using the side scatter/forward scatter lymphocyte gate. Within those parameters the markers were set on the isotype control to define the negative population. The B-CLL population was defined by lymphocyte (as above) gating followed by gating of CD20+CD19+CD79+CD38+ cells. The percentage of CD38+ cells among the CD20+CD38+ population was then determined. A cutoff point for threshold of at least 20% CD38 expression was chosen according to publication.[8]

Statistical Analysis

Patient characteristics were compared using Wilcoxon and Fisher exact tests. ZAP-70 expression was considered a continuous variable and an ordered categorical variable based on the following groups: 0 to <10%, ≥10% to <20%, ≥20 to <30%, and ≥30%. MFIR was also considered a continuous variable and an ordered categorical variable based on the following groups: 0 to <1.5, ≥1.5 to <2, and ≥2. The relationship between continuous variables ZAP-70 and MFIR was analyzed using a linear-regression method. The time from diagnosis to the initiation of therapy was estimated using the Kaplan-Meier method[23] and assessed with the log-rank test. Data from patients who were still untreated were censored. Cox proportional hazards models[24] were applied to assess the potential association between ZAP-70 and CD38 expression and the interval from B-CLL diagnosis to treatment onset. Differences established by comparative tests were considered significant when $P<.05$. All statistical analyses were performed using S-PLUS 2000 (MathSoft) software.

Results

Evaluation of ZAP-70 Expression Using a Flow Cytometry Method in B-CLL Cells

Flow cytometry analysis quantified the percentages of B-CLL cells (CD19+CD5+) expressing ZAP-70 protein (Figure 1). The percentage of B-CLL cells expressing ZAP-70 in the 94 samples ranged from 0.3 to 99. Four subgroups could be discerned based on the percentage of CD19+CD5+ B-CLL cells exceeding the ZAP-70 positivity threshold of ≥30%: 16 samples (17.2%) contained <10% ZAP-70+ cells; 20 samples (21.2%) had ≥10% but <20% exceeding the threshold; 10 samples (10.6%) contained ≥20% but <30% ZAP-70+ cells; and ≥30% of the B-CLL cells in 45 samples (47.8%) exceeded the ZAP-70 positivity threshold.

The MFIs of gated CD19+CD5+ cells labeled with anti–ZAP-70 or the IgG2a isotype control monoclonal antibody were calculated for 92 patients. MFIR values were distributed as follows: <1.5 for 9 samples (9.9%), ≥1.5 but <2 for 21 samples (23.0%), and ≥2 for 62 samples (68.1%). Linear-regression analyses showed a strong significant association ($R=-5.57$; $P<.00001$) between MFIR and ZAP-70 expression percentages (Figure 2).

Association Between ZAP-70 and Time to Initial Therapy

Initially, CD19+CD5+ B-CLL cells were considered ZAP-70+ when ≥20% of the gated population expressed this protein, according to Crespo at al.[14] Comparisons of

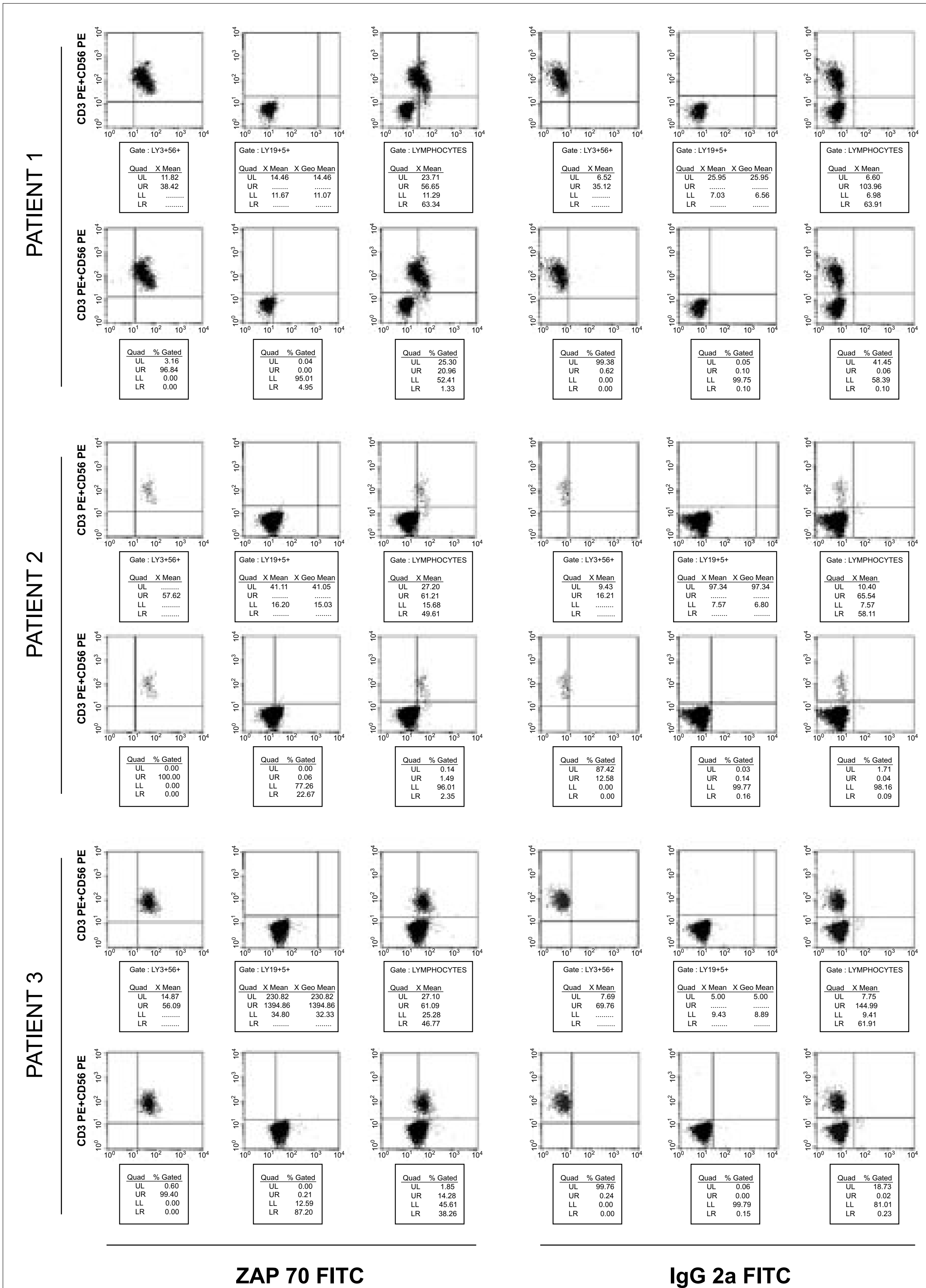
PATIENT 1
CD3 PE+CD56 PE
Gate : LY3+56+ Quad X Mean UL 11.82 UR 38.42 LL .... LR ....
Gate : LY19+5+ Quad X Mean X Geo Mean UL 14.46 14.46 UR .... LL 11.67 11.07 LR ....
Gate : LYMPHOCYTES Quad X Mean UL 23.71 UR 56.65 LL 11.29 LR 63.34
Gate : LY3+56+ Quad X Mean UL 6.52 UR 35.12 LL .... LR ....
Gate : LY19+5+ Quad X Mean X Geo Mean UL 25.95 25.95 UR .... LL 7.03 6.56 LR ....
Gate : LYMPHOCYTES Quad X Mean UL 6.60 UR 103.96 LL 6.98 LR 63.91
Quad % Gated UL 3.16 UR 96.84 LL 0.00 LR 0.00
Quad % Gated UL 0.04 UR 0.00 LL 95.01 LR 4.95
Quad % Gated UL 25.30 UR 20.96 LL 52.41 LR 1.33
Quad % Gated UL 99.38 UR 0.62 LL 0.00 LR 0.00
Quad % Gated UL 0.05 UR 0.10 LL 99.75 LR 0.10
Quad % Gated UL 41.45 UR 0.06 LL 58.39 LR 0.10
PATIENT 2
Gate : LY3+56+ Quad X Mean UL .... UR 57.62 LL .... LR ....
Gate : LY19+5+ Quad X Mean X Geo Mean UL 41.11 41.05 UR .... LL 16.20 15.03 LR ....
Gate : LYMPHOCYTES Quad X Mean UL 27.20 UR 61.21 LL 15.68 LR 49.61
Gate : LY3+56+ Quad X Mean UL 9.43 UR 16.21 LL .... LR ....
Gate : LY19+5+ Quad X Mean X Geo Mean UL 97.34 97.34 UR .... LL 7.57 6.80 LR ....
Gate : LYMPHOCYTES Quad X Mean UL 10.40 UR 65.54 LL 7.57 LR 58.11
Quad % Gated UL 0.00 UR 100.00 LL 0.00 LR 0.00
Quad % Gated UL 0.00 UR 0.06 LL 77.26 LR 22.67
Quad % Gated UL 0.14 UR 1.49 LL 96.01 LR 2.35
Quad % Gated UL 87.42 UR 12.58 LL 0.00 LR 0.00
Quad % Gated UL 0.03 UR 0.14 LL 99.77 LR 0.16
Quad % Gated UL 1.71 UR 0.04 LL 98.16 LR 0.09
PATIENT 3
Gate : LY3+56+ Quad X Mean UL 14.87 UR 56.09 LL .... LR ....
Gate : LY19+5+ Quad X Mean X Geo Mean UL 230.82 230.82 UR 1394.86 1394.86 LL 34.80 32.33 LR ....
Gate : LYMPHOCYTES Quad X Mean UL 27.10 UR 61.09 LL 25.28 LR 46.77
Gate : LY3+56+ Quad X Mean UL 7.69 UR 69.76 LL .... LR ....
Gate : LY19+5+ Quad X Mean X Geo Mean UL 5.00 5.00 UR .... LL 9.43 8.89 LR ....
Gate : LYMPHOCYTES Quad X Mean UL 7.75 UR 144.99 LL 9.41 LR 61.91
Quad % Gated UL 0.60 UR 99.40 LL 0.00 LR 0.00
Quad % Gated UL 0.00 UR 0.21 LL 12.59 LR 87.20
Quad % Gated UL 1.85 UR 14.28 LL 45.61 LR 38.26
Quad % Gated UL 99.76 UR 0.24 LL 0.00 LR 0.00
Quad % Gated UL 0.06 UR 0.00 LL 99.79 LR 0.15
Quad % Gated UL 18.73 UR 0.02 LL 81.01 LR 0.23
ZAP 70 FITC
IgG 2a FITC

Figure 1. Detection of ZAP-70+ B-CLL cells by flow cytometry. Dot plots of gated lymphocytes, labeled with ZAP-70-FITC or the Ig-isotype control antibody from 3 B-CLL patients. For each patient, the first vertical column shows gated lymphocytes. In column 2 line 1 for each patient, T cells and NK cells with high ZAP-70 expression appear in the upper right (UR) quadrant. In column 3, markers were placed so that the ZAP-70+ or IgG2a+ B-CLL cells are in the lower left (LL) quadrant (line 1). Using their respective MFI (reported for LL in the box beneath the dot plot) as numerator and denominator, yields the MFIR for patients 1–3: 1.66 (11.67/7.03), 2.14 (16.20/7.57), and 3.69 (34.80/9.43). After the exclusion of T cells and NK cells (second line of dot plots), cells in the lower right (LR) quadrant were considered B-CLL cells with high ZAP-70 expression and their percentages are reported for LR in the box underneath the plot.

B-CLL=B-cell chronic lymphocytic leukemia; FITC=fluorescein isothiocyanate; MFIR=mean fluorescence intensity ratio; NK=natural killer.

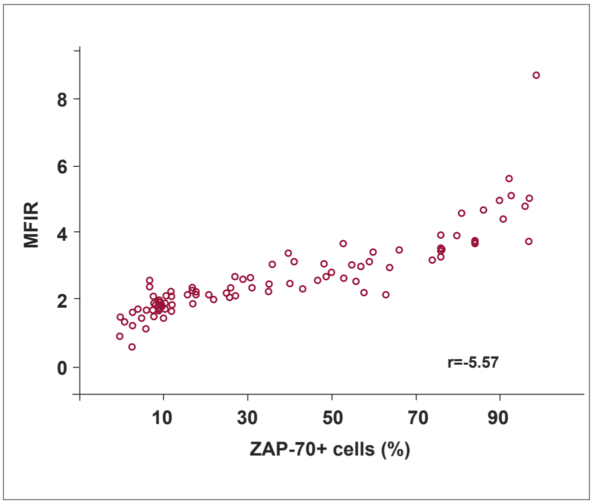

Figure 2. The relationship between the mean fluorescence intensity ratio and the percentage of ZAP-70+ B-CLL cells assessed by linear regression analysis expression.

MFIR=mean fluorescence intensity ratio.

additional clinical parameters between the 40 patients with less than 20% ZAP-70+ CD19+CD5+ cells gating and the 54 patients with at least 20% ZAP-70 positivity are shown in Table 1. Notably, Binet stage A was significantly more common at diagnosis for patients with less than 20% ZAP-70+ B-CLL cells.

Analyses of the time from diagnosis to initial therapy as a function of ZAP-70 positivity (<20% or ≥20%) showed that the median time to treatment onset had not yet been reached for the 40 patients with lower ZAP-70 percentages, but was 30 months for the 54 patients with at least 20% ZAP-70 positivity (*P*=.00001; Figure 3).

Moreover, for the 29 patients whose MFIR was less than 2, the median time from diagnosis to initial therapy had not yet been reached, whereas for the 62 patients with MFIR 2 or higher, the median time was 42.6 months (*P*=.001). The median time from diagnosis to initial therapy had not yet been reached for the 8 patients whose MFIR was less than 1.5 and the median time was 68 months for patients whose MFIR was 1.5 or higher (*P*=0.09). The optimal MFIR for classifying patients as ZAP-70+ was 2.

Relationship Between ZAP-70 and CD38 Expressions

Data on CD38 expression were available for all B-CLL patients. Among these 94 patients, 32 (34.0%) had at least 20% CD38+CD20+ cells, and their median time from diagnosis to initial treatment (19 months) was significantly shorter than that for patients with <20% CD38+CD20+ cells (median time not been reached; *P*=.0001).

Regardless of the CD38+CD20+ subgroup examined, CD38+CD20+ expression was significantly associated with ZAP-70 positivity (defined as at least 20% positive B-CLL cells; *P*=.049). Among the 94 patients, 31 (33%) were concordant double-negative and 23 (24.5%) were concordant double-positive, whereas 40 (42.6%) were discordant, with 31 (32.9%) expressing only ZAP-70 and the remaining 9 (9.6%) exclusively CD38+.

When the median time from diagnosis to treatment for these patients was analyzed, it had not been reached for concordant double-negative patients, it was 60 months for all discordant patients, and was merely 16.2 months for concordant double-positive patients (*P*=.00001; Figure 4). Strikingly, the median times to treatment for patients with discordant ZAP-70+CD38- or ZAP-70-CD38+ phenotypes, respectively 49 and 99.4 months, did not differ significantly (*P*=.56).

The median times to treatment for patients with discordant ZAP-70+CD38- or ZAP-70-CD38+ phenotypes were 48.7 and 99.4 months, respectively. Similar results were obtained when an MFIR over 2, rather than at least 20% ZAP-70+ B-CLL cells, was combined with CD38 for the analyses of time to treatment onset (*P*=.0001; Figure 5).

Discussion

Initially, ZAP-70 mRNA expression was detected by means of reverse-transcriptase polymerase-chain reac-

Table 1. Comparison of CLL Patients' Clinical Parameters as a Function of the Percentage CD19+CD5+ Cells Expressing ZAP-70 and MFIR (n=94)

Parameter	Males*	Age,* years[1]	Binet Stage* A	Matutes* 3	Matutes* 4	Matutes* 5	Patients treated*	Diagnosis-treatment,* months[1]
Total, n (%)	57 (60.6)	59 [52–83]	82 (87.2)	10 (10.6)	23 (24.4)	61 (64.8)	42 (44.6)	30.4 [12.1–78.1]
ZAP-70, n (%)								
<20% (n=40)	22 (55)	58 [51–63]	40 (100)†	5 (12.5)	14 (35.0)	21 (52.5)	11 (27.5)†	64 [22.8–102.4]†
≥20% (n=54)	35 (64.8)	63 [55–67]	63 [55–67]	5 (9.2)	9 (16.6)	40 (74.0)	31 (57.4)	17.2 [6.3–41.5]
MFIR, n (%)								
<2 (n=29)	15 (51.7)	59 [50–67]	29 (100)‡	6 (20.6)	10 (34.4)	13 (44.8)	6 (20.6)†	23.3 [8.1–54.7]†
≥2 (n=62)	40 (64.5)	61 [55–66]	50 (80.6)	3 (4.8)	12 (19.3)	47 (75.8)	36 (58.0)	17.2 [14.7–29.4]

[1]Median (1st Qu.–3rd Qu.).

*As determined by c2 and Fisher exact tests; †*P*<.01; ‡*P*<.05.

MFIR=mean fluorescence intensity ratio.

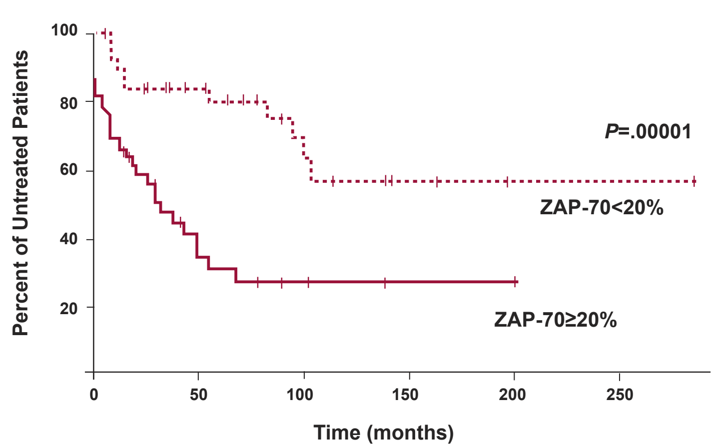

Figure 3. The relationship between the percentage of ZAP-70+ B-CLL cells and the time from diagnosis to initial therapy.

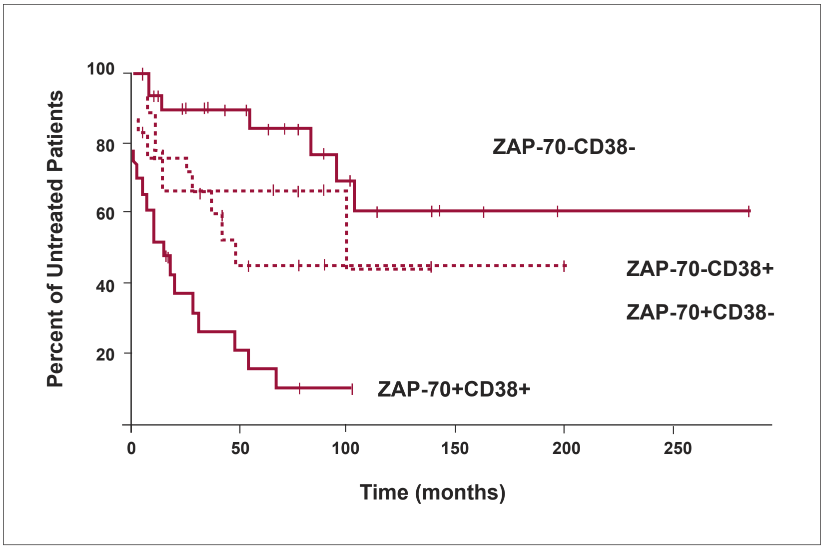

Figure 4. Analysis of the time from diagnosis to initial therapy as a function of combined ZAP-70 and CD38 expression. Positivity for both markers was defined as ≥20%.

tion[16,25] and ZAP-70 protein levels were visualized by western blotting or immunohistochemical labeling.[26] ZAP-70 expression as determined by flow cytometry was identified by several groups as a predictor of disease progression and overall survival of B-CLL patients.[14,15] Two main antibody clones to ZAP-70 have been used for cytofluorometry: 2F3.2 and 1E7.2. The use of indirect immunofluorescence was reported in most reference articles. However, monoclonal ZAP-70 antibodies directly conjugated to fluorochromes surpass the need for indirect immunofluorescence and current studies extend previous observations that direct immunofluorescence can be used for ZAP-70 determination.[15,27,28] This method presents the advantage of a simplified technique, which is also used in other staining procedures (eg, CD38). Herein we studied ZAP-70 expression using a conjugated monoclonal antibody 2F3.2 clone and assessed the percentages and MFIRs of labeled B-CLL cells. The gating strategy for defining ZAP-70 positivity is dependent on the methodology employed and the threshold for positivity may vary according to cell lineage. The cutoff was 20% for Crespo et al and Rassenti et al.[14,15] They compared, respectively, the expression of ZAP-70 B-CLL to T and NK cell populations and B lymphocytes. However, Crespo et al used an unconjugated monoclonal antibody clone and Rassenti et al used conjugated monoclonal antibody clone 1E7.2. Orchard and coworkers compared the expression of ZAP-70 B-CLL to isotype control by using unconjugated monoclonal antibody clone 2F3.2, and the threshold for ZAP-70 positivity was 10%.[16] Passam and associates used monoclonal antibody 1E7.2 clone conjugated with PE and Alexa fluorochromes. The cutoff for positivity varies regarding the use of fluorochrome, according to the receiver operating characteristic curves, and a cutoff ZAP-70-PE of 26% and a cutoff for ZAP-70-Alexa of 33% would have to apply for 100% specificity.[28] Herein, the cutoff for positivity was 20% in accord with Crespo et al and Rassenti et al.[14,15]

As with indirect immunolabeling, fresh or frozen mononuclear cells isolated from peripheral blood or bone marrow cells from B-CLL patients were used for direct immunolabeling in this study. This procedure shortens the incubation times and requires fewer washes, thereby making this method more amenable to routine use than indirect immunolabeling.[29]

In accordance with pioneering studies, the median times from diagnosis to initial therapy were shorter for ZAP-70+ than ZAP-70- patients when the threshold of ZAP-70 positivity was set at 20% or higher. Furthermore, linear regression analysis shows a significant association between the percentage of ZAP-70+ B-CLL cells and MFIR over 2 in this study. The comparison between the MFI of B-CLL cells (CD19+CD5+), normal B lymphocytes (CD19+), and T cells (from B-CLL patients and normal volunteers) has been used.[30] Moreover, Folarin et al analyzed the MFIR in B-CLL patients and found a correlation between that ratio and IgV_H mutational status.[31] In this study, the median times from diagnosis to initial therapy for our B-CLL patients with either at least 20% ZAP-70+ B-CLL cells or an MFIR of at least 2 were shorter than for those patients with less than 20% ZAP-70+ or an MFIR of less than 2.

In accordance with other studies, our investigation of the prognostic value of combining CD38 and

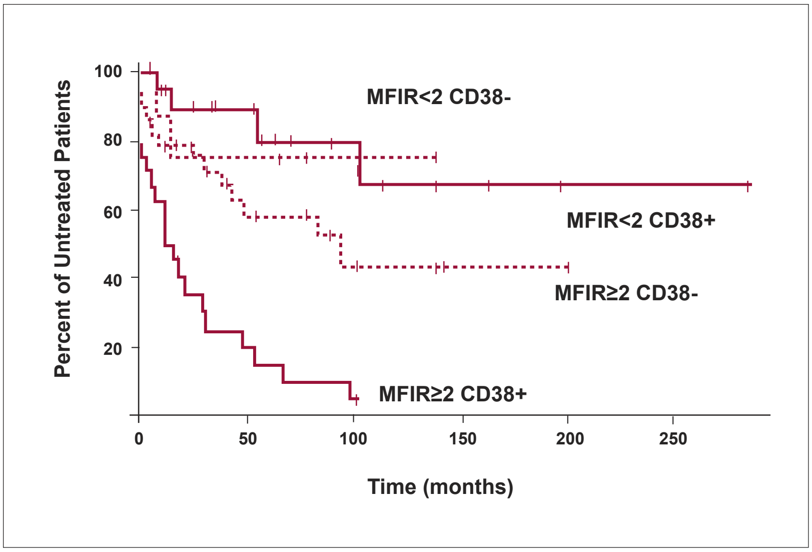

Figure 5. The relationship between the time from diagnosis to initial therapy as a function of MFIR and CD38 expression. CD38 positivity was set as ≥20%.

MFIR=mean fluorescence intensity ratio.

Table 2. Median Survival in Three Studies Based on ZAP-70 and CD38 Status

Study	Threshold of ZAP-70/CD38	Follow-up		*P* value
		ZAP-70+/CD38+	ZAP-70-/CD38-	
Del Giudice et al (n=197)[35]	20/7	TFS (months): 12	TFS (months): 54	<.00001
Schroers et al (n=252)[32]	20/30	TFS (months): 43	TFS (months): 130	<.001
Del Principe et al (n=256)[18]	20/30	OS at 16 yrs 18% PFS at 12 yrs 8%	OS at 16 yrs 94% PFS at 12 yrs 60%	<.001 <.001

OS=overall survival; PFS=progression-free survival; TFS=treatment-free survival.

ZAP-70 (using the ≥20% threshold to define positivity) expression showed median times from diagnosis to treatment for the concordant subgroups (double-negative or double-positive) to be respectively favorable and unfavorable. Both a shorter median treatment-free survival and decreased overall survival were observed in patients with ZAP-70+CD38+ status as compared with ZAP-70-CD38- status (Table 2). Furthermore, ZAP-70+CD38+ status was associated with shorter median treatment-free survival within Binet stage A cases as compared with ZAP-70-CD38- status (38 months vs not reached).[32] However, 42.5% of patients exhibited discordant CD38 and ZAP-70 expressions in our study compared to 29.0% for Schroers et al,[32] 17% for Dürig et al,[33] and 41.6% for Del Principe et al.[18] Discordant patients—ZAP-70+CD38-or ZAP-70-CD38+—showed an intermediate outcome. Furthermore, no significant association between ZAP-70/CD38–discordant patients and IgV_H mutational status was observed.[18] CD38 expression changes over time,[7,9] and the stability of ZAP-70 expression remains controversial.[14,32,33] Nevertheless ZAP-70 expression evaluation represents an acceptable surrogate for IgV_H mutation and can be easily immunolabeled simultaneously with other markers.

Identification of prognostic markers in addition to Binet stage is still a major concern for physicians caring for B-CLL patients. New treatments are able to obtain complete remissions[34] and the wait-and-watch policy may no longer be easy to propose to patients now that we know the probability of disease progression. In any case, incorporation of an easy test for ZAP-70/CD38 determination into routine immunophenotyping should help guide the clinician's decision-making.

References

1. Fais F, Ghiotto F, Hashimoto S, et al. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. *J Clin Invest*. 1998;102:1515-1525.
2. Maloum K, Pritsch O, Magnac C, et al. VH gene expression in CD5 positive and CD5 negative B cell chronic lymphoid malignancies. *Leuk Lymphoma*. 1997;24:437-448.
3. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood*. 1999;94:1848-1854.
4. Krober A, Seiler T, Benner A, et al. V(H) mutation status, CD38 expression level, genomic aberrations, and survival in chronic lymphocytic leukemia. *Blood*. 2002;100:1410-1416.
5. Oscier DG, Gardiner AC, Mould SJ, et al. Multivariate analysis of prognostic factors in CLL: clinical stage, IGVH gene mutational status, and loss or mutation of the p53 gene are independent prognostic factors. *Blood*. 2002;100:1177-1184.
6. Deaglio S, Vaisitti T, Bergui L, et al. CD38 and CD100 lead a network of surface receptors relaying positive signals for B-CLL growth and survival. *Blood*. 2005;105:3042-3050.
7. Durig J, Naschar M, Schmucker U, et al. CD38 expression is an important prognostic marker in chronic lymphocytic leukaemia. *Leukemia*. 2002;16:30-35.
8. Ibrahim S, Keating M, Do KA, et al. CD38 expression as an important prognostic factor in B-cell chronic lymphocytic leukemia. *Blood*. 2001;98:181-186.
9. Hamblin TJ, Orchard JA, Ibbotson RE, et al. CD38 expression and immunoglobulin variable region mutations are independent prognostic variables in chronic lymphocytic leukemia, but CD38 expression may vary during the course of the disease. *Blood*. 2002;99:1023-1029.
10. Rosenwald A, Alizadeh AA, Widhopf G, et al. Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. *J Exp Med*. 2001;194:1639-1647.
11. Chen L, Widhopf G, Huynh L, et al. Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. *Blood*. 2002;100:4609-4614.
12. Elder ME, Lin D, Clever J, et al. Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. *Science*. 1994;264:1596-1599.
13. Iwashima M, Irving BA, van Oers NS, Chan AC, Weiss A. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science*. 1994;263:1136-1139.
14. Crespo M, Bosch F, Villamor N, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. *N Engl J Med*. 2003;348:1764-1775.
15. Rassenti LZ, Huynh L, Toy TL, et al. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *N Engl J Med*. 2004;351:893-901.
16. Orchard JA, Ibbotson RE, Davis Z, et al. ZAP-70 expression and prognosis in chronic lymphocytic leukaemia. *Lancet*. 2004;363:105-111
17. Bakke AC, Purtzer Z, Leis J, Huang J. A robust ratio metric method for analysis of Zap-70 expression in chronic lymphocytic leukemia (CLL). *Cytometry B Clin Cytom*. 2006;70:227-234.
18. Del Principe MI, Del Poeta G, et al. Clinical significance of ZAP-70 protein expression in B-cell chronic lymphocytic leukemia. *Blood*. 2006;108:853-861.
19. Tinhofer I, Rubenzer G, Holler C, Hofstaetter E, Stoecher M, Egle A, Steurer M, Greil R. Expression levels of CD38 in T cells predict course of disease in male patients with B-chronic lymphocytic leukemia. *Blood*. 2006;108:2950-2956.
20. Matutes E, Owusu-Ankomah K, Morilla R, et al. The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. *Leukemia*. 1994;8:1640-1645.
21. Binet JL, Auquier A, Dighiero G, et al. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. *Cancer*. 1981;48:198-206.
22. Cheson BD, Bennett JM, Grever M, et al. National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. *Blood*. 1996;87:4990-4997.
23. Kaplan EL, Meier P. Non parametric estimation from incomplete observations. *Journal of the American Statistical Association*. 1958;53:457-481.
24. Cox D. Regression model and life tables. *J R Statist Soc B*. 1972;34:187-220.
25. Del Poeta G, Maurillo L, Venditti A, et al. Clinical significance of CD38 expression in chronic lymphocytic leukemia. *Blood*. 2001;98:2633-2639.
26. Wiestner A, Rosenwald A, Barry TS, et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. *Blood*. 2003;101:4944-4951.
27. Gibbs G, Bromidge T, Howe D, Hopkins J, Johnson S. Comparison of flow cytometric methods for the measurement of ZAP-70 expression in a routine diagnostic laboratory. *Clin Lab Haematol*. 2005;27:258-266.
28. Passam F, Tachynopoulou V, Skoumi D, et al. Feasibility of an easily applicable method of ZAP-70 measurement in chronic lymphocytic leukemia in the routine flow cytometry setting: a methodological approach. *Ann Hematol*. 2006;85:795-805.
29. Bojarska-Junak A, Rolinski J, Kawiak J. Modification of immunocytochemical ZAP-70 assay for potential clinical application in B-cell chronic lymphocytic leukemia. *Folia Histochem Cytobiol*. 2005;43:19-23.
30. Herishanu Y, Kay S, Rogowski O, et al. T-cell ZAP-70 overexpression in chronic lymphocytic leukemia (CLL) correlates with CLL cell ZAP-70 levels, clinical stage and disease progression. *Leukemia*. 2005;19:1289-1291
31. Folarin NI, Baker RJ, Duke V, et al. Detection of ZAP-70 by flow cytometry using the highly fluorescent alexa fluor dye in cells from patients with chronic lymphocytic leukaemia (CLL). *The Hematology Journal*. 2004;5:S23.
32. Schroers R, Griesinger F, Trumper L, et al. Combined analysis of ZAP-70 and CD38 expression as a predictor of disease progression in B-cell chronic lymphocytic leukemia. *Leukemia*. 2005;19:750-758.
33. Dürig J, Nuckel H, Cremer M, et al. ZAP-70 expression is a prognostic factor in chronic lymphocytic leukemia. *Leukemia*. 2003;17:2426-2434.
34. Keating MJ, O'Brien S, Albitar M, et al. Early results of a chemoimmunotherapy regimen of fludarabine, cyclophosphamide, and rituximab as initial therapy for chronic lymphocytic leukemia. *J Clin Oncol*. 2005;23:4079-4088.
35. Del Giudice I, Morilla A, Osuji N, et al. Zeta-chain associated protein 70 and CD38 combined predict the time to first treatment in patients with chronic lymphocytic leukemia. *Cancer*. 2005;104:2124-2132.