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Adhesion molecules expression in CLL: Potential impact on clinical and hematological parameters

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KEYWORDS

CLL;
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Abstract *Background:* B-cell chronic lymphocytic leukemia (CLL) is marked by the accumulation of CD5+ B lymphocytes within the blood, bone marrow (BM), and secondary lymphoid tissues. Abnormalities in the expression and function of cell adhesion molecules may account for the patterns of intra-nodal growth and hematogenous spread of the malignant cells. Chemokines and integrin-mediated adhesion and trans-endothelial migration (TEM) are central aspects in trafficking and retention of hematopoietic cells in the BM and lymphoid organs.

Aim of the work: This work was conducted to study adhesion molecules status in CLL and its potential impact on both hematological and clinical parameters.

Patients and methods: The study included 78 newly diagnosed CLL patients. Immunophenotyping was performed on peripheral blood using the chronic lymphoid panel. Adhesion molecules (CD11a, CD11b, CD49d, CD49C, CD29 and CD38) were tested using monoclonal antibodies and analyzed by Flow Cytometry.

Results: Positive correlation was encountered between adhesion molecules: CD38 with CD49d ($r = 0.25$, $p = 0.028$), CD11a with CD11b, CD49d and CD29 ($r = 0.394$, $p = 0.001$; $r = 0.441$, $p = <0.01$ and $r = 0.446$, $p < 0.01$ respectively) and CD29 with CD49c and CD49d ($r = 0.437$, $p < 0.01$; $r = 0.674$, $p < 0.01$ respectively).

CD49c showed negative correlation with Rai staging ($r = -0.269$, $p = 0.033$). CD11a and CD29 showed a significant relation with splenomegaly ($p = 0.04$ and 0.03 respectively) and CD49d showed a significant relation with lymphadenopathy ($p = 0.02$).

Conclusion: The level of different adhesion molecules expression in CLL is apparently reflected on the potential migratory behavior of the leukemic cells to different organs.

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Introduction

CLL is a malignant disease of B-cells with characteristic phenotype. In USA, it accounts for ~4.5%/100,000 person per year [1]. In Egypt, according to NCI hospital-based registry (2002–2010), CLL accounts for 0.5% of all cancers and 3.08% of lymphohemopoietic malignancies. Most cases do not entail treatment until evident progression of disease is encountered [2]. Mere staging by Binet [3] or Rai [4] is not satisfactory to expect which clinical course the patient will experience. Numerous, more recently suggested, markers apparently contribute to the cellular processes involved in CLL pathogenesis [5]. Chemokine and integrin-mediated adhesion and trans-endothelial migration (TEM) are central aspects in trafficking and retention of hematopoietic cells in the BM and lymphoid organs. To enter these organs, circulating cells need to arrest on specific endothelial barriers, to locomote over the endothelial surface toward inter-endothelial junctions, and to cross these junctions while resisting disruptive shear forces [6]. Both firm adhesion and ability to locomote and transmigrate across endothelial barriers depend on the ability of circulating cells to establish dynamic adhesive interactions through VLA-4 and LFA-1 integrins. During these interactions, the integrins undergo reversible activation by endothelial-presented chemokines [7]. From this prospect, many adhesion molecules have been linked to natural history of CLL, the pattern of organ and bone marrow invasion as well as various hematological and clinical parameters.

In this work, we have studied a number of adhesion molecules (CD11a, CD11b, CD49c, CD49d, CD29 and CD38) in a cohort of newly diagnosed CLL patients. We aimed to determine the level of expression of these markers in CLL patients and to verify their impact on clinical and hematological parameters.

Material and methods

Patients

The study was conducted on 78 newly diagnosed CLL patients attending at the National Cancer Institute, Cairo University, during the period from March 2013 to December 2014. Patients were selected on the basis of standard clinical, hematological and immunophenotypic criteria. They included 52 males and 26 females; their age ranged from 25 to 85 with a mean of 59.58 ± 10.98 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 (CBC) using Cell Dyn 37000, Leishman-stained peripheral blood (PB) smear examination, laying stress on lymphocyte percentage and absolute lymphocyte count. Bone marrow (BM) aspiration with examination of Leishman-stained smears for lymphocyte percentage and morphology. The work was conducted according to Helsinki declaration for studies involving human beings. It was approved by the IRB of the NCI, Cairo University and exempted from obtaining patient's consent. The study was performed at the Bone Marrow Transplantation Laboratory Unit (BMT Lab Unit), NCI, Cairo University.

Clinical data were available for 67 and hematological data for 69 patients.

Flow Cytometry analysis

Immunophenotyping was performed on peripheral blood. Chronic lymphoproliferative panel was applied. It includes CD5, CD19, CD20, CD22, CD23, CD79b, FMC7, CD10, CD11C, CD103, CD25, CD123, CD3, CD4, CD8, CD16, CD56, Cyclin D1, IgM, CD2, CD7 as well as κ and λ light chains; all were purchased from Beckman Coulter. Additional adhesion molecule panel was used including CD38 (T16) phycoerythrin (PE), CD11b (Bear 1) PE, CD49d (HP211) fluorescin isothiocyanate (FITC) and CD29 (K20) FITC were supplied from Beckman Coulter; CD11a (HI 111) FITC and CD49c (ASC-1) PE were obtained from Biolegend (San Diego, CA, USA). Whole blood staining method was applied as previously described. Twenty microliter labeled Mo Ab were dispensed into appropriately labeled tubes. One hundred-microliter sample was added and incubated in the dark for 20 min. Three and half milliliter lysing reagent was added to each tube, inverted once and kept for 5 min. Centrifugation at 1500*g* (3200 rpm) for 3 min was done, 3.5 ml PBS added, and centrifugation at 1500*g* (3200 rpm) for 3 min repeated. Samples were suspended in PBS, tubes vortexed and analyzed using a Coulter Epics XL Hilah Flow Cytometer.

The instrument set up was checked daily using QC check beads Flow Cytometry purchased from Beckman Coulter (Miami, USA). Forward scatter and side scatter measurements were made using linear amplifiers, while mean fluorescence measurements were made with logarithmic amplifiers. Flow cytometric two-parameter dot plots and quadrant statistics were generated. A region around the lymphocyte population on a forward scatter versus side scatter dot-plot was created manually. A second gate was subsequently put on the CD19 positive B-lymphocyte population.

A marker was considered positive at a cutoff of 20%.

Statistical analysis

Statistical package for Social Science (SPSS 15.0.1 for windows; SPSS Inc, Chicago, IL, 2001) was used for data management and analysis. Mean and Standard deviation described quantitative data with median and range when appropriate. Number and percentage described qualitative data. Chi-square and Fisher exact tested proportion independence. p value was set significant at 0.05 level. Correlation analysis (using Pearson's method) was used to assess the strength of association between two quantitative variables. The correlation coefficient denoted symbolically "r" defines the strength and direction of the linear relationship between two variables.

Results

The study was performed on 78 CLL patients including 52 (66.67%) males and 26 (33.33%) females; their age ranged

from 25 to 85 with a mean of 59.58 ± 10.98 and a median of 60 years. Clinical and hematological parameters are presented in (Tables 1 and 2). Using the scoring system, the majority of our cases had a score of 5 (50/78, 64.1%) and 4 (21/78, 26.9%); only 7/78 (9%) had a score of 3.

In our study, Rai staging was distributed as follows: 1 case (1.4%) stage 0, 14 (20.3%) stage I, 21(30.4%) stage II, 24 (34.8%) stage III, 9 (13%) stage IV.

Adhesion molecules expression

The frequency and percentage expression of the various adhesion molecules are presented in (Table 3).

The adhesion molecules showed marked variability in their expression, the most frequent was CD49d followed by CD29, CD38, both CD49c and CD11a and the least was CD11b expressed on two cases (2.56%) only. Also there was marked variability in percentage expression in positive cases from just achieving the cutoff up to expression on more than 90% of the cells.

Table 1 Clinical findings in 67 chronic lymphocytic leukemia (CLL) patients.

Parameter	CLL
Age: mean ± SD	59.58 ± 10.98
Median (range)	60 (25.00–85.00)
Gender: No (%)	
Male	52 (66.67%)
Female	26 (33.33%)
Hepatomegaly: No (%)	33/67 (49.30%)
Splenomegaly: No (%)	43/67 (64.20%)
Lymphadenopathy: No (%)	56/67 (83.60%)

Correlation between adhesion molecules with each other as well as with prognostic hematological parameters and Rai stage are presented in (Table 4).

In our study Rai staging showed a positive correlation with ALC ($r = 0.438$, $p = <0.01$) and a negative correlation with Hb level ($r = -0.766$, $p = <0.01$) and CD49c ($r = -0.269$, $p = 0.033$).

CD11a showed a positive correlation with CD11b ($r = 0.394$, $p = 0.001$), CD49d ($r = 0.441$, $p = <0.01$) and CD29 ($r = 0.446$, $p = <0.01$) (Fig. 1). CD29 showed a positive correlation with CD49d ($r = 0.674$, $P = <0.01$), CD49c ($r = 0.437$, $P = <0.01$) and CD11a ($r = 0.446$, $P = <0.01$) (Fig. 2). D38 showed a positive correlation with CD49d ($r = 0.250$, $P = 0.028$) (Fig. 3).

Splenomegaly showed a significant relation with CD11a ($P = 0.04$) and CD29 ($P = 0.03$). Lymphadenopathy showed a significant relation with CD49d ($P = 0.02$).

Discussion

Extreme clinical heterogeneity is one of the hallmark features of CLL. Despite the identification of genetic and phenotypic markers that correlate with prognosis, the biological basis of this clinical variability remains unclear. Progressive CLL is defined by the expansion of the neoplastic clone and extravascular accumulation in lymphoid tissues, bone marrow and other organs.

Infiltration at these sites gives rise to the characteristic clinical picture of immune dysfunction, lymphadenopathy, splenomegaly and hematopoietic failure; features that correlate with survival [3,4].

Adhesion molecules are a group of ligands and receptors involved in several biological processes, particularly cell migra-

Table 2 Hematological findings in 69 chronic lymphocytic leukemia patients.

Parameter	Mean ± SD Median (range)	Parameter	Mean ± SD Median (range)
Total leukocyte count × 10^9/L	113.57 ± 131.93 71.37 (4.90–567.00)	Absolute lymphocyte count × 10^9/L	103.17 ± 126.30 66.37(3.19–561.30)
Hemoglobin: gm/dL	10.65 ± 2.78 11.1 (4.20–15.80)	B.M cellularity: Normocellular	21/61 (34.40%)
Platelets × 10^9/L	188.54 ± 118.79 172.00 (17.00–846.00)	Hypocellular Hypercellular	2/61 (3.30%) 38/61 (62.30%)
Lymphocyte%	81.55 ± 15.89 88 (44–99)	Bone marrow lymphocyte%	71.00 ± 20.15 72.00 (18.00–98.00)

Table 3 Expression of adhesion molecules on 78 chronic lymphocytic leukemia patients.

Adhesion molecule	Frequency No (%)	Expression in positive cases		
		Mean ± SD	Median	Range
CD49d	27/78 (34.61%)	61.9 ± 23.41	63.90	22.50–94.70
CD49c	15/78 (19.23%)	62.43 ± 21.75	64.00	26.50–96.00
CD11a	14/78 (17.95%)	43.99 ± 17.72	36.50	20.40–74.40
CD11b	2/78 (2.56%)	37.90 and 71.00[a]	54.45	37.90–71.00
CD29	23/78 (29.49%)	59.41 ± 19.56	59.00	23.40–89.30
CD 38	19/78 (24.36%)	56.62 ± 18.70	53.50	32.00–82.00

[a] Actual values for the 2 cases.

Table 4 Correlation matrix for different adhesion molecules in 78 chronic lymphocytic leukemia patients.

Parameter		Hemoglobin	Platelets	ALC	Rai stage	CD49d	CD49c	CD11a	CD11b	CD29
Platelets[a]	*r*	0.053								
	p	0.665								
ALC[a]	*r*	**−0.632**	−0.175							
	p	**0.000**	0.150							
Rai stage[a]	*r*	**−0.766**	−0.198	**0.438**						
	p	**0.000**	0.103	**0.000**						
CD49d	*r*	0.005	−0.107	−0.166	0.006					
	p	0.965	0.382	0.173	0.964					
CD49c	*r*	0.203	−0.003	−0.202	**−0.269**	0.167				
	p	0.111	0.984	0.111	**0.033**	0.165				
CD11a	*r*	−0.130	−0.050	−0.064	0.137	**0.441**	0.101			
	p	0.305	0.696	0.616	0.279	**0.000**	0.407			
CD11b	*r*	0.079	0.010	0.106	−0.093	0.107	0.138	**0.394**		
	p	0.528	0.939	0.398	0.455	0.363	0.251	**0.001**		
CD29	*r*	−0.002	0.009	−0.178	0.009	**0.674**	**0.437**	**0.446**	0.170	
	p	0.987	0.944	0.143	0.941	**0.000**	**0.000**	**0.000**	0.144	
CD38	*r*	0.081	0.079	−0.064	−0.097	**0.250**	0.085	−0.033	−0.024	0.213
	p	0.506	0.517	0.602	0.430	**0.028**	0.479	0.784	0.841	0.061

ALC: absolute lymphocytic count, bold font denotes significance.
[a] For platelets, ALC and Rai staging, correlations were done for 69 cases only.

tion [8]. Given that some B-NHL subtypes are considered to be the malignant counterparts of distinctive steps in normal B-cell development, it is reasonable to infer that the malignant lymphoid cells use the same mechanisms of lymphocyte migration as normal B cells to disseminate from their primary sites. In fact, a number of in vitro [9] and preclinical experiments [10], as well as clinical observations [11] give support to this hypothesis. Matos et al. [12] studied the expression of 10 adhesion molecules in tumor cells from the peripheral blood of patients with leukemic B-NHL (chronic lymphocytic leukemia, mantle-cell lymphoma, nodal or splenic marginal B-cell lymphoma) to determine how the down-regulation of specific adhesion markers inherently associated with malignant transformation contributes to the biogenesis of the leukemic phase observed in some B-NHL subtypes.

In our study Rai staging showed a positive correlation with ALC and a negative correlation with Hb level; this is expected as ALC and Hb level contribute to Rai staging. A negative correlation was also encountered with CD49c; this negative correlation is in harmony with the previously addressed good prognostic value of CD49c [13].

In our series, CD29 showed a positive correlation with CD49d and CD49c; this is because CD29 is the common β1 chain of α subunits of integrins α3/CD49c and α4/CD49d [8,12].

A positive correlation was also encountered between CD11a with CD11b, CD49d and CD29. This positive correlation might reflect the contribution of more than one adhesion molecule to the trafficking and infiltrating pattern of the malignant cells. It has to be noted that only two cases were

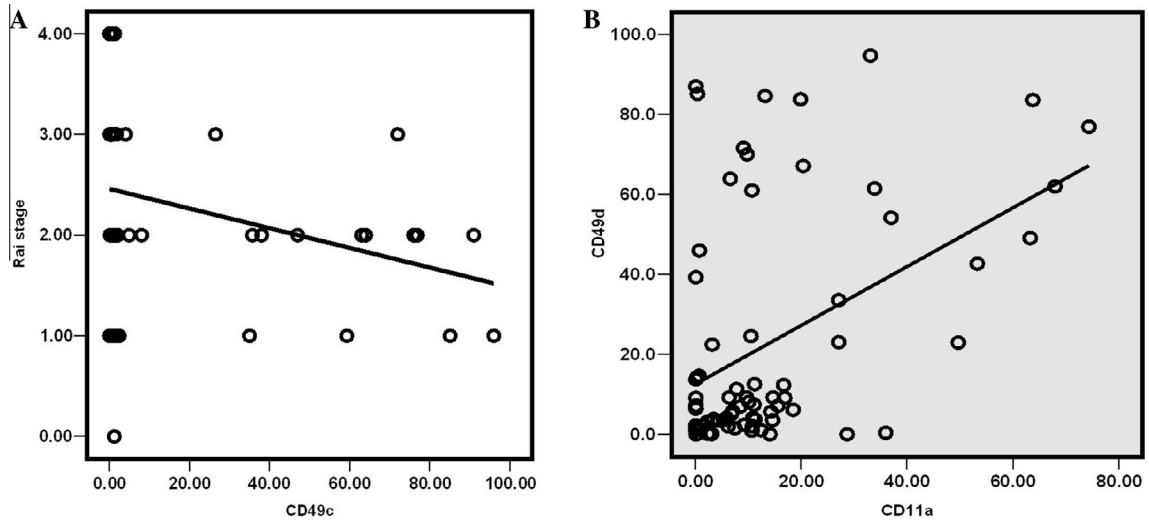

Figure 1 (A) Correlation between CD49c and Rai staging in 67. (B) Correlation between CD49d and CD11a in 78 chronic lymphocytic leukemia patients.

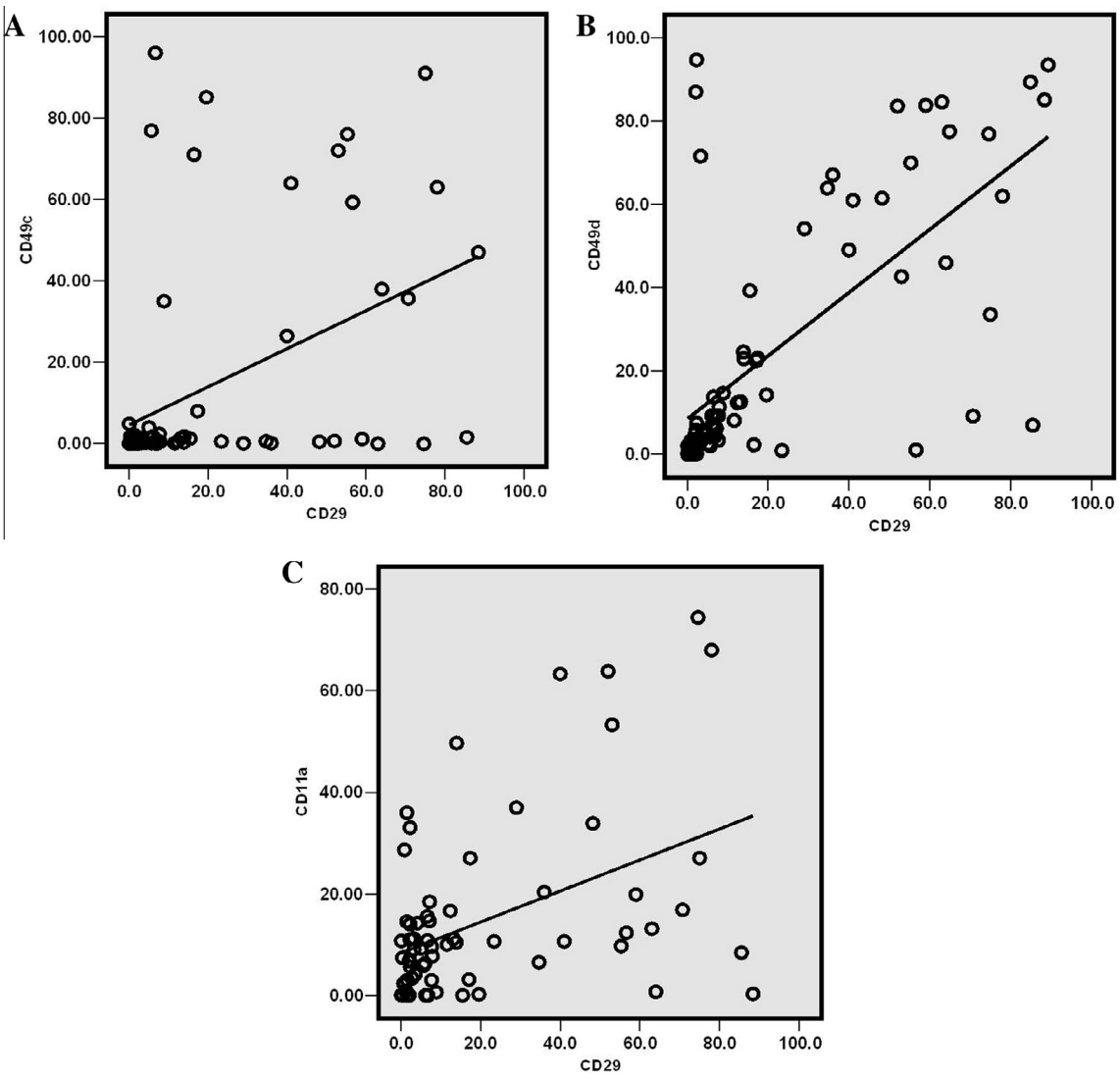

Figure 2 CD29 correlations in 78 chronic lymphocytic leukemia patients (A) with CD49c, (B) with CD49d, (C) with CD11a.

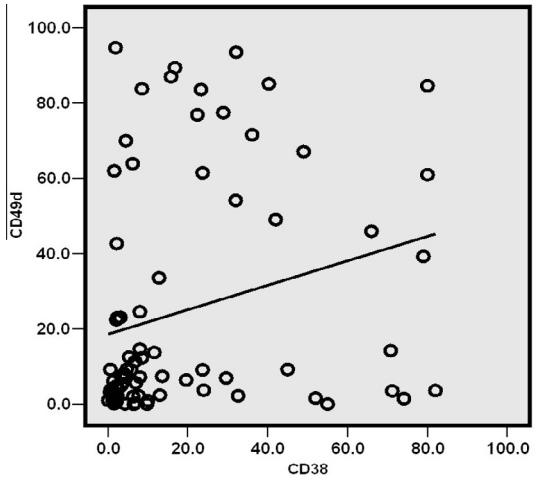

Figure 3 CD49d correlations with CD38 in 78 chronic lymphocytic leukemia patients.

considered positive for CD11B expression ($\geqslant$20%). However the direct correlation involves the true% expression regardless if the case is considered positive ($\geqslant$20%) or not and it could still be meaningful. CD11a and CD49d were both reported to have lower expression in chronic lymphocytic leukemia [12] suggesting that these adhesion molecules are probably responsible for the different compartments the disease infiltrates compared to its related entity, the small lymphocytic lymphoma. It was suggested that the higher frequency of blood invasion by tumor cells in CLL, when compared with the other B-NHL, could be explained by specific profiles of adhesion molecule expression. Since the structure of normal lymphoid follicles in lymph nodes depends on the appropriate association between B lymphocytes and dendritic follicular cells through the interaction of CD11a and ICAM-1 (an intercellular adhesion molecule), and of CD49d and VCAM1 (a vascular cell adhesion molecule) respectively [14], the lower expression of CD11a and CD49d on tumor cells of CLL could facilitate their detachment from the lymph node, while the expression of these adhesion molecules is preserved in small

lymphocytic lymphoma [15]. In fact, the down-regulation of CD11a and CD49d in chronic lymphocytic leukemia could also explain the peripheral blood invasion by these malignant cells, as compared with the occasional infiltration observed in mantle-cell lymphoma and marginal B-cell lymphoma [12]. It is worth mentioning that we observed a trend for higher CD49d values in MCL than in CLL ($P = 0.06$) [unpublished data].

In the current study, splenomegaly showed a significant relation with CD11a and CD29. Hartmann et al. [16] found that CLL samples expressed markedly reduced LFA-1 levels and failed to home to LNs but exhibited spleen tropism. Lymphocyte spleen homing does not necessarily require integrin activation [17], also they observed that CLL cases with high LFA-1 expression and trans-migratory capacity can enter LNs at low numbers.

In this study, we found significant relation between lymphadenopathy and CD49d. This is consistent with other studies that linked CD49d to the presence of bulky lymphadenopathy and/or adverse outcome in CLL [18,19].

In addition to their role in tissue invasion, interactions between VLA4 and its ligand VCAM1 have been shown to promote the viability of CLL cells and high levels of CD49d were associated with increased numbers of VCAM1 expressing micro-vessels within lymph nodes [20].

The combination of a strong expression of CD49c and a weak expression of CD49d is the hallmark of chronic lymphocytic leukemia and can be used to differentiate this disease from other B-NHL in leukemic phase. It has been shown that imbalance in CD49c/CD49d expression in chronic lymphocytic leukemia contributes to the peripheral blood invasion [21]. Moreover, other studies have demonstrated prognostic relevance of the combination of high CD49c and low CD49d expression in chronic lymphocytic leukemia [22].

Our results revealed a positive correlation between CD38 and CD49d; this is in agreement with other studies [16,23,24]. Zucchetto et al. [24] observed statistically significant correlation between CD49d and CD38 ($r = 0.59$, $p = \leqslant 0.001$); lower but still significant associations were found between CD49d expression and Rai staging.

Hartmann et al. [16] observed a highly significant higher VLA-4 expression in CD38 high-risk groups compared with CD38 low risk groups with nearly total loss of VLA-4 in the low-risk group. The low expression of both VLA-4 and LFA-1 in low-risk CLL groups might explain the more favorable clinical course of these patients because it obviously restricts circulating CLL cells from re-entering the BM and from homing to the LNs and other organs where these cells are likely to encounter anti-apoptotic factors. Simultaneous over expression of CD38 and CD49d was claimed to be a part of the signature characterizing the CLL subgroup with the worst outcome [22,24].

The migration of lymphocytes into tissues is initiated by chemokine-induced activation of integrins, which increases their affinity for endothelial ligands such as intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) [25]. This results in firm adhesion and migration though the endothelial barrier. In some cells integrins are arranged in supra-molecular complexes termed podosomes [26] that are linked to the cytoskeleton and contain other proteins involved in tissue invasion such as matrix metalloproteinases. Other studies suggested that CLL cells, but not normal B cells, possess podosomes containing a4b1 integrin (VLA4) [27].

These observations are paralleled by the work of Patten group [28] showing that CD38-positive cases of CLL have increased numbers of micro-vessels in lymph nodes and Buggins et al. study [29] in which interactions with vascular endothelial cells were shown to inhibit CLL cell apoptosis through nuclear factor NFKB-mediated regulation of Bcl-2 family proteins.

Comparing the integrin expression on CD38 high and low CLL cells, Pittner et al. [30] found an increased VLA-4 (CD49d subunit) and LFA-1 (CD18 subunit) expression in the CD38 high subtype, making it difficult to ascribe enhanced migration functions solely to CD38.

A number of molecules involved in migration through endothelium and tissue invasion have also been correlated with disease progression in CLL including CCR7, CD49d and matrix metalloproteinase 9 (MMP-9) [19]. In addition, the membrane of CLL cells has been shown to contain a supramolecular complex involved in tissue migration and metastasis.

A possible molecular basis for the high correlation of CD49d and CD38 expression in CLL could be their physical association in multi-protein-complexes. Buggins et al. [23] reported a multimer-complex involving CD38, CD49d, MMP9, and CD44 and observed a co-immunoprecipitation of CD38 with CD49d in the majority.

CD38/CD49d/MMP9/CD44 complex plays a role in migration into the tissues and in pro-survival signaling. Accumulation of leukemic cells in the tissues is responsible for many of the clinical features of CLL and defines a more advanced and progressive disease with an adverse outcome [3,4]. Tissue invasion also exposes the tumor cells to other components of the leukemic microenvironment such as T cells, nurse-like cells and other stromal elements that promote expansion of the leukemic clone [28,29].

The identification of the CD38/CD49d complex thus suggests a biological basis for the correlation between the expression of these molecules and prognosis. As the structure is not present in normal B cells, it also presents a novel leukemia-specific target for the therapy of this common disorder.

These structures contain CD49d, CD44 and MMP-9 and are not present in normal B cells [27]. Given the clinical correlation noted between CD38 and CD49d expressions in CLL [19,30] and the known involvement of both in tissue migration, Buggins et al. [23] hypothesized that CD38 might be physically associated with CD49d as part of this supramolecular complex in CLL cells.

In conclusion our results demonstrated the extreme variability in the level of expression of various adhesion molecules on CLL cells. The expression level of the adhesion molecules (CD38, CD11a, CD11b, CD49C and CD49d) might be reflected on the pattern of trafficking and infiltration by the malignant cells. Furthermore some may be potential therapeutic targets.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

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