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Evaluation of ZAP-70 Expression by Flow Cytometry in Chronic Lymphocytic Leukemia: A Multicentric International Harmonization Process

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The clinical course of patients with chronic lymphocytic leukemia (CLL) is heterogeneous with some patients requiring early therapy whereas others will not be treated for years. The evaluation of an individual CLL patient's prognosis remains a problematic issue. The presence or absence of somatic mutations in the IgVH genes is currently the gold-standard prognostic factor, but this technique is labor intensive and costly. Genomic studies uncovered that 70 kDa zeta-associated protein (ZAP-70) expression was associated with unmutated IgVH genes and ZAP-70 protein expression was proposed as a surrogate for somatic mutational status. Among the available techniques for ZAP-70 detection, flow cytometry is most preferable as it allows the simultaneous quantification of ZAP-70 protein expression levels in CLL cells

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and residual normal lymphocyte subsets. However, several factors introduce variability in the results reported from different laboratories; these factors include the anti-ZAP-70 antibody clone and conjugate, the staining procedure, the gating strategy, and the method of reporting the results. The need for standardization of the approach led to the organization of an international working group focused on harmonizing all aspects of the technique. During this workshop, a technical consensus was reached on the methods for cell permeabilization and immunophenotyping procedures. An assay was then designed that allowed comparison of two clones of anti-ZAP-70 antibody and the identification of the expression of this molecule in B, T, and NK cells identified in a four multicolor analysis. This procedure was applied to three stabilized blood samples, provided by the UK NEQAS group to all participating members of this study, in order to minimize variability caused by sample storage and shipment. Analysis was performed in 20 laboratories providing interpretable data from 14 centers. Various gating strategies were used and the ZAP-70 levels were expressed as percentage positive (POS) relative to isotype control or normal B-cells or normal T-cells; in addition the levels were reported as a ratio of expression in CLL cells relative to T-cells. The reported level of ZAP-70 expression varied greatly depending on the antibody and the method used to express the results. The CLL/T-cell ZAP-70 expression ratio showed a much lower interlaboratory variation than other reporting strategies and is recommended for multicenter studies. Stabilization results in decreased expression of CD19 making gating more difficult and therefore stabilized samples are not optimal for multicentric analysis of ZAP-70 expression. We assessed the variation of ZAP-70 expression levels in fresh cells according to storage time, which demonstrated that ZAP-70 is labile but sufficiently stable to allow comparison using fresh samples distributed between labs in Europe. These studies have demonstrated progress toward a consensus reporting procedure, and further work is underway to harmonize the preparation and analysis procedures. © 2006 International Society for Analytical Cytology

Key terms: ZAP-70; flow cytometry; CLL; harmonization

The clinical course of patients with CLL is heterogeneous. Some patients have aggressive disease requiring therapy within a relatively short time after diagnosis, while others have indolent, asymptomatic disease that might not require therapy for many years. Because of the difficulty in identifying such patients at diagnosis, only patients with progressive and/or symptomatic disease currently are recommended for therapy despite the advent of new treatment modalities that might be more effective in patients with low tumor burden. Although easily characterized by diagnostic markers, the evaluation of an individual CLL patient's prognosis remains a problematic issue. There is a crucial need for reliable prognostic factors in a disorder with such an heterogeneity as B-CLL, and since 1999, the complex and time consuming determination of the mutational status of the variable genes of the immunoglobulin heavy chains (IgVH) has been considered as the gold standard prognostic factor (1,2). Since the discovery of its expression in CLL by microarray (3), 70 kDa zeta-associated protein (ZAP-70) expression has been the focus of great interest. Besides its importance for a better understanding of the disease, ZAP-70 has appeared of a promising prognostic factor. As ZAP-70 was identified by comparison of CLL cases with or without IgVH mutations, it was first considered as a potential surrogate for IgVH mutational status (4,5). When data on ZAP-70 accumulated, the concordance with mutational status decreased to around 75–80% (6), though ZAP-70 expression maintained its independent prognostic significance (7–9). It is therefore important to develop a reliable and reproducible routine test for detecting expression of ZAP-70.

DETECTION OF ZAP-70 EXPRESSION AT THE PROTEIN LEVEL AND POTENTIAL PROBLEMS

As protein is the effector, determination of protein expression is usually considered as the level of choice. This may be achieved either by flow cytometry, immunohistochemistry, cytochemistry, or Western blotting.

When antibodies are available, flow cytometry is always an attractive option since this technique is readily accessible in most routine diagnostic laboratories, and is currently used for CLL diagnostic procedures, and therefore the introduction of this prognosis parameter in the diagnostic panel would be easy. Flow cytometry allows the simultaneous evaluation of ZAP-70 protein expression levels in CLL cells and residual normal lymphocyte subsets. This cellular discrimination is particularly important in the analysis of ZAP-70 because this protein is a well-known essential tyrosine kinase in T-lymphocytes (10,11). Its strong expression in T and NK cells implicates that any technique used for ZAP-70 determination must be either able to either identify the ZAP-70 expression related to T and NK cells or conducted on purified B cells.

ZAP-70 expression has been considered for a long time as restricted to T cell settings, and absent in B cell lineage. However, it has recently been shown to be present at early stages of B cell development (12). In addition, other reports show its expression in mature B cells (13) after activation or in subpopulations from tonsil and spleen (12,14). ZAP-70 expression in circulating normal mature B-lymphocytes may be also detected (13), though it remains controversial.

ZAP-70 is a member of the syk tyrosine kinase family, and its protein sequence is closely related to syk (15). The generation of antibodies able to recognize ZAP-70 sequence and not syk has proven to be difficult. The staining procedure, the antibody used to detect ZAP-70, and the interpretation of the results remain still controversial. The staining procedure requires a permeabilization step due to the intracellular localization of ZAP-70. However, the method employed to permeabilize cells differs between groups (5,8,16). Several antibodies adapted to flow cytometry are commercially available. The previously published reports used two different anti-ZAP-70 antibodies (2F3.2 and 1E7.2), unconjugated or conjugated with different fluorochromes. ZAP-70 positivity in the CLL clone was assessed using various gating strategies and a different threshold for positivity: 20% relative to T-lymphocytes (5,17), 10% relative to isotype control (16), 20% relative to B-lymphocytes (8). As the intensity of expression of ZAP-70 is highly variable among CLL samples, defining the threshold between negative (NEG) and positive (POS) cases is challenging. Therefore, standardization and validation are warranted before a flow cytometry technique can be generally adopted in routine laboratories.

CONSENSUS MEETING

This led to the organization of an international working group in an attempt to harmonize the technique. Many technical issues were considered during the initial workshop, including: the nature of the sample (i.e., fresh vs. stabilized, peripheral blood vs. bone marrow); the fixation and permeabilization procedure; the choice of antibody clone and fluorochrome; the specificity of the secondary antibody; the combination of surface markers for the identification of the cell populations; the gating strategy; and the method of expressing of the results. An assay was then designed that allowed comparison of two clones of anti-ZAP-70 antibody.

A technical consensus was reached on the use of Fix& Perm (Caltag Laboratories) kit for the fixation and permeabilization steps in order to achieve homogeneity among laboratories and not precluding the use of other commercial reagents after harmonization. Immunophenotyping procedures were designed to identify B, T, and NK cells based on the expression of specific surface markers. The combination was defined for a four-color flow cytometer as shown in Table 1 and used antibodies of IgG1 isotype.

Concerning the anti-ZAP-70 antibodies, as the quality of the conjugation of antibody and fluorochrome is critical, some were not taken into account based on previous results obtained by some members of this workshop. On the basis of the published data and previous French national collaborative effort (iwCLL 2005), two clones were selected: the 1E7.2 and 2F3.2. The 1E7.2 Alexa fluor 488 conjugated (IgG1) was kindly provided by Caltag Laboratories and the 2F3.2 (IgG2a) was purchased from UpState and used with indirect FITC labeling. To allow membrane staining prior to fixation and permeabilization steps, the indirect procedure used a specific anti-IgG2a secondary antibody (Southern Biotechnologies).

Table 1
Antibodies and Fluorochromes

Surface markers		Intracytoplasmic marker: FL1
FL3	CD19 PerCP/ PE-Cy5.5	1E7.2 Alexa fluor 488 conjugated from Caltag Laboratories
FL4	CD5 APC	2F3.2 unconjugated from Upstate (indirect labeling) with a FITC conjugated goat anti mouse IgG2a secondary antibody
FL2	CD3, CD56, CD16 all PE	

The issue of the sample on which the first trial should be carried on was discussed. As the problem of conservation while shipping arose, it was decided to use stabilized blood samples prepared by UK NEQAS in order to avoid cell variability and shipment difficulties. For the first step of the study, three samples were selected based on their characteristic pattern of ZAP-70 expression: one normal blood sample and two CLL cases, one ZAP-70 POS (>60%), one clearly NEG (<10%). Upon receiving the sample, each laboratory stored them according the UK NEQAS recommendations.

Finally, detailed procedures for the gating of the different cellular subsets and the mode of expression of the results (as detailed below) were established in order to reduce interlaboratory variability.

RESULTS

General Data

The procedure described earlier was applied to the three stabilized blood samples. 21 experienced laboratories from 10 European countries, USA, and Canada participated in this study. Analysis was performed in 20 laboratories providing results for direct comparison. After centralized review of the graphs, the analysis of the results showed informative data for 14 centers. Conversely, in six experienced laboratories technical difficulties resulted in the absence of interpretable data. In most of these six cases, the samples were unsuitable for ZAP-70 evaluation primarily because of impaired membrane marker detection caused by stabilization.

Optimal Mode of Expression of the Results

In 14 centers, discriminating lymphocyte subsets on stabilized blood samples proved possible although B cell marker (CD19) appeared more sensitive to stabilization procedure as compared to T/NK-cell markers (CD3, CD5, CD56) as shown in Figure 1. Moreover, Zap-70 expression, even in the T cells, was significantly reduced compared with the expected values in fresh blood. Various gating strategies were used and the results were expressed according to four different methods (Table 2) exemplified on Figure 2.

Concerning the expression of the results (Table 3), it appeared that expression levels derived using a threshold set on isotype control (%/CTRL) or normal B cells

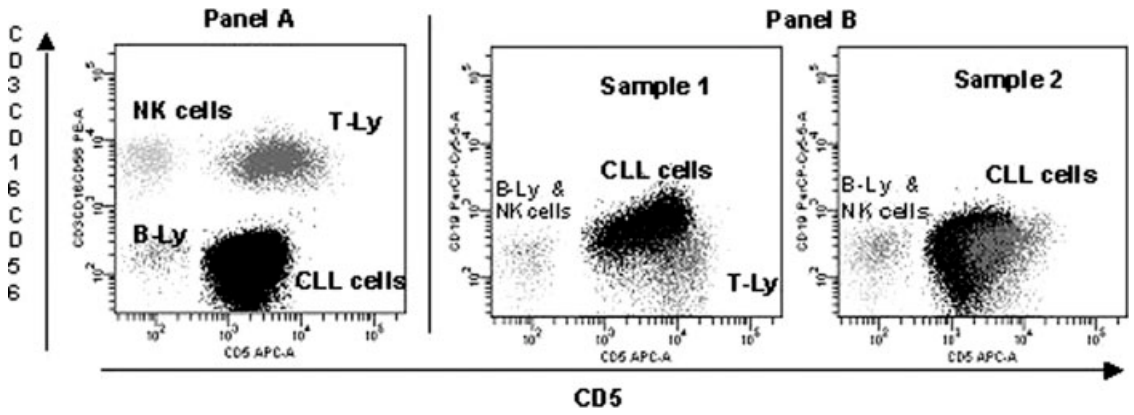

FIG. 1. Panel **A** shows conserved expression of T-cell markers exemplified on Sample 2. Panel **B** shows altered expression of CD19 on CLL cells particularly on Sample 2.

(%/B-Ly) were less reproducible with broad CV and showed a high rate of false NEG results. Regarding Method 4, the enlargement of CVs observed in the ZAP-70 NEG sample probably reflects the difficulty in setting the threshold on ZAP-70 expression in T-lymphocytes. As expected, MFI raw data were inadequate for interlaboratory comparison because of the use of different types of flow cytometer and acquisition software. The most accurate and reproducible parameter appeared to be the mean fluorescence intensity (MFI) ratio (T-lymphocyte MFI of ZAP-70/ CLL MFI of ZAP-70). This method showed the best CVs in either POS or NEG CLL samples.

Comparison of the Two Clones: 1E7.2 Versus 2F3.2

In this study, besides the evaluation of the most accurate mode of expression of the results, we also aimed to compare two different clones of anti ZAP-70 antibody. Comparison between the two antibodies was available in 8/14 centers. When examining ZAP-70 expression in residual T-lymphocytes, the direct technique showed a low mean percentage of ZAP-70 expressing T cells relative to isotype control (45%, SD = 23) with a high interlaboratory variability (CV, 53%). Conversely, the indirect method showed a mean percentage of ZAP-70 POS T-lymphocytes at 96% (SD = 4) with a narrow coefficient of variation (CV 4%). In line with these results, as shown in Table 3, when considering T/CLL MFI ratio, the optimal mode of expression of the results, although the results were hampered by the use of stabilized blood, direct staining exhibited a slightly higher variability as compared to indirect technique.

ZAP-70 Stability in Fresh Blood Cells

As stabilized blood induced problems in the gating strategy and was not optimal for multicenter evaluation, we determined the rate of ZAP-70 decay in fresh samples according to storage time along with different anticoagulants. Six fresh CLL samples (3 ZAP+ and 3 ZAP−) were tested at different time points (Days 1, 2, 3 in all cases and Day 5 in 3/6 cases). Blood samples were obtained on EDTA, heparin, and a preservative medium (BCT, BD Biosciences). The variation of morphology scattergrams, surface labeling, fluorescence background, and ZAP-70 labeling were evaluated over time and according to the anticoagulant used. A decrease in SSC was observed without drastic alteration of morphology at the different time points. Lymphocyte gating remained easy and no impact of the anticoagulant was noted on either morphology or membrane marker fluorescence intensity. Conversely, surface labeling intensity was decreased on CLL but not on normal B and T-lymphocytes, whereas fluorescence background varied over time on normal B-lymphocytes and CLL cells. When considering ZAP-70 expression, T Ly/B-CLL MFI ratio were found higher with EDTA as compared to Heparin suggesting a best discrimination between T and CLL cells in the presence of EDTA, although no impact was noted on the conclusion on ZAP-70 status. Finally, ZAP-70 fluorescence intensity was found variable on both T and B cells with different kinetics suggesting a faster degradation in T-lymphocytes.

DISCUSSION

The results of the first experiments of this international effort have been hampered by the use of stabilized

Table 2
Modes of Expression of the Results

Method	Description
Method 1: %/CTRL	Percentage of ZAP-70 positive B-CLL cells according to isotype control
Method 2: %/B-Ly	Percentage of ZAP-70 positive B-CLL cells according to normal B-lymphocytes from normal blood
Method 3: T/CLL MFI ratio	ZAP-70 mean fluorescence intensity T-Ly / B-CLL ratio
Method 4: %/T-Ly	Percentage of ZAP-70 positive B-CLL cells according to ZAP-70 expression in T-lymphocytes

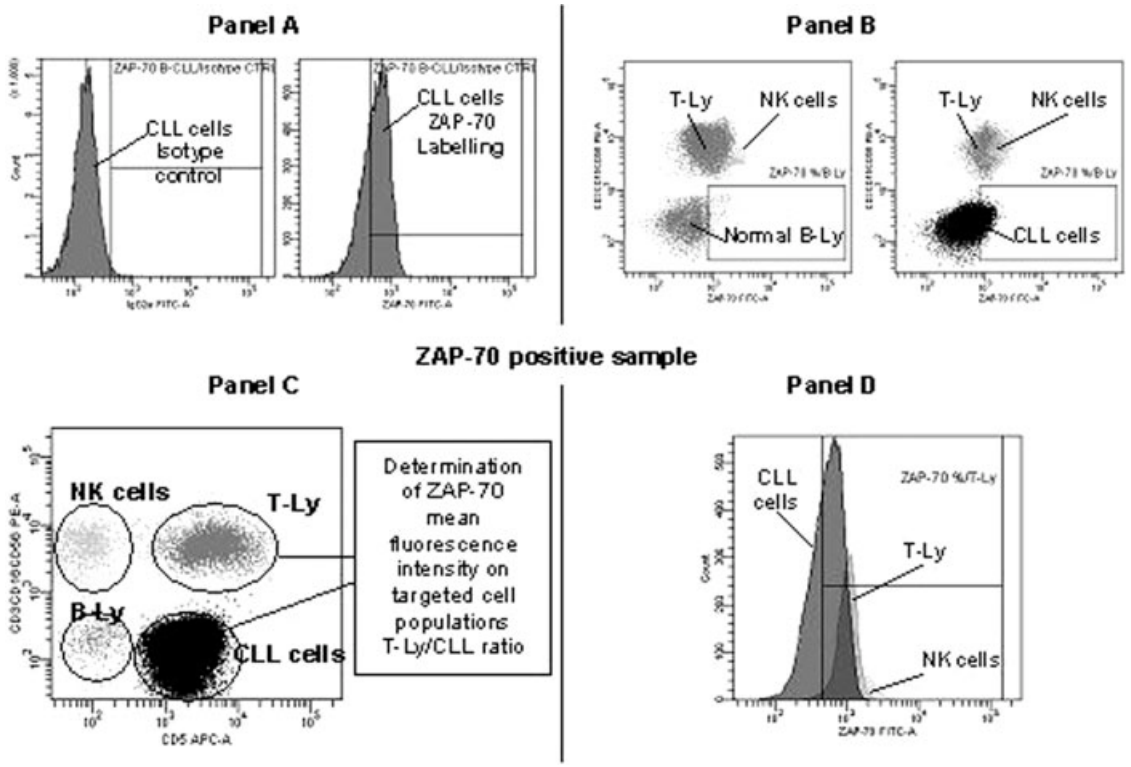

FIG. 2. Modes of expression of the results. Panel **A** exemplifies the Method 1 reporting ZAP-70 expression relative to isotype control on the same population. Panel **B** exemplifies the Method 2 reporting ZAP-70 expression in CLL relative to ZAP-70 expression in B-Ly from normal donor. Panel **C** exemplifies the Method 3 reporting ZAP-70 MFI T-Ly/CLL ratio. Panel **D** exemplifies the Method 4 reporting ZAP-70 expression in CLL relative to ZAP-70 expression in patient's residual T-Ly.

blood samples. The discrimination of the lymphocyte subsets on these samples actually proved possible, but suboptimal with a high rate of unsuitable data (in 6 out o 20 participating laboratories: 30%). This evidence suggests the need for an alternative cellular material before further studies are carried on. To this purpose, ZAP-70 stability was evaluated according to storage time in different lymphocyte subsets. The results suggested that ZAP-70 expression could be accurately detected on fresh cells within 24 h after sampling. A maximum delay of 48 h could be accepted in the context of the harmonization protocol. Both heparin and EDTA may be used, while our experience with the preservative medium BCT is not encouraging, according to the results obtained on stabilized blood. Thus, exchanging fresh blood samples across Europe appears feasible. However, shipment delays should be carefully monitored and taken into account in the final interpretation of the results.

Several methods were described in the literature for the expression of the results, and four different procedures were tested in this study. Surprisingly, the methods using isotype control or normal B-lymphocytes as reference population proved a high variability from one center to another. In addition, setting a positivity threshold on the expression of T-lymphocytes also appeared problematic. In contrast, our preliminary results encourage

Table 3
Analysis of the Results

	Sample 1 ZAP-70 positive		Sample 2 ZAP-70 negative	
Method	1E7.2 Direct	2F3.2 Indirect	1E7.2 Direct	2F3.2 Indirect
M 1: %/CTRL	POS = 17 CV 129	POS = 60 CV 101	NEG = 100 CV 55	NEG = 100 CV 89
M 2: %/B-Ly	POS = 64 CV 58	POS = 88 CV 59	NEG = 100 CV 81	NEG = 100 CV 72
M 3: T/CLL	POS = 89	POS = 100	NEG = 92	NEG = 80
MFI ratio	CV 17	CV 9	CV 22	CV 19
M 4: %/T-Ly	POS = 83 CV 21	POS = 88 CV 13	NEG = 90 CV 45	NEG = 88 CV 68

M, method; POS, positive; NEG, negative.
All values given are in percentages.

the use of fluorescence ratio for which the best coefficients of variation were observed in either POS or NEG CLL samples. This method is attractive since it takes into account the variability of the technique among different laboratories and operators by providing an internal control for ZAP-70 labeling on T-cell population. These results are in line with the results obtained in a French national multicentric protocol for the evaluation of prognostic markers in CLL.

Unfortunately, comparison between 2F3.2 and 1E7.2 clones was available only for a limited number of participating centers and was carried out on suboptimal cellular samples. Therefore, no definitive conclusion can be drawn at the moment regarding the comparison between the two antibodies. However, the results suggested that 2F3.2 clone might provide a higher sensitivity for ZAP-70 expression at least in T-cells by repeatedly identifying as ZAP-70 POS more than 90% of the T-lymphocytes. In contrast, the 1E7.2 clone appeared less sensitive. Moreover, the overall decrease of ZAP-70 MFI when using the 1E7.2 clone hampered to precisely evaluate its expression in the T-cell subset. In the meantime, different clones became available on the market that are now under scrutiny by the various participating laboratories, and might be also selected in a future to enter a comparative analysis.

In conclusion, though ZAP-70 is undoubtedly a valuable prognostic factor in CLL, its determination and interpretation remains difficult. This present study provided valuable information, though several technical issues did not allow to reach any international consensus yet on the most appropriate technique and the expression of the results. Some new reagents are likely to be soon available, and will probably help reaching a consensus. These preliminary results appear promising and are the first steps toward a consensus reporting procedure. Further work is underway to harmonize the preparation and analysis procedures.

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