Evaluation of prognostic variables in chronic lymphocytic leukemia and association with disease stage

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Abstract. The aim of the present study was to investigate different biological prognostic markers to identify high-risk patients with chronic lymphocytic leukemia (CLL) with a higher tumor burden, in order to ensure appropriate management. A total of 81 Egyptian patients with CLL were enrolled in the present study, with 75 healthy subjects serving as the control group. The expression of CD49d, CD38 and ZAP-70 in CLL cells was assessed using flow cytometry. The fluorescence *in situ* hybridization technique was employed to evaluate *TP53* (del17p), ataxia-telangiectasia (del11q) and *13q14* (del13q14) genes and the presence of trisomy 12. The serological markers β2 microglobulin (B2M) and sCD23 were measured by ELISA. The *CD49d* gene was highly expressed in 25.9% and cytogenetic aberrations were observed in 66.6% of all recruited CLL patients. The patients were categorized according to the Binet staging system and a significant increase in the expression of sCD23, CD49d and ZAP-70 was detected in group C (P=0.008, 0.034 and 0.017, respectively) when compared to groups A and B. CD49d$^+$ patients exhibited significantly higher expression of CD38 (P=0.002) and trisomy 12 (P=0.015) and lower expression of del13q14 (P=0.001). Patients who were CD49d$^+$ with B2M>3.5 μg/ml exhibited higher total leukocyte count (P=0.048), higher absolute lymphocyte count (P=0.036), higher expression of CD38 (P=0.002) and trisomy 12 (P=0.034) and lower expression of del13q14 (P=0.002). Therefore, sCD23, CD49d and ZAP-70 may be considered as an optimal prognostic marker combination to be evaluated in the early stages of CLL and throughout disease management. Integrating both serological markers and CD49d expression by flow cytometry may add to the prognostic value of each marker alone and help identify high-risk patients with a higher tumor burden.

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Introduction

Chronic lymphocytic leukemia (CLL) is a clinically heterogeneous disease, as evidenced by the fact that a proportion of the patients have refractory disease and succumb within 2-3 years after the diagnosis, whereas others display an indolent disease course without need for treatment and may survive for decades (1). Due to this heterogeneity, CLL is considered suitable as a disease model for personalized medicine. In addition, malignant cells are easily obtainable, and the patient characteristics, including clinical parameters (e.g., age and comorbidities) and biological parameters (e.g., genomic aberrations and mutations), are diverse. A large number of cellular biomarkers have been found to be correlated with the prognosis of CLL patients. Certain genetic lesions, such as loss of function of the DNA-damage response by tumour protein P53 (*TP53*) gene (del17p), or ataxia-telangiectasia (*ATM*) gene (del11q), particularly with respect to DNA-damaging chemotherapy (e.g., fludarabine), have a highly negative prognostic impact (2). Furthermore, markers such as CD49d, CD38 and intracytoplasmic ZAP-70, which are not only correlated with prognosis, but also with genetic aberrations, are possibly involved in the mobilization and homing of CLL cells (3,4). CD49d is the α4 subunit of the integrin heterodimer α4β1. Its expression promotes microenvironment-mediated proliferation of CLL cells and identifies a subgroup of CLL patients who develop progressive disease (5,6). The levels of soluble serum markers, such as thymidine kinase, β2-microglobulin (B2M) and soluble CD23, are important predictors of prognostic

relevance in patients with CLL (7,8). With the recent advent of new, more effective therapies, the known survival predictors, which require continuous research to properly stratify patients for treatment, are becoming obsolete (9). The aim of the present study was to evaluate the most commonly used genetic and serological prognostic markers known to have survival predictive value for identifying high-risk CLL patients with a higher tumor burden to ensure appropriate management, and to validate CD49d as a predictive prognosticator in a sample of Egyptian patients with CLL.

Patients and methods

Subjects. A total of 81 Egyptian patients with CLL were recruited from the National Cancer Institute and Kasr El-Aini Hospital between December 2017 and October 2019. A total of 75 healthy subjects who were age- and sex-matched to the studied patients were randomly recruited as the control group. All participants provided informed verbal consent before being included in the study, in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments involving human subjects. The study was approved by the Ethics Committee of the National Research Center (registration no. 17-147). The diagnosis of CLL was based on the World Health Organization classification of tumors of hematopoietic and lymphoid tissues (10). Full history taking and thorough clinical examination of the patients were performed to evaluate the presence of pallor, petechiae, jaundice, lymphadenopathy, splenomegaly or hepatomegaly. Treatment was initiated in 58% of patients with disease-related symptoms, advanced or progressive disease according to criteria described by recent guidelines (11). At least one of these criteria should be met: Evidence of progressive marrow failure as manifested by the development or worsening of anemia and/or thrombocytopenia; massive (≥6 cm below the left costal margin) or progressive or symptomatic splenomegaly; massive nodes (≥10 cm in longest diameter) or progressive or symptomatic lymphadenopathy; progressive lymphocytosis with an increase of ≥50% over a 2-month period, or lymphocyte doubling time <6 months; autoimmune complications, including anemia or thrombocytopenia, poorly responsive to corticosteroids; symptomatic or functional extranodal involvement; disease-related symptoms including unintentional weight loss ≥10% within the previous 6 months, significant fatigue, fever (≥38.0˚C) for ≥2 or weeks without evidence of infection, night sweats for ≥1 month without evidence of infection.

Patients were evaluated clinically according to the Binet and Rai staging systems (11) regardless of treatment type or disease duration.

Sample collection. Peripheral blood samples (6 ml) were collected in K2-EDTA, sodium heparin and plain blood collection tubes (Becton, Dickinson and Company), in order to perform diagnostic and prognostic evaluation of patients with CLL.

Flow cytometric analysis. Monoclonal antibodies for diagnostic and prognostic evaluation (all from Beckman Coulter Immunotech) included: CD19-PC7 (cat. no. IM3628U); CD5-FITC (cat. no. IM0468U); CD10-PC5.5 (cat. no. B16490); CD49d-FITC (cat. no. IM1404U); CD38-PE (cat. no. IM2371U) and ZAP-70-PE (cat. no. B57658). Monoclonal antibodies (all from Agilent Technologies, Inc.) included: CD20-FITC (cat. no. F0799); CD79b-PE (cat. no. R7272); anti-kappa-FITC (cat. no. F0434); and anti-lambda-PE light chains (cat. no. R0437).

Assessment of CD49d, CD38 and cytoplasmic ZAP-70 (cytoZAP-70) markers was performed using Beckman Coulter Navios Flow Cytometer, as described by Chen *et al* (12). Labeled Mo Ab (20 μl) were dispensed into appropriately labeled tubes, followed by the addition of 100 μl sample and incubation in the dark for 20 min. Three and half milliliter Lysing reagent (3.5 ml) was added to each tube, inverted once and kept for 5 min. Centrifugation was performed at 300 x g for 3 min at room temperature, 3.5 ml PBS was added, and centrifugation was repeated at 300 x g for 3 min. CD49d and CD38 were tested in two different tubes and were gated on $CD19^+$ B cells. IntraPrep Permeabilization Reagent (cat. no. A07803; Beckman Coulter Immunotech) was used

Table I. Clinical characteristics of patients with CLL.

Characteristics	Patients (n=81)
Course of disease, n (%)	
Newly diagnosed	10 (12.35)
Indolent	32 (39.51)
Aggressive	39 (48.14)
Binet stage at diagnosis, n (%)	
A	11 (13.6)
B	29 (35.8)
C	41 (50.6)
Rai stage at diagnosis, n (%)	
I and II (intermediate risk)	40 (49.4)
III and IV (high risk)	41 (50.6)
Clinical findings, n (%)	
Lymphadenopathy	59 (72.8)
Splenomegaly	56 (69.1)
Hepatomegaly	34 (41.9)
Light chain subtype, n (%)	
Lambda (λ-restricted)	30 (37.04)
Kappa (κ-restricted)	51 (62.96)
Scheme of therapy of 46 treated patients, n (%)	
FC (purine analogue-based) or FCR chemotherapy	26 (55.3)
Chemotherapy other than FC, including CVP and CHOP	16 (34.0)
Targeted therapy[a]	4 (8.5)

[a]Oral targeted therapies (ibrutinib) or anti-CD20 monoclonal antibodies (obinutuzumab). CLL, chronic lymphocytic leukemia; FC, fludarabine + cyclophosphamide; FCR, fludarabine + cyclophosphamide + rituximab; CVP, cyclophosphamide + vincristine + prednisone; CHOP, cyclophosphamide + doxorubicin + vincristine + prednisone.

Table II. Hematological and serological variables in patients with CLL and controls.

Variables	CLL patients (n=81)	Controls (n=75)	P-value
Sex (n), male/female	47/34	46/29	0.362 (NS)
Age (years)	58.25±9.67	51.18±7.0	0.125 (NS)
Hemoglobin (gm/dl)	3.8-15.7 (10.8±2.5)	12.0-18.6 (15.5±1.9)	0.000[a]
Total leukocyte count (x10^9/l)	2.0-407.2 (78.1±105.9)	4.0-10.0 (6.6±1.6)	0.000[a]
Platelet count (x10^9/l)	30-412 (166.2±88.9)	160-418 (262.9±55.1)	0.000[a]
PB lymphocytes (%)	14-98 (61.4±28.6)	15-60 (41.57±9.8)	0.001[a]
Absolute lymphocyte count (x10^9/l)	0.6-371.7 (60.75±96.4)	1.0-5.5 (2.6±0.8)	0.000[a]
sCD23 (U/ml)	13.8-20032 (1.443E3±96.0)	9.9-95.0 (43.01±2.7)	0.000[a]
B2M (μg/ml)	2.0-17.8 (5.79±3.4)	1.2-3.0 (2.6±0.49)	0.000[a]

[a]Highly statistically significant. Values are presented as range (mean ± SD). CLL, chronic lymphocytic leukemia; PB, peripheral blood; B2M, β2 microglobulin.

for cytoZAP-70 detection. Specific isotypic controls for FITC and PE-conjugated monoclonal antibodies were used. The expression results were reported as the percentage of $CD49d^+/CD38^+$/cytoZAP-70^+ cells over the corresponding isotypic control within the gated $CD19^+$ B-cell population for each patient and control subject.

Fluorescence in situ hybridization (FISH). FISH was performed for all fresh heparinized peripheral blood samples obtained from the patients according to the manufacturer's instructions using the Vysis CLL FISH Probe Kit (cat. no. 04N02-021; Abbott Molecular) (13). The SpectrumGreen-labeled (LSI) ATM probe specific for del11q22.3 contains the ATM gene, the SpectrumOrange-labeled LSI-D13S319 probe is located at 13q14.3 and the SpectrumOrange-labeled LSI TP53 probe is located at 17p13.1. In addition, the SpectrumGreen-labeled CEP 12 probe is located at the centromere of chromosome 12. A total of 200 interphase cells were examined for each probe. The number of observed orange and green signal patterns from each of two readers were added together to generate the count for 200 nuclei for each specimen. Normal/abnormal determination was made for each specimen by comparing the number of nuclei with observed abnormal patterns per 200 scoreable nuclei to the normal determined cut-off value. Cut-off points were determined by the main laboratory of Kasr El-Aini Hospital from cytogenetically normal controls among patients' relatives.

Serology. Serum samples from all recruited subjects were quantitatively analyzed for sCD23 and B2M levels using the human sCD23 ELISA kit (Invitrogen; Thermo Fisher Scientific, Inc.; Bender MedSystems GmbH; cat no. BMS 227-2) and the B2M ELISA kit, ORGENTEC Diagnostika GmbH; cat. no. ORG 5BM), according to the manufacturer's protocols.

Statistical analysis. Data were coded and entered into the SPSS analysis program (version 25; IBM Corp.). Data are presented as mean with standard deviation, minimum and maximum for quantitative data and as frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were performed using the non-parametric Kruskal-Wallis and Mann-Whitney U tests (14). Categorical data were compared using the χ^2 test. Fisher's exact test was used instead when the expected frequency was <5 (15). Correlations between quantitative variables were assessed using Spearman's correlation coefficient. P<0.05 was considered to indicate statistically significant differences.

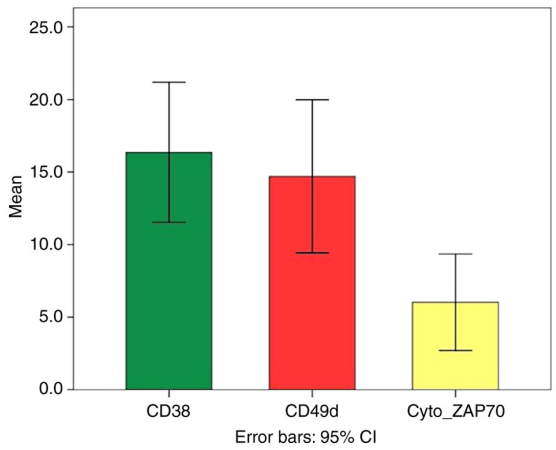

Figure 1. Expression levels of prognostic markers in patients with chronic lymphocytic leukemia (n=81). Flow cytometric data are presented as the percentage of cells positively expressing the marker within $CD19^+$ gated population.

Results

Patient characteristics. The clinical data of patients with CLL are summarized in Table I. A total of 32 patients (39.51%) had an indolent disease course during follow-up, whereas 39 patients (48.14%) had progressive disease with clinical symptoms. A total of 10 patients (12.35%) were newly diagnosed at the time of recruitment. According to the Binet staging system, 50.6% of the patients were stage C, 35.8% were stage B and 13.6% were stage A at diagnosis. In the present study, patients with CLL had clonal neoplastic cells with the morphological characteristics of small mature B lymphocytes in the peripheral blood. The hematological and serological findings of patients

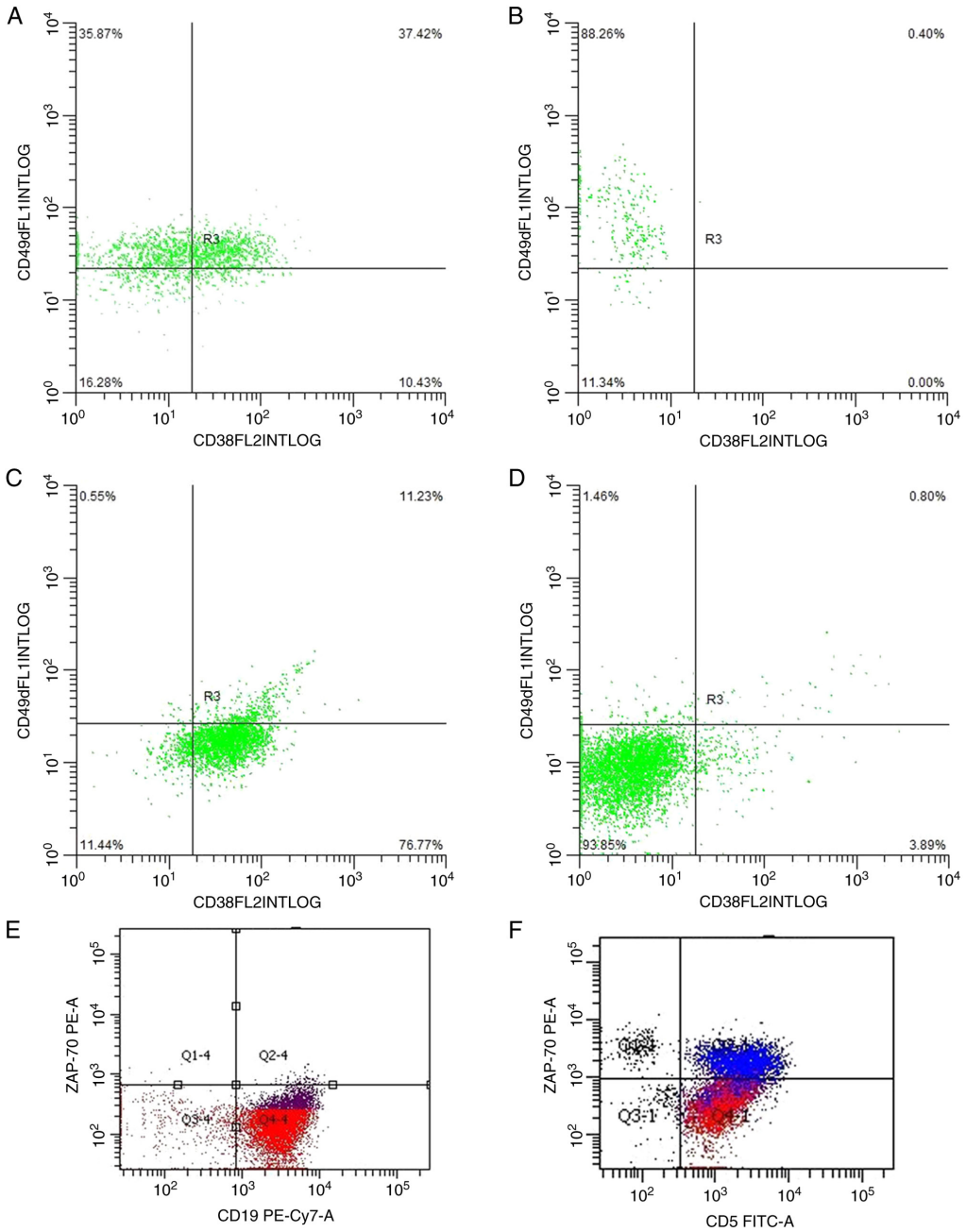

Figure 2. (A-F) Representative flow cytometric dot plots of different expression patterns in CD19+ patients with chronic lymphocytic leukemia. (A) Dot plot of a double-positive CD49d and CD38 case; (B) dot plot of a single CD49d expression case (CD38-negative); (C) dot plot of a single CD38 expression case (CD49d-negative); (D) dot plot of a double-negative case for CD38 and CD49d; (E) dot plot of a ZAP-70-negative case; (F) dot plot of a ZAP-70-positive case.

Table III. Prognostic variables by flow cytometry in patients with CLL and controls.

Prognostic markers	Expression levels[a] in CLL patients (n=81)		Positive expression[b] in CLL (%)	Expression levels in controls (n=75)		P-value
	Range (%)	Mean ± SD (%)		Range (%)	Mean ± SD (%)	
CD49d	0.1-94.0	15.7±22.7	25.9	0.5-3.9	2.2±1.1	0.000[c]
CD38	0.1-90	15.8±19.2	28.4	0.6-13.6	7.5±4.4	0.005[c]
ZAP70	0.1-62	6.0±13.3	8.6	0.0-2.9	0.8±1.0	0.004[c]

[a]Flow cytometric data are presented as the percentage of cells positively expressing the marker within the CD19+ gated population. [b]Positive expression was considered as >30% for CD49d and CD38, and as >20% for ZAP-70. [c]Statistically significant. CLL, chronic lymphocytic leukemia.

Table IV. Prognostic variables by FISH in patients with CLL.

Prognostic markers	Positive expression, n (%)	Expression range (%)	Cut-off[a] (%)
Trisomy 12	13 (16)	0.0-67.0	9
del17p	10 (12.3)	0.0-81.0	9
Del11q	13 (16)	0.0-88.0	9
Del13q14	18 (22.2)	0.0-93.0	9

[a]Cut-off values were determined in the main laboratory of Kasr El-Aini Hospital from cytogenetically normal controls among patients' relatives. CLL, chronic lymphocytic leukemia.

Table V. Significant prognostic variables in CLL patient groups according to the Binet staging system.

Variables	Stage A (n=11)	Stage B (n=29)	Stage C (n=41)	P-value (stage C vs. A and B)
sCD23 (U/ml)	864.17±202.7	1.270±176.6	1.698±138.9	0.008[b]
CD49d (%)	19.3±12.03	14.3±5.7	20.06±4.05	0.034[a]
ZAP-70 (%)	0.5±0.2	1.1±0.4	8.7±3.0	0.048[a]

[a]Statistically significant. [b]Highly statistically significant. Values are presented as mean ± SE. CLL, chronic lymphocytic leukemia.

Table VI. Significant prognostic variables in patients with CLL according to CD49d (CD49d$^+$, n=21; CD49d$^-$, n=60) and trisomy 12 (trisomy 12$^+$, n=113; trisomy 12$^-$, n=68) expression.

	Expression		
Variables	Positive	Negative	P-value
CD49d			
Absolute lymphocyte count (x10^9/l)	75.9±30.9	47.1±13.6	0.022[a]
CD38 (%)	25.5±4.7	13.5±2.7	0.01[a]
Trisomy 12 (%)	21.6±7.03	4.06±1.3	0.015[a]
Del13q14 (%)	2.79±0.47	23.4±5.8	0.001[b]
Trisomy 12			
CD49d (%)	39.3±5.9	16.6±4.1	0.004[b]
Del13q14 (%)	2.9±0.46	20.8±4.6	0.000[b]

[a]Statistically significant. [b]Highly statistically significant. Values are presented as mean ± SE. CLL, chronic lymphocytic leukemia.

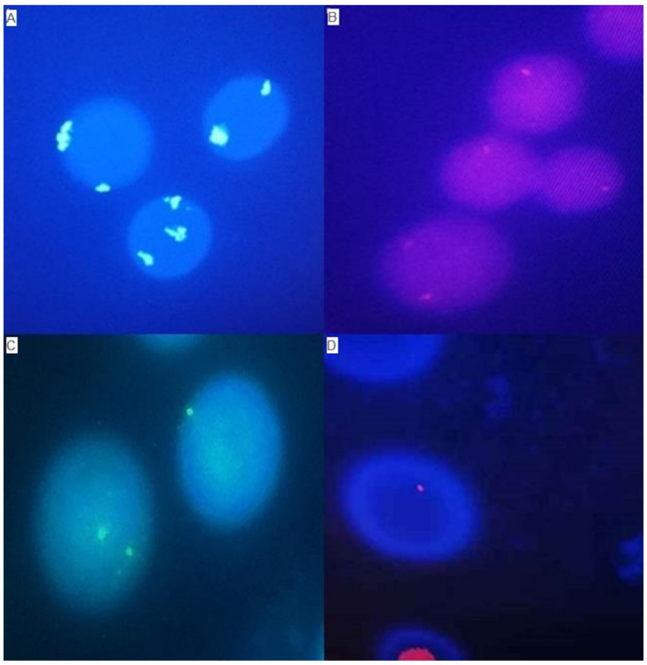

Figure 3. (A-D) Fluorescence *in situ* hybridization analysis in patients with chronic lymphocytic leukemia positive for cytogenetic aberrations. (A) Trisomy 12-positive cells with three green signals; (B) 13q deletion with one orange signal; (C) ATM del with one green signal; (D) p53 del with one orange signal.

with CLL and the age- and sex-matched controls are listed in Table II. The wide range in these parameters in the CLL group was expected due to the presence of different categories of patients as regards disease stage and therapeutic interventions. i) On flow cytometry, the patient samples displayed 100% positive expression of CD19, 25 and 20, as well as clonal restriction (λ-restricted, 37.04% and κ-restricted, 62.96% of the samples). ii) Prognostic workup: The expression levels (%) of prognostic markers by flow cytometry are demonstrated in Table III and Fig. 1. Representative flow cytometric dot plots of different prognostic variables on CD19$^+$ B-CLL cases are shown in Fig. 2A-F. A total of 54 patients with CLL (66.6%) exhibited genetic aberrations using the FISH technique (Table IV and Fig. 3A-D).

The patients were categorized according to the Binet staging system, and a significant increase in the expression of sCD23, CD49d and ZAP-70 was detected in group C (P=0.008, 0.034 and 0.017, respectively) when compared to both groups A and B, as shown in Table V and Figs. 4 and 5.

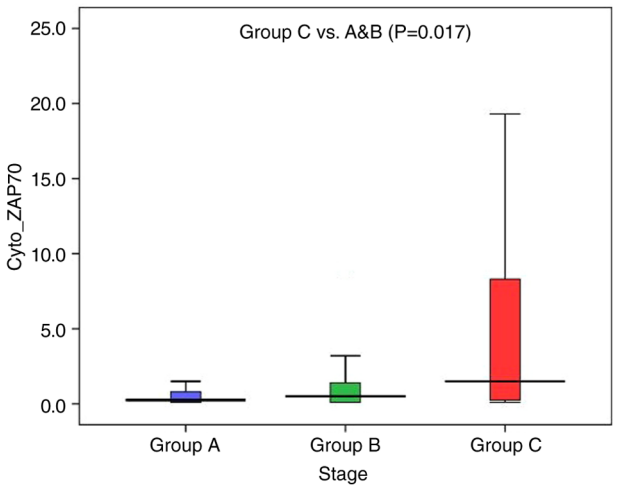

Figure 4. Mean expression of ZAP-70 (%) in patients with Binet stage A (n=11), B (n=29) and C (n=41) chronic lymphocytic leukemia.

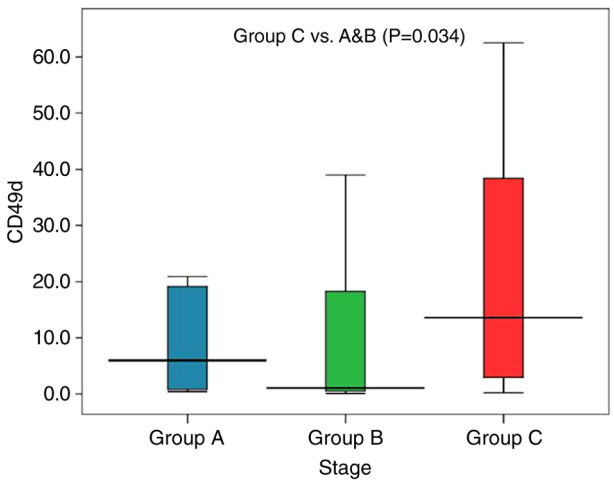

Figure 5. Mean expression level of CD49d (%) in patients with Binet stage A (n=11), B (n=29) and C (n=41) chronic lymphocytic leukemia.

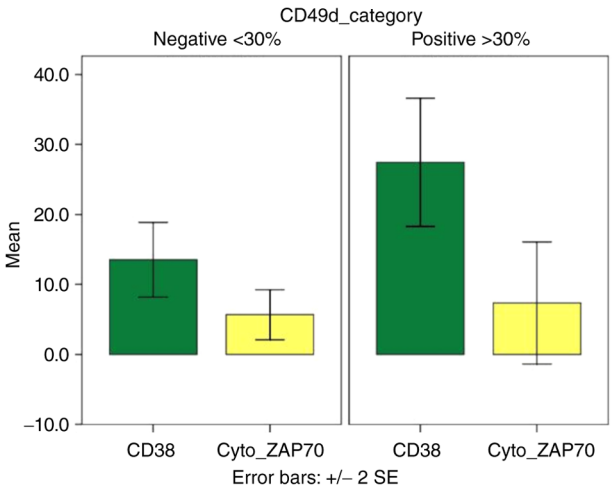

Figure 6. Changes in the mean expression levels (%) of CD38 (P=0.002) and ZAP-70 in both positive (>30%; n=21) and negative (<30%; n=60) CD49d cases by flow cytometry.

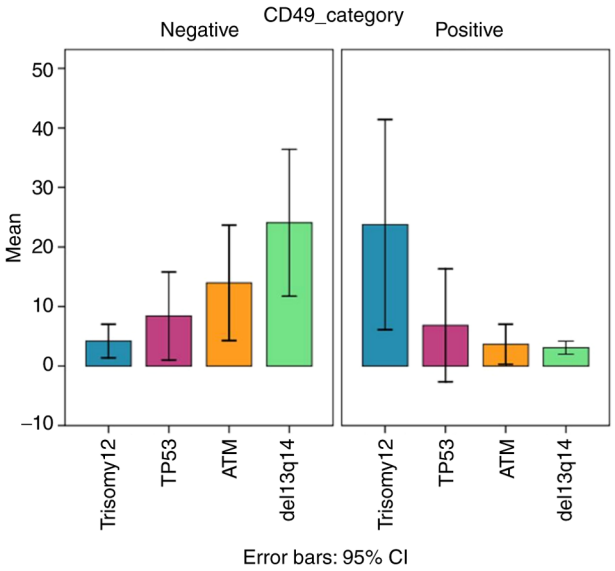

Figure 7. Cytogenetic aberrations (mean expression, %) by fluorescence *in situ* hybridization analysis in both CD49d$^+$ (n=21) and CD49d$^-$ (n=60) patients with chronic lymphocytic leukemia.

Elevated B2M (>3.5 μg/ml) was more frequently detected in group B and C patients (91.7 and 97.1%, respectively; P=0.004). In addition, patients under treatment with advanced disease and aggressive course exhibited higher B2M levels compared with those with an indolent course (P=0.025). The B2M levels were significantly elevated in group C patients when compared with group A patients (P=0.034).

Higher frequency of CD49d positivity was found in patients with positive CD38 expression (P=0.027). CD49d was considered positive when its level was >30% according to Bulian *et al* (3). Higher incidence of hepatomegaly and splenomegaly was detected among CD49d$^+$ patients (57.1 and 85.7% with P=0.049 and 0.016 respectively). Furthermore, CD49d$^+$ patients exhibited significantly higher CD38 levels (P=0.002; Table VI and Fig. 6). Additionally, CD49$^+$ patients exhibited higher expression of trisomy 12 (P=0.015) and lower expression of del13q14 (P=0.001), as shown in Table VI and Fig. 7.

Variations in the levels of CD38 and ZAP-70 in relation to the different categories of sCD23 and CD49d are shown in Fig. 8. Higher CD38 expression was observed in patients with levels of sCD23 >95 U/ml and CD49d positivity >30% (P=0.012).

In trisomy 12$^+$ patients (n=13; 16% of patients with CLL), CD49d positivity was more frequently observed (75%; P=0.015), as was a higher mean level of CD49d (P=0.004) and lower mean expression of del13q (P=0.000; Table VI and Fig. 9).

Patients positive for CD38 had a higher total leukocyte count (TLC), absolute lymphocyte count (ALC) and sCD23 levels (P=0.038, 0.02 and 0.000, respectively).

Patients with both CD49d$^+$ and B2M>3.5 μg/ml exhibited higher TLC (P=0.048), higher ALC (P=0.036), higher expression of CD38 (P=0.002) and trisomy 12 (P=0.034), and lower expression of del13q14 (P=0.002; Fig. 10).

In correlation studies, s-CD23 and CD49d were positively correlated with CD38 (r=0.267 and 0.371; P=0.037 and 0.002, respectively); sCD23 was positively correlated with B2M

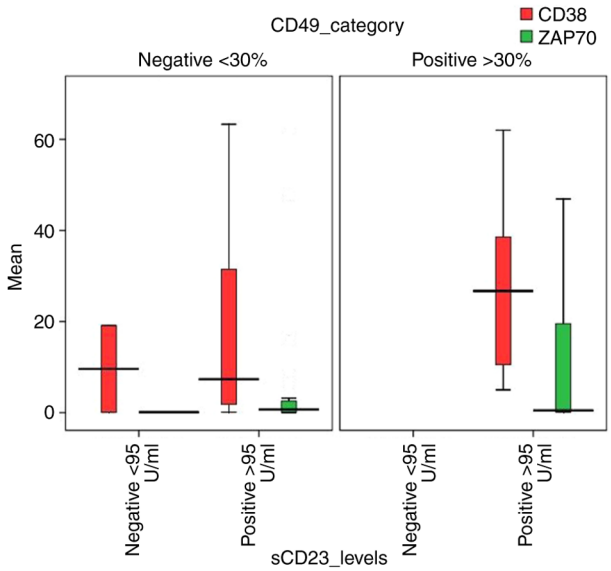

Figure 8. Variation in the expression levels of CD38 and ZAP-70 in patients with chronic lymphocytic leukemia exhibiting different sCD23 levels and CD49d expression. CD49d$^+$ patients (n=21) exhibited 90.47% positive expression of sCD23 (>95 U/ml); and CD49d$^-$ patients (n=60) exhibited 91.6% positive expression of sCD23 (>95 U/ml). Higher CD38 expression was observed in patients with high levels of sCD23 (>95 U/ml) and CD49d >30% (P=0.012).

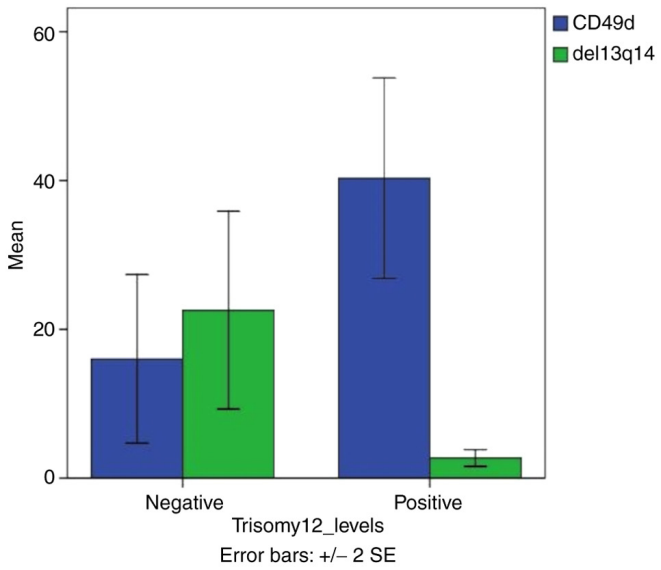

Figure 9. Mean expression levels of CD49d and del13q14 in both trisomy 12$^+$ (n=13) and trisomy12$^-$ patients with chronic lymphocytic leukemia (n=68). CD49d positivity was more commonly observed among trisomy 12$^+$ patients (75%; P=0.015), and those with higher mean expression of CD49d (P=0.004) and lower mean expression of del13q (P=0.000).

(r=0.344, P=0.003); and CD38 was positively correlated with the presence of del11q (r=0.314, P=0.036).

Discussion

The present study investigated different clinical and biological prognosticators in patients with CLL. Patients on novel therapeutic agents were included in the study.

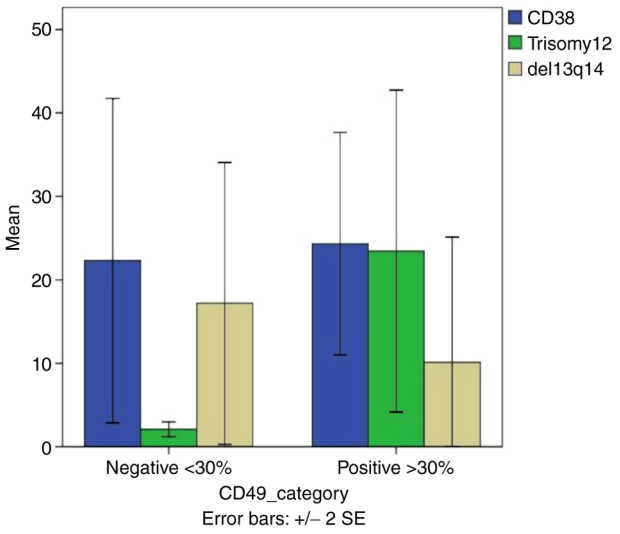

Figure 10. Mean expression levels of CD38 (%), trisomy 12 (%) and del13q14 (%) in patients with chronic lymphocytic leukemia with different categories of CD49d and B2M >3.5 μg/ml. Patients with B2M levels >3.5 μg/ml (n=62) who expressed positive CD49d, had higher expression of CD38 (P=0.002), trisomy12 (P=0.034) and lower expression of del13q14 (P=0.002). B2M, β2 microglobulin.

Patients were categorized according to the Binet clinical staging system. sCD23, CD49d and ZAP-70 exhibited significantly higher expression in group C when compared with groups A and B. Therefore, the expression of these prognostic markers in the early stages and throughout the disease management may help identify progression. Popova *et al* (16) reported that flow cytometric markers were negative in the early stages of CLL and underwent changes in 10-25% of cases with disease progression. Previously, Saka *et al* (17) found that sCD23 determination at diagnosis and during the course of the disease may help identify patients who rapidly progress to more advanced stages.

Detection of CD49d by flow cytometry was considered superior to CD38 and ZAP-70 in predicting overall survival (18). In the present study, the frequency of positive CD49d and CD38 cases was 25.9 and 28.4% of the total number of cases, whereas that of ZAP-70 was 8.6%. Due to the lower frequency of positive ZAP-70 expression among CLL patients, the interpretation of the results was easier for CD49d and CD38.

Patients with CLL were stratified according to their CD49d levels to identify variations in different prognosticators. In CD49d$^+$ patients, higher incidence of hepatomegaly and splenomegaly was observed, whereas higher expression of CD38, sCD23 and trisomy 12 and significantly lower expression of del13q14 were detected by FISH. These findings were in agreement with those of previous investigators who found that positive CD49d expression >30% was associated with advanced clinical stage and aggressive CLL course (3,18-20). Recently, Tissino *et al* identified a bimodal expression of CD49d with distinct clinicobiological characteristics in 20% of CLL cases. They recommended that the pattern of CD49d expression should also be considered to improve its prognostic impact in CLL (21).

In the subgroup of CD38$^+$ patients, higher tumor burden with higher TLC, ALC and increased sCD23 levels were

detected. These associations were in agreement with previous findings (22). Serological markers have been considered as predictors of tumor burden in addition to disease progression. In the present study, when patients were stratified according to sCD23 expression levels, high levels were associated with increased expression of CD38 and CD49d, indicating its role in disease progression. In patients with high levels of sCD23, higher CD38 expression was observed in the presence of positive CD49d expression compared with patients negative for CD49d expression. Patients with B2M levels >3.5 μg/ml and positive CD49d expression exhibited increased tumor burden, higher expression of CD38 and trisomy 12. These findings highlighted that integrating both serological markers and CD49d may add to the prognostic value of each marker alone. A number of researchers have investigated the integration of prognostic markers into a combined risk score due to discrepancies found in the same patient, with some prognostic factors suggesting shorter time to first treatment and overall survival compared with others (23-25).

Trisomy12$^+$ CLL patients represent a distinctive subset of patients with CLL who exhibit higher expression of CD49d and lower expression of del13q14. The association between trisomy 12 and CD49d expression was previously explained (21). Trisomy 12$^+$ CLL is characterized by high mutation frequency of the *NOTCH1* gene, and an association between higher CD49d expression and the presence of *NOTCH1* mutations through nuclear factor-κB pathway involvement has also been reported (26,27). In the future, the assessment of *TP53* aberrations, in the form of both gene mutations and del17p, may become mandatory, as patients carrying such gene disruptions are defined as high-risk. In addition, the analysis of *TP53* mutations can identify 30-40% of CLL patients who only carry the gene mutations and may be missed by FISH. Cohen *et al* proposed that *TP53* aberrations, unmutated *IGHV*, mutated *NOTCH1* and CD49d expression are powerful prognosticators and have predictive significance in CLL before treatment initiation to guide therapeutic decisions (28). *IGHV* mutational status is a stable CLL prognostic marker and can be measured at any time throughout the disease course. It may also help with the selection of novel targeted therapies in unmutated cases. Further studies are recommended on a larger scale to assess and integrate the *IGHV* mutational status and *TP53* mutations to optimize patient stratification.

Expression of the sCD23, CD49d and ZAP-70 prognostic markers in early-stage CLL and throughout the disease management may help identify any progression to a more advanced stage. CD49d is considered superior as a prognostic marker and CLL patients positive for CD49d expression exhibited higher incidence of hepatomegaly and splenomegaly, higher expression of CD38, sCD23 and trisomy 12, and significantly lower expression of del13q14. CD38 expression was associated with higher tumor burden, higher TLC and ALC, and increased sCD23 levels. CLL patients with high levels of sCD23 exhibited higher CD38 expression in the presence of CD49d-positive expression compared with CD49d-negative patients. Patients with high B2M levels (>3.5 μg/ml) and CD49d-positive expression exhibited increased tumor burden, higher expression of CD38 and trisomy 12. Therefore, integrating serological markers and CD49d expression by flow cytometry may add to the prognostic value of each marker alone and help identify high-risk patients with a higher tumor burden.

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Authors' contributions

HRMA and AHA contributed to the project preparation, study design and submission for funding. HRMA, MHI and SHAEA were responsible for the management of purchasing tasks and schedules. MHI coordinated specimen collection and transport and implemented a quality policy throughout the laboratory analysis workflow. MHI and SHAEA contributed to serological analysis and tabulation of final result. Patient selection and data collection were performed by NMH, MES, RA, RR and OYAED. FISH was performed by MEY and HAAH. Flow cytometric analysis was performed by RAO. NE contributed to appropriate selection of CD markers and supervision of flow cytometric procedures. Analysis of data and the first draft of the manuscript were prepared by HRMA. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All participants provided verbal informed consent prior to being included in the present study in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments on humans and the study was approved by ethical committee of National Research Center, Egypt, (registration no. 17-147).

Patient consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed in the present study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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