Published in final edited form as:

Leuk Lymphoma. 2013 March ; 54(3): . doi:10.3109/10428194.2012.715349.

Quantitative detection of zeta-chain-associated protein 70 expression in chronic lymphocytic leukemia

Peixuan Zhu¹, Heba A. Degheidy², Gerald E. Marti², Shuhong Li¹, Fatima Abbasi², Adrian Wiestner³, Platte Amstutz¹, and Cha-Mei Tang¹

¹Creatv MicroTech, Inc., Potomac, MD, USA

²Center for Biologics Evaluation and Research, FDA, Bethesda, MD, USA

³NHLBI, NIH, Bethesda, MD, USA

Abstract

Overexpression of zeta-chain-associated protein 70 (ZAP-70) was recently recognized as an independent prognostic marker for the aggressive form of chronic lymphocytic leukemia (CLL). The objective of this study was to demonstrate the feasibility and implementation of quantitative detection of ZAP-70 protein in B cells to clearly distinguish patients with CLL with the aggressive form of the disease. B cells were isolated from patient blood and lysed. Released ZAP-70 protein was detected using an immunomagnetic fluorescence assay. The assay protocol was developed using Jurkat cells and recombinant ZAP-70 (rZAP-70). The limit of detection was determined to be lower than 125 Jurkat cells and 39 pg of rZAP-70 protein. The signal response was linear over a wide dynamic range, from 125 to 40 000 Jurkat cells per test ($R^2 = 0.9987$) and from 0 to 40 000 pg rZAP-70 protein per test ($R^2 = 0.9928$). The results from 20 patients with CLL correlated strongly with flow cytometry analysis. Concordance between the two methods for positive and negative results was 100% (7/7) and 92% (12/13), respectively, while the overall concordance between the two methods was 95%. The assay reported here is a simple, reliable and reproducible method for quantitative detection of ZAP-70 in patient leukemic cells, without the need for cell fixation or permeabilization. The ZAP-70 signal was linear over a wide dynamic range, which we believe enables quantitative assessment of small changes in ZAP-70 expression over the course of the disease and in response to therapeutic intervention.

Keywords

Chronic lymphocytic leukemia (CLL); ZAP-70; flow cytometry; immunomagnetic fluorescence assay; Signalyte-II spectrofluorometer

Introduction

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with high individual variability in the clinical course. Some patients survive for decades without needing

© 2013 Informa UK, Ltd.

Correspondence: Peixuan Zhu, Creatv MicroTech, Inc., 11609 Lake Potomac Drive, Potomac, MD 20854, USA. Tel: 001-240-450-2467. Fax: 001-301-983-6264. pzhu@creativmicrotech.com.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahhealthcare.com/lal.

Supplementary material available online

Figures and Tables giving supplementary information and further results

treatment, whereas in other patients the disease progresses rapidly and requires early aggressive therapy. In the past 10 years, a number of prognostic markers have been reported for prediction of outcome and response in early-stage CLL. These prognostic markers include somatic mutational status of the immunoglobulin variable heavy chain region gene (*IgV_H*) [1], genomic aberrations such as deletions of 11q and 17p [2,3], loss or mutation of the p53 gene [4], microRNA expression profiles [5 – 7], C-334 methylation status [8], CD38 antigen expression [9,10], zeta-chain-associated protein 70 (ZAP-70) [11,12] and lipoprotein lipase (LPL) [13]. The most established molecular predictor of disease progression is the presence or absence of somatic mutations in the *IgV_H* region of CLL leukemic cells. Absence of mutations in leukemic cells (*IgV_H*-unmutated CLL) often correlates with aggressive disease, whereas the presence of mutations in leukemic cells (*IgV_H*-mutated CLL) more often has longer survival in 50–70% of patients with CLL.

Because *IgV_H* mutation analysis is not routinely available in many clinical laboratories, protein surrogate markers have become attractive as an indication of CLL status. ZAP-70, a member of the Syk family of tyrosine kinases, plays an important role in T and natural killer (NK) cell activation (Supplementary Figure 1 to be found online at <http://www.informahealthcare.com/doi/abs/10.3109/10428194.2012.715349>). Three isoforms of ZAP-70 have been identified by DNA sequencing (Supplementary Figure 2 to be found online at <http://www.informahealthcare.com/doi/abs/10.3109/10428194.2012.715349>). Several studies have found that ZAP-70 is more highly expressed in Ig-unmutated B cells of patients with CLL (“CLL cells”) than in Ig-mutated CLL cells [11,12,14,15]. Overall survival was significantly better for patients with ZAP-70 negative than ZAP-70 positive CLL cells, suggesting that the ZAP-70 expression level provides important prognostic information for patients with CLL. Several methods have been described for detection of ZAP-70 expression, including microarray-based gene expression profiling [11,12,14,16]; flow cytometry [11,12], enzyme-linked immunosorbent assay (ELISA) [17], quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) [18,19], immunohistochemistry [20,21] and Western blot analysis [22].

Flow cytometry is commonly used to measure ZAP-70 in CLL cells, because it allows specific gating on different cell populations of interest [11,15,23]. The principle of measuring ZAP-70 in B cells by flow cytometry is based on counting the number of B cells that express ZAP-70 at a level considered as positive based on normal B cells, T or NK cells. A continuous distribution of ZAP-70 expression levels was observed in CLL cells from 307 patients, ranging from 0.3 to 98% [15]. The continuous nature of ZAP-70 distribution suggests why it has been difficult to establish a clear-cut percentage of ZAP-70 expression that can definitively confirm ZAP-70 overexpression. A second problem associated with the use of flow cytometry to determine ZAP-70 expression is the difficulty of standardizing results across different laboratories [21]. Currently, a reliable and quantitative method for assessing ZAP-70 in leukemic cells is lacking. An improved method for quantitative detection of ZAP-70 would make this marker more widely available for diagnosis and prognosis of CLL.

In this study, we demonstrate a quantitative assay to determine directly the amount of ZAP-70 in leukemic B-cells. We collect a defined number of B cells from the blood of a patient with CLL and then lyse these cells to release all intracellular ZAP-70 proteins. ZAP-70 in the cell lysate is detected. Immunomagnetic fluorescence assay (IMFA) is used to quantitate ZAP-70.

Immunomagnetic separation is a rapid, technically straightforward, specific and efficient method that can be used for isolation and/or concentration of selected targets from complex sample matrices. In our previous study, we developed an IMFA system by combining

immunomagnetic separation with sandwich antibody fluorescence in a single assay [24]. Our data demonstrated that this technology was feasible for detection of pathogenic *Escherichia coli* O157:H7 cells, with a limit of detection of 10 colony forming units (CFU) per assay [24]. The current study investigated the use of a similar approach for quantitative detection of ZAP-70 proteins. Our objective was to combine sample preparation, IMFA detection and signal measurement on Signalyte™-II for quantitative detection of ZAP-70 in leukemic B cells from patients with CLL.

Materials and methods

Cell line, antibodies and reagents

A human T lymphocyte-derived cell line, Jurkat (ATCC No. TIB-152), was obtained from the American Type Culture Collection (ATCC). This cell line expresses a high level of ZAP-70 protein and served as a positive control. The cell line was grown in the complete growth medium RPMI-1640 according to ATCC's protocols. All antibodies and reagents are listed in online Supplementary Table 1 to be found online at <http://www.informahealthcare.com/doi/abs/10.3109/10428194.2012.715349>. The biotinylated antibodies, fluorescence-conjugated antibodies and immunoaffinity magnetic beads were prepared using standard protocols as previously described [24,25].

Human whole blood samples and preparation of T and B cells

Peripheral blood samples were obtained from the National Heart, Lung, and Blood Institute (NHLBI) at the National Institutes of Health (NIH) from patients with CLL. These patients were enrolled on a NHLBI Institutional Review Board (IRB) approved clinical study registered with ClinicalTrials.gov under identifier NCT00923507, and under National Cancer Institute (NCI) study 97-C-0178 (ClinicalTrials.gov identifier NCT00019370). Healthy blood controls were collected from anonymous donors by the Department of Transfusion Medicine at the Warren Grant Magnuson Clinical Center in the National Institutes of Health. The study involving healthy control participants was under the IRB's oversight with written informed consent (Protocol No. 99CC0168-H). Viable peripheral blood mononuclear cells (PBMCs) were isolated from 12 mL of blood sample by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ). The recovered PBMCs were counted with a hemocytometer and adjusted to a final concentration of 10^7 cells/mL. T and B cells were individually isolated from PBMCs by magnetic activated cell sorting (MACS) using Human CD3 and CD19 MicroBead Systems (Miltenyi, Auburn, CA) according to the manufacturer's instructions, respectively. These purified cells were used for the preparation of cell lysate or stored at -80°C in cryo-fluid until use. The purity of the isolated B-cells was confirmed by staining with fluorescent antibodies and flow cytometry analysis. Briefly, cells were fixed in 1% paraformaldehyde–saline solution and stained with fluorescein isothiocyanate (FITC)-labeled anti-CD19 antibody to identify B cells (BioLegend), allophycocyanin (APC)-labeled anti-CD3 antibody to identify T cells (eBioscience) and phycoerythrin (PE)-labeled anti-CD20 antibody also to identify B cells (eBioscience). This flow cytometry analysis showed that the B-cell purity was >98%.

Preparation of cell lysates

Purified B cells were counted with a hemocytometer. To prepare cell lysate, one million of the purified B-cells were suspended in 200 μL of $1\times$ Cell Lysis Buffer (Cell Signaling Technology; Cat# 7172) and incubated on ice for 5 min. The lysate was sonicated in a water-bath sonicator (Model 450 ultrasonic cleaner; E/MC Corp.) for 1 min. The lysate sample was heated at 95°C in a water bath for 5 min, placed on ice for 1 min, and then centrifuged at $12\,000 \times g$ for 1 min to bring down all liquid evaporates. The lysate was then transferred to a QIAamp Mini Column (Qiagen) and centrifuged at $12\,000 \times g$ for 2 min to

remove genomic DNA and cell debris. The pass-through fraction in the collection tube (the final cell lysate sample) was stored at 20 °C until use for further analysis.

Assessment of ZAP-70 expression

Expression of ZAP-70 protein in lymphocytes was assessed by three different methods: immunomagnetic fluorescence detection, quantitative RT-PCR and flow cytometry. Instrumentation of the Signalyte™-II spectrofluorometer and the principle of the immunomagnetic fluorescence detection of ZAP-70 (VeriZAP system) are described in Supplementary Figures 3 and 4 to be found online at <http://www.informahealthcare.com/doi/abs/10.3109/10428194.2012.715349>, respectively. Quantitative RT-PCR for detection of ZAP-70-specific mRNA transcription levels was performed as described [12]. Flow cytometry analysis for measurement of ZAP-70 was performed as previously described [26,27].

Results

Selection of anti-ZAP-70 capture antibodies

Three different anti-ZAP-70 monoclonal antibodies were evaluated in this study (clones 2F3.2, SBZAP and 1E7.2). The locations of the immunogens used to generate these antibodies are shown in Supplementary Figure 2 to be found online at <http://www.informahealthcare.com/doi/abs/10.3109/10428194.2012.715349>. The clones 2F3.2 and SBZAP were used as a capture antibody, whereas 1E7.2 was used as a detector antibody, forming two combinations of antibody pairs: 2F3.2/1E7.2 and SBZAP/1E7.2. The capture antibody 2F3.2 or SBZAP was immobilized on the surface of magnetic beads through biotin/avidin interaction as previously described [24,25]. PE-Cy5.5-conjugated 1E7.2 (eBioscience) was used as the detector antibody. Two ZAP-70-positive controls, Jurkat cell lysate and CLL cell lysate (P8), were tested with these antibody pairs. The results showed that antibody pair 2F3.2/1E7.2 produced a higher fluorescence signal than antibody pair SBZAP/1E7.2 for Jurkat cell lysate (1.6-fold) and for CLL cell lysate (4.3-fold) (Figure 1). Both antibody pairs were highly specific to ZAP-70, as demonstrated by a very low fluorescence signal for the negative control. The antibody pair 2F3.2/1E7.2 showed better sensitivity and was selected for detection of ZAP-70 protein in the following experiments. The 2F3.2 antibody has been most often used in flow cytometry and immunohistochemistry analyses for detection of ZAP-70 [28]. Previous studies also demonstrated that this antibody bound only to the human ZAP-70 protein, with no detectable cross-activity to other human proteins [12,29]. Thus, selection of 2F3.2 as the capture antibody in this study would help ensure the assay specificity.

Selection of fluorescence-conjugated detector antibody

In order to minimize non-specific background signal, four fluorescence-conjugated 1E7.2 antibodies were evaluated in this study. The PE-Cy5.5- and APC-conjugated 1E7.2 antibodies were obtained from eBioscience. Cy5- and Dylight 649-conjugated 1E7.2 antibodies were prepared in-house. Jurkat cell lysate and phosphate buffered saline (PBS) were used as positive and negative controls, respectively. For Jurkat cell lysate, the fluorescence signal was determined as Cy5-1E7.2 (51 014) > Dylight 649-1E7.2 (17 007) > PE-Cy5.5-1E7.2 (5995) > APC-1E7.2 (97). The signal-to-noise (S/N) ratio of the Jurkat cell lysate to the PBS control was determined as PE-Cy5.5-1E7.2 (90.1) > Dylight 649-1E7.2 (89.8) > Cy5-1E7.2 (11.78) > APC-1E7.2 (10.7) (Figure 2). Although Cy5-conjugated antibody produced the highest fluorescence signal, its background noise was also higher, which significantly reduced the S/N ratio for this dye. PE-Cy5.5- and Dylight 649-conjugated antibodies produced a relatively high signal for ZAP-70 and low background, yielding the highest S/N ratio. PE-Cy5.5-conjugated antibody could be excited by

Signalyte™-II at two different wavelengths, either 470 nm or 635 nm, to produce an emission peak at 692 nm. Although the detection sensitivity was similar for the two excitation wavelengths, excitation at 635 nm produced better linearity at high concentrations of ZAP-70. Based on these results, we selected PE-Cy5.5-1E7.2 antibody as the detector antibody and used excitation/emission wavelengths at 635/692 nm for the measurement of ZAP-70 signals.

Detection of ZAP-70 in Jurkat cell lysate

To determine the detection sensitivity, two-fold serial dilutions of Jurkat cell lysate were prepared with a dilution solution (PBS containing 2% lysis buffer) to obtain final concentrations ranging from 63 to 1000 cells/reaction. These serial dilutions were tested using PE-Cy5.5-conjugated detector antibody. The fluorescence intensity (FI) of each sample was measured with Signalyte™-II. In order to obtain a very low limit of detection, it was necessary to normalize the FI signal by subtracting the FI of a buffer control from the FI of each sample. This resulted in the normalized fluorescence intensity (NFI) of each sample. The relationship between NFI and Jurkat cell concentration is plotted in Figure 3(A), showing a strong linear relationship ($R^2 = 0.9908$). The equation describing the relationship is $y = 8.6265x + 768.87$. Based on average NFI and standard deviation (SD) of the negative control (79 ± 53.74), a threshold NFI value (average + 3SD) was set at 240.22. The input concentration of 125 cells resulted in a significantly higher signal (395.5 ± 10.61) than that of the negative control [Figure 3(A)]. Thus, the limit of detection (LOD) of ZAP-70 in Jurkat cell lysate was determined to be lower than 125 cells. To determine the assay dynamic range, serial dilutions including higher concentrations (up to 100 000 cells/reaction) were tested. To avoid signal saturation at high cell concentrations, a shorter excitation time [10 ms, equivalent to 1/50 in Figure 3(A)] was used for measurement of fluorescence signals. The relationship between NFI and higher cell concentrations of the Jurkat cells is plotted in Figure 3(B). A linear signal response was observed over a wide range of 63–40 000 cells/reaction ($R^2 = 0.9987$).

Detection of recombinant ZAP-70 protein

Purified recombinant ZAP-70 protein (rZAP-70) from R&D Systems was used as a positive control in serial dilution tests and spiking experiments to validate assay specificity (see Supplementary Table 1 to be found online at <http://www.informahealthcare.com/doi/abs/10.3109/10428194.2012.715349>). A strong linear relationship ($R^2 = 0.99996$) between NFI and rZAP-70 concentrations was observed from 0 to 3125 pg/reaction [Figure 4(A)]. The equation describing the relationship is $y = 1.371x + 585.12$. Based on average NFI and SD of the negative control (533.50 ± 24.75), a threshold NFI value was set at 607.75. The input concentration of 39 pg/reaction resulted in a significantly higher signal (697.5 ± 157.68) than that of the negative control. The LOD for rZAP-70 was thus determined to be lower than 39 pg per reaction. In addition, the relationship between NFI and higher concentrations of rZAP-70 was plotted on a log-log scale [Figure 4(B)]. A linear signal response was observed over a wide range of 0–40 000 pg/capillary ($R^2 = 0.9928$) [Figure 4(B)]. Overall, these results demonstrate that immunomagnetic fluorescence detection of ZAP-70 is extremely sensitive and specific. This technology also produced a wide linear detection range, making it suitable for quantitation of low levels of ZAP-70 in clinical samples.

Effect of sample matrix on ZAP-70 analysis

The sample matrix of B cell lysate contains components that might affect assay response differently from the PBS buffer used for construction of the standard curve. A spiking experiment was designed to assess this difference in assay response. Three known amounts of rZAP-70 (R&D Systems), 1500 pg, 12 500 pg and 25 000 pg per reaction, were spiked in

the B-cell lysate of a healthy control (100 000 cells/reaction) and the standard PBS diluent, respectively. Two sets of samples were analyzed under the same experimental conditions (Figure 5). The recovery efficiencies for the three spiked concentrations were 81.7%, 108.8% and 107.2%, respectively, with an average recovery efficiency of $99.2 \pm 15.2\%$. The result shows that B-cell sample matrix was valid for the ZAP-70 assay. The presence of B-cell sample matrix in the test samples had no adverse impact on the ZAP-70 assay. In addition, the fluorescence signal was still linear from the samples containing rZAP-70 and cell lysate matrix. This confirms that the assay using the antibody sandwich format is highly specific for the detection of ZAP-70 in cell lysate samples.

ZAP-70 assay variability

The assay was validated for intra-assay variability and inter-assay variability (Table I). To minimize the variation caused by cell lysis and sample preparation, multiple vials of Jurkat cell lysate with the same lot number were obtained from Millipore (Cat# 12-303, Lot# DAM1641050) and tested by immunomagnetic fluorescence detection of ZAP-70. Two concentrations of Jurkat cell lysate, 6.25 $\mu\text{g/mL}$ and 25 $\mu\text{g/mL}$, were repeatedly tested. A negative control of dilution buffer only was included in the experiment and subject to all assay procedures for measurement of the assay non-specific signals. A buffer control with Elution Buffer only was included in the final reading step for measurement of background noise caused by the Elution Buffer and SignalyteTM-II instrument. Table I shows the resulting assay variability. Coefficient variations of intra-assay variability were 3.72%, 15.72% and 5.68% for the zero control, 6.25 $\mu\text{g/mL}$ and 25 $\mu\text{g/mL}$ of Jurkat cell lysate. Coefficient variations of inter-assay variability were 5.48%, 17.81% and 13.00% for the zero control, 6.25 $\mu\text{g/mL}$ and 25 $\mu\text{g/mL}$ of Jurkat cell lysate.

Comparison of VeriZAP with flow cytometry analysis

Twenty whole blood samples from patients with CLL were used for the comparative analysis (P1–P20). ZAP-70 expression levels in these patients were determined by flow cytometry analysis as described [26,27]. The VeriZAP system starts with the preparation of B cell lysate from a fixed number of purified B cells from patients with CLL and is followed by immunomagnetic fluorescence detection of ZAP-70. Purified T and B cell samples from a healthy control were used as positive and negative controls (PC and NC) for calculation of the ZAP-70 ratio of each CLL cell sample. ZAP-70 expression was reported as the ratio of the average NFI of a CLL sample to the average NFI of the healthy control. ZAP-70 expression was defined as positive if the CLL/NC ratio was ≥ 2.0 and as negative if the CLL/NC was <2.0 . The results of flow cytometry analysis and VeriZAP are summarized in Figure 6 and Supplementary Table 2 to be found online at <http://www.informahealthcare.com/doi/abs/10.3109/10428194.2012.715349>. All seven samples that had been determined as ZAP-70-positive by flow cytometry were also determined as ZAP-70-positive by VeriZAP (100%). Further, 12 of 13 samples that had been determined as ZAP-70-negative by flow cytometry were also found negative by VeriZAP. Only one sample of the 20 (P6) was discordant, measuring negative by flow cytometry, but positive by VeriZAP, for an overall concordance of 95% between the two methods. We performed further analysis on the discrepant P6 sample to determine the ZAP-70-specific mRNA transcription level by quantitative RT-PCR (Supplementary Figure 5 to be found online at <http://www.informahealthcare.com/doi/abs/10.3109/10428194.2012.715349>). The results showed that the ZAP-70-mRNA transcription level in B cells from P6 was 7.84-fold higher than that in B cells from the healthy control, indicating that patient P6 had mRNA levels consistent with increased ZAP-70 expression. Overall, the flow cytometry and VeriZAP methods were highly correlated, confirming that VeriZAP is a valid alternative method for detection of ZAP-70 in CLL.

Discussion

Several methods have been developed to assess CLL patient-status. Microarray analysis was initially used to differentiate gene expression profiles of patients with mutated and unmutated *IgV_H* status [14,16]. The ZAP-70 -specific mRNA transcription level was observed to be five-fold higher in patients with unmutated *IgV_H* genes than in those with mutated genes. Subsequently, expression of ZAP-70 protein in CLL cells was detected by several studies using different flow cytometry methods, including direct and indirect fluorescent antibody staining [11,12,15,21,23]. These studies confirmed the association between ZAP-70 expression and *IgV_H* mutation status and showed that a high percentage of ZAP-70-positive CLL cells could provide prognostic information. Flow cytometry has since become a method of choice for analysis of ZAP-70 expression in CLL cells. There are, however, some severe limitations that confound the use of this technique as a routine diagnostic tool in clinical laboratories. Flow cytometry analysis requires red blood cell lysis, cell fixation and permeabilization to allow access of the fluorescent antibody to subcellular structures. These steps not only increase the complexity of the assay procedure, but also cause significant variations from laboratory to laboratory. In addition, the use of different antibody clones in flow cytometry can produce conflicting results for an individual patient. This discrepancy can be explained by varying the accessibility of antibody to different epitopes and subcellular locations of the ZAP-70 protein. A previous study in human epithelial and T cells showed that a large amount of ZAP-70 resides in the nucleus of quiescent and activated cells [30]. This suggests that free access of antibody to all subcellular locations is critical to detect both cytoplasmic and nucleic ZAP-70. The variations and discrepancies in flow cytometry analysis of ZAP-70 may be attributed to detrimental effects of fixation and permeabilization, such as fixation-induced masking of antigenic sites or insufficient accessibility of detector antibody to nucleic ZAP-70.

The VeriZAP system developed in this study overcomes the drawbacks of the flow cytometry method, because it directly detects ZAP-70 protein, rather than counting cells with detectable levels of the marker. There is no need for cell fixation and permeabilization. This assay was designed for use of whole cell lysis to effectively release all cytoplasmic and nucleic ZAP-70 protein to a homologous supernatant. The interaction between ZAP-70 and anti-ZAP-70 antibody was uninhibited in the supernatant, allowing maximum access and binding of antibody to the target antigen. Subsequently, ZAP-70 protein is specifically captured on the surface of the magnetic beads, and unbound proteins and other contaminants are washed away. Without this isolation of ZAP-70 by capture antibody, other proteins and contaminants in the system may interfere with assay sensitivity and specificity. Use of antibody-coated magnetic beads eliminates the need to purify ZAP-70 from complicated mixtures before measurement, simplifying the assay and increasing the specificity and the sensitivity. Thus, interference from other cell components is minimized. With this assay format, it was possible to increase detection sensitivity and reduce inter-laboratory variation. Our results demonstrated that VeriZAP is a very efficient assay that can detect ZAP-70 at levels lower than 39 pg of purified ZAP-70 protein and 125 Jurkat cells. This is much more sensitive than existing methods.

The immunomagnetic fluorescence detection of ZAP-70 measures the amount of ZAP-70 in a complex of two antibodies (capture and detector antibodies). This requires that each ZAP-70 molecule contains at least two antigenic sites capable of binding to antibody in the sandwich format. Clones 2F3.2/1E7.2 were selected as the capture/detection antibody pair for optimal performance in this study. These two antibodies bind ZAP-70 at two distinct sites. The clone 2F3.2 antibody was generated against glutathione-S-transferase (GST)-fusion protein containing the two tandem SH2 domains of human ZAP-70, corresponding to amino acids 1–254. The specific binding sites of this clone have not yet been mapped on the

ZAP-70 amino acid sequences (Millipore technical support, personal communications). If the binding sites located at the regions are associated with the SH2 domains, use of clone 2F3.2 may pull down as much of the ZAP-70 as possible. Clone 2F3.2 is the antibody most commonly used in flow cytometry and immunohistochemistry for detection of ZAP-70 [28]. Our results further supported that the clone 2F3.2 was an effective antibody for capture of ZAP-70 in cell lysate samples. The clone 1E7.2 antibody was generated against a keyhole limpet hemocyanin (KLH)-peptide sequence corresponding to the human ZAP-70 amino acids 282–307. As 1E7.2 recognizes a single epitope, it allows specific detection and quantification of small differences in antigen. Our results showed that this clone worked well as detector antibody when conjugated with either PE-Cy5.5 or Dylight 649. Although three iso-forms of ZAP-70 have been reported, little is known about their expression and distribution in different cell types. According to the known antibody binding sites, the selected antibody pair of 2F3.2/1E7.2 could detect isoforms 1 and 3, but might not be efficient to detect isoform 2, a truncated version of ZAP-70. It would be worthwhile to investigate the expression pattern of all ZAP-70 isoforms in CLL cells. If CLL cells produce any truncated ZAP-70 proteins, additional antibody should be included in our assay to further improve assay efficiency.

In our previous study, we investigated the feasibility of immunomagnetic fluorescence detection and identification of *E. coli* O157:H7 in water and food samples [24]. The results showed that the new approach was feasible due to the use of an ultra-sensitive Signalyte™-II fluorescent instrument platform [31–33], allowing for a small number of bead-captured bacteria (LOD 10 CFU) to be directly detected by a fluorescent detector antibody without the need of a pre-enrichment step. In this study, we demonstrated that the same approach could be used for detection of ZAP-70 protein in CLL with a modified assay protocol. Many experimental parameters may influence the results for detection of ZAP-70. For example, presence of the cell lysis buffer in the test sample might influence the ZAP-70 signal. We found that the final concentration of cell lysis buffer should be less than 2% in order to reduce or avoid the compound inferences in the capture reaction. Multiple experimental parameters have also been extensively optimized in this study, such as antibodies and fluorescence dyes, incubation temperature, incubation time, blocking and elution reagents. The immunomagnetic fluorescence detection of ZAP-70 can be completed in less than 1.5 h, since colorimetric substrate is not required, as compared to existing ELISA techniques that typically require 3–4 h incubation and washes, followed by color development and reaction termination. Since the magnetic separation was done manually in this study, we believe the assay reproducibility can be further improved by using an automated magnetic bead separation system.

In addition, signal response to ZAP-70 protein in the VeriZAP system was linear over a wide dynamic range, enabling a quantitative detection of ZAP-70 in a small numbers of leukemic cells. Since significant change in ZAP-70 expression was recently observed during the course of chronic lymphocytic leukemia, repeated assessment of ZAP-70 expression might be especially useful at the time of disease progression or relapse in patients who were initially ZAP-70-negative [34]. Our results suggest that the VeriZAP system is very suitable for quantitative monitoring the dynamic changes of ZAP-70 expression over the disease course and the response to therapeutic intervention.

In conclusion, the VeriZAP system is a sensitive and quantitative method for detection of ZAP-70 in leukemic cells from patients with CLL. This new technology addresses the problems caused by cell fixation and permeabilization associated with flow cytometry, since all of the nucleic and cytoplasmic ZAP-70 are completely released to the cell lysate, making them freely accessible for the capture and detector antibodies. Our results showed that VeriZAP system was able to detect ZAP-70 at levels lower than 125 Jurkat cells and 39 pg

of rZAP-70 protein. This is much more sensitive than other conventional methods, such as ELISA. The VeriZAP system results from 20 cases of CLL correlated strongly with flow cytometry analysis. Moreover, the ZAP-70 fluorescence signal from the VeriZAP system was linear over a wider dynamic range, allowing quantitative assessment of small changes of ZAP-70 expression during the disease course and response to therapeutic intervention. This method may be applied in the clinical laboratory as an alternative tool to flow cytometry analysis for the diagnosis and prognosis of CLL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Susan Soto in Dr. Wiestner's laboratory for assistance in the collection of CLL blood samples and Yong-Qiang Wang for assistance in preparation of PBMCs from the samples. This research was supported in part by the intramural research program of NHLBI, NIH.

This work was supported by a grant R44 CA094430-02 from the National Institutes of Health.

References

1. Hamblin TJ, Davis Z, Gardiner A, et al. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood*. 1999; 94:1848–1854. [PubMed: 10477713]
2. Döhner H, Stilgenbauer S, Benner A, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med*. 2000; 343:1910–1916. [PubMed: 11136261]
3. Kröber 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. [PubMed: 12149225]
4. Stilgenbauer S, Döhner H. Campath-1H-induced complete remission of chronic lymphocytic leukemia despite p53 gene mutation and resistance to chemotherapy. *N Engl J Med*. 2002; 347:452–453. [PubMed: 12167696]
5. Calin GA, Liu CG, Sevignani C, et al. MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. *Proc Natl Acad Sci USA*. 2004; 101:11755–11760. [PubMed: 15284443]
6. Calin GA, Ferracin M, Cimmino A, et al. A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med*. 2005; 353:1793–1801. [PubMed: 16251535]
7. Moussay E, Wang K, Cho JH, et al. MicroRNA as biomarkers and regulators in B-cell chronic lymphocytic leukemia. *Proc Natl Acad Sci USA*. 2011; 108:6573–6578. [PubMed: 21460253]
8. Corcoran M, Parker A, Orchard J, et al. ZAP-70 methylation status is associated with ZAP-70 expression status in chronic lymphocytic leukemia. *Haematologica*. 2005; 90:1078–1088. [PubMed: 16079107]
9. Damle RN, Wasil T, Fais F, et al. IgVH gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood*. 1999; 94:1840–1847. [PubMed: 10477712]
10. Dürig J, Naschar M, Schmücker U, et al. CD38 expression is an important prognostic marker in chronic lymphocytic leukaemia. *Leukemia*. 2002; 16:30–35. [PubMed: 11840260]
11. 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. [PubMed: 12724482]
12. 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. [PubMed: 12595313]

13. Van Bockstaele F, Pede V, Janssens A, et al. Lipoprotein lipase mRNA expression in whole blood is a prognostic marker in B cell chronic lymphocytic leukemia. *Clin Chem.* 2007; 53:204–212. [PubMed: 17158192]
14. 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. [PubMed: 11733578]
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. [PubMed: 15329427]
16. Klein U, Tu Y, Stolovitzky GA, et al. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. *J Exp Med.* 2001; 194:1625–1638. [PubMed: 11733577]
17. Pryshchep S, Goronzy JJ, Parashar S, et al. Insufficient deactivation of the protein tyrosine kinase lck amplifies T-cell responsiveness in acute coronary syndrome. *Circ Res.* 2010; 106:769–778. [PubMed: 20035083]
18. Stamatopoulos B, Meuleman N, Haibe-Kains B, et al. Quantification of ZAP-70 mRNA in B cells by real-time PCR is a powerful prognostic factor in chronic lymphocytic leukemia. *Clin Chem.* 2007; 53:1757–1766. [PubMed: 17702857]
19. Stamatopoulos B, Haibe-Kains B, Equeter C, et al. Gene expression profiling reveals differences in microenvironment interaction between patients with chronic lymphocytic leukemia expressing high versus low ZAP-70 mRNA. *Haematologica.* 2009; 94:790–799. [PubMed: 19377082]
20. Carreras J, Villamor N, Colomo L, et al. Immunohistochemical analysis of ZAP-70 expression in B-cell lymphoid neoplasms. *J Pathol.* 2005; 205:507–513. [PubMed: 15685592]
21. Admirand JH, Knoblock RJ, Coombes KR, et al. Immunohistochemical detection of ZAP-70 in chronic lymphocytic leukemia predicts immunoglobulin heavy chain gene mutation status and time to progression. *Mod Pathol.* 2010; 23:1518–1523. [PubMed: 20657554]
22. 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. [PubMed: 12393534]
23. Preobrazhensky SN, Bahler DW. Optimization of flow cytometric measurement of ZAP-70 in chronic lymphocytic leukemia. *Cytometry B Clin Cytom.* 2008; 74:118–127. [PubMed: 17948236]
24. Zhu P, Shelton DR, Li S, et al. Detection of E. coli O157:H7 by immunomagnetic separation coupled with fluorescence immuno-assay. *Biosens Bioelectron.* 2011; 30:337–341. [PubMed: 22005594]
25. Zhu P, Shelton DR, Karns JS, et al. Detection of water-borne E. coli O157 using the integrating waveguide biosensor. *Biosens Bioelectron.* 2005; 21:678–683. [PubMed: 16202883]
26. Degheidy HA, Venzon DJ, Farooqui MZ, et al. Methodological comparison of two anti-ZAP-70 antibodies. *Cytometry B Clin Cytom.* 2011; 80B:300–308. [PubMed: 21472849]
27. Degheidy HA, Venzon DJ, Farooqui MZ, et al. Improved ZAP-70 assay using two clones, multiple methods of analysis and clinical correlation. *Cytometry B Clin Cytom.* 2011; 80B:309–317. [PubMed: 21472850]
28. Orchard J, Ibbotson R, Best G, et al. ZAP-70 in B cell malignancies. *Leuk Lymphoma.* 2005; 46:1689–1698. [PubMed: 16263570]
29. Sup SJ, Domiati-Saad R, Kelley TW, et al. ZAP-70 expression in B-cell hematologic malignancy is not limited to CLL/SLL. *Am J Clin Pathol.* 2004; 122:582–587. [PubMed: 15487457]
30. Sloan-Lancaster J, Zhang W, Presley J, et al. Regulation of ZAP-70 intracellular localization: visualization with the green fluorescent protein. *J Exp Med.* 1997; 186:1713–1724. [PubMed: 9362531]
31. Li, S.; Amstutz, P.; Tang, CM., et al. Integrating waveguide biosensor.. In: Rasooly, A.; Herold, KE., editors. *Biosensors and biodetection methods and protocols, Vol. 1: optical-based detectors.* Humana Press; New York: 2009. p. 89-401.

32. Li, S.; Zhang, Y.; Amstutz, P., et al. Multiplex integrating waveguide sensor–Signalyte™-II.. In: Rasooly, A.; Herold, KE., editors. Biosensors and biodetection methods and protocols, Vol. 1: Optical-based detectors. Humana Press; New York: 2009. p. 423–434.
33. Li, S.; Kiefer, L.; Zhu, P., et al. Ultra-sensitive detection using integrated waveguide technologies.. In: Somerset, V., editor. Biosensors for health, environment and biosecurity, Book 3, Environmental biosensors. InTech; Rijeka, Croatia: 2011.
34. Vroblova V, Vrbacky F, Hrudkova M, et al. Significant change in ZAP-70 expression during the course of chronic lymphocytic leukemia. Eur J Haematol. 2010; 84:513–517. [PubMed: 20132303]

Figure 1.

Selection of antibody for detection of ZAP-70. Clones 2F3.2 and SBZAP antibodies were used as capture antibodies, whereas clone 1E7.2 was used as detector antibody. Two ZAP-70-positive samples (Jurkat cell lysate, P8 CLL cell lysate) and a PBS buffer control were tested. Clone 2F3.2 antibody produced higher detection signal than clone SBZAP antibody for both Jurkat cell lysate (1.6-fold) and P8 cell lysate (4.3-fold). PBS control showed low background signal, indicating that the assay was highly specific for detection of ZAP-70.

Figure 2.

Selection of fluorescence dyes for detector antibody. Clone 1E7.2 antibody was used as detector antibody. This detector antibody was conjugated with four different fluorescence dyes, PE-Cy5.5, APC, Cy5 and Dylight 649, respectively. Jurkat cell lysate was used as a positive control to evaluate detection sensitivity, whereas PBS solution was used as negative control to evaluate non-specific background noise. Y-axis indicates signal-to-noise ratio (S/N). Although Cy5-conjugated antibody produced the highest signal, it also produced higher background noise, resulting in lower S/N ratio. PE-Cy5.5- and Dylight 649-conjugated antibodies produced lower background noise, resulting in better S/N ratios.

Figure 3.

Detection of ZAP-70 in Jurkat cell lysate. Plots show relationships of normalized fluorescence signal (NFI) and number of Jurkat cells used for preparation of cell lysate samples. (A) Low cell concentration range from 0 to 1000 cells/reaction. (B) High cell concentration range from 0 to 40 000 cells/reaction. To precisely measure each sample and maximize detection sensitivity without saturation, two different measurement times were used for the lower concentration range (3000 ms) and high concentration range (10 ms).

Figure 4.

Detection of recombinant ZAP-70 protein. Plots show relationships of NFI and input concentrations of recombinant ZAP-70 protein (R&D Systems). Two different measurement times were used for the lower concentration range (500 ms) and high concentration range (100 ms). (A) Low ZAP-70 concentration range from 0 to 3125 pg/reaction. (B) High ZAP-70 concentration range from 0 to 40 000 pg/reaction. Relationships at the high concentration range are plotted on a log-log scale.

Figure 5.

Effect of sample matrix on ZAP-70 analysis. Three known amounts of rZAP-70, 1500 pg, 12 500 pg and 25 000 pg per reaction, were spiked in the B-cell lysate of a healthy control and standard PBS diluent control, respectively. Spiked samples were analyzed under the same experimental conditions. NFI, normalized fluorescence intensity.

Figure 6.

Detection of ZAP-70 expression level in 20 peripheral blood samples from patients with CLL. Y-axis displays normalized ZAP-70 signal (ratio of CLL cells to negative control cells, CLL/NC). X-axis indicates blood sample numbers. ZAP-70 results from flow cytometry analysis are shown below chart.

Table I

VeriZAP assay variability^{*} of detection of ZAP-70 in Jurkat cell lysate.

Jurkat cell lysate ($\mu\text{g/mL}$)	Intra-assay variability ($n = 12$)				Inter-assay variability ($n = 6$)			
	Buffer control [†]	0	6.25	25	Buffer control [†]	0	6.25	25
Average FI	1595.50	2303.50	5501.25	18 894.92	1643.93	2271.50	6586.04	18 993.92
Standard deviation	22.42	85.80	864.65	1072.51	62.40	124.52	1173.04	2468.99
Coefficient of variation (%)	1.41	3.72	15.72	5.68	3.80	5.48	17.81	13.00

FI, fluorescence intensity.

^{*} To assess intra-assay variability, 12 assays were performed on the same sample on the same day. To assess inter-assay variability, assays were performed on the same samples each day over 6 days.

[†] Buffer control: a blank tube containing only Elution Buffer which was used for evaluation of background signals.