Plasma Levels of CXCL 9, 10, 11 and 12 and Their Impact on Overall Survival in Chronic Lymphocytic Leukemia

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Abstract: Background/Aims: Background/Aims: The expression of chemokines is altered in chronic lymphocytic leukemia (CLL) due to inactivation of the tumor suppressor genes or constitutive activation of the oncogenes.The aim of this study was to measure plasma levels of chemokines CXCL9,10, 11 and 12 in CLL and relate that, if any, with abnormal immunophenotype considered with bad prognosis. **Methods:** Plasma from 40 CLL patients and 20 healthy age and gender-matched controls were analyzed for CXCL 9, 10, 11 and 12 by enzyme-linked immunosorbent assay. **Results:** CXCL11 and 12 plasma concentrations were significantly higher in CLL patients compared to controls (*P*=0.013 and 0.0015) respectively. CLL patients with higher CXCL11 or 12 levels (median >128.4, 1006.65 pg/mL respectively) before treatment had worse prognosis for overall survival (OS) (P=0.0342, 0.0229). **Conclusion:** High plasma levels of CXCL11 and 12, is associated with high grade CLL leukemia and may be useful as a predictive indicator for OS.

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1. Introduction

Although improvement in survival of CLL patients after administering combination of chemo-immunotherapy regimens, the disease remains incurable with residual malignant cells remaining in the bone marrow and lymph nodes[1,2]. Chemokines play an important role in the evolution of cancers as they are involved in tumor growth, angiogenesis, metastasis and immune evasion[3]. Also, the expression of chemokines and their receptors is altered in many malignancies and subsequently leads to aberrant chemokine receptor signaling. This alteration occurs due to inactivation of the tumor suppressor genes or constitutive activation of the oncogenes that play a role in the regulation of the chemokines. Deregulated expression of the transcription factors affect the levels of chemokines and promote tumor genesis[4,5].

In CLL patients stromal cells in the bone marrow and lymph nodes secrete chemokines such as CXCL 9, CXCL 10, CXCL 11, CXCL12, CXCL13, which bind to a variety of corresponding receptors on the CLL cell surface[6-8]. These interactions lead to CLL cell chemotaxis into the tissue microenvironments, where the malignant cells are then subject to survival and proliferation signals through the cell receptor and other pathways[9,10]. Once in the tissues, CLL cells are surrounded by a supportive microenvironment that includes cells expressing CD40L, fibronectin, and vascular adhesion molecule 1 (VCAM-1), all of which provide additional survival and anti-apoptotic signals to the CLL cells[11,12]. Now, novel strategies being undertaken to disrupt stroma-mediated survival signals as part of a new therapeutic approach in CLL[13].

In the current study, we measured plasma levels of CXCL9, 10, 11 and 12 in CLL patients and healthy controls and monitored their OS to determine their potential role as a predictive indicator for CLL patients.

2. Methods

This prospective study was conducted in Tanta University Hospital from March 2011 to December 2014. Following approval of Local Research and Ethics Committee and obtaining written informed consent, forty CLL patients and twenty healthy controls were prospectively enrolled in the study.

CLL was diagnosed according the diagnostic criteria for CLL defined by the National Cancer Institute (NCI) and International Workshop on CLL (IWCLL) & Scoring System for Diagnosis of CLL[14-16].Patients were staged according to Rai's staging system[17]. National Cancer Institute-Working Group (NCI-WG) revised criteria were used to define the response to chemotherapy[18].Patient' samples used for this study were collected after diagnosis but prior to treatment initiation. Full history talking, complete physical examination, routine laboratory investigations were done for all patients

Peripheral blood sample was collected into ethylene diamine tetra-acetic acid (EDTA) tube by

venipuncture, and plasma was separated after centrifuge and subsequently stored at -80°C. Plasma levels of CXCL9, CXCL10, CXCL11 and CXCL12 were determined using commercially available ELISA kits provided by Abcam (USA) according to manufacture instructions (catalog numbers ab119588, ab83700, ab100580 and ab100637 respectively). The kits are sandwich enzyme linked immunosorbant assay. The sample were done in duplicate and compared to standard curve. Complete blood count (CBC) was done using Sysmex XT- 1800i (Sysmex, Japan).Another portion of blood was centrifuged and serum stored at -80 °C until it was needed for routine testing of renal and liver function tests, lactate dehydrogenase (LDH) and β2-microglobulin.

Routine immunophenotyping was done for CLL cases using FACS Calibur flow cytometry and Cell Qust Pro software (BD, USA). Panel of labeled monoclonal antibodies were used. FITC- conjugated CD20, CD38, CD5, FMC7, CD3, and kappa light chain and PE-conjugated CD23, ZAP-70, CD79b, CD10, CD19 and Lambda light chain. The ZAP-70 tube was permeabilised before adding the monoclonal antibodies. All reagents (monoclonal antibodies, lysing solution and permeabilising solution) were provided by BD. Patients with <30% of the clone expressing CD38 were considered CD38 negative, and those with ≥30% of the clone expressing CD38 as CD38 positive, but as regards to ZAP-70, patients were considered ZAP-70 positive when 20% or more of their CD19+ cells expressed ZAP-70[19] as shown in (Figure 1).

The standard protocol chemotherapy for CLL which used in this study was FCR (cyclophosphamide, fludarabine, rituximab)[20]but in frail patients with significant comorbidities not able to tolerate purine analogs, we used chlorambucil[21].

The collected data were analyzed using SPSS version 17 software (SPSS Inc, Chicago, ILL Company). Comparison of continuous data between two groups was made by using Mann-Whitney test for non-parametric data. Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. The accepted level of significance in this work was stated at 0.05 ($P \leq 0.05$ was considered significant).

3. Results

Forty CLL patients were recruited in this study. There were 24 men (60%) and 16 women (40%). At the time of diagnosis, patient ages ranged from 38 to 73 years (mean 54±10.66 years) (median 53.5 years). The mean ± SD for hemoglobin, total leucocyctic count, platelets and β-2 microglobulin for CLL patients were (9.08±1.91 gm/dl), (91.6±86.99 x109/L), (249.73±129.82 x109/L) and (3.79±2.47 mg/L) respectively. The demographic, clinical and laboratory data of CLL patients are shown in (Table 1).

All patients received treatment either due to high grade disease (III and IV) or grade II with indications to start therapy (two patients with autoimmune thrombocytopenia and autoimmune hemolytic anemia not respond to corticosteroids, five patients with B symptoms and the remaining seven patients had high total lucocyctic count with lymphocyte doubling time less than 6 months).

Plasma levels for all 4 chemokines (CXCL9-12) were greater in CLL patients when compared with the age and sex-matched healthy subjects. However only plasma levels of CXCL11, CXCL12 were significantly higher (P =0.013, 0.0015 respectively) (Table 2).

Our study showed that CD38 or ZAP-70 positive patients were (13/40) (32.5%), while the percent was 30% (12/40) for patients who were both CD38 and ZAP-70 positive. Analysis of CD38, ZAP-70 and the line of treatment as independent variables for disease outcome (OS) showed that OS significantly decreased only in truly positive ZAP-70 (Table 3, Figure 2). Also, CLL patient's chemokine levels were analyzed as independent variables for disease outcome (OS). CLL patients were divided into 2 categories for each plasma cytokine: those with levels above and below the median. CXCL11 and CXCL12 levels were independently associated with shorter survival (Table 4, Figure 3). Comparison between chemokines (CXCL11 and CXCL12) in patients with CD38 or ZAP-70 positive patients (14 patients) and others with CD38 and ZAP-70 negative (26 patients) showed significantly higher levels in patients with CD38 or ZAP-70 positive (Table 5).

4. Discussion

Our study demonstrated elevated plasma levels of CXCL9-12 in CLL patients compared to age and gender matched control group however, only CXCL11, CXCL12 levels were significant.. Although elevated plasma levels of CXCL 9 and 11 in CLL patients were shown previously[22], nevertheless, this is the first demonstration, for our knowledge, that CXCL12 exists at higher levels in CLL plasma, suggesting new molecules that can predict disease progress.

Chemokines and their receptors organize the recruitment and positioning of cells at each stage of the immune response, a system critically dependent upon coordination to get the right cells to the right place at the right time. Chemokine receptors expressed on CLL cells are thought to function in a similar fashion, regulating the trafficking of the leukemia cells between blood, lymphoid organs, and the bone marrow, and within sub compartments within these tissues, in concert with adhesion molecules and other guidance cues[7].

Table 1: Demographic, clinical and laboratory characteristics of CLL patients.

variable		N.	%
Sex	**Female**	16	40.00
	Male	24	60.00
B symptoms	**Negative**	25	62.50
	Positive	15	37.50
Lymphadenopathy	**Negative**	15	37.50
	Positive	25	62.50
Splenomegaly	**Negative**	14	35.00
	Positive	26	65.00
Performance status	**1**	23	57.50
	2	17	42.50
Rai Stage	**Stage II**	14	35.00
	Stage III	17	42.50
	Stage IV	9	22.50
Lactate dehydrogenase (LDH)	**Normal**	24	60.00
	High	16	40.00
ZAP-70	**Negative**	27	67.50
	Positive	13	32.50
CD38	**Negative**	27	67.50
	Positive	13	32.50
Line of treatment	**FCR**(Fludarabin+ Cyclophosphamide + Rituximab)	29	72.5
	Chlorambucil	11	27.5
Treatment response	**Complete remission (CR)**	18	45
	Partial remission (PR)	14	35
	Progressive disease (PD)	8	20
Fate of the patients	**Survival**	33	82.50
	Non survival	7	17.50

Table 2:Plasma chemokines level in CLL patients versus healthy control (pg/mL).

Variables	Groups	Range			Median	95% confidence interval	Mann-Whitney Test (P-value)
CXCL 9	**Patients**	54	-	9120.7	524.15	1277.5 - 3284.9	0.3119
	Controls	66.9	-	1021.9	377.9	307.39 - 643.08	
CXCL 10	**Patients**	12.7	-	562.4	100.8	125.18 – 230.04	0.2273
	Controls	19.3	-	387.2	62.1	66.523 – 168.85	
CXCL 11	**Patients**	17.5	-	1621	128.4	253.59 – 574.62	0.013*
	Controls	9.1	-	245.1	67.2	54.532 – 128.05	
CXCL 12	**Patients**	547.9	-	3211.8	1006.65	1126.9 – 1597.7	0.0015*
	Controls	152.7	-	2019	829.25	571.08 – 954.58	

Table 3: Overall survival in CLL patients in relation to CD 38, ZAP-70 and line of treatment.

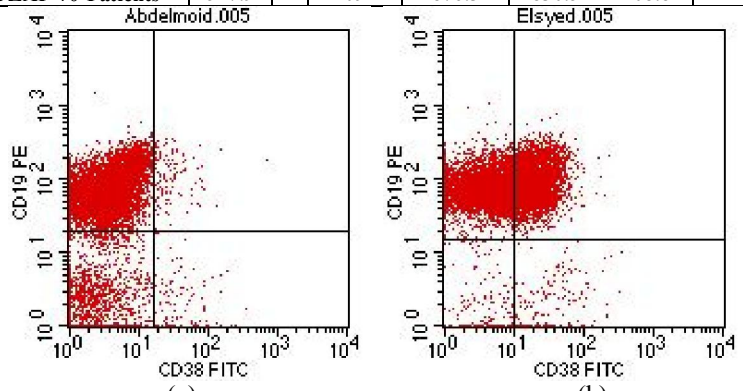
	Number of patients	Events (N)	Censored (N) (%)	Survival (Mean ± SE) (Months)	Statistic test for equality of survival distributions (Log Rank)	
					Statistic	*P*-value (Significance)
Patients with negative ZAP-70	27	2	25(92.59%)	33.96 ± 1.38	8.68	**0.0032***
Patients with positive ZAP-70	13	5	8 (61.54%)	17.53 ± 1.92		
Patients with negative CD38	27	3	24 (88.89%)	32.9 ± 1.67	3.5	**0.0615**
Patients with positive CD38	13	4	9 (69.23%)	24.57± 3.38		
Patients received Chlorambucil	11	2	9 (81.82%)	31.34 ± 2.95	0.02	0.8868
Patients received FCR(Fludarabin+ Cyclophosphamide + Rituximab)	29	5	24 (82.76%)	30.74 ± 2.11		

Table 4: Overall survival in CLL patients in relation to CXCL 9-12.

	Number of patients	Events (N)	Censored (N) (%)	Survival(Mean ± SE)(Months)	Statistic test for equality of survival distributions (Log Rank)	
					Statistic	*P*-value (Significance)
Patients with CXCL 9 < 524.15	20	3	17 (85%)	27.48 ± 2.34	0.00	0.9962
Patients with CXCL 9 ≥ 524.15	20	4	16 (80%)	30.21 ± 2.56		
Patients with CXCL 10 < 100.8	19	3	16 (84.21%)	31.3 ± 2.44	0.09	0.7609
Patients with CXCL 10 ≥ 100.8	21	4	17 (80.95%)	30.56 ± 2.43		
Patients with CXCL 11 < 128.4	20	1	19 (95%)	34.61 ± 1.35	4.48	**0.0342***
Patients with CXCL 11 ≥ 128.4	20	6	14 (70%)	27.1 ± 2.92		
Patients with CXCL 12< 1006.65	20	1	19 (95%)	34.61 ± 1.35	5.18	**0.0229***
Patients with CXCL 12 ≥1006.65	20	6	14 (70%)	26.8 ± 3.01		

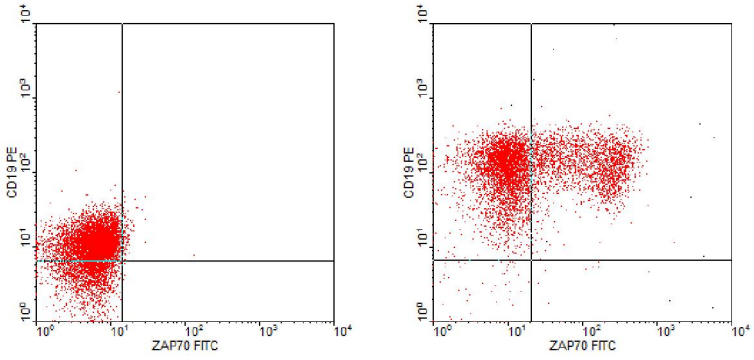
Table 5: Plasma chemokines (CXCL 11 and CXCL 12) levels in CLL patients in relation to CD38 and ZAP-70

Variables	Groups	Range			Median	95% confidence interval	Mann-Whitney Test (P-value)
CXCL 11	**$CD38^+$ or $ZAP\text{-}70^+$ Patients**	132.9	-	1621	994.05	774.09 - 1239.4	**< 0.0001***
	CD38- and ZAP-70-Patients	17.5	-	321.1	76.5	64.231 – 125.77	
CXCL 12	**$CD38^+$ or $ZAP\text{-}70^+$ Patients**	894.2	-	3211.8	2001.9	1677.2 – 2505.8	**< 0.0001***
	CD38- and ZAP-70-Patients	547.9	-	2091	876.3	830.51 – 1108.8	



(a) (b)

Legend for flow1: Flow cytometry dot plots show a) CD38 negative CLL case b) CD38 positive CLL case. Gates were done on lymphocytes populations.



(a) (b)

Legend for flow2: Flow cytometry dot plots show a) ZAP70 negative CLL case b) ZAP-70 positive CLL case. Gates were done on lymphocytes populations.

Figure 1: Flow cytometry images

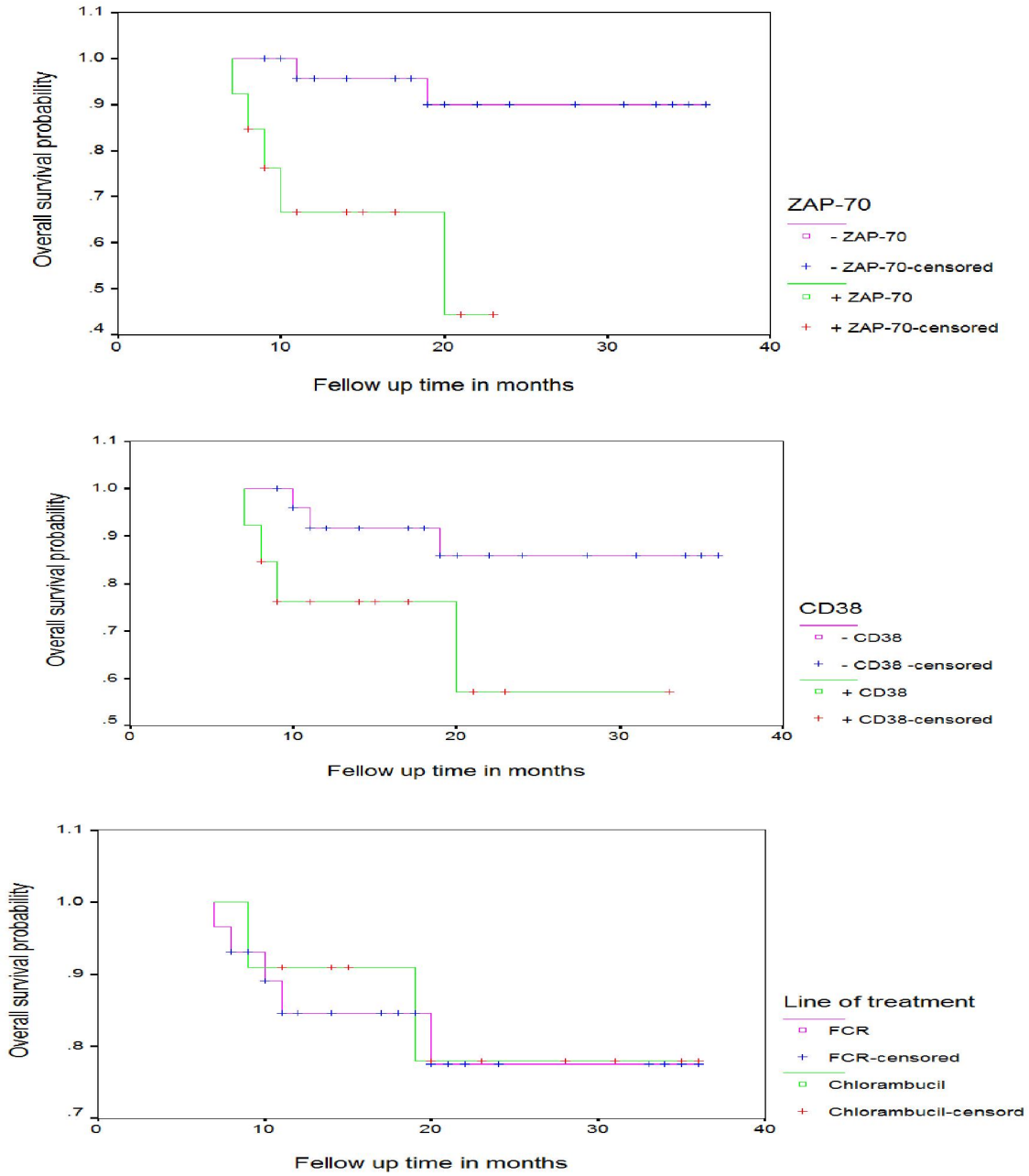
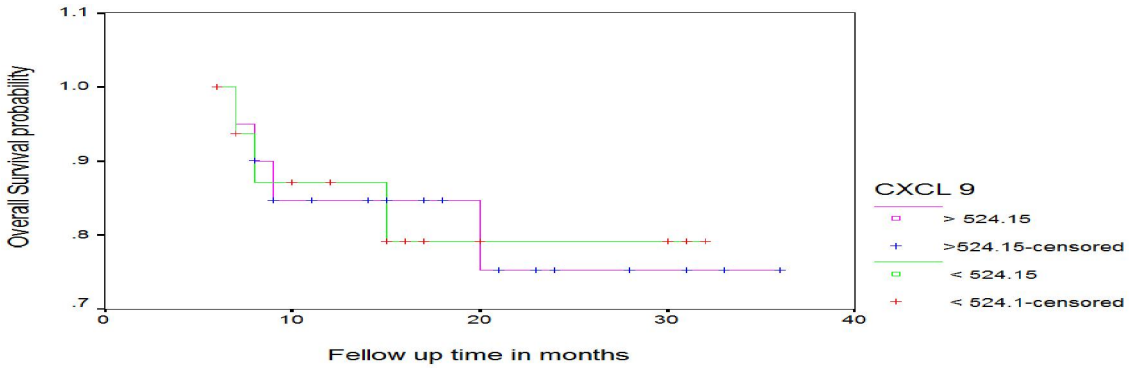


Figure 2: Overall survival in CLL patients in relation to CD38, ZAP-70 and line of treatment.



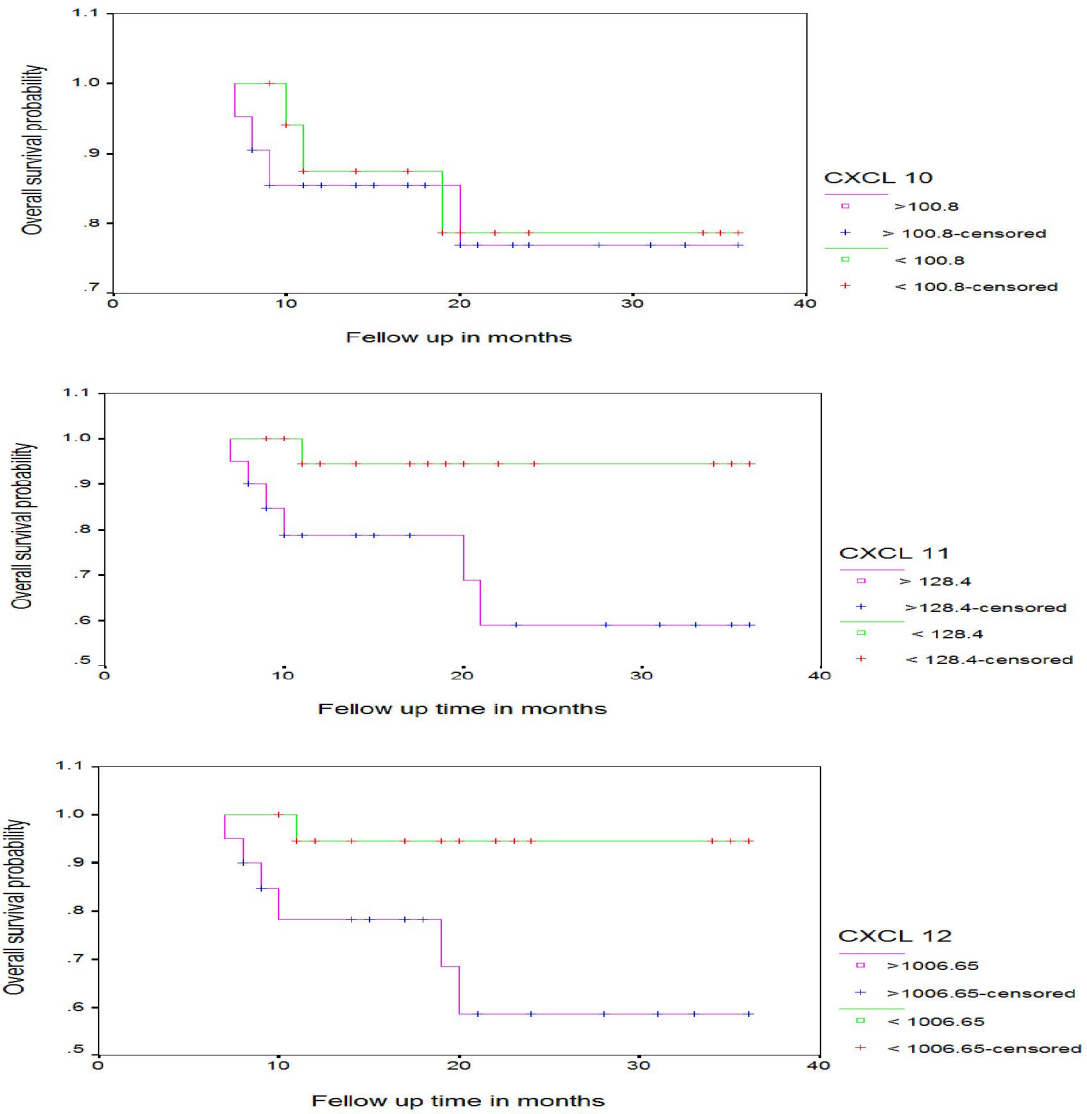


Figure 3: Overall survival in CLL patients in relation to CXCL 9-12.

CLL cells have also been shown to express certain receptors like CXCR3, CXCR4 and CXCR5 on cell surface. Both CXCL9 and CXCL11 are ligands for CXCR3, a receptor highly expressed on CLL cells. These chemokines may regulate B-CLL cell homing to tissues where they receive growth and survival signals[23]. Ocaña *et al.* [24] investigated the prognostic role of CXCR3 expression by chronic lymphocytic leukemia cells. Their analysis revealed that low CXCR3 expression by CLL cells was independently a strong predictor of a poor clinical progression. In addition, low CXCR3 expression goes with Rai stages III-IV and a diffuse pattern of bone marrow infiltration. Similar results were found by Vaisiti *et al.*[25] who investigated any specific associations between clinical signs and immune cells in CLL patients, They found that CXCR3 proportion within the CLL pool was lower in the occurrence of LN/ organ involvement. Grigore *et al.*[26] found that only patients with early stage CLL showed over-expression of CXCR3 while low CXCR3 expression was associated with advanced stage and poorer prognosis in CLL patients[26].

CXCL12 (formerly called stroma-derived factor-1 (SDF-1), originally characterized as a pre-B cell growth factor[27].CXCL12 has at least two major effects on CLL cells. It causes migration towards stromal cells and it independently and directly provides survival signals Burger *et al.*, 2000[28]. Both of these effects are mediated through the CXCR4 receptor on the surface of the CLL cell[29]. Thus, CXCR4 cell surface expression can be used as a

marker of CXCL12 exposure, with circulating CLL cells in the peripheral blood typically expressing high levels of surface CXCR4, and CLL cells resident in the bone marrow or lymph nodes having lower levels of surface CXCR4[30]. Upon stimulation by CXCL12, signaling through the CXCR4 receptor has pleotropic effects on CLL cells, including activation of through different pathways as serine phosphorylation of signal transducer and activator of transcription 3 (STAT3)[31].

The CXCL12/CXCR4 axis is involved in some aspects of tumor progression that include angiogenesis, metastasis, and survival[32,33]. The microenvironment in bone marrow enables the survival, differentiation, and proliferation of normal and malignant hematopoietic cells, and epithelial tumor cell bone metastasis. Bone marrow produces factors such as CXCL12, that mediate homing, survival, and proliferation of tumor cells, and integrin-mediated adhesion sequesters tumor cells to this place. The CXCL12/CXCR4 pathway is guilty for withholding of acute lymphoid leukemia and acute myeloid leukemia cells in the bone marrow[34,35].

Our study showed that CD38 or ZAP-70 positive patients were (13/40) (32.5%), while the percent was 30% (12/40) for patients who were both CD38 and Zap-70 positive. This is comparable with Schroers *et al.*[36] who done combined analysis of ZAP-70 and CD38 yielded discordant results in 29.0% of patients, whereas 47.6% of patients were concordantly negative and (23.4% of patients) were concordantly positive for ZAP-70 and CD38 expression.

In our study, analysis of CD38, ZAP-70 and the line of treatment as independent variables for disease outcome (OS) showed that OS significantly decreased only in truly positive ZAP-70. Also, CLL patients with higher CXCL11 and CXCL12 levels were independently associated with shorter overall survival. Comparison between chemokines (CXCL11 and CXCL12) in patients with CD38 or ZAP-70 positive patients and others with CD38 and ZAP-70 negative, showed significantly higher levels in patients with CD38 or Zap-70 positive.

Grigore *et al.*[26] studied the effect of the combination of CXCR3 and CD38 parameters and showed that the low CXCR3, high CD38 group had a shorter survival than other groups: high CXCR3 low CD38, low CXCR3 low CD38 group or high CXCR3 high CD38.

An interesting study performed by Burger et al[31] demonstrated that migration of CLL cells in response to CXCL12/CXCR4 depend on CD38 expression on cell surface these results indicate that CD38 synergizes with the CXCL12/CXCR4 pathway. Moreover Deaglio *et al.*[37] showed that patients with CD38 (+)/ZAP-70(+) are characterized by enhanced migration toward Stromal derived factor-1alpha (SDF-1alpha)/CXCL12 and combined CD38 and ZAP-70 expression in patients with CLL have more aggressive disease.

CD38 is an ectoenzyme, an enzyme present on the cell surface. It participates in cell adhesion, signal transduction, and calcium regulation. CD38 is a useful and reliable factor because it typically remains stable over time, even in the face of chemotherapy, and it can be measured easily in flow cytometry laboratories[19,38]. Damle *et al.*[39] showed that high levels of CD38 (≥ 30%) identified those with unmutated IgVH genes in 100% of cases and that OS was significantly improved in truly negative CD38 cases. Also, Crespo et al[40] demonstrated that ZAP-70, a tyrosine kinase associated with T-cell signaling, can also be evaluated by flow cytometry and that, at a cutoff of 20%, ZAP-70 positivity clearly separated CLL patients into two groups; those with < 20% ZAP-70 had increased survival time and decreased chance of disease progression.

In conclusion; increased plasma levels of CXCL 11 and 12 is predictive of higher risk of disease progression with decreased OS in CLL.

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Conflict of Interest: We declare no conflict of interest.

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