Evaluation of CD38 expression in Sudanese patients with chronic lymphocytic leukemia

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Abstract
Objective: The objective of this study was to evaluate the cluster of differentiation-38 (CD38) expression in Sudanese patients with chronic lymphocytic leukemia (CLL) and to determine its association with clinical and laboratory characteristics of the disease.

Results: We conducted a cross-sectional study on 99 patients diagnosed with CLL in Khartoum Oncology Hospital in Khartoum, Sudan. Immunophenotyping and CD38 expression levels were measured with four-color flow cytometry. The results of physical examination and blood analyses were used for assigning a modified Rai clinical staging system. The collected data were analyzed using the Statistical Package for the Social Science, version 22 (SPSS Inc., Chicago, IL, USA). According to our findings, the frequencies of 7%, 20%, and 30% cutoff levels of CD38 expressions were 68.7%, 41.4%, and 36.4% respectively. CD38 cutoff level of 7% showed a significant association with hemoglobin concentration (P = 0.04), whereas other cutoff levels showed insignificant results. All the three cutoff levels showed insignificant associations with the other clinical and laboratory variables. In conclusion, the CD38 expression at a cutoff level of 7% seems to be more valuable clinically than higher cutoff levels in Sudanese CLL patients.

Keywords: CD38, Leukemia, CLL, Flowcytometer

Introduction
Chronic lymphocytic leukemia (CLL) is a chronic lymphoproliferative malignancy characterized by an accumulation of cluster of differentiation-5 (CD5) monoclonal B cells in both primary and secondary lymphoid tissues [1]. The distinct immunophenotype of CLL lymphocytes is essential to confirm the diagnosis [2]. The 1994 scoring system was based on the evaluation of five parameters: CD5, CD23, FMC7, an intensity of kappa/lambda chains, and CD22/CD79b. The B-CLL score would range between five (typical B-CLL cases) and three (less typical B-CLL cases). Lower scores (0–2) would exclude the diagnosis of B-CLL [3].

CD38 is a type II transmembrane glycoprotein that acts as a complex ectoenzyme, a receptor molecule, and a signaling factor in lymphocytic cells [4]. It participates in many cellular activities that include cell adhesion, signal transduction, and calcium regulation [5]. CD38 expression, as well as zeta-chain-associated protein kinase-70 (ZAP70) and the mutation status of immunoglobulin variable region heavy chain (IgVH), are important prognostic indicators in CLL. The advantage of CD38 is its stability over time and its easy measurement [6, 7].

The expression of CD38 by CLL cells is associated with an aggressive clinical presentation that is confirmed by many studies [8–13]. The progression-free intervals in CD38 +ve patients are shorter, and they die sooner when compared with the CD38 –ve patients [8]. This observation encouraged many clinical centers to adopt the determination of CD38 percentage expression as part of the regular investigations of CLL patients [14].
Flowcytometry is a useful tool for population analysis. It enables measurements of multiple characteristics in single cells. Light scattering can detect differences in size and internal complexity, and the fluorescence emitted from labeled antibodies can locate cell surface and intracellular antigens [15]. The analysis of both CD38 and ZAP-70 would provide valuable information in the diagnostic work-up of B-CLL patients [16]. CD38 expression also correlates with the IgVH mutation status [17, 18]; however, the two parameters were found to be independent prognostic factors in B-CLL [19, 20]. Besides, CD38 is an independent predictor of progression-free survival (PFS) in Binet stage A patients [21].

Many articles described the dynamic expression of CD38 that indicates the proliferative activity of the leukemic cells at the time of analysis [22, 23]. The expression could be a parallel indicator of clonal evolution, which ultimately determines the patient’s prognosis [24]. On the other hand, the best threshold for CD38 positivity is unclear. Early reports suggested 30% as a cutoff level of interest, but lower figures (7%, 20%) might be more clinically relevant [25].

There is a lack of data regarding the prevalence of CD38 positivity or its clinical application in the management of CLL patients in Sudan. We carried out this study to estimate the level of CD38 expression in CLL patients, to find its association with baseline clinical and laboratory parameters at presentation, and to determine a cutoff level for a CD38 appearance that is more likely to be associated with the clinical and laboratory findings of CLL patients.

Main text

Methods

We conducted a cross-sectional study at Khartoum Oncology Hospital/Sudan, during the period from September 2016 to February 2017. The study included 99 newly diagnosed, untreated B-CLL patients (69.7% males, 30.3% females), diagnosed according to the International CLL Workshop Criteria [26] and staged according to the modified Rai system [27]. A structured questionnaire was used to collect clinical and demographic data. Physical examination was performed to determine the dimensional diameters of lymph nodes in the neck, axilla, supraclavicular, inguinal, and femoral regions. The size of the liver and spleen were assessed by chest radiography and abdominal ultrasound. Three ml of peripheral blood (PB) were withdrawn from each participant, collected in EDTA tubes, and preserved at room temperature (22–24 °C). All the samples were processed within 6–24 h of collection. Complete blood count (CBC) was obtained by automated hematology analyzer (Sysmex XE-2100™, Kobe, Japan). Four color flowcytometer (COULTER EPICS XL-MCLTM Flowcytometer—Miami, Florida—USA) with SYSTEM II software was used to determine the immunophenotyping and CD38 expression for the study population. The instrument set up was checked daily using QC check beads Flowcytometry (Beckman Coulter, Miami, USA).

The immunophenotyping (IPT) of lymphocytes (from lysed whole peripheral blood samples) was carried out to confirm the diagnosis of CLL using the following monoclonal antibodies (Beckman Coulter); CD45, CD5, CD19, CD20, CD22, CD23, kappa and lambda light chains, FMC7, and CD79b. A marker was considered positive at a cutoff level of 20%. The Matutes scoring system allocates one point each for the expression of weak SmIg, CD5, CD23, and absent or low expression of CD79b and FMC7 [3, 28].

The CD38 was analyzed in peripheral blood using anti CD38 monoclonal antibodies (Mo Ab), (from Beckman Coulter, Miami, USA). The cell surface staining method was applied according to the following protocol: Twenty microliters labeled Mo Ab were dispensed into appropriately labeled tubes. A sample of 100 µl was added containing no more than 1 × 10⁴ leukocytes/ml. Each tube was vortexed for 5 s. The tubes were incubated at room temperature in a dark place for 10 min. One milliliter of the RBCs lysis solution was added to each tube and then incubated at room temperature in a dark place for 10 min. Centrifugation at 1500g (3200 rpm) for 3 min was done, 3.5 ml PBS added, and centrifugation at 1500g (3200 rpm) for 3 min repeated. Samples were suspended in PBS, tubes vortexed and analyzed using flowcytometry. CD38 was measured by flowcytometry and plotted against B cell marker CD19 expression. The CD38 expression was considered positive at three cutoff levels; 7%, 20%, and 30%.

The collected data were analyzed using the software program of the Statistical Package for Social science for Windows (SPSS), version 22 (SPSS Inc., Chicago, IL, USA). Both quantitative variables (mean and standard deviation) and qualitative variables (number and percentage) were described. Chi square test was used for Comparison of categorical variables. Statistical significance was defined for a P-value less than 0.05.

The study was approved by the Ethical Research Committee of the Sudan Medical Specialization Board (SMSB, 8/2016). Official letters were sent to the Director of Khartoum Oncology Hospital. Informed written consent was obtained from each participant before sampling. The study followed the general principles of medical research and the stated guidelines of the Ethical Committee.
Results

Ninety-nine CLL patients, including 69 (69.7%) males and 30 (30.3%) females, participated in this study. Their mean age (SD) was 62.0 (11.14) year, with a median of 63 (range 36–95) years. The clinical, hematological and flowcytometry findings are presented in Tables 1 and 2. The majority of the enrolled patients presented with lymphadenopathy (71.7%) and splenomegaly (52.5%). Complete blood count results showed anemia (72.7%), and thrombocytopenia (40.4%). The study results showed that nearly two-thirds of the participants (64.7%) were a high-risk group, 30.3% were intermediate risk group, and only 5.0% were low-risk group (Table 1). Using the scoring system, the majority of our patients had a score of 5.0 (53.5%), followed by a score of 4.0 (31.3%), (Table 1).

The diagnosis of B-CLL was confirmed by flowcytometry. It revealed a typical immunophenotype; (kappa (κ)/ lambda (λ) staining) weak, CD5+, CD19+, CD20 weak, CD23+, FMC7 (a CD20 epitope) and CD79b were absent or weakly expressed) (Table 2).

There was marked variability in the percentage expression of CD38 (range 0.09–96.1), with a median 13.00 and mean (SD) expression of 36.6 (37.92). The CD38 expression at cutoff levels of 7%, 20%, and 30%, were 68.7%, 41.4%, and 36.4% respectively (Table 3). CD38 cutoff level of 7% showed a significant association with the different classes of hemoglobin concentration (P = 0.04). The association with the other variables (age, sex, lymphadenopathy, splenomegaly, hepatomegaly, TWBC, platelets, and modified Rai staging systems was statistically insignificant (P > 0.05, Table 3).

Table 1 Characteristics of CLL patients

| Parameter                        | Frequency no (%) | Parameter                        | Frequency no (%) |
|----------------------------------|------------------|----------------------------------|------------------|
| Age                              |                  | Hemoglobin (g/dl)*               |                  |
| ≥ 60                             | 65 (65.7%)       | Severe anemia                    | 12 (12.1%)       |
| < 60                             | 34 (34.3%)       | Moderate anemia                  | 32 (32.3%)       |
| Sex                              |                  | Mild anemia                      | 28 (28.3%)       |
| Male                             | 69 (69.7%)       | No anemia                        | 27 (27.3%)       |
| Female                           | 30 (30.3%)       | Leukocyte count (x 10³/µl)       |                  |
| LN (no of sites involved)        |                  | ≤ 50                             | 33 (33.3%)       |
| Absent                           | 28 (28.3%)       | > 50                             | 66 (66.6%)       |
| 1 site                           | 4 (4.0%)         | Modified Rai staging system      |                  |
| 2 sites                          | 10 (10.1%)       | High risk group                  | 64 (64.7%)       |
| 3 sites                          | 2 (2.0%)         | Intermediate risk group          | 30 (30.3%)       |
| > 3 sites                        | 55 (55.6%)       | Low risk group                   | 5 (5.0%)         |
| Splenomegaly                     |                  | Scoring system                   |                  |
| Present                          | 52 (52.5%)       | Score 3                          | 6 (6.1%)         |
| Absent                           | 47 (47.5%)       | Score 3.5                        | 5 (5.1%)         |
| Hepatomegaly                     |                  | Score 4                          | 31 (31.3%)       |
| Present                          | 25 (25.3%)       | Score 4.5                        | 4 (4.0%)         |
| Absent                           | 74 (74.7%)       | Score 5                          | 53 (53.5%)       |
| Platelets count (x 10³/µl)       |                  | CD38 positivity                  |                  |
| < 150                            | 40 (40.4%)       | At 7% cutoff level               | 68 (68.7%)       |
| 150–450                          | 57 (57.6%)       | At 20% cutoff level              | 41 (41.4%)       |
| > 450                            | 2 (2.0%)         | At 30% cutoff level              | 36 (36.4%)       |

* WHO classification of anemia

Table 2 Flowcytometry findings in CLL patients

| Variables | Mean ± SD | Median (range) |
|-----------|-----------|----------------|
| CD45      | 85.05 ± 13.09 | 88.4 (0–98.20) |
| CD19      | 88.39 ± 8.94 | 90.60 (49.70–99.70) |
| CD20      | 81.62 ± 15.25 | 87.60 (12.90–98.40) |
| CD22      | 10.71 ± 16.67 | 3.94 (0–78.10) |
| CD23      | 64.40 ± 25.65 | 71.0 (15–96) |
| CD5       | 71.81 ± 30.99 | 88.4 (1.44–99.0) |
| CD79b     | 23.08 ± 30.71 | 19.30 (0–94.90) |
| FMC7      | 9.86 ± 15.65 | 3.70 (0.04–92.60) |
| Kappa     | 8.05 ± 15.42 | 2.05 (0–92.30) |
| Lambda    | 5.90 ± 15.58 | 0.59 (0–86.10) |

Mean and median expression of immunophenotyping markers in the study population

Distribution of the most important clinical and laboratory findings. Nearly two-thirds of the participants are within the high-risk group at presentation
The CD38 is a glycoprotein expressed on the surface of many white blood cells, including lymphocytes of CLL, and it has been linked to aggressiveness and poor prognosis of the disease. The determination of CD38 expression and IgVH mutation status are ordinary laboratory investigations in CLL patients [29]. In the clinical setting, CD38 is an independent predictor of progression-free survival (PFS) rate in Binet stage “A” patients [30]. However, the cutoff value for CD38-positivity that is most likely to predict a poor prognosis has been a source of extensive discussion.

In our study, CD38 expression at cutoff levels of 7%, 20%, and 30%, were 68.7%, 41.4%, and 36.4% respectively. CD38 (7%) showed the highest positive expression, and a significant association (*) with hemoglobin level.

Table 3 Association between the three CD38 cutoff levels and the study variables

| Variables                  | 7% cutoff level | 20% cutoff level | 30% cutoff level |
|----------------------------|-----------------|------------------|------------------|
|                            | CD38+ | CD38− | P value | CD38+ | CD38− | P value | CD38+ | CD38− | P-value |
| Age (≥ 60)                 | 46.5  | 19.2  | 0.64    | 28.3  | 37.4  | 0.41    | 25.3  | 40.4  | 0.53    |
| < 60                       | 22.2  | 12.1  | 0.09    | 13.1  | 21.2  | 0.28    | 11.1  | 23.2  | 0.39    |
| Sex (Male)                 | 51.5  | 18.2  | 0.09    | 31.3  | 38.4  | 0.28    | 27.3  | 42.4  | 0.39    |
| Lymphadenopathy (Not found)| 20.2  | 8.1   | 0.49    | 11.1  | 17.2  | 0.68    | 10.1  | 18.2  | 0.37    |
| One site                   | 2.0   | 10.1  | 0.04    | 0.0   | 5.1   | 0.0     | 0.0   | 1.0   | 0.0     |
| Two sites                  | 9.1   | 1.0   | 0.0     | 5.1   | 5.1   | 0.0     | 5.1   | 5.1   | 0.0     |
| Three sites                | 1.0   | 1.0   | 0.0     | 0.0   | 2.0   | 0.0     | 0.0   | 2.0   | 0.0     |
| More than three sites      | 36.4  | 19.2  | 0.76    | 24.2  | 31.3  | 0.21    | 21.2  | 34.3  | 0.36    |
| Splenomegaly (Present)     | 35.4  | 17.2  | 0.76    | 22.2  | 30.3  | 0.86    | 20.2  | 32.3  | 0.65    |
| Absent                     | 33.3  | 14.1  | 0.09    | 19.2  | 28.3  | 0.08    | 16.2  | 31.3  | 0.31    |
| Hepatomegaly (Present)     | 18.2  | 7.1   | 0.68    | 13.1  | 12.1  | 0.21    | 11.1  | 14.1  | 0.36    |
| Absent                     | 50.5  | 24.2  | 0.09    | 28.3  | 46.5  | 0.29    | 25.3  | 49.5  | 0.47    |
| Hemoglobin concentration (g/dl) (%) | 0.04* | 0.08  | 0.09    | 0.08  | 0.10  | 0.09    | 0.08  | 0.10  | 0.09    |
| Severe                     | 11.1  | 1.0   | 0.04    | 6.1   | 6.1   | 0.04    | 6.1   | 6.1   | 0.04    |
| Moderate                   | 18.2  | 14.1  | 0.04    | 11.1  | 12.1  | 0.04    | 11.1  | 12.1  | 0.04    |
| Mild                       | 17.2  | 11.1  | 0.04    | 11.1  | 17.2  | 0.04    | 11.1  | 20.2  | 0.04    |
| No anemia                  | 22.2  | 5.1   | 0.04    | 13.1  | 14.1  | 0.04    | 11.1  | 16.2  | 0.04    |
| TWBCs count × 10⁹ (%)      | 0.10  | 0.09  | 0.09    | 0.09  | 0.09  | 0.09    | 0.09  | 0.09  | 0.09    |
| < 50                       | 26.3  | 8.1   | 0.10    | 13.1  | 21.2  | 0.09    | 12.1  | 22.2  | 0.09    |
| ≥ 50                       | 42.4  | 23.2  | 0.28    | 28.3  | 37.4  | 0.28    | 24.2  | 41.4  | 0.28    |
| Platelet count × 10⁹ (%)   | 0.94  | 0.94  | 0.94    | 0.94  | 0.94  | 0.94    | 0.94  | 0.94  | 0.94    |
| < 150                      | 26.3  | 13.1  | 0.94    | 16.1  | 23.2  | 0.97    | 14.1  | 25.3  | 0.97    |
| ≥ 150                      | 42.4  | 18.2  | 0.94    | 25.3  | 35.4  | 0.94    | 22.2  | 38.4  | 0.94    |
| Modified Rai staging system (%) | 0.54 | 0.54 | 0.54 | 0.54 | 0.54 | 0.54 | 0.54 | 0.54 | 0.54 |
| High risk group            | 44.4  | 20.2  | 0.54    | 26.3  | 37.4  | 0.10    | 18.2  | 46.5  | 0.99    |
| Intermediate risk group    | 22.2  | 8.0   | 0.54    | 13.1  | 18.2  | 0.54    | 17.2  | 13.1  | 0.54    |
| Low risk group             | 2.0   | 3.0   | 0.54    | 2.0   | 3.0   | 0.54    | 1.0   | 4.0   | 0.54    |

The CD38 expression at cutoff levels of 7%, 20%, and 30%, were 68.7%, 41.4%, and 36.4% respectively. CD38 (7%) showed the highest positive expression, and a significant association (*) with hemoglobin level.
marker that can be used in the classification of CLL patients into different prognostic groups.

The present study showed an insignificant association between CD38 positivity and many of the clinical and laboratory parameters that included age, gender, lymphadenopathy, splenomegaly, hepatomegaly, WBC count, platelet count, and modified Rai staging system. This might be related to the late presentation of most of our patients; as about two-thirds of them are a high-risk group at the time of diagnosis, 30% were an intermediate-risk group, and only 5% are a low-risk group. In contrast to our findings, a significant association was found between advanced disease and surface expression of CD38 in >30% of B-CLL cells [29]. Contradictory results were also reported by Damle et al. who showed that high levels of CD38 (>30%) are associated with unmutated IgVH genes [8], whereas the mutated IgVH genes that indicate good prognosis, were found in patients with <30% CD38-positive cells. Our finding that CD38 positivity in CLL at a cutoff level of 30% was 36% goes in line with the published literature that reported comparable results [32–36].

In conclusion, the CD38 expression at a cutoff value of 7% is significantly associated with low hemoglobin concentration, and therefore, more likely to predict poor outcomes than the higher CD38 cutoff levels in Sudanese CLL patients. Further studies are highly recommended for determining the “progression-free survival” and the most likely predictors of prognosis in Sudanese patients.

Limitations
The present study has many limitations. The sampling method that depended on the voluntary participation of participants, the late presentation of our patients compared to other studies, and the lack of follow-up information, should all be considered for interpretation of the results. The follow-up information is particularly relevant because it gives valuable information about the prognosis regarding survival rate and response to treatment.

Abbreviations
CLL: chronic lymphocytic leukemia; CD: cluster of differentiation; IgVH: immunoglobulin variable region heavy chain; ZAP70: zeta-chain-associated protein kinase-70; TWBCs: total white blood cells count; PFS: progression-free survival.

Authors’ contributions
EAA conceived the study, participated in data collection, performed the statistical analysis, interpreted the results, and revised the manuscript. NHE participated in data collection, participated in the statistical analysis, and drafted the manuscript. TAE, OAA, and EAF participated in the data collection, carried out the laboratory work, and prepared the results. EMA participated in study conception, supervised the laboratory work, and revised the manuscript. THM participated in the supervision, data interpretation, and drafting the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The individual data are available in the archives of the Khartoum Oncology Hospital-Sudan and can be obtained from the corresponding author on request.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The study was approved by the Ethical Committee of the Sudan Medical Specialty Board (SMSB, 8/2016). Official letters were sent to the Director of Khartoum Oncology Hospital. Informed consent was obtained from each participant before data collection.

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