International Journal of Clinical and Diagnostic Pathology

ISSN (P): 2617-7226
ISSN (E): 2617-7234
www.patholjournal.com
2023; 6(2): 17-22
Received: 01-02-2023
Accepted: 05-03-2023

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Study of CD73 Expression on B Lymphocytes in patients with chronic lymphocytic leukemia

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DOI: https://doi.org/10.33545/pathol.2023.v6.i2a.513

Abstract
Background: A subgroup of T and B cells express the extracellular enzyme cluster of differentiation (CD73), which hydrolyzes adenosine monophosphate to adenosine. This work aimed to evaluate CD73 expression and its correlation with other disease characteristics in leukemia of chronic lymphocytic type (CLL) patients.
Methods: This cross-sectional research was done on 30 CLL cases diagnosed by laboratory investigations or presented with fatigue and fever with or without lymphadenopathy and organomegaly, and a healthy control group of 30 age- and sex-matched individuals. All patients had subjected to estimation of β2 microglobulin, flowcytometric detection of CD73 and CD19 in peripheral blood.
Results: In neither group did CD19/CD73 expression and haemoglobin significantly correlate. microglobulin in control group. While, the CD19/CD73 expression had positive significant correlation with B2 macroglobulin in CLL group. In the CLL group, haemoglobin level, platelet count, and CD19/CD73 expression were all noticeably reduced. B2 microglobulin, however, was noticeably greater in the CLL group. CD19/CD73 expression can diagnose CLL with high accuracy (100%) at cut-off 16.8, sensitivity, specificity, PPV and NPV were 100%.
Conclusions: When compared to controls, most CLL patients have less expression of CD19CD73, making it useful in standard diagnostic immunophenotyping workup. At the cut-off point of 16.8, CD19/CD73 expression has a high sensitivity (100%) for the diagnosis of CLL.

Keywords: CD73 Expression, B Lymphocytes, chronic lymphocytic leukemia

Introduction
The most prevalent adult leukaemia is chronic lymphocytic leukaemia (CLL), a lymphoproliferative illness with a very varied clinical history. Mature antigen-stimulated Cluster of Differentiation (CD5+/CD23) + B-lymphocyte clonal growth in blood, secondary lymphoid tissue, and bone marrow are characteristics of CLL [1].
The clinical staging systems created by Rai and Binet are still the de facto techniques for assessing risk in CLL, however they do not provide forecasts of the risk of disease progression in patients with early-stage illness, which is the majority of patients. Numerous research looked at prognostic markers, which are useful for forecasting a person's risk at an early stage of the disease [2].
The immunoglobulin variable gene segments (IgVH), CD38 expression, the -associated protein of 70-kD (ZAP-70), and cytogenetic risk groups are the most extensively recognised and utilised prognostic indicators in CLL [3].
With more understanding of the molecular and biological mechanism underlying the pathophysiology in CLL, there has been a remarkable change in the approach towards the disease management and many new prognostic markers have emrged [4].
A membrane-bound enzyme called ecto-5 0-nucleotidase (CD73) catalyses the transformation of adenosine monophosphate into adenosine. Adenosine is believed to have cytoprotective effects, primarily through aiding immune escape and having an immunosuppressive impact. Adenosine increases tumour growth and CD73-expressing cancer cell lines are more aggressive, hence it has been hypothesised that CD73 plays a significant role in carcinogenesis. However, additional research has revealed that active adenosine receptors may also prevent cell growth [5].

The objective of this study was to assess CD73 expression in CLL patients and its relationship to other illness features.

Patients and Methods

A cross-sectional analysis of 30 CLL patients was conducted who diagnosed by laboratory investigations or presented with fatigue and fever ± lymphadenopathy ± organomegaly aged from 44 to 66 years, and a healthy control group of 30 age- and sex-matched volunteers, ranging in age from 40 to 61 years.

After receiving clearance from the ethical committee at Tanta University Hospitals in Tanta, Egypt, the study was carried out. The patient gave written, fully informed consent.

All patients underwent the following: full medical history and examination, abdominal with chest CT scan for detection of internal organs or lymph node involvement, immunophenotypic scoring system was obtained to differentiate CLL cases from other lymphoproliferative disorders, laboratory investigations (complete blood picture and blood film, estimation of β2 microglobulin, flowcytometric detection of CD73 and CD19 in peripheral blood).

Five millilitres of venous blood were drawn by a sterile venipuncture; two millilitres were put into a plain test tube provided by the vacutainer and allowed to clot; the serum was then separated after centrifugation for ten minutes at three thousand revolutions per minute; and a measurement of two microglobulins was then made. K2-EDTA (Dipotassium Ethylene Diamine Tetra Acetic acid) is administered in three millilitres of plastic vacutainer tubes for CBC, blood film, CD73, and CD19 analyses.

Estimation of β2 microglobulin by Microparticle Enzyme Immunoassay (MEIA) technology on Abbott AXSYM system. Reaction between antibody coated microparticles and the sample antigen. Enzyme labeled antibody is then added to form a sandwich. Finally, a substrate is added and a fluorescent signal is detected. The Advia 2120's comprehensive blood image and haematological analyzer employ laser light scatter technology to compute the blood count and differential. For each cell, the low angle (2–3) and high angle (5–15) scatters are converted into volume and inner structure values (for haemoglobin, leukocytes' nuclei, and platelets' granules) [6].

Flow cytometric analysis of CD73 and CD19 expression on Becton Dickinson FACSC alibur.

Two ml blood were added gently to an equal volume of ficoll and centrifuged for 20 minutes at 1200 rpm. The lymphocytes were deposited in a white band at the interface between plasma and ficoll and separated into another centrifuge tube. The separated cells were centrifuged for 5 minutes at 2000 rpm after being rinsed three times with Phosphate Buffered Saline (PBS). We carefully aspirated the supernatant and threw it away. In 500 l of PBS, the pellet was resuspended. Two polystyrene test tubes (12x75mm) were prepared. Dual stains with 5 microns of CD73 FITC and 10 microns of CD19 PE were placed in the first tube. A negative control was employed with the second tube. Each tube received 100 l of the suspended cells, which were then gently blended using a vortex mixer. The tubes were incubated at 4 c for 30 min. gating on the lymphocyte region allowed the Becton Dickinson FACS Calibur flow cytometer to be used for analysis. The number of events to be analyzed was adjusted to 10,000. The sample was mixed properly before acquisition. Calibration was done every time by an installed calibration program. A specialist cheks up this program every 3 months and adjusts it by calibration beads.

Statistical analysis: SPSS (Statistical Package for Social Sciences) version 26.0 and Microsoft Excel 2016 were used for the statistical analysis. Descriptive statistics were calculated for categorical data using number and percentage, while they were done for numerical parametric data using mean, SD (standard deviation), minimum and maximum of the range, and numerical nonparametric data using median and interquartile range. For quantitative variables, independent t-tests are used when there are two independent groups with parametric data while Mann Whitney U tests are used when there are two separate groups with non-parametric data. Inferential studies for qualitative data use the Chi square test for independent groups. In order to establish the diagnostic use of CD19/CD73 expression, receiver operating characteristic (ROC) analysis was conducted. Spearman correlation test was used to compare the research groups. P 0.05 is considered significant.

Results

There was insignificant difference regarding demographic characteristics among the studied groups. Table (1)

Table 1: Information about the study groups' demographics

		CLL patients' group (Group I) n = 30	Control group (Group II) n = 30	Test value	p-value
Age (years)		57.23± 6.47	54.63± 7.92	1.491	0.136
Sex	Male	12(40.0%)	19(63.3%)	3.27	0.071
	Female	18(60.0%)	11(36.7%)		
Residence	Rural	15 (50.0%)	16(53.3%)	0.067	0.798
	Urban	15 (50.0%)	14(46.7%)		
DM	No	18(60.0%)	16(53.3%)	0.271	0.602
	Yes	12(40.0%)	14(46.7%)		
Hypertension	No	18(60.0%)	20(66.7%)	0.287	0.592
	Yes	12(40.0%)	10(33.3%)		
Dyslipidemia	No	21(70.0%)	25(83.3%)	1.49	0.222
	Yes	9(30.0%)	5(16.7%)		
Smoking	No	23(76.7%)	22(73.3%)	0.089	0.766
	Yes	7(23.3%)	8(26.7%)		

Data are shown as Mean, SD, or frequency (%); DM stands for diabetes mellitus.

According to the clinical data and stages of CLL, 14 (46.7%) patients presented with lymphadenopathy, 14 (46.7%) patients presented with splenomegaly and 12 (40%) patients presented with hepatomegaly. 8 (26.7%) patients presented with stage 1 & III, 11 (36.7%) patients presented with stage II and 3 (10%) patients presented with stage IV. Table 2

Table 2: Distribution of clinical data and stages in CLL patients

	CLL patients group (Group I) (n= 30)
Lymphadenopathy	14(46.7%)
Splenomegaly	14(46.7%)
Hepatomegaly	12(40.0%)
CLL stage	
Stage I	8(26.7%)
Stage II	11(36.7%)
Stage III	8(26.7%)
Stage IV	3(10.0%)

Data are presented as frequency (%).

When compared to the control group, the CLL patient group's haemoglobin level, platelet count, and CD19/CD73 expression count were all considerably lower (p 0.001). When compared to the control group, the B2 microglobulin level in the CLL patient group was considerably higher (p=0.002 and 0.043, respectively). Table (3).

Table 3: Shows a comparison of the study groups' CD19/CD73 expression results from the lab.

Parameters	CLL patients' group (Group I) (n=30)	Control group (Group II) (n=30)	Test value	p-value
Hb. (gm/dl)	11.27± 1.85	13.57± 1.55	T= 5.24	<0.001
<10	11(36.7%)	0	X^2= 13.47	<0.001
>10	19(63.3%)	30(100.0%)		
Platelets count (*10^3/L)	153.57± 55.44	227.30± 32.36	T= 6.29	<0.001
<100	9(30.0%)	0	X^2= 10.59	0.001
>100	21(70.0%)	30(100.0%)		
B2 Microglobulin (mg/L)	1.95 (1.30 – 2.40)	1.25 (0.9 – 2.0)	^{Z}MWU= 3.03	0.002
Normal	24(80.0%)	30(100.0%)	X^2= 6.67	0.012^{FET}
High	6(20.0%)	0		
CD19/CD73 expression	11.91± 2.62	37.53± 7.80	^{Z}MWU= 6.65	<0.001
<17.9	0	30(100.0%)	X^2= 60.0	<0.001
>17.9	30(100.0%)	0		

The data is shown as Mean ±S D, median, or frequency (%); ZMWU = Mann- Whitney U test; X2 = Chi-Square test; FET: Fischer exact test. It is statistically significant if p 0.05.

Haemoglobin expression and CD19/CD73 expression did not significantly correlate. In the group of CLL patients, there was a strong positive connection between the expression of CD19/CD73 and B2 microglobulin (p=0.011). When compared to CLL patients without splenomegaly, CD19/CD73 expression was considerably greater in CLL patients with splenomegaly (p<0.001). Figure (1).

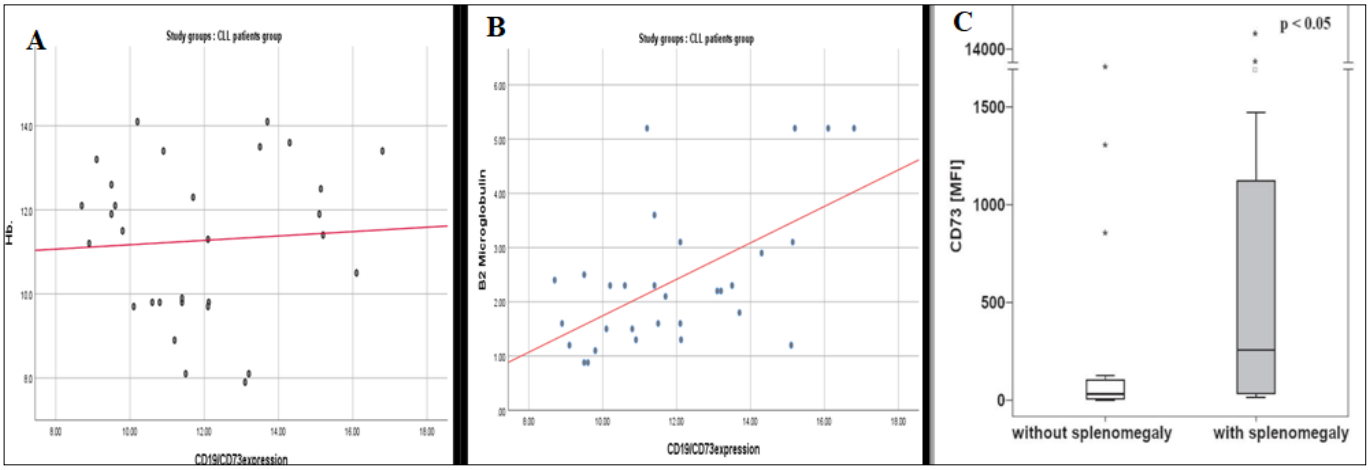

Fig 1: Connections that exist between CD19/CD73 expression and (A) Hemoglobin, (B) B2 macroglobulin (C) splenomegaly in CLL patients group

In order to establish the diagnostic use of CD19/CD73 expression, receiver operating characteristic (ROC) analysis was conducted. Using ROC-curve analysis and a cut-off point of 16.8, CD19/CD73 expression can diagnose CLL with 100% accuracy. Sensitivity, specificity, positive predictive value (NPV), and specificity (PPV) were all 100% (p<0.001). Figure 2

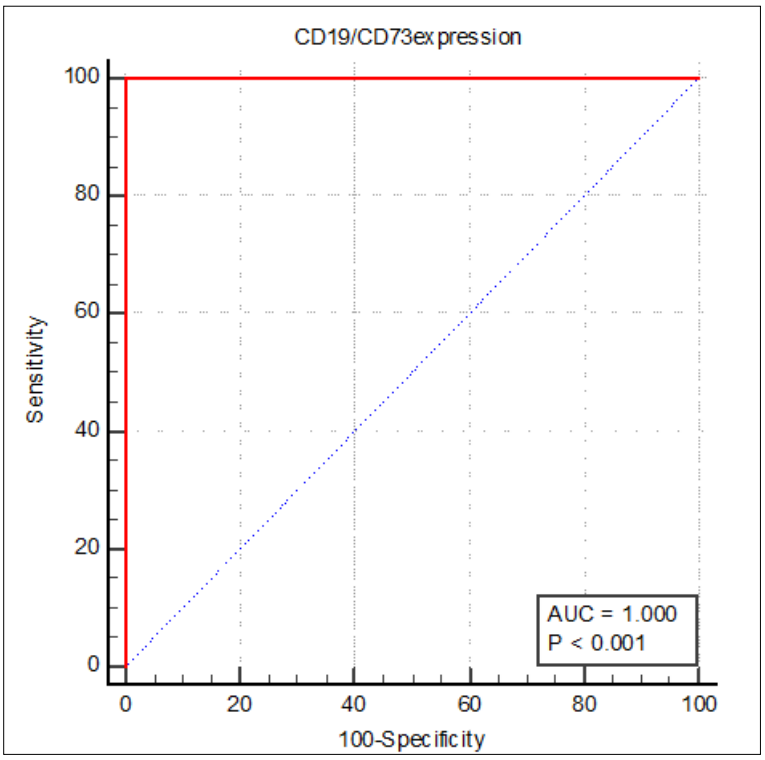

Fig 2: ROC curve of CD19/CD73 expression in diagnosis of CLL.

Discussion

By converting ADP/ATP to AMP and AMP to adenosine, respectively, the enzymatic activities of CD39 and CD73 play crucial roles in regulating the length, intensity, and chemical make-up of purinergic signals transmitted to immune cells Antonioli *et al.* [7]

With reference to distribution of stages in CLL patients, the present study showed that 8 (26.7%) patients presented with stage 1 & III, 11 (36.7%) patients presented with stage II and 3 (10%) patients presented with stage IV. Patients in these places frequently present at more advanced stages, as seen by statistics from other developing nations from Payandeh *et al.* [8] and Gogia *et al.* [9] (38.5%, 33.3%, and 41%), respectively.

With reference to comparison between the studied groups regarding CD19/CD73 expression, flow cytometric immunophenotyping has shown that CLL B-cells exhibit a decreased expression of CD73 compared to normal B-cells (p<0.001).

Similar findings were made by Serra *et al.* [11], who found that 60% of their study group had less than 10% of CD19+/CD73+ cells in the peripheral blood, and Rosi *et al.* [10], who found that the percentage of CD73 positive cells in CD19+ CD5+ populations were reduced at least by 95% in CLL lymphocytes compared to controls. Additionally, Pulte *et al.* [12] demonstrated that malignant B lymphocyte CD73 expression was often lower than that of normal B lymphocytes (19.3% overall for CLL vs. 77% in normal B cells) and the Egyptian study Osman *et al.* [13] found that CD73 expression on CD19+ B lymphocytes was significantly lower in untreated CLL patients compared with controls (mean 3.74 vs. 29.35%, respectively) than in controls. The study involved 25 newly diagnosed CLL patients and 15 apparently healthy normal individuals, p<0.001)

The beta-2-microglobulin concentration is associated with the extent of the disease and its more aggressive course [14]. Stronger CD73 expression was seen in our CLL patients with greater beta-2-microglobulin levels, which may signify a more advanced stage or more aggressive course of the illness. This is in line with a study by Serra *et al.* [11], which assessed the clinical effects of CD73 in CLL and discovered that high expression of CD73 is linked to more aggressive clinical behaviour, larger CLL clones, and poor prognostic markers, such as higher expression of Ki-67, CD38, and ZAP70. As opposed to CD73 CLL, which was linked to more advanced illness, Pulte *et al.* [12] reported that patients with CD73+ CLL have a less aggressive trajectory. Similar to this, Kicova *et al.* [15] found that elevated beta-2-microglobulin levels in CLL patients are substantially linked with CD73 expression (p 0.05). Unlike Osman *et al.* [13], who demonstrated that there was no significant association between blood levels of 2 microglobulin and CD73 expression on B or T cells (p>0.05). In line with a research by Kicova *et al.* [15], it was discovered in the study that CLL patients with splenomegaly showed considerably greater levels of CD73 expression on pathogenic B-lymphocytes than CLL patients without splenomegaly,

In our research we found that higher B-lymphocyte CD73 levels are associated with later stage disease. As Sera *et al.* [11], It has been proven that extracellular ADO, which is mostly produced by CD73, has powerful effects in preventing CLL cells from dying naturally or as a result of etoposide. When laboratory data from the examined groups were analysed, it was discovered that B2 microglobulin levels in the CLL patient group were considerably greater than those in the control group (p= 0.002 & 0.043 respectively). This agreed with Osman *et al.* [13], who found significant difference between the studied group.

According to Validity of CD19/CD73 expression in diagnosis of CLL, CD19/CD73 expression can diagnose CLL with high accuracy (100%) at 16.8 as the cutoff. 100% (p 0.001) were the sensitivity, specificity, PPV, and NPV. This was in line with Youssef *et al.*'s [16] Osman *et al.*'s [13] claim that a cut-off value of 19% for CD73 expression was able to differentiate between CLL patients and healthy

controls with 96% diagnostic accuracy study that a cut-off value of 19% for CD73 expression who found that CD 73/19 expression to have a sensitivity and specificity of 100% when 14.79% was used as a cutoff in our CLL patients. This discovery might justify the inclusion of surface expression of CD73 in the first diagnostic workup for routine CLL diagnosis with a cutoff for positive that is almost identical to other markers utilised (around 20%), and it could also assist further research into CD73 in other lymphoproliferative disorders (LPD).

There were several studies that involved CD73 role in other malignancies. Unlike in CLL, Supernat *et al*. [5], examined CD73 expression by immunohistochemistry on sections obtained from patients with breast carcinoma. 74% of the time, CD73 status was positive. Breast cancer CD73 expression did not correlate with any of the disease-related variables, such as tumour size, lymph node status, histologic type, or oestrogen or progesterone receptor status. The prolonged disease-free survival (DFS) and overall survival (OS) were shown to be correlated with positive CD73 expression, suggesting that CD73 expression may be a good predictor of a favourable prognosis in breast cancer. According to Aliagas *et al*. [17], both the stroma and epithelial structures of endometrial adenocarcinomas showed elevated levels of CD73 expression.

Sadej *et al*. [18], displayed that advanced metastatic melanoma cell lines have much higher levels of CD73 expression than normal melanocytes and initial tumour cell lines. Furthermore, they proposed that CD73 acts as an adhesion molecule on melanoma cells, identifying the most aggressive variants and promoting metastasis.

CD 73 plays a diverse role in different malignancies. Our study, among others, has demonstrated that CLL patients had B cells with reduced CD73 expression.

Limitations: We were unable to identify whether B-lymphocyte CD73 levels rise as the disease develops or if greater levels represent an early prognostic indicator for worse disease because this was a cross-sectional research therefore, it was recommended that Quantitative CD73 analysis should be carried out on many CLL patients and on other B-lymphoproliferative disorders to examine the possibility of adding it to CLL scoring system

Conclusions

Whenever juxtaposed with controls, most CLL patients have a decreased level of CD19CD73, which makes it useful for conventional diagnosing immunophenotyping workups and enhances the probability of immune response failure and subsequent proliferation and clonal expansion of the implicated clone. CD19/CD73 expression can diagnose CLL with high accuracy (100%) at cut-off point at 16.8.

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How to Cite This Article
Zohery HMKE, Gawaly AM, Mohamed WS, Said AAE. Study of CD73 Expression on B Lymphocytes in patients with chronic lymphocytic leukemia. International Journal of Clinical and Diagnostic Pathology. 2023;6(2):17-22.