Correlation study between CD49d expression and disease stages in patients with Chronic Lymphocytic Leukemia

Samaher Abbas Marhoon*, Alaa Salah Jumaah**, Ebtihal Chaid Abbas***, Raheem Mahdi Raheem****

*** College of Medicine, AL-Qadisiyah University.**
**** Department of Pathology/ College of Medicine, Kufa University.**
*****Department of Pathology/ College of Medicine, Kufa University.**

Abstract

BACKGROUND: Chronic lymphocytic leukemia a proliferative clinical heterogenic disease had shown a variable clinical course, so it is important to categorize the heterogeneity by development of clinical, genetic and biological parameters and to evaluate the prognosis of the patients with CLL.

OBJECTIVE: To evaluate the expression of CD49d in CLL patients, correlates them with Modified Rai staging system and find the significance of CD49d immunohistochemical expression in bone marrow biopsy as a prognostic marker.

PATIENTS AND METHODS: This is an analytic study conducted on sixty four formalin fixed paraffin embedded blocks of chronic lymphocytic leukemia cases collected from teaching laboratories of medical city in Baghdad, during November 2018 to June 2019. Bone marrow biopsy sections have been regularly processed and examined by a light microscope using immunohistochemical stain for CD49d, by using EnVision+ systems method.

RESULTS: Lymphadenopathy and splenomegaly were the main clinical findings, followed by hepatomegaly and pallor. Leukocytosis, absolute lymphocytosis and anemia, were primary laboratory results. Most of cases were within the intermediate risk group (51.6%) by applying modified Rai staging system. CD49d expression was detected in 43.75% our study has been revealed that there was no statistical association between CD49d expression and the clinical stage of the disease using Modified Rai system (P= 0.072). Furthermore, there was no statistical association between CD49d expression and clinical findings, and hematological parameter including Hb, PCV%, platelets, total white blood cell count and lymphocyte % in peripheral blood, while there was statistical association between CD49d expression and lymphocyte % in bone marrow (p=0.014). Also there was no statistical association between CD49d expression and bone marrow histological lymphocytes involvement.

CONCLUSION: CD49d expression on chronic lymphocytic leukemia B-cells was identifying in less than half of patients. More than half of chronic lymphocytic leukemic patients according to modified Rai staging system were within the intermediate stage. There was no significant correlation between CD49d expression and clinical stage.

Keywords: chronic lymphocytic leukemia; CD49d expression; modified Rai staging system

Introduction:

Chronic lymphocytic leukemia a proliferative clinical heterogenic disease characterized by the accumulation of monoclonal Population of small, homogenous, mature CD5 + B lymphocytes in the peripheral blood (> 5 x 10^9/L), secondary lymphoid organs and bone marrow. (1)

Recently the CLL has shown a variable clinical course to be highly heterogeneous diseases that certain patients show aggressive and refractory disease that lead to short survival and death within 2 to 3 years after diagnosis while others show

indolent disease not require any treatment and survival reaching decades. For this reason it is important to categorize the heterogeneity by development of clinical, genetic and biological parameters and to evaluate the prognosis of the patients with CLL. [2]

CD49d, the α4 subunit of the integrin heterodimer α4β1 [3], together with CD29 (β1subunit) forms the α4β1 integrin. [4]

It is surface molecule act as adhesion structures for extracellular matrix component or mediating cell-cell interactions through the binding with fibronectin or vascular cellular adhesion molecules-1 (VCAM-1) respectively [5,6]. In this aspect the CLL cell expressing CD49d shown to have high propensity to adhere to fibronectin substrate, and an increased CD49d protein expression was demonstrated in CLL cells from advanced Rai stage patients [7]. Expression of which facilitates CLL leukemic cell growth micro-environmentally, [4] and identify progressive and short-lived patients subgroups. [8]

Aim of the study:

1. Evaluate the expression of CD49d in the bone marrow biopsy of newly diagnosed patients with CLL by immunohistochemistry.
2. Correlate the CD49d expression with Modified Rai staging system (low, intermediate and high risk groups).
3. Find the significance of CD49d immunohistochemical expression in bone marrow biopsy as a prognostic marker .

Patients, materials, and methods:

A- Data Base:

We collected all data available about all CLL patients registered in the teaching laboratories of medical city in Baghdad, including 64 data sheet involving patient name, age, address, occupation, date of diagnosis, clinical presentation (lymphadenopathy, splenomegaly, hepatomegaly), base line and serial records of hematological findings (CBC, blood film, bone marrow aspirate and bone marrow biopsy). Some additional information was completed for those without complete data available in their sheets by phone calling or direct questionnaire.

B- Study Design and Setting:

This is an analytic study amongst a collection of patients with CLL registered in the teaching laboratories of medical city in Baghdad. This study was achieved from November 2018 to June 2019.Cross-sectional study, planned to assess the expression of CD49d in bone marrow biopsy of CLL patients, correlates them with Modified Rai staging system (low, intermediate and high risk groups), and find the significance of CD49d as a prognostic marker. This study conducted in Middle Euphrates unit of Research Cancer and the Department of Pathology and Forensic Medicine in the Faculty of Medicine, University of Kufa. A total of 64 CLL patients were registered in the teaching laboratories of medical city in Baghdad. All patients were eligible for the study including those have complete laboratory data as CBC, blood film, bone marrow aspirate and bone marrow biopsy.

C- Cases definition and ascertainment:

C.1 Inclusion Criteria:

- Patients diagnosed with CLL by hematopathologist.
- Diagnosis depending on CBC, blood film and BM examination with or without flowcytometry.
- Available full data and materials of CBC, blood film, BM aspirate slides and formalin- fixed, paraffin embedded BM biopsy.
- All cases have been available and diagnosed with CLL in teaching laboratories of medical city in Baghdad through period of year 2017 and 2018.

C.2 Exclusion Criteria:

We exclude patients with CLL who are on treatment or lost their data or laboratory materials.

C.3 Positive control slides:

In each set of immunostaining parallel positive control sections have been processed. Positive controls basal layer of epidermis tissue sections, which are known to express CD49d, were used with each run.[9]

C.4 Negative control slides:

Normal bone marrow biopsies obtained from laboratories of AL-Sader teaching hospital in Najaf, were taken in every run as negative control.

Immunohistochemical staining protocol

The immunostaining method used in the current study was EnVision+ systems technique which was applied for CD49d staining.

Statistical Analysis:

Data were analyzed with SPSS software (Statistical Package of Social Sciences (SPSS), version 23, (Inc., Chicago, IL, USA) Independent samples T test, Chi-square test were used to evaluate the statistical difference between groups. P value < 0.05 was considered to indicate statistical significance (Al-Ukaelii and Al-Shaeb, 1998).

Results:

The current study included 64 patients diagnosed as having CLL of age ranging 36-76 years with a Mean= 59.12 ± 11.326 (Mean ± SD) years. The majority of the patients are within the age group of 60-67 (42.18%).In our study male constitute 67.18 % (43/64) while females constitute 33.8% (21/64),so male to female ratio was aroun d 2.04:1.

Lymphadenopathy and splenomegaly were the main clinical findings, followed by hepatomegaly and pallor.

Hematological parameters of patients with CLL in this study:

The average of Hb and PCV levels in the patients was 10.8 ± 2.7 % (Mean ±SD) and 33.7 ± 7.8 respectively. About 49% of the patients had been anemia than normal range to age and gender. The average of platelet count was 187.6 ± 75.2 $x10^9$/L and platelet count in 32.8% of cases was lower than normal range. The average of total white blood cells count was 74.45 ± 84.26 $x10^9$\L and lymphocyte percent in peripheral blood represent (57.48 ± 62.26) % of circulating white blood cells. The mean lymphocyte percent in bone marrow aspirate was (75.5 ± 16.6) % of all nucleated cells. Bone marrow histological lymphocytes involvement was diffuse pattern in 42.18% of CLL cases, interstitial in 20.3% of cases and mixed in 37.5% of cases, as display in table 1.

Table 1: The hematological parameters of the patients with CLL included in the study (n=64)

Parameter	Range	Mean	SD
Hb	4.2-15.3	10.8	2.7
PCV %	13.2%-45.6%	33.7%	7.8
Platelet count($x10^9$\L)	29-377	187.6	75.2
WBC count	8.62-274	74.45	84.26
Lymphocytes % in peripheral blood	8.3-177.7	57.48	62.26
Lymphocytes % in bone marrow aspirate	40%-97%	75.5	16.6

Clinical staging of patients with CLL:

The studied patients categorized in accordance with the Modified Rai staging system into: The low risk group included 0 patients (0 %), the intermediate risk group included 33 patients (51.6%) and the high risk group included 31 patients (48.4%), as display in Figure 1:

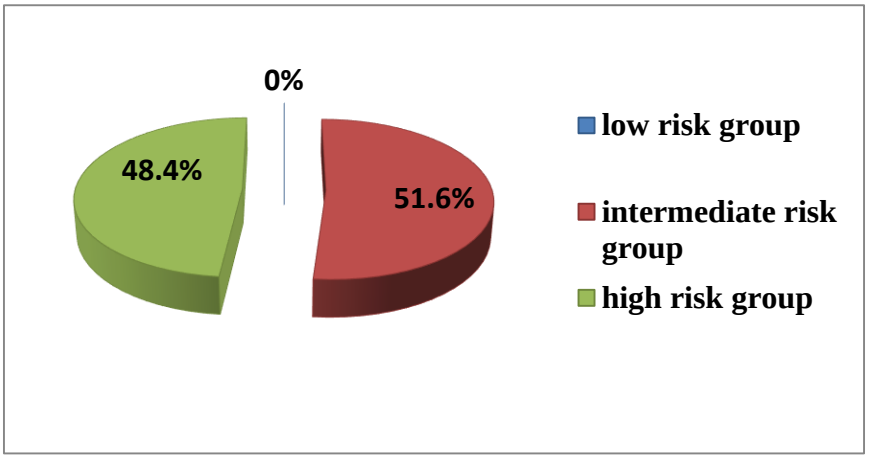

Figure 1: The distribution of patients with CLL according to modified Rai staging system

Immunohistochemical findings:
Staining findings of CD49d expression:

The presence of membranous brown stain is regarded as positive finding. The scoring system for CD49d was scored positive if 10% and more of the cells within an aggregate showed membranous stain. [10] So the patients in this study categorize as following:

- 36/64 cases (56.25%) were negative CD49d.
- 28/64 cases (43.75%) were positive CD49d, as display in table 2:

Table 2: CD49d score distribution for the patients with CLL

CD49d score	Number of cases	%
Negative	36/64	56.25%
Positive	28/64	43.75%
Total	64	100 %

The staining character and localization of CD49d in CLL cases in our study are shown in the following figures:

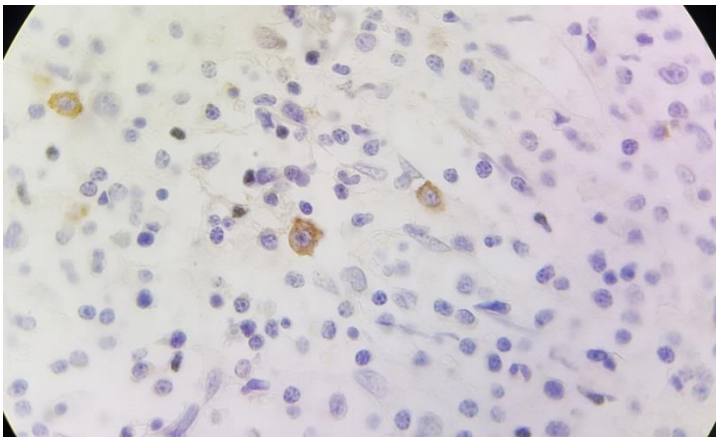

Figure 2: Bone marrow tissue of CLL stain with CD49d, (10-25%) of cells show brown membranous stain (CD49d positive stain, weak intensity, proportion score +1) (x100)

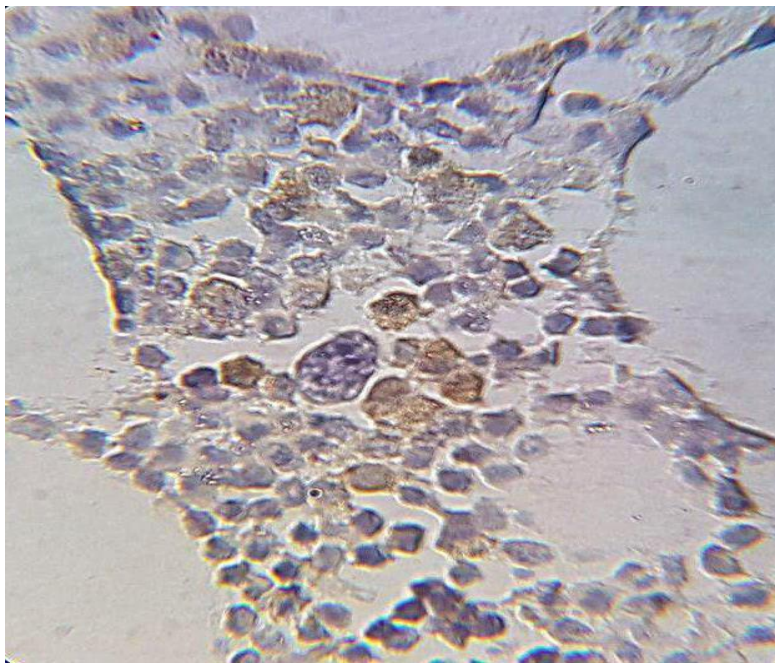

Figure 3: Bone marrow tissue of CLL stain with CD49d, (25-50%) of cells show brown membranous stain (CD49d positive stain, moderate intensity, proportion score +2) (x100)

There was no statistical association between CD49d expression and the clinical stage of the disease using Modified Rai system (P= 0.072) (r = ‑ 0.225) by, using chi square test and spearmann test respectively, as displayed in table 3.

Table 3: The association between CD49d expression and Modified Rai stage in patients with CLL

Modified Rai stage	CD49d score			P value
	Negative	Positive	Total	
High risk	21	10	31	0.072 (NS)
Intermediate risk	15	18	33	
Low risk	0	0	0	
Total	36	28	64	

NS: Non-significant difference at p <0.05

Furthermore, there was no statistical association between CD49d expression and age, gender, clinical findings, histological lymphocytes involvement and hematological parameter including Hb, PCV%, platelets, total white blood cell count and lymphocyte % in peripheral blood, while there was statistical association between CD49d expression and lymphocyte % in bone marrow (p=0.014) as display in table 4 by using t-test.

Table 4: The association between CD49d expression and hematological parameters in patients with CLL

	CD49d	N	Mean	SD	Minimum	Maximum	p-value
Hb	Negative	36	10.73	2.52	4.2	14	0.695(NS)
	Positive	28	11.01	3.06	4.2	15.3	
Platelet count ($x10^9$/L)	Negative	36	178.3	71.74	29	286	0.289(NS)
	Positive	28	199.07	79.36	71	311	
PCV (%)	Negative	36	33.01	7.38	15.3	45.6	0.396(NS)
	positive	28	34.7	8.4	13.2	43.3	
WBC count ($x10^9$/L)	Negative	36	63.54	8.7	11	250	0.285(NS)
	Positive	28	88.47	21.27	8.62	274	
LC % in peripheral blood	Negative	36	52.99	8.08	10	237.5	0.541(NS)
	positive	28	63.26	14.55	8.3	177.7	
LC % in BM aspirate	Negative	36	80.14	14	45	97	0.014(S)
	positive	28	69.57	18	40	95	

Discussion:

In this study, the mean age of all patients included was 59.12 + 11.326 years which was comparable to other Iraqi studies in 2011, 2012 and 2013, [11,12,13] Egyptian study in 2007[14] and Turkish study in 2012.[15]

The clinical staging of patients with CLL in this study, by applying Modified Rai staging, showing that more patients are in the intermediate stage and no patient was found in low risk stage. Those results show the higher percentage of the intermediate stage over high-risk stage were comparable with other Iraqi studies [12,13, 16,17,18,19,20], and these variations could have attributed to sampling or to the increase in the patient's awareness about the health and early clinical counseling or may related to expansion in general health service covering in our country in last few years. But western countries studies have shown that 40-60% of all CLL patients were at the time of diagnosis at the early clinical stage of the disease [21].

Among the patients with CLL included in this study, around 44% of the patients showed positive CD49d expression close to the result of V. Gattei et al. 2008 [8] who reported that CD49d was expressed in 47% of CLL cases. But this study result was slightly higher than the 39% reported by Zucchetto A et al. 2013 [22], the 38% reported by Bulian P et al. 2014 [23], 29% reported by Dal Bo M et al.2016 [24] and lower than that reported by Iraqi studies [15,25, 26]. These variations can have explained by the racial differences of some studies, sample size, method for detection, in addition to the higher cut off value for positivity they used depending on available kits for immunohistochemical study like 30% for example.

In our study, there was no statistical significant association of CD49d expression with modified Rai staging system. This was agreement with Zaki, Eman Mosaad, et al. 2019[27] who reported there was no significant association between CD49d expression and modified Rai staging, while disagreement with V. Gattei et al. 2008 [8], Ibrahim et al. 2015[26], both reported there was significant association between CD49d expression and clinical stage. This can have related to relatively small sample size, detection method for CD49d expression (immunohistochemistry) and low cut off value (10%) we used in our study for CD49d

positivity, although, most of our patients were within intermediate stage which consider as advance stage making the CD49d as adverse prognosticator for CLL.

CLL cells survival and proliferation is well established for the interaction between CLL cells and the micro environment in secondary lymphoid organs and bone marrow, CD49d has important roles to frame these interactions. It influences the trafficking in CLL between blood and lymphoid organs, in addition influence survival and proliferation within lymphoid tissue, thus affecting the disease's pathophysiology. [28]

Many studies showing the relation of CD49d expression and a series of factors affect the CLL cells migration and invasion properties with subsequent increase in CD49d expressive cell survival[29] and reduce drug induce apoptosis [30,31] However, the results of this marker in this study do not give the full impression of the relationship between advanced disease and the existence of this indicator and its expression still of doubtful association with disease progression and may have a direct or indirect effect.

Conclusion:

1. CD49d expression on CLL B-cells was identified in less than half of patients.
2. More than half of CLL patients according to modified Rai staging system were within the intermediate stage.
3. There was no significant correlation between CD49d expression and clinical stage.

References:

1. Salawu L, Bolarinwa R, Durosinmi M. Chronic lymphocytic leukaemia: a-twenty-years experience and problems in Ile-Ife, South-Western Nigeria. African health sciences. 2010; 10(2).
2. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute–Working Group 1996 guidelines. Blood. 2008 Jun 15; 111(12):5446-56.
3. Burger JA. Targeting the microenvironment in chronic lymphocytic leukemia is changing the therapeutic landscape. Current opinion in oncology. 2012 Nov 1; 24(6):643-9.
4. Buggins AG, Levi A, Gohil S, Fishlock K, Patten PE, Calle Y, et al. Evidence for a macromolecular complex in poor prognosis CLL that contains CD38, CD49d, CD44 and MMP-9. British journal of haematology. 2011 Jul; 154(2):216-22.
5. Ruoslahti E. Integrins. The Journal of clinical investigation. 1991 Jan 1; 87(1):1-5.
6. Rose DM, Han J, Ginsberg MH. α4 integrins and the immune response. Immunological reviews. 2002 Aug; 186(1):118-24.
7. Eksioglu-Demiralp E, Alpdogan O, Aktan M, Firatli T, Ozturk A, Budak T, et al. Variable expression of CD49d antigen in B cell chronic lymphocytic leukemia is related to disease stages. Leukemia. 1996 Aug 1; 10(8):1331-9.
8. Gattei V, Bulian P, Del Principe MI, Zucchetto A, Maurillo L, Buccisano F, et al. Relevance of CD49d protein expression as overall survival and progressive disease prognosticator in chronic lymphocytic leukemia. Blood. 2008 Jan 15; 111(2):865-73.
9. ositive controle of CD49d.Available athttps://www.pathologyoutlines.com
10. Charafe-Jauffret E, Tarpin C, Bardou VJ, Bertucci F, Ginestier C, Braud AC, et al. Immunophenotypic analysis of inflammatory breast cancers: identification of an 'inflammatory signature'. The Journal of pathology. 2004 Mar; 202(3):265-73.
11. Jaafar AM, Al-Rubaie HA, Mustafa SA, Majeed BA. mRNA in situ hybridization analysis of p-53 cancer suppression gene and Bcl-2 oncogene in chronic lymphocytic leukemia. Journal of the Faculty of Medicine Baghdad. 2010 Jul 4; 52(2):175-9.
12. AL-Mosawy MS. ZAP 70 expression in bone marrow biopsy of Chronic Lymphocytic Leukemia among Iraqi people. KUFA MEDICAL JOURNAL. 2012; 15(2):24-31.
13. Jasim HN, Al-Mudallal SS. Immunohistochemical expression of Bcl2 and Ki67 in Chronic Lymphocytic Leukemia (CLL) (Doctoral dissertation, MSc. Thesis, Al-Nahrain University. Baghdad, Iraq).
14. Sorour A, ElSorady M. Expression of CD38 and ZAP-70 in Chronic Lymphocytic Leukemia and relation to clinical stage. Journal of Medical Research Institute JMRI. 2007; 28(2):179.
15. Uzay A, Toptaş T, Kaygusuz I, Ekşioğlu-demiralp E, Fıratlı Tuğlular T, Bayık M. The

prognostic value of CD49d expression in Turkish patients with chronic lymphocytic leukemia.
16. Albanaa AM, Almudalal SS, Almamoori HS. Evaluation of CD69 Expression and Plasma Interleukin-6 in Chronic Lymphocytic Leukemia. Journal of Pharmaceutical Sciences and Research. 2019 Mar 1; 11(3):935-41.
17. Yousif MH, Musa RJ. Immunohistochemical expression of CD38, CD23 and serum beta 2 microglobulin in CLL and their correlation with Rai staging. M.Sc.thesis (Path). Al-Nahrain University. 2012.
18. Mahir S, Helmmi F, AL-Allawi N. P53 expression in CLL. M.Sc. thesis (path.). University of Baghdad. 2002.
19. Abdulkareem MJ, Ban AA, Al-Rubaie HA. In Situ Hybridization Analysis of p-53 and Bcl-2 Oncogenes and Angiogenesis Factors VEGF and MMP-9 in Chronic Lymphocytic Leukemia. Ph.D. thesis (path.) University of Baghdad.2008.
20. Mohammed S. *Immunohistochemical Analysis of BCL-2 to assess the Significance of Dysregulated Apoptosis and CD34 to Evaluate Angiogenesis in Chronic Lymphocytic Leukemia* (Doctoral dissertation, M. Sc. thesis (Path). University of Baghdad).
21. Montserrat E. Classical and new prognostic factors in chronic lymphocytic leukemia: where to now? The Hematology Journal. 2002 Mar; 3(1):7-9.
22. Zucchetto A, Caldana C, Benedetti D, Tissino E, Rossi FM, Hutterer E, et al. CD49d is overexpressed by trisomy 12 chronic lymphocytic leukemia cells: evidence for a methylation-dependent regulation mechanism. Blood. 2013 Nov 7; 122(19):3317-21.
23. Bulian P, Shanafelt TD, Fegan C, Zucchetto A, Cro L, Nückel H, et al. CD49d is the strongest flow cytometry–based predictor of overall survival in chronic lymphocytic leukemia. Journal of Clinical Oncology. 2014 Mar 20; 32(9):897.
24. Dal Bo M, Bulian P, Bomben R, Zucchetto A, Rossi FM, Pozzo F, et al. CD49d prevails over the novel recurrent mutations as independent prognosticator of overall survival in chronic lymphocytic leukemia. Leukemia. 2016 Oct; 30(10):2011.
25. Al-Rubaie HA, Thabit ZA, Jawad AM. CD49d as Prognostic Marker in B-Cell Chronic Lymphocytic Leukemia in Correlation with the Expression of CD38, ZAP-70 and Clinical Binet Stage. Iraqi Academic Scientific Journal. 2016; 15(4):486-92.
26. Ibrahem L, Elderiny WE, Elhelw L, Ismail M. CD49d and CD26 are independent prognostic markers for disease progression in patients with chronic lymphocytic leukemia. Blood Cells, Molecules, and Diseases. 2015 Aug 1; 55(2):154-60.
27. Zaki EM, El-Gammal SA, Sayed NG, Sediek MF, Mohamed AA. The Prognostic Implication of CD49d Expression by Flow Cytometry and Trisomy 12 Detection by Fluorescent in situ Hybridization in Chronic Lymphocytic Leukemia. Journal of Advances in Medicine and Medical Research. 2019 Mar 20:1-9.
28. Brachtl G, Hofbauer JP, Greil R, Hartmann TN. The pathogenic relevance of the prognostic markers CD38 and CD49d in chronic lymphocytic leukemia. Annals of hematology. 2014 Mar 1; 93(3):361-74.
29. Zucchetto A, Benedetti D, Tripodo C, Bomben R, Dal Bo M, Marconi D, et al. CD38/CD31, the CCL3 and CCL4 chemokines, and CD49d/vascular cell adhesion molecule-1 are interchained by sequential events sustaining chronic lymphocytic leukemia cell survival. Cancer research. 2009 May 1; 69(9):4001-9.
30. De la Fuente MT, Casanova B, Cantero E, del Cerro MH, Garcia-Marco J, Silva A, et al. Involvement of p53 in α4β1 integrin-mediated resistance of B-CLL cells to fludarabine. Biochemical and biophysical research communications. 2003 Nov 21; 311(3):708-12.
31. De la Fuente MT, Casanova B, Moyano JV, Garcia-Gila M, Sanz L, Garcia-Marco J, et al. Engagement of α4β1 integrin by fibronectin induces in vitro resistance of B chronic lymphocytic leukemia cells to fludarabine. Journal of leukocyte biology. 2002 Mar; 71(3):495-502.