Association of CD49d and CD44 in chronic lymphocytic leukemia patients and their effects on response to fludarabine, cyclophosphamide, and rituximab therapy

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Introduction

Chronic lymph ocytic leukemia (CLL) is a clonal expansion of small mature lymphocytes accumulating in the blood, bone marrow, and lymphoid organs. The interactions of the CLL microenvironment are known to promote CLL cell survival and proliferation. CD49d, an adhesion molecule belonging to the integrin family mediates cell-to-extracellular matrix and cell-to-cell interactions. CD44 is a glycoprotein and also a major hyaluronan receptor that mediates the response of cells to their cellular microenvironment.

Aim

We aimed to evaluate the value of CD49d and CD44 in CLL patients, and their effect on response to fludarabine, cyclophosphamide, and rituximab therapy, to study the relation of each to the other and its relation to the poor prognostic marker CD38.

Patients and methods

The study included 30 newly diagnosed CLL patients with measurement of the expression of CD49d and CD44 by flow cytometry before and after therapy.

Results

CD49d expression was increased in advanced risk stages according to modified Rai staging, but CD44 had no different expression in the different stages. There is a positive correlation between CD44 with CD49d, CD38 (documented poor prognostic marker) with CD49d, and CD38 with CD44. Lymphadenopathy showed a significant relation with CD49d only. Patients without complete response had a significant higher expression of CD49d, CD38, and CD44 before and after therapy.

Conclusion

The study has shown that the expression of CD49d and CD44 in newly diagnosed CLL patients is related to resistance to the fludarabine, cyclophosphamide, and rituximab therapy and their expression is related to each other and to CD38.

Keywords:
CD44, CD49d, chronic lymphocytic leukemia

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Introduction

Chronic lymphocytic leukemia (CLL) is a clonal expansion of small mature lymphocytes accumulating in the blood, bone marrow (BM), and lymphoid organs. CLL is characterized by an extremely variable clinical course with survivals ranging from one to more than 15 years [1]. The cellular and molecular interactions of CLL cells with the microenvironment in secondary lymphoid tissues and the BM are known to promote CLL cell survival and proliferation [2]. The staging systems of Rai and Binet are used to estimate the prognosis based on the clinical presentation [3,4]. It has been recognized that the Rai or Binet clinical staging systems alone are not sufficient to estimate individual prognosis because there is a great variation in disease progression and survival of patients belonging to the same stage [5]. The original Rai classification was modified to reduce the number of prognostic groups from five to three [6]. In addition to clinical staging, there are some simple clinical and laboratory parameters, such as age, sex, absolute lymphocytic count, lymphocytic doubling time, and serum beta 2 microglobulin which have been show an improved prognostic value in early-stage CLL [7]. Several serologic markers such as thymidine-kinase and soluble CD23 provide valuable information about disease progression and survival [8]. Also, several biological markers can predict therapeutic outcomes and disease progression in CLL patients including cytogenetic abnormalities, IGHV mutational status,

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ZAP-70, and CD38 [9]. Recently, newer impressive, effective therapies have been developed, which hold the potential of increasing individualized treatments if patient risk could be accurately characterized by the emerging prognostic factors [10].

CD49d, an adhesion molecule belonging to the integrin family, has a critical role in leukocyte trafficking, activation, and survival. It mediates cell-to-extracellular matrix and cell-to-cell interactions, through binding with fibronectin or vascular cell adhesion molecule-1 (VCAM-1), respectively [11,12]. CD49d can also affect B-cell survival via the upregulation of Bcl-2 family members which was suggested to be related to the chemoresistant phenotype of CLL [13,14]. Some studies reported that CD49d expression on B-CLL cells is lower than in normal B cells, and is related to the presence of lymphadenopathy and disease stage as well as prognostic stratification of CLL [15–17].

CD44 as class I transmembrane glycoproteins and also as a major hyaluronan receptor mediates the response of cells to their cellular microenvironment [18]. CD44 is also being described as an adhesion molecule expressed in various cell types and it participates in a number of signaling pathways mediating proliferation, migration, hematopoiesis, lymphocyte activation, and lymph node homing [19]. In various tumors it has been described as a cancer stem cell marker and tumor marker [20,21]. Some studies suggested that CLL cells express high levels of CD44. CD44 is important for leukemic cell survival, proliferation, and for clinical aggressiveness of CLL [22]. Natural-ligand-based CD44 engagement on CLL cells seems protective toward spontaneous and therapeutic-induced apoptosis [23,24]. CD44 has little role in the prognosis of CLL.

Aim of the work

We aimed to evaluate the expression of membrane CD49d and CD44 on CLL leukemic cells by flow cytometry, to highlight the effect of these markers on response to fludarabine, cyclophosphamide, and rituximab (FCR) therapy in CLL patients, and to study the relation of each one of these to the other.

Patients and methods

Patients

The study was conducted on 30 newly diagnosed CLL patients attending the Clinical Hematology Department, Outpatient Clinic and South Egypt Cancer Institute, during the period from May 2016 to March 2018. The samples were collected according to the frequency of cases in the period between May 2016 and March 2017 and were followed up for 12 months to assess their response and the studied markers were remeasured after receiving the FCR regimen. Informed consent was obtained from every patient for laboratory studies according to the guidelines of the Committee of Medical Ethics of Assiut University Hospitals. This study was approved by the Committee on Human Research at Assiut University. Patients were selected on the basis of standard clinical, hematological, and immunophenotypic criteria. They included 23 men and seven women; their age ranged from 33 to 80 years with a mean of 60.73 ± 10.42 years and a median of 60 years.

Methods

All patients were subjected to history taking and clinical examination stressing on the presence of lymphadenopathy, splenomegaly, hepatomegaly, and symptoms and signs of anemia and/or thrombocytopenia. Imaging studies were performed for assessing organomegaly. Laboratory investigations included complete blood count using CELL DYN (1700), Leishman-stained peripheral blood (PB) smear examination [25,26], laying stress on lymphocyte percentage, and absolute lymphocyte count [27]. BM aspiration with examination of Leishman-stained smears was used for lymphocyte percentage and morphology [26,28]. The studied patients were classified according to the modified Rai staging into high risk, moderate risk, and low risk [29]. Clinical and hematological data were available for 30 patients at the Assiut University Hospital and South Egypt Cancer Institute.

Flow cytometry analysis

Immunophenotyping was performed on PB. Chronic lymphoproliferative panel was applied. It includes using CD5, CD10, CD19, CD4, CD8, CD3, CD16, CD56 as the primary panel and κ/λ/CD19/, FMC7/CD23/CD19, CD38/CD79b, sIgm/CD19, CD20/CD200 as the secondary panel [30,31] using BD FACSCalibur Flow Cytometer from Beckman (Indianapolis, Indiana 46268, United States) Coulter and Becton Dickenson. Additional studied adhesion molecule mAbs panels were CD44 (FITC) and CD49d (PE) together in one tube. The two were obtained from MACS Miltenyi Biotec (Bergisch Gladbach, Germany). Flow cytometric two-parameter dot plots and quadrant statistics were generated. In the first dot plot a region around the lymphocyte population on a forward scatter versus side scatter dot plot was created manually. Lymphocytes are gated for further analysis of the studied monoclonal antibodies. The second plot displayed the antibody

CD49d marker on the Y axis versus the other marker CD44 on the X axis [32,33]. A marker was considered positive at a cutoff of 20% except CD49d more than or equal to 30% [34].

Dot-plot analysis of a CLL case (Figs. 1 and 2).

Statistical analysis

Data were collected and analyzed using the Statistical Package for the Social Sciences (version 20; IBM, Armonk, New York, USA). Continuous data were expressed in the form of mean ± SD or Student's *t* test was used to compare the means of two different groups and analysis of variance test for more than two groups while nominal data were expressed in the form of frequency (%) and compared with the χ^2 test. Spearman's correlation was used to determine the correlation between CD38, CD44, and CD49d with each other and with different variables in the study. The *P* value was significant if less than 0.05.

Results

The study was performed on 30 CLL patients including 23 (76.7%) men and seven (23.3%) women; their age ranged from 33 to 80 years with a mean of 60.73 ± 10.42 years and a median of 60 years. Clinical and hematological parameters are presented in Tables 1 and 2. In our study, modified Rai staging was distributed as follows: high risk, 18 (60%) cases, intermediate risk, six (20%) cases, and low risk six (20%) cases. The diagnostic CDs (CD5, CD19, CD23, and CD200) have no significant difference in the studied different risk stages of CLL. The documented prognostic marker CD38 shows significantly higher levels in those with high-risk CLL in comparison to other groups (high vs. intermediate, $P = 0.02$; intermediate vs. low, $P = 0.02$; high vs. low, $P = 0.01$).

The mean expression of CD44- and CD49d-positive cells in the studied patients was 95.56 ± 6.68 and 71.20 ± 22.05, respectively. Also the expression of CD44 and CD49d in the studied patients according to the modified Rai staging system is presented in Table 3. The response of the studied patients to FCR chemotherapy is presented in Table 4. The characteristics of patients and expression of CDs (CD44, CD49d, CD38) before and after therapy were based on the type of response (Table 5). The correlation between white blood cells and BM lymphocytes with CD38, CD44, and C49d and other parameters is presented in Table 6.

In our study, there is a positive correlation between CD44 with CD49d ($r = 0.30$; $P = 0.02$), CD38

Table 1 Clinical data of the studied patients

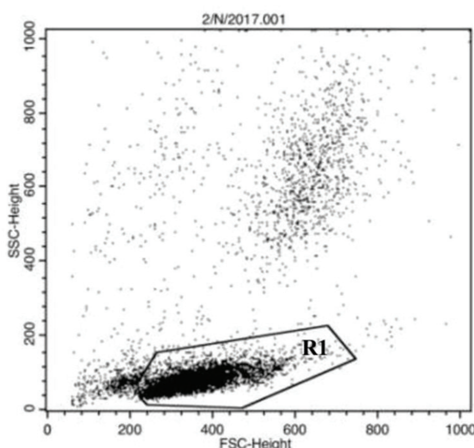
Variables	*n*=30
Lymphadenopathy	19 (63.3)
Hepatomegaly	7 (23.3)
Splenomegaly	14 (46.7)
Bleeding tendency	7 (23.3)
Fever	11 (36.7)
Fatigue	23 (76.7)

Data was expressed in the form of frequency (%).

Table 2 Hematological data of the studied patients

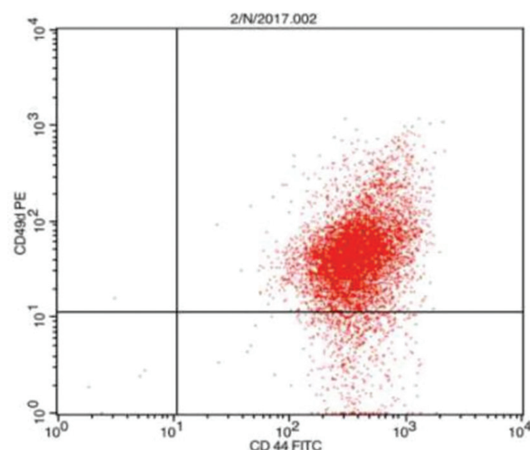
Parameters	Mean±SD
Hemoglobin (g/dl)	9.78±2.59
Platelets (×10³/μl)	108.1±31.51
White blood cells (×10³/μl)	140.35±15.08
Neutrophil (×10³/μl)	19.07±4.89
Lymphocytes (×10³/μl)	120.1±34.57
Monocytes (×10³/μl)	4.24±1.09
Eosinophil (×10³/μl)	2.17±0.65
Reticulocyte (%)	1.86±1.06

Figure 1



Dot plot of Forward scatter (FSC) versus Side scatter (SSC) showing a distinct population (R1) of mononuclear cells (lymphoid cells).

Figure 2



Dot plot of CD44 versus CD49d gated on R1.

with CD49d (r = 0.38; P = 0.01), and CD38 with CD44 (r = 0.34; P = 0.02). Also, the expression of CD49d is significantly higher in patients with lymphadenopathy with the mean of expression in relation to the presence and absence of lymphadenopathy being 82.89 ± 18.78 and 51.02 ± 10.56, respectively, with a P value of 0.02.

Discussion

Extreme clinical heterogeneity is one of the hallmark features of CLL. Progressive CLL is defined by the expansion of the neoplastic clone and extravascular accumulation in lymphoid tissues, BM, and other organs [35]. CLL is a malignancy of mature B cells that depend on host factors in the tissue microenvironment for survival and proliferation [36]. Adhesion molecules, particularly integrins, for example, VLA-4 (CD49d) play a key role in the adhesion of CLL cells to stromal cells and the extracellular matrix in the BM and lymph node microenvironment. Integrins along with other important adhesion molecules, for example, CD44 are contributing in shaping these microenvironmental interactions. These interactions affect drug resistance that may be responsible for residual disease after conventional therapy which also affect the prognosis [37].

Table 3 CD44 and CD49d in the studied patients based on the modified Rai staging

	High risk (H)	Intermediate risk (I)	Low risk (L)	P
CD44	96.38±6.59	95.37±9.98	98.27±2.45	0.76
CD49d	86.89±13.45	81.03±24.31	14.31±9.13	H vs. I=0.01 I vs. L=0.00 H vs. L=0.01

Data are expressed in the form of mean±SD. CD, cluster of differentiation.

Table 4 Response of the studied patients to fludarabine, cyclophosphamide, and rituximab chemotherapy

Type of responses	n=30
Patients with complete response	22 (73.3)
Patients without complete response	8 (26.7)
Stationary course	3 (10)
Progressive course	5 (16.7)

Data are expressed in the form of frequency (%).

We tried to evaluate the possible effect of CD49d and CD44 on response to FCR therapy in CLL patients and their correlation with each other or with other prognostic factors as the expression of CD38.

There was a higher expression of CD49d related to advanced disease stages (high risk and intermediate risk) as shown in Table 3 and also there was a significant correlation between the presence of lymphadenopathy and CD49d expression in our patients. Possibly this is due to the higher expression of CD49d-α subunit of the VLA-4 integrin receptor that could enhance its binding to adhesion molecules VCAM-1. This differential homing patterns could in turn allow CD49d-positive cells to easily populate proliferation centers located in lymph nodes and BM, with a distinct, protective, and prosurvival microenvironment. In addition, high levels of CD49d may be associated with increased numbers of VCAM-1 expressing microvessels within the lymph nodes [15,35,38–40]. In PB VLA-4 (CD49d) high-risk CLL cells increase proliferation rates upon activation of CD4+ T-cells through CD40L which, as well as being a costimulatory molecule, is required for CLL cells to undergo transendothelial migration and enter the proliferation centers of the lymph nodes [41].

There was significant higher CD49d expression in patients without complete response [Table 5]. This may be due to the signals via CD49d in CLL cells as it mediates both cell–cell and cell–matrix interactions,

Table 5 Characteristics of patients and expression of CDs (CD44, CD49d, CD38) before and after therapy based on the type of response

	With complete response	Without complete response[a]	P
Age (years)	58.13±9.33	60.12±10.53	0.09
Sex (male)	17 (77.3)	5 (62.5)	0.11
High-risk level	13 (59)	5 (62.5)	0.34
Baseline WBCs	113.33±19.1	105.99±25.01	0.52
Baseline peripheral lymphocytes	22.17±5.09	19.65±6.98	0.65
Baseline BM lymphocytes	5.67±1.45	6.47±1.03	0.66
CDs before therapy			
CD44	60.09±14.87	80.87±9.76	0.01
CD49d	15.11±5.07	66.70±1.42	0.01
CD38	30.11±9.45	41.11±8.67	0.04
CDs after therapy			
CD44	58.11±12.90	77.60±9.32	0.01
CD49d	14.11±2.33	59.11±11.45	0.03
CD38	29.12±10.45	40.09±9.65	0.03

Data were expressed in the form of mean±SD and frequency (%). CD, cluster of differentiation; BM, bone marrow; WBCs, white blood cells. [a]No complete response included those patients with stationary course and those with progressive course. P value was significant if <0.05.

Table 6 Correlation between white blood cells and bone marrow lymphocytes with CD38, CD44, and C49d and other parameters

	CD38		CD44		CD49d	
	r	*P*	*r*	*P*	*r*	*P*
Age	0.05	0.09	0.04	0.22	−0.11	0.08
ESR (1st h)	0.33	0.30	0.02	0.06	−0.45	0.09
ESR (2nd h)	0.05	0.07	0.11	0.45	0.21	0.41
Urea			No correlation			
Creatinine			No correlation			
Serum calcium			No correlation			
Prothrombin time			No correlation			
Prothrombin concentration			No correlation			
International normalized ratio			No correlation			
Bilirubin	0.23	0.06	0.34	0.05	−0.11	0.12
Direct bilirubin	0.22	0.11	0.21	0.33	0.24	0.09
Aspartate transaminase	0.43	0.34	−0.34	0.05	−0.43	0.11
Alanine transaminase	−0.23	0.65	0.03	0.09	0.11	0.48
Alkaline phosphatase	−0.11	0.31	−0.11	0.12	−0.10	0.23
Lactate dehydrogenase	0.21	0.51	−0.21	0.34	0.09	0.81
Hemoglobin	0.30	0.98	0.09	0.41	0.30	0.42
Platelets	0.01	0.65	0.12	0.51	0.03	0.51
Reticulocytes	−0.10	0.32	0.34	0.09	0.45	0.09
BP lymphocytes	−0.01	0.95	−0.14	0.06	0.11	0.54
Bone marrow lymphocytes	0.02	0.98	−0.13	0.46	0.04	0.81

r, coefficient of correlation. *P* indicates significance of correlation and considered significant if <0.05.

which facilitate the resistance to both spontaneous and drug-induced apoptosis [42,43]. In addition, the high CD49d expression associated with other high-risk aggressive biological markers, for example, CD38 or its presence in the macromolecular complex implies that leukemic clones may receive enhanced stromal support with the resultant effects on leukemic cell viability, migration, and apoptotic resistance [16,44,45].

In our study, the documented poor prognostic marker CD38 was significantly expressed in advanced modified Rai stages. It was suggested that CD38 expression and tumor proliferation in CLL are linked through interactions in the leukemic microenvironment involving T cells and the vascular endothelium that lead to enhanced leukemia-cell proliferation and/or survival [46–48].

Some studies suggested that the assessment of CD49d may be informative in early diagnosed patients in addition to the assessment of the usual prognostic marker, for example, CD38 because CD49d is characterized by a higher expression than CD38 as CD49d is involved in more microenvironmental interactions [49–51].

In the current study, CD44 expression had no significant difference between different stages of the disease but was high in all stages [Table 3]. High CD44 expression is most probably due to its high expression in cells of the hematopoietic system. Also, it was described as a lymphocyte homing receptor which was typically found in the microenvironment of CLL cells which express high levels of CD44. Some studies suggested that it is also expressed in cancer-initiating and leukemia-initiating cells [52–55].

Other studies suggested that CD44 has a multipotent role in tumorigenesis by binding to hyaluronan which abundantly accumulates into the stroma of various human tumors leading to the overexpression of CD44. CD44 was suggested to be an established cancer stem cell marker or tumor marker [20,56–58].

Also CD44 in our studied CLL patients had a significant higher expression in patients without complete response [Table 5]. Homing of leukemia stem cells in BM may be assisted by CD44 which is the primary cause for the relapse or resistance of leukemic cells after chemotherapy. Down-expression of CD44 could impair leukemia stem cells' self-renewability and promote its sensitivity to chemotherapy [59].

There was a significant correlation of CD44 with CD49d and CD44 with CD38. This correlation may be due to that the membrane of CLL cells has been shown to contain a macromolecular complex which is strongly involved in tissue migration and metastasis. This complex contains CD49d, CD44, and CD38 and is not present in normal B cells. All these molecules have physical relationship and overlapping functions [60,61]. There is a significant correlation between CD49d and the aggressive prognostic CLL factor CD38, such a relation may be due to some synergistic physical and functional interactions as the CD49d/CD29 complex is linked with CD38, through the production of specific soluble factors, in a circuitry regulating adhesion and survival of $CD49d^{+}$ $CD38^{+}$ CLL [62].

Actually, there is no significant change in the level of expression of the three studied CDs (CD44, CD49d, and CD38) before and after therapy, but all three CDs have a significant difference between patients with complete response and patients without complete response to therapy [Table 5]. This is possibly due to the fact that the FCR is a chemo-immuno-based therapy regimen as fludarabine is a Purine analog, cyclophosphamide is an alkylating agent [63], and rituximab is an anti-CD20 monoclonal antibody [64]. Although it has been proved that microenvironmental interactions have a key role in cellular proliferation, survival, and drug resistance which are controlled by various molecules like VLA-4 integrins family (CD49d), adhesion molecules like CD44, and ectoenzymes CD38 [37,61], this frequently used first-line therapy (FCR) does not include specific

mechanisms toward those very important studied molecules. So, if these studied molecules are not targeted in the used regimen of therapy, their pretreatment level at least could be informative about the response of FCR therapy in such newly diagnosed patients. All of this directs us toward new therapeutic approaches of CLL treatment and we suggest that targeting of such important microenvironmental molecules could be a potential therapeutic strategy.

This study has shown that the expression of CD49d and CD44 in newly diagnosed CLL patients is related to resistance to FCR therapy. Also, there was a positive correlation between the expression pattern of both CD49d and CD44. CD49d and CD44 have association with the poor prognostic marker CD38 which may have a role in tumor burden of CLL.

It would be promising to start clinical trials using specific monoclonal antibodies against CD49d and CD44 in CLL patients for a better response to therapy.

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Conflicts of interest
There are no conflicts of interest.

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