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Original article

Clinical Significance of CD69 Expression in Chronic Lymphocytic Leukemia

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ABSTRACT

Background: Increased proliferation and survival of leukemic B-lymphocytes can be induced by CD69 overexpression, which resembles B cells at an earlier and larger stage of activation. This could potentially indicate an aggressive and progressive clinical outcome. Increased CLL stages, diffuse marrow infiltration and a brief overall survival are linked to overexpression of CD69. The aim of our study was to identify the prevalence of CD69 in patients with chronic lymphocytic leukemia and its value as a predictor for prognosis. **Methods:** 50 newly diagnosed CLL patients were included. Immunophenotyping and detection of CD69, CD38 and CD200 expression by flowcytometry was performed. **Results:** There was a statistically significant relation between presence of positive CD69, CD38 expression level and positive Del p17. There was a statistically significant positive correlation between CD-69 and CD-200, CD-38, spleen, liver diameters and LN size. Regarding response to treatment, 20% showed no response and there was a statistically significant relation between response and CD 69 (significantly higher in those with no response). By applying the ROC curve, the best cutoff of CD 69 in prediction of non-response is ≥58.15% with area under curve 0.958, sensitivity 90%, specificity 87.5%, positive predictive value 64.3%, negative predictive value 97.2% and overall accuracy 88%. There was a statistically significant association between time till response and expression of CD-69. All those with positive expression of CD-69 had significantly higher time till response. **Conclusion:** CD69 determined by flowcytometry could be considered a novel important independent prognostic parameter in B-CLL.

Keywords: Chronic lymphocytic leukemia, CD69, Response

INTRODUCTION

The cancer of mature B cells that affects the blood, bone marrow (BM) and lymphoid organs is known as B cell-chronic lymphocytic leukemia (B-CLL). The disease's cells originate from the polyclonal expansion of CD5+ B lymphocytes that have been mutated to become a monoclonal population. B-CLL is a diverse illness with a fluctuating clinical trajectory. While some patients have an aggressive disease with a brief morbidity and overall survival, others are asymptomatic and have an indolent course that requires no treatment [1].

When it comes to CLL patient risk categorization, somatic mutation of the immunoglobulin variable heavy chain is thought to be the gold standard. However, it is costly, time-consuming, and not available for routine analysis; the inapplicability of the techniques and their difficulty in developing countries leads to the identification of other alternative markers that are reliable, easy to apply and have similar prognostic values. These markers could aid in the stratification and risk assessment of those patients [2].

Type II integral membrane protein CD69 is a member of the C-type lectin family of surface receptors and has a single transmembrane domain [3]; Except for erythroid lineage, it functions as an immunoregulatory protein expressed on several hematopoietic cells [4].

Leukemic B-lymphocytes can proliferate and survive longer when CD69 is overexpressed, which

is similar to B cells at an earlier and larger level of activation [5]. This could indicate an aggressive and progressive course of the disease. The overexpression of CD69 has been linked to diffuse marrow infiltration, more advanced stages of CLL and a shorter overall survival rate [6]. Evaluation of CD69 expression in CLL offers a straightforward technique that could be added to standard immunophenotyping and integrated into the CLL scoring system to help stratify patients who are early progressors and make timely treatment decisions that would enhance results [7]. The aim of this study was to identify the prevelance of CD69 in patients with chronic lymphocytic leukemia and its value as a predictor for prognosis.

METHODS

This study was performed in Clinical Pathology and Hematology unit of Internal Medicine departments. Faculty of Medicine, Zagazig University Hospitals, during the period from October 2021 to October 2023. In this study, we included 50 newly diagnosed CLL patients. They were 18 females and 32 males. Their ages ranged from 42 to 82 years.

Each individual had seven milliliters of venous blood extracted aseptically via venipuncture, of which one milliliter was placed in a sterile tube containing ethylene diamine tetra acetic acid (EDTA) for CBC testing. For PT analysis, 2 milliliters were transferred into a sterile vacutainer tube containing trisodium citrate and 3 milliliters were transferred into a sterile plain vacutainer tube with a stopper. The samples were allowed to coagulate for 10 minutes at 37°C before being centrifuged for 10 minutes at 3000 rpm. The serum was then utilized to measure LDH, liver and kidney functions.

Immunophenotyping by Flowcytometry for detection of CD69 expression was performed using the same sample for CBC examination. Samples were processed within two hours from collection. Finally, one ml was delivered into a sterile vacutainer tube containing lithium heparin for cytogenetic analysis.

Participants included in our study were subjected to the following: complete history taking, clinical examination, radiological examination includes abdominal ultrasonography, complete blood count was identified by cell counter (Sysmex XN1000, Japan) and Prothrombin time (PT) was done on automated blood coagulation analyzer, model CS 2100, (Sysmex Corporation, Japan). Liver, kidney function tests and Lactate dehydrogenase (LDH) were measured spectrophotometrically using automated analyzer “Roche Cobas 8000-c702” (Roche Diagnostics, Germany). The International System for Human Cytogenetic Nomenclature (ISCN) was followed in the conventional karyotyping process, which involved bone marrow aspiration and inspection, as well as the use of the G banding technique and an image analyzer Imstar (Paris, France).

Immunophenotyping and detection of CD69 expression by ***flowcytometry****:* Multicolor flow cytometry for immunophenotyping (BD FACSCantoTM II flow Cytometry, Becton Dickinson, San Jose, USA) was done to confirm the diagnosis. Using flow cytometry, the expression of CD69 was measured. Clonal lymphocytes are first identified using forward and side scatter. Positive CD45 population of lymphocytes are then gated and mononuclear specification is carried out using various fluorochrome-conjugated monoclonal antibodies, such as CD69 FITC, CD19 PE, and PerCP CD5. Moreover, to distinguish it from other lymphoproliferative disorders, various diagnostic monoclonal antibodies such CD23, FMC7, CD20, CD10, CD79b, CD38 and CD200 should be used. To verify clonality, a specific kappa and lambda ratio was computed. all purchased from BD Bioscience (Becton Dickinson Biosciences, San Diego, CA) [8].

Ethical consideration: The study was conducted in compliance with the Declaration of Helsinki, the World Medical Association's code of ethics for human subjects' research. There was no risk or injury to our research groups, and the patient data was kept private. The investigated groups were informed about the purpose and nature of the study and an informed written consent, for the required investigations, was taken from all the patients. Moreover, an approval from the ethical committee in Faculty of Medicine, Zagazig University was done.

Statistical analysis: The SPSS computer program (version 20; SPSS Inc. Chicago, Illinois, USA) was used to analyze the data. The $\chi 2$-test was used to compare qualitative data. Mann-Whitney and t tests were used to compare the quantitative data. To determine the optimal cutoff value, receiver operating characteristic curve (ROC) analysis was employed. Risk was estimated using the odds ratio (OR) and its 95% confidence interval (CI). Additionally, a Spearman rank correlation test was used to examine the relationship between the expression of CD69 and other lab data. Utilizing the Kaplan-Meier technique, survival was estimated. $P<0.001$ was regarded as very significant, and P values less than 0.05 were regarded as statistically significant.

RESULTS

Table 1: show clinical data, immunophenotyping pattern and prevalence of organomegaly among the studied patients. RIA III and IV prevailed in 40% and 34%; respectively. Positive Coomb's test was reported in seven patients (14%). CD69 and CD38 were positive in 58% and 34% of patients; respectively. splenomegaly, hepatomegaly and enlarged lymph nodes were prevalent in 74%, 60% and 90% of patients; respectively. Among 31 examined patients who had Del p17, 19.4% were ranked as positive.

Table 2: show that there was no statistically significant difference was observed between CD 69 +ve and CD69 -ve patients as regard age, gender, organomegaly, enlarged lymph node, coomb's test, or RIA. hemoglobin, WBCs, lymphocytes, platelet, ESR, LDH, CD 200, or CD 23. While, there was a statistically significant relation between presence of positive CD69, CD38 expression level and positive Del p17 among studied patients as all patients with positive Del p17 had positive CD69.

Table 3 and Figure 1: reveal that there was a statistically significant positive correlation between CD-69 and CD-200, CD-38, spleen, liver diameters and LN size. While there was a statistically non-significant positive correlation between CD-69 and either age, WBCs, lymphocytes, hemoglobin, platelet count, ESR, LDH or CD-23.

Table 4 and 5 : Regarding response to treatment and follow up of the studied patients, which ranged from 6 to 12 months, 20% showed no response and there was a statistically significant relation between response and CD 69 (significantly higher in those with no response). In addition, there was a statistically significant relation between response and positive CD 69 (all non-responders had positive CD 69 versus 27.5% of responders). In addition, higher levels of CD 69 significantly independently increased risk of no repsonse by 1.071 folds (one % increase in CD 69 increase risk by 1.071 folds).

Table 6 and Figure 2: By applying the ROC curve, the best cutoff of CD 69 in prediction of non-response is ≥58.15% with area under curve 0.958, sensitivity 90%, specificity 87.5%, positive predictive value 64.3%, negative predictive value 97.2% and overall accuracy 88%. Also, performance of CD 200 and CD 38 in prediction of response to therapy among the studied patients were evaluated, The best cutoff of CD 200 and CD38 in prediction of non-response is ≥89%, ≥34% , with area under curve 0.725, 0.783, sensitivity 80%, 80%, specificity 70%, 66.7%, positive predictive value 40%, ≥34% , negative predictive value 93.3%, 88.9% and overall accuracy 72% (p=0.155), 70.3% (p=0.073); respectively

Table 7 and Figure 3: Regarding relation between time till response and CD-69 marker expression. There was a statistically significant association between time till response and expression of CD-69. All those with positive expression of CD-69 had significantly higher time till response.

Table (1) Clinical data and immunophenotyping pattern of the studied patients

	N=50	%
Coomb's test:		
Negative	43	86%
Positive	7	14%
Spleen		
Normal	13	26%
Enlarged	37	74%
Liver		
Normal	20	40%
Enlarged	30	60%
Lymph node		
Normal	5	10%
Enlarged	45	90%
RIA		
0	2	4%
I	8	16%
II	3	6%
III	20	40%
IV	17	34%
CD69 +ve	29	58%
CD69 -ve	21	42%

CD38 +ve	17	34%
	N=50	**%**
CD38 -ve	33	66%
Del p17	N=31	%
Negative	25	80.6%
Positive	6	19.4%

Table 2: Comparison between CD-69 positive and CD-69 negative patients as regard demographic and clinical and lab data:

	Negative CD-69	**Positive CD-69**	
	Mean ± SD	**Mean ± SD**	**p**
Age	60.38 ± 8.86	58.57 ± 10.57	0.514
	N=29(%)	**N=21(%)**	**p**
Gender			
Female	13 (44.8%)	5 (23.8%)	0.126
Male	16 (56.2%)	16 (76.2%)	
RIA			
0	1 (3.4%)	1 (4.8%)	
I	7 (24.1%)	1 (4.8%)	
II	1 (3.4%)	2 (9.5%)	0.568
III	9 (31%)	11 (52.4%)	
IV	11 (37.9%)	6 (28.6%)	
LN			
Enlarged	27 (93.1%)	20 (95.2%)	>0.999
Not	2 (6.9%)	1 (4.8%)	
Liver			
Enlarged	16 (55.2%)	14 (66.7%)	0.413
Not	13 (44.8%)	7 (33.3%)	
Spleen			
Enlarged	19 (65.5%)	18 (85.7%)	0.108
Not	10 (34.5%)	3 (14.3%)	
Coomb's			
Negative	25 (86.2%)	18 (85.7%)	>0.999
Positive	4 (13.8%)	3 (14.3%)	
	Negative CD-69	**Positive CD-69**	p
	Mean ± SD	**Mean ± SD**	
Hemoglobin (g/dl)	9.98 ± 2.47	9.17 ± 2.63	0.269
	Median (IQR)	**Median (IQR)**	p
WBCs (10^3/mm^3)	71(48.95 – 157.4)	47(36.15 – 163)	0.275
Lymph (10^3/mm^3)	60(43.1 – 147.35)	41(25.93 – 149.5)	0.178
Platelet (10^3/mm^3)	170(63.5 – 205)	153(78.5 – 173)	0.623
ESR (mm/hr)	36(27 – 65)	40(32 – 50)	0.88
LDH	300(202 – 335)	275(232.5 – 385.5)	0.651
CD-200	85(63.5 – 90)	89(75 – 92)	0.306
CD-23	50(29 – 62.5)	56(45.5 – 62.65)	0.212
CD-38	28(25.45 – 31.5)	57(38.5 – 80)	0.003*
Del 17			
Negative	18 (100%)	7 (53.8%)	<0.001**
Positive	0 (0%)	6 (46.2%)	

χ^2Chi square test ¥ Chi square for trend test t independent sample t test
t independent sample t test Z Mann Whitney test *p<0.05 is statistically significant

Table 3: Correlation between CD69 and the studied parameters

	r	P
Age (year)	-0.051	0.726
WBCs (10^3/mm^3)	-0.057	0.695
Lymph (10^3/mm^3)	-0.085	0.558
Hemoglobin (g/dl)	-0.167	0.246
Platelet (10^3/mm^3)	-0.169	0.241
ESR (mm/hr)	-0.018	0.902
LDH	-0.072	0.618
CD-200	**0.287**	**0.04***
CD-23	0.12	0.407
CD-38	**0.621**	**0.008***
Liver	0.486	<0.001**
Spleen	**0.742**	**<0.001****
Lymph node	**0.412**	**0.003***
RAI	0.121	0.403

r Spearman rank correlation coefficient *p<0.05 is statistically significant

Table 4: Distribution of the studied patients according to response to treatment and follow-up period
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	N=50	%
Response to treatment Non-responder Responder	 10 40	 20% 80%
	Mean ± SD	**Range**
Follow up (month)	8.32 ± 1.87	6 – 12 months

Table 5: Relation between CD-69 and response to therapy

	No response	Response	Z	P
	Median (IQR)	**Median (IQR)**		
CD-69	96.5(78.75 – 98.5%)	22(16.93 – 32.15%)	-4.439	<0.001**
	N=10 (%)	**N=40 (%)**	**χ^2**	**P**
Positive **Negative**	10 (100%) 0 (0%)	11 (27.5%) 29 (72.5%)	Fisher	<0.001**

Z Mann Whitney test. **p≤0.001 is statistically highly significant IQR interquartile range.

Table 6: Performance of CD 69, CD 200 and CD 38 in prediction of response to therapy among the studied patients

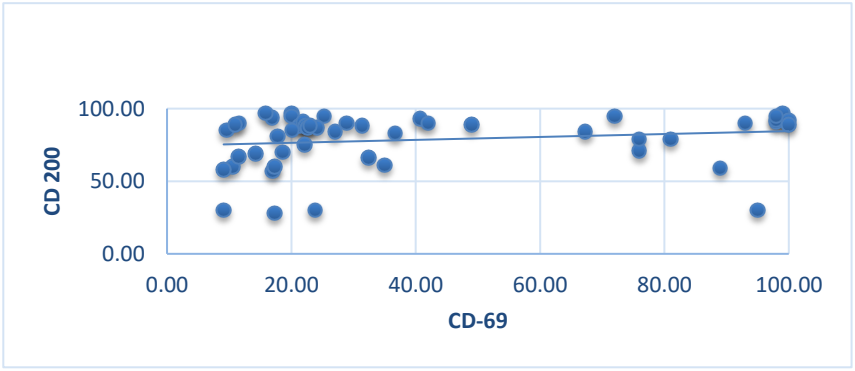
	Cutoff	AUC	Sensitivity	Specificity	PPV	NPV	Accuracy	P
CD 69	58.15%	0.958	90%	87.5%	64.3%	97.2%	88%	<0.001**
CD 200	89%	0.725	80%	70%	40%	93.3%	72%	0.155
CD 38	34%	0.783	80%	66.7%	50%	88.9%	70.6%	0.073

**p≤0.001 is statistically highly significant AUC area under curve PPV positive predictive value NPV negative predictive value
AUC area under curve PPV positive predictive value NPV negative predictive value

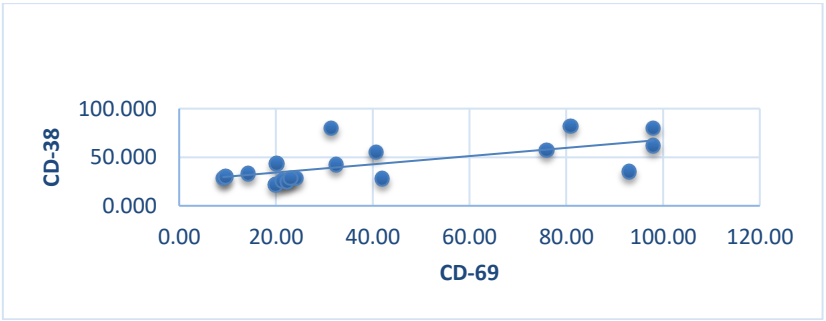
Table 7: Relation between time till response and CD-69 marker expression

		Total N	N of Events	Censored		Survival time, Months		P
				N	%	Mean		
						Estimate ± SD	95% CI	
CD-69	**Negative**	29	29	0	0.0%	7.62 ± 0.25	7.13 – 8.11	<0.001**
	Positive	21	11	10	47.6%	9.8 ± 0.49	8.83 – 10.76	
Overall		50	40	10	20.0%	8.5 ± 0.29	7.93 – 9.07	

*p<0.05 is statistically significant

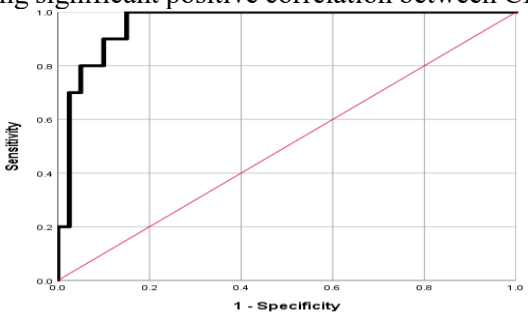


(a)



(b)

Figure (1) Scatter dot plot showing significant positive correlation between CD 69, CD 200 (a) and CD 38 (b)



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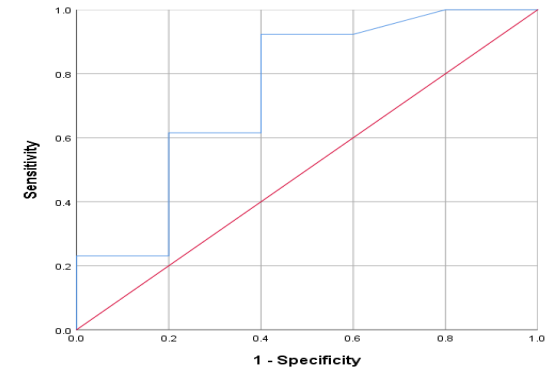

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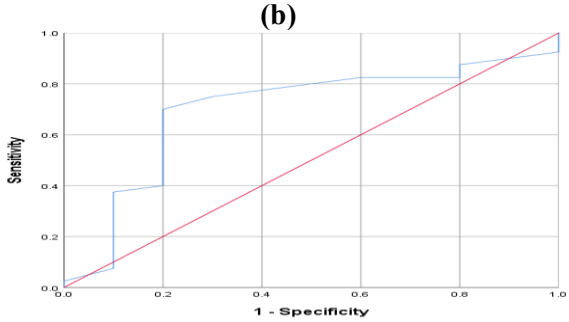

(c)

Figure (2) ROC curve showing performance of CD69 (a), CD38 (b) and CD 200 (c) in prediction of no response to therapy among the studied patients.

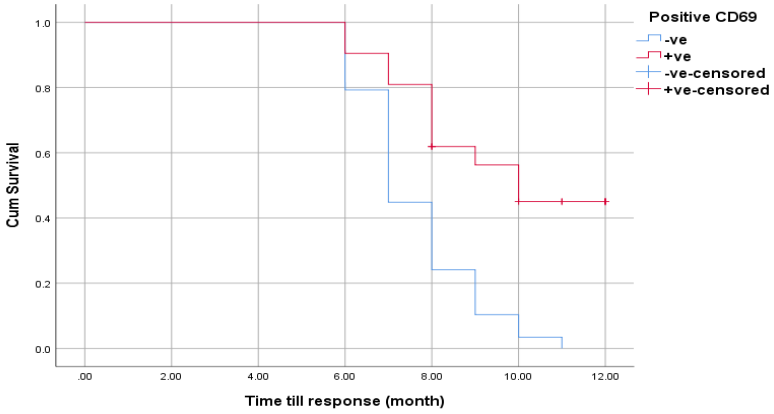

Figure (3) Kaplan Meier plot showing relation between time till response and CD-69 expression among studied patients

DISCUSSION

The accumulation of morphologically mature monoclonal B cells with the CD19+/CD5+/CD23+ phenotype in lymphoid tissue, peripheral blood and bone marrow is a hallmark of chronic lymphocytic leukemia. Patients with CLL have a variety of clinical outcomes, from aggressive to indolent [9]. A crucial membrane protein, CD69 is a member of the lectin family. With the exception of erythrocytes, it is expressed in all BM-derived cells after activation. Features of activated B cells exposed to antigens and upregulation of CD69 are seen in CLL [10]. Given that CD69 has a strong negative correlation with both poor clinical and biological prognostic variables, it should be included in routine laboratory evaluations and, if necessary, in a prognostic score system for CLL following a sufficient standardization process [11]. Our study's objective was to determine the prevalence of CD69 in individuals with chronic lymphocytic leukemia and the significance of this marker for prognostic purposes. According to interlaboratory repeatability and the stability of CD69 antigen expression over time, the ideal cutoff point of 30% was required for positive CD69 antigen expression in our cohort analysis. This standardizing method is in agreement with Abd El-hadi et al. [11] and Aref et al. [12].

In CLL, CD38 is a potent prognostic marker that predicts both an aggressive clinical course and survival. As a result, CD38 expression serves as a gauge for cell division and an indicator of in vivo growth [13].

In our study CD69 was positive in (42%) of patients, CD38 was positive in (34%) of patients. Similarly, Aref et al. [12] and Albanaa et al. [14] reported that CD69 and CD38 was positive in (30.7%), (31.4%) and (46.7%), (50%) of patients; respectively.

The most common genomic abnormalities in CLL include trisomy 12, TP53 mutations, deletion of 11q, loss of 13q and deletion of 17p. While the remaining anomalies are linked to a progressively worse outcome, CLL with 13q deletions is connected with a good prognostic effect [15].

The 17p deletion affects another tumor suppressor gene TP53 gene. Since it causes cell cycle arrest and encourages DNA repair or apoptosis when the cell has accumulated DNA damage, this gene is a crucial regulator of the cell cycle [16]. In patients who are chemorefractory, the 17p deletion accumulates over time and increases in stages [15].

In the current study, 17p deletion was positive in (12%) of patients, which corresponds with the report of Arafa et al. [17] (10%), it was higher than that found by Gutierrez et al., [18] (2%). While, it was lower than that reported by Wu et al., [19] (26.6%), which included both 17p deletion and TP53 mutation.

In our study, there was a statistically significant positive correlation between CD69 and CD38 (p=0.008). This finding was confirmed with Abd El-hadi et al. [11], Montraveta et al. [20], Noreldin et al. [10] who reported that there was a statistically significant positive correlation between CD69 and CD38.

Thymocytes, activated T cells, B cells, dendritic cells, endothelial cells, and neurons all express the type I glycoprotein CD200 [21]. CD200 expression has recently become a valuable technique for improving the differentiation between mantle cell lymphoma and conventional CLL [22]. Studies on the prognostic importance of this marker in patients with CLL are scarce, despite its utility in the diagnostic context [23].

A statistically significant positive correlation (p=0.04) was found between CD69 and CD200 in our study. This finding is consistent with reports from Miao et al. [21] and Aref et al. [23], who found that patients with high sCD200 in the CLL subgroup had higher levels of CD69 than patients with low sCD200.

In the current study, the existence of a positive 17p deletion and positive CD69 were statistically significantly correlated (p>0.001). among studied patients (all patients with Del positive had positive CD69), which is in agreement with that reported by Montraveta et al. [20].

In our study, follow up period ranged from 6 to 12 months with mean 8.32 months. 80% showed response and 20% showed no response. After therapy, it was noted that CD69 expression was statistically significantly higher in those with no response, and we reported that all non-responders had positive CD69 versus 27.5% of responders. In addition, this CD69 high expression significantly independently increased the risk of no response by 1.071 folds, indicating that this high expression confers a poor outcome. Same findings were reported by Montraveta et al. [20] who observed that patients with high CD69 expression prior to treatment were associated with low response to therapy.

Montraveta et al. [20] documented that the expression of CD69 may be able to predict the reaction to bendamustine. Bendamustine's clinical effects in CLL patients vary greatly, and there is a lack of information on particular markers that could indicate a patient's susceptibility to the medication. The in vitro activity of bendamustine and the gene expression profile in primary CLL cells were examined in order to find response indicators. The strongest predictor of bendamustine response is found to be the mRNA expression of CD69. The expression of the activation marker CD69 was the most accurate indicator of bendamustine sensitivity when compared to the functions of the other cell surface proteins. A decrease in bendamustine sensitivity was noted in conjunction with an increase of CD69 in CLL cells co-cultured with different subtypes of stromal cells.

By applying the ROC curve in this study, the best cutoff of value of both CD69 and CD38 for prediction of non-response to therapy was ≥58.15% and ≥34%; respectively, with area under curve of 0.958 and 0.783, sensitivity 90% and 80%, specificity 87.5% and 66.7, positive predictive value 64.3% and 50%, negative predictive value 97.2% and 88.9%, and overall accuracy 88% and 70.3%; respectively.

Noreldin et al. [10] identified a cutoff value of CD38, CD69 to best predict cases need to start chemotherapy. They showed that CD69% and CD38% at cut off levels of > 45% and > 40%; respectively can significantly detect the need to start chemotherapy with sensitivity and specificity for CD69 of 88.89% and 77.27% and for CD38 of 88.89% and 81.82%; respectively.

Also, we used ROC curve to the optimal CD200 cutoff for non-response prediction was found to be ≥89%, with an area under the curve of 0.725,

sensitivity of 80%, specificity of 70%, positive predictive value of 40%, negative predictive value of 93.3%, and overall accuracy of 72% (p=0.155). Aref et al. [23] who measured soluble CD200 in serum, reported that the best cuttoff leve of sCD200, by applying ROC curve, to predict treatment response was (752.5 pg/ml) with sensitivity 72.1%, and specificity 75.6%.

Unfortunately, the current study lacks information about PFS and OS because of the short duration of the study, so we tried to detect relation between time till the patient respond to treatment and expression of CD69.

In the present study, with one year follow up period and by applying Kaplan Meier analysis to detect relation between time till response and CD69 expression among studied patients. There was a statistically significant association between time till response and expression of CD69 ($p<0.001$). All those with positive expression of CD69 had significantly higher time till response.

Also, Montraveta et al. [20] stated that the molecular processes underlying bendamustine resistance are still largely unknown and that CLL cells' responsiveness to the drug varies substantially. They discovered that one of the most accurate indicators of bendamustine resistance in CLL cells was the mRNA expression of the activation marker CD69.

In addition, Aref et al. [12] revealed that elevated CD69 expression was linked to a short-duration response with a quick relapse, a short overall survival, and a poorer result. This was demonstrated by survival analysis using Kaplan-Meier analysis. Additionally, there was a strong correlation found between the combination and shorter PFS and OS. Compared to CD38 expression %, CD69 expression exhibited a considerably stronger prediction of PFS and OS (hazard ratio of 1.7, 1.8 vs 1.5, 1.5) ($p = 0.03, 0.03$ vs 0.08, 0.1), respectively.

This increased disease progression which associated with high CD69 expression could be explained by the more aggressive disease course seen in these patients may be explained by the fact that CD69, which is up-regulated quickly with cellular activation similar to B cells at an earlier state of activation, may be better able to transduce BCR-mediated signals with the assistance of simultaneous ZAP-70 expression. This elevated intracellular signaling may impact CLL cell survival or proliferation, which could result in a propensity for the disease to advance [24]

Aref et al. [12] stated that the strength of CD69 expression as an independent prognostic factor was evaluated using a Cox proportional hazard regression model. In comparison to CD38 expression percent, CD69 expression showed a considerably stronger prediction of PFS and OS (hazard ratio: 1.7, 1.8 vs. 1.5, 1.5) ($p = 0.03, 0.03$ vs. 0.08, 0.1). It was discovered that CD69 positive expression is an independent prognostic factor comparable to the immunoglobulin variable heavy chain mutational status. The OS had a hazard ratio (HR) of 1.8 vs 1.9 ($p = 0.03$ vs 0.01), whereas the PFS's HR for CD69 relative to IgVH was 1.7 VS 1.6 ($p = 0.04$ vs 0.02) Del Poeta et al. [24] proved that CD69 and ZAP-70 have independent predictive values for both PFS and OS using multivariate analysis. Further multivariate analysis verified CD69 as an independent prognosticator, along with CD49d, FISH cytogenetics and IGHV mutational status.

CONCLUSION

It was discovered that CD69 expression is a separate predictor of prognosis for CLL. CD69 determined by flowcytometry could be considered a novel important independent prognostic parameter in B-CLL. It is easy and rapid laboratory evaluation allows early identification of progressive patients, enabling timely therapeutic decisions.

Declaration of interest

The authors report no conflicts of interest. The authors along are responsible for the content and writing of the paper.

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