The Role of Expression of Lymphoid Enhancer-Binding Factor-1 (LEF-1) in Patients with Chronic Lymphocytic Leukemia

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Abstract

Background: Lymphoid Enhancer-Binding factor-1 (LEF-1) is a 48-kD nuclear protein that is expressed in pro-B cells and mature T cells but not in mature B cell. LEF-1 binds to a functionally important site in the T-cell receptor alpha (TCR α) enhancer and confers maximal enhancer activity; LEF-1 belongs to a family of regulatory proteins that share homology with high mobility group protein-1 (HMG1). Lymphoid Enhancer-Binding factor-1 LEF-1 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, LEF-1 is highly associated with CLL/SLL among small B-cell lymphomas and therefore can serve as a useful immune-histo-chemistry (IHC) marker for diagnosis and differential diagnosis of CLL/SLL.

Aim of the Study: Our aim is to evaluate the role of Lymphoid Enhancer-Binding factor-1 (LEF-1) expression by flow cytometry in patients with chronic lymphocytic leukemia.

Methods and Material: This study was carried out on 45 newly diagnosed B-CLL patients attending the hematology oncology clinic of Tanta University Hospitals. The patients were selected for the study on the basis of standard clinical, hematological and immune phenotypic criteria for diagnosis of CLL, In addition to 15 apparently healthy subjects serving as healthy control group.

Subjects included in this study were classified into the following groups:

Chronic Lymphocytic Leukemia Group I: Included 45 patients with chronic lymphocytic leukemia.

Group I: Healthy Control Group.

This group included 15 apparently healthy subjects.

Results: There was no significant correlation between LEF-1 expression and age and sex, there was a significant negative correlation between LEF1 expression and Hb and platelet count, there was a significant positive correlation between LEF-1 expression and TLC, ALC and LDH. According to LEF1 expression, there was 100% of patients positively expressed LEF-1 and negative in control cases and there was significant positive correlation between LEF-1 expression and Zap 70 expression.

Conclusions: LEF-1 expression on CLL cells represent an important adverse diagnostic marker and therefore its expression should be routinely investigated for a better diagnostic and prognostic assessment of CLL patients and showed be taken in consideration in designing further therapeutic strategies based on patient-specific risk factors.

Key Words: Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) – Flow cytometry – LEF1.

Introduction

CHRONIC lymphocytic leukemia (CLL) is the most common type of leukemia in the world, with an average incidence of 4.1 new patients per 100 000 populations annually [1]. The median age at diagnosis lies between 67 and 72 years and the male/female ratio is approximately 2:1 [2].

Chronic lymphocytic leukemia (CLL) is a hematologic malignancy characterized by the clonal expansion and significant accumulation of immunophenotypically similar mature B lymphocytes. It is clear that CLL is a disease marked by both increased leukemic cell survival and proliferation. Of interest, it has recently been demonstrated that 3% to 5% of the healthy middle-aged adult population has a circulating clonal population of CLL-phenotype B lymphocytes, and this has been designated as monoclonal B-cell lymphocytosis (MBL) [3,4].

Finding new prognostic markers for CLL has always been and still is challenge for investigations. ZAP-70 & CD38 expression is the first markers to be discovered and chromosomal abnormalities of CLL lymphocytes are also commonly used [5,6].
Lymphoid Enhancer-Binding factor-1 (LEF-1) is a 48-kD nuclear protein that is expressed in pro-B cells and mature T cells but not in mature B cell [7,8]. LEF-1 binds to a functionally important site in the T-cell receptor alpha (TCR\(\alpha\)) enhancer and confers maximal enhancer activity; LEF-1 belongs to a family of regulatory proteins that share homology with high mobility group protein-1 (HMG1) [9,10].

LEF-1 acts as a mediator of the canonical Wnt signaling pathway by recruitment of \(\beta\)-catenin to promoter regulatory elements via an N-terminal binding domain. The canonical Wnt pathway is initiated by binding of soluble glycoprotein Wnt ligands to frizzled family surface receptors along with the coreceptors LRP5/6. These receptors signal through disheveled to block the multimolecular \(\beta\)-catenin destruction complex. Inhibition of the destruction complex allows accumulation of \(\beta\)-catenin with subsequent translocation to the nucleus where it regulates transcription in conjunction with members of the TCF/LEF family. Of interest, the canonical Wnt signaling pathway has been found to regulate genes that control survival and the cell cycle [11].

**Material and Methods**

Most CLL cases developed from monoclonal B lymphocytes, and T-CLL occurs rarely (less than 1%), so this study was conducted on 45 newly diagnosed B-CLL patients attending the hematology oncology clinic of Tanta University Hospitals. The patients were selected for the study on the basis of standard clinical, hematological and immune phenotypic criteria for diagnosis of CLL. In addition to 15 apparently healthy subjects serving as healthy control.

**Subjects included in this study were classified into the following groups:**

**Group I: CLL patients:**

This group included 45 patients with chronic lymphocytic leukemia. They were 28 males and 17 females with a male to female ratio of 2:1. Their ages ranged from 38-79 years, with a mean value 57.45 ± 11.13.

**Group II: Healthy control group:**

1- This group included 15 apparently healthy subjects. They were 10 males and 5 females. Their mean age was 55.45 ± 10.54 years.

**Subject:** History taking, clinical examination, Abdominal ultrasonography, Routine laboratory investigation as Complete blood count (CBC), Lactate dehydrogenase (LDH), Erythrocyte Sedimentation Rate (ESR), Routine Flow cytometric analysis for CLL scoring (CD5, CD23, FMC7, SmIg and CD22/CD79b+), CD 38 & ZAP70.

**And Specific laboratory investigations:** Flow cytometric analysis of lymphocyte Enhancer binding factor (LEF-1) expression on peripheral blood samples for all cases using BD bioscience kits.

**Method:**

**Sample collection:**

Samples were collected using sterile vacutainers and divided as follows:

- Two mL of PB was delivered into an EDTA vacutainer tube for complete blood count and stained with giemsa to determine the differential leucocytic count.
- Two ml of PB on EDTA was used for immunophenotypic determination.
- One ml of clotted blood was collected into an empty sterile tube for measurement of serum lactate dehydrogenase enzyme.

**Procedure:**

**Sample preparation:**

1- 100 µl of EDTA peripheral blood were pipetted in the standard flow cytometric tube (flacon tube, 5ml polyestrene round bottom tube 12x75mm non pyrogenic and sterile form BD immunocytometry system). Each tube was labeled with the patient's name.
2- 1 ml of FACS lysing solution (diluted 1:10) was added to each tube, vortex mixer was immediately used at low speed for 5 seconds.
3- Tubes were incubated at 4ºC for 25 minutes.
4- After incubation, tubes were centrifuged for 5 minutes at 3000 r.p.m.
5- Supernatant was aspirated leaving approximately 50 µl of residual fluid to avoid disturbing the pellet.
6- Vortex mixer was used to resuspend the cell in the residual fluid and then 2ml of PBS were added to each tube for washing.
7- The tubes were centrifuged at 3000 r.p.m for 5 minutes.
8- 250 µl of anti-LEF1 monoclonal antibodies were added to positive tubes together with 250 µl of cytofix-cytoperm was added to each tube.
9- Tubes were incubated at 4ºC for10 minutes.
10- The supernatant was discarded and the washing step was repeated twice.
11- 20 µl of anti-LEF1 monoclonal antibodies were added to positive tubes together with 250 µl of...
2nd Ab (anti rabbit IgG) prediluted 1/2000 with PBS to both negative and positive tubes.

12- The Tubes were vortexed and incubated for 25 minutes in the dark at 4ºC.

13- 1.5ml of lysing solution was added to each tube.

14- The Tubes were vortexed and incubated for 25 minutes in the dark at 4ºC.

15- The tubes were centrifuged at 3000 r.p.m for 5 minutes and supernatant was discarded.

16- 0.5ml of PBS as washing solution was added to each tube and thoroughly.

17- The tubes were centrifuged at 3000 r.p.m for 5 minutes and supernatant was discarded, this step was repeated.

18- In the last wash, after discarding the supernatant, the cells were resuspended in 0.5ml of 1% paraformaldehyde which is added to each tube and then vortexed.

19- Cells were ready for direct cytometric analysis.

**Flow cytometric analysis:**

After warming up the argon laser (488) for 30 minutes, the full alignment procedure were performed using standard immunocheck alignment flurospheres for adjusting forward scatter, side scatter and photomultiplier tube (PMT).

Control samples (PE IgG2b) were introduced in the machine and forced in the sheath by the sample pressure (run button) where the laser scatter was received on both forward scatter detectors and scale to show the cell population in a basic histogram and to adjust the regions.

10000 events (cells) at least were passed in front of the laser for each case from which the lymphocytes were selectively gated (surrounded by a line to separate them from other cells in the basic histogram) for immunophenotyping analysis.

The sample tubes were then introduced and processed in the same way as the control, where the monoclonal cells tagged with PE were analyzed on PMT2 (green). The fraction of cells coated by monoclonal antibodies were determined inside the gated population of lymphocytes and assessed in a single histogram.

**Measurements:** Statistical presentation and analysis of the present study was conducted, using the mean, standard error, student t-test Chi-square, ROC curve and Linear Correlation Coefficient by SPSS V17.

**Demographic data of studied CLL patients:**

This study was conducted on 45 newly diagnosed CLL patients, their age ranged from 51 to 80.35/45 CLL patients were older than 60 years, 10/45 CLL patients were less than 60 years. They were 28 (62.2%) males and 17 (37.8%) females with male to female ratio 1.6:1.

**Results**

There was no significant correlation between LEF-1 expression and age and sex, there was a significant negative correlation between LEF 1 expression and Hb and platelet count, There was a significant positive correlation between LEF-1 expression and TLC, ALC, ESR and LDH, There is significant higher level in LEF-1 expression in patients with Zap70 positive compared to patients with Zap70 negative and there is insignificant difference in LEF-1 expression in patients with CD38positive and CD38negative.

Regarding the clinical examination, lymphadenopathy was observed in 41/45 (91.1%), splenomegaly was observed in 27/45 (60%) and hepatomegaly was observed in 11/45 (24.4%).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients (n = 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>Age:</td>
<td></td>
</tr>
<tr>
<td>&gt;60</td>
<td>35</td>
</tr>
<tr>
<td>&lt;60</td>
<td>10</td>
</tr>
<tr>
<td>Sex:</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>28</td>
</tr>
<tr>
<td>Female</td>
<td>17</td>
</tr>
<tr>
<td>Splenomegaly:</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>18</td>
</tr>
<tr>
<td>Present</td>
<td>27</td>
</tr>
<tr>
<td>Hepatomegaly:</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>34</td>
</tr>
<tr>
<td>Present</td>
<td>11</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td></td>
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<tr>
<td>Non</td>
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</tr>
<tr>
<td>Present</td>
<td>41</td>
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<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range</th>
<th>Mean</th>
<th>Std. Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (gm/dl)</td>
<td>7.8-11.2</td>
<td>9.12</td>
<td>0.88</td>
</tr>
<tr>
<td>Platelet (x 10^9/L)</td>
<td>68-155</td>
<td>101.31</td>
<td>22.04</td>
</tr>
<tr>
<td>Total Leucocytic Count (x 10^9/L)</td>
<td>15-503</td>
<td>79.88</td>
<td>105.2</td>
</tr>
<tr>
<td>Absolute Lymphocytic Count (x 10^9/L)</td>
<td>13.5-407</td>
<td>65.9</td>
<td>84.69</td>
</tr>
</tbody>
</table>
The Role of Expression of Lymphoid Enhancer-Binding Factor-1 (LEF-1)

Table (3): CBC findings in control (group II).

<table>
<thead>
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<th></th>
<th>Range</th>
<th>Mean</th>
<th>Std. Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (gm/dl)</td>
<td>10.5-13.5</td>
<td>11.8</td>
<td>0.81</td>
</tr>
<tr>
<td>Platelet (x 10^9/L)</td>
<td>150-450</td>
<td>251.13</td>
<td>91.5</td>
</tr>
<tr>
<td>Total Leucocytic Count</td>
<td>4.5-11.3</td>
<td>8.17</td>
<td>2</td>
</tr>
<tr>
<td>(x 10^9/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute Lymphocytic</td>
<td>0.9-4.0</td>
<td>2.39</td>
<td>0.81</td>
</tr>
<tr>
<td>Count (x 10^9/L)</td>
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Table (4): Correlation between Zap70 and LEF-1 expression.

<table>
<thead>
<tr>
<th>LEF</th>
<th>ZAP 70</th>
</tr>
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<tbody>
<tr>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>55-98</td>
</tr>
<tr>
<td>Mean ± S.D</td>
<td>81.58 ± 12.58</td>
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<tr>
<td></td>
<td>16.194</td>
</tr>
<tr>
<td></td>
<td>0.001*</td>
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Table (5): Correlation between LEF-1 expression and CD38.

<table>
<thead>
<tr>
<th>LEF</th>
<th>CD38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>35-98</td>
</tr>
<tr>
<td>Mean ± S.D</td>
<td>76.7 ± 19.5</td>
</tr>
<tr>
<td></td>
<td>0.639</td>
</tr>
<tr>
<td></td>
<td>0.428</td>
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Discussion

CLL is a B-cell neoplasm, characterized by indolent course and presence of lymphadenopathy and/or splenomegaly and Presence in the peripheral blood of ≥5000 monoclonal B Lymphocytes/µl. The clonality of the circulating B lymphocytes needs to be confirmed by flow cytometry. The leukaemia cells found in the blood smear are characteristically small, mature-appearing lymphocytes with a narrow border of cytoplasm and a dense nucleus lacking discernible nucleoli, and having partially aggregated chromatin [12].

LEF-1 in hematopoietic development have been mainly restricted to the lymphoid lineages, where LEF-1 has functions in T-cell development and affects proliferation and apoptosis in pro-B cells [13]. Specific functions of LEF-1 independent of Wnt signaling, suggesting a more complex role of LEF-1 in the development of hematopoietic tissues [14].

Lymphoid Enhancer-Binding factor-1 (LEF-1) is highly associated with CLL/SLL among small B-cell lymphomas [15]. Beside that LEF-1 has been shown to be down regulated in NK cells because LEF-1 and TCF-1 (T-cell-specific transcription factor) play a redundant role in the regulation of NK cells and it is believed that TCF activity is up regulated, while LEF-1 is suppressed in normal NK cells [16].

In this study; we investigated the potential role of LEF-1 in CLL by analyzing the level of LEF1 expression on leukemic B cells in peripheral blood in correlation with clinical and laboratory parameters characterizing disease activity.

The present study included 45 newly diagnosed cases of CLL selected from Internal Medicine Department of Tanta University Hospital.

All included subjects were submitted to full history taking, clinical examination, and laboratory investigations. The diagnosis of CLL was based on clinical history, lymphocyte morphology, and immunophenotypic criteria. Staging of disease was done using Modified Rai staging system and all included subjects were submitted to measurement of LEF1 expression by flowcytometry.

In the present study, there was predominance of CLL in males than females. This was in agreement with [17,18] who reported a male predominance in patients with CLL. This explained by [19] who noticed anti proliferative effect of estradiol in human leukemia cell lines.

As regard age, most of CLL patients were older than 60 years. This was in agreement with [20], who reported that most CLL cases were more than 60 years. In addition our study revealed, lymphadenopathy and hepatosplenomegaly were present in most CLL patients. This was in agreement with [21] who reported that the spleen is the most common site of involvement by CLL after bone marrow and lymph node.

In the current study, Hb level showed significant lower in most CLL patients. This finding was in consistent with the results of [22] who reported decrease in Hb in CLL cases.

Also, there was statistically significant lower in platelets count in CLL patients. This was in accordance with [23] who reported that decrease platelets count in CLL patients due to marrow replacement and hypersplenism.

Our study revealed that the mean value of total leukocytic count showed significant higher in patients with CLL. In agreement with these results [24] reported leukocytosis in CLL patients.

In addition, our study revealed that the mean value of peripheral lymphocytic count showed significant higher in CLL a patient which was in accordance with [25] who reported that patients...
with CLL showed higher absolute lymphocytic count.

In addition, our study revealed that Lactate dehydrogenase showed significant higher in CLL patients when compared to control group. In agreement with [25] who reported that patients with CLL showed higher Lactate dehydrogenase.

In addition, our study revealed that Erythrocyte Sedimentation Rate showed significant higher in CLL patients when compared to control group. In agreement with [25] who reported that patients with CLL showed higher Erythrocyte Sedimentation Rate.

We found that LEF 1 expression in CLL patients is associated with high percentages of lymphocytes and this suggests a significant role of LEF-1 in prognosis and disease progression in CLL, this was in agreement with [26-28] who also reported the diagnostic utility of LEF-1 immunohistochemistry as a sensitive and specific marker for CLL and is helpful in the diagnosis of small B-cell lymphomas.

As regard LEF1 expression, there was 100% of patients positively expressed LEF-1. In agreement with our results [29] reported that expression of LEF 1 in all his studied cases, [30] found that strong evidence for the aberrant activation of LEF 1 in MBL and CLL cells, identified LEF 1 as an adverse prognostic marker in CLL patients and indicate LEF 1 may serve as an attractive therapeutic target for future CLL therapies and [31] showed 100% of their CLL neoplastic samples express nuclear LEF 1 strongly. Interestingly, [32] indicated that 70% of CLL cases express LEF- 1.

In the present study, There is significant higher level in LEF-1 expression in patients with Zap70 positive compared to patients with Zap70 negative and these results are in accordance with [33] who reported that there is significant relevance has been found between LEF 1 expression and Zap70 positive. Interestingly, [30] found that no statistically significant relevance has been found between LEF 1 expression and ZAP70 positivity.

In the present study, there is insignificant difference in LEF-1 expression in patients with CD38 positive and CD38 negative and these result are in accordance with [30,33] found that no statistically significant relevance has been found between LEF 1 expression and CD38 level.

Conflicts of interest:
No conflicts of interest declared.

Authors' contributions:
All authors had equal role in design, work, statistical analysis and manuscript writing.

References


دراسة دور تعبير عامل المرتبط المحفز للخلايا الليمفاوية في مرضى سرطان الدم الليمفاوي المزمن

العامل المرتبط المحفز للخلايا الليمفاوية حجمه 48 كيلو دالتون بروتين في الخلايا البائية الليمفاوية الفخامة والخلايا البائية الليمفاوية (تي) الليمفاوية الفخامة ولا يوجد في الخلايا البائية الليمفاوية الفخامة ويوجد مستقبلات ألفا المحفز على خلايا (تي) الليمفاوية تعتبر مكان وظيفي مهم للإرتباط به وتمتاز أقصى نشاط تفعيلته وأنه ينتمي إلى عائلة البروتينات المنظمة التي تشارك بالتجارب مع مجموعة بروتينات عاملة الحركة وأنه مرتبط ارتباطاً وثيقاً بسرطان الدم الليمفاوي المزمن وأنه من دلالات المناعة المفيدة للتشخيص والتشخيص التجريبي للسرطان.

الهدف من البحث: قياس دور العامل المرتبط المحفز في تشخيص سرطان الدم الليمفاوي المزمن.

طريقة البحث: اشتملت الدراسة على حالة من مرضى سرطان الدم الليمفاوي المزمن الذين تم تشخيصهم حديثاً أخباراً من مستشفى طنطا العامي وسيتم إجراء هذه الدراسة في قسم البيولوجيا الإلكترونية بكلية الطب جامعة طنطا وسوف يتم تصنيف الافراد إلى مجموعتين
- مجموعة (1): عدد 5 من مرضى سرطان الدم الليمفاوي المزمن.
- مجموعة (2): عدد 10 من الاصحاب المتطوعين كمقياس طبيعي للدراسة.

وقد أسفرت النتائج عن بناء على نسبة العامل المرتبط المحفز التعبيري وجد ارتفاع نسبة تعبير العامل المرتبط المحفز في 45 حالة لا توجد علاقة بين كل من (العمر والسن) والعامل المرتبط المحفز وتوجد علاقة طردية بين كل من (كرازات الدم البضاء والخلايا الليمفاوية) مع عدد الالتهاب ديهجبين وزيتا بروتين 70 والعامل المرتبط المحفز وتوجد علاقة عكسية مع كل من الهيموجلوبين والصفائح الدمئية والعامل المرتبط المحفز.