Flow Cytometric Analysis of Lymphoid Enhancer-Binding Factor 1 in Diagnosis of Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

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ABSTRACT

Objectives: Nuclear overexpression of lymphoid enhancer-binding factor 1 (LEF1) assessed by immunohistochemistry has been shown to be highly associated with chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) among small B-cell lymphomas. The purpose of this study was to evaluate the utility of flow cytometric analysis of LEF1 in the diagnosis of CLL/SLL.

Methods: Normal peripheral blood was used to validate the test. Flow cytometric analysis of LEF1 was performed in 64 patient samples qualitatively and quantitatively by comparing the staining intensity and the ratios of the median fluorescence intensities (MFIs) of LEF1 in B cells of interest to the internal reference cell populations. The results were correlated with the pathologic diagnosis.

Results: Proper sample processing ensured sufficient separation of positive LEF1 staining in T cells from negative staining in normal B and natural killer (NK) cells. Qualitative analysis of patient samples showed that all 25 cases of CLL/SLL but none of the other small B-cell lymphomas were positive for LEF1. Using a B/NK MFI ratio of 1.5 and B/T MFI ratio of 0.45 separated CLL/SLL cases from non-CLL lymphomas.

Conclusions: Flow cytometric analysis of LEF1 is sufficient to differentiate CLL/SLL from other small B-cell lymphomas and may serve as a useful tool in the diagnosis of CLL/SLL.

The diagnosis of chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) can be established in most cases based on the morphologic and immunophenotypic characteristics. However, the differential diagnosis of CLL/SLL from its mimickers can be challenging in some cases due to overlapping morphologic and immunophenotypic features, particularly when analyzing peripheral blood or limited tissue samples.1-3 It is well recognized that CLL/SLL and mantle cell lymphoma (MCL), the two major categories of CD5+ small B-cell lymphomas, may show significant overlap in immunophenotype.4 The differential diagnosis between the two entities can usually be facilitated by positive cyclin D1 staining in MCL using immunohistochemistry (IHC) on tissue sections. When the differential diagnosis is raised in peripheral blood samples, however, fluorescence in situ hybridization (FISH) analysis for t(11;14)-CCND1/IgH is often performed to rule out or rule in MCL since IHC is not routinely performed in blood samples. Occasionally, other small B-cell lymphomas, such as marginal zone lymphoma (MZL) and lymphoplasmacytic lymphoma (LPL), may be included in the differential diagnosis, particularly in leukemic phase and when expressing CD5.5-7 It has been reported that CD200 is strongly expressed in CLL/SLL and absent in MCL and therefore can be used as a marker to distinguish CLL/SLL from MCL. However, CD200 expression has also been found in other B-cell neoplasms, including hairy cell leukemia and LPL.8-10

We have recently reported that lymphoid enhancer-binding factor 1 (LEF1) is highly associated with CLL/SLL among small B-cell lymphomas and therefore can serve as a useful IHC marker for diagnosis and differential diagnosis of CLL/SLL.11 LEF1 belongs to the LEF/TCF (T-cell–specific
transcription factor) family and is involved in early lymphocyte development as well as regulation of T-cell receptor α enhancer.\textsuperscript{12,13} LEF1 is normally expressed in pro-B cells and mature T cells but not in mature B cells.\textsuperscript{14,15} Its expression has also been downregulated in natural killer (NK) cells.\textsuperscript{16} Interestingly, gene expression profiling revealed overexpression of LEF1 in CLL/SLL.\textsuperscript{17,18} Our previous immunohistochemical study demonstrated strong nuclear staining of LEF1 in all 92 cases of CLL/SLL but in none of the other types of small B-cell lymphoma, including 53 cases of MCL.\textsuperscript{11} A subset of diffuse large B-cell lymphoma (DLBCL) did show variable staining for LEF1; however, DLBCL rarely causes diagnostic confusion with nontransformed CLL/SLL. Despite the value of IHC for LEF1 in the diagnosis of CLL/SLL, its utility is limited in the evaluation of lymphocytosis in the peripheral blood or other fluid samples. In addition, the interpretation of LEF1 staining in the B-cell subset in tissue section can be difficult in cases in which abundant reactive T cells are present since LEF1 is normally expressed in T cells.\textsuperscript{11}

The purpose of this study was to develop a flow cytometric assay to analyze LEF1 expression in lymphocytes and apply it to clinical samples to evaluate its utility in the diagnosis and differential diagnosis of CLL/SLL. We anticipated that flow cytometric analysis would be as sensitive as IHC in detecting nuclear LEF1 and could be used in analyzing samples such as peripheral blood. In addition, by taking advantage of gating strategies and the internal positive and negative reference cell populations, flow cytometry would be more specific in assessing LEF1 staining in the B-cell subset in cases in which abundant T cells are present, which may interfere with IHC interpretation.

**Materials and Methods**

**Study Cases and Pathologic Evaluation**

This study was approved by the institutional review board of Northwestern University (Chicago, IL). A total of 64 cases submitted for lymphoma evaluation between February 2013 and September 2013 were included in this study (22 lymph node biopsy specimens, 18 bone marrow aspirates, and 24 peripheral blood samples). Cases eventually diagnosed as DLBCL, Hodgkin lymphoma, or lymphomas of non–B-cell lineage were excluded from the study.

Routine histologic evaluation, IHC stains, and flow cytometric analysis were performed by hematopathologists independent of LEF1 status assessed by flow cytometry, and the subclassification of lymphoma was based on the current World Health Organization (WHO) criteria.\textsuperscript{19} In addition, IHC for LEF1 was performed on paraffin-embedded bone marrow and lymph node tissues, and FISH for t(11;14)-CCND1/IgH was performed on all peripheral blood samples with a CD5+ monotypic B-cell population, as previously described.\textsuperscript{11,20}

**Flow Cytometric Analysis of LEF1**

**LEF1 Staining Procedure and Data Acquisition**

Normal donor blood samples were used to validate the flow cytometric assay for LEF1. All samples were processed and analyzed within 24 hours of collection. The fixation and permeabilization procedure used in this study was slightly modified from Marvin et al.\textsuperscript{21} Briefly, an aliquot (typically 100 μL) of peripheral blood or bone marrow aspirate samples containing 500,000 to 2,000,000 cells or single-cell suspension prepared from lymph node tissue containing approximately 1,000,000 cells was used for flow cytometric analysis of LEF1. The cell suspension was mixed with antibodies to cell surface antigens (CD45, CD5, CD19) and incubated at room temperature for 15 minutes. The antibodies used were allophycocyanin-labeled anti-CD45 (J.33; Beckman Coulter, Miami, FL), phycoerythrin (PE)–labeled anti-CD5 (BL1a; Beckman Coulter), and PE-Cy7–labeled anti-CD19 (SU25C1; Becton Dickinson Biosciences, San Jose, CA). The sample was then fixed in formaldehyde (final concentration of 3%) at room temperature for 10 minutes. Subsequently, the cells were treated with 1 mL Procleen buffer (PerFix-P Cell Preparation Reagent Kit; Beckman Coulter) for 15 minutes at 37°C, centrifuged at 400 g, washed in phosphate-buffered saline (PBS), and resuspended in 1 mL PBS. The permeabilization of the cell and nuclear membranes was performed by dropwise addition of ice-cold methanol to the sample to a final concentration of 80% and incubation at –20°C for 30 minutes. The sample was centrifuged (400 g), washed with 2 mL PBS, and resuspended in 350 μL of the buffer containing 0.5% formaldehyde (PerFix-P Cell Preparation Reagent Kit; Beckman Coulter).

Data acquisition was performed on a standard Canto II eight-color flow cytometer (Becton Dickinson Biosciences) employing 405-nm, 488-nm, and 635-nm excitation lasers using a standard optical detection configuration. Data analysis was performed using Kaluza software (Beckman Coulter) and FACSDiva software (Becton Dickinson Biosciences).

**Qualitative Analysis of LEF1 Staining**

Based on the staining of the surface antigens (CD45, CD19, and CD5), three lymphocyte populations were
defined: B cells (bright CD45+/low side scatter/CD19+/CD5– or bright CD45+/low side scatter/CD19+/CD5+), T cells (bright CD45+/low side scatter/CD19–/CD5+), and the residual non-B (CD19–), non-T (CD5–) events, predominantly NK cells (bright CD45+/low side scatter/CD19–/CD5–). Figure 1.

LEF1 staining in the patient samples was analyzed and interpreted by two reviewers (C.L.G. and K.L.W.) independent of the histologic diagnosis and the results of routine flow cytometric analysis. The internal normal T cells served as a positive reference population, and the NK cells and/or CD5– B cells, in cases with CD5+ B cells, served as negative reference populations. The LEF1 staining was considered positive in B cells when there was a significant overlap in LEF1 staining distribution between B cells and T cells and no significant overlap with NK cells, whereas the LEF1 staining was considered negative in B cells when there was a significant overlap in staining intensity between B cells and NK cells or between CD5+ B cells and CD5– B cells when both populations were present.

Quantitative Analysis of LEF1 Staining

To provide an objective assessment of LEF1 staining in the B cells of interest, we performed a quantitative analysis by using the ratio of the median fluorescence intensities (MFIs) of LEF1 staining in B cells to that of NK cells (negative reference) and T cells (positive reference). Ratios of MFIs of B cells over NK cells (B/NK), B cells over T cells (B/T), and CD5+ B cells over CD5– B cells (CD5+ B/CD5– B) were calculated. The ratios were rank ordered, and a threshold value was determined to allow best separation of CLL/SLL from non-CLL/SLL cases.

Statistical Analysis

Unpaired Student t tests were used to analyze differences of MFIs between B cells, T cells, and NK cells in the normal peripheral blood samples. Analysis of variance was used to compare B/NK and B/T ratios between different diagnostic groups in the study cohort. Statistical analyses were performed using Minitab 16 (Minitab, State College, PA), and a significance level of P less than .05 was used.

Results

Case Information

Sixty-four samples from 52 patients (31 men and 21 women; age range, 30-90 years; median age, 63 years) were included in the study Table 1. Of these, 46 samples were diagnosed as B-cell lymphoma based on the WHO criteria, including 25 CLL/SLLs, six MCLs, four follicular lymphomas (FLs), four MZLs, three LPLs, and four cases of low-grade B-cell lymphoma/leukemia (3 CD5+/CD10– and 1 CD5–/CD10–) that could not be further classified. The remaining 18 samples included five cases of monoclonal B-cell lymphocytosis (MBL) with CLL phenotype and 13 cases with no evidence of lymphoma. Of the 25 cases of CLL/SLL (five lymph node, eight bone marrow, and 12 peripheral blood samples), four were new diagnoses (two lymph node, one bone marrow, and one peripheral blood sample), and 21 had a history of CLL/SLL. FISH for t(11;14) CCND1/IgH or IHC for cyclin D1 was negative in all 25 cases of CLL/SLL.

The three samples of CD5+, unclassifiable low-grade B-cell lymphoma were from two patients (patient 1: one peripheral blood sample; patient 2: one peripheral blood and one bone marrow sample) with lymphocytosis (patient 1: 7000/μL; patient 2: 25,000/μL). The phenotypes of the monoclonal B cells (patient 1: bright CD20+, CD5+, CD10, dim CD23+, FMC7+, CD79b+; patient 2: bright CD20+,
dim CD5+, CD10−, CD23−, FMC7+, CD79b+) were unusual for CLL/SLL, and FISH for t(11;14) was negative in both cases. The subsequent bone marrow biopsy specimens showed 10% (patient 1) and 60% (patient 2) involvement by a low-grade B-cell lymphoma with identical phenotype to that identified in the corresponding peripheral blood sample. Patient 1 had no lymphadenopathy or organomegaly, and patient 2 had mild splenomegaly with no lymphadenopathy. A diagnosis of CD5− MZL, especially splenic MZL, was favored in both patients.

**LEF1 Staining Pattern in Normal Lymphocytes by Flow Cytometry**

Flow cytometric analysis of intracellular LEF1 staining in 20 normal peripheral blood samples demonstrated brighter staining in T cells than in B and NK cells. The MFIs of LEF1 staining in T cells, B cells, and NK cells were 7.13 (range, 4.85-10.40; SD, 1.58; coefficient of variation [CV], 22%), 2.83 (range, 1.18-5.22; SD, 0.94; CV, 33%), and 2.52 (range, 1.30-4.31; SD, 0.75; CV, 30%), respectively. There were significant differences in the mean MFIs of LEF1 staining between T cells and B cells (P < .001) and between T cells and NK cells (P < .01), but no significant difference was found between B cells and NK cells (P = .31). The LEF1 staining distribution patterns were sufficient to separate positive staining in T cells from negative staining in NK or B cells by visual inspection (Figure 1).

It was noted that in normal donor peripheral blood samples, the T cells demonstrated a predominant peak of positive LEF1 staining; however, a small peak overlapping with the negative staining in normal B and NK cells was also present (Figure 1B,C).

**Quantitative Flow Cytometric Analysis of LEF1 Staining in Patient Samples**

The qualitative analysis of LEF1 staining in 64 patient samples by visual comparison of staining in the B cells of interest to the internal reference cell populations was performed independent of routine immunophenotyping results, histologic findings, and genetic information. A correlation of LEF1 staining results with the pathologic diagnosis showed that all 25 cases of CLL/SLL were LEF1 positive by flow cytometric analysis. In addition, five cases of MBL were also positive for LEF1. However, no LEF1-positive B cells were identified in the remaining cases, including 21 cases of other types of small B-cell lymphomas (six MCLs, four FLs, four MZLs, three LPLs, and four unclassifiable low-grade B-cell lymphomas) and 13 cases with no evidence of lymphoma (Table 1). In this study, 11 patients had two or more specimens analyzed, and the flow cytometric analysis of LEF1 yielded consistent results in all samples across different sample types in each individual patient. In addition, LEF1 status assessed by flow cytometry was consistent with LEF1 staining by IHC in all 12 cases that had tissue available for both studies (Table 1). The results of flow cytometric analysis of LEF1 in representative cases of CLL/SLL, MCL, and LPL are shown in Figure 2, Figure 3, and Figure 4, respectively.

It was interesting to note that although most T cells in normal donor blood were positive for LEF1, the T cells in approximately half (48%) of patients with CLL/SLL showed a distinct bimodal staining pattern for LEF1, with a prominent negative LEF1 peak in addition to the positive peak (Figure 2D). Of note, the brighter T-cell peak was used as the positive reference in this study.

**Quantitative Flow Cytometric Analysis of LEF1 Staining in Patient Samples**

To provide an objective assessment of LEF1 staining in patient samples, calculation of the ratios of MFIs of B cells to NK cells (B/NK), CD5+ B cells to internal CD5− B cells (CD5+ B/CD5− B), and B cells to T cells (B/T) was performed.
Flow cytometric analysis of lymphoid enhancer-binding factor 1 (LEF1) in chronic lymphocytic leukemia/small lymphocytic lymphoma. **A**, CD19 vs CD5 dot plot shows a CD5+ and a CD5− B-cell population, as well as a T-cell and a natural killer (NK)-cell population. **B**, CD5+ B cells show positive LEF1 staining compared with the NK cells. **C**, CD5+ B cells show positive LEF1 staining compared with the internal CD5− B cells. **D**, T cells in this case show a distinct bimodal staining for LEF1. The LEF1 staining in CD5+ B cells overlaps with the LEF1+ T-cell peak. **E**, Bone marrow aspirate showing sheets of small lymphocytes with condensed chromatin and scant cytoplasm. **F**, Bone marrow core biopsy specimen showing abnormal lymphoid infiltrate with a proliferation center. The lymphocytes are positive for LEF1 by immunohistochemical stain (inset).

In the 25 CLL/SLL samples, the B/NK ratio was calculated in 19 samples (one lymph node, seven bone marrow, and 11 peripheral blood samples) since most lymph node samples lacked a distinct NK cell population. The results showed that all 19 cases of CLL/SLL had a B/NK ratio greater than 1.6 (range, 1.61-3.73; mean [SD], 2.31 [0.60]). In contrast, all 11 cases of non-CLL/SLL lymphomas had a B/NK ratio equal to or less than 1.4 (range, 0.82-1.40; mean [SD], 1.07 [0.18]). Seven cases with no evidence of lymphoma had a B/NK ratio equal to or less than 1.25 (range, 0.54-1.25; mean [SD], 1.05 [0.25]). The B/NK ratio in CLL/SLL was significantly higher than in other small B-cell lymphomas as well as cases with no evidence of lymphoma (P < .01). The five cases of MBL had a B/NK ratio greater than 1.4, similar to CLL/SLL (range, 1.43-1.95; mean [SD], 1.66 [0.19]) Figure 5A.

All 64 patient samples in this study had sufficient T cells to be used as a positive reference. All 25 cases of CLL/SLL had a B/T MFI ratio equal to or greater than 0.45 (range, 0.45-1.44; mean [SD], 0.75 [0.23]), whereas all the remaining 21 cases of non-CLL/SLL lymphomas (range, 0.10-0.41; mean [SD], 0.24 [0.12]; P < .01) and 13 cases with no evidence of lymphoma (range, 0.09-0.42; mean [SD], 0.20 [0.12]) had a B/T MFI ratio less than 0.45. The B/T MFI ratio in CLL/SLL was significantly higher than that in non-CLL lymphomas and cases with no evidence of lymphoma (P < .01). The five cases of MBL had a B/T MFI ratio greater than 0.45, similar to CLL/SLL (range, 0.49-0.64; mean [SD], 0.56 [0.05]) Figure 5B.

In this study, only seven of 25 cases of CLL/SLL had a distinct internal CD19+/CD5− B-cell population as negative reference, which were insufficient to determine a valid cut-off for the CD5+ B/CD5− B MFI ratio to predict CLL/SLL.

**Discussion**

LEF1 is normally expressed in T cells and pro-B cells but not in mature B cells. However, our previous IHC study demonstrated universal, strong nuclear staining of LEF1 in CLL/SLL but not in other small B-cell lymphomas, including MCL, MZL, FL, and LPL. Therefore, LEF1 can serve as a valuable IHC marker for diagnosis and differential diagnosis of CLL/SLL, particularly when morphologic features and immunophenotype are atypical or unavailable. However, IHC does not allow for routine analysis of peripheral blood samples in which CLL/SLL is often first identified. Occasionally, a definitive diagnosis of CLL in a peripheral blood sample requires FISH analysis for t(11;14) to rule out MCL. When a low-level CD5+ monotypic B-cell population is identified in a peripheral blood sample, the question may arise as to whether this represents MBL or blood involvement by an MCL. Furthermore, discerning
LEF1 staining in B-cell populations by IHC in tissue sections can be difficult in cases with abundant reactive T cells since they normally express LEF1. This study aimed to develop a flow cytometric assay to detect nuclear expression of LEF1 and to evaluate its utility in the diagnosis and differential diagnosis of CLL/SLL.

The expression of LEF1 in normal peripheral T cells and lack of expression in normal mature B cells provide internal reference populations for flow cytometric analysis of LEF1 staining in the neoplastic B lymphocytes.23-25 LEF1 has been shown to be downregulated in NK cells because LEF1 and TCF1 play a redundant role in the regulation of NK cells,
and it is believed that TCF1 activity is upregulated, while LEF1 is suppressed in normal NK cells. Therefore, NK cells may also serve as a negative reference population for LEF1 staining. In our validation test using normal donor peripheral blood samples, we demonstrated consistently positive LEF1 staining in the T-cell population and consistently negative staining in both B- and NK-cell populations. In our experience, permeabilization of cell and nuclear membranes with methanol, as described in the Materials and Methods, is the key to successful intracellular LEF1 staining that is sufficient to differentiate positive and negative staining in the corresponding reference cell populations. In addition, the staining quality may be compromised when the specimen is older than 24 hours (data not shown).

In this study, LEF1 staining was analyzed by flow cytometry in patient samples using both a qualitative and a quantitative approach independent of the histologic findings and the results of routine flow cytometric analysis. In the qualitative analysis, LEF1 staining in the B-cell population was visually compared with the staining of internal T cells (positive reference) and NK cells (negative reference). The staining of CD5+ B cells was also compared with that of an internal CD5− B-cell population, if present. Using this method, all cases of CLL/SLL showed positive LEF1 staining, while all other small B-cell lymphomas and cases with no evidence of lymphoma were negative. In the quantitative analysis performed using the ratio of the MFI of LEF1 staining in B cells to that of NK cells and T cells, all cases with a ratio equal to or above the established cutoffs were corresponding to a final pathologic diagnosis of CLL/SLL, whereas all non-CLL/SLL small B-cell lymphomas and cases with no evidence of lymphoma had a B/NK ratio less than the cutoffs. In both approaches, the presence of a reliable control plays a key role for an accurate and reproducible interpretation of LEF1 staining. In cases that lack appropriate internal reference cell populations, a normal blood sample spiked into the patient sample can serve as robust controls to aid in the data analysis (data not shown).

Our results demonstrated that flow cytometric analysis of LEF1 accurately predicts CLL/SLL among small B-cell lymphomas in a variety of sample types, including lymph node, bone marrow, and peripheral blood. It is particularly useful when encountering CD5+ B-cell lymphoma/leukemias involving peripheral blood that do not show classic morphologic and/or immunophenotypic features of CLL/SLL. This is exemplified in the present study by three samples from two patients who were diagnosed with CD5+ small B-cell lymphomas/leukemia that could not be further subclassified. The patients had lymphocytosis, and the monotypic B cells demonstrated a phenotype highly unusual for CLL. FISH...
analysis was negative for t(11;14). Independent flow cytometric analysis for LEF1 was negative in all three samples, including a peripheral blood sample and a bone marrow aspirate from the same patient. The results of LEF1 added additional supporting evidence to indicate that these cases are unlikely to represent CLL/SLL, and the possibility of a CD5+ MZL was entertained. Consistent with the previous report, our study also demonstrated positive LEF1 staining in MBL. Therefore, flow cytometric analysis for LEF1 cannot be used to distinguish between CLL and MBL but may aid in the distinction of MBL with low-level blood involvement by MCL, particularly in cases with atypical phenotype.

Although our study showed high association of positive LEF1 staining by flow cytometry with CLL/SLL in small B-cell lymphomas, it is important to realize that a diagnosis of CLL/SLL should not be made solely on the basis of flow cytometric results on LEF1. As has been reported in our previous study, approximately one-third of DLBCLs are variably positive for LEF1, and LEF1 positivity has also been reported in acute lymphoblastic leukemia/lymphoma and Burkitt lymphoma. Therefore, correlation with morphologic findings and the overall immunophenotypic profiles is important for an accurate diagnosis. It is also noted that LEF1 is expressed in the normal B-cell precursors (hematogones) in the bone marrow, which is keeping in line with its role in early B-cell development. In our study, a very small population of LEF1+ B cells was observed in a subset of bone marrow samples that had B cells with an immunophenotype consistent with hematogones (data not shown).

It is interesting that although most T cells in normal donor blood are positive for LEF1, the T cells in nearly half of the patients with CLL/SLL showed a bimodal staining pattern with a prominent T-cell population negative for LEF1. Further studies are needed to explain the phenomenon. It has been reported that LEF1 is downregulated in naive CD8+ T cells following antigen encounter, and LEF1 is the most differentially expressed transcription factor between naive T cells and antigen-experienced CD8+ T cells. Several lines of evidence indicate that T-cell function is profoundly dysregulated in patients with CLL/SLL. A common finding is a significant reduction in naive CD4+ and CD8+ T cells and accumulation of terminally differentiated effector memory T cells. Marked abnormalities in the expression of certain key activation and interaction molecules on the T cells of patients with CLL have also been demonstrated. Therefore, the decreased or lost LEF1 expression in a significant population of T cells observed in our study may reflect the altered T-cell function in patients with CLL/SLL.

In summary, flow cytometric analysis of LEF1 is sufficient to separate CLL/SLL from other small B-cell lymphomas and may serve as a useful tool to aid in the diagnosis and differential diagnosis of CLL/SLL, particularly in samples in which IHC has limited utility. However, a diagnosis of CLL/SLL cannot be based solely on the flow cytometric results on LEF1 and should be interpreted in correlation with histologic findings and the immunophenotypic profile of the monotypic B cells.

References


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