Expanded and highly active proliferation centers identify a histological subtype of chronic lymphocytic leukemia ("accelerated" chronic lymphocytic leukemia) with aggressive clinical behavior

Eva Giné,[1] Antoni Martinez,[2] Neus Villamor,[2] Armando López-Guillermo,[1] Mireia Camos,[2] Daniel Martinez,[2] Jordi Esteve,[1] Xavier Calvo,[1] Ana Muntañola,[1] Pau Abrisqueta,[1] Maria Rozman,[2] Ciril Rozman,[1] Francesc Bosch,[1] Elias Campo,[2] and Emili Montserrat[1]

[1]Hematology Department, Institute of Hematology and Oncology, Hospital Clinic, Barcelona, Spain, and [2]Hematopathology Unit, Hospital Clínic, Barcelona, Spain

ABSTRACT

Background
The concept of "accelerated" chronic lymphocytic leukemia is frequently used by both pathologists and clinicians. However, neither histological criteria to define this form of chronic lymphocytic leukemia nor its clinical correlates and prognostic impact have been formally defined in large series of patients.

Design and Methods
Tissue biopsies from 100 patients with chronic lymphocytic leukemia were analyzed for the size of proliferation centers and their proliferation rate as assessed by mitosis count and Ki-67 immunostaining. Histological patterns were correlated with main clinico-biological features and outcome.

Results
A suspicion of disease transformation was the main reason for carrying out tissue biopsy, which was performed at a median time of 14 months (range, 0 to 204 months) after the diagnosis of chronic lymphocytic leukemia. The biopsy showed histological transformation to diffuse large B-cell lymphoma in 22 cases. In the remaining 78 patients, the presence of expanded proliferation centers (broader than a 20x field) and high proliferation rate (either >2.4 mitoses/proliferation center or Ki-67 >40%/proliferation center) predicted a poor outcome and were selected to define a highly proliferative group. Thus, 23 patients with either expanded proliferation centers or high proliferation rate were considered as having "accelerated" chronic lymphocytic leukemia. These patients displayed particular features, including higher serum lactate dehydrogenase levels and more frequently elevated ZAP-70 than "non-accelerated" cases. The median survival from biopsy of patients with "non-accelerated" chronic lymphocytic leukemia, "accelerated" chronic lymphocytic leukemia and transformation to diffuse large B-cell leukemia was 76, 34, and 4.3 months, respectively (P<0.001).

Conclusions
The presence of expanded and/or highly active proliferation centers identifies a group of patients with "accelerated" chronic lymphocytic leukemia characterized by an aggressive clinical behavior.

Key words: accelerated chronic lymphocytic leukemia, lymph node biopsy, ZAP-70.

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Correspondence:
Francesc Bosch, MD, Department of Hematology, Hospital Clínic Villarroel nº 170, 08036-Barcelona, Spain.
E-mail: fbosch@clinic.ub.es

Current address of F. Bosch Department of Hematology, University Hospital Vall d'Hebron, P. Vall d'Hebron 119-129, 08035 Barcelona, Spain

Introduction

Chronic lymphocytic leukemia (CLL) is the most frequent form of leukemia in western countries and is considered to be mainly due to the accumulation of neoplastic CD5+ B lymphocytes with a typical CD19+, CD23+ immunophenotype.[1,2] According to the World Health Organization classification[3] CLL and small lymphocytic lymphoma are considered to be the same disease, primarily involving peripheral blood in the case of CLL and lymph nodes in small lymphocytic lymphoma. A number of parameters, particularly disease stage, lymphocyte doubling time, immunoglobulin heavy chain (*IGVH*) mutational status, ZAP-70 expression and cytogenetics allow the prognosis of the disease to be established at diagnosis.[2,4-6] During the course of the disease, refractoriness to treatment indicates a poor prognosis. Likewise, disease transformation implies a very short survival.

Although considered as a cumulative rather than a proliferative CD5+ B-cell neoplasm, CLL cells have a proliferation rate higher than previously recognized, particularly in the lymphoid tissues.[1,7] Enlarged nodes from patients with CLL show effacement of the lymphoid architecture with a pseudofollicular pattern of pale areas on a dark background of small cells. These pale areas correspond to proliferation centers[7-10] and are predominantly composed of clusters of prolymphocytes and paraimmunoblasts. Proliferation centers contain numerous T cells, most of which are CD4+, and in some cases a fine network of dendritic cells.[11] Notably, as compared to the non-proliferation center component of CLL, cells clustered in the proliferation centers have increased expression of the proliferation-associated markers Ki-67 and CD71, co-expression of survivin and BCL-2 and also higher expression of CD20, CD23, and MUM1/IRF-4.[11-18]

The histology of lymph nodes in CLL is heterogeneous and the relationship between different histological patterns and clinical outcome has been poorly studied.[8-10,12,18-20] The main reason for this is that tissue biopsy is not a standard procedure in the diagnostic work-up of patients with CLL. The commonest reason for performing a biopsy is to rule out histological transformation. In general, two major histological patterns in lymph nodes of patients with CLL have been recognized: typical CLL involvement and transformation of CLL to diffuse large B-cell lymphoma (DLBCL). However, the existence of other, not clear-cut histopathological patterns not pertaining to these two broad categories has been known for many years;[10] these patterns are poorly characterized and belong to a kind of "gray zone" in the histopathology of CLL. Although the term "accelerated" CLL is frequently used to describe most of these cases, no histological criteria for identifying this form have yet been proposed and its biological background and clinical significance are largely unknown. In the present study we report criteria to identify "accelerated" CLL, its clinical correlates and prognostic impact.

Design and Methods

Patients and samples

From January 1990 to December 2008, 146 patients fulfilling the diagnostic criteria of CLL according to National Cancer Institute-sponsored Working Group guidelines[21,22] underwent a tissue biopsy (except bone marrow). These cases represented 24% of the 616 patients diagnosed with CLL during this period in our institution. One hundred patients with biopsies available for pathological review were the basis of this study. Forty-six patients were not included in the study for various reasons: the biopsy was too small to allow a histological review or immunohistological analysis (31 cases), incidental diagnosis of CLL in patients with solid tumors (3 cases), composite lymphomas (3 cases), transformation to prolymphocytic leukemia (2 cases), T-cell lymphomas (2 cases) and Hodgkin's lymphoma (5 cases). The tissue analyzed was lymph node in 89 cases, spleen in 1, Waldeyer's ring in 1 and different extranodal tissues in 9.

The following biological and clinical data of each patient were evaluated and recorded both at diagnosis and at the time of the tissue biopsy: age, gender, performance status (according to the Eastern Cooperative Oncology Group [ECOG] scale), presence of B-symptoms, presence of splenomegaly or hepatomegaly, extranodal involvement, hemoglobin concentration, white blood cell count, lymphocyte count, lymphocyte doubling time, platelet count, serum albumin, lactate dehydrogenase (LDH), and β2-microglobulin levels, Rai's and Binet's staging systems, degree of lymphocytic bone marrow infiltration, ZAP-70 and CD38 expression, *IGVH* mutational status and fluorescence *in situ* hybridization (FISH) analysis for recurrent genetic abnormalities in CLL. Moreover, treatment-related variables (type of treatment and response to therapy) and the M.D. Anderson score for Richter's syndrome[23] were also recorded and evaluated.

FISH studies were performed on peripheral blood lymphocytes with the LSI p53/LSI ATM and LSI D13S319/LSI 13q34/CEP 12 Multicolor Probe Sets provided by Vysis (Downers Grove, IL, USA) using defined cut-off levels.[5] ZAP-70 expression was assessed in peripheral blood samples by flow cytometry (42 cases) or by immunostaining in lymph nodes (23 cases) as previously described, with 95% concordance between the two methods.[4,24] CD38 expression was analyzed by flow cytometry in peripheral blood and considered as increased when equal to or greater than 30%. The mutational status of the *IGVH* genes was analyzed in 27 cases following protocols described elsewhere.[4] Clonality was assessed by studying immunoglobulin heavy chain gene rearrangement from peripheral blood and paraffin-embedded tissue samples following BIOMED-2 protocols in 12 patients.[25]

Histological analyses

Tissue samples were analyzed for the presence and size of proliferation centers, and for proliferation status as assessed by the mitotic index and Ki-67 immunostaining. Histological review was performed by three investigators (AM, DM, EC) blinded to clinical records. The samples were examined in a BX51 Olympus microscope (Olympus Tokio, Japan) with a 20x objective Plan CN20x/0.40 and an ocular WHN 10x/22. Immunostaining was performed on formalin-fixed, paraffin-embedded tissue sections with p27 (1B4, Novocastra, Newcastle Upon Tyne, UK), Ki-67 (Mib1, Dako, Glostrup, Denmark), and p53 (DO7, Dako) in an automated immunostainer (BondMax, Vision BioSystems, Mount Waverley, Australia).

Proliferation centers were defined as pale areas containing large cells, prolymphocytes and paraimmunoblasts, surrounded by a dark background of small lymphocytes. Proliferation centers were also identified and delineated by the fact that they did not stain with p27.[26] Proliferation centers were arbitrarily considered as expanded when any of the major dimensions of two or more of the centers exceeded a 20x power field, which is approximately equivalent to an area of 0.95 mm^2 in the microscope used to evaluate the samples. Proliferation within the proliferation centers was

determined by both mitotic count and Ki-67 immunostaining and quantified in eight to ten proliferation centers per case. In addition, global Ki-67 and p53 were quantified in eight to ten 40x high power fields (*hpf*) randomly selected in the whole tissue section that included both proliferation centers and areas of the small cell component. The threshold for considering p53 staining as positive was 30%.

The diagnosis of transformation to diffuse large cell lymphoma was based on the World Health Organization 2008 criteria.[3] Ki-67 and p53 immunostaining was performed in cases of transformation and analyzed separately.

Statistical analysis

The correlation between different clinical and biological variables and the histological pattern was examined by means of Fisher's exact test or non-parametric tests when necessary.

Parameters obtained in the histological analysis, namely the size of proliferation centers and the proliferation index, were correlated with patients' survival. To select the optimal cut-off of the quantitative histological variables (mitotic index and Ki-67 expression) for predicting survival, a maximally selected rank statistics test was performed using the Maxstat package (R statistical package, v. 2.8.1, Vienna, Austria)[27] and the cut-off was ultimately delimitated by the Kaplan and Meier method.[28] To minimize a possible selection bias, survival was calculated from the time of biopsy and analyzed according to the method described by Kaplan and Meier and the curves compared by the log-rank test.[29] All prognostic variables in the univariate analysis for which a sufficient number of cases was available were included in the multivariate analysis using a stepwise proportional hazard Cox regression model.[30] All statistical tests were two-sided and the level of statistical significance was 0.05.

Results

Patients and tissue biopsies

The main initial clinico-biological characteristics of the patients are detailed in Table 1. The median age at diagnosis was 59 years old. Forty-six per cent of patients were in Binet stage B or C and 10% in Rai stage III-IV. The proportion of patients with increased ZAP-70 (≥20%) and CD38 (≥30%) expression was 67% and 74%, respectively. Forty-four patients were treated immediately after diagnosis and 50 additional cases required treatment during evolution with an overall median time to progression of 5 months (range, 0 to 112 months).

The reason for performing a tissue biopsy in 73 patients was the clinical suspicion of transformation to aggressive lymphoma (i.e. general symptoms, rapid lymph node enlargement, bulky extranodal involvement or increasing serum LDH levels). Other reasons for carrying out a biopsy were the diagnostic work-up of a lymphoproliferative disorder without overt peripheral blood involvement (12 cases), re-assessment of the disease before treatment (9 cases) and biopsies routinely obtained during surgical procedures (6 cases). The median time from diagnosis to biopsy was 14 months (range, 0 to 204 months) and the median survival from the time of tissue biopsy was 49 months (95% confidence interval [95% CI]: 24 to 74 months). The clinical features and outcomes of patients with CLL who did not undergo biopsy (median overall survival, 154 months) were more favorable than those who did have a biopsy (median overall survival, 90 months) (*detailed data not shown*).

Impact of expanded and highly active proliferation centers on the patients' outcome

Among the 100 patients included in this series, the diagnosis of CLL was established in 78 cases, whereas the remaining 22 patients were diagnosed as having transformation into DLBCL (DLBCL-t).

In the 78 cases included in this study not showing disease transformation to DLBCL, proliferation centers were observed in 69 and were absent in 9. In the latter, isolated prolymphocytes and paraimmunoblasts could be recognized at high magnification and corresponded to the diffuse CLL pattern described by K. Lennert.[10,31] These cases (n=9) did not exhibit other particular histological or clinical features.

Overall, 22 cases (28%) showed expanded and confluent proliferation centers broader than a 20x field. The median number of mitoses per proliferation center was 0.9 (range, 0 to 12 mitoses) and the median Ki-67 proliferation index per proliferation center was 10% (range, 0 to 75%). The median Ki-67 in the whole tissue section was 2% (range, 0 to 30%). In addition, p53-positive immunostaining was observed in 5% of the samples.

The following histological features were analyzed for their relation to the patients' survival: presence of two or more expanded and confluent proliferation centers, mitotic index, Ki-67 expression inside proliferation centers and global Ki-67 expression. Patients with expanded and con-

Table 1. Initial characteristics of 100 patients with chronic lymphocytic leukemia.

Parameters	Number
Median age (range), years	59 (24-85)
Gender	58M/42 F
ECOG score ≥2	6 (6%)
B symptoms	12 (12%)
Bulky disease	7 (7%)
Binet stage	
A	54 (54%)
B	39 (39%)
C	7 (7%)
Rai stage	
0	24 (24%)
I-II	66 (66%)
III-IV	10 (10%)
Increased serum LDH*	19 (22%)
Increased β2-microglobulin*	28 (38%)
ZAP-70 (≥20%)*	42 (67%)
CD38 (≥30%)*	45 (74%)
Cytogenetics*	
13q-	16 (43%)
11q-	7 (19%)
17p-	11 (30%)
+12	18 (48%)
IGVH mutational status (unmutated> 98%)*	23 (85%)
First-line treatment (n=92)	
Alkylators	38 (41%)
Purine analogs	34 (37%)
CHOP regimen	18 (20%)
Others	2 (2%)

*M: male; F: female. * Available in 88, 74, 63, 61, 37 and 27 patients, respectively.*

fluent proliferation centers broader than a 20x field (n=22) had a shorter survival than those with small or absent proliferation centers (hazard ratio [HR] 2.72, 95% CI 1.47-5.04; *P*=0.001) (Figure 1A). Moreover, patients with a mitotic index greater than 2.4 (n=14) had a shorter survival than patients with 2.4 or fewer mitoses (HR 2.31, 95% CI 1.1-4.5; *P*=0.016) (Figure 1B). In addition, patients with 40% or less Ki-67 expression inside proliferation centers lived longer than patients with greater than 40% Ki-67 expression (n=6) (HR 3.36, 95% CI 1.27-8.87; *P*=0.014) (Figure 1C). Global Ki-67 expression analysis did not yield a significant cut-off for survival. Among patients with expanded proliferation centers, 65% had either a mitotic rate greater than 2.4 or Ki67 expression greater than 40% within the proliferation centers.

Patients with any of the histological parameters with adverse prognosis, namely expanded proliferation centers, a mitosis count greater than 2.4 or Ki67 expression greater than 40% per proliferation center, were considered as having "accelerated" CLL (n=23). The remaining patients with no adverse histological features were considered as having "non-accelerated" CLL (n=55). The main histological features of patients with "non-accelerated" CLL, "accelerated" CLL, and DLBCL-t are summarized in Table 2. Representative histological images are shown in Figure 2. Among other histological variables studied, the global Ki-67 immunostaining was higher in DLBCL-t than in "accelerated" CLL and "non-accelerated" CLL (mean ± SD of Ki-67: 68%±14% *versus* 12%±8.5% *versus* 2%±2.4%, respectively; *P*=0.001). In addition, immunostaining for p53 was positive in 67%, 18% and 2% of patients with DLBCL-t, "accelerated" CLL and "non-accelerated" CLL, respectively (*P*<0.0001).

Analysis of clonality

To ascertain whether tissue samples of "accelerated" CLL were clonally related to the CLL neoplastic lymphocytes from peripheral blood, *IGVH* rearrangement was analyzed in eight patients. The same kind of analysis was also performed in four patients with DLBCL-t. All patients with "accelerated" CLL showed the same clonal rearrangement in tissue and peripheral blood. In contrast, one out of four DLBCL-t had clonally unrelated disease.

Clinico-biological features and outcome of "accelerated" chronic lymphocytic leukemia

The main characteristics of the patients at the time of tissue biopsy are listed in Table 3. Patients with "accelerated" CLL had similar characteristics to those with "non-accelerated" CLL cases with regard to the presence of B symptoms, performance status, bulky disease or clinical stage. However, patients with "accelerated" CLL" had higher serum LDH levels and more elevated ZAP-70 expression (*P*<0.01), than patients with "non-accelerated" CLL.

On the other hand, patients with DLBCL-t more frequently had a poor performance status and B symptoms than did patients with "accelerated" or "non-accelerated" CLL. It is of note that in nine out of 22 cases (41%) with DLBCL-t, disease transformation was diagnosed in an extranodal site, whereas in patients with CLL (either "accelerated" or "non-accelerated") only one extranodal case (breast mass) was observed (*P*<0.001). Serum LDH levels were found to increase progressively from "non-accelerated" CLL to "accelerated" CLL and to DLBCL-t

Table 2. Histological characteristics of "non-accelerated", "accelerated" chronic lymphocytic leukemia and DLBCL-t cases.

Features	Non-accelerated CLL (n=55)	Accelerated CLL (n=23)	DLBCL-t (n=22)
Proliferation centers			
Absent	9 (16%)	0	
Small	46 (84%)	1 (4%)	NA
Expanded	0	22 (96%)	
Mitoses/PC*			NA
Median (range)	0.4 (0-2.4)	2.5 (1-12)	
≤2.4	46 (100%)	6 (27%)	
>2.4	0	16 (73%)	
Ki67/PC (%)†			NA
Median (range)	5 (0-30)	35 (3-75)	
≤40%	39 (100%)	16 (73%)	
>40%	0	6 (27%)	
Ki67 global (%)			
Median (range)	1 (0-10)	13 (5-30)	70 (50-90)

*PC: proliferation center; DLBCL-t: diffuse large B cell lymphoma transformation; NA: not apply; *evaluable in 69 cases; †evaluable in 61 cases.*

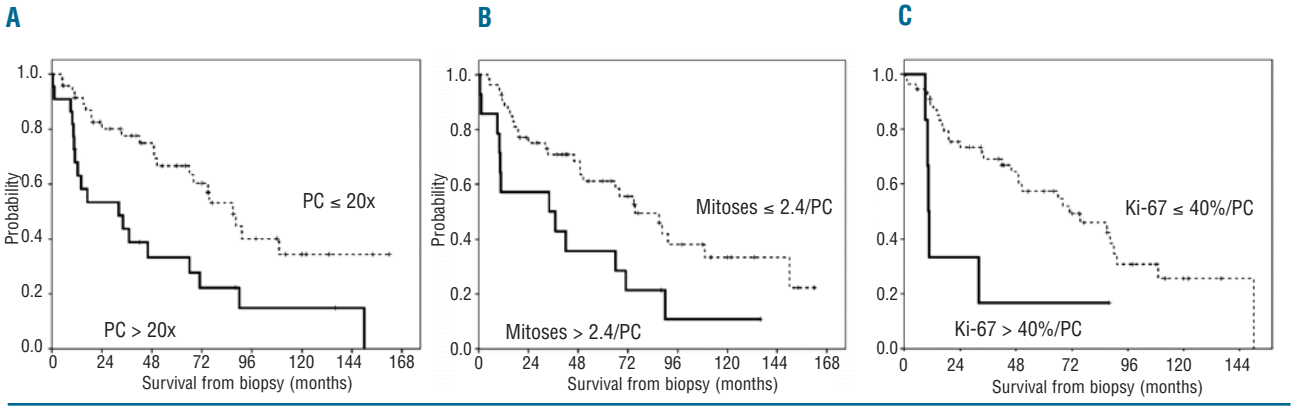

Figure 1. Survival from the time of tissue biopsy of patients with non-transformed CLL according to the characteristics of proliferation centers (PC): (A) presence of expanded PC broader than a 20x field (17 *versus* 75 months, PC broader than a 20x field *versus* PC not broader than a 20x field, respectively; *P*=0.001); (B) >2.4 mitoses per PC (34 *versus* 75 months, >2.4 mitoses/PC *versus* ≤2.4 mitoses/PC, respectively; *P*=0.016); (C) >40% Ki-67 positivity per PC (11 *versus* 68 months, >40%/PC *versus* ≤40%/PC, respectively; *P*=0.014).

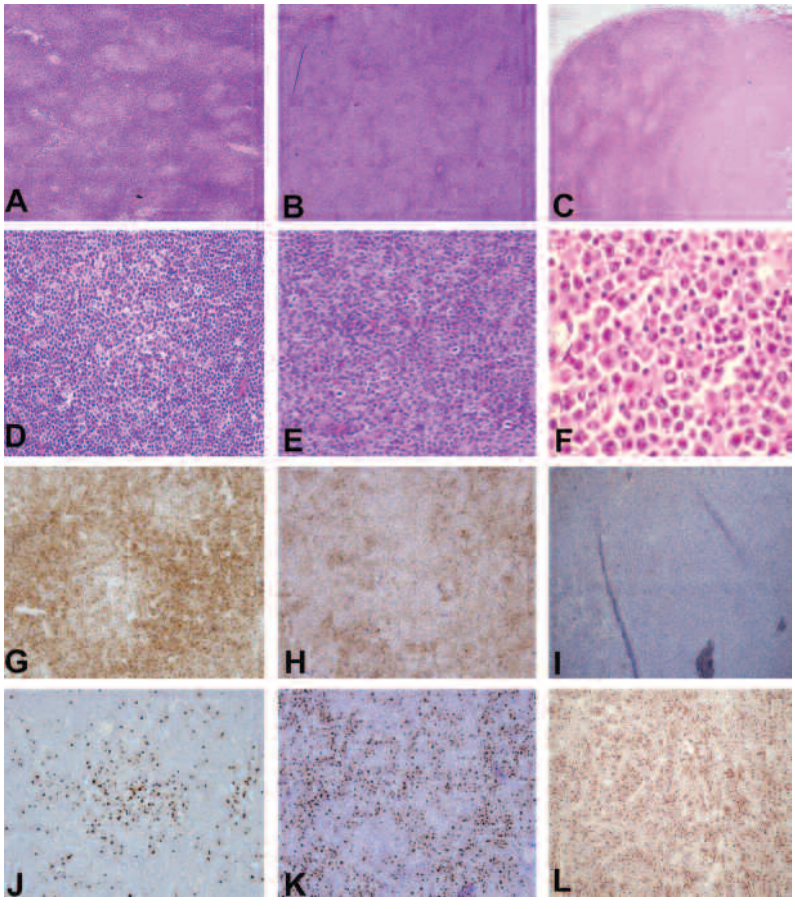

Figure 2. (A-D-G-J). A representative case of lymph node involvement by chronic lymphocytic leukemia. (A) At low magnification the tumor shows the typical biphasic growth pattern with dark areas representing the small lymphocytic component and the clear areas corresponding to small proliferation centers (PC) (hematoxylin-eosin stain, Olympus DP70, X10). (D) The PC are small (lesser than 20x) and contain some large prolymphocytic cells. Scattered mitoses may also be seen (hematoxylin-eosin stain, Olympus DP70, X20). PC are highlighted by negative staining with p27 (G), and low Ki-67 labeling index (J). (B-E-H-K): A representative CLL case with expanded proliferation centers (B, E) wider than a 20x field. PC are highlighted by negative staining with p27 (H). The mitotic count as well as the Ki-67 index (K) is high. (C-F-I-L): Representative sections of DLBCL transformation of a CLL case. (C) Partial involvement of the lymph node by a diffuse proliferation (bottom) of large immunoblastic cells (F). p27 is negative in the tumor cells (I) that shows a very high proliferative rate (L).

Table 3. Main features of patients at biopsy according to histological findings.

Characteristics	Non-accelerated CLL (n=55)	Accelerated CLL (n=23)	DLBCL-t (n=22)
Median age (range), years	61 (34-85)	59 (34-84)	63 (39-83)
Gender	34M/21 F	11M/12 F	13M/9 F
ECOG ≥2	8 (17%)	4 (20%)	7 (41%)
B symptoms	11 (22%)	3 (16%)	8 (44%)
Bulky disease	12 (22%)	3 (14%)	5 (28%)
Binet stage			
A	19 (39%)	7 (35%)	3 (18%)
B	19 (39%)	4 (20%)	6 (35%)
C	11 (22%)	9 (45%)	8 (47%)
Rai stage			
0*	1 (2%)	–	2 (13%)
I	24 (49%)	6 (30%)	3 (17%)
II	12 (25%)	5 (25%)	3 (17%)
III	6 (12%)	4 (20%)	3 (17%)
IV	6 (12%)	5 (25%)	6 (36%)
Extranodal involvement	1 (0.01)	0‡	9 (41%)
LDH (>450 UI/L)	19 (38%)	16 (73%)†	12 (71%)
β2-microglobulin (>2.3 mg/dl)	21 (48%)	11 (69%)	7 (70%)
IGVH mutational status (unmutated> 98%)	10 (77%)	7 (100%)	6 (86%)
ZAP-70 (≥20%)	19 (53%)	16 (89%)†	7 (78%)
Prior therapies			
0	39 (74%)	10 (45%)†	5 (25%)
1	4 (7%)	5 (23)	5 (25%)
>1	10 (19%)	7 (32%)	10 (50%)
Resistance to the last treatment¶	4 (29%)	6 (50%)	5 (33%)

*DLBCL-t: Diffuse large B-cell lymphoma transformation. * corresponding to abdominal mass. ¶ % of previously treated patients. Significant P values for accelerated* versus *non-accelerated cases (†): increased serum LDH (P=0.01), elevated ZAP-70 (P=0.014) and ≥1 lines of treatment (P=0.032). Significant P value for accelerated* versus *DLBCL-t (†) cases: extranodal involvement (P=0.0006)*

(mean values ± SD in IU/L: 455±194 *versus* 543±198 *versus* 820±538, respectively; *P*=0.008). Among the three histological groups, no differences in the distribution of poor risk cytogenetic alterations (17p and 11q deletions) were observed.

Time from diagnosis to tissue biopsy was longer in patients with DLBCL-t than in those with CLL (mean: 69 *versus* 45 months, respectively, *P*=0.001). While nine out of the 23 "accelerated" CLL cases were observed at diagnosis, only two out of the 22 DLBCL-t cases were observed at that moment. Forty-five percent of patients with "accelerated" CLL were diagnosed before starting treatment.

In two patients with "accelerated" CLL new tissue biopsies were obtained during follow-up. In one case the pattern of "accelerated" CLL remained stable over time, whereas in the second one transformation to DLBCL was demonstrated after 5 years.

The presence of expanded or highly active proliferation centers did not influence the treatment decision. Treatment for patients with "accelerated" CLL varied over time. Whereas 52% of patients received doxorubicin-containing regimens, 30% of patients were treated with purine analogs alone or in combination. In contrast, 73% of patients diagnosed with DLBCL-t were treated with doxorubicin-containing regimens. Therapy intensification with stem cell transplantation was carried out in 16 patients, including six patients with "non-accelerated" CLL, six patients with "accelerated" CLL, and four patients with DLBCL-t.

After a median follow-up of 63 months (range, 5.5 to 162 months) from tissue biopsy, 64 of the 100 patients had died. The cause of death was related to the disease in the 18 patients with DLBCL-t who died, in 17/19 "accelerated" cases and in 26/29 cases of "non-accelerated" CLL. The median survival from the time of tissue biopsy for patients with DLBCL-t, "accelerated" CLL and "non-accelerated" CLL was 4.3, 34 and 76 months, respectively (*P*<0.001) (Figure 3). In addition, when cases without suspicion of transformation at the time of biopsy (n=27) were excluded from the analysis, the median survival was 4.3 months in patients with DLBCL-t (n=22), 34 months in those with "accelerated" CLL (n=21) and 49 months in those with "non-accelerated" CLL (n=30) (*P*=0.032).

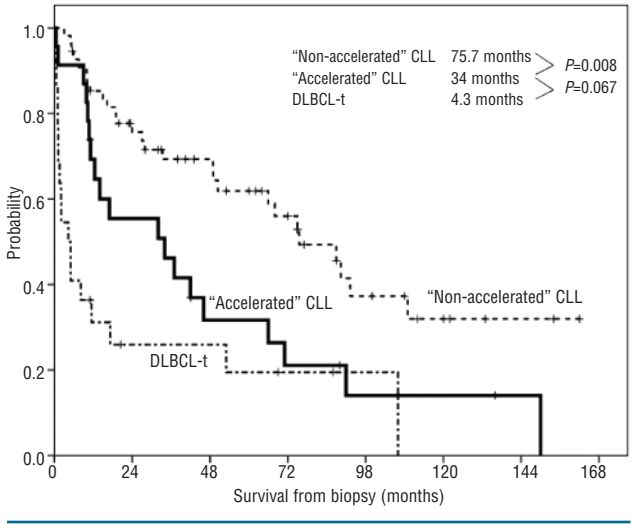

Figure 3. Survival from biopsy according to the histological patterns of "non-accelerated" CLL, "accelerated" CLL and DLBCL transformation: median survival 76 months, 34 months and 4.3 months, respectively (*P*<0.001).

Other variables that, at the time of biopsy, correlated with poor survival were: age (>60 years), ECOG performance status (≥2), Binet's and Rai's advanced clinical stage, B symptoms, bulky disease, hemoglobin level (<110 g/L), platelet count (<100×10^9/L), elevated serum levels of LDH and beta-2-microglobulin, high ZAP-70 expression and 17p deletion. The M.D. Anderson score for Richter's syndrome was also predictive for survival in the whole series and in the subsets of patients with DLBCL-t and "accelerated" CLL.

In a multivariate survival analysis, the histological subtype ("non-accelerated" *versus* "accelerated" *versus* DLBCL-t) (*P*≤0.001; relative risk [RR] 2.7 and 5 .5 for "accelerated" and DLBCL-t *versus* "non-accelerated", respectively) together with age (*P*≤0.001; RR 1.05) and the M.D. Anderson score for Richter's syndrome (0-1 *versus* ≥2; *P*<0.001; RR 3.9) were the most important prognostic variables in the global series (analysis performed in 94 patients for whom all the data were available). When the same analysis was performed after excluding patients with DLBCL-t, the histological subtype ("non-accelerated" *versus* "accelerated") (*P*=0.007; RR 2.5) along with age (*P*=0.002; RR 1.05) and M.D. Anderson score for Richter's syndrome (*P*=0.001; RR 3.6) maintained their prognostic importance.

Discussion

An increasing body of data suggests that proliferative centers play an important role in the biology of CLL as they constitute its proliferative compartment.[1,7] However, the complexity of the microenvironment of proliferation centers has not been completely elucidated.[32] In addition, the clinical significance of the number and size of proliferation centers, where proliferation of CLL takes place, remains to be established. Historically, Lennert[10,31] recognized a histological "tumor-forming" type of CLL characterized by an excessive prolymphocyte proliferation, resulting in large lighter regions when examined under the microscope. These "tumor-forming" type CLL cases were considered to have a poor prognosis but no clinical studies were performed at that time. Since then, studies have been conducted in an attempt to correlate histological characteristics of lymph nodes with prognosis but most were flawed for a number of reasons, including lack of immunophenotypic studies,[8,9,20] limited numbers of subjects and short follow-up.[12,18,19]

In our series, proliferative centers were found in 88% of the biopsies and their size and proliferation activity were important predictors of duration of survival from the time of biopsy. Thus, patients with either proliferation centers broader than 20x *hpf* or an increased proliferation rate within the proliferation centers as assessed by a mitotic count greater than 2.4 per proliferation centers and/or Ki-67 index greater than 40% per proliferation centers, had a particularly poor outcome.

From a clinical point of view, the features of "non-accelerated" and "accelerated" CLL patients were comparable except for the observed higher frequency of elevated LDH levels and ZAP-70 expression in "accelerated" cases. While

the latter were also clinically undistinguishable from DLBCL-t cases, "accelerated" CLL was observed in around half of the cases early on in the clinical course of the disease, in untreated patients and mainly with nodal involvement. In contrast, DLBCL-t tended to occur later in the course of the disease, usually in pretreated patients and frequently in those with extranodal involvement. Moreover, survival curves for patients with "accelerated" CLL or DLBCL-t were different, although this difference did not reach statistical significance (34 months *versus* 4 months, P=0.07).

A predominance of poor prognostic markers was found in our series compared to in patients with standard CLL.[33,34] This is not unexpected since the main reason for performing a biopsy was the suspicion of histological transformation. An increasing number of p53-positive cases was found when comparing patients with "non-accelerated" CLL to those with "accelerated" CLL and to those with DLBCL-t. Although the high frequency of p53 alterations has been extensively recognized in DLBCL transformation,[35,36] the relative small number of cases in the latter two subsets precludes any definitive conclusion.

"Accelerated" CLL probably reflects a biological state of tumor CLL cells characterized by a high proliferation resulting in an aggressive form of the disease and, hence, a poor prognosis.

Data obtained in the present work emphasize the importance of lymph node biopsy in CLL. As a general recommendation, all patients with a suspicion of clinical transformation and/or an aggressive clinical behavior (refractoriness to treatment, high cell turnover) should undergo tissue biopsy. Moreover, the identification of "accelerated" CLL is easy in clinical practice since it is based on standard laboratory techniques and it should improve the clinical and therapeutic management of patients with CLL. Ideally, these observations should be confirmed by other groups. On the other hand, whether or not patients with "accelerated" CLL should immediately receive intensive treatment needs to be addressed in prospective clinical trials.

In conclusion, in this study we present easy-to-apply histological criteria to identify "accelerated" CLL and show that this form of disease is associated with poor prognostic features and short survival. Further studies are needed to clarify the molecular events underlying this form of the disease, its boundaries with disease transformation, and the most appropriate treatment approach for the patients.

Authorship and Disclosures

AM, DM and EC performed the histological review; EG and XC collected clinical data; NV, MR, MC, JE, AL, AM, and PA provided study materials and patients; FB, AL, CR and EG performed the statistical analysis; EG, AM, FB, AL and EC designed the study; EG, AL, FB, EC and EM wrote the paper.

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