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Original Article

Association of CD49d expression with clinicopathological features of chronic lymphocytic leukemia patients in the Iranian population

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Abstract: Background: Identification of factors affecting prognosis in chronic lymphocytic leukemia (CLL) is important for risk stratification of patients. Methods: In the present study, CD49d expression was analyzed by multicolor flow cytometry in 98 newly diagnosed and untreated CLL patients at the hematopathology ward. The patients were divided into two subgroups according to CD49d expression (30% cut off) and the association of this marker with the patients' clinicopathological properties were evaluated. Results: In this study, CD49d expression exhibited significant association with the Rai stage of the disease (P<0.0001), CD38 status (P<0.0001), hemoglobin level (P=0.0006), and platelet count (P=0.0016). The CD49d-positive patients presented in higher stages in comparison with CD49d-negative patients. Although only 1% of the CD49d-negative patients were CD38-positive, this proportion for CD49d-positive group was 69%. However, no significant correlation was observed between CD49d expression and patients' age (P=0.2031), gender (P=0.8119), and absolute lymphocytes count (P=0.1073). Conclusion: Therefore, CD49d is a grateful biomarker with high association with clinicopathological parameters in CLL patients.

Keywords: CD49d, chronic lymphocytic leukemia, CD38, clinicopathological parameters

Introduction

Chronic lymphocytic leukemia (CLL) is a markedly heterogeneous disease and prediction of the patients' disease behavior and prognosis is extremely hard, even at the same clinical risk category [1-3]. Although, many different genetic and phenotypic biomarkers have been identified to have association with the CLL patients' prognosis, the biological basis and accuracy of them remains unclear. Therefore, identification of prognostic biomarkers with high efficacy are necessary and can significantly improve the treatment planning, therapeutic efficacy and outcome.

Progressive CLL is defined by expansion of the neoplastic clone. The CLL cells will proliferate and accumulate at the lymphoid tissues, the bone marrow and other organs. This phenomenon causes immune dysfunction, lymphadenopathy, splenomegaly and hematopoietic failure [4]. Therefore, CLL cells infiltration can determine the clinical picture and survival of the patients [5]. Recent studies have reported that this highly variable clinical course in CLL can be predicted by new biomarkers, including the mutational status of immunoglobulin heavy chain variable (IGHV) gene [6, 7], expression of the surface marker CD38 [8, 9], the intracytoplasmic protein ZAP-70 [10] and CD49d [11].

CD49d is a 150 kDa protein that belongs to the integrin family, specifically the 4-integrin chain [12]. It is directly involved in mononuclear leukocyte trafficking by acting as an adhesion molecule for cell-to-cell and cell-to-extracellular-matrix interactions. CD49d has been found on the surface of CLL cells. Recent studies have shown that low levels of CD49d expression are associated with a favorable outcome for CLL patients [13]. Also, this biomarker has emerged as a negative prognosticator in CLL patients which is positive in approximately 40% of the

CLL patients with a more aggressive clinical course [14].

According to the best of our knowledge, CD49d has exhibited significant association with CLL patients' clinicopathological findings and overall survival, however, our knowledge about its prognostic efficacy for the Iranian CLL patients is extremely limited. Therefore, we planned to investigate the association of this biomarker with clinicopathological properties of the CLL patients in Iranian population.

Materials and methods

Patients

This study was reviewed and approved by Institutional Review and Ethical Committee. In this cross-sectional study, 98 CLL patients were included. All the patients were diagnosed at the Hematopathology ward in Seyed Alshohada hospital affiliated to Isfahan University of Medical Science, Iran, during 2016 to 2018. Informed consent was obtained in accordance with the Declaration of Helsinki. The patients involved in the study according to the defined inclusion criteria including: (1) Characteristic immunophenotyping of CLL by multicolor flow cytometry, (2) Diagnosis made during last 12 months, (3) Untreated disease, according to the current criteria [13]. 4 mL peripheral blood [15] sample was obtained for immunophenotypic analysis.

Immunophenotyping analysis

In this study, FACSCalibur flow cytometer (Partec space, Germany) was employed for immunophenotyping analysis of the PB samples. Whole blood was used to FACS scan. The patients' blood samples were collected in the EDTA tubes. The samples were adjusted to approximately 500,000 cells/100 µL with PBS. We used three-color flow cytometry analyses in three tubes 1-anti-CD19 (PerCP)/anti-CD5 (FITC)/anti-CD49d (PE) 2-anti-CD20 (PE)/anti-CD38 (FITC)/anti-CD5 (PerCP) 3-anti-FMC7 (FITC)/anti-CD23 (PE)/anti-CD19 (PreCP). All antibodies and other solutions were purchased from Exbio, CZ/SK company, Germany. Recommended amount of samples and antibodies were incubated for 10 min at room temperature and darkness. Then, red blood cells lysis buffer was added to the tubes and incubated for 10 min at the room temperature and darkness. The samples were analyzed by flow cytometry after 2 times centrifuging and PBS washing. At least 30,000 events were acquired and analyzed. Small and large lymphoid cells was gated based on forward and side scatter grams which were shown in the first dot plots of each figures and expression of markers were evaluated. CD20/CD5+ populations were selected for analysis of CD38 (**Figure 1**) and CD19/CD5+ populations also were selected for analysis of CD49d (**Figure 2**), CLL phenotype was confirmed as B cells with expression of CD23 and negative for FMC7 (**Figure 3**). First of all again remind that patients with lymphocytosis as CLL phenotype (B cells with weak expression of CD19 and CD20 and aberrant expression of CD5, which are positive for CD23 and negative for FMC7 include this study). The 30% was purposed as the cut off for discrimination of CD49d-positive and CD49d-negative patients.

Statistical analysis

All statistical analyses were performed using JMP software version 11.0. Fisher's exact test was used to analyze the association between CD49d expression and other variables. A p-value <0.05 was considered significant.

Results

The clinicopathological properties of the studied patients

98 CLL patients were involved in the present study. Median age of the patients was 68 years old at the time of diagnosis, between 41 to 90 years. Most of the patients were male (75 males, 23 females). The distribution of clinical stages according to the Rai staging system was as follows: 33 patients stage 0, 16 patients stage I, 7 patients stage II, 17 patients stage III, 25 patients stage IV. Also, 63% of the patients were CD38-negative. All the clinicopathological properties of the CLL patients are illustrated in **Table 1**.

Association of CD49d expression with clinicopathological parameters in CLL patients

The patients with CLL phenotype CD20/CD5+ CD19/CD5+ which confirmed by expression of CD23 and negative for FMC7 were selected

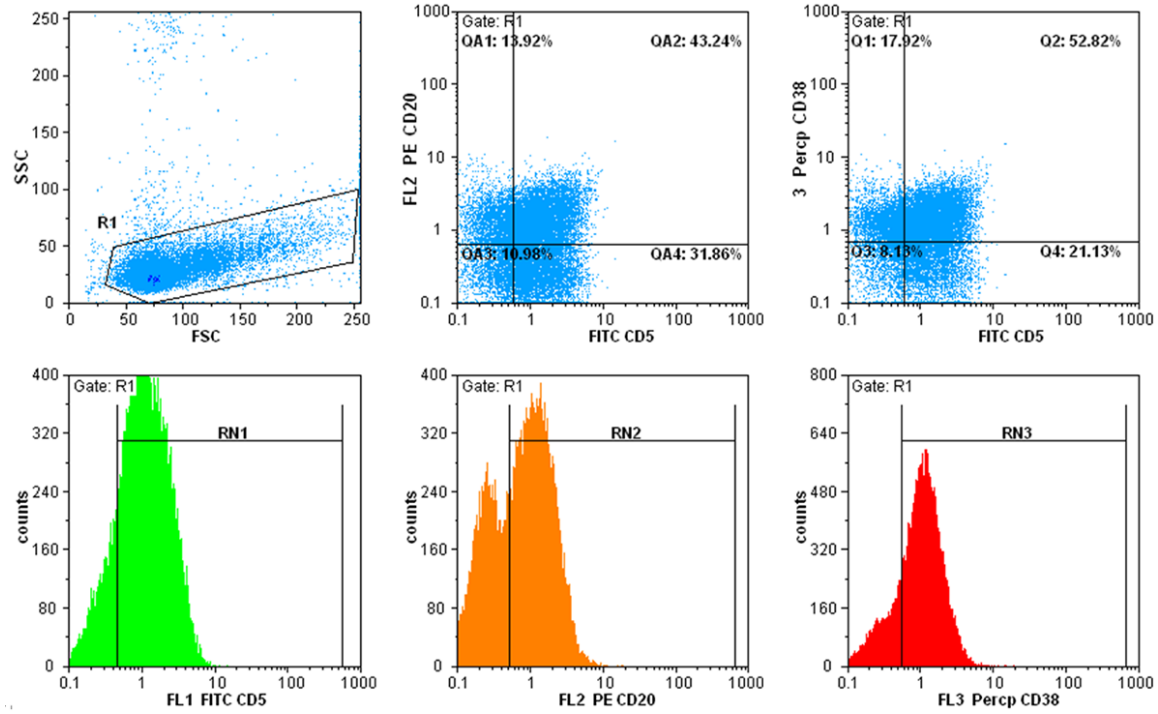

Figure 1. Dot plot and histogram charts of multicolor flow cytometry, anti-CD20 (PE)/anti-CD5 (FITC)/anti-CD38 (PerCP). On the CD20/CD5+ CLL population, note that CD38+ population above the gating threshol.

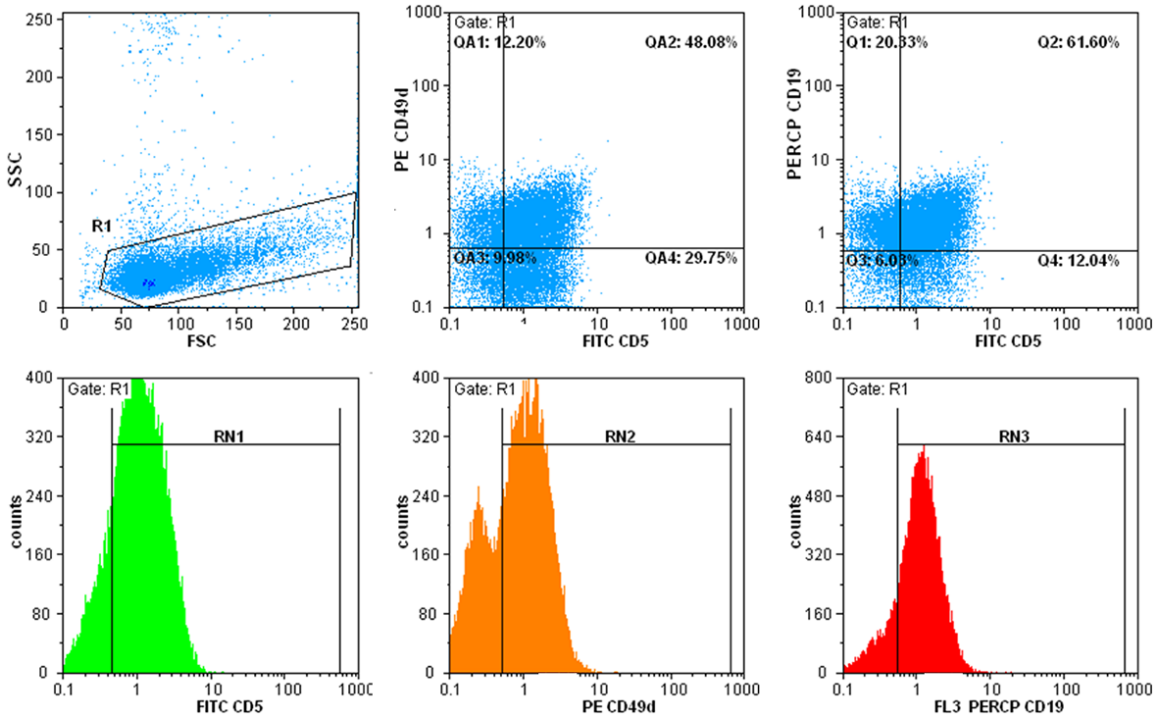

Figure 2. Dot plot and histogram charts of multicolor flow cytometry, anti-CD19 (PerCP)/anti-CD5 (FITC)/anti-CD49d (PE). On the CD19/CD5+ CLL population, note that CD49d+ population above the gating threshold.

and analyzed for expression of CD38 and CD49d (**Figures 1**-**3**). The patients were divided into two groups according to CD49d expression (**Table 2**). 51 patients (52%) were positive for

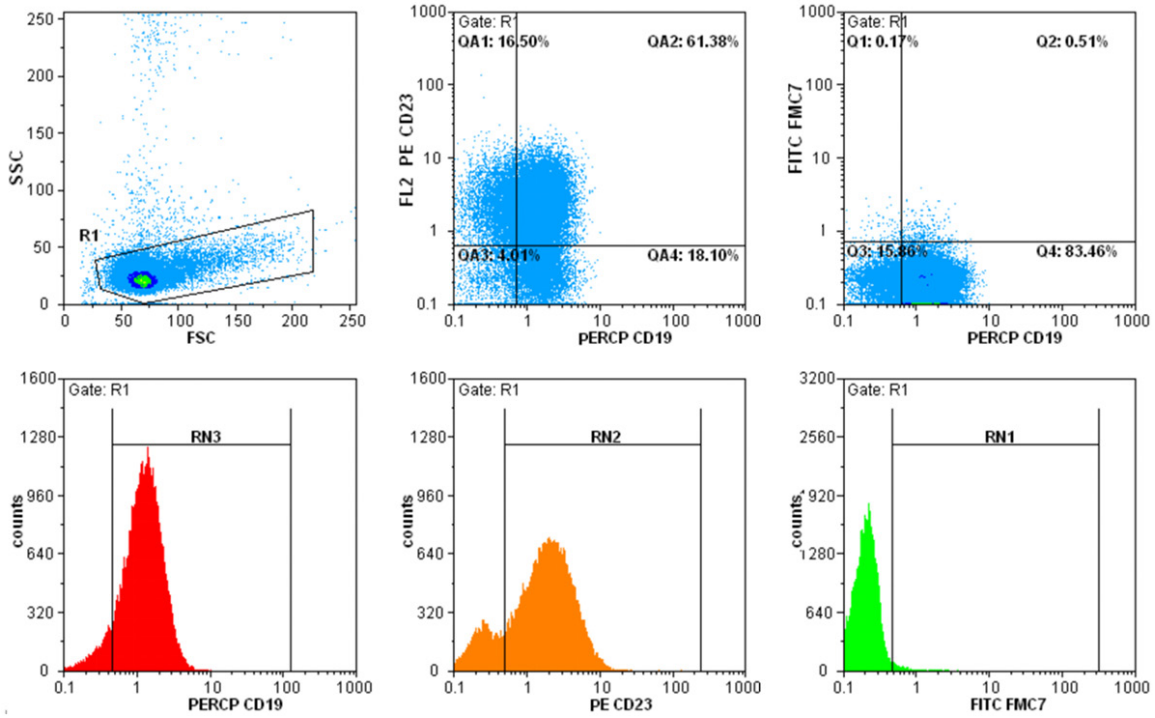

Figure 3. Dot plot and histogram charts of multicolor flow cytometry, anti-FMC7 (FITC)/anti-CD23 (PE)/anti-CD19 (PreCP). Lymphoid cells with CLL phenotype express CD23 and negative for FMC7.

Table 1. Clinicopathological features of the CLL patients

Clinicopathological parameters	Patient number (n=98)	Proportion (%)
Age		
Median	68	-
Mean	65	-
*ALC (× 10^9/L)	27.22 [5.5-168]	-
Hemoglobin (g/L)	120 [32-187]	-
Platelet count (× 10^9/L)	145.5 [20-293]	-
Gender		
Male	75	77%
Female	23	23%
Rai stage		
0	33	34%
I	16	16%
II	7	7%
III	17	17%
IV	25	26%
CD38 status		
CD38-positive	36	37%
CD38-negative	62	63%

CLL: Chronic lymphoid leukemia, *Absolute lymphocyte count: ALC.

CD49d (≥30% expression by multicolor flow cytometry), 47 patients express CD49d <30% and placed in the CD49d-negative group. The relation between CD49d expression and clinicopathological parameters was investigated. The median age of positive CD49d group was 63 and negative CD49d group was 67 which shows no significant association of age and CD49d expression (*p*-value: 0.2031). The ALC of negative and positive CD49d groups were 42.2/Microliter and 59.1/Microliter respectively with no significant statistical difference (*p*-value: 0.1073). The mean Hb level of negative CD49d group was 129 g/L while in positive CD49d group was 107 g/L which difference is significant (*p*-value: 0.0006). The platelets (plt) count of negative and positive CD49d groups were 167,000/Microliter and 126,000/Microliter respectively which difference statistically is significant (*p*-value: 0.0016). Expression of CD49d shows significant correlation with CD38 status (only 1% of the CD49d-negative patients were CD38-positive, this proportion for CD49d-positive group was 69%) and the Rai stage of the patients (The CD49d-positive patients had higher stages in comparison with CD49d-ne-

Table 2. Association of CD49d expression and prognostic parameters of the CLL patients

Clinicopathological parameters	CD49d-negative (n=47)	CD49d-positive (n=51)	*P*-value
Age	67	63	0.2031
ALC* (× 10^9/L)	42.2	59.1	0.1073
Hemoglobin (g/L)	129	107	0.0006
Platelet count (× 10^9/L)	167	126	0.0016
Gender			
Male	35 (74%)	40 (78%)	0.8119
Female	12 (26%)	11 (22%)	
Rai stage			
0	27 (57%)	6 (12%)	<0.0001
I-II	14 (30%)	10 (20%)	
III-IV	6 (13%)	35 (68%)	
CD38 status			
CD38-negative	46 (98%)	16 (31%)	<0.0001
CD38-positive	1 (2%)	35 (69%)	

CLL: Chronic lymphoid leukemia, *Absolute lymphocyte count: ALC.

gative patients), there is no correlation between CD49d expression and gender (*p*-value: 0.8119).

Discussion

Each cluster designation [16] antigen can play a role in the CLL cells behavior. As the tissue infiltration is the main determinative factor for CLL progression, many of the proteins which are involved in the vascular endothelium and cancer cells interaction, extravasation, and migration of CLL cells can be potential prognostic biomarkers [17]. CD38 is one of the most well-known prognostic markers which shows high association with CLL cells migratory potential [18]. Several other molecules which are involved in migration through endothelium and tissue invasion have also been correlated with disease progression in CLL including C-C chemokine receptor type 7 (CCR7), matrix metalloproteinase 9 (MMP-9) [19], and CD49d. In addition, the membrane of CLL cells has been shown to contain a supramolecular complex involved in tissue migration and metastasis. These structures which do not present in normal B cells contain CD49d, CD44, and MMP-9 [20]. In addition to their role in tissue invasion, these structures have also been shown to promote the viability of CLL cells [21]. The previous studies have demonstrated role of CD49d in the adherence of CLL cells to fibronectin and induces a resistance phenotype. Given the known association between CD49d and trans-endothelial migration [22], they examined the relationship between CD49d and the chemokine receptor CXCR4. They demonstrated a strong association between CD49d and CXCR4, suggesting that CD49d and CXCR4 may be functionally linked or up regulated in a coordinated fashion. The CXCR4 chemokine receptor is expressed in several human cancers, including hematological malignancies as well as breast and lung cancer [23, 24]. CLL cells express high levels of CXCR4, which may have a role in the migratory potential of neoplastic B-cells between the blood and lymph node or other supportive microenvironments, thus enhance survival of these cells [11, 25].

Based on previous studies, CD49d is an independent predictor of overall survival, along with age, IGHV mutation status, del 17p, sex, and ALC. Moreover, as demonstrated by bivariate analyses, CD49d expression reliably identified subsets of patients with poorer outcome independent of CD38 or ZAP-70 status [26-28]. According to these facts, CD49d can be a good potential prognostic biomarker for CLL patients. Although this fact has been demonstrated by different studies, based on our knowledge no study has reported the correlation of this biomarker with Iranian CLL patients' clinicopathological properties. Therefore, we tried to evaluate expression of CD49d on 98 newly diagnosed and untreated CLL patients and correlate it with demographic data, laboratory findings and stage of disease at presentation. 51 patients were CD49d-positive and exhibited high level of CD49d expression. Also, 47 patients had CD49d expression lower than 30% and placed in the CD49d-negative group. CD49d expression shows significant correlation with the Rai stage of the patients as CD49d-positive patients had higher stages in comparison with CD49d-negative patients. In addition, the CD38

expression was significantly higher in CD49d-positive groups in comparison with CD49d-negative patients (P<0.0001). No significant correlation was observed between CD49d expression and patients' age, ALC, and gender. However, CD49d-positive patients exhibited significantly lower levels of hemoglobin and platelet count in comparison with CD49d-negative patients which both can identify the negative prognostic features of the CD49d expression. Therefore, CD49d is a grateful biomarker with high association with clinicopathological parameters of CLL patients in Iranian population.

Conclusion

Nowadays some molecular and genetic analysis are available in developed countries such as mutation analysis of IGHV genes which have prognostic significance in CLL patients. Molecular tests are expensive and are not available in most centers of developing countries so another tests such as evaluation of cytoplasmic or surface cellular markers are cost benefit and may be prognostic importance. However, there is limited information about the association of CD49d expression and CLL patients' clinicopathological features, especially in the Iranian population. Previous studies described CD49d to play a permissive role in the cancerous cells' development and growth. Therefore, this surface marker has gained many attentions as a potential prognostic biomarker for CLL. In this study, analysis of CD49d expression exhibited its significant correlation with Rai stage of the patients, CD38 status, hemoglobin, and platelet count. Therefore, CD49d is a grateful biomarker with high association with clinicopathological parameters of CLL patients.

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Disclosure of conflict of interest

None.

Abbreviations

CLL, Chronic lymphocytic leukemia; PB, Peripheral blood; ALC, Absolute lymphocytes count; CD, Cluster designation; Plt, platelet; Hb, Hemoglobin; PBS, Phosphate buffered saline; IGHV, Immunoglobulin heavy chain V.

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