Downregulation of Lymphoid enhancer-binding factor 1 (LEF-1) expression (by immunohistochemistry and/ flow cytometry) in chronic Lymphocytic Leukemia with atypical immunophenotypic and cytologic features

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Abstract

Introduction: Lymphoid enhancer-binding factor 1 (LEF-1) overexpression has been recently remarkably reported in chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) and has shown utility in distinguishing CLL/SLL from other B-cell lymphomas. CLL has a well-defined immunophenotype, yet, some cases of CLL demonstrate atypical morphology/phenotype reflected by low Matutes score (atypical CLL). Till date, LEF1 expression has not been systematically studied in cases of CLL with atypical features.

Methods: In this study, LEF-1 expression was assessed by two different techniques, (immunohistochemistry and flow cytometry), to investigate the expression profile of LEF-1 in cases of CLL/SLL, in comparison with other low-grade B-lymphomas and CLL with atypical features, including atypical immunophenotype and CLL with increased prolymphocytes or morphologically atypical cells.

Results: We found that LEF-1 expression is downregulated in CLL with atypical immunophenotype/features compared to classic CLL; Chi-Square $P < .0001$. The ratio for LEF-1 expression in malignant B-cells/NK (by flow cytometry) in CLL/SLL with classic immunophenotype was higher than atypical CLL and is significantly higher in other small B-cell lymphomas ($P < .01$). Absence of LEF-1 expression in CLL/SLL is correlated ($P < .05$) with downregulation of CD5, CD23, CD200, expression of FMC7, brighter expression of CD79b, brighter expression of surface light chain, increased prolymphocytes and lower Matutes score.

Conclusion: As downregulation of LEF-1 expression is well correlated with atypical CLL, we suggest adding LEF-1 to Matutes score as a beneficial marker to differentiate...
INTRODUCTION

B-cell non-Hodgkin lymphoma (NHL) is a heterogeneous group of lymphoproliferative malignancies with different clinical behavior and treatments. Lymphomas are diagnosed based on histopathologic features and their specific immunophenotypic profile. B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) is the most prevalent adult leukemia in the Western world, characterized by progressive accumulation of monotypic mature B-cells and show a relatively heterogeneous clinical course. This pathologic, immunophenotypic and clinical heterogeneity has significant influence on clinical judgments, treatment protocols, and even the overall survival.\(^1\)

CLL has a distinct immunophenotype (IPT) (CD19+, CD5+, CD23+, CD20 dim+, CD79b dim/-, FCN7–, CD10–, surface IgM dim + and CD200–), this is to a part defined by the most widely accepted scoring tool for the immunophenotypic characterization of CLL, defined more than 20 years ago (Matutes score), which mandates strong expression of CD5 and CD23, lack of FMC7 as well as low or absent expression of CD79b and weak surface IgM (sigM).\(^2\)\(^3\) Yet, there are some equivocal cases with less typical IPT, especially those with a low Matutes score (including rare cases of CD5-negative CLL/SLL), these cases are referred to as atypical CLL (aCLL). According to Matutes score, CLL is usually correlates with score 4,5 scores of ≤ 3 seen with atypical CLL and score of 0-2 is unusual in CLL.

The differential diagnosis of CLL/ SLL from its mimickers of CD5-positive small B-cell lymphoma can be challenging in some cases due to overlapping morphologic and immunophenotypic features, particularly when analyzing peripheral blood or limited tissue samples.\(^4\) Even with the introduction of CD200 as a useful marker for CLL, some cases remain diagnostically challenging emphasizing the necessity for additional markers in order to confirm or exclude the CLL diagnosis.

Several relatively recent studies have demonstrated dysregulation of WNT/\(\beta\)-catenin signaling in CLL.\(^5\) The ultimate mediator of this pathway is a nuclear complex of lymphoid enhancer-binding factor (LEF)/T-cell factor (TCF) and \(\beta\)-catenin.\(^6\)\(^7\) Lymphoid enhancer-binding factor (LEF-1) is a pivotal transcription factor that regulates cell proliferation and survival and has a critical role in lymphopoiesis. LEF-1 is normally expressed in T-cells and precursor B-cells and is switched off in mature B-cells; however, gene expression profiling studies have revealed overexpression of LEF-1 in CLL/SLL in comparison with normal B-cells.\(^8\) LEF-1 has been shown to be suppressed in normal natural killer (NK) cells, therefore, NK may also serve as a negative reference population for LEF-1 staining.\(^9\)

Few studies had recently evaluated the expression of LEF-1 by immunohistochemistry (IHC) and concluded that nuclear staining of LEF-1 is expressed in almost all cases of B-cell CLL/SLL, and negative in nearly all other small B-cell lymphomas.\(^10\)\(^11\)

Although LEF-1 expression has shown utility in distinguishing CLL from other small B-cell lymphomas, yet its use in the diagnostic setting is still limited. In addition, a substantial number of CLL patients with negative LEF-1 expression has been reported in the literature. However, the morphologic, immunophenotypic and cytogenetics features of LEF-1 negative CLL cases have not been studied before.

This is a single center study of LEF-1 expression using two different techniques IHC and/or flowcytometry (FCM) analysis to test the utility of using LEF-1 (by either method) for optimizing the diagnosis B- cell lymphoma including CLL/SLL. In this study, we will investigate the expression profile of LEF-1 in cases of classic CLL/SLL, in comparison to low-grade B-non-Hodgkin’s lymphoma (other than CLL), and CLL with atypical features (atypical CLL; aCLL): including those with atypical IPT, CLL with increased prolymphocytes/atypical lymphoid cells and CLL with complex karyotype.

In addition, we will present and discuss the morphologic, immunophenotypic, genetic features/markers associated with LEF-1 expression in patients with CLL, in order to find out the LEF-1 expression profile in patients with aCLL and whether LEF-1 would be of diagnostic importance in differentiating these patients from non-CLL patients.

MATERIAL AND METHOD

This is a mixed design (includes both prospective and retrospective data), diagnostic study with no interventions. All patients were diagnosed in National Center for Cancer Care & Research (NCCCR), a member of Hamad Medical Corporation (HMC) in Doha-Qatar between January 2016 and January 2019. The NCCCR is the only cancer treatment center in Qatar and is part of the government funded national health system. The study had ethical approval from IRB (Institutional research board; # MRC-16164/16). Informed consents are taken for patients in whom peripheral blood (PB) samples were collected to perform LEF-1 by FCM. Patients with already collected tissues were waived from consenting.

The study included patients diagnosed as CLL/SLL, Mantle cell lymphoma (MCL), Follicular lymphoma (FL), Marginal zone lymphoma...
(MZL), Lymphoplasmacytic lymphoma (LPL), Hairy Cell leukemia (HCL). Only patients who had FCM immunophenotyping (IPT) and or tissue biopsy to confirm the lymphoma diagnosis were included in the study.

The diagnosis was made on PB and or BM/LN tissue biopsy, according to WHO 2016 diagnostic criteria. All patients included in our study, were originally diagnosed and classified as (CLL, atypical CLL or B-cell lymphoma other than CLL) at a time prior to data analysis. LEF-1 is not routinely used in our diagnostic service. LEF-1 was performed blindly without a prior information about the cases’ diagnosis or subclassification. Lymphoma diagnosis on P.B &/or tissues was independently done & concurred by two hematopathologists and/or two histopathologists respectively. No modifications in patients’ classification were done later in the study based on LEF-1 result or any other variable.

Morphologic features, multiparameter FCM analysis, immunohistochemical data and cytogenetic/ Fluorescence in situ hybridization (FISH) results were extracted and reviewed individually by the research team. FISH panel (including del13q, trisomy CEP12P, ATM11q22.3 del, lgh4/CCND1 and TP53) was performed for all cases of CLL (including atypical CLL). Modified Matutes scoring system was calculated for all cases of CLL for which flow cytometry result was available (ie, diagnosis made on peripheral blood). Matutes score includes one score point for each of: surface Ig weak, CD5, CD23, CD38, light chain intensity), FISH markers (del 13q, trisomy 1q), karyotype (KT) findings.

LEF-1 expression was assessed by IHC and/or FCM technique. The choice of the technique or combination of more than one technique for a single case; was decided based on the type of the available material submitted at the time of diagnosis.

For cases with classic CLL and aCLL, LEF-1 expression was correlated with morphologic features: increased atypical lymphoid cells (clefting/ folding, larger less mature forms) and cases with increased prolymphocytes, different immunophenotypic markers (CD5, CD79, CD200, CD23, CD38, light chain intensity). FISH markers (del 13q, trisomy 12) and karyotype (KT) findings.

### 2.2 Flow cytometric analysis of LEF1

EDTA blood samples were lysed using BD Pharm Lyse™ lysing solution (BD Biosciences), washed and then fixed using BD Cell Fix solution (BD Biosciences). Afterward, resuspended in Recovery™ Cell Culture Freezing Medium (Thermo Fisher Scientific) and stored in −80°C until immunostaining was performed. Briefly, 100 μL samples containing 1-2x10^6 cells were used for LEF1 flow cytometric analysis using the BB515-labeled anti-CD45 (HI30; BD Biosciences), BV421-labeled anti-CD5 (UCHT2; BD Biosciences), PE-labeled anti-CD19 (HIB19; BD Biosciences) and Alexa Fluor 647-labeled anti-LEF1 (C12A5; Cell Signal Technologies). Initially, the cell suspension was incubated with antibodies against cell surface antigens (CD45, CD5, CD19) at room temperature for 30 minutes. Cells were washed by adding 1 ml of FBS containing staining buffer (BD Biosciences) per tube and centrifuged (300g, for 5 min). The cell pellet was resuspended in 100 μL of BD Cytofix/Cytperm Fixation/Permeabilization Solution (BD Biosciences) per tube, incubated for 30 minutes at room temperature and then washed with 1 ml of 1X BD Perm/Wash Buffer and resuspended in BD Perm/Wash Buffer. The cell suspension was mixed with 2 μL of anti-LEF1 antibody, incubated at room temperature for 30 minutes. The sample was washed in PBS, resuspended in 300 μL of the staining buffer for analysis on flow cytometer. Data acquisition was performed on an 18 color BD LSRFortessa (BD Biosciences) flow cytometer equipped with 355-nm, 405-nm, 488-nm, 561-nm and 640-nm excitation lasers and a standard optical detection configuration. Data analysis was performed using FACSDiva software V8 (BD Biosciences).

The normal T-cell population (bright CD45+/low side scatter/CD19-/CD5-) was used as an internal positive reference population, and the NK cell population (bright CD45+/low side scatter/CD19-/CD5-) and/or CD5- B-cell population (bright CD45+/low side scatter/CD19+/CD5+) was used as a negative reference populations in the analysis of CD5 + B-cell population (bright CD45+/low side scatter/CD19+/CD5+).

A quantitative analysis for the assessment of LEF1 staining in the malignant B-cells of interest was carried out by calculating the ratio of the median fluorescence intensities (MFI) of LEF1 staining in malignant B- cells to that of NK cells (negative reference) and T-cells.
(positive reference). Ratios of MFI's of malignant B-cells over NK cells (malignant B/NK) and malignant B-cells over T-cells (B/T), were calculated to achieve best separation of CLL/SLL from non-CLL/SLL cases.

In difficult cases, correlation with FCM results (done at diagnosis) was performed for accurate separation of malignant B-cells, validation of precise percentages of residual normal B, T-cells and NK cells.

### 2.3 | Statistical analysis

Categorical and continuous values were expressed as frequency (percentage) and mean ± SD or median and range as appropriate. Descriptive statistics were used to summarize demographic, laboratory, morphologic, flow cytometry and cytogenetics features of the patients. The Kolmogorov-Smirnov (K-S) test was used to assess the normality of the data distribution. Associations between two or more qualitative variables were assessed using Chi-square test or Fisher Exact test as appropriate (to examine the association between morphologic, immunophenotypic, genetic features/markers with LEF-1 expression, if any). Quantitative data between the two independent groups were analyzed using unpaired “t” and Mann Whitney U tests as appropriate. To assess diagnostic and predictive accuracy of LEF-1 expression (in cases of CLL and atypical CLL), the sensitivity, specificity, positive and negative predictive values (along with their corresponding 95% confidence limits) were calculated. ROC analysis was performed to determine an optimum and suitable cutoff value for respective test/markers to evaluate accuracy of such diagnostic markers against the gold standard. A two-sided P-value < 0.05 was considered to be statistically significant. Scatter diagram and box plot were constructed to depict the distribution of malignant B/NK ratio values for LEF-1 expression by flow cytometry across CLL, atypical CLL and non-CLL cases. All Statistical analyses were done using statistical packages SPSS 24.0 (SPSS Inc Chicago, IL) and Epi InfoTM 2000 (Centres for Disease Control and Prevention, Atlanta, GA).

### 3 | RESULTS

Our cohort includes a total number of 108 cases encompassing different B-lymphoma entities categorized into three groups: classic CLL, aCLL and B-cell lymphomas other than CLL. Our cohort is especially enriched in CLL/SLL (66 case, 61%) and 42 case (38.5%) of other B-cell lymphomas: (MZL; 17 case, FL; 12 case, MCL; 7 cases, LPL; 3 cases, and one case of each HCL, DLBCL (on top of FL) and mononodal B-cell lymphocytosis (MBL). Within the CLL group (including aCLL), 33 patients were diagnosed by peripheral blood flow cytometry alone without tissue diagnosis. Cases are summarized in (Table 1). Among cases of CLL, 24 cases out of 66 (36%) were classified as atypical CLL (aCLL). Typical CLL had classic morphology of small mature-looking lymphoid cells with scanty cytoplasm, clumped chromatin and no increase in prolymphocytes. All cases classified as atypical CLL in our cohort are negative for t(11;14)(q13;q32), IgH/CCND1 by FISH &/or Cyclin D1 immunostain and do not fulfill the diagnostic criteria of any B-cell lymphomas other than CLL.

#### 3.1 | Cytogenetics findings within cases of CLL and atypical CLL

del13q (17/51) (33.3%), trisomy CEP12P:17/61 (5.8%), ATM11q22.3del: 3/51 (5.8%), IgHCCND1 1/65(1.5%) (composite MCL and CLL lymphoma), TP53/ del 17p was not detected in any tested patient in our cohort: 0/47 (0%). Two patients with atypical CLL had more than one cytogenetic abnormality detected by FISH; deletion of both ATM11q22.3del and del13q in one case and combined del13q and trisomy CEP12P in the other case.

KT results were available for 35 out of 66 cases. 9 patients showed normal KT, a complex KT (with 3 or more chromosomal rearrangement) was detected in 4 patients who were all classified as atypical CLL. Two cases of otherwise typical CLL showed t(14;18)(q32;q21)/IGH/BCL-2, two cases of typical CLL had unusual chromosomal translocation del(6)(q21q23) in one case and t(1;8)(p36.1;q24.1) in the other one. KT did not show any added abnormality other than those detected by FISH in 18 patients.

| Diagnosis               | No. of cases, % Total N = 109 | LEF-1 + by FCM | LEF-1 + by IHC | No of positive/ Total No. (%) |
|------------------------|-------------------------------|----------------|---------------|-----------------------------|
| CLL/SLL: 66/108 (61.1%)|                               |                |               |                             |
| Classic CLL/SLL        | 42 (38.5)                     | 19             | 21            | 40/42 (95.2)               |
| Atypical CLL, CLL/PLL  | 24 (22)                       | 7              | 6             | 13/24 (54.2)               |
| Non-CLL: 42/108 (38.8%)|                               |                |               |                             |
| MZL                    | 17 (15.6)                     | 1              | 2             | 3/17 (17)                  |
| FL                     | 12 (11)                       | 0              | 0             | 0/12 (0)                   |
| MCL                    | 7(6.4)                        | 0              | 0             | 0/7 (0)                    |
| HCL                    | 1(0.9)                        | 0              | 0             | 0/1 (0)                    |
| LPL                    | 3(2.8%)                       | 0              | 0             | 0/3(0)                     |
| DLBCL/FL               | 1(0.9)                        | 0              | 0             | 0/1 (0)                    |
| MBL                    | 1(0.9)                        | 1              | 0             | 1/1(100)                   |

**TABLE 1** Results of LEF1 Immunohistochemistry and Flow cytometry in Various Lymphoma Entities
3.2 | \textit{LEF-1} Immunohistochemistry results

3.2.1 | Results for \textit{LEF-1} expression among CLL and non-CLL cases are summarized in Table 1

IHC was performed on 72 cases (LN and BM biopsy) (66.6%), including CLL/SLL (35 case), other B-cell lymphomas other than CLL (37 cases). The great majority of cases of CLL/SLL show nuclear positivity for \textit{LEF-1}, however, 7 cases (20%) were negative for \textit{LEF-1} by IHC. All cases of CLL/SLL with negative \textit{LEF-1} by IHC had been classified as atypical CLL. The latter included two cases of CLL with increased prolymphocytes > 10 (CLL/PLL) and one case with Richter’s transformation of CLL/PLL.

\textit{LEF-1} showed heterogenous expression pattern; scored II and III (strong) in the great majority of CLL cases with classic

\textbf{FIGURE 1} Cases with classic CLL with typical IPT (Matutes score 4-5), showing diffuse strong \textit{LEF-1} expression graded as (III) (A-D). On lymph node (10x) (A), on BM trephine biopsy (50 x)(B), and on lymph nodes (50x) (C,D). T-cells showed stronger \textit{LEF-1} positivity compared to malignant B-cells. Examples of \textit{LEF-1} heterogenous expression in cases of CLL (E-H). Weak \textit{LEF-1} in a LN of a case of CLL with atypical IPT (grade I) (Matutes score 3)(20x) (E). Heterogenous \textit{LEF-1} expression on BM biopsy (50x), in cases of CLL with classic IPT scored I-II, II & II-III according to strength and extent of \textit{LEF-1} expression (20X)(F-H). Negative \textit{LEF-1} in non-CLL lymphomas (I-L). A LN of a FL showing a whole follicle negative \textit{LEF-1} immunostain (10x) with scattered interfollicular positive T-cells (I). BMB (50X) of FL with negative \textit{LEF-1} (J). LN of MZL (10x) with negative \textit{LEF-1} (K). BMB of a LPL showing a lymphoid aggregate negative for \textit{LEF-1}(20x) (L)
immmunophenotype (Figure 1; A-D) while weaker intensities I-II were shown in the great majority of cases of atypical CLL including three cases of CLL with negative CD5 (Figure 1; E-H).

All non-CLL cases were negative for LEF-1 by IHC (Figure 1; I-L), except one MZL case which was LEF-1-positive.

### 3.3 LEF-1 results by flow cytometry

FCM for LEF-1 was performed on PB for 47 cases (37 CLL and 10 non-CLL cases), for which the MFI for LEF-1 in malignant cells /NK cells was calculated. Since LEF-1 was tested for its reliability to detect CLL out of other B-cell lymphomas, we postulated that CLL to...
be the defining variable in a receiver operating characteristic (ROC) curve model plotting LEF-1’s sensitivity versus 1-specificity. Using ROC curve, the discriminative ability of the model was found to be good with an area under the ROC curve (as a diagnostic marker for CLL) as 0.767 (95% CI 0.58 to 0.96; P = .010). The suggested optimum cutoff value for LEF-1 positivity determined by the Youden’s index was ≥ 1.458 to be most relevant for the diagnosis of CLL (sensitivity 73.7% and specificity 80%). Cases were considered positive for LEF-1 if the ratio of LEF-1 MFI in malignant cells/NK cells ≥ 1.458. (Supplementary 1; A).

The malignant cells/NK ratio for LEF-1 expression in CLL/SLL with classic IPT was higher than CLL with atypical immunophenotypic/cytologic features (aCLL) and is significantly higher in other small B-cell lymphomas (P < .01). This was evident by both scatter diagram and box plot (Supplementary 1; B, C). Among CLL group, 27 cases were positive for LEF-1 by FCM while 10 patients were negative with malignant B/NK ratio < 1.458 (range, 0-1.4; mean [1.0]). CLL with negative FCM LEF-1 encompasses: 7 cases classified as atypical CLL, two cases of CLL/PLL and one case with typical CLL.

For non-CLL cases, 8 out of 10 cases, were negative for LEF-1 FCM and had a malignant B/NK ratio lesser than 1.458 (range, [0-3.8]; mean [1.5]). In contrast, 2 of non-CLL/SLL lymphomas were positive for LEF-1 by FCM (ie, had a malignant B/NK ratio ≥ 1.458). These included one MZL, and one case of MBL. One CLL case showed discrepant LEF-1 expression; negative by IHC and positive by FCM (B/NK ratio = 2), this case was classified as atypical CLL (Matutes score 3) and had trisomy 12. Figure 2 shows examples of LEF-1 expression by flow cytometry in different groups.

The diagnostic performance of LEF-1 Expression by each IHC and FCM test used alone for the detection of CLL in the setting of a small B-Cell lymphoma is summarized in Table 2. IHC showed a sensitivity, specificity, Positive Predictive Value % (PPV), Negative Predictive Value % (NPV), and positive likelihood ratio of 77.1%, 97.3%, 96.4%, 81.8%, and 28.54 respectively. FCM had a sensitivity, specificity, PPV, NPV, and positive likelihood ratio of 70.3%, 88.9%, 96.3%, 42.1%, and 6.32 respectively. Also, both LR + and NPV were found to have significantly higher values (P < .05) in IHC analysis compared to FCM, other diagnostic indices were also noted to be higher for IHC analysis, though their differences were statistically insignificant (P > .05).

LEF-1 (either by FCM or IHC) was positive in 51 out of 66 cases of CLL (77.2%), most of the negative cases were cases with atypical immunophenotype with low Matutes score and CLL with increased prolymphocytes. Detailed clinical and pathological features of CLL cases (typical and atypical) with negative LEF-1 expression were summarized in (Supplementary 2).

### 3.4 Association between various markers and LEF-1 expression was summarized in Table 3

CDS, CD200, CD23 positivity percentage and dim light chain expression had significantly higher proportions in patients with positive LEF-1 expression (P < .05). In contrast, CD79, FMC7 positivity percentages were found to be significantly lower in patients with LEF-1 positive expression (P < .05). No statistically significant association between any of cytogenetics findings and LEF-1 expression. The proportion of Trisomy 12 was found to be similar in both LEF-1

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**Table 2** Various Diagnostic indices (Sensitivity, Specificity, PPV and NPV) of LEF1 Expression for Diagnosis of CLL in the Setting of a Small B-Cell Lymphoma and its morphologic mimics

|                  | LEF1 by IHC | LEF1 by FCM | Difference (P-value) | LEF1 by IHC and/or FCM |
|------------------|-------------|-------------|----------------------|------------------------|
| **Sensitivity %, (95% CI)** | 77.1 (60.98, 87.93) | 70.27 (54.22, 82.51) | 6.83 (0.514) | 78.46 (67.03, 86.71) |
| **Specificity %, (95% CI)** | 97.3 (86.18, 99.52) | 88.89 (56.5, 98.01) | 8.41 (0.260) | 92.86 (80.99, 97.54) |
| **Positive Predictive Value % (PPV) (95% CI)** | 96.43 (82.29, 99.37) | 96.3 (81.72, 99.34) | 0.13 (0.979) | 94.44 (84.89, 98.09) |
| **Negative Predictive Value % (NPV) (95% CI)** | 81.82 (68.04,90.49) | 42.11 (23.14, 63.72) | 39.71 (0.002) | 73.58 (60.42, 83.56) |
| **Diagnostic Accuracy (95% CI)** | 87.5 (77.92, 93.28) | 73.91 (59.74, 84.4) | 13.59 (0.061) | 84.11 (76.02, 89.84) |
| **Likelihood ratio of a Positive Test (95% CI)** | 28.54 (3.94, 20.7) | 6.32 (0.86, 46.35) | 22.22 (0.003) | 10.98 (5.66, 21.34) |
| **Likelihood ratio of a Negative Test (95% CI)** | 0.235 (0.18, 0.30) | 0.335 (0.27, 0.41) | -0.10 (0.919) | 0.232 (0.20, 0.27) |
positive and negative patients, and this difference was noted to be statistically insignificant ($P = .865$). CLL Matutes score was found to be positively correlated with malignant B/NK ratio; however, the strength of the correlation/linear relationship was very low and statistically insignificant (Pearson ($r$) = 0.148; $P = .396$). Unpaired statistical t-test indicates that the mean CLL Matutes score was significantly higher among CLL patients with positive LEF-1 expression detected by IHC, FMC, and either by IHC or FCM compared to LEF-1-negative CLL group (4.41 ± 0.67 vs 3.08 ± 1.24; $P < .0001$). Supplementary table (3).

3.5 | Correlation between different clinical and pathologic features of typical CLL compared to atypical CLL (aCLL) are summarized in supplementary (4)

The proportion of Trisomy 12 was observed to be significantly lower (6/40, 15%) in patients with typical CLL compared to aCLL (11/21, 52.4%); Chi-Square $P = .002$. Similar, was the case when CD38 positivity was compared between CLL and aCLL (22% vs 47.6%; $P = .038$). Mean CLL score was observed to be significantly higher among CLL cases compared to aCLL (4.68 ± 0.47 vs 3.26 ± 0.92; $P < .0001$). Other features such as age, gender, CD200 and del 13q (positivity and partial combined) were insignificantly associated with CLL and aCLL ($P > .05$). LEF-1 expression was found to be significantly higher in CLL with typical features compared to aCLL; Chi-Square $P < .0001$.

4 | DISCUSSION

In this mixed design study, we evaluated the usefulness and applicability of LEF-1 in the diagnostic setting of a small B-cell lymphoma. Since IHC for LEF-1 does not allow routine testing of PB samples in which CLL is usually first identified hence, LEF-1 assessment by peripheral blood FCM was added as a supplementary method. Till date, a single recently published study had demonstrated the value of LEF-1 expression assessed by multiparameter FCM in the diagnosis of CLL/SLL. Up to our knowledge, a comparison between LEF-1 expression by FCM and IHC methods has not been conducted before. We found out that various diagnostic accuracy indices strongly indicate that LEF-1 IHC had much better and accurate diagnostic performance compared to FCM for diagnosing CLL. It also differentiates it from other small B-Cell Lymphomas. However, one of the limitations for IHC are cases rich in T-cell background which precludes proper assessment of LEF-1 expression within the malignant B-cells. On the other hand, FCM can be easily performed on fresh blood samples, which makes it an applicable method to be utilized in the majority of CLL cases, for which the initial diagnosis is made on peripheral blood samples.

We are frequently challenged with a considerable number of atypical CLL, as a relatively large proportion of CLL diagnosed in our center exhibit one or more of atypical cytologic or immunophenotypic features, estimated by ~ 36% of the total number of CLL cases. Immunophenotypically, aCLL usually exhibit less common features

### TABLE 3 showing the association between various immunophenotypic and cytogenetics markers and LEF-1 expressions. CD5, CD200, CD23 positivity percentage and dim light chain expression had significantly higher proportions in patients with positive LEF-1 positive expression ($P < .05$). In contrast, CD79, FMC7 positivity percentages were found to have significantly lower in patients with LEF-1 positive expression ($P < .05$). However, the proportion of Trisomy 12 was found to be similar in both LEF-1 positive and negative expressions, and this difference was noted to be statistically insignificant ($P = .865$). No statistically significant difference for CD38 expression or del13q between LEF-1 positive and LEF-1 negative cases

| Marker     | Positive LEF-1 (%) | Negative LEF-1 (%) | P-value |
|------------|--------------------|--------------------|---------|
| CD5        |                    |                    | <0.0001 |
| Positive   | 50 (92.6%)         | 16(53.3%)          |         |
| Negative   | 2(3.7%)            | 10(33.3%)          |         |
| Partial    | 2 (3.7%)           | 4 (13.3%)          |         |
| CD79       |                    |                    | 0.004   |
| Positive   | 15 (28.3%)         | 17(68%)            |         |
| Negative   | 25 (47.2%)         | 6 (24%)            |         |
| Dim        | 13 (24.5%)         | 2 (8%)             |         |
| FMC7       |                    |                    | <0.0001 |
| Positive   | 5 (9.4%)           | 16(64%)            |         |
| Negative   | 36 (67.9%)         | 3 (12%)            |         |
| Partial    | 12 (22.6%)         | 6(24%)             |         |
| CD200      |                    |                    | 0.004   |
| Positive   | 32 (82.1%)         | 7 (41%)            |         |
| Negative   | 2 (5.1%)           | 6 (35.3%)          |         |
| Partial    | 5 (12.8%)          | 4 (23.5%)          |         |
| CD23       |                    |                    | 0.001   |
| Positive   | 46 (86.8%)         | 12 (48%)           |         |
| Negative   | 1 (1.9%)           | 5 (20%)            |         |
| Partial    | 6(11.3%)           | 8(32%)             |         |
| CD38       |                    |                    | 0.934   |
| Positive   | 9 (17%)            | 5 (20.8%)          |         |
| Negative   | 34 (65%)           | 15(62.5%)          |         |
| Partial    | 9 (17%)            | 4 (16.6%)          |         |
| Del 13q    |                    |                    | 0.07    |
| Positive   | 15 (36.5%)         | 1 (9%)             |         |
| Negative   | 26 (63.4%)         | 10 (90.9%)         |         |
| Light chain|                    |                    | <0.0001 |
| Dim        | 45 (86.5%)         | 7 (29.2%)          |         |
| Bright     | 7 (13.5%)          | 17(70.8%)          |         |
| Trisomy 12 |                    |                    | 0.865   |
| Yes        | 12 (24.5%)         | 4 (26.7%)          |         |
| No         | 37 (75.5%)         | 11 (73.3%)         |         |

*Bright indicates brighter than dim expression which is usually seen in CLL and this includes (normal and bright).
(such as brighter expression of light chain, FMC7 expression, CD79 upregulation and/or downregulation of CD5, CD23 or CD200) and, accordingly associated with a potentially lower Matutes score. In addition, trisomy 12 (frequently detected in aCLL) acts a confounder. Due to the trisomy 12 frequent correlation with less favorable clinical course, distinction between CLL and aCLL is clearly clinically relevant.

In 1989, The French-American-British (FAB) group, defined three different CLL cytologic subtypes; including a classical form and two other types, CLL/ prolymphocytic leukemia (with 10%-55% prolymphocytes), and a second subtype described by atypical looking lymphoid cells (clefted or folded cells), subsequently called aCLL.

It had been suggested by Marc Sorigue that the lack of well-standardized definition of aCLL over years, could be partially due to absence of a well-defined entity in the WHO over its successive versions. Obviously, there is a lack of consensus on aCLL in the literature. Some groups defined it by MS < 4 or a lack of CD23, while other groups, such as Marionneaux S, et al defined aCLL according to the cytologic features. Given the inconsistency of the cytological and, particularly, immunophenotypic features of what has been called aCLL in different studies, it is likely that aCLL is not a uniform biological entity. Atypical CLL cases in our cohort were defined as those having Matutes score 2-3 or cases with Matutes score 4 in addition to the presence of one or more of the following features: negative CD5 or increased prolymphocytes/morphologically atypical cells.

In concordance with previous literature, we confirmed that LEF1 expression is primarily confined to CLL among small B-cell lymphomas. Upon literature review, we found a considerable difference in the percentage of LEF-1 positivity in CLL among different studies. While Tandon et al. reported a 100% expression of LEF-1 in CLL by IHC; Thomas Menter reported a relatively low sensitivity (70%) with about 30% of CLL cases did not express LEF-1. In our cohort, the sensitivity of LEF-1 expression by IHC was 77.1 and 78.46% by either FCM or IHC.

In contrast to a previous LEF-1 immunohistochemical studies which had reported a uniform staining pattern in all CLL cases or in the majority of cases, a heterogenous LEF-1 staining pattern was demonstrated in our CLL cohort (Figure 1, A-H). While, this could be in part technically related; however, a weaker staining intensity (I-II) was recorded in most cases of aCLL, including three cases of CLL with negative CD5. We did not detect any significant difference in LEF-1 intensity among different tissues. However, the interpretation in lymphoid tissue (ie, LN, spleen) was easier, compared to BM especially in cases with interstitial/subtle marrow infiltration.

The most frequent cytogenetic abnormality within CLL group in our cohort, was del13q (17/51) (33.3%) identified by FISH followed by trisomy 12 detected in 17/61 (26.2%). Similarly, Döhner H, et al. had reported that trisomy 12 as the second most frequent cytogenetic abnormality in CLL, (in 16% of patients at initial diagnosis). Patients with CLL and trisomy 12 frequently have unmutated IGHV, ZAP-70 positivity, frequent NOTCH1 mutations which usually indicates a worse prognosis and an increased risk of Richter syndrome. Deletion 17p (del 17p) is a rare genomic aberration found in patients with CLL. The incidence of del 17p varies from 5% to 9% in newly diagnosed CLL while it is up to 50% in patients with relapsed/refractory CLL. Interestingly, TP53/ del 17p was not detected in any tested patient of our cohort: 0/47 (0%). However, we did not screen for TP53 mutation by molecular genetics. It is worth noting that all CLL cases with complex karyotype in our cohort showed negative LEF-1 and were associated with one or more of atypical immunophenotypic/ cytologic features and all of them showed unfavorable clinical course. This could indicate that LEF-1 downregulation could serve as potential poor prognostic marker.

No previously published data regarding the clinicopathologic features of CLL cases with negative for LEF-1. The detailed pathological and clinical characteristics of 14 CLL cases with negative LEF-1 expression summarized in Supplementary (2) were as follows: One case with classic CLL (score 5). Nine cases were classified as aCLL: 6 cases with atypical immunophenotypic features (score 2-3) (including one case with complex KT and 4 cases with trisomy 12). Three cases of CD5-negative aCLL (score 2-4). An example of a case of atypical CLL (score 3) with many clefted/folded lymphoid cells and trisomy 12 was shown in supplementary 5; A, B.

Among CLL patients with negative LEF-1, four cases were classified as CLL/PLL (with > 10% prolymphocytes) with trisomy 12 in two cases and complex KT detected in the other two cases; including a case progressed into Richter’s transformation developed during the disease course (Supplementary 5: C, D).

Correlation between different immunophenotypic markers and LEF-1 expression has not been systematically studied before. However, Menter et al, reported no observed correlation between LEF-1 expression and trisomy 12, p53 or CD38. We studied the association between some selected immunophenotypic and cytogenetics markers and LEF-1 expression in CLL cases (Table 3). While CD5, CD200, CD23 positivity percentage and dim light chain expression had significantly higher proportions in patients with positive LEF-1 expression (P < .05). In contrast, CD79, FMC7 positivity percentages were found to be significantly lower in patients with positive LEF-1 expression (P < .05). On the other hand, the proportions of Trisomy 12, CD38 expression and del13q were found to be similar in both LEF-1 positive and negative cases with statistically insignificant difference (P = .865).

Previously published data showed that other hematolymphoid malignancies which express LEF1 include Burkitt lymphoma, certain subsets of T-cell lymphomas, and acute lymphoblastic leukemias/lymphomas of T- and B-cell lineage. In our study, we found out Reed Sternberg cell in Hodgkin Lymphoma (HL) also expresses LEF-1. However, in contrast to what is reported before by Thomas Menter, LRF-1 was negative in the transformed cells in a single case of Richter's transformation on top of CLL/PLL.
4.1 | Expression of LEF-1 in some interesting cases in our cohort

We found out that LEF-1 is a useful marker in cases of composite lymphomas (CL). In our cohort, we had a case of CL (CLL and Mantle cell lymphoma), LEF-1 expression by FCM had nicely separated the two different clones and served as an additional interesting tool to differentiate both types of lymphoma (Figure 2D); MCL clone (lambda restricted) and CLL clone (kappa restricted). The LEF-1 MFI ratio for lambda restricted (MCL) clone was 1.4 which is (< cutoff value) while the LEF-1 MFI ratio for kappa restricted (CLL) clone is 2.5. Another case of CL (HL and CLL/SLL) where LEF-1 immunostain on lymph node biopsy showed many scattered strongly positive T-cells, some scattered CLL B-cells (weaker LEF-1 staining) and few Reed Sternberg cells positive for LEF-1 (Supplementary 6).

5 | CONCLUSION

LEF-1 is not expressed in the great majority of low-grade lymphomas and is a reliable marker for distinguishing them from CLL/SLL with an added value in supporting the diagnosis of cases of discordant and composite lymphomas. In this study we document that LEF-1 expression is downregulated in a significant number of cases of CL with atypical immunophenotype/features compared to classic CLL; Chi-Square $P < .0001$. As downregulation of LEF-1 expression is well correlated with atypical CLL, we suggest adding it to Matutes score as a beneficial marker to differentiate classic from atypical CLL. LEF-1 could also serve as a potential prognostic indicator for CLL clinical course. Further studies are desirable to support our data regarding LEF-1 expression in atypical CLL, and whether its downregulation has any prognostic/predictive value.

ACKNOWLEDGEMENT

We would like to acknowledge the great contribution of technical staff in hematology & flow cytometry laboratory especially Miss Suhair Hassan El ajez and Miss Somayya Rahhal for their hard work and continuous support. We would also like to acknowledge the crucial role and the great contribution of cytogenetics laboratory staff especially Miss Farzana Murad, staff of the interim research laboratory and histopathology laboratories in HMC.

CONFLICTS OF INTEREST

“The authors have no conflicts of interest to declare.”

AUTHORS CONTRIBUTIONS

Soliman D S created the research design, patients’ selection, performed data collection, hematopathologic examination, data analysis, wrote the manuscript and formulated and submitted the paper.

Al-Kuvari E performed the histopathologic examination and reviewed the data.

Siveen K, performed flow cytometry analysis and reviewed the manuscript.

Al-Abdulla R, performed the immuno-histochemical analysis.

Chandra P, performed the statistical analysis and reviewed the manuscript.

Yassin P, participated in patients’ recruitment, consenting and clinical assessment.

Ibrahim F, shared in data collection and reviewed the manuscript.

Nashwan A, participated in research protocol submission, patients’ recruitment and data collection.

Taha R Y, participated in patients’ recruitment, and clinical assessment.

Nawaz Z, performed the cytogenetic analysis.

El-Omri H participated in clinical assessment.

Mateo J M, processed the samples for flow cytometry analysis.

Al-Sabbagh A reviewed the manuscript.

ETHICAL APPROVAL

The study was approved by HMC ethics committee on human research (Medical Research Centre-MRC).

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How to cite this article: Soliman DS, Al-Kuwari E, Siveen KS, et al. Downregulation of Lymphoid enhancer-binding factor 1 (LEF-1) expression (by immunohistochemistry and/ flow cytometry) in chronic Lymphocytic Leukemia with atypical immunophenotypic and cytologic features. *Int J Lab Hematol.* 2021;43:515-525. https://doi.org/10.1111/ijlh.13420