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Improved ZAP-70 Assay Using Two Clones and Multiple Methods of Analysis

Heba A. Degheidy, MD, PhD¹, David J. Venzon, PhD², Mohammed Z.H. Farooqui, D.O.³, Fatima Abbasi, MS, MPH¹, Diane C. Arthur, MD⁴, Adrian Wiestner, MD, PhD³, MA Stetler-Stevenson, MD, PhD⁴, and Gerald E. Marti, MD, PhD¹

¹Center for Biologics Evaluation and Research, FDA, Bethesda, MD, USA

²Biostatistics and Data Management Section, NCI, MD, USA

³NHLBI, NIH, Bethesda, MD, USA

⁴Laboratory of Pathology, NCI, Bethesda, MD, USA

Abstract

Introduction—In a companion methodological study, we compared two anti-ZAP-70 clones (1E7.2 AF 488 and SBZAP PE) and four selected methods of analysis. Clinical correlations are required for validation.

Methods—Multicolor flow-cytometric evaluation of ZAP-70, CD38, CD69, CD26, CD49d, and CD27 were tested in 45 untreated-CLL patients. Four methods of ZAP-70 expression analysis and a scoring system were designed. A correlation analysis between ZAP-70 score, IGHV mutational status, FISH, and these biomarkers was undertaken.

Results—There is a strong correlation between ZAP-70 expression and IGHV mutational status. The scoring system for a single reagent ($p=0.0006$ or 0.0002) favors the use of multiple methods of analysis. The combined score was substantially equivalent ($p=0.0003$). There was also a correlation with del 13q14 ($p=0.017$) and trisomy12 ($p=0.011$). A correlation for CD38 and ZAP-70 score was seen using both 1E7.2 AF488 and SBZAP PE when 20% or 7% cutoff were used. A positive correlation was seen for CD49d expression using both reagents. CD26 showed a correlation with ZAP-70 expression but it was dependent upon the method of analysis. CD69 and CD27 showed no statistically significant correlation.

Conclusion—In our study population, ZAP-70 expression is the better predictor of the IGHV mutational status. The correlation analysis confirms that the use of four methods of analysis with a single reagent or both reagents is superior to the use of a single method of analysis. The routine use of CD38, CD49d, and CD26 will require standardization.

Keywords

chronic lymphocytic leukemia; ZAP-70; CLL Score System; flow cytometry; prognostic marker

Introduction

Although clinical staging remains the basis for assessing prognosis in CLL (1, 2), many efforts have been made in the last decade to identify prognostic markers in CLL. This has

Correspondence to: Gerald E. Marti, MD, PhD, CDR (retired PHS), Cellular and Tissue Therapy Branch, Division of Cell and Gene Therapies, Office of Cellular, Tissues and Gene Therapies, CBER FDA NIH Bldg 29B Rm 1NN010, 8800 Rockville Pike, Bethesda, MD 20892, 301-827-0453, gemarti@mac.com.

led to a large number of reports describing the predictive value of different parameters with regard to overall survival, disease progression and response to therapy. Each of these markers could segregate patients into subgroups with different rate of disease progression.

The mutational status of immunoglobulin heavy chain variable region genes (IGHV) has been identified as a robust indicator for disease outcome. IGHV status separates CLL into two different variant forms of the disease (3, 4). Patients with IGHV un-mutated genes (U IGHV) have a shorter time to treatment than those with mutated IGHV genes (M IGHV) (3). Equally important, cytogenetic abnormalities identify groups of patients with different times to progression and survival. Broadly, three risk groups are recognized: 1) low-risk (del 13q14); 2) intermediate risk group (normal cytogenetics or trisomy 12); and 3) high-risk patients with del 17p, or del 11q. The prognostic significance of cytogenetic abnormalities and IGHV mutational status are clinical stage independent (5–9).

Zeta-chain-associated protein kinase 70 (ZAP-70) is a cytoplasmic tyrosine kinase linked T-cell receptor, initially believed to be restricted to the T and natural killer (NK) cells. Detection of ZAP-70 expression in CLL by flow cytometry was found to correlate with IGHV mutational status, and now is considered an independent prognostic marker for time to treatment in CLL (10). CD38 expression on leukemic lymphocytes was the one of first surface marker that was studied for its correlation with IGHV mutational status and discordance was noticed (9). ZAP-70 and CD38 may provide complementary prognostic information. Patients who express both markers would have a poor prognosis, while those in whom both of these markers are negative would have good outcome. An intermediate-risk category would consist of ZAP-70⁻, CD38⁺ individual (11–13).

In addition to ZAP-70 and CD38, several other prognostic biomarkers have been proposed. These include CD49d (14, 15), CD26 (15), CD69 (16), and CD27 (17). In the present study, we have combined these markers with ZAP-70, IGHV mutational status, and FISH. In a separate methodological paper, we compared two ZAP-70 clones and proposed a ZAP-70 scoring system. We now report the clinical significance of using two reagents and multiple methods of analysis.

Material & Methods

Patients

This study includes 45 untreated CLL patients with their median age 62.4, and 1.14:1 male:female ratio. Rai stages, Binet stage, lymphocyte doubling time (LDT), serum level for β_2 -microglobulin, serum Ig levels were determined from the clinical record (see Table 1). These patients were enrolled on NHLBI IRS approved clinical study registered with clinicaltrials.gov under identifier (NCT00923507), and under NCI study 97-C-0178 (clinicaltrials.gov identifier: NCT00019370). The diagnosis of CLL was made on the basis of clinical examination, as well as morphological and immunological criteria according to Hallek et al. (18). Anonymous normal donor blood samples were obtained from the NIH Department of Transfusion Medicine and used as controls.

Antibody Staining Panels for CLL samples

A seven-color flow cytometric panel was designed to analyze the expression of ZAP-70, CD38, CD49d, CD26, CD69, and CD27. Tubes (1–3) were performed to study CD38, CD69, CD26, CD49d, CD27; tube (4–9) was the panel used for ZAP-70 assessment including ZAP-70 normal donor control (4–5), isotype-matched antibody control (6–7), and patient sample (8–9). See Table 2 for panel used in this study.

Analysis and Staining for Cell Surface antigens

Surface staining for CD38, CD49d, CD26, CD69, CD27 were performed as following: 100 µl of washed whole blood (Phosphate Buffer Saline (PBS) x2) was added to pre-wetted tubes containing the designated antibody [See Table 1 (tube 1–3)]. The cells were stained for 30 minute at room temperature in the dark, lysed for 10 minute with 2 ml of (1x BD FACS™ Lysing Solution), then the cells were washed with PBS/Azide, and resuspended in 0.5 ml of 1% paraformaldehyde. Flowcytometric expression for the studied prognostic surface biomarkers were analyzed on the CLL clone (CD19+, CD5+, and CD20 dim). The expression data were reported as a percentage of positive cells using the following cutoff values: 7% and 20%, 30%, 10%, 30%, and 30% for CD 38, CD49d, CD26, CD69, and CD27 respectively.

Intracellular ZAP-70 Staining, Gating Strategy and Scoring

After cell surface staining with the designated antibodies [see Table-2 tubes (4–9)], The cells were fixed with 4% paraformaldehyde for 10 minutes, and permeabilized following the optimized saponin based method of Rassenti et al (10). The cells were stained with the manufacturer's recommended concentration of 1E7.2 AF 488, and SBZAP-PE or corresponding isotype control for 30 min at room temperature. After intracellular staining, cells were washed twice with 2 ml of cold 2% bovine serum albumin (BSA) in Duple phosphate buffered saline (DPBS), re-suspended in the same buffer and acquired immediately. Two other tubes were prepared; one stained using normal donor blood. [See Table 2 (tube 4–5)]; and the other stained with the corresponding isotypic control [Table 2 (tube 6–7)] and run in parallel with each patient sample. Nine different methods (M1–M9) were used for ZAP-70 expression analysis. Four methods were selected to design a scoring system (See the companion paper for ZAP-70 analysis and scoring).

All flow cytometric analyses were performed on a FACSCanto II (Becton Dickinson, CA, USA) flow cytometer. FACS Diva software was used for acquisition. Cytometry setup and tracking bead set (CST, BD) was used to initialize PMT settings. Individual compensation controls were prepared for each reagent using normal donor blood. In some experiments, rat anti-mouse kappa light chain Comp beads (BD) were used to set the compensation and were stained according to the manufacturer's instruction. The number of events collected in these experiments varied from 500, 000 to 1 million events. FlowJo software version 8.8.6 (Tree Star, Ashland, OR) was used for data analysis and display.

IGHV Gene Analysis

Amplification of the IGHV gene was performed as previously described by Wiestner et al., 2003 (19). In brief, 500 ng mRNA or 1 to 5 µg total RNA was used to generate oligo-dT primed cDNA using Superscript (Invitrogen, Carlsbad, CA). cDNA was amplified by polymerase chain reaction (PCR) using a mixture of 5' oligonucleotides specific for each leader sequence of the VH1 to VH7 IgVH families as forward primers and either a 3' oligonucleotide complementary to the consensus sequence of the joining region or the constant region of the IgM locus as reverse primers. PCR was performed in 50 µl reactions with Taq polymerase (Sigma, St Louis, MO) and 20 pmol of each primer. Cycling conditions were 94° C for 30 seconds, 60° C for 20 seconds, and 72° C for 30 seconds for up to 35 cycles. Products were purified (MinElute PCR Purification kit; Qiagen, Valencia, CA) and sequenced directly with the appropriate 3' oligonucleotide using Big Dye terminator and analyzed using automated DNA sequencer (Applied Biosystems, Foster City, CA). Nucleotide sequences were aligned to the V-Base sequence directory (<http://www.mrc-cpe.cam.ac.uk>). Percentage of homology was calculated by counting the number of mutations between the 5' end of FR1 and the 3' end of FR3. Sequences with 2% or less deviation from any germ line IGHV sequence were considered un-mutated.

Interphase fluorescence in-situ hybridization (FISH)

Interphase FISH was performed as previously described (20). Briefly, buffy coat cells from fresh, heparinized peripheral blood were cultured in duplicate overnight without mitogens and for 96 hours with B-cell mitogens. Cells were harvested and fixed in 3:1 methanol:glacial acetic acid. Fresh slides were made from the fixed cell pellets and hybridized with commercially available probes (Abbott Molecular, Inc., formerly Vysis, Downers Grove, IL) to detect deletions in 13q14.3 (D13S319, D13S25), 11q23 (MLL) or 11q22.3 (ATM), and 17p13.1 (TP53); and to detect trisomy 12 (CEP 12 DNA). Minimums of 200 interphase nuclei were scored for hybridization signals for each probe.

Statistical analysis

The association between IGHV mutational status and ZAP-70 expression levels by individual methods were assessed using Fisher's exact test, and trends across combined CLL score categories (negative, equivocal and positive) were tested by the exact Cochran-Armitage method. Associations between the positivity of biomarkers and ZAP-70 measures employed the same methods. Receiver operating characteristic (ROC) analysis was also conducted (21). For the ROC analysis, the sensitivity (ratio of positive tests to all positive patients) was plotted against the specificity (ratio of negative tests to all negative patients) where the cutoff between negative and positive tests takes successively each value of a measure from lowest to highest.

Results

Patient Demographics

This study involved 45 untreated CLL patients who were evaluated at the time of diagnosis or during the subsequent years before treatment. These patients were 24 males and 21 females with their age range of 47–81 years (median 62.4). The median WBCs of 24.6×10^9 , ALC of 19.9×10^9 , Hg of 12.9 g/dl, platelet of 164×10^9 , and mean LDT of 36.6 months. The medians of the serum Ig levels were 636 mg/dl, 85 mg/dl, and 33 mg/dl for IgG, IgA, and IgM respectively. In all, 28 were Rai Stage A, 14 Rai Stage B & C, and 3 not determined; 24 had elevated levels (2.2 g/l or higher) serum β 2-microglobulin. The majority of the patients had early or intermediate stage of disease (Binet A+B, 93%, Rai 0+I+II, 93%). See Table 1 for the Summary of patient's demographic characteristic.

Association between CLL ZAP-70 scoring and IGHV mutational status

The results of ZAP-70 expression values obtained by the four methods of analysis and ZAP-70 score are reported in a companion paper (Degheidy et al, Clinical Cytometry part B, in press). The significance levels of the correlations between IGHV mutational status and each of the four selected methods of analysis were as follows: $p=0.016$, 0.018 , 0.018 , and 0.0010 for M1, M3, M7, and M9 respectively using the 1E7.2 clone. Whereas for the SBZAP clone, the p values were as follows: 0.0010 , 0.0039 , 0.016 , and 0.0010 for M1, M3, M7, and M9 respectively. When ZAP-70 was scored for each clone separately, the p value obtained was 0.0006 for 1E7.2/AF488 and 0.0002 for SBZAP/PE clone. The correlation between the combined ZAP-70 score and IGHV mutational status for both reagents is essentially the same ($p=0.0003$), thereby supporting the use of either reagent alone or both reagent together. [See Table 3].

To investigate these associations without the cutoffs recommended in the literature, we constructed ROC curves for each of the methods of analysis of ZAP-70 expression using the two clones separately (see Fig. 1). It is evident that good discrimination is possible with these methods, although optimal cutoffs derived from this study population might be imprecise and not generally applicable. Since the area under the ROC curve quantifies the

degree of discrimination, the SBZAP clone is slightly more effective for methods M1 and M3, and the 1E7.2 clone is slightly more effective for methods M7 and M9, but these differences are not significant.

Seven CLL samples were U-IGHV: six showed a combined positive score P/P and one showed a combined P/E score and was scored as positive. On the other hand, 14 had a M-IGHV gene in their CLL cells. Of the 14 cases 11 were scored negative [N/N (9 cases) or E/N (2 cases)] with the combined ZAP-70 score. Discordant results were observed in 2 cases (1 case showed P/P combined score and the other case showed P/E score and was considered positive with the combined score). And only one case (7%) was scored equivocal (E/E). See Table 4.

CLL ZAP-70 Score and Cytogenetic Abnormalities

Forty of the 45 patients were tested for cytogenetic abnormalities. Overall, 23 cases were found to have 13q14 deletion. Five of these cases, however, had additional FISH abnormalities including three with 11q deletion and two with 17p deletion. Four of these cases had less than 15% of the unfavorable deletion in their CLL clone and were ZAP-70 negative. The fifth had 95% 13q14 deletion and 95% 11q deletion and showed bimodal ZAP-70 expression with a positive ZAP-70 combined score.

Eighteen CLL cases thus had del 13q14 as a sole abnormality in their CLL clone. The correlation between del 13q14 as a sole abnormality and ZAP-70 expression was tested for each of the four selected methods of analysis separately and the p values were as follow: 0.013, 0.010, 0.54, and 0.073 for M1, M3, M7, and M9 respectively using the 1E7.2 clone. Whereas for the SBZAP clone, the p values were: 0.0043, 0.20, 0.33, and 0.20 for M1, M3, M7, and M9 respectively. When ZAP-70 was scored for each clone separately, the p value obtained was 0.027 for 1E7.2 and 0.0048 for SBZAP clone. For the combined ZAP-70 score using both reagents the p value was 0.017. See Table 3.

Of these 18 cases with del 13q14 as a sole abnormality, 17 cases were scored negative with the combined 1E7.2/SBZAP score [N/N (14 cases), E/N (2 cases), and N/E (1 case)]. The remaining case was scored combined positive score [P/E]. See Table 4.

Regarding trisomy 12, the correlation with ZAP-70 expression showed the following p values 0.015, 0.0036, 0.43, and 0.024 for M1, M3, M7, M9 respectively using the 1E7.2 clone. Whereas for the SBZAP clone, the p values were: 0.0048, 0.042, 0.44, and 0.082 for M1, M3, M7, and M9 respectively. When ZAP-70 expression was scored in each clone separately, the p value obtained was 0.018 for 1E7.2 and 0.0073 for SBZAP clone. For the combined ZAP-70 score the p value was 0.011. See Table 3.

Eight cases had trisomy 12. Five of these eight showed positive combined score [P/P (4 cases), and P/E (one case)] and three showed combined negative [N/N (2 cases), and N/E (one case)]. See Table 4.

ZAP-70 Score and CD38, CD49d, CD26, CD69, CD27

We examined the immunophenotypic expression of CD38, CD49d, CD26, CD69, and CD27 on CLL cells (see Fig. 2) and correlated the expression of these prognostic biomarkers with the CLL ZAP-70 score. Two threshold values were used to assess CD38 positivity: 20% and 7% positive cells. The correlation between ZAP-70 expression by the four methods of analysis and CD38 using the 20 % cutoff revealed that CD38 positivity was associated with only the M3 method of analysis using the 1E7.2 clone ($p = 0.0041$); the other three methods of analysis were not statistically significant ($p > 0.1$). For the SBZAP clone, M1 showed a statistically significant correlation with CD38 ($p = 0.013$), while the other three

methods showed no correlation ($p > 0.28$). However, using the 7% cutoff, the following p values were obtained: 0.0098, 0.0009, 0.071, and 0.017 using 1E7.2 clone. For the SBZAP clone, only M1 achieved statistical significance ($p=0.0017$), while the other three methods did not (See Table 3 for p values). The 1E7.2 score showed a significant correlation ($p=0.0067$), as did the SBZAP score ($p=0.0090$). Marginal statistical significance was observed between CD38 expression and the combined ZAP-70 score using 20% or 7% cutoff ($p=0.039$, and 0.012 respectively). See Table 3.

We found that CD49d achieved a statistically significant correlation with the ZAP-70 expression for the 1E7.2 score, the SBZAP score, and combined 1E7.2/SBZAP score with p values of 0.0025 and 0.0053, and 0.0050 respectively. (See Table 3 for p values for individual methods of analysis). Regarding the correlation between CD26 and the ZAP-70 score, CD26 showed a correlation with ZAP-70 expression, but it was dependent upon either the M1 method of analysis or the individual reagent score. No statistically significant correlation was observed between ZAP-70 expression and CD69 or ZAP-70 expression and CD27 using either single individual method or the score method (see Table 3).

Discussion

Two Anti-ZAP-70 clones (1E7.2 and SBZAP) were examined in this study in the context of IGHV mutational status, cytogenetic, and five biomarkers in 45 untreated CLL patients. ZAP-70 expression was assessed on (CD20 dim, CD19+, and CD5+) CLL cells using four methods of analysis (M1, M3, M7, and M9, see the companion paper for details). A CLL ZAP-70 scoring system that integrated these four methods of analysis for each individual reagent as well as a combined score for both reagents was used to further clarify the assignment of ZAP-70 expression. Five other prognostic biomarkers were studied including CD38, CD49d, CD26, CD69, and CD27. Analysis of all these biomarkers was performed blindly without knowledge of clinical, molecular or cytogenetic data using fresh heparinized whole blood.

Previous reports showed that there was a difference in ZAP-70 evaluation using various methods of analysis even if the same clone was used (22, 23). Moreover, other studies observed a difference in the percentage of CLL cells stained positive for ZAP-70 when different monoclonal antibodies were used even when the same gating strategy was used on the same CLL population (24). This raised the idea that a scoring system combining four methods of analysis (including percentage positive, and ratio metric methods), and using two clones may resolve the assignment of ZAP-70 expression. If one clone was unable to clearly assess ZAP-70 expression by four methods, the addition of a second clone might aid in this assessment. And even if the ZAP-70 expression is positive by three or four methods of analysis, the confirmation by a second reagent enhances the analytic certitude. In our companion paper, we report the basis of how these two clones, and the four methods of ZAP-70 analysis were selected. In this paper, we correlated the proposed scoring system with the Ig gene mutational status, interphase FISH cytogenetics and other prognostic biomarkers.

In the present study, IGHV mutational status was available for 21 patients. Seven cases were U-IGHV and 14 cases were M-IGHV. Using the combined 1E7.2/SBZAP score there was no discordant results for the seven U-IGHV samples as all were scored positive (six cases showed P/P score, and one case showed P/E score). For the 14 M-IGHV, two samples were discordant based on the positive combined 1E7.2/SBZAP score. And only one remaining case (7%) showed E/E score. Others have noted discordant results particularly when mutated IGHV cells express ZAP-70. ZAP-70 expression takes president in terms of clinical prognosis (25). Multiple studies have found that ZAP-70 expression correlates with IGHV

mutational status (26, 27). Our study confirms this correlation. In addition, we found that using the combined ZAP-70 score integrating both clones validated the use of a single ZAP-70 score for each clone separately (See Table 3). And the use of four methods of analysis was superior to any single method of analysis for either reagent.

It is well established that trisomy 12 and del 11q are associated with a poor prognosis in CLL, whereas del 13q14 is associated with good prognosis disease (28). In our study population, we observed a significant correlation between 13q14 deletion as a sole abnormality and low ZAP-70 expression, and between trisomy 12 and high ZAP-70 expression. Even though there were insufficient numbers of del 11q cases in our study, the non-del 13q14 abnormality, trisomy 12 was associated with ZAP-70 expression.

Regarding CD38 and ZAP-70 expression, a highly statistically significant correlation was observed using the M3 method and a cutoff 20% when 1E7.2 clone was used. Rassenti et al. reported a similar observation previously using the same clone and the same method (M3) of ZAP-70 expression analysis (27). M1 and M3 were statistically significant when 7% was used as a cut-off for CD38 positivity using the 1E7.2 clone. These data confirm that using 7% cut-off is important for identification of early CLL patients with more aggressive disease as previously reported by Gentile et al. 2005 (29). The SBZAP clone also supported the more discriminate use of 7% cut-off (See Table 3). Others also observed discordant results between CD38 and ZAP-70 depending on method of analysis and the percent of cutoff (30). Not all patients in this study were tested at the time of diagnosis. Others have suggested that CD38 expression may change with disease progression or evolution (31). Regardless, these observations concerning the method of analysis and difference in clones, urge the standardization of this important biomarker in CLL.

From a technical standpoint the pattern of CD49d and CD26 expression would seem to warrant further investigation as prognostic markers. It will be of interest to see how they complement the pattern of CD38 expression. Our data does not support the use of either CD69 or CD27 as a surrogate biomarker for ZAP-70 as these three markers do not show a significant correlation to ZAP-70 expression. This does not rule out the correlation with other clinical markers.

During the course of this study, it was noted that either reagent was useful in detecting or distinguishing inter- and intra-clonal heterogeneity (data not shown). Also in this study the use of a normal donor as an external control achieved significant importance. This approach could be applied to other biomarkers such as CD38, and CD49d. Given the complexity of analysis and the use of two reagents in this study, effort should be made to simplify this assay. Combining the normal donor and patient sample might be the next step in the development of this assay (32).

In conclusion, our results suggest that integrating four methods for ZAP-70 assessment improve its correlation with the two major primary prognostic CLL biomarkers: IGHV, and interphase FISH abnormalities. It is important to note that even in using one reagent, several methods of analysis, i.e. isotype method or normal donor (ND) T-cell as a reference control, could be used to improve ZAP-70 expression assignment. The combined ZAP-70 score using two reagents permitted the assignment and resolution of equivocal cases that could not be solved using one reagent. The simultaneous estimation of ZAP-70, CD38, CD49d, CD26 and expression might distinguish different CLL subgroups in term of disease progression or chemotherapy requirement and this combined evaluation may confirm prognosis if one predictive factor is borderline.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Receiver operating characteristic (ROC) analysis of M1, M3, M7, and M9 for 1E7.2 (plotting symbol A) and SBZAP (plotting symbol B).

Figure 2. Representative two parameter histograms for prognostic biomarkers

In all examples, gating has been carried out on CD19+ve, CD20 dim, CD5+ve cells. CD23 was conjugated with FITC; CD38, CD49d, and CD26 were conjugated with PE; CD69 was conjugated with APC; CD27 was conjugated with APC-Alexa FluorR 750 (APC Cy7).

Table 1

Demographic characteristics and clinical data for the 45 CLL cases

Features	Total
Age: mean 62.4 (47–81)	45
Sex:	
Male	24 (53%)
Female	21 (47%)
M/F ratio: 1.14/1	
Race:	
White	41(91%)
Other	4(9%)
Binet stage:	
A	28(67%)
B	11(26%)
C	3 (7%)
Unknown	3
Rai stage:	
0	7(17%)
1	19 (45%)
2	13 (31%)
3+4	3 (7%)
Unknown	3
$\beta 2$-microglobulin: 2.57 g/l (1.1–6.5 g/l)	
Lower than 2.2 g/l	21(47%)
2.2 g/l or higher	24 (53%)
LDT: 36.6 months (4.8–140.6)	
Less than 12 months	9 (26%)
12 months or greater	25 (74%)
Unknown	11
WBC ($\times 10^9$): median 24.6 (1.81–245.7)	
$< 30 \times 10^9$	25 (56%)
$> 30 \times 10^9$	20 (44%)
ALC ($\times 10^9$): median 19.9 (0.05–236)	
Hg (g/dl): median 12.9 g/dl (8.9–16.6)	
Plt ($\times 10^9$): median 164 (68–447)	
Serum Ig:	
IgG (mg/dl): median 636 mg/dl (<140–1220mg/dl)	
IgA (mg/dl): median 85 mg/dl (<10–350mg/dl)	
IgM (mg/dl): median 33 mg/dl (<21–2680 mg/dl)	

Table 2

Panel Used for Intracellular ZAP-70 Staining and CLL Prognostic Markers

Tube #	FITC	PE	Per CP	APC	PE Cy7	APC Cy7	Pacific blue
#1	CD23	CD38	CD19	CD69	CD5	CD27	CD20 *
#2	CD23	CD49d	CD19	CD69	CD5	CD27	CD20 *
#3	CD23	CD26	CD19	CD69	CD5	CD27	CD20 *
#4	1E7.2		CD19	-----	CD5	CD3	CD20 *
#5		SBZAP	CD19	-----	CD5	CD3	CD20 *
#6	Isotype control	-----	CD19	-----	CD5	CD3	CD20 *
#7	-----	Isotype control	CD19	-----	CD5	CD3	CD20 *
#8	1E7.2	-----	CD19	-----	CD5	CD3	CD20 *
#9	-----	SBZAP	CD19	-----	CD5	CD3	CD20 *

* CD20 e Fluor 450. **Reagents.** The 1E7.2 clone and its isotype matched IgG1 control were obtained from Invitrogen (1E7.2 AF488 Camarillo, California). The SBZAP clone conjugated with phycoerythrin (PE) and its isotype matched control IgG1-PE were obtained from Beckman Coulter, Inc. (SBPE, Fullerton CA). Saponin, was purchased from (Sigma, St. Louis, MO). CD20-eFluor 450 was obtained from eBiosciences (San Diego, CA). BD FACS™ Lysing Solution, CD49d-PE, CD19-PerCPy 5.5, CD5 PE-Cy7, CD23 FITC, CD26-PE, CD23 APC-Cy7 were obtained from BD Biosciences (San Diego, USA). CD38-PE, CD69-APC, CD27- APC-Alexa FluorR 750, and CD23-APC were obtained from Invitrogen (Camarillo, California). Tubes (1–3) were performed to study CD38, CD69, CD26, CD49d, CD27; tube (4–9) was the panel used for ZAP-70 assessment including ZAP-70 normal donor control (4–5), isotype-matched antibody control (6–7), and patient sample (8–9). The unstained control tubes and a single stained tube for each fluorochrome were not included in the table.

Table 3

Correlation between four methods of ZAP-70 expression analysis, ZAP-70 score with other CLL prognostic biomarkers

		IGHV	del 13q14 only	Trisomy12	CD38 20%	CD38 7%	CD49d	CD69	CD26
1E7.2	M1	0.016	0.013	0.015	0.19	0.0098	0.017	0.46	0.0011
	M3	0.018	0.010	0.0036	0.0041	0.0009	0.0006	0.065	0.096
	M7	0.018	0.54	0.43	0.72	0.071	0.14	0.53	0.38
	M9	0.0010	0.073	0.024	0.10	0.017	0.0062	0.30	0.035
	1E7.2 ZAP score	0.0006	0.027	0.018	0.057	0.0067	0.0025	0.27	0.0045
SBZAB	M1	0.0010	0.0043	0.0048	0.013	0.0017	0.0062	0.30	0.0033
	M3	0.0039	0.20	0.042	0.28	0.071	0.14	0.53	0.069
	M7	0.016	0.33	0.44	0.46	0.12	0.050	0.20	0.65
	M9	0.0010	0.20	0.082	0.64	0.41	0.13	0.23	0.23
	SB ZAP score	0.0002	0.0048	0.0073	0.045	0.0090	0.0053	0.18	0.014
Combined	1E7.2/SBZAP	0.0003	0.017	0.011	0.039	0.012	0.0050	0.43	0.012

M1, isotype control to determine negative; M3, normal donor (ND) T-cell to determine positive; M7, clone/ND B- cell ratio; M9, modified CLL-Z score.

Table 4

Correlation of CLL Combined ZAP-70 Score with IGHV, and FISH

CLL score					P value
	Negative	Equivocal	Positive	Total	
IGHV					
M	9	4	1	14	0.0003
U	0	1	6	7	
13q14 deletion					
Present	14	4	0	18	0.017
Non/complex*	11	3	8	22	
Trisomy 12					
Absent	23	5	4	32	0.011
Present	2	2	4	8	

Negative group (N/N): scored negative with both clone and showed score of 0 or 1 with each one. Equivocal group: showed equivocal results with one of the two clone with a score of 2. Positive group: (P/P): scored positive with both clone and showed score of 3 or 4 with each one.

* Non means no 13q14 deletion, while complex means 13q14 deletion plus other cytogenetic abnormality. Infact, there are 5 cases with complex cytogenetic abnormalities (see Table. 1s in the supplementary data).