

Evaluation of CD69 as a prognostic factor and its relation with other prognostic factors in a sample of Iraqi patients with chronic lymphocytic leukemia

Maysem Mouayad Alwash F.I.B.M.S / Hematology

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Address for Correspondence:

Dr. Maysem Mouayad Alwash,
Department of Pathology & Forensic
Medicine, College of Medicine, AL-
Mustansiriya University, Baghdad,
Iraq.

Abstract

Background: Chronic lymphocytic leukemia (CLL) is clinically heterogeneous disease with variable prognosis. It is important to take into consideration the prognosis of presenting with CLL before starting suitable therapeutic option. Different studies proved that leukemic CLL cells have the surface membrane phenotype of activated and antigen experienced B lymphocytes with the overexpression of the activation markers such as CD69. In one previous study, CD69 was assumed to be considered as a new promising immunologic prognostic factor in B-CLL.

Aim: In this study we aimed to detect CD69 expression in newly diagnosed patients with CLL by flow cytometry and correlate it with some other clinical and laboratory parameters in order to evaluate its role as a prognostic factor.

Patients and Methods: CD69 expression was investigated by flow cytometry in 26 untreated newly diagnosed patients with chronic lymphocytic leukemia, and compared this with other standard prognostic parameters (β 2-microglobulin, lymphocyte count, Rai stage and CD38).

Results: The present study shows that about (34.61%) of the CLL patient were positive for CD69 expression, while (65.38%) of patient were negative. CD69 expression is significantly associated with CD38 (P=0.002), Rai stages (p=0.0001), lymphocyte count (p=0.0001), β 2-microglobulin (P=0.012).

Conclusion: The results of this study demonstrated that CD69 is significantly associated with poor prognostic factors. This supports its introduction in a laboratory assessment of newly diagnosed patient with CLL and, possibly, in a prognostic scoring system for chronic lymphocytic leukemia.

Key words: Chronic lymphocytic leukemia, CD69.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a monoclonal disorder of progressive accumulation of CD5+ B-lymphocytes in peripheral blood, bone marrow, lymph nodes and spleen.^(1,2)

Chronic lymphocytic leukemia (CLL) notoriously shows heterogeneity regarding disease progression, response to therapy and outcome.^(3,4)

The interaction of genetic and epigenetic abnormalities together with the influence of microenvironment involved in CLL pathogenesis were considered to be responsible for the clinical heterogeneity seen in CLL.⁽⁵⁾

The variable prognosis and the absence of a curative therapy influence the decision when planning the management of patients with CLL, as it is important to take into consideration their prognosis before starting suitable therapeutic option.⁽⁶⁾

The two major clinical staging systems (Rai, Binet) are unable to prospectively differentiate an indolent or aggressive course especially within the low and intermediate risk groups.⁽⁷⁾

Therefore researches on CLL aimed to understand its pathogenesis, incorporate new prognostic factors and to discover new treatment modalities of CLL.⁽⁸⁾ Several parameters have been added to the staging

systems to differentiate prognostic subsets as^(9,10) shown in table 1 ⁽¹¹⁾

Table (1). Most important Outcome Predictors in CLL.

| Prognostic factors | Consolidated Complementary value |
|-------------------------------|----------------------------------|
| Clinical stages | Blood lymphocyte count |
| <i>IGHV</i> mutational status | ZAP70 |
| Cytogenetics | CD38 |
| LDT | Beta-2 microglobulin |

Different studies proved that leukemic CLL cells have the surface membrane phenotype of activated and antigen experienced B lymphocytes with the overexpression of the activation markers CD23, CD25, CD69 and CD71. ⁽¹²⁾

CD69 is a type II transmembrane protein present on platelets, CD4-positive or CD8-positive lymphocytes , activated lymphocytes , and activated T or natural killer cells that functions as a signal transducer , enhancing cell activation and / or platelet aggregation. ⁽¹³⁾

An association has been demonstrated between mutation status and CD69 expression. ⁽¹⁴⁾ In one previous study , CD69 was assumed to be considered as a new promising immunologic prognostic factor in B-CLL. However, the exact pathogenetic role of CD69 in the clinical behavior of B-CLL remains to be better established, needing more work and investigations. ⁽¹⁵⁾

Aim of the study :

In this study we aimed to detect CD69 expression in newly diagnosed patients with CLL to correlate CD69 expression with some other clinical and laboratory parameters in order to evaluate its role as a prognostic factor

PATIENTS AND METHODS

Twenty six newly diagnosed untreated patients with B-CLL were included in this study were attending the laboratory department of Al- Yarmuk teaching hospital ,The National Center of Hematology and private lab during the period between December 2014 to December 2015.They were 17 male and 9 female. Their ages range was from 56 to 79 year with mean age of 64.57 ± 6.13 year . A verbal consent were taken from each patient. The diagnosis of CLL was based on a lymphocyte count of at least 5x 10⁹/L and typical immunophenotype or bone marrow morphology. ⁽¹⁶⁾ Every Patient was subjected to the followings:

1. Clinical sheet details (name, age, sex, chief compliant with special concern to organomegaly, lymphadenopathy).
2. Complete blood count with examination of peripheral blood smears stained with Leishman stain, bone marrow aspiration, flowcytometric immunophenotyping and B2-Microglobulin measurement.
3. Rai staging according to disease burden and degree of bone marrow involvement.
4. A total of 3 ml of venous blood was collected by clear venipuncture and divided into 2 tubes, 2ml in an EDTA tube and 1 ml in a plane tube. Complete blood count was done for each sample by automated Abbot Ruby hemoanalyzer at Al-Yarmouk teaching lab, blood film slides were revised for some of the patients, then the samples were sent within six hours to private lab for immunophenotyping.The remaining 1 ml was put in plane tube to obtain serum for β2- microglobulin measurement.
5. Immunophenotyping was done to detect CD5,CD23 markers as diagnostic , and CD 69 and CD38 as prognostic markers by using four –color Cyflow® Cube ⁶ flow cytometry device (Partec Cyflow®, German), which is a fully equipped desktop Flow Cytometer (FCM). Mononuclear cells are obtained from peripheral blood by Ficoll density centrifugation. The cells are Washed twice in phosphate buffered saline(PBS) and re suspended in PBS containing 1% bovine serum Albumin (BSA) (Sigma),005% sodium azide , and 2% human AB serum (pH: 7.6) (PBS-BSA- azide -AB buffer). Identification of cells was performed using forward scatter (FSC) versus side scatter (SSC) parameters . Antigen expression was considered to be positive when the percentage of positive cells was equal or greater than 20%⁽¹⁷⁾. Markers which shows expression value more than 20% was considered positive while less than 20% was considered negative.
6. B2-Microglobulin measurement by ELISA . A value of more than 2.3 mg/l is considered to be associated with poor prognosis.⁽¹⁶⁾
7. Bone marrow (BM) aspiration was withdrawn for morphological examination .

Statistical analysis

Analysis of data was carried out using the available statistical package of SPSS-18 (Statistical Packages for Social Sciences- version 18).

Statistical significance was considered whenever the P value was equal or less than 0.05.

RESULT

This study includes 26 adult patients with newly diagnosed chronic lymphocytic leukemia. The mean age of all patients included in this study was 64.57 ± 6.13 , and a range of (56-79) years old. The patients were 17 male and 9 females with a male to female ratio of about 2:1. According to Rai stages at diagnosis, 5 patients belong to Rai stage I, 14 patients with Rai stage II and 7 patients with Rai stage III. (see table 2).

Table (2): distribution of patients according to Rai staging

| Rai stage | No. of patients | Percentage of patients |
|-----------|-----------------|------------------------|
| I | 5 | 19.2% |
| II | 14 | 53.8% |
| III | 7 | 26.9% |

On the basis of a 20% cut off value, all patients in the present study who were diagnosed morphologically as CLL were positive for CD5 and CD23.

Nine out of 26 patients (34.61%) were CD69 positive, 17 patients (65.39%) were negative. 11 out of 26 patients (46.15%) were CD38 positive, 15 (53.84%) patient were negative for CD 38 expression as seen in table (3).

The mean lymphocyte count (17.16 ± 3.49) was significantly higher (P-value 0.000) in patients who were CD69 positive (CD69 >20%) than mean lymphocyte count (10.59 ± 2.76) in CD69 negative (CD69 <20%) patients, as shown in table (4).

A significant association has been found between CD69 and CD38 expression, P-value is 0.002, as seen in table (5).

Table (3): Distribution of CLL patients according to CD38 and CD69 expression.

| Marker | No. of patient with Positive expression (>20%) | Percentage of patient with Positive expression (>20%) | No. of patient with Negative expression (<20%) | Percentage of patient with Negative Expression |
|--------|--|---|--|--|
| CD38 | 11 | 46.15% | 15 | 53.84% |
| CD69 | 9 | 34.1% | 17 | 65.39% |

Table (4): CD69 expression and lymphocyte count.

| | | Lymphocyte count (cell /l) | | |
|------|-----------------|----------------------------|------|--------|
| | | Mean | SD | P |
| CD69 | Negative (<20%) | 10.59×10^9 | 2.76 | 0.0001 |
| | Positive (>20%) | 17.16×10^9 | 3.49 | |

Table (5): The association between CD69 expression and CD38, β_2 Microglobulin & Rai stage

| | | CD69 | | Sig (P) |
|-------------|-----------------|----------------------------------|------------------------------------|---------|
| | | Negative <20% No. of patients | Positive (>20%) No. of patients | |
| CD38 | Neg <20% | 12 82.4% | 1 11.12% | 0.002 |
| | Positive (>20%) | 3 17.6% | 8 88.88% | |
| β_2 M | <2.3 mg/l | 14 82.4% | 3 33.3% | 0.012 |
| | >2.3 mg/l | 3 17.6% | 6 66.7% | |
| Rai Stage | I | 5 | 0 | 0.0001 |
| | II | 12 | 2 | |
| | III | 0 | 7 | |

DISCUSSION

Nowadays, interest in applying prognostic parameters which may predict survival and direct the plan of management in patients with CLL has been increased. This increasing interest is the result of the difficulty in determining the time of onset and choice of therapy as CLL shows a highly heterogeneous clinical course.⁽¹⁸⁾

Besides the established clinical and biological prognostic factors e.g. Rai and Binet staging, lymphocyte count, serum β_2 -microglobulin, CD38), there are several clinical and biological parameters which have been assumed to predict the outcome of CLL patients when assessed at presentation of the disease.⁽¹⁹⁾

In the present study, we aimed to validate the clinical impact of CD69 as a prognostic factor for CLL and its relationship with other standard prognostic factor (lymphocyte count, β_2 M, RAI clinical stages, CD38) which are all associated with inferior prognosis on multivariate analysis⁽²⁰⁾.

In spite of appearing as small resting cells when viewed by microscope, CLL cells express different surface molecules which are linked to B-cell activation as revealed by immunophenotyping, which is important in predicting clinical outcome in CLL patients, special attention has been directed to CD38 together with ZAP-70 expressed by activated T and B cells and a subset of CLL cells^(21,22).

In addition, activation- and maturation- associated markers can be displaced on a subset of circulating CLL cells such as CD69⁽¹²⁾, which was evaluated in the present study as prognostic factor.

The current study demonstrated that CD69 expression was positive in about 34.61% of CLL patients and significantly correlated with CD38.

A similar association had been mentioned by D'Arenaon *et al*⁽¹⁵⁾. Also Del Poeta *et al*⁽⁷⁾ has found that CD69 expression was positive in about 26.6% of CLL patient and it was significantly correlated with CD38. The higher percentage of CD69 expression in the present study may be attributed to the higher cut off value for positivity which had been chosen by Del Poeta *et al* (30%) than that applied in our study (20%). In addition to larger sample size taken by Del Poeta *et al*. It is an interesting thing to be mentioned that when applying a cut-off value of 30%, there was no significant change and the same results were reproduced as none of the samples available for this study had a value between 20% and 30% for CD69 and CD38 expression. The significant correlations found between CD69 expression and high lymphocyte counts,

B2-microglobulin and advance Rai stage are in agreement with results of several previous studies.^(7,15)

The rapidly up-regulated CD69 when cellular activation started, may be responsible for the transduction of (B-cell Receptor) BCR-mediated signals in a better way with the co-expression of CD38 that has an important role to transduce B-cell receptor (BCR)-mediated signal.⁽²³⁾

Such increased intracellular signaling may reflect ongoing stimulation, thereby influence the proliferation or survival of CLL cells leading to a tendency toward disease progression, and advanced stages, and hence explaining the more aggressive disease course observed in these patients.⁽⁷⁾

CLL is a dynamic disease comprised of birthing and dying cells.⁽²⁴⁾ Cell cycling and not longevity may be responsible for clonal evolution in CLL and disease worsening^(25,26). It has been suggested by Messmer *et al* that more active disease will present in patients with the higher daily birth rates of CLL cells,⁽²⁵⁾ therefore, it is cell cycling and proliferation but not longevity that are responsible of clonal evolution in CLL and disease worsening^(25,26).

It has been assumed by the studies done by Messmer *et al*. that as the division rate of a CLL clone is higher and faster, the more likelihood that patient will have an aggressive disease with advance stage and higher lymphocyte count.⁽²⁵⁾

CD69 was correlated with high B2-microglobulin, which in turn correlated with adverse prognostic features such as advance stage, high tumor burden, and overall survival as demonstrated by different studies.⁽²⁷⁾

Interestingly, Grund *et al*. had studied the association of CD69 expression with IgVH mutation status and found that CD69 expression was negative in all mutated cases of CLL, while positive CD69 had been found in all unmutated cases except one. Thus we can speculate that high CD69 expression has a negative impact on survival in CLL patients, since it is well known that patients with unmutated *IgVH* genes have a decreased survival compared with patients mutated *IgVH* genes.⁽²⁸⁾ Recent studies show that the CD69 molecule is in fact a negative regulator of the immune response in part through production of transforming growth factor - β (*TGF- β*). Autoimmune and antitumor responses can be promoted by CD69 deficiency.^(29,30) An optimal antitumor response can be developed by blockage of CD69 as Esplugues *et al* had demonstrated in their studies, they found that the use of anti-CD69 monoclonal antibodies can activate resting natural killer cells (NK) in an Fc receptor-independent

manner. This leads to increase interferon production and NK-cell cytolytic activity. ⁽³¹⁾

Conclusion : CD69 is an important prognostic factor that is significantly associated with other poor prognostic parameters (CD 38, B₂ –microglobulin , high lymphocyte count and advance Rai stage) .

Recommendation: It is recommended to do further studies on larger samples to support the role of CD69 as prognostic factor and to introduce it in routine

laboratory assessment of CLL patients at time of presentation.

Also it is recommended to perform follow up studies to see the effect of treatment on CD69 expression and the association with other parameters.

More attention is recommended to be directed toward the application of CD69 as powerful new target for the development of immune therapies in patient presented with CLL.

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