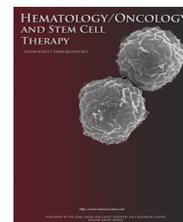




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ORIGINAL RESEARCH REPORT

Impact of CD39 expression on CD4+ T lymphocytes and 6q deletion on outcome of patients with chronic lymphocytic leukemia



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KEYWORDS

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Abstract

Objective/Background: Chronic lymphocytic leukemia is one of the commonest leukemias affecting adults. CD39 inhibits T-cell and Natural killer (NK) cell responses by hydrolyzing adenosine triphosphate and adenosine diphosphate, suppressing the immune system. We investigated expression of CD39 on CD4+ T Lymphocytes in chronic lymphocytic leukemia (CLL) patients and its relationship with deletion 6q, its association with disease stage and survival. **Methods:** Thirty CLL patients and 20 matched controls were included in the study. Bone marrow studies with immunophenotyping, CD39, CD38, and ZAP-70, and detection of del 6q by FISH were performed.

Results: CD39+ CD4+ T helper cells in CLL patients were significantly expressed compared with the controls ($p < .001$). Levels of CD39+ CD4+ T cells were significantly expressed in high risk CLL patients. Del 6q was detected in 63.3% of patients and it correlated with CD39, CD38, and ZAP-70, and advanced stage disease. There was a significant relation between response to treatment and CD39 expression and del 6q, also there was a significant difference in overall survival (OS) between patients with and without Del 6q.

Conclusion: CD39 expression on CD4+ T cells and del 6q act as prognostic markers in CLL. Blocking or inhibition of CD39 may be a target for new immune therapy for CLL.

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Introduction

In the West, chronic lymphocytic leukaemia (CLL) is considered one of the most common leukemias affecting the adult population. However, it is infrequent in the Eastern world. Most of the patients can be asymptomatic at diagnosis whereas others can present with systemic B symptoms, lymphadenopathy, hepatosplenomegaly, and infiltration of bone marrow leading to cytopenias [1].

Up to now, there has been no definite cure for CLL, despite advances in understanding the underlying pathology of the disease [2]. Different mechanisms are used by tumors to cause suppression of the host immune system. Adenosine triphosphate (ATP) hydrolysis by CD39 plays an important role in tumor mediated immune suppression [3].

CD39, which is the main ectoenzyme in the immune system, is expressed by most B cells, subsets of T cells, and dendritic cells. It aids in inhibiting T-cell and Natural killer (NK) cell responses by hydrolyzing ATP and adenosine diphosphate (ADP) generating adenosine, thus suppressing the immune system. This immunosuppressive pathway protects cancer cells and favors tumor growth [4].

In this study, we hypothesized that elevated levels of the highly immune-suppressive CD39+ T cell population may support and promote the unopposed growth of CLL cells, thereby contributing to a more aggressive clinical course of the disease. We investigated the expression of CD39 on CD4+ T Lymphocytes obtained from patients with CLL by using flow cytometry and (del 6q) by fluorescence *in situ* hybridization (FISH), its correlation with the disease stage and survival.

Materials and methods

This prospective nonrandomized study was conducted at Clinical Haematology Unit, Internal Medicine Department, Faculty of Medicine and South Egypt Cancer Institute, Assiut University, Assiut, Egypt between January 2013 and December 2017. This study included thirty patients with CLL in addition to 20 healthy controls. The CLL group included 16 males (53.3%) and 14 females (46.7%) with a mean age of (58.5 ± 10.8 years) and a range of 36–82 years. The mean age of the control group was (59.3 ± 9.6 years) and the age range was 38–81 years. In the control group, 12 (60%) participants were males and eight (40%) were females. Written informed consents were taken from all participants. This study was approved by the ethical committee of Assiut University.

All patients were diagnosed as CLL based on clinical and morphological data in addition to immunophenotyping according to the following criteria: patient presented with (1) absolute lymphocytosis in peripheral blood > 5000/cmm; (2) lymphocytes were small, mature-looking with high N/C (N = nuclear, C = cytoplasmic) ratio, round to oval nuclei and clumped chromatin; smudge cell is a characteristic feature; (3) characteristic immunophenotyping: CD19+, CD20+(dim), CD23+, CD5+, surface membrane immunoglobulin + (dim), monoclonal κ or λ light chain, FMC7-, and CD10-; and (4) bone marrow showed increased numbers (\geq 30%) of mature, small lymphocytes. Prolymphocytes are \leq 55%. All patients and controls were evaluated by history taking, clinical examination, complete blood count (CBC), ery-

throcyte sedimentation rate (ESR), liver and kidney function tests, abdominal and chest computed tomography (CT).

All CLL patients were submitted to bone marrow aspirate, biopsy, immunophenotyping including: CD5, CD10, CD19, λ , κ , FMC7, CD23, CD39, CD4, ZAP-70, and CD38, and FISH detection of del 6q.

All patients were evaluated for presence of an indication to treatment as constitutional symptoms in the form of severe fatigue, night sweats, weight loss or fever without infection, the presence of threatened end organ function, progressive bulky disease, progressive anaemia, or progressive thrombocytopenia. Fit patients with indication to treatment received chemotherapy in the form of fludarabine and cyclophosphamide (FC) with or without rituximab and frail patients or young patients with significant comorbidities received alkylating agent in the form of chlorambucil with or without rituximab.

Flowcytometric analysis of CD39, CD4, CD38, and ZAP-70 expression

The sample was washed with phosphate buffered saline (PBS) and the supernatant was removed. Surface antibodies conjugates CD39, CD4, and CD38 were dispensed into appropriately labeled tubes.

Next, 50 μ L of cell suspension was added to monoclonal mixture in each tube. Tubes were agitated gently to mix and incubated in the dark for 20 min at 4 °C. Then, 3 mL of the lysing reagent was added to each tube, inverted once, and kept for 5 min. For detection of monoclonal 50 μ L were added of reagents A (fixation medium) and incubated for 10 min at room temperature and washing was performed once in 3 mL PBS. Next, 50 μ L of permeabilization reagent was added, then 10 μ L of conjugated antibody was added. Samples were analyzed by multicolor flow cytometry (FACSCalibur, Becton Dickinson, San Jose, California, USA; Fig. 1).

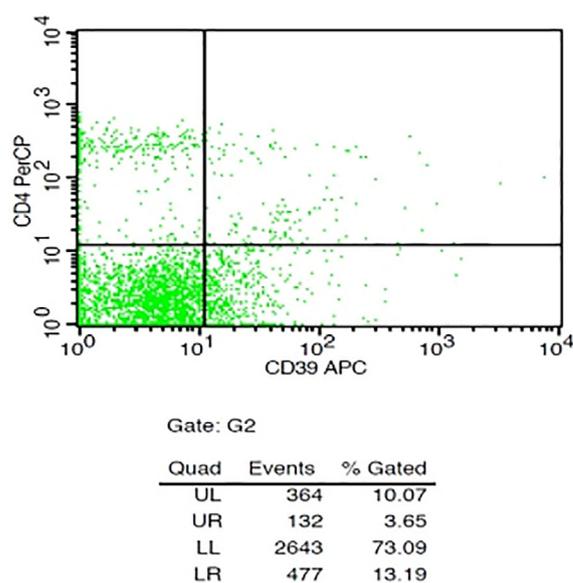


Fig. 1 Flow cytometry show CD39 expression on CD4 T helper cells.

Detection of del 6 by FISH

A solution of 0.7 mL of heparinized blood was added to 10 mL RPMI 1640 full medium containing 0.2 mL phytohemagglutinin in T-25 flasks, and incubated for 72 hours at 37 °C in a cell culture incubator. Cells were resuspended in the remaining medium and ~2 mL of prewarmed (37 °C) 0.075 M KCL was carefully added. The cells were resuspended and fixed by adding 10 mL of fixative; the first 2 mL were added dropwise while agitating gently. The suspension was dropped into a cleaned microscope slide. Poseidon Whole chromosome probes (product Kreotech Diagnostics, Amsterdam, The Netherlands, KBI-30001-30024) are direct-labeled DNA probes provided at 5× concentrated and diluted. DNA probes were labelled with Platinum Bright 495 (Green). Then, 10 µL of probe or probe-mix per 22 × 22 mm field were applied. Sample and probe were denatured on a hot plate at 75 °C for 5–0 min. Hybridization was continued in a humid, lightproof container at 37 °C (± 1 °C) overnight. Posthybridization washes were performed by removing the coverslip and all traces of glue carefully. The slide was immersed in 0.4 × SSC (pH 7.0) at 72 °C (± 1 °C) for 2 min without agitation. The slide was drained and immersed in 2 × SSC, 0.05% Tween-20 at RT (pH 7.0) for 30 s without agitation, covered with a coverslip, and viewed with a fluorescence microscope (Fig. 2).

Statistical analysis

Data entry and data analysis were performed using SPSS software version 20 (Statistical Package for the Social Science, version 20, IBM, and Armonk, New York). Data were presented as number, percentage, mean, standard deviation, standard error, median, and range. The Mann–Whitney *U* test was used to compare quantitative variables between two groups. A *p* value < .05 was considered statistically significant. Kaplan–Meier Survival analysis and log rank test were used to detect survival curves.

Results

This study included 30 patients diagnosed as CLL and 20 healthy controls. The CLL group included 16 males (53.3%) and 14 females (46.7%) with a mean age of (58.5 ± 10.8)

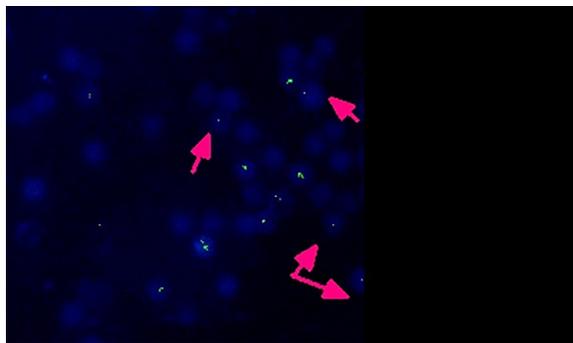


Fig. 2 Detection of del 6q in one of our studied CLL patients. CLL = chronic lymphocytic leukemia.

and a range of 36–82 years. The mean age of the control group was 59.3 ± 9.6 years and the age range was 38–81 years. In the control group, 12 (60%) participants were males and eight (40%) were females.

As shown in Table 1 staging of the cases was performed according to the Standard scoring system (Rai staging). Six patients were at low risk (20%), 13 patients were at intermediate risk (43.3%), and 11 patients were at high risk (36.7%).

In our study, CD 39 expression on CD4 T cells in CLL patients was significantly higher compared with the control group (*p* < .001). According to Rai staging, CD39 expression was significantly higher in high risk group of patients than low and intermediate group with a *p* value = .04 (Table 2).

In the studied group of CLL patients, 17 patients were CD38 positive (56.7%) and 13 patients were CD38 negative (43.3%). As regards to ZAP-70, 21 patients were ZAP-70 negative (70%) and nine patients were ZAP-70 positive (30%).

There was a statistical significant correlation between CD39 expression on CD4+ T cells and CD38 and ZAP-70 (Table 3).

Del 6q was detected in 19 patients (63.3%) with a range of 2–75% and a mean of 32.97 ± 14.85. As regards to the staging system, del 6q was significantly higher in high risk group of patients (*p* = .01) as shown in Table 1, also there was a significant correlation between del 6q, CD39, CD38, and ZAP-70 (Table 3).

Twenty-five (83.3%) CLL patients had an indication to treatment whereas five (16.6%) patients had no indication to treatment. Regarding the course of the disease, there was a significant relation between expression of CD39 and response to treatment with one patient (8%) achieving

Table 1 Patient characteristics.

Characteristic	No. of patients (%)
Age	58.5 ± 10.8
Sex	
Males	16 (53.3)
Females	14 (46.7)
White blood cells	148.2 ± 100
Hemoglobin	8.9 ± 2.8
Platelets	143 ± 72
Lactate dehydrogenase	545 ± 308.7
Absolute lymphocytic count	60.5 ± 55.3
Rai	
0	6 (20)
1 + 2	13 (43.3)
3 + 4	11 (36.7)
ZAP-70	
Positive	21 (70)
Negative	9 (30)
CD38	
Positive	17 (56.7)
Negative	13 (43.3)
Treatment	
Treated	25 (83.3)
Untreated	5 (16.6)

Table 2 The relation between Rai staging system and del 6q, CD39+ expression on CD4+ T helper cells, CD4+ T cells, CD38, and ZAP-70 expression.

%	Grade			<i>p</i>
	Low	Intermediate	High	
Del 6q	17.67 ± 3.46	25.15 ± 3.53	50.73 ± 15.54	.010
CD38	10.92 ± 4.74	15.62 ± 2.22	46.05 ± 37.43	.02
ZAP-70	2.83 ± 3.94	13.28 ± 23.68	36.7 ± 29.79	.0467
CD39+ expression on CD4+ cells	14.78 ± 7.11	22.3 ± 4.97	56.69 ± 10.56	.04
CD4+ cells	23.7 ± 8.5	28.99 ± 9.6	50.68 ± 12.4	<.001

CD = clusters of differentiation; Del 6q = deletion 6q; ZAP70 = zeta-chain-associated protein kinase 70.

Table 3 The correlation between CD39+ expression on CD4+ T helper cells, del 6q, and other parameters.

%	Del 6q		CD39+ expression on CD4+ Tcells	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
CD39+ expression on CD4+ Tcells	.41	.023		
CD38+	.61	.001	.47	.009
ZAP-70+	.42	.020	.36	.048

complete remission (CR), three patients had partial remission (PR) and eight patients (66.7%) had refractory disease (RD) with ($p = .04$). According to presence of 6q deletion, three patients (15.7%) had CR, five patients (26.3%) had PR, and 11 patients (57.8%) had RD with p -value of .03 as shown in Table 4.

With a median follow up of 36 months, median OS in patients with positive CD39 was 30 months (range, 20.7–41.9 months) with no significant difference between patients with positive and negative CD39 ($p = .385$) as shown in Fig. 3. Median DFS was 17.5 months in CD39 positive patients versus 15 months in CD39 negative ($p = .8$) as shown in Fig. 4. There was a significant difference in OS between patients with positive and negative del6q with mean OS in patients with del 6q was 22.4 ± 22.6 months versus 46 ± 14 months in patients with negative del 6q with significant difference ($p = .047$) as shown in Fig. 5, however, there was no significant difference in DFS between both groups (Fig. 6).

Discussion

The main hallmark of cancer cells now is their ability to cause host immunosuppression by the activation of multiple immune checkpoints. Blockage of these pathways using several antibodies has led to durable responses and increased survival rates in cancer patients [5].

Recently, CD39/CD73-adenosine pathway has been recognized as an important immune function regulator. It plays an important role in tumor mediated immunosuppression by removing proinflammatory ATP and production of adenosine which causes inhibition of T cell proliferation [4].

In this study, CD39+ expression on CD4+ T helper cells in CLL patients was significantly higher compared with the control group. This finding supports the recent observation that CD39 is significantly higher expressed in many different types of cancer cells than in normal tissues by infiltrating lymphocytes, tumor cells, and tumor stroma [4].

Table 4 The relation between the disease course in the CLL studied group, the percentage of del 6q and CD39+ expression on CD4+ T helper cells.

%	Disease course, <i>n</i> (%)			<i>p</i>
	CR	PR	R	
Del 6q	Positive 3 (15.7)	Positive 5 (26.3)	Positive 11 (57.8)	.03
	Negative 5 (83.3)	Negative 1 (16.7)	Negative 0 (0)	
CD39 + expression on CD4 + T cells >20%	Positive 1 (8)	Positive 3 (25)	Positive 8 (66.7)	.04
	Negative 7 (53.8)	Negative 3 (23)	Negative 3 (23)	
Total	8	6	11	25

CD = cluster of differentiation; CR = complete remission; Del 6q = deletion 6q; PR = partial remission; R = refractory.

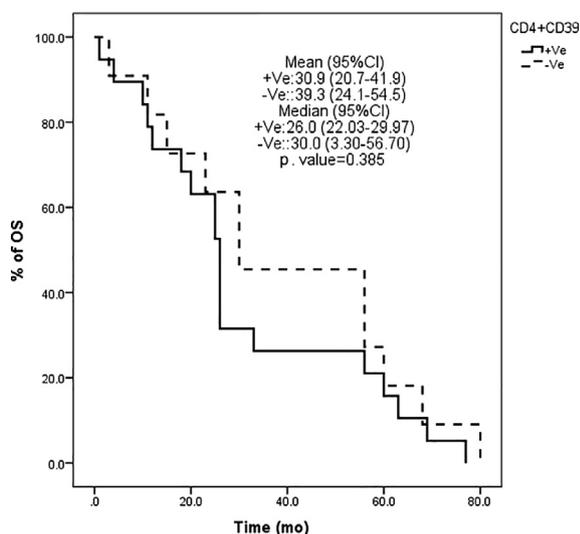


Fig. 3 Kaplan–Meier curves showing overall survival in patients with and without CD39+ expression on CD4+ T helper cells.

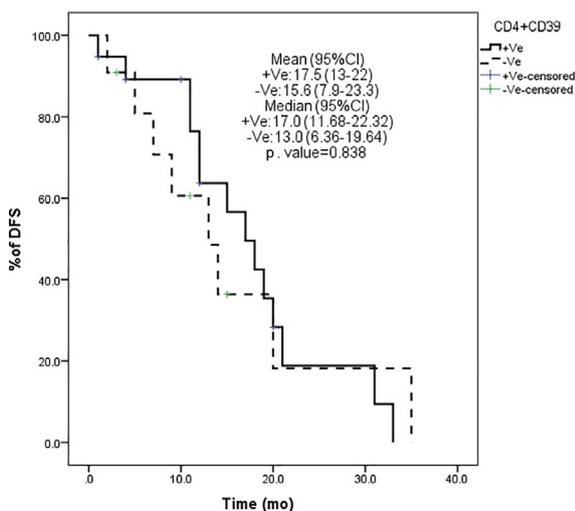


Fig. 4 Kaplan–Meier curves showing disease free survival (DFS) and patients with or without CD39+ expression on CD4+ T helper cells.

Levels of CD39+ CD4+ T cells in this study were higher in advanced stage disease which is in agreement with previous studies which demonstrated a significant difference between early and late stages of the disease [6,7].

Also, in this study, expression of CD39+ CD4+ T cells was positively correlated with CD38 and ZAP-70 expression which are well known as prognostic markers of advanced disease which supports previous results of Pulte et al. [7] but contrasted the results of Perry et al. [6] who found no correlations between them. However, in this study, there were no significant differences in overall survival or disease-free survival between patients with positive and negative CD39.

The nature of gene predisposition in CLL is still unclear. Most of the reported genetic abnormalities in CLL are not

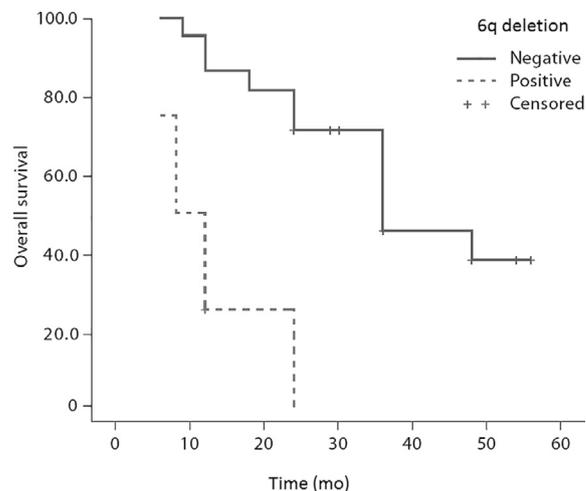


Fig. 5 Kaplan–Meier curves of the relation between overall survival (OS) and patients with or without del 6q.

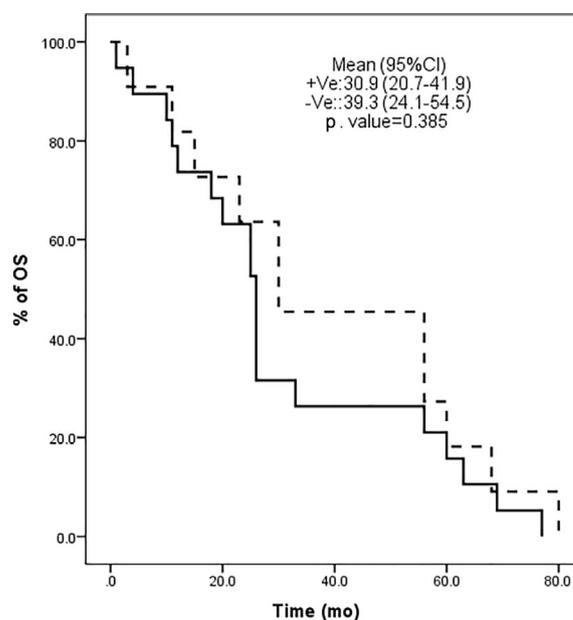


Fig. 6 Kaplan–Meier curves showing relation between disease free survival in patients with positive and negative Del6q.

constant and it is not known whether they occur as early events or during evolution of the disease. The most common abnormalities in CLL are trisomies, mutations, or deletions [8]. We examined the prognostic significance of 6q- in CLL patients. 6q- was found positive by FISH in 63.3% of patients. This is more or less in agreement with Zhang et al. [9], who found that deletions of 6q23-q24 detected in four of nine B-CLL patients. However, Stilgenbauer et al. [10] and Merup et al. [11], found that deletions involving 6q were found in 7% and 6%, respectively. The discrepancy between our results and the previous results could be explained by the lower number of patients in our study. In addition, on reviewing their study, we found that the majority of CLL patients who were included in that study had early-stage disease. By contrast, only 20% (6/30) of

the patients included in our study were in the low risk group in the modified Rai staging system, with the majority (80%) in the intermediate and high-risk group, which were associated with significant positive correlation with CD39+ expression on CD4+ T cells. This agrees with Cuneo et al. [12] who found that CLL patients with del 6q were an intermediate-risk group. Also, Welson et al. [13] observed that all CLL patients (100%) with 6q deletion were allocated to Stage C and Stages III and IV of Binet and Rai staging systems, respectively. In our study, presence of 6q- was associated with significant positive correlation with Rai staging, CD39+ expression on CD4+ T cells, CD38, and ZAP-70 expression and this was translated into a statistical significant difference in OS between patients with positive 6q- and negative 6q-.

In conclusion, this study showed that CD39 expression on CD4+ T cells and 6q- were associated with advanced stages of disease and were positively correlated with CD38 and ZAP-70 expression. However, their use as independent prognostic markers needs further studies including a larger number of patients. Blocking or inhibition of CD39 may be a target for new immune therapy for CLL.

Limitations of the study

This study has some limitations; the most significant are the small number of patients included, absence of correlation with 17p deletion and IGVH mutation, and both are due limited resources available.

Recommendations

More studies are needed with a larger number of patients and measurement of CD39 levels before and after therapy.

Conflicts of interest

The authors declare no conflict of interest.

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