The prognostic role of CXCR3 expression by chronic lymphocytic leukemia B cells

Esther Ocaña, Luis Delgado-Pérez, Antonio Campos-Caro, Juan Muñóz, Antonio Paz, Rafael Franco, José A. Brieva

Servicio de Inmunología and Unidad de Investigación (EO, LD-P, AC-C, JAB); Servicio de Hematología, Hospital Universitario Puerta del Mar, Cádiz, Spain (JM); Servicio de Hematología, Hospital Universitario de Puerto Real, Cádiz, Spain (AP); Servicio de Hematología, Hospital Punta Europa, Algeciras, Cádiz, Spain (RF)

Funding: this work was supported by grants 225/03 and 91/04 from the Consejería de Salud de Andalucía, Spain.

Manuscript received August 14, 2006. Manuscript accepted January 26, 2007.

Correspondence: José A. Brieva, Servicio de Inmunología, Hospital Universitario Puerta del Mar, Avenida Ana de Viya 21, 11009 Cádiz, Spain. E mail: josea.brieva.sspa@juntadeandalucia.es

ABSTRACT

Background and Objectives

Chemokine receptors are involved in tumor progression and several of these receptors, including CXCR3, are expressed by chronic lymphocytic leukemia (CLL) B cells. This study was aimed to examine a possible relationship between CXCR3 expression in CLL and the clinical evolution of the disease.

Design and Methods

Using flow activated cell sorting (FACS), we analyzed the level of expression of CXCR3 on blood CLL B cells from 76 consecutive patients. The results were correlated with CD38 expression, IgV_H gene status and clinical outcome.

Results

CXCR3, measured as mean fluorescence intensity (MFI), was unimodally expressed by blood tumor cells at various levels (range, 3.5 to 232.3) but levels within individual patients were remarkably stable over time. Low CXCR3 expression by CLL B cells was strongly associated with Rai disease stages III and IV ($p<0.0001$) and a pattern of diffuse tumor infiltration of the bone marrow ($p<0.0001$). In the 28 cases available for genetic studies, low CXCR3 expression also showed good concordance with tumor unmutated IgV_H gene status ($p<0.04$), and tended to correlate with high CD38 expression ($p<0.06$). Patients with low CXCR3 expression (MFI ≤15) had a shorter survival ($p<0.0001$) and, in multivariate analysis, low CXCR3 expression (MFI ≤15) was an independent predictor of poor outcome (hazard ratio 24.5; $p<0.01$).

Interpretation and Conclusions

CXCR3 expression by CLL B cells appears to be stable within individual patients. Tests to assay this chemokine receptor are cheap and easy to perform and the results could be of prognostic value in CLL.

Key words: chronic lymphocytic leukemia, CXCR3 expression by CLL B cells, prognostic factors.

Haematologica 2007; 92:349-356

B-cell chronic lymphocytic leukemia (CLL), the most common leukemia in adulhood, is a malignancy of B lymphocytes immunophenotypically characterized by the expression of CD19, CD5, CD23 and low-intensity monoclonal immunoglobulin (Ig) on their cell surface.[1] This disorder presents at older ages and its course is typically indolent. However, a substantial number of patients die of progressive disease and it is now clear that CLL is a heterogeneous entity, not only at the clinical level but also at molecular and cellular levels, as evidenced by differences in the number of somatic mutations present in the Ig variable heavy-chain (*IgV$_H$*) gene and by a variety of cytogenetic alterations. These findings are leading researchers to consider that the clinical heterogeneity of CLL originates from B lymphocytes that may differ in differentiation stages or B cell subsets.[2]

Clinical staging systems, introduced by Rai and Binet, have been useful for classifying patients with CLL into broad prognostic groups.[3-5] However, since these classifications are based on clinical features, they fail to predict the course of the disease at the onset. Therefore, in recent years, considerable attention has been given to finding parameters that could anticipate the accelerated accumulation of and invasive behavior by CLL B cells. This has led to the analysis of many possible prognostic factors in this disease, including lactate dehydrogenase levels, β_2-microglobulin, lymphocyte doubling time, diffuse pattern of bone marrow invasion, soluble CD23, CD38 surface expression, cytogenetic abnormalities, *IgV$_H$* gene somatic mutations and ZAP-70 expression.[2,6]

Chemokines and their receptors are essential in the initial driving of hematopoietic cells to their homing locations in physiological as well as in pathological conditions, including tumor invasion.[7,8] CLL B cells have been shown to express CXCR3, CXCR4 and CXCR5.[9,11] The two latter receptors are a common feature of most normal human B lymphocytes.[7,12] In contrast, CXCR3 is only expressed by a minor fraction of normal B lymphocytes, including CD5 positive and negative subsets.[9,11] This chemokine receptor has been shown to play a critical role in the mobilization of activated T lymphocytes, NK cells, dendritic cells and other leukocytes towards an inflammatory focus, where their interferon-γ-inducible ligands CXCL9, CXCL10 and CXCL11 (Mig, IP-10 and ITAC, respectively) are abundantly produced by several cell types.[7,13] The unexpected and distinctive expression of CXCR3 by CLL B cells led us to explore its possible role in the disease.

Design and Methods

Patients and samples

Seventy-six consecutive patients diagnosed with B-cell chronic lymphocytic leukemia (CLL) were included in the study. Twenty-seven of these patients were recruited at diagnosis. In all the cases, the monoclonal B cell expansion was CD19$^+$, CD23$^+$ and CD5$^+$. Clinical data corresponding to these cases are summarized in Table 1. CLL was diagnosed in all cases according to international CLL workshop criteria and staged according to the Rai system.[4] Patients` peripheral blood samples were obtained by venipuncture, and CLL B lymphocytes were isolated as previously reported.[14] All patients were informed about the objectives and methods of the study and gave their consent according to the Declaration of Helsinki. Approval for this study was obtained from the institutional review board (Comisión Ética, Hospital Universitario Puerta del Mar). The median follow-up time since the first CXCR3 determination was 17.2 (range 6-31) months, and that since the diagnosis was 45 (range 8-193) months. Fifteen patients had died due to CLL by the time of the last evaluation.

Materials

Lymphocyte isolation medium was purchased from ICN (Costa Mesa, CA, USA). Monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC) anti-CD5 (clone L17F12) , to phycoerythrin (PE) anti-CD38 (clone HB-7) and anti-CXCR3 (clone IC6), to allophycocyanine (APC) anti-CD5 (clone L17F12) and to peridinin chlorophyll protein (PerCP) anti-CD19 (clone 4G7), and FITC-, PE-, PerCP- and APC-conjugated monoclonal antibodies of appropriate isotype used as negative controls, were obtained from Becton Dickinson (San Jose, CA, USA). The FITC-labeled anti-CXCR3 monoclonal antibody (clone 49801) was from R & D Systems, Inc. (Minneapolis, MN, USA).

Immunofluorescence staining and flow cytometry analysis

Three or four-color labeling experiments were performed as previously reported.[15] Briefly, mononuclear cells were isolated from blood samples by density centrifugation on Ficoll and 0.5×10^6 peripheral blood mononuclear cells in 100 μL of phosphate-buffered saline (PBS) were incubated with saturating quantities of the appropriate FITC-, PE-, PerCP- and APC-conjugated monoclonal antibodies for 30' in the dark at 4°C. After staining, cells were washed twice in PBS. Isotype-matched FITC, PE-, PerCP- and APC-conjugated irrelevant monoclonal antibodies were used as negative controls. Labeled cells were analyzed within 1 hour in a FACScalibur instrument (Becton Dickinson) equipped with an air-cooled argon ion laser that operated at 488 nm and a red diode laser that operated at 635 nm. A minimum of 5000 cells were collected and analyzed by using the CELLQUEST software (Becton Dickinson), and the percentage, and mean fluorescence intensity (MFI) of the cells for each examined molecule were monitored. Lymphocytes were selected in the forward

versus side scatter dot plot, and additionally gated as CD19 CD5 positive cells corresponding to either FL3 – FL1 or FL4- FL3 dot plots depending on the monoclonal antibody combination used. The color combination commonly used in the present study was PercP-anti-CD19, PE-anti-CXCR3 and FITC-anti-CD5. The percentage of CD38 CLL B cells was also explored using the same combination with PE-anti-CD38 instead of PE-anti-CXCR3. In some experiments, the combination PercP-anti-CD19, APC-anti-CD5 and FITC-anti-CXCR3 was used as an additional control for the fluorochrome and the anti-CXCR3 monoclonal antibody employed, and gave equivalent results. Background fluorescence was set so that less than 1% of the cells were positively stained with the corresponding negative control. The expression of CXCR3 by blood CLL B cells from all patients exhibited a rather homogeneous uni-modal peak (Figure 1A). Accordingly, the MFI parameter was chosen to compare the differences of expression by the patients` cells.

IgV$_H$ gene somatic mutations analysis

The *IgV$_H$* gene was amplified as previously described.[16] Briefly, RNA was obtained from purified CLL B cells using a Total RNA Isolation Kit (Roche, Barcelona, Spain), and random-hexamer primed cDNA was generated using a First Strand cDNA Synthesis Kit (Roche). cDNA was amplified by polymerase chain reaction (PCR) using a mixture of complementary 5′ oligonucleotides specific for frame region 1 (FR1) corresponding to all the VH1-VH6 *IgV$_H$* families, and a consensus 3′ oligonucleotide complementary for the JH region.[16] PCR was performed with the Expand High Fidelity Plus PCR System in 50 µL (Roche). Cycling conditions were 94°C for 30 second, 60°C for 30 seconds and 72°C for 60 seconds up to 35 cycles, followed by a final period of 7 minutes at 72°C. Products were purified and directly sequenced using the same JH primer in a Genetic Analyzer ABI-310 (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were analyzed with the IMGT *IgV$_H$* data base (*http://imgt.cines.fr/*). Sequences with 2% or less deviation from any germ line *IgV$_H$* sequence were considered unmutated.[16-18] Repeated PCR and sequencing reactions from cDNA samples from five CLL patients (two unmutated and three with a large number of mutations) were performed, and identical results were obtained.

Statistical analysis

Group-wise comparisons of clinical and biological data were performed using the Mann-Whitney U test and the Kruskal-Wallis test (for continuous variables) and the two-tailed Fisher´s exact test or χ^2-test (for categorical variables). Survival was defined as the time from diagnosis to death or to the last observation. Overall survival was estimated according to the Kaplan-Meier method, and comparisons between curves were performed using the two-sided log rank test. Associations between overall survival and uni- and multivariate analyses were performed according to the Cox´s proportional hazard model.

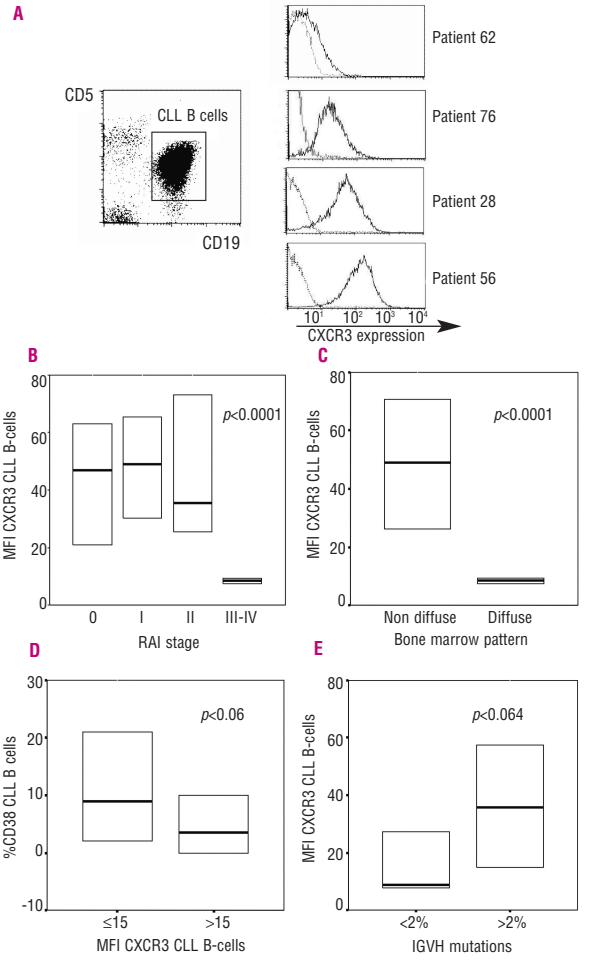

Figure 1. CXCR3 expression by peripheral blood CLL B cells and its association with patients' clinical data. A. Peripheral blood CLL B cells were selected as lymphocytes staining positive for CD19 and CD5 (left dot-plot histogram). The CXCR3 expression exhibited by four different patients is depicted in the right histogram. B. Association between CXCR3 expression (MFI) by peripheral blood CLL B cells and Rai stages. C. Association between CXCR3 expression (MFI) by peripheral blood CLL B cells and the pattern of tumor bone marrow infiltration (diffuse and non-diffuse). D. Association between CXCR3 expression by peripheral blood CLL B cells and CD38 expression (%). E. Association between CXCR3 expression by peripheral blood CLL B cells and *IgV$_H$* gene configuration status (<2% or>2% of mutations). In figures 1B, 1C, 1D and 1E results are expressed as the median (thick lines) and interquartile range (boxes) for 76, 41, 63 and 28 patients, respectively.

Results

Association between CXCR3 expression by CLL B cells and clinical data

The expression of CXCR3 by CLL B cells was examined in blood samples from 76 consecutive patients. Table 1 summarizes the expression results along with

the clinical data of these cases. As previously reported,[9-11] in all the CLL cases blood tumoral cells expressed this chemokine receptor. Nevertheless, the level of CXCR3 expression (MFI) was rather variable from patient to patient, ranging from 3.5 to 232.3 (median, 31.6). The CLL B-cell expression of the corresponding PE-conjugated isotypic negative control was stable (range 2.01-3.40). These observations are illustrated in Figure 1A. During the 31 months over which the present study was conducted, blood samples of 19 of these patients were repeatedly analyzed (3 to 6 times; mean 3.5 times); eight of these patients showed low CXCR3 expression, whereas 11 had high expression. The medians of the time intervals during which the repeated analyses were performed were 17 and 24 months for the $CXCR3^{low}$ and $CXCR3^{high}$ groups, respectively. The results obtained in these repeated analyses showed that similar values of CXCR3 expression were observed for every case (coefficients of variation < 6), thus indicating that this parameter was highly stable over time. During the period of observation, two patients with low CXCR3 expression progressed into more severe stages (one from Rai stage 0 to Rai stage II, and another from Rai stage I to Rai stage III). Two additional patients of this group (one with Rai stage III the other with Rai stage IV, respectively) died from their disease. The clinical situation in the remaining four patients (three with Rai stage 0 and one with Rai stage II) of this group, as well as in the 11 patients showing high CXCR3 expression did not change during the observation period. We then explored the possible association of CXCR3 expression with the patients' disease stage at the moment of the first detection of this parameter. Figure 1B shows that patients in Rai stages III and IV had CLL B cells showing lower levels of CXCR3 expression (Figure 1B; $p<0.0001$; Kruskal Wallis test). A feature clearly associated with CLL progression is the presence of diffuse CLL B-cell infiltrates in the bone marrow.[19] Bone marrow biopsy studies were available for 41 patients (Table 1), and Figure 1C shows that those with a diffuse infiltration pattern also exhibited lower levels of CXCR3 expression on their blood tumor cells ($p< 0.0001$; Mann-Whitney U test). Figures 1B and 1C suggest that CLL cases could be distributed into two groups according to the MFI of CXCR3 expression (those with ≤15 and those with >15). Thus, 30 patients were included in the former group ($CXCR3^{low}$) and 46 in the latter ($CXCR3^{high}$). Table 1 (right part) summarizes the results obtained by studying the patients according to this parameter, and, as expected, they confirmed previously described differences. No correlation was observed between CXCR3 expression and the values of leukocyte

Table 1. Clinical data and prognostic factors.

Variable	*All patients*	*CXCR3 MFI ≤15 CLL B cells*	*CXCR3 MFI >15 CLL B cells*	
No. of patients	76	30	46	
		Median		*p*[a]
Age (years)	72.0	73.0	70.5	NS
Leukocytes ($\times10^9$/L)	22.7	28.6	21.2	NS
Lymphocytes ($\times10^9$/L)	17.6	21.9	17.1	NS
Hemoglobin (g/L)	129.0	126.0	134.0	0.005
Platelets ($\times10^9$/L)	194.0	206.0	191.5	NS
CXCR3 expression (MFI)	31.6	8.5	54.9	0.0001
		Number of patients (%)		*p*[b]
Rai Stage				
0	25 (33%)	6 (20%)	19 (41%)	0.0001
I	21 (28%)	5 (17%)	16 (35%)	
II	12 (16%)	2 (7%)	10 (22%)	
III-IV	18 (23.7%)	17 (56%)	1 (2%)	
Bone marrow pattern				
Non diffuse	31 (75%)	6 (37%)	25 (100%)	0.0001
Diffuse	10 (25%)	10 (63%)	0 (0%)	
CD38				
<30%	52 (82%)	18 (72%)	34 (89%)	0.06
>30%	11 (18%)	7 (28%)	4 (11%)	
IgVH mutational status				
<2%	13 (46%)	9 (64%)	4 (29%)	0.05
>2%	15 (54%)	5 (36%)	10 (71%)	

NS indicates not significant; [a]The p-values are for comparisons between the two subgroups (CXCR3 MFI >15 and ≤15) and were calculated using the Mann-Whitney U test; [b]The p-values are for comparisons between the two subgroups using the two-tailed Fisher's exact test.

and lymphocyte counts, hemoglobin concentration and platelet count in these patients, although hemoglobin concentration was lower in the group of patients with CXCR3 MFI ≤15. Interestingly, 11 out of the 46 patients (24%) with Rai stage 0 or I also had low CXCR3 expression. (MFI ≤15, Table 1). The use of a MFI of 20 as a cut-off limit for CXCR3 expression gave a similar patient group distribution and, accordingly, identical results.

Relationship of CXCR3 expression with CD38⁺ CLL B cell percentage and with IgV_H gene mutational status

A high percentage (>30%) of CD38⁺ CLL B cells has been previously associated with poor prognosis.[20-22] In the present study, samples from 63 patients were available for analysis of CD38⁺ percentages. The median percentage of CD38⁺ CLL B cells was 5 (range, 0 to 90; n=63). When CD38 expression was explored in these cases separated according to their corresponding CXCR3 expression into groups with MFI ≤15 and >15, the CD38 values tended to be significantly higher in the group of patients with low CXCR3 expression (Figure 1D; $p<0.06$). In addition, when the patients were separated into cases with CD38 percentages either higher or lower than 30% ($CD38^{high}$ and $CD38^{low}$, respectively), and their corresponding CXCR3 expression into groups with MFI ≤15 and >15 MFI, the differences observed also tended to be significant (Table 1, bottom part; $p<0.06$). Similar results were obtained using 20% as the cut-off. Only one discordant case moved from the $CXCR3^{low}$ $CD38^{low}$ group to the $CXCR3^{low}$ $CD38^{high}$ group when the the CD38 cut-off was set at 20% instead of 30%. Concordance between the two parameters was observed for 41 patients (65.1%): 34 $CXCR3^{high}$ $CD38^{low}$ cases and seven $CXCR3^{low}$ $CD38^{high}$ cases. The number of patients without this concordance was 22 (34.9%), and these patients could be separated into two groups, one of 18 cases (81.8%) showing $CXCR3^{low}$ $CD38^{low}$ expression and another of four cases (18.1%) exhibiting $CXCR3^{high}$ and $CD38^{high}$ expression. Clinical data from CXCR3/CD38 combined subgroups are presented in Table 2. As can be seen, the $CXCR3^{high}$/$CD38^{low}$ group had more favorable clinical data, whereas the $CXCR3^{low}$/$CD38^{high}$ group showed worse indicators, as expected. Interestingly, the main group of patients with discordant CXCR3/CD38 expression, consisting of $CXCR3^{low}$/$CD38^{low}$ cases, presented intermediate clinical data. Finally, the small $CXCR3^{high}$/$CD38^{high}$ group (four patients) seemed to be older and have more favorable clinical data.

The mutational status of IgV_H gene is considered a good predictor of the clinical outcome of CLL, since it has been demonstrated that patients with somatic mutations in their tumoral IgV_H gene have survival times similar to those of age-matched healthy controls, whereas those harboring tumoral IgV_H genes in germ line configuration (< 2% mutations) have a worse prognosis, with a median survival of about 8 years.[2, 6, 16-18] Accordingly, CXCR3 expression by the tumoral cells of CLL patients was correlated with the mutational status of their corresponding IgV_H gene. Cells from only 30 cases of those included in the present study were available for this analysis. In two cases the study was not conclusive. The results of the valid 28 cases are given in Table 1 (bottom part). The majority of cases showing low CXCR3 expression (MFI ≤15) on blood tumor cells (9 out of 13) had unmutated IgV_H genes; in contrast, most cases with high CXCR3 levels (MFI >15) (10 out of 15) presented somatically mutated IgV_H genes ($p<0.003$). In addition, Figure 1E shows that the levels of CLL B-cell CXCR3 expression, measured as MFI, observed in the patients separated into two groups according to the number of IgV_H mutations were 8.7 and 35.6 for those with a germinal line (< 2%) and somatically mutated (> 2%) IgV_H configuration, respectively (median, $p<0.04$; Mann-Whitney U test). A specific pattern of IgV_H-family usage was not observed in these patients (*data not shown*).

CXCR3 expression level by blood CLL B cells and survival

A Kaplan-Meier analysis was performed to assess the possibility that CXCR3 expression by blood CLL B cells was associated with overall survival. Figure 2A shows that the survival time from diagnosis for CLL cases showing CXCR3 MFI ≤15 was clearly shorter than that of the cases exhibiting MFI >15 (median: 65 months; 95% CI: 40-90; $p<0.0001$). In addition, Figure 2B shows that a similar analysis of the 46 patients in Rai stages 0 and I also revealed a significant association of those CLL cases with low CXCR3 values (MFI ≤15) with an unfavorable course (median survival 108 months; 95% CI 41-175; $p<0.002$). The $CD38^{high}$ group of patients presented shorter survival (*data not shown*), as previously reported.[20-22] The effect of the combination of CXCR3 and CD38 parameters was examined in the 63 cases available. Figure 2C shows that the $CXCR3^{low}$ $CD38^{high}$ group also had a shorter survival (median: 34 months; 95% CI 10-58; $p<0.0001$), the $CXCR3^{high}$ $CD38^{low}$ combination appeared to confer a good prognosis (the median survival time was not reached), and an intermediate prospect for survival was obtained for the $CXCR3^{low}$ $CD38^{low}$ group (median: 84; 95% CI: 30-138; $p<0.0001$). The small number of cases showing $CXCR3^{high}$ $CD38^{high}$ expression apparently had a favorable prognosis.

The fact that a considerable proportion of the patients in Rai stages 0 and I exhibited a low level of CXCR3 expression (MFI ≤15) led us to explore the possible role of CXCR3 expression as a predictor of the clinical outcome for these cases. To confirm the possibility that CXCR3 expression by CLL B cells had a predictive value, a Cox proportional regression model analysis was performed. All variables analyzed were individually

Table 2. Clinical data of patients divided according to combined CXCR3/CD38 expression.

Variable	*CXCR3^{low} CD38^{low}*	*CXCR3^{low} CD38^{high}*	*CXCR3^{high} CD38^{high}*	*CXCR3^{high} CD38^{low}*	
No. of patients	18	7	4	34	
		Median			*p*[a]
Age (years)	69.0	71.0	81.0	70.0	NS
Leukocytes (×10^9/L)	25.7	66.8	33.4	20.3	0.005
Lymphocytes (×10^9/L)	17.3	52.3	28.5	16.2	0.003
Hemoglobin (g/L)	129.5	109.5	127.0	140.0	0.03
Platelets (×10^9/L)	202.5	207.5	238.0	194.0	NS
		Number of patients (%)			*p*[b]
Rai Stage					
0	6 (33%)	0 (0%)	2 (50%)	17 (49%)	0.0001
I	2 (11%)	0 (0%)	1 (25%)	10 (28%)	
II	1 (6%)	0 (0%)	1 (25%)	6 (17%)	
III-IV	9 (50%)	7 (100%)	0 (0%)	1 (6%)	

NS indicates not significant; [a] The p-values are for comparisons among the three subgroups, and were calculated using the Kruskall-Wallis test.; [b]The p-values are for comparisons among the three subgroups, and were calculated using the χ^2 - test.

evaluated, with the exception of IgV_H mutational status, which was not included due to the limited number of cases available for the assessment of this parameter. As shown in Table 3 (upper part), hemoglobin concentration, CD38 (%) and CXCR3 (MFI) expression by blood CLL B cells were significant factors in determining overall survival. In addition, Rai stages III-IV and a diffuse pattern of bone marrow infiltration also tended to be significant. Variables significantly related to survival were then included in the multivariate Cox proportional hazard regression model. As can be seen (Table 3, bottom part), this analysis revealed that low CXCR3 expression (MFI ≤15) by blood CLL B cells was the only parameter that independently and strongly predicted a poor clinical evolution (HR 24.52).

Table 3. Univariate and multivariate analyses.

	Univariate study of patients' characteristics predicting survival *Hazard ratio*	*95%CI*	*p*
Rai stage III-IV	6.8	0.861-54.23	0.06
Lymphocytes	1.005	0.995-1.014	0.33
Leukocytes	1.006	0.996-1.015	0.23
Hemoglobin	0.973	0.950-0.996	0.02
Platelets	0.996	0.987-1.005	0.38
Diffuse bone marrow infiltration	0.21	0.04-1.074	0.06
CD38 >30%	3.41	1.028-1.31	0.007
CXCR3 MFI ≤15	18.46	2.423-140.723	0.005

	Multivariate Cox proportional hazard model on CLL patients *Hazard ratio*	*95%CI*	*p*
Rai stage III-IV	0.874	0.081- 9.494	0.912
Hemoglobin	0.97	0.931- 1.031	0.999
CD38 > 30%	2.29	0.568-9.253	0.234
CXCR3 MFI ≤15	24.52	2.051 293.112	0.01

Discussion

It is now clear that CLL is a complex condition that presents with different clinical outcomes and survival rates. As a result, increasing efforts are being made to find parameters that are readily detectable at reasonable cost for general use as prognostic factors. The present study shows that CXCR3 expression is a stable parameter easily detectable on blood CLL B cells. Furthermore, low CXCR3 expression by blood CLL B cells is associated with clinical features clearly indicative of disease progression, such as diffuse bone marrow tumor infiltration, as well as with higher disease stage categories. Kaplan-Meier analysis of the patients separated into two groups according to whether their blood CLL B cells showed low (MFI ≤15) or high (MFI >15) expression of CXCR3 revealed that the former group had a significantly shorter survival. The question arises as to whether these findings only indicate an association between low CXCR3 expression and advanced stages of the disease, or whether this parameter can actually predict a poor prognosis at the onset of disease. In favor of the latter assumption is the fact that, during the period of this study (31 months), and after repeated evaluation of a large number of these patients at different times, CXCR3 expression was stable and no transition

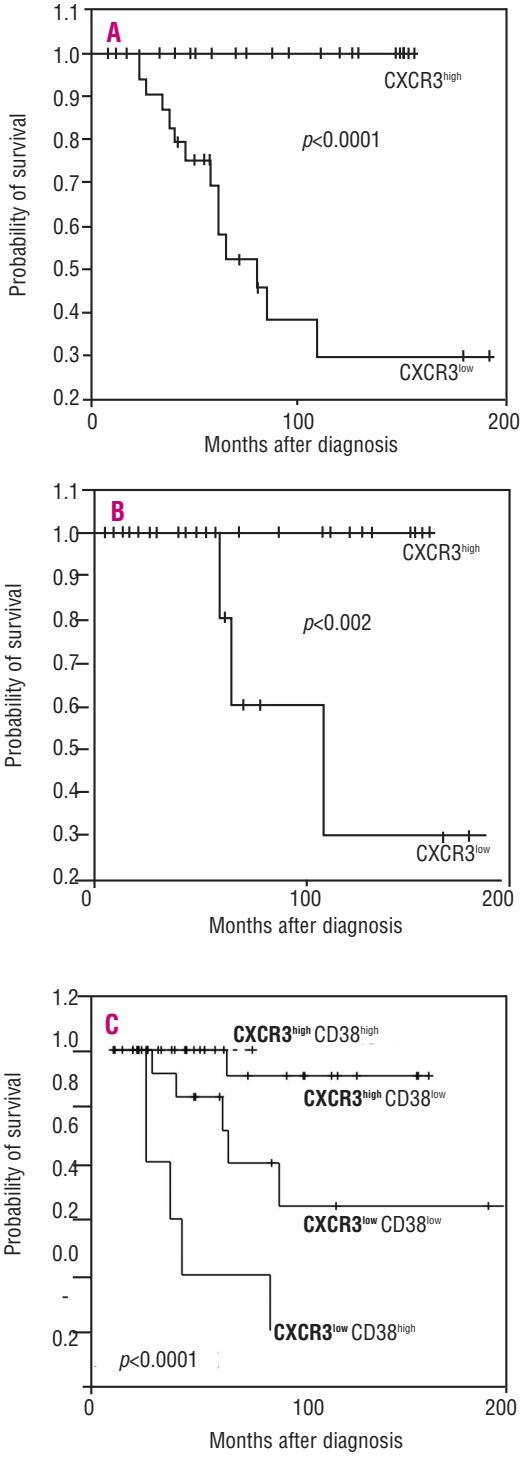

Figure 2. Kaplan-Meier estimated curves of survival probability from time of diagnosis of the CLL patients distributed according to various parameters. **A.** Survival curves of CLL patients separated into two groups according to their CXCR3 expression (MFI ≤15 or >15). N=76. **B.** Survival curves of CLL patients in Rai 0 and I stages separated into the same two groups. N=46. **C.** Survival curves of CLL patients separated according to the combination of CXCR3 expression (MFI ≤15 or >15) and CD38 expression (< or > 30%). The combination generates four different groups: $CXCR3^{low}$ $CD38^{high}$, $CXCR3^{high}$ $CD38^{low}$, $CXCR3^{low}$ $CD38^{low}$ and $CXCR3^{high}$ $CD38^{high}$ N=63.

from high to low CXCR3 expression or *vice versa* across the MFI threshold of 15 was observed. In addition, 24% of the CLL patients with Rai stage 0 or I included in this cohort of patients exhibited low expression of CXCR3 positive cells, and a Kaplan-Meier analysis of these cases also showed significant association with shorter survival. Taken together, these data indicate that low expression of CXCR3 appears to be associated with rapidly progressive CLL.

Unmutated *IgV*$_H$ gene configuration and high expression of CD38 are two parameters associated with poor prognosis in CLL.[2,6,7,16-18,20,21] Data obtained from the available 28 samples in this study revealed a good correlation between low CXCR3 expression and germline *IgV*$_H$ gene configuration, although the association between these two parameters was partial, and the overall concordance was 78% (22 of 28 samples analyzed). Therefore, mutational *IgV*$_H$ gene status and CXCR3 expression appear to be widely overlapping parameters but not totally coincidental phenomena. The present results also show that low CXCR3 expression tends to correlate with high CD38 expression. Examined in combination, the group of patients exhibiting low CXCR3 and high CD38 expression had short survival times, while the group with high CXCR3 and low CD38 expression had a favorable prognosis. However, the proportion of CXCR3/CD38 discordant cases (34.9%) was considerable, and most of these (81.8%) corresponded to patients with low CXCR3 and low CD38 expression. Moreover, this latter group had intermediate clinical data and survival probabilities (Table 2 and Figure 2C), a finding that allowed the identification of a sub-group of patients in whom low expression of CXCR3 appeared to indicate a poor prognosis. This observation is similar to those previously reported for CLL cases without concordance between CD38 expression and either ZAP-70 expression or *IgV*$_H$ mutational status.[21-23] More importantly, multivariate analysis revealed that low expression (MFI ≤15) of CXCR3 by blood CLL B cells was a clear, independent predictor of death. Collectively, these findings lead us to propose that CXCR3 expression by CLL B cells could be considered a predictive factor for prognosis.

It is well established that chemokine receptors participate in tumor invasion.[8] Nevertheless, the mechanisms by which CXCR3 might act in the pathogenesis of CLL remain unknown. CLL B cells express CXCR3, CXCR4 and CXCR5 receptors in a functional way.[9-11] In addition, the chemokine CXCL12 (SDF-1), the ligand of CXCR4, is produced by stromal cells of many organs, included the bone marrow, and it exerts not only chemotactic, but also anti-apoptotic effects on CLL B cells in *in vitro* systems.[24] Furthermore, CCR7 expression on the tumor cells of certain CLL patients has been associated with lymph node infiltration.[25] In the light of this, it has been proposed that these chemokine receptors participate in the

invasion of the bone marrow and lymph nodes by CLL B cells. Hence, the prognostic value of CXCR4 and CCR7 expression in CLL has been examined, and conflicting results have been reported. Although low CXCR4 expression has been associated with enhanced survival,[26] and high CXCR4 expression with advanced disease stages,[27] two studies including larger numbers of patients[28, 29] did not provide evidence of any relationship between these parameters and either disease stage or a diffuse pattern of bone marrow infiltration, and only showed a correlation with the number of circulating CLL B cells. Moreover, it has recently been found that tumor cells from a variety of non-Hodgkin's B-cell lymphomas, including CLL, express an intricate profile of CC and CXC chemokine receptors that exhibit marked functional heterogeneity.[30] Therefore, the mechanisms underlying the invasive capacity of CLL B cells still remain largely unknown, and factors additional to CXCR4 and CCR7 expression are likely to be implicated in the progression of the disease. Further work will be required to ascertain whether low CXCR3 expression has a role in the invasive capacity and progression of this tumor.

Author Contributions

JAB acted as principal investigator: he designed the protocols and wrote the report; EO: conducted most laboratory work and helped in manuscript preparation; LD-P: conducted laboratory genetic work; JM, AP, RF participated in the data collection; AC-C: analysis and interpretation of the genetic results.

Conflict of Interest

The authors reported no potential conflicts of interest.

References

1. Rozman C, Montserrat E. Chronic lymphocytic leukemia. N Engl J Med 1995;333:1052-7.
2. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. N Engl J Med 2005;352:804-15.
3. Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. Blood 1975; 46:219-34.
4. Rai. A critical analysis of staging in CLL. In: Gale RP, editor. Chronic lymphocytic leukemia: recent progress, future direction. Vol. 253. New York; 1987.
5. Binet JL, Auquier A, Dighiero G, Chastang C, Piguet H, Goasguen J, et al. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. Cancer 1981;48:198-206.
6. Shanafelt TD, Geyer SM, Kay NE. Prognosis at diagnosis: integrating molecular biologic insights into clinical practice for patients with CLL. Blood 2004;103:1202-10.
7. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. Immunity 2000; 12:121-127
8. Balkwill F. Cancer and the chemokine network. Nat Rev Cancer 2004;4:540-50.
9. Trentin L, Agostini C, Facco M, Piazza F, Perin A, Siviero M, et al. The chemokine receptor CXCR3 is expressed on malignant B cells and mediates chemotaxis. J Clin Invest 1999;104:115-121
10. Burger JA, Burger M, Kipps TJ. Chronic lymphocytic leukemia B cells express functional CXCR4 chemokine receptors that mediate spontaneous migration beneath bone marrow stromal cells. Blood 1999;94:3658-67.
11. Jones D, Benjamin RJ, Shahsafaei A, Dorfman DM. The chemokine receptor CXCR3 is expressed in a subset of B-cell lymphomas and is a marker of B-cell chronic lymphocytic leukemia. Blood 2000;95:627-632
12. Roy MP, Kim CH, Butcher EC. Cytokine control of memory B cell homing machinery. J Immunol 2002; 169:1676-82.
13. Sauty A, Colvin RA, Wagner L, Rochat S, Spertini F, Luster AD. CXCR3 internalization following T cell-endothelial cell contact: preferential role of IFN-inducible T cell alpha chemoattractant (CXCL11). J Immunol 2001;167:7084-93.
14. Sampalo A, Navas G, Medina F, Segundo C, Camara C, Brieva JA. Chronic lymphocytic leukemia B cells inhibit spontaneous Ig production by autologous bone marrow cells: role of CD95-CD95L interaction. Blood 2000;96:3168-74.
15. Medina F, Segundo C, Campos-Caro A, Gonzalez-Garcia I, Brieva JA. The heterogeneity shown by human plasma cells from tonsil, blood, and bone marrow reveals graded stages of increasing maturity, but local profiles of adhesion molecule expression. Blood 2002;99:2154-61.
16. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003;17: 2257-317.
17. Hamblin TJ, Davies Z, Gardiner A, Oscier DJ, Stevenson FK. Unmutated Ig VH genes are associated with more aggressive forms of chronic lymphocytic leukemia. Blood 1999;94:1848-54.
18. Damie RN, Wasil T, Fais F, Ghiotto F, Valetta A, Allen SL, et al. IgV gene mutation status and CD38 expressionas novel prognostic indicators in chronic lymphocytic leukemia. Blood 1999; 94:1840-7.
19. Rozman C, Montserrat E, Rodriguez-Fernandez JM, Ayats R, Vallespi T, Parody R, et al. Bone marrow histologic pattern--the best single prognostic parameter in chronic lymphocytic leukemia: a multivariate survival analysis of 329 cases. Blood 1984;64:642-8.
20. Deaglio S, Vaisitti T, Aydin S, Ferrero E, Malavasi F. In-tandem insight from basic science combined to clinical research: CD38 as both marker and key component of the pathogenetic network underlying chronic lymphocytic leukemia. Blood 2006;108:1135-44.
21. Domingo-Domenech E, Domingo-Claros A, Gonzalez-Barca E, Beneitez D, Alonso E, Romagosa V, et al. CD38 expression in B-chronic lymphocytic leukemia: association with clinical presentation and outcome in 155 patients. Haematologica 2002;87:1021-7.
22. Schroers R, Griesinger F, Trumper L, Haase D, Kulle B, Klein-Hitpass L, et al. Combined analysis of ZAP-70 and CD38 expression as a predictor of disease progression in B-cell chronic lymphocytic leukemia. Leukemia 2005;19:750-8.
23. Del Principe MI, Del Poeta G, Buccisano F, Maurillo L, Venditti A, Zucchetto A, et al. Clinical significance of ZAP-70 protein expression in B-cell chronic lymphocytic leukemia. Blood 2006;108:853-61.
24. Burger JA, Tsukada N, Burger M, Zvaifler NJ, Dell'Aquila M, Kipps TJ. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. Blood 2000;96:2655-63.
25. Till KJ, Lin K, Zuzel M, Cawley JC. The chemokine receptor CCR7 and a4 integrin are important for migration of chronic lymphocytic leukemia cells into lymph nodes. Blood 2002;99:2977-84.
26. Ishibe N, Albitar M, Jilani IB, Goldin LR, Marti GE, Caporaso NE. CXCR4 expression is associated with survival in familial chronic lymphocytic leukemia, but CD38 expression is not. Blood 2002;100:1100-1.
27. Ghobrial IM, Bone ND, Stenson MJ, Novak A, Hedin KE, Kay NE, et al. Expression of the chemokine receptors CXCR4 and CCR7 and disease progression in B-cell chronic lymphocytic leukemia/ small lymphocytic lymphoma. Mayo Clin Proc 2004;79:318-25.
28. Barretina J, Junca J, Llano A, Gutierrez A, Flores A, Blanco J, et al. CXCR4 and SDF-1 expression in B-cell chronic lymphocytic leukemia and stage of the disease. Ann Hematol 2003;82:500-5.
29. Dao-Ung LP, Sluyter R, Fuller SJ, Taper J, Wiley JS. CXCR4 but not CXCR3 expression correlates with lymphocyte counts in B-cell chronic lymphocytic leukemia. Ann Hematol 2004;83:326-7.
30. Trentin L, Cabrelle A, Facco M, Carollo D, Miorin M, Tosoni A, et al. Homeostatic chemokines drive migration of malignant B cells in patients with non-Hodgkin lymphomas. Blood 2004;104:502-8.