The prognostic significance of dipeptidyl peptidase IV (CD26) expression in B-cell chronic lymphocytic leukemia

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Background B-cell chronic lymphocytic leukemia (B-CLL) has a highly variable clinical course. The leukemic transformation is initiated by specific genomic alterations, impairing apoptosis of clonal B-cells. Many prognostic factors have been identified, yet research studies continue to identify new prognostic factors to predict the course of B-CLL.

Aim This study aimed to evaluate the prognostic value of CD26 expression in Egyptian patients with B-CLL and assess its correlation to other clinical and laboratory parameters with known prognostic significance.

Patients and methods A total of 30 Egyptian adults with newly diagnosed B-CLL and 15 healthy participants (control group) were subjected to full study of clinical, laboratory, flow cytometry, and cytogenetic data. Testing of CD26% expression was done by flow cytometry-immunophenotyping. Detections of ataxia telangiectasia mutated, P53, 13q14 deletions, and trisomy 12 were performed using fluorescent in-situ hybridization. Immunoglobulin heavy-chain variable mutational status was done by PCR. Moreover, smudge cells % was calculated by microscopic evaluation of stained blood smears.

Results A statistically significant increase of CD26% expression in patients with B-CLL in comparison with normal participants was documented ($P<0.001$). CD26% expression on B-CLL was statistically significantly correlated with advanced modified Rai's clinical stage of B-CLL at diagnosis ($P<0.001$), hepatomegaly ($P=0.003$), low hemoglobin level ($P=0.049$), low platelets count ($P=0.034$), high lactate dehydrogenase blood levels ($P=0.001$), and atypical lymphocytes % ($P=0.049$). Correlation studies with other prognostic factors revealed statistically significant correlations between CD26% expression and CD38% ($P<0.001$), ZAP-70% ($P=0.007$), the unmutated status of immunoglobulin heavy-chain variable ($P<0.001$), and low % of smudge cells ($P<0.001$).

Conclusion CD26% expression could be considered as an adverse prognostic predictor being associated with high-risk markers in B-CLL. It can be routinely used, as a part of immunophenotyping panel, in third world countries at diagnosis, for prognosis, risk-stratification, and tailoring of therapy.

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Introduction

Chronic lymphocytic leukemia (CLL) is characterized by clonal proliferation and accumulation of mature, typically CD5-positive B cells within the blood, bone marrow (BM), lymph nodes (LNs), and spleen. Recently, it has been reported that, in CLL, the capacity to generate clonal B-cells might be acquired at the hematopoietic stem cell stage, suggesting that the primary leukemogenic event, in CLL, might involve multi-potent self-renewing hematopoietic stem cells [1].

Despite homogeneous morphology, transcriptional profile, and immunophenotype (IPT), CLL is clinically a heterogeneous disease, where some patients never require therapy, whereas others display an aggressive course with poor response to therapy [2]. Recently, some investigators have tried to develop and validate a 17-gene expression signature that distinguished immunoglobulin (Ig) heavy-chain variable gene (IgHV)-unmutated patients, to identify which of them is likely to achieve durable remissions with fludarabine, cyclophosphamide, and rituximab chemoimmunotherapy from those who might benefit from alternative front-line regimens [3].

To address heterogeneity and predict prognosis of patients, several prognostic markers were studied, based on genetic phenotyping or molecular characteristic of B-cell chronic lymphocytic leukemia (B-CLL) [4,5]. However, owing to the limited availability, high cost of markers, and methodological complexity, some of these markers cannot be used for routine evaluation. Thus, researchers continue to identify new prognostic factors, targeting to predict the CLL course [6].

Various biological and genetic markers also have prognostic value. Deletions of short arm of

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chromosome 17[del (17p)] and/or mutations of tumor protein '53' (TP53) gene [7], predict resistance to chemoimmunotherapy and a shorter time to progression, with most targeted therapies. A comprehensive, international prognostic index/score (CLL-IPI) integrates genetic, biological, and clinical variables to identify distinct risk groups of patients with CLL [1,8,9].

Dipeptidyl peptidase IV (DPPIV/CD26) is a unique multifunctional 110-kDa membrane-bound glycoprotein that belongs to the serine protease family. It acts as a receptor, binding, and proteolytic molecule. DPPIV is widely distributed, with the highest expression in kidney, lung, liver, and small intestine, whereas low expression is found in brain, heart, and skeletal muscle. It is mainly found on endothelial and epithelial cells in the body, and is also present on immune cells like T-cells, activated B-cells, activated natural killer cells, and myeloid cells [10]. It plays an important role in immune regulation, signal transduction, and apoptosis [11]. In addition, DPPIV has roles in nutrition, metabolism, the immune and endocrine systems, BM mobilization, cancer growth, and cell adhesion [12].

Recent works have suggested that CD26 plays a regulatory role in neoplastic transformation and progression of various tumors. It was proposed as an important tumor biomarker in melanoma, lung and prostate cancer, as well as gastric and colorectal cancer. In hematological malignancies, CD26 expression was defined as a marker of aggressiveness in T-cell malignancies, for example, T-ALL, and was linked to poor prognosis and survival [13]. Some investigators reported variable expression of CD26 in B-CLL, yet the importance of its expression on B-lymphocytes is not completely clarified [14].

Patients and methods

This study was conducted on 30 newly diagnosed patients with CLL. They were enrolled from the Hematology/Oncology Unit, Internal Medicine Department, Ain-Shams University Hospitals, Ain-Shams Faculty of Medicine, and Misr University for Science and Technology, during the period from July 2018 to July 2019. There were 22 (73.3%) males and eight (26.7%) females, with a male to female ratio of 2.7 : 1. Their ages ranged from 51 to 89 years (mean: 70.1±11.5 years). Moreover, 15 age-matched and sex-matched healthy participants were enrolled as a control group (11 males and four females), ranging in age from 50 to 80 years.

Patients were diagnosed on the basis of the following:

(1) Thorough history and full clinical examination.
(2) Laboratory investigation, including complete blood count (Sysmex SX-800i, Kobe, Japan) and examination of Leishman-stained peripheral blood films, for morphology and % of smudge cells (ratio of smudge to intact cells+smudge lymphocytes). A cutoff level of 30% of smudge cells was considered to differentiate between low-risk and high-risk groups [15,16].
(3) BM aspiration for morphology and BM lymphocyte %.
(4) Flow-cytometric (FCM) IPT and expression of CD26 on B-CLL (BD Biosciences, California, USA) was measured at diagnosis, using a FACS Calibur FCM (Becton Dickinson, USA) [17] for all patients and controls.
(5) Fluorescent in-situ hybridization (FISH) analysis, using locus-specific identifier (LSI) probes, was done for detection of ataxia telangiectasia mutated (ATM) and protein 53 (P53) genes, and 13q14 deletions. For trisomy 12 detection, centromeric enumeration probe was also used (Vysis, UK; Abbott Laboratories, Abbott Park, IL, USA). Two age-matched healthy volunteers were used as controls to check the intensity of signals of the used probes [16].
(6) IgHV mutational status was evaluated by PCR amplification of IgHV transcript, and comparing the transcript to known germline genes available in Ig databases. IgHV sequences were considered mutated or not using the conventional cutoff of 2% mismatch from germline IgHV sequences [18].

Clinical staging of the patients with B-CLL was done according to the Modified Rai staging system (2016) [19]. The Rai classification (1975) [20] was later modified to reduce the number of prognostic groups from 5 to 3 [19]. It described three major prognostic groups with discrete clinical outcomes: defining low-risk B-CLL as patients who have lymphocytosis with leukemic cells in blood and/or BM (lymphoid cells >30%) (former Rai stage 0). Patients with lymphocytosis, enlarged LNs in any site, splenomegaly and/or hepatomegaly (±palpable nodes) are defined as intermediate-risk disease (former Rai stage I or stage II). High-risk disease includes patients with CLL-related anemia (hemoglobin <11 g/dl) (former stage III) or thrombocytopenia (platelet $<100 \times 10^9$/l) (former stage IV) [19].

An informed consent was obtained from each patient before participation in this study. The study-applied procedures were approved by the Ethical Committee of

Human Experimentation of Ain-Shams University and are in accordance to the Revised Helsinki Declaration of 1975 (2004) [21].

Sample collection

The peripheral blood and BM samples were collected on EDTA (1.2 mg/ml) for morphology and IPT. The BM aspirates were collected in sterile preservative-free lithium heparin-coated vacutainer tubes for cytogenetic analysis (FISH).

Cytogenetic study

Cytogenetic aberrations were detected by FISH technology to determine deletion status of probe targets LSI P53 (containing tumor protein p53 gene, located on 17p), LSI ATM (containing ATM gene, located on 11q), and LSI D13S319 (containing marker D13S319, located on 13q), plus determining trisomy 12 with centromeric enumeration probe 12. At least 100 interphase nuclei and/or 20 metaphases were scanned under fluorescence microscope for the detection of ATM, P53, 13q14 deletions, and trisomy 12. In mono-allelic ATM deletion, one red signal was reported in more than 10% of examined cells, and biallelic deletion was reported by absence of red signals in at least 10% of examined interphase cells. The P53 deletion was recorded positive, if one red signal was noticed in more than 10% of cells. In case of 13q14.3 deletion, the results were considered clonal when the % of cells with the abnormality exceeded the established cutoff value of 10% for the (13q14.3) deletions. Trisomy 12 was documented if three red signals were observed in at least more than 10% of interphase cells, and/or more than 2% of metaphase [16].

Statistical analysis

Data were collected, revised, coded, and entered to the Statistical Package for Social Science (IBM SPSS, Armonk, New York, United States), version 20. Qualitative data were presented as numbers and percentages, whereas quantitative data were entered into Kolmogorov–Smirnov test of normality. Parametric distribution data were presented as mean, SDs, and ranges, whereas nonparametric distribution data were presented as median with interquartile range. To compare parametric quantitative variables between two groups, Student t test was applied. Nonparametric quantitative variables between two groups were compared using Mann–Whitney test. For comparison between two groups with qualitative data, χ^2 test was used. Receiver operating characteristics (ROC) curve analysis identifies accurate cutoff value that is capable of discriminating between healthy individuals and patients. Spearman's correlation was used to correlate among parameters. Multiple logistic regression analysis was used to statistically define independent prognostic variables, among all studied parameters, using the modified Rai staging system [19] as a dependent one, as it provides a basic framework for estimating prognosis; it is used to predict median survival and is factored into the current International Workshop on CLL guidelines for initiation of treatment [22]. For all statistical analysis, P value less than 0.05 was considered statistically significant, P value less than 0.01 was highly significant, and P value less than 0.001 was considered statistically very highly significant.

Results

Descriptive data, laboratory data, and the prognostic markers routinely used to predict the B-CLL progression and to assess tumor burden are listed in

Tables 1 and 2. The presently investigated potential prognostic indicators included age, sex, total leukocyte count, BM lymphocytes, lactate dehydrogenase (LDH), CD26, CD38, ZAP-70, IgVH, smudge cell %, del (11q), P53, del (13q), and trisomy 12.

In this study, CD26% expression was positive in 18 (60%) patients with B-CLL, and it ranged from 0.2 to 46.0%, with a mean of 22.3±16.5%.

The best cut-off value for CD26% expression that can discriminate patients with B-CLL from the controls was sought by constructing ROC curves. The area under the curve of 0.878, 95% confidence interval=0.779–0.977, P value less than 0.001, and cutoff more than or equal to 7.0% had perfect specificity but moderate sensitivity (Fig. 1). According to CD26% expression, patients with B-CLL were divided into CD26+ and CD26– subgroups.

Table 1 Demographic and clinical characteristics of studied patients with B-cell chronic lymphocytic leukemia

Characteristics	Mean±SD	Range
Age (years)	70.1±11.5	51.0–89.0
Sex [*n* (%)]		
Male	22 (73.3)	
Female	8 (26.7)	
Lymphadenopathy	10 (33.3)	
Hepatomegaly	15 (50.0)	
Splenomegaly	21 (70.0)	
Modified Rai stage		
Stage 0 (low risk)	6 (20.0)	
Stages I-II (intermediate risk)	12 (40.0)	
Stages III–IV (high risk)	12 (40.0)	

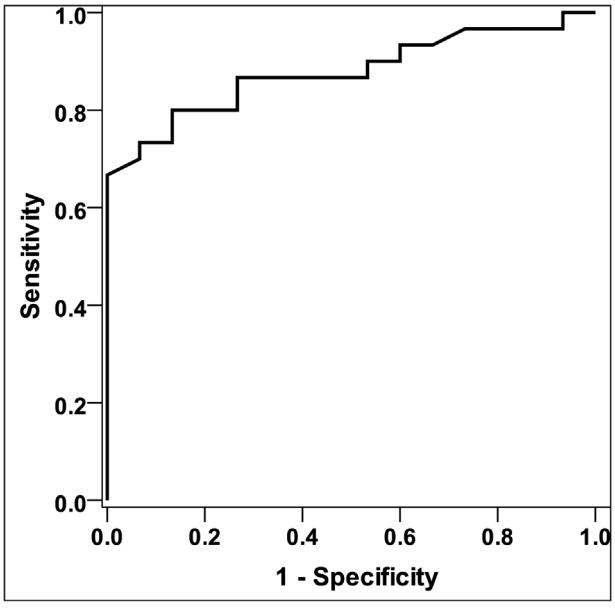
Total=30.

Table 2 Laboratory findings of studied patients with B-cell chronic lymphocytic leukemia

Characteristics	Mean±SD	Range
Hb (g/dl)	11.1±1.9	7.3–15.1
Platelets count (×10^3/μl)	158.4±83.6	16.0–315.0
TLC (×10^3/μl)	81.6±73.2	11.9–226.0
Absolute Lymphocytic count (×10^3/μl)	64.3±59.3	8.7–191.5
BM lymphocytes (%)	83.4±11.0	53.0–97.0
Atypical lymphocytes (%)	9.9±6.2	1.0–31.0
Prolymphocytes (%)	3.5±1.5	1.0–6.0
LDH (U/l)	567.0±385.9	142.0–1805.0
CD26 (%)	22.3±16.5	0.2–46.0
CD38 (%)	25.0±17.0	2.5–49.0
ZAP-70 (%)	20.1±15.4	0.3–44.3
Positive CD26 [*n* (%)]	18 (60.0)	
Negative CD26	12 (40.0)	
IPT score		
Score 4	10 (33.3)	
Score 5	20 (66.7)	
Smudge cells < 30.0	20 (66.7)	
Genetic aberrations [*n* (%)]		
Deletion 11q	4 (13.3)	
Deletion 13p	7 (23.3)	
Deletion P53	7 (23.3)	
Trisomy 12	5 (16.7)	

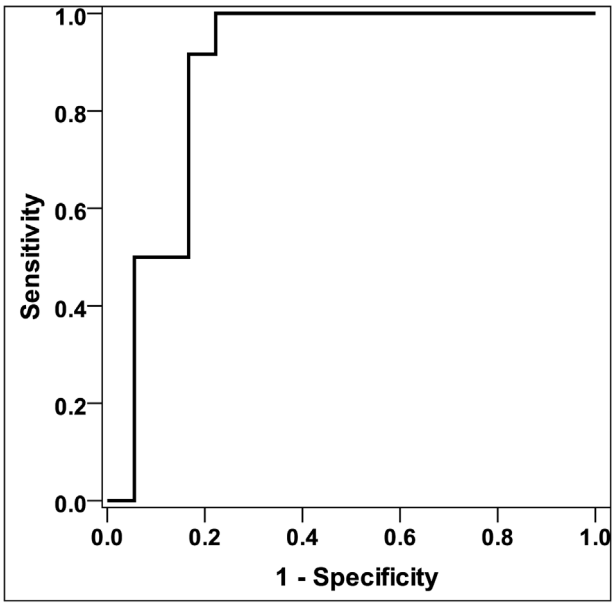
BM, bone marrow; IPT, immunophenotyping score; LDH, lactate dehydrogenase; TLC, total leukocyte count.

Figure 1



ROC curve for CD26% in differentiating studied patients with B-CLL from control group. B-CLL, B-cell chronic lymphocytic leukemia; ROC, receiver operating characteristics.

Figure 2



ROC curve for CD26% in differentiating Rai high-risk from intermediate/low-risk groups in the studied patients with B-CLL. B-CLL, B-cell chronic lymphocytic leukemia; ROC, receiver operating characteristics.

The diagnostic ability of CD26% expression to differentiate patients with B-CLL at high risk from those with intermediate or low risk was done by a cutoff value of more than or equal to 19.5%, which was sought by the ROC curve, with area under the curve =0.884, 95% confidence interval=0.731–1.000, *P* value less than 0.001 (Fig. 2).

Comparison between studied CD26% positive versus negative B-cell chronic lymphocytic leukemia subgroups

CD26%-positive subgroup of patients with B-CLL revealed no statistically significant difference versus negative expression subgroup regarding age (*P*=0.810) and sex (*P*=1.000). Lymphadenopathy (*P*=0.694), hepatomegaly (*P*=0.003), and splenomegaly

Table 3 Demographic and clinical characteristics of CD26% positive versus negative expression in B-cell chronic lymphocytic leukemia subgroups

Characteristics	Positive CD26 (*N*=18)	Negative CD26 (*N*=12)	*P* value
Age (years)	70.6±10.7	69.5±13.0	0.810
Sex [*n* (%)]			
Male	13 (72.2)	9 (75.0)	1.000
Female	5 (27.8)	3 (25.0)	
Lymphadenopathy [*n* (%)]	7 (38.9)	3 (25.0)	0.694
Hepatomegaly	13 (72.2)	2 (16.7)	0.003*
Splenomegaly	14 (77.8)	7 (58.3)	0.418
Modified Rai Stage [*n* (%)]			
Low risk	1 (5.6)	5 (41.7)	<0.001*
Intermediate risk	5 (27.8)	7 (58.3)	
High risk	12 (66.7)	0	

*Significant.

Table 4 Laboratory findings characteristics of CD26% positive versus negative expression in B-cell chronic lymphocytic leukemia subgroups

Characteristics	Positive CD26 (*N*=18)	Negative CD26 (*N*=12)	*P* value
Hb (g/dl)	10.6±2.3	11.8±0.6	0.049*
Platelets ($\times10^3$/μl)	129.4±60.6	201.8±96.7	0.034*
TLC ($\times10^3$/μl)	78.1±76.4	86.8±71.0	0.756
Absolute Lymphocytic count ($\times10^3$/μl)	59.6±59.7	71.5±60.6	0.598
BM lymphocytes (%)	82.9±10.4	84.2±12.3	0.772
Atypical lymphocytes (%)	11.7±7.1	7.2±3.1	0.049*
Prolymphocytes (%)	3.8±1.6	3.2±1.4	0.290
LDH (U/l)	730.0±410.5	322.4±153.5	0.001*
CD38 (%)	33.4±14.5	12.3±12.0	<0.001*
ZAP-70 (%)	26.0±13.6	11.2±14.0	0.007*
Low smudge cells [*n* (%)]	18 (100.0)	2 (16.7)	<0.001*
q11 deletion [*n* (%)]	3 (16.7)	1 (8.3)	0.632
q13 deletion [*n* (%)]	6 (33.3)	1 (8.3)	0.193
p53 deletion [*n* (%)]	6 (33.3)	1 (8.3)	0.193
Trisomy 12 [*n* (%)]	5 (27.8)	0	0.066
IgHV mutation [*n* (%)]	2 (11.1)	11 (91.7)	<0.001*
IPT score [*n* (%)]			
4	4 (22.2)	6 (50.0)	<0.001*
5	14 (77.8)	6 (50.0)	

BM, bone marrow; IgHV, immunoglobulin heavy-chain variable; IPT, immunophenotyping score; LDH, lactate dehydrogenase; TLC, total leukocyte count. *Significant.

(*P*=0.418) were more frequent in positive CD26% expression subgroup, but the differences were statistically significant only for hepatomegaly. Advanced modified Rai group (high-risk) showed highly statistically significant difference in the positive CD26% expression (*P*<0.001) subgroup (Table 3).

Additionally, the studied B-CLL subgroups showed that hemoglobin (*P*=0.049), platelets count (*P*=0.034), low % of smudge cell (*P*<0.001), and IgHV mutation status (*P*<0.001) were statistically significantly lower in CD26%-positive patients, whereas atypical lymphocytes % (*P*=0.049), LDH (*P*=0.001), CD38% (*P*<0.001), ZAP-70% (*P*=0.007), and IPT score 5 (*P*<0.001) were statistically significantly higher in the CD26%-positive subgroup (Table 4).

Correlations of CD26% expression with studied known prognostic factors

In the studied patients with B-CLL, positive CD26% expression showed statistically significant positive correlations with LDH (*P*<0.001), CD38% (*P*<0.001), and ZAP-70% (*P*=0.006), besides statistically significant negative correlations with hemoglobin (*P*=0.046), platelets count (*P*=0.039), and low % of smudge cell (*P*=0.001) (Table 5).

Multiple logistic regression analysis

The test was used for all items as model 1 (Table 6); the test was repeated as model 2 for only items whose *P* values are the best and so on as model 3 (Tables 7 and 8). The best model that has maximum *F* ratio and *P* was model 3 (Table 8).

Table 5 Correlations of CD26% with known prognostic factors in studied B-cell chronic lymphocytic leukemia patients

Variables	B-CLL (*N*=30)	
	r	*P* value
Age (years)	−0.034	0.860
Hb (g/dl)	−0.367	0.046*
Platelets count (×10^3/μl)	−0.379	0.039*
TLC (×10^3/μl)	−0.078	0.683
Absolute lymphocytic count (×10^3/μl)	−0.146	0.440
BM lymphocytes (%)	−0.089	0.639
Atypical lymphocytes (%)	0.248	0.187
Prolymphocytes (%)	0.233	0.215
LDH (U/l)	0.626	<0.001*
CD38 (%)	0.708	<0.001*
ZAP-70 (%)	0.486	0.006*
Smudge cells (%)	−0.587	0.001*

B-CLL, B-cell chronic lymphocytic leukemia; BM, bone marrow; LDH, lactate dehydrogenase; *r*, correlation coefficient; TLC, total leukocyte count. *Significant.

In the current work, logistic regression found out that del(11q) and CD26% expression were the best independent prognostic factors to predict advanced disease (Rai stage III/IV) and poor survival (Table 8).

Discussion

Extreme clinical heterogeneity is one of the hallmark features of CLL. Thus, it is more important than ever to develop sensitive stratification parameters to identify patients with poor prognosis [11]. Attempts to identify new factors related to disease activity, or parameters for predicting the course of CLL were assumed [5]. These factors included B_2-microglobulin concentration or soluble CD23 antigen in blood; thymidine kinase activity; B-cell expression of CD38, CD49d, and intra-cytoplasmic ZAP-70; cytogenetic aberrations by FISH; or gene mutational status of IgVH by PCR [23].

In the present study, the expression of CD26% was higher in patients with B-CLL in comparison with normal participants. This was in agreement with Hodeib and Shahbah [24]. The present results showed a positive CD26% expression in 60% of the studied patients with B-CLL, and this was in accordance with Ghannam *et al.* [11] and Matuszak *et al*. [6] The earlier investigators found that CD26+ B-CLL constituted 50% of their patients [11]. Cro *et al.* [25] investigated the role of DPPIV/CD26 in the pathogenesis of some hematological malignancies, including B-CLL, stating that it can interact with extracellular matrix, via fibronectin and collagen, thus, influence one of the postulated mechanisms of preventing cell apoptosis in B-CLL.

On evaluating the effect of CD26% expression on demographic and clinical data of patients with B-CLL, the present work revealed a significant correlation between CD26+ expression and advanced Rai clinical stage ($P<0.001$), as there was a statistically significant increase in the CD26% expression in the high-risk group of CLL. This was in accordance with Ibrahim *et al.* [26] and Hodeib and Shahbah [24]. Moreover, Matuszak *et al* [6] noticed that CD26 expression, on B-CLL, correlates with the Rai's clinical stage of the disease at diagnosis. However, conflicting results were reported by Ghannam *et al* [11].

The two similar clinical staging systems (Rai and Binet) create prognostic information by using results of physical examination and blood counts. Both systems are simple, inexpensive, and do not require ultrasound, computed tomography, or MRI [1]. Both staging systems provide a basic framework for estimating prognosis and are factored into the current International Workshop on CLL guidelines for initiation of treatment. Based on these guidelines, individuals with Rai stage III/IV or Binet stage C would meet the criteria for therapy [27]. These systems are still widely used in clinical practice, but they do not predict disease progression or response to therapy. To fill these voids in the management of CLL, there are numerous ongoing efforts to identify additional prognostic markers at the cellular and molecular levels [22].

The first prognostic marker to be used in the clinical management of CLL was the Rai clinical staging system [20], which can be used to predict median survival: stage 0, more than 150 months; stage I: 101 months; stage II: 71 months; and stages III or IV: 19 months each. This staging system provides a basic framework for prognosis estimation and is factored into the current International Workshop on CLL guidelines for initiation of treatment. Based on these guidelines, individuals with Rai stage III/IV would meet the criteria for therapy [22].

The present findings (Table 3) showed that CD26% expression does not correlate with the patients' age or sex. This was in accordance with the exploratory studies of Molica *et al.* [14], Matuszak *et al.* [6] and Hodeib and Shahbah [24]. Meanwhile, the currently most relevant prognostic score (CLL-IPI) still uses a weighted grading of five independent prognostic factors, including clinical stage and age [9].

Table 6 Multiple logistic regression analysis: model 1, the test was used for all items

Item (constant)	Regression coefficient 5.58	SE 2.855	*P*=0.086	Significant NS	*F* ratio	*P*	Significance
Age	–0.004	0.015	0.807	NS			
TLC	–0.008	0.02	0.711	NS			
BM lymphocytes	–0.028	0.012	0.046	S			
LDH	0	0.001	0.894	NS			
Sex	0.125	0.459	0.792	NS			
CD26	0.325	0.155	0.036	S			
CD38	0.478	0.762	0.548	NS			
ZAP-70	–0.205	0.647	0.76	NS			
IgVH	–0.834	0.647	0.233	NS			
Smear cell	–0.494	0.854	0.579	NS			
del (11q)	1.376	0.53	0.032	S			
del (p53)	–0.816	0.506	0.146	NS			
del (13q)	–0.645	0.458	0.196	NS			
Trisomy 12	–2.037	0.822	0.038	S			
					7.923	0.003	HS

BM, bone marrow; HS, highly significant; LDH, lactate dehydrogenase; NS, nonsignificant; TLC, total leukocyte count; S, significant.

Table 7 Multiple logistic regression analysis: 'model 2' the test was repeated only for items whose *P* values are the best

Item (constant)	Regression coefficient 2.648	SE 1.024	*P*=0.016	Significant S	*F* ratio	*P*	Significance
BM lymphocytes	–0.008	0.008	0.35	NS			
11q.del	1.711	0.666	0.017	S			
Trisomy 12	–0.605	0.507	0.244	NS			
CD26	1.785	0.545	0.003	HS			
					9.214	0	HS

BM, bone marrow; HS, highly significant; NS, nonsignificant; S, significant.

Table 8 Multiple logistic regression analysis: 'model 3,' the best model that had maximum *F* ratio and *P*

Item (constant)	Regression coefficient 1.823	SE 0.732	*P*=0.02	Significance S	*F* ratio	*P*	Significance
del (11q)	1.83	0.653	0.009	HS			
CD26	1.863	0.536	0.002	HS			
					14.983	0	HS

HS, highly significant.

To explore the correlation between CD26% expression and other laboratory data of B-CLL patients, detailed statistical analyses were initiated (Table 3). The most important findings were the significant negative correlations between CD26% expression and hemoglobin level and platelets count, besides a significant positive correlation between CD26% expression and LDH levels. These results were in agreement with Ibrahim *et al.* [26] and Hodeib and Shahbah [24] but were conflicting to Molica *et al.* [14] who failed to detect a relation between CD26 expression in CLL and any clinicohematological biomarkers of prognostic relevance. However, this latter study was performed on serum levels of soluble CD26. The source and mechanism of soluble CD26 in patients with B-CLL much remains unproven [14]. Yet, treatment of patients with B-CLL is still significantly and independently influenced by clinical disease stage (according to Rai), LDH activity, and CD26 expression on B-CLL cells [6].

The present results (Table 3) documented a more frequent expression of CD38%, ZAP-70%, and unmutated IgHV among CD26+ patients, confirming the prior work of Ghannam *et al.* [11]. The assessment of these adverse prognostic indicators is increasing used in B-CLL. The intriguing question is whether these combined reactivates are merely markers of prognostic significance or functionally involved in the disease biology. Yet, Malavasi *et al.* [28] stated that CD38 is not simply a marker but a signaling molecule in B-CLL, which induces proliferation and survival of leukemic cells.

CD26 has many biological functions and may be involved in the sIgM-mediated signal competence, which, *in vitro*, seems to unite three important B-CLL prognostic markers: CD38%, ZAP-70%, and VH-gene mutational status. Moreover, CD26% expression (particularly its DPPIV enzyme activity) induces constitutive phosphorylation of p38, which,

in B-cell tumors, contributes to tumor growth. In CLL with B cells expressing ZAP-70, it is possible that activation (phosphorylation) of ZAP-70 via CD26 signaling may trigger down-stream events with enhanced survival signals and cell-growth, causing a more aggressive phenotype [11].

To reduce the awesome and ample prognostic information to a few clinically relevant crucial prognostic parameters, comprehensive prognostic scores were constructed that combine clinical, biological, and genetic information. Currently, the most relevant prognostic score is the CLL-IPI [8]. It uses a weighted grading of five independent prognostic factors: age, clinical stage, serum β_2-microglobulin (>3.5 mg/l is associated with shorter response to treatment and overall survival), TP53 dysfunction (TP53 deletion and/or mutation), and IgHV mutational status. The CLL-IPI separates four groups with different overall survival at 5 years. However, the prognostic value of the CLL-IPI will need to be revised when trials with targeted agents and a longer follow-up will become available [1].

The simultaneous estimate of CD26%, ZAP-70%, and CD38% might distinguish different B-CLL patient subgroups, at least in terms of chemotherapy requisite and outcome prediction. Moreover, these markers have many advantages: they can be easily evaluated by FCM (a widely used technique, easy to perform, and less expensive) [29]. Their combined evaluation, in patients with B-CLL, may confirm the worst prognosis. The ZAP-70 is independently more indicative of the prognosis than CD38 in Egyptian patients with CLL. The concordant ZAP-70 and CD38 positive expression appears to be more useful than ZAP-70 and CD38 alone [29,30]. The relationship between IgVH and ZAP-70 is more concordant than the relationship between IgHV and CD38 [31].

IgHV mutation was documented by PCR in 13 (43.3%) of the presently studied Egyptian patients with CLL (Table 2). Based on the IgHV mutational status, CLL can be divided into two groups that have significantly different clinical outcomes. Patients with mutated IgHV have significantly superior outcomes, compared with unmutated ones. The cells with unmutated IgHV genes present different capacities to proliferate and differentiate into antibody-secreting plasma cells, after in-vitro stimulation, compared with other B-cell subsets [2]. For physically fit patients more than 65 years (especially who present with a mutated IgVH gene), fludarabine, cyclophosphamide, and rituximab chemoimmunotherapy remains the standard therapy, as it may have curative potential [1].

Evaluation of all these markers and their reactivity became a routine panel for prognostic stratification [11]. Both CD38 and ZAP-70 were used as surrogate markers for IgHV mutation status, as they are available for clinical use in immunohistochemistry and FCM analysis. Some markers cannot be used for routine hematological evaluation [1] owing to the limited availability, high cost of markers, and methodological complexity. Sequencing for mutation status is clearly not easily available in most clinical laboratories [32]. Interestingly, CD26%, ZAP-70% and CD38% can be done by FCM, using a single blood sample, rendering very precise results in a short-time, mainly suitable for the third world countries that cannot afford genetic laboratories [29], and especially with the worldwide financial crises of COVID-19.

For FCM-IPT, markers considered as 'required' for the diagnosis of CLL are CD19, CD5, CD20, CD23, Kappa, and Lambda, according to current diagnostic criteria and practice [17]. Simultaneous use of both polyclonal and monoclonal antibodies can prove clonality in 85% of patients with CLL at diagnosis, and increase the sensitivity of light-chain detection by FCM [33]. Recently, a reproducible approach to validate and apply these markers in individual laboratories has been identified. Finally, to refine diagnosis in borderline cases, a consensus 'suggested' a panel of defined markers (CD43, CD79b, CD81, CD200, CD10, and ROR1) that will be prospectively evaluated by the International Clinical Cytometry Society (2017) [17].

Additionally, smear or 'smudge cells' (Gumprecht nuclear shadows, shadow cells, basket cell) found as cell debris, are other characteristic morphologic features seen in CLL [1]. They lack any identifiable cytoplasmic membrane or nuclear structure. In the present work, smudge cells less than 30.0% were found in 66.7% of the studied Egyptian patients with B-CLL. In newly diagnosed B-CLL, lower % of smudge cells (<30%) was significantly correlated with age, lymphadenopathy, organomegaly, and advanced staging. It was also associated with high total leukocyte count, low hemoglobin and platelets count, and high absolute and atypical lymphocytic count. A larger % of smudge cells is a better prognostic factor. Correlation with other prognostic factors revealed an association between low % of smudge cells and CD38 expression, short lymphocyte doubling time, P53, and ATM deletions. Patients with more than 30% smudge cells show longer times before requiring treatment and longer survival rates than patients with fewer smudge cells [16].

The present results (Table 4) documented a highly significant negative correlation (r=–0.587) between CD26% expression and low % smudge cell. This strong significant correlation provided an extra evidence of the poor prognostic effect of CD26% expression on the disease progression. This was in accordance with Nowakowski *et al.* [15] who reported that % of smudge cells, in patients with B-CLL, is a prognostic factor, as smudge formation is related to the content of the cytoskeletal protein 'vimentin' present in leukemic cells [15]. The physiologic role of vimentin may extend beyond retaining cell integrity. Rearrangement of vimentin fibers participates in cell activation and signal transduction. It is equally possible that tumorigenic events including tumor migration and invasion are a consequence of vimentin over-expression in these cells. Therefore, high vimentin expression (low % of smudge cells) was related to poor outcome and a shortened time to first treatment (TFT) [34].

Acquired genetic aberrations have an important role in B-CLL pathogenesis. FISH technology raised sensitivity of detection of chromosomal aberrations (found in >80% of patients), and identification of candidate genes. The most frequent chromosomal anomalies are partial losses of one affected chromosome, for example, deletions of 11q22, 17p13, or 13q14; gains of entire chromosomes, for example, trisomy 12, are less frequent. Approximately 50% of patients with CLL show single abnormalities, 25% display two abnormalities, and the rest of patients exhibit complex chromosomal changes [2].

(1) Deletion 11q was confirmed in four (13.3%) of presently studied patients with CLL. Loss of chromosome 11q22 is considered a poor prognostic cytogenetic event, as this location harbors the ATM gene. The ATM protein is the main integrator of cellular response after DNA double-strand breaks, and is responsible for cell-cycle arrest and apoptosis [35]. Interestingly, both del(17p) and del(11q) and inactivating somatic mutations in TP53 and ATM are enhanced in patients with secondary resistance to DNA damaging chemotherapy [36,37].
(2) Deletions (17p) were found in 23.3% of the presently studied newly diagnosed Egyptian patients with CLL (Table 2) versus 5–8% of chemotherapy-naïve patients. These deletions almost always include band 17p13 (location of the prominent tumor suppressor gene, TP53). The patients with CLL carrying a del (17p) clone show marked resistance against genotoxic chemotherapies that cannot be overcome by the addition of anti-CD20 antibodies in the context of chemo-immunotherapy [38]. Patients with 17p13 deletion were always included into the highest risk prognostic category, owing to cell-cycle deregulation caused by loss of TP53, and unusual chemotherapy requirement [39].
(3) Deletion 13q14 constituted 23.3% of studied patients with CLL (Table 2). It presents, as the sole abnormality, in 45% of patients with CLL and predicts a more favorable prognosis. Patients harboring 13q14 deletion constitute a heterogeneous group. The anatomic heterogeneity is reflected in large versus small deletions, monoalleleic versus biallelic deletions, and various deletion extensions into centromeric and telomeric 13q regions. This heterogeneity provides clues to underlying effects of various 13q14 deletions on CLL biology and clinical behavior. So, more than one pathobiological mechanism may underlie the existence of various 13q14 deletions in CLL.

Clinically, 13q14 deletions were separated into subtypes that are defined as follows:

(1) Good prognosis, type I [exclusive of retinoblastoma gene (RB1); ~30% of all CLL].
(2) Poor prognosis, type II [inclusive of RB1; ~20% of all CLL] lesions. The CLL clinical course is accelerated in patients with large (type II) 13q14 deletions that span the RB1 gene, thus justifying routine identification of 13q14 subtypes in CLL management [40].

Patients with CLL with del(13q) (that show a form of reciprocal translocation with deletion at the 13q14 breakpoint) show higher rates of concomitant 17p deletion. The t(13q) and del(13q) patients constitute a subgroup within the 13q-deleted CLL cases associated with a worse clinical outcome. Moreover, Van Dyke *et al.* [41] showed that patients with heterozygous (13q-x1) and homozygous (13q-x2) deletion of 13q have similar survival and TFT, but the patients with a higher % of nuclei (>65–85% of their cells) with the deletion show worse prognosis with significantly shorter TFT. Interestingly, among patients with 13q- plus one other FISH abnormality, concomitant 13q- appears to attenuate the shorter survival associated with del17p. Those patients with larger deletions including the RB1 gene have a significantly poorer clinical outcome [2].

(a) Mutations of TP53 are found in 23.3% of the presently studied newly diagnosed Egyptian

patients with CLL (Table 2), versus 4 to 37% of patients with CLL, as previously reported [7]. It was associated with very poor prognosis [7]. Among patients with confirmed del (17p), the majority shows mutations in the remaining TP53 allele (>80%). In CLL without del (17p), TP53 mutations are much rarer but have a similarly detrimental effect on chemotherapy response and overall survival [4]. The TP53 mutations are also associated with higher genomic complexity, indicating that a crippled DDR promotes a 'mutator phenotype' in CLL [4].

(a) Patients with del (17p) or TP53 mutation respond well to targeted inhibitors of the B-cell receptor pathway and BCL-2 pathway, for example, Ibrutinib and venetoclax, respectively. Hence, patients with these aberrations at diagnosis or progression should be treated with these targeted inhibitors [42].

(b) Trisomy 12 was observed in 16.7% of the presently studied patients with CLL (Table 2) versus 10–20% of patients with CLL reported in the literature [1,4]. However, the genes involved in the pathogenesis of CLL carrying trisomy 12 are largely unclear, probably amplification of MdM2 gene (a mouse double-minute-2 homolog) is assumed [43]. MdM2 is a primary repressor of P53. Targeting this protein is an attractive therapeutic approach for nongenotoxic reactivation of P53 [44].

The prognostic relevance of trisomy 12 remains a matter of debate [4]. It was considered to as an intermediate-risk; however, associated trisomy 12, with an aggressive clinical course, poor predicted prognosis, and poor disease outcome in Egyptian patients with CLL was previously claimed [43]. Some authors claimed that it carries a bad prognostic effect when it is associated with NOTCH1 mutation, as well as CD38, ZAP-70, and the integrins (CD11a and CD49d) [29,43,45].

Regarding the correlation between these high-risk cytogenetic aberrations and CD26% expression, the present work showed a nonsignificant correlation between them, probably owing to the limited number of cytogenetically studied patients, or may also be attributed to difference in sample size; ethnic, racial, and genetic backgrounds; and environmental factors.

However, logistic multiregression analysis was done to provide complementary prognostic information identifying the most significant independent factors that can predict advanced disease (Rai stage III/IV) and poor survival: del (11q) and CD26% positive expression (Tables 6–8). Del (11q) is a high-risk chromosomal aberration; its incidence increases over time, thus highlighting the need to test for it at diagnosis, as well as at clinical progression. Usually del (11q) is generally seen in young male patients and tends to present with bulky LNs. It is associated with rapid disease progression and a shorter progression-free survival. Some studies reported that combination chemoimmunotherapy containing alkylating agents may overcome the negative prognostic implications of del (11q). Interestingly recent follow-up data with new drugs, like ibrutinib, showed durable responses in treatment-naïve and relapsed-refractory patients with del (11q) abnormality. More importantly, pooled data from three randomized phase three studies showed that del (11q) was not a prognostic factor for adverse outcomes for patients with CLL.

Moreover, the present work proved that CD26 is the second best independent adverse prognostic predictor to predict advanced disease (Rai stage III/IV) and poor survival in CLL (Tables 6–8). In alignment, Ibrahim *et al.* [26] and Matuszak *et al.* [6] praised its use as a predictor for shorter progression-free survival, among the biologic risk factors. The former investigators evaluated CD26 by 3-color FCM in a series of 103 untreated patients with CLL. Both researchers stated that CD26 has independent prognostic value, and they suggested its use as a part of routine panel for prognostic stratification of CLL [26]. Moreover, treatment is significantly influenced by CD26 expression [6].

Finally, serum CD26 level was proved to be an important emerging prognostic marker of B-CLL. In fact, Molica *et al.* [14] reported a shorter TFT in B-CLL that exhibited higher serum CD26 levels and simultaneously demonstrated absence of mutation in *IgVH*. The overall survival and disease-free survival were shorter in patients with B-CLL with positive CD26 expression compared with patients with negative CD26 expression. Patients with positive CD26 expression had significantly shorter overall survival (16 vs. 20 months) and significantly lower disease-free survival (6 vs. 16.5 months) at 24 months compared with those with negative CD26 expression [24]. Recently, CD26/DPPIV is potentially useful as a therapeutic target in specific malignancies [13].

Conclusion

In conclusion, the present work documented that the del (11q) and surface expression of CD26% are considered the worst independent prognostic factors, in newly diagnosed Egyptian patients with CLL at diagnosis, and at clinical progression. Both markers

predict advanced disease (Rai stage III/IV) and poor survival. CD26% expression significantly correlated with high-risk prognostic markers (advanced modified Rai staging, low hemoglobin and platelets count, and high % of atypical lymphocytes and serum LDH levels). The present study established significantly correlations between CD26% expression and other relevant prognostic factors, for example, CD38%, ZAP-70%, low % of smudge cells (<30%), and the unmutated status of IgHV. CD26% is recommended as a part of routine future panel for prognostic stratification of Egyptian patients with CLL at diagnosis. Detection of CD26% expression by FCM can replace more tedious, laborious, and time-consuming molecular genetics and cytogenetic techniques, mainly in the third world countries, especially during the economic crises of COVID-19.

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Conflicts of interest
There are no conflicts of interest.

References

1 Hallek M. Chronic lymphocytic leukemia: 2020 update on diagnosis, risk stratification and treatment. *Am J Hematol* 2019; **94**:1266–1287.

2 Darwiche W, Gubler B, Marolleau J-P, Ghamlouch H. Chronic lymphocytic leukemia B-cell normal cellular counterpart: clues from a functional perspective. *Front Immunol* 2018; **9**:683.

3 Herling CD, Coombes KR, Benner A, Bloehdorn J, Barron LL, Abrams ZB, *et al.* Time-to-progression after front-line fludarabine, cyclophosphamide, and rituximab chemo-immunotherapy for chronic lymphocytic leukaemia: a retrospective, multicohort study. *Lancet Oncol* 2019; **20**:1576–1586.

4 Seiffert M, Dietrich S, Jethwa A, Glimm H, Lichter P, Zenz T. Exploiting biological diversity and genomic aberrations in chronic lymphocytic leukemia. *Leuk Lymphoma* 2012; **53**:1023–1031.

5 Hendy OM, El Shafie MA, Allam MM, Motalib TA, Khalaf FA, Gohar SF. The diagnostic and prognostic value of CD38 expressions in chronic lymphocytic leukemia. *Egypt J Haematol* 2016; **41**:70–76.

6 Matuszak M, Lewandowski K, Czy A, Kiernicka-Parulska J, Przybyłowicz-Chalecka A, Jarmuz-Szymczak M, Lewandowska M, Komarnicki M. The prognostic significance of surface dipeptidyl peptidase IV (CD26) expression in B-cell chronic lymphocytic leukemia. *Leuk Res* 2016; **47**:166–171.

7 Zenz T, Vollmer D, Trbusek M, Smardova J, Benner A, Soussi T, *et al.* European Research Initiative on CLL (ERIC): TP53 mutation profile in chronic lymphocytic leukemia: evidence for a disease specific profile from a comprehensive analysis of 268 mutations. *Leukemia* 2010; **24**:2072–2079.

8 International CLL IPI working group. An international prognostic index for patients with chronic lymphocytic leukaemia (CLL-IPI): a meta-analysis of individual patient data. *Lancet Oncol* 2016; **17**:779–790.

9 Hallek M, Cheson BD, Catovesky D, Caligaris-Cappio F, Dighiero G, Döhner H, *et al.* iwCLL guidelines for diagnosis, indications for treatment, response assessment, and supportive management of CLL. *Blood* 2018; **131**:2745–2760.

10 Wagner L. Dipeptidyl peptidase 4. In: Choi S editor: *Encyclopedia of signaling molecules*. 2nd ed. Switzerland: Springer 2010. p. 1383–1396.

11 Ghannam DME, Taalab MM, Ghazy HF, Salam EMA, Fawzy IM. CD26 expression in mature B-cell neoplasms and its prognostic impact on B-cell chronic lymphocytic leukemia. *J Blood Disorders Transf* 2014; **5**:222–229.

12 Bassindin MF, Bridge SH, McCaughan GW, Gorrell MD. COVID-19 and comorbidities: a role for dipeptidyl peptidase 4 (DPP4) in disease severity?. *J Diabetes* 2020; **12**:649–658.

13 Enz N, Vliegen G, Ingrid De Meester I, Jungraithmayr W. CD26/DPP4–a potential biomarker and target for cancer therapy. *Pharmacol Ther* 2019; **198**:135–159.

14 Molica S, Digiesi G, Mirabelli R, Cutrona G, Antenucci A, Molica M, *et al.* Serum level of CD26 predicts time to first treatment in early B-chronic lymphocytic leukemia. *Eur J Hematol* 2009; **83**:208–214.

15 Nowakowski G, Hoyer HD, Shanafelt TD, Zent CS, Call TG, Bone ND, *et al.* Percentage of smudge cells on routine blood smear predicts survival in chronic lymphocytic leukemia. *J Clin Oncol* 2009; **27**:1844–1849.

16 Mohamed AA, Safwat NA. New insights into smudge cell percentage in chronic lymphocytic leukemia: a novel prognostic indicator of disease burden. *Egypt J Med Human Genet* 2018; **19**:409–415.

17 Rawstron AC, Kreuzer KA, Soosapilla A, Spacek M, Stehlikova O, Gambell PN, *et al.* Reproducible diagnosis of chronic lymphocytic leukemia by flow cytometry: an European research initiative on CLL (ERIC) & European Society for Clinical Cell Analysis (ESCCA) Harmonization Project. *Cytometry* 2018; **94**:121–128.

18 Rozovskia U, Michael J, Estrova KZ. Why is the immunoglobulin heavy chain gene mutation status a prognostic indicator in chronic lymphocytic leukemia?. *Acta Haematol* 2018; **140**:51–54.

19 Koffman B. *Rai staging of chronic lymphocytic leukemia (CLL)*. Available at: https:// cllsociety.org/2016/03. [Accessed date 16 March 2016].

20 Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. *Blood* 1975; **46**:219–234.

21 Carlson RV, Boyd MK, Webb DJ. The revision of the Declaration of Helsinki: past, present and future. *Br J Clin Pharmacol* 2004; **57**:695–713.

22 Parker TL, Strout MP. Chronic lymphocytic leukemia: prognostic factors and impact on treatment. *Discov Med* 2011; **11**:115–123.

23 Martens D, Stilgenbauer S. Prognostic and predictive factors in patients with chronic lymphocytic leukemia: relevant in the era of novel treatment approaches?. *J Clin Oncol* 2014; **32**:869–872.

24 Hodeib H, Shahbah A. Dipeptidyl peptidase-4 (CD26): a prognostic marker in patients with B-cell chronic lymphocytic leukemia. *Egypt J Haematol* 2017; **41**:206–210.

25 Cro L, Morabito F, Zucal N, Fabris S, Lionetti M, Cutrona G. CD26 expression in mature B-cell neoplasia: its possible role as a new prognostic marker in B-CLL. *Hematol Oncol* 2009; **26**:140–147.

26 Ibrahim L, Elderiny WE, Elhelw L, Ismail M. CD49d and CD26 are independent prognostic markers for disease progression in patients with chronic lymphocytic leukemia. *Blood Cells Mol Dis* 2015; **55**:154–160.

27 Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, *et al.* International Workshop on Chronic Lymphocytic Leukemia. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood* 2008; **111**:5446–5456.

28 Malavasi F, Deaglio S, Damle R, Cutrona G, Ferrarini M, Chiorazzi N. CD38 and chronic lymphocytic leukemia: a decade later. *Blood* 2011; **118**:3470–3478.

29 Abaza HMH, Abd El-Halim AZ, Ismail MM, Youssef SR, Mahmoud MA. Clinical relevance of ZAP-70 expression by lymphocyte subpopulations in CLL patients. *Egypt J Hematol* 2010a; **35**:233–239.

30 D'Arena G, Tarnani M, Rumi C, Vaisitti T, Aydin S, De Filippi R. Prognostic significance of combined analysis of ZAP-70 and CD38 in chronic lymphocytic leukemia. *Am J Hematol* 2007; **82**:787–791.

31 Cruz GM, Lewis RE, Webb RN, Sanders CM, Suggs JL. ZAP-70 and CD38 as predictors of IgVH mutation in chronic lymphocytic leukemia. *Exp Mol Pathol* 2007; **83**:459–461.

32 Kaur P. Prognostic indicators in CLL: clinical, cytogenetics, novel mutations, microRNAs, hierarchal. In: Coleman WB, Tsongalis GJ editors. *Chronic lymphocytic leukemia: pathobiology, B cell receptors, novel mutations, clonal evolution, molecular and translational medicine*. Switzerland: Springer Nature, Springer International Publishing AG; 2018. p. 35–60.

33 Abaza HMH, Mohammed MT, Boshra MG. *Flow cytometric analysis of surface light chain patterns using monoclonal and polyclonal antibodies [MSc thesis]*. Cairo, Egypt: Faculty of Medicine, Ain-Shams University; 2015.

34 Ngan C, Yamamoto H, Seshimo I, Tsujino T, Mani M, Ikeda J, *et al.* Quantitative evaluation of vimentin expression in tumor stroma of colorectal cancer. *Br J Cancer* 2007; **96**:986–992.

35 te Raa D, Moerland P, Leeksma A, Derks A, Yigittop H, Laddach N, *et al.* Assessment of p53 and ATM functionality in chronic lymphocytic leukemia by multiplex ligation-dependent probe amplification. *Cell Death Dis* 2015; **6**:1852–1860.

36 Puente XS, Pinyol M, Quesada V, Xose S, Puente XS, Pinyol M, *et al.* Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature* 2011; **475**:101–105.

37 Quesada V, Conde L, Villamor N, Villamor N, Gonzalo R, Ordóñez GR, *et al.* Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia. *Nat Genet* 2012; **44**:47–52.

38 Hallek M, Fischer K, Fingerle-Rowson G, Fink AM, Busch R, Mayer J, *et al.* Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomized, open-label, phase 3 trial. International Group of Investigators; German Chronic Lymphocytic Leukaemia Study Group. *Lancet* 2010; **376**:1164–1174.

39 Rossi D, Rasi S, Spina V, Bruscaggin A, Monti S, Ciardullo C, *et al.* Integrated mutational and cytogenetic analysis identifies new prognostic subgroups in chronic lymphocytic leukemia. *Blood* 2013; **121**:1403–1412.

40 Ouillette P, Collins R, Shakhan S, Li J, Li C, Shedden K, *et al.* The prognostic significance of various 13q14 deletions in chronic lymphocytic leukemia. *Clin Cancer Res* 2011; **17**:6778–6790.

41 Van Dyke DL, Shanafelt TD, Call TG, Zent CS, Smoley SA, Rabe KG, *et al.* Comprehensive evaluation of the prognostic significance of 13q deletions in patients with B-chronic lymphocytic leukaemia. *Br J Haematol* 2011; **148**:544–550.

42 Kamdar M. *Prognostic factors in CLL, 2017*. Available at: https://cllsociety.org/2017/09/prognostic-factors-cll/. [Accessed date 27 September 2017].

43 Abaza HMH, Ezzat NI, Fouad DA, Mohamed AA, Abdel-Gaber SM. *Trisomy 12, 17p(P53) deletion and 14q32(IgHV) in B-chronic lymphocytic leukemia: clinical and immunophenotypic correlation [MD thesis]*. Cairo, Egypt: Faculty of Medicine, Ain-Shams University; 2010.

44 Ciardullo C, Aptullahoglu E, Woodhouse L, Lin WY, Wallis JP, Marr H, *et al.* Non-genotoxic MdM2 inhibition selectively induces a pro-apoptotic p53 gene signature in chronic lymphocytic leukemia cells. *Haematologica* 2019; **104**:2429–2442.

45 Puiggros A, Blanco G, Espinet B. Genetic abnormalities in chronic lymphocytic leukemia: where we are and where we go. *Biomed Res Int* 2014; **2014**:435983.