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Association between the proliferative rate of neoplastic B cells, their maturation stage, and underlying cytogenetic abnormalities in B-cell chronic lymphoproliferative disorders: analysis of a series of 432 patients

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Limited knowledge exists about the impact of specific genetic abnormalities on the proliferation of neoplastic B cells from chronic lymphoproliferative disorders (B-CLPDs). Here we analyze the impact of cytogenetic abnormalities on the proliferation of neoplastic B cells in 432 B-CLPD patients, grouped according to diagnosis and site of sampling, versus their normal counterparts. Overall, proliferation of neoplastic B cells highly varied among the different B-CLPD subtypes, the greatest numbers of proliferating cells being identified in diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma (BL). Compared with normal B cells, neoplastic B-CLPD cells showed significantly increased S + G_2/M-phase values in mantle cell lymphoma (MCL), B-chronic lymphocytic leukemia (B-CLL), BL, and some DLBCL cases. Conversely, decreased proliferation was observed in follicular lymphoma, lymphoplasmacytic lymphoma/Waldenström macroglobulinemia (LPL/WM), and some DLBCL patients; hairy cell leukemia, splenic marginal zone, and MALT-lymphoma patients showed S + G_2/M phase values similar to normal mature B lymphocytes from LN. Interestingly, in B-CLL and MCL significantly higher percentages of S + G_2/M cells were detected in BM versus PB and in LN versus BM and PB samples, respectively. In turn, presence of 14q32.3 gene rearrangements and DNA aneuploidy, was associated with a higher percentage of S + G_2/M-phase cells among LPL/WM and B-CLL cases, respectively. (Blood. 2008;111:5130-5141)

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Introduction

Malignant transformation of neoplastic cells, including B lymphocytes, has long been associated with genetic abnormalities involving cell proliferation and death signaling pathways.[1-5] Accordingly, high grade B-cell non-Hodgkin lymphomas (NHLs; eg, Burkitt lymphoma) have been considered to have an abnormally increased cell proliferation rate, linked to constitutive activation of proto-oncogenes (eg, *C-MYC*).[6-8] In contrast, an increased cell survival because of a prominent antiapoptotic phenotype (eg, high bcl2 or cyclin D1 expression)[9-13] has been associated with low-grade B-NHL and most chronic lymphoid leukemias.[1] However, a careful analysis of the literature shows very limited information about the real impact of specific genetic abnormalities associated with different subtypes of both high- and low-grade B-cell chronic lymphoproliferative disorder (B-CLPD) on the proliferation rate of primary tumor cells, in comparison with their normal counterparts.

Such comparison could contribute to a better understanding of the real impact of specific genetic abnormalities in the proliferation rate of tumor cells arrested at specific stages of maturation; in turn, this information is also of potential clinical relevance, since measurement of the proliferative activity of neoplastic cells by semiquantitative immunohistochemical techniques (eg, ki67 or PCNA immunostaining),[14-19] ^{3}H thymidine incorporation,[20] and single-parameter flow cytometric analysis of paraffin-embedded tumor samples[21-27] is considered to be of great help for the prognostic stratification of B-cell chronic lymphoproliferative disorders (B-CLPDs),[21-23,26-33] even within specific histologic and WHO subtypes (eg, mantle cell lymphoma).[34]

Despite this, evaluation of tumor cell proliferation has only partially translated into routine clinical practice. This is due to a great variability with respect to numbers of cells analyzed, the use of different sampling (eg, fresh tissue vs paraffin-embedded material), and sample preparation and staining techniques. In addition, in the case of flow cytometry, the coefficients of variation of the G_0/G_1 peaks and the mathematical models for cell-cycle

Submitted October 19, 2007; accepted March 4, 2008. Prepublished online as *Blood* First Edition paper, March 12, 2008; DOI 10.1182/blood-2007-10-119289.

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phase calculations are highly variable[21,23,32]; moreover, assessment of the overall percentage of proliferating cells in the whole sample, and not exclusively among neoplastic B cells, leads to an additional increasing degree of variability.[35]

Here, we analyze the potential impact of the most frequent genetic abnormalities on the proliferative rate of neoplastic B cells from a series of 432 B-CLPD patients grouped according to diagnosis and site of sampling in comparison with their normal counterparts. For that purpose, a detailed analysis of the proliferative rate of different compartments of maturation-associated subsets of bone marrow (BM), peripheral blood (PB), and reactive lymph node (RLN) B cells is provided as a frame of reference for the identification of an altered cell-cycle distribution in B-CLPD.

Methods

This study was approved by the Ethical Committee of the University Hospital of Salamanca. Informed consent was obtained in accordance with the Declaration of Helsinki.

Patients and samples

A total of 432 untreated patients (260 males and 172 females with a mean age of 66 ± 13 years; range: 19 to 95 years), newly diagnosed with B-CLPD between September 2000 and February 2007, were included in this study. In all cases, diagnosis was established according to the WHO criteria[36,37] with the following distribution: B-cell chronic lymphocytic leukemia (B-CLL), 210 patients; hairy cell leukemia (HCL), 7; mantle-cell lymphoma (MCL), 39; splenic marginal zone B-cell lymphoma (SMZL), 16; MALT lymphoma (MALT-NHL), 20; follicular lymphoma (FL), 71; diffuse large B-cell lymphoma (DLCL), 19; Burkitt lymphoma (BL), 14; and lymphoplasmacytic lymphoma/Waldenström macroglobulinemia (LPL/WM), 36. Immunophenotypic analyses, DNA cell content measurements, and interphase fluorescence in situ hybridization (iFISH) studies were performed on EDTA-anticoagulated PB (39%), BM (43%), and fine needle–aspirated (FNA) lymph node (LN; 18%) samples.

Immunophenotypical studies

Immunophenotypic analysis of neoplastic B cells was performed in all 432 B-CLPD cases after staining of erythrocyte-lysed BM and PB as well as LN samples, according to procedures that have been previously described in detail.[38] The following fluorocrome-conjugated—fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/peridinin chlorophyll protein-cyanin 5.5 (PerCPCy5.5)/allophycocyanin (APC)—combinations of monoclonal antibodies (MAbs) were used: CD8-sIgλ/CD56-sIgκ/CD4-CD19/CD3, CD22/CD23/CD19/CD20, CD103/CD25/CD19/CD11c, FMC7/CD24/CD19/CD34, cytoplasmatic (Cy) bcl2/CD10/CD19/CD38, surface immunoglobulin (sIg)λ/sIgκ/CD19/CD5, sIgμ/CD27/CD19, and CD43/CD79b/CD19. All MAb reagents were purchased from BD Biosciences (San Jose, CA) except anti-CD79b, CD24, CD10, and CD43, which were obtained from Immunotech (Marseille, France) and antihuman sIgμ, antihuman sIgλ, and antihuman sIgκ, which were purchased from DAKO (Glostrup, Denmark). For the staining of Cybcl2, the Fix & Perm reagent kit (Invitrogen, Burlingame, CA) was used, strictly following the recommendations of the manufacturer. In all cases, one tube stained with CD19-PerCPCy5.5 was used as a control for the specific evaluation of baseline green, orange, and deep red autofluorescence of B cells.

Immediately after sample preparation was completed, data acquisition was performed on a FACSCalibur flow cytometer using the CellQUEST software program (BD Biosciences). For each combination of MAbs, information about 5×10^4 events corresponding to the whole sample cellularity was acquired and stored. In a second step, information on events acquired through an electronic live gate containing only CD19$^+$ cells was specifically stored (median of 201 643 and 95% confidence interval of 169 641 to 233 645 events). For data analysis, the PAINT-A-GATE-PRO software program (BD Biosciences) was used. For each sample, all major subpopulations of lymphocytes were measured including B cells, T cells, and their major CD3$^+$/CD4$^+$ and CD3$^+$/CD8$^+$ subsets and CD56$^+$/CD3$^-$ natural killer (NK) cells. CD19 was used for the specific identification of B cells present in all sample aliquots, and their percentage was calculated after excluding cell debris and platelets according to conventional procedures.[38] Identification of B-cell precursors versus mature B lymphocytes was performed on the basis of a low to intermediate CD45 expression on CD19$^+$ cells. In 57 B-CLPD cases showing dim CD19 expression, CD20 was used to calculate the percentage of B cells in the sample.

Table 1. Chromosomal abnormalities detected by iFISH in B-CLPD subtypes

B-CLPD/gene	Chromosomal abnormality	Probe
B-CLL		
MDM2?[39]	trisomy 12	CEP12*
RB1/D13S25[40]	del(13q)	LSI 13/RB1 (13q14)/LSI D13S25 (13q14.3)*
ATM/MLL[40,41]	del(11q)	LSI ATM (11q22.3)/LSI MLL (11q23.3)*
P53[40]	del(17p)	LSI P53 (17p13.1)*
MCL		
CYCLIN D1[42,43]	t(11;14)	LSI IGH/CCND1*
MALT		
MLT1[44,45]	t(18q21)	LSI MALT1*
IGH	t(14q32)	LSI IGH*
FL, BCL2[43,46]	t(14;18)	LSI IGH/BCL2*
DLBCL, BCL6[47]	t(3q27)	LSI BCL6*
BL, C-MYC[48]	t(8;14)	LSI IGH/MYC/CEP8*
LPL/WM		
IGH[49]	t(14q32)	LSI IGH*
BLIMP1[50,51]	del(6q21)	6q21 cocktail probe†

B-CLPD indicates B-cell chronic lymphoproliferative disorders; B-CLL, B-cell chronic lymphocytic leukemia; MCL, mantle-cell lymphoma; MALT, mucosa-associated lymphoid tissue lymphoma; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; BL, Burkitt lymphoma; and LPL/WM, lymphoplasmacytic lymphoma/Waldenström macroglobulinemia.

*Vysis.

†Q-BIOgene.

Interphase fluorescence in situ hybridization (iFISH) studies

Analysis of the genetic alterations associated with B-CLPD was systematically performed by multicolor iFISH. For the detection of trisomy 12, del(11q22.3), del(11q23), del(17p13.1), and del(13q14), the following DNA probes purchased from Vysis (Downers Grove, IL) were used in multicolor stainings (Table 1): CEP12, LSI ATM (11q22.3) and LSI MLL (11q23.3) dual-color probe, LSI p53 (17p13.1) and both the LSI 13/RB1 gene (13q14) and LSI D13S25 (13q14.3) DNA probes. In turn, presence of t(14q32), t(18q21), t(14;18), t(11;14), and *c-MYC* gene rearrangements was assessed using the LSI IgH dual-color, LSI MALT1 dual-color, LSI IgH/Bcl2 dual-color, LSI IgH/CCND1 dual-color and both the LSI MYC dual-color breakapart and LSI IgH/MYC/CEP8 3-color probes, respectively. DNA probes for the detection of *BCL6* gene rearrangements (LSI BCL6 dual-color breakapart probe; Vysis) and del(6q21) (6q21 cocktail probe; Q-BIOgene, Amsterdam, The Netherlands) were also used (Table 1).

FISH studies were performed on freshly obtained, fluorescence-activated cell sorting (FACS)–purified neoplastic B cells (purity of 98% ± 0.82%) fixed in 3:1 (vol/vol) methanol–acetic acid, prepared, and hybridized as previously described in detail.[52] The number of hybridization spots was evaluated using a BX60 fluorescence microscope (Olympus, Hamburg, Germany) equipped with a 100× oil objective. For each slide, the number of hybridization spots with a similar size, intensity, and shape was counted for at least 200 nuclei, the number of unhybridization cells in the areas assessed being less than

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1%. Cutoff values used for the definition of each chromosomal abnormality were established according to the iFISH patterns observed in a group of 10 age- and sex-matched normal control BM samples studied with the same probes (mean percentage + 2 standard deviations [SD]): chromosome 12, more than 1%; del(13q14), more than 4%; del(17p13.1), more than 7%; del(11q22.3), more than 4%; del(11q23.3), more than 1%; t(14;18), more than 1%; *c-MYC* gene rearrangements, more than 1%; t(11;14), more than 1%; and; del(6q21), more than 6%.

Flow cytometric analysis of B-cell DNA contents

The DNA content of neoplastic B cells was measured in a FACSCalibur flow cytometer (BD Biosciences) using the Cycloscope NHL-B reagent kit (Cytognos, Salamanca, Spain), strictly following the recommendations of the manufacturer. DNA aneuploidy was defined by the observation of a different DNA content for G_0/G_1 tumor B cells compared with normal residual (T/NK) lymphocytes; DNA index of neoplastic B cells was calculated as the ratio between the modal fluorescence channel of G_0/G_1 tumor B cells and that of the normal residual G_0/G_1 diploid T/NK lymphocytes present in the same sample. Analysis of the distribution of B-CLPD cells in the G_0/G_1 and $S + G_2/M$ cell-cycle phases was performed according to well-established methods, after specifically selecting CD19/CD20/CD22/CD23 FITC-stained B cells and excluding debris and cell doublets.

In parallel, analysis of the cell-cycle distribution of normal B cells was performed on a total of 20 samples—10 BM, 5 PB, and 5 reactive LNs—obtained from an identical number of adult volunteers without any known hematologic disorder and normal blood cell counts. For this purpose, normal BM, PB, and RLN samples were stained with DRAQ5 (Cytognos), together with CD45-FITC/CD19-PE, CD38-FITC/CD19-PE for B17, CD20-FITC/CD23-PE for PB, and CD38-FITC/CD20-PE (all purchased from BD Biosciences), for LN sacrophage as previously described in detail.[53] Briefly, after being stained with saturating amounts of the above-listed monoclonal antibodies (MAbs) and lysed (Quicklysis; Cytognos), cells were resuspended in 0.5 mL PBS to which 3 μL DRAQ5 (Vitro SA, Madrid, Spain) was added; afterward, an incubation was performed for 10 minutes in the dark at room temperature (RT). Immediately after this incubation period, sample aliquots were measured in a FACSCalibur flow cytometer using the CellQUEST software program. For each sample aliquot, information about 5×10^5 cells was stored. For data analysis, the PAINT-A-GATE software program (BD Biosciences) was used. For each sample, the following cell populations were identified after excluding dead cells and cell doublets in a sideward light scatter (SSC) versus DRAQ5 fluorescence area and a DRAQ5 fluorescence area versus DRAQ5 fluorescence width bivariate dot plot (Figure 1): early $CD45^{lo}/CD19^{lo}$ B-cell progenitors, late $CD45^{+}/CD19^{hi}$ B-cell precursors, mature $CD45^{hi}/CD19^{+}$ B lymphocytes and $CD38^{hi}/CD19^{-/+}$ plasma cells in BM samples (Figure 1A-D); mature $CD20^{+}/CD23^{+}$ and $CD20^{+}CD23^{-}$ B lymphocytes in PB (Figure 1E-F); and mature $CD38^{lo}/CD20^{+}$, $CD38^{+}CD20^{hi}$ B lymphocytes and $CD38^{hi}/CD20^{-/lo}$ plasma cells in RLN samples (Figure 1G-I).

Statistical methods

For all continuous variables studied, their means and standard deviations (SDs), medians, and ranges were calculated using the SPSS software program (SPSS 12; Chicago, IL). To establish the statistical significance of the differences observed between groups, either the ANOVA or the Student *t* test and either the Kruskal-Wallis or Mann-Whitney *U* tests were used for continuous variables showing a parametric and nonparametric distribution, respectively. For categoric variables, frequencies were calculated and the chi-square test was used to establish the statistical significance of differences observed between groups. Survival curves were plotted according to the method of Kaplan and Meier, and the log-rank test was used to assess the statistical significance of the differences observed between curves. *P* values less than .05 were considered to be associated with statistical significance.

Results

Cell-cycle distribution of different normal B-cell compartments

In normal BM, the proliferative rate of the B-cell populations significantly ($P < .001$) decreased with B-cell maturation from early $CD45^{lo}/CD19^{lo}$ B-cell progenitors, to the late $CD45^{+}/CD19^{hi}$ B-cell precursors, and the more mature $CD45^{hi}/CD19^{+}$ B lymphocytes ($S + G_2/M$ 7% ± 2.4%, 3% ± 1.2%, and 0.5% ± 1.1%, respectively; Table 2). In PB, even lower percentages of $S + G_2/M$ cells were found among mature $CD20^{+}/CD23^{+}$ (0.1% ± 0.8%) and $CD20^{+}CD23^{-}$ (0.1% ± 0.05%) B lymphocytes (Table 2).

Mature $CD38^{lo}/CD20^{+}$ B lymphocytes from reactive lymph nodes (RLNs) showed a higher proliferative rate (% of $S + G_2/M$ cells of 2% ± 3%) than mature BM and PB B lymphocytes ($P < .005$; Table 2). In turn, RLN B lymphocytes showing a germinal center–associated phenotype ($CD38^{+}CD20^{hi}$) represented the compartment of mature B cells showing the highest proliferation ($P < .005$ vs other mature B lymphocytes and plasma cells) with a mean percentage of $S + G_2/M$-phase cells of 13.5% plus or minus 8% (Table 2). The proliferative rate of more mature B cells decreased thereafter with maturation into $CD38^{hi}/CD20^{-/lo}$ LN and $CD38^{hi}/CD19^{-/+}$ BM plasma cells—percentage of $S + G_2/M$ plasma cells of 5.4% plus or minus 2% ($P < .001$) and of 3% plus or minus 1.1%, ($P < .005$), respectively.

Cell-cycle distribution of neoplastic B cells according to the type of sample analyzed

Comparative analysis of the proliferative rate of neoplastic B cells from B-CLPD patients in different tissues (BM, PB, and LN) showed significant differences in B-CLL and MCL cases (Table 3). Accordingly, in B-CLL, a significantly ($P = .003$) higher percentage of cells in the $S + G_2/M$ cell-cycle phases was detected in BM versus PB (0.7% ± 1% vs 0.4% ± 0.5%, respectively); in turn, significantly higher percentages of $S + G_2/M$ cells were detected in LN (7.7% ± 8%) versus BM (2% ± 1.3%; $P = .02$) and PB (2.4% ± 4.3; $P = .004$) samples from MCL patients. By contrast, no significant differences were observed for the other B-CLPD subgroups once the proliferative rate of neoplastic B cells from different types of samples was compared. However, both BL and LPL/WM showed a tendency toward a higher proliferation in LN versus PB and LN versus PB and BM, respectively (Table 3).

Cell-cycle distribution of neoplastic B cells from patients with different diagnostic subtypes of B-CLPD

Overall, large B-cell lymphomas (BL and DLBCL) were those subgroups of B-CLPD showing the highest percentage of $S + G_2/M$-phase neoplastic B cells, with no significant differences being observed in the proliferative rate between these 2 subgroups of B-NHL. However, in comparison with normal $CD38^{+}/CD20^{hi}$ germinal center B cells, half of all BL cases—7/14—while only 3 (16%) of 19 DLBCL cases showed an abnormally increased proliferative rate ($P = .001$ and $P = .03$, respectively); in addition 4 (21%) of 19 ($P = .01$) DLBCL cases, but none of the BL patients, displayed decreased $S + G_2/M$ cell percentages. In contrast, despite showing a germinal center immunophenotype, FL cases displayed an overall intermediate proliferative rate that was significantly ($P < .001$) lower than the proliferative rate of normal $CD38^{+}/CD20^{hi}$ RLN B cells

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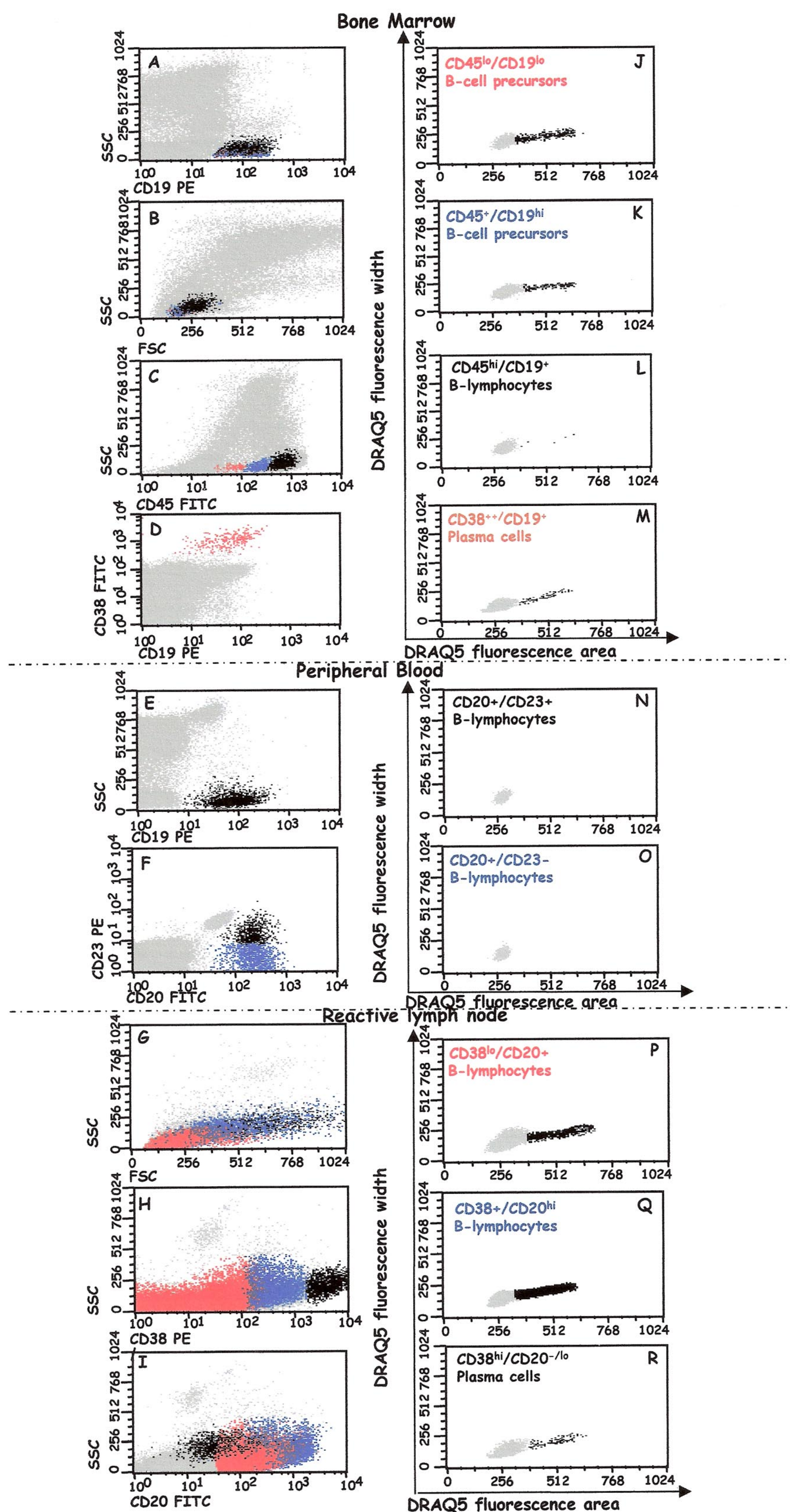

Figure 1. Representative bivariate dot plots illustrating the gating strategy used for the inmunophenotypic identification of each B-cell subpopulation identified and the analysis of their cell-cycle distribution. The strategy used for the identification of B-cell subsets is shown for normal bone marrow (BM; panels A-D), peripheral blood (PB; panels E,F) and reactive lymph node (LN; panels G-I) samples (left columns), together with the strategy used for the analysis of their cell-cycle distribution according to their DNA cell contents (DRAQ5 fluorescence area: panels J-R in the right column). In these dot plots for each B-cell population represented by the corresponding colored label, G0/G1 cells are depicted as grey events while, whenever present (panels J-M and P-R) S+G2/M cells are displayed as black dots.

(% of S + G_2/M cells of 13.5% ± 8% vs 2.4% ± 2%, respectively), the percentage of S + G2/M B cells being decreased in 66 (93%) of 71 cases (Figure 2).

Other B-CLPDs showing intermediate numbers of S + G_2/M-phase cells were HCL (2.4% ± 3%), MCL (3.3% ± 5%), MALT-NHL (1.4% ± 1.5%), and LPL/WM (2.5% ± 5%). Once compared

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Table 2. Proliferative rate of different maturation-associated B-cell compartments from normal adult bone marrow (BM), peripheral blood (PB), and reactive lymph node (RLN) samples

Type of sample/ B-cell compartment	Proliferative rate (% of S+G2/M cells)	P
BM		<.001*
CD45^{lo}/CD19^{lo}	7 ± 2.4	
CD45^{+}/CD19^{hi}	3 ± 1.2	
CD45^{hi}/CD19^{+}	0.5 ± 1.1	
CD38^{hi}/CD19$^{-/+}$	3 ± 1.1	
PB		NS
CD20^{+}/CD23^{+}	0.1 ± 0.8	
CD20^{+}/CD23^{-}	0.1 ± 0.05	
RLN		<.05†
CD38^{lo}/CD20^{+}	2 ± 3	
CD38^{+}/CD20^{hi}	13.5 ± 8	
CD38^{hi}/CD20$^{-/lo}$	5.4 ± 2	

Results are expressed as means plus or minus 1 standard deviation (range).
NS indicates no statistically significant differences: $P > .05$.
*$P < .001$ for all BM B-cell compartments versus CD45^{lo}CD19^{lo} B-cell precursors.
†$P < .05$ for all LN B-cell compartments versus CD38^{+}CD20^{hi} B lymphocytes.

with normal mature CD38^{lo}/CD20^{+} LN B cells (% of S + G_2/M cells of 2% ± 3%) no significant differences were found for HCL and MALT-NHL; in contrast, 6 (16%) of 39 MCL cases ($P = .001$) and 3 (37%) of 8 LN samples from MCL ($P = .01$) showed abnormally increased percentages of S + G_2/M cells (13.8% ± 5% and 17% ± 5%, respectively). Among LPL/WM cases, 30 (83%) of 36 patients showed a significantly ($P < .001$) lower percentage of S + G_2/M-phase cells (1.2% ± 1%) than normal reactive CD38^{hi}/CD20^{lo} LN and BM plasma cells (5.4% ± 2% and 3% ± 1.1%, respectively; Figure 2). In contrast, S + G2/M cell percentages detected in LPL/WM cases were similar to those of CD38^{lo}/CD20^{+} mature RLN B lymphocytes (Figure 2).

B-CLL together with SMZL were those B-CLPDs showing the lowest proportion of S + G_2/M-phase cells (0.6% ± 0.7% and 0.9% ± 1.6%, respectively). Despite this, 73 (60%) of 123 PB B-CLL and 58 (74%) of 78 BM B-CLL cases showed a significantly higher proportion of S + G2/M-phase cells (0.6% ± 0.5% and 2.7% ± 2.1%; respectively) compared with mature CD20^{+}/CD23^{+} PB B cells and with mature CD45^{hi}/CD19^{+} BM B cells ($P < .001$), respectively; in contrast, no significant differences were observed between neoplastic B cells from SMZL patients and normal/reactive CD38^{lo}CD20^{+} LN B cells (Figure 2).

Overall, there was an association between the proliferative rate of neoplastic B cells as evaluated by the percentage of S + G2/M phase cells and the Ki67 score by immunohistochemistry assessed in a subset of 76 lymph node biopsies from an identical number of patients, particularly when comparing cases with high Ki67 expression (> 90% of neoplastic B cells; % of S + G_2/M of 17% ± 15%) with those showing low (< 10%; % of S + G_2/M of 2% ± 2%; $P < .001$), intermediate (10%-60%; % of S + G_2/M of 2% ± 1.5%; $P = .002$) and intermediate-high (60%-90%; % of S + G_2/M of 3% ± 1.5%; $P = .01$) Ki67 expression.

Impact of common recurrent cytogenetic abnormalities in the proliferative rate of neoplastic B cells

Most (385/432; 89%) B-CLPD patients included in the present study showed a diploid DNA content, while the remaining 47 cases (11%) were DNA hyperdiploid. The highest frequency of DNA aneuploid cases was observed among DLBCL (42%), BL (29%), MCL (21%), and FL (18%) cases, while all HCL and SMZL analyzed had a diploid DNA cell content. In turn, the frequency of DNA aneuploid cases detected by flow cytometry among MALT-NHL (10%), B-CLL (5%), and LPL/WM cases (6%) was low. Interestingly, upon comparing the DNA index (DI) of aneuploid cases, significant differences were observed between B-CLL cases (DI of 1.05 ± 0.04) in comparison with MCL (DI of 1.6 ± 0.4; $P < .001$), FL (DI of 1.3 ± 0.4; $P < .001$), DLBCL (DI of 1.4 ± 0.4; $P < .001$), and BL (DI of 1.4 ± 0.2; $P = .01$) patients, in line with previous observations.[21,24,28,29,54] In addition, FL cases also showed a significantly lower DNA index than MCL cases ($P = .02$). Among B-CLL patients, DNA aneuploidy was associated with a higher percentage of S + G2/M-phase cells (1% ± 0.7% vs 0.5% ± 0.7%; $P = .002$) as previously reported.[55] In contrast, no statistically significant differences were found as regards the proliferative rate of neoplastic B cells in the other subgroups of B-CLPD with respect to the presence or absence of DNA aneuploidy.

Among B-CLL cases, de1(13q) and trisomy 12 were those chromosomal abnormalities more frequently observed (37% and 22% of the cases, respectively) followed by del(11q) (9%) and del(17p) (7%). DNA aneuploid B-CLL patients showed a higher

Table 3. Proliferative rate of neoplastic B-cells in B-CLPD patients according to the type of sample analyzed: bone marrow (BM), peripheral blood (PB) and lymph node (LN)

Subtype of B-CLPD/ type of sample (no. of cases)	Proliferative rate, % of S+G2/M cells	P
B-CLL		.003*
BM (n=78)	0.7 ± 1	
PB (n=123)	0.4 ± 0.4	
LN (n=9)	0.5 ± 0.5	
SMZL		NS
BM (n=7)	1.7 ± 2.4	
PB (n=9)	0.5 ± 0.2	
HCL		
BM (n=2)	1.7 ± 2.4	
LN (n=5)	0.5 ± 0.2	
MALT		NS
BM (n=15)	1.4 ± 1.4	
PB (n=4)	0.7 ± 0.5	
LN (n=1)	4.6	
MCL		.04†
BM (n=15)	2 ± 1.3	
PB (n=16)	2.4 ± 4.3	
LN (n=8)	7.7 ± 8	
FL		NS
BM (n=37)	3 ± 2.5	
PB (n=9)	2.1 ± 2	
LN (n=25)	3 ± 2	
BL		NS
PB (n=8)	15 ± 3.4	
LN (n=11)	26 ± 13	
DLBCL		NS
BM (n=4)	12.6 ± 19	
LN (n=15)	14 ± 7	
LPL/WM		NS
BM (n=31)	2 ± 2	
PB (n=2)	1.1 ± 1.3	
LN (n=3)	10.6 ± 16.7	

Results expressed as means plus or minus 1 standard deviation. P values provided correspond to comparisons indicated.
B-CLPD indicates B-cell chronic lymphoproliferative disorders; B-CLL, B-cell chronic lymphocytic leukemia; SMZL, splenic marginal zone B-cell lymphoma; NS, statistically not significantly different ($P > .05$); HCL, hairy cell leukemia; MCL, mantle-cell lymphoma; MALT, mucosa-associated lymphoid tissue lymphoma; FL, follicular lymphoma; BL, Burkitt lymphoma; DLBCL, diffuse large B-cell lymphoma; and; LPL/WM, lymphoplasmacytic lymphoma/Waldenström macroglobulinemia.
*BM versus PB samples.
†LN versus BM and PB samples.

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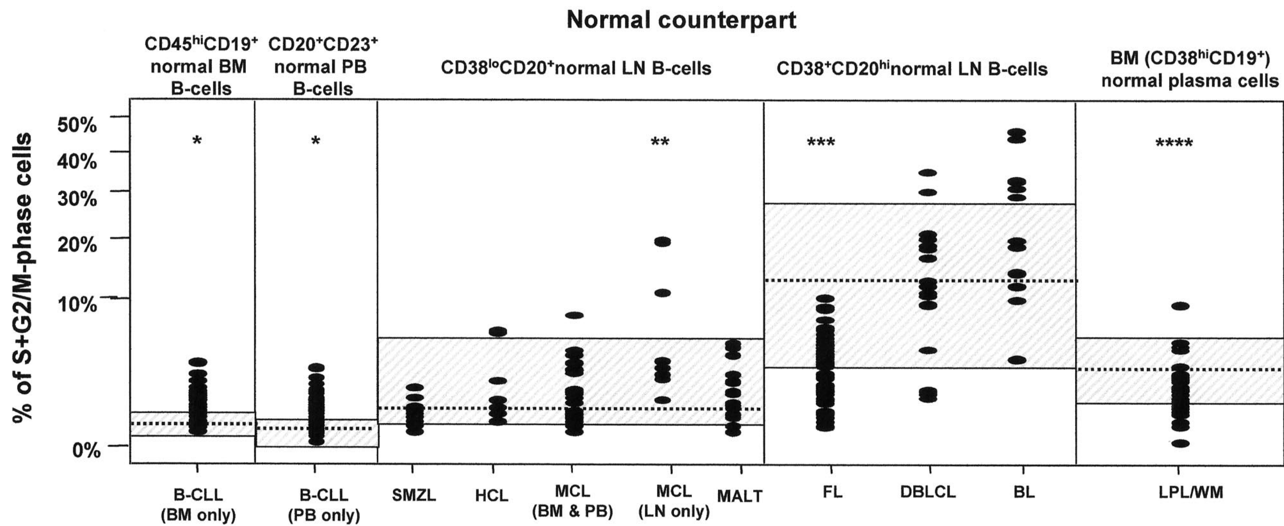

Figure 2. Proliferative rate of neoplastic B cells in individual B-CLPD patients in comparison with their normal counterparts. The range of S + G2/M-phase cells for the corresponding normal B-cell counterpart is shown as a gray box, and the dotted line corresponds to normal median values. *$P < .05$ for B-CLL versus normal mature bone marrow (BM) CD45hiCD19+ B cells and CD20+CD23+ peripheral blood (PB) B lymphocytes; **$P = .01$ for MCL LN samples versus CD38loCD20+ reactive LN B lymphocytes; ***$P < .001$ for FL versus CD38+CD20hi reactive LN B lymphocytes; and ****$P < .05$ for LPL/WM versus normal BM and lymph node (LN) plasma cells. B-CLL indicates B-cell chronic lymphocytic leukemia; HCL, hairy cell leukemia; MCL, mantle-cell lymphoma; SMZL, splenic marginal zone B-cell lymphoma; MALT, mucosa-associated lymphoid tissue lymphoma; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; BL, Burkitt lymphoma; and LPL/WM, lymphoplasmacytic lymphoma/Waldenström macroglobulinemia.

frequency of trisomy 12 versus diploid B-CLL cases (63% vs 20%; $P = .003$). In turn, in MALT-NHL cases, t(18q21) was negative in 16 of 16 cases studied, while gene rearrangements involving the 14q32.3 chromosome region were detected in 4 (25%) of 16 patients. In addition, t(14;18), *BCL6* gene rearrangements, and t(8;14) were found in 86% (n = 61/71), 17%, and 6% of all FL patients, respectively; t(8;14) was identified in 13 (93%) of 14 BL cases, t(11;14) was found in 30 (77%) of 39 MCL, and *BCL6* gene rearrangements were present in 5 (38%) of 13 DLBCL patients, none of the later cases showing t(14;18). Finally, del(6q21) was identified in 9 (30%) of 30 LPL/WM patients, and translocations associated with the 14q32.3 region were detected in 2 (8%) of 24 cases analyzed. Interestingly, once we analyzed the potential impact of the presence versus absence of these different cytogenetic abnormalities within the specific subgroups of B-CLPD patients on the proliferative rate of neoplastic B cells, presence of translocations associated with the 14q32.3 region in LPL/WM cases was associated with a higher percentage of S + G2/M-phase cells in comparison with negative cases (17% ± 18% vs 1.3% ± 0.7%; $P = .006$). In contrast, no statistically significant differences were found between cases showing a distinct cytogenetic pattern for any of the other diagnostic subgroups of B-CLPD analyzed except for a higher proliferative rate among FL cases with t(8;14) ($P = .05$).

Association between the proliferative rate of neoplastic B cells and other clinical and biologic disease features

Among B-CLPD patients as a whole, a significant association was observed between a worse performance status (ECOG ≥ 2; ($P < .001$), presence of infiltration of 2 or more extranodal sites ($P = .001$), thrombocytopenia (< 100 × 10^9/L; $P = .01$), increased LDH serum levels ($P < .001$), and a higher tumor cell proliferation (Table 4). In addition, FL patients with histologic grade 1 ($P = .003$) and grade II ($P = .01$) had lower percentage of S + G2/M phase cells compared with grade 3 cases; in line with this, absence of superficial adenopathies ($P = .03$) and infiltration of 2 or more extranodal sites ($P < .001$) in FL was also associated with a higher percentage of S + G2/M neoplastic B cells (Table 4). Similarly, a significantly higher proliferation ($P = .03$) was observed among stage B and C versus stage A B-CLL cases (Table 4). In contrast, presence of leukocytosis (> 10 × 10^9/L; $P < .001$) and lymphocytosis (> 5 × 10^9/L; $P < .001$) was associated with lower percentages of S + G2/M cells, particularly among B-CLL ($P \leq .06$) and low-grade B-CLPD other than B-CLL, MCL, and FL ($P \leq .09$). Regarding patient outcome, a clear association between higher percentages of S + G2/M cells and both progressive disease and shorter survival was observed after a median follow-up of 25 months ($P = .005$) for the whole B-CLPD group as well as for MCL patients ($P = .03$; Table 4).

Discussion

The proliferative activity of neoplastic B cells has long been suggested to be a relevant prognostic factor in B-CLPD associated with both response to chemotherapy and patient survival,[17,27,30-32,34,35,56] as also found here. However, evaluation of cell proliferation has only partially translated into routine clinical practice through the use of semiquantitative immunohistochemical stains for individual cell cycle–associated proteins such as Ki67 and PCNA.[14-19,34,57-59] Although, Ki67 immunohistochemistry alone is a powerful tool to measure the distribution of proliferating cells and has been found to correlate with worse clinical outcome,[59,60] to the best of our knowledge, no study has been reported so far in which the proliferative rate of neoplastic B-CLPD cells is analyzed in a reproducible and quantitative way in comparison with that of their normal counterpart. Such comparison would provide further insight on the impact of specific genetic abnormalities on the proliferation of neoplastic B cells in specific subtypes of both chronic lymphoid leukemias and B-NHL.

Early BM B-cell development concludes when a B-cell precursor successfully rearranges and expresses Ig heavy and light chain genes coupled with CD79 at the cell surface membrane, to form a functional antigen receptor (BCR) capable

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Table 4. B-CLPD: relationship between the proliferative rate of neoplastic B cells and the clinical and biologic characteristics of the disease

	B-CLL, n=151			MCL, n=27			FL, n=54			Other low-grade B-CLPD*, n=51		
	% of cases	% of S+G2/M cells	P	% of cases	% of S+G2/M cells	P	% of cases	% of S+G2/M cells	P	% of cases	% of S+G2/M cells	P
Patient characteristic												
Performance status												
ECOG 2 or less	97	0.5 ± 0.3	NS	84	3±4	NS	86	2.7±2.1	NS	87	1.5±2	**.03**
ECOG more than 2	3	0.5±0.5		16	7±6		14	2.6±2.3		13	6.5±11	
Adenopathies												
No	45	0.5±0.4	NS	11	4±6	NS	4	5.7±1.2	**.03**	71	1.5±1.6	NS
Yes	55	0.5±0.3		89	3±5		96	2.5±2		29	4±7	
Infiltration of 2 or more extranodal sites												
No	95	0.4±0.4	**<.01**	83	2.4±3	NS	73	2.4±1.6	**<.001**	83	4±6	NS
Yes	5	3.5±6		17	1.5±1		26	3.3±3		17	10±13	
B-CLL Binet stage												
A	75	0.4±0.3										
B	15	0.6±0.7	**.03**									
C	10	0.7±0.7										
FL histologic grade												
Grade I							57	2.3±1.6				
Grade II							32	2.1±1.5	**<.05§**			
Grade III							11	5±2				
Standard IPI												
Low (0;1)	84	0.4±0.3		21	3.8±5		45	2.2±2		41	1.3±1.3	
Low to intermediate (2)	11	0.5±0.4	**<.001**	47	1.5±1.1	NS	24	2.3±1.4	NS	37	2±2.4	NS
High to intermediate (3)	5	2.4±1.2		32	6.5±8		24	3.3±2.6		18	7±13	
High (4;5)	0			0			7	3.8±2		4	2.2	
Laboratory parameters‡												
WBC count less than 10×10^9/L	14	1.1±2	**.03**	57	5±6	.06	83	2.5±1.6	.06	24	3±5	.09
Leukocytosis more than 10×10^9/L	86	0.4±0.4		43	2.4±4		17	2±2.5		76	1±1.4	
Lymphocyte count less than 5×10^9/L	10	1.4±2.4	.06	55	4±4	.09	83	2.7±2	NS	24	3±6	**.01**
Lymphocytosis more than 5×10^9/L	90	0.5±0.6		45	4±7		17	1.5±2		76	0.6±0.4	
Hemoglobin												
Anemia less than 100 g/dL	8	0.5±0.4	NS	5	4±5	NS	4	0.4±0.1	**.03**	13	7±13	NS
More than 100 g/dL	92	0.5±0.9		95	0.2		96	2.5±2		87	2±2	
Platelet count												
Thrombocytopenia less than 100×10^9/L	7	1.4±3	**.01**	10	7±8	NS	8	2.5±3	NS	13	8±12	NS
More than 100×10^9/L	93	0.5±0.5		90	3±5		92	2.5±2		87	2±2	
Serum LDH												
Normal	94	0.5±0.8	.06	72	3±4	NS	23	2.3±2	NS	87	2±2	**.01**
Increased more than 450 U/L	6	0.7±0.8		28	4±7		77	3.6±2.6		13	7±11	
Patient follow-up												
Outcome												
Stable disease	64	0.4±0.3	NS	19	1.3±1	NS	14	2±1.6	NS	50	1.3±1.4	NS
Progressive disease	8	0.4±0.3		33	6±7		25	2.7±1.7		8	1.2±1	
Total deaths												
No	90	0.5±0.8	NS	50	1.3±1.1	**.007**	80	2.4±2	NS	84	2±5	NS
Yes	10	0.5±0.8		50	6.5±6.2		20	3±1.6		16	2±3	
Median survival	**Cutoff value¶**	**Median survival, mo**	**P**	**Cutoff value¶**	**Median survival, mo**	**P**	**Cutoff value¶**	**Median survival, mo**	**P**	**Cutoff value ¶**	**Median survival, mo**	**P**
Low proliferative rate	<0.34%	NR	NS	<1.4%	NR	**.03**	<1.8%	NR	NS	<1%	NR	NS
High proliferative rate	≥0.34%	NR		≥1.4%	12		≥1.8%	NR		≥1%	34	

Significant differences were not found for sex, Ann Arbor stage, B symptoms, hepatomegaly, splenomegaly, bulky disease, and b-2 microglobulin serum levels.

B-CLPD indicates B-cell chronic lymphoproliferative disorders; B-CLL, B-cell chronic lymphocytic leukemia; MCL, mantle-cell lymphoma; FL, follicular lymphoma; LDH, lactic dehydrogenase; NS, no statistically significant differences found ($P > .05$); and NR, not reached.

‡Results expressed as mean plus or minus 1 standard deviation.

§*P* value provided corresponds to comparisons between histologic grade I and II versus grade III FL, respectively.

*Other low-grade B-NHL: hairy cell leukemia; splenic marginal zone B-cell lymphoma; mucosa-associated lymphoid tissue lymphoma; and lymphoplasmacytic lymphoma/Waldenström macroglobulinemia.

†High-grade B-NHL: diffuse large B-cell lymphoma; Burkitt lymphoma; and other unclassifiable high-grade B-NHLs.

‖Only 1 case showing 5 x 10^9/L or more lymphocytes.

¶The cutoff value used for grouping into the low and high proliferative rate categories in each diagnostic subtype corresponds to the median value of the percentage of S+G2M cells.

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Table 4. B-CLPD: relationship between the proliferative rate of neoplastic B cells and the clinical and biologic characteristics of the disease (continued)

	High-grade B-CLPD,† n=21			All B-CLPD patients, n=304		
	% of cases	% of S+G2/M cells	*P*	% of cases	% of S+G2/M cells	*P*
Patient characteristic						
Performance status						
ECOG 2 or less	44	20±13	NS	11	2±4	**<.001**
ECOG 2 or more	56	15±8		89	6±8	
Adenopathies						
No	25	19±7	NS	42	1.5±3	NS
Yes	75	17±12		58	3.1±6	
Infiltration of 2 or more extranodal sites						
No	45	12±7	NS	86	2±3.5	**.001**
Yes	55	20±13		14	3.5±6	
B-CLL Binet stage						
A						
B						
C						
FL histologic grade						
Grade I						
Grade II						
Grade III						
Standard IPI						
Low (0;1)	40	21±13		39	5±8	
Low to intermediate (2)	40	11±8	NS	35	3.5±5	NS
High to intermediate (3)	13	24±8		22	5.7±8	
High (4;5)	7	31		4	3.4±2	
Laboratory parameters‡						
WBC count less than 10×10⁹/L	74	15±12	NS	45	4.7±7	**<.001**
Leukocytosis more than 10×10⁹/L	26	14±13		55	1±2	
Lymphocyte count less than 5×10⁹/L	96		**NS**	45	5.1±7	**<.001**
Lymphocytosis more than 5×10⁹/L	4‖			55	1±2.1	
Hemoglobin						
Anemia less than 100 g/dL	11	30±1.4	.07	8	4±9	NS
More than 100 g/dL	89	16±10		92	2.5±5	
Platelet count						
Thrombocytopenia less than 100×10⁹/L	11	7±5	NS	9	4.3±7	**.01**
More than 100×10⁹/L	89	18±11		91	2.4±5	
Serum LDH						
Normal	63	17±11	NS	86	2±4.6	<**.001**
Increased more than 450 U/L	37	18±1o		14	5±7	
Patient follow-up						
Outcome						
Stable disease	0	-	NS	46	0.7±0.9	**<.001**
Progressive disease	28	20±10		14	3.4±5	
Total deaths						
No	57	15±12	NS	82	2±5	**0.009**
Yes	43	19±8		18	5±6	
Median survival	**Cutoff value¶**	**Median survival, mo**	***P***	**Cutoff value¶**	**Median survival, mo**	***P***
Low proliferative rate	<13.7%	NR	NS	<0.7%	NR	**.005**
High proliferative rate	≥13.7%	NR		≥0.7%	68	

Significant differences were not found for sex, Ann Arbor stage, B symptoms, hepatomegaly, splenomegaly, bulky disease, and b-2 microglobulin serum levels.

B-CLPD indicates B-cell chronic lymphoproliferative disorders; B-CLL, B-cell chronic lymphocytic leukemia; MCL, mantle-cell lymphoma; FL, follicular lymphoma; LDH, lactic dehydrogenase; NS, no statistically significant differences found ($P > .05$); and NR, not reached.

‡Results expressed as mean plus or minus 1 standard deviation.

§*P* value provided corresponds to comparisons between histologic grade I and II versus grade III FL, respectively.

*Other low-grade B-NHL: hairy cell leukemia; splenic marginal zone B-cell lymphoma; mucosa-associated lymphoid tissue lymphoma; and lymphoplasmacytic lymphoma/Waldenström macroglobulinemia.

†High-grade B-NHL: diffuse large B-cell lymphoma; Burkitt lymphoma; and other unclassifiable high-grade B-NHLs.

‖Only 1 case showing 5 x 10^9/L or more lymphocytes.

¶The cutoff value used for grouping into the low and high proliferative rate categories in each diagnostic subtype corresponds to the median value of the percentage of S+G2M cells.

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of recognizing non–self-antigens.[3,61] During this period, B-cell maturation is associated with a significant expansion of the B-cell compartment until large numbers of mature, naive, resting B cells are generated.[3] Afterward, mature resting B cells leave the BM[62] and recirculate through PB and the LN.[63] Under appropriate T cell–dependent stimulatory conditions, antigen recognition through the BCR induces the activation, (mono/oligo) clonal expansion, and further maturation of B cells; in the germinal center (GC) B-cell responses are associated with an increase affinity of the BCR for the antigen recognized and Ig class switch through the occurrence of both hypermutation and rearrangements of the Ig genes.[62,64-66] In line with this, mature PB and marginal zone B lymphocytes are characterized by signals that help to maintain a quiescent state (eg, low expression of cyclins such as CCNA, CDK1, and CCNB1), while GC cells have a high proliferation gene expression signature (eg, high expression of *CCNB1*, *PCNA*, and *Ki67*).[67] Further maturation of GC B cells into plasma cells (PCs) capable of producing high-affinity antibodies is followed by a progressive decreased proliferation[3,63,66,68] also supported by the observation that $CD45^{hi}/bcl2^{lo}$ PCs from secondary lymphoid organs and PB are highly proliferating, while $CD45^{dim}/bcl2^{+}$ PCs from BM have a lower proliferative rate decreasing in parallel to CD45 expression.[69]

In this study, we accurately establish the magnitude of the variations in the proliferative fraction of B cells at different maturation stages providing a frame of reference for the understanding of the impact of neoplastic transformation of mature B cells in their proliferative rate. Accordingly, our results show the existence of a progressively lower proportion of S + G2/M cells from the more immature $CD45^{lo}/CD19^{lo}$ and $CD45^{+}/CD19^{hi}$ BM B-cell precursors to the more mature BM $CD45^{hi}/CD19^{+}$ and PB $CD20^{+}CD23^{+}$ and $CD20^{+}CD23^{-}$ B lymphocytes. In turn, RLN B cells displaying a germinal center–associated immunophenotype ($CD38^{+}CD20^{hi}$ B cells) showed a higher proliferative rate than that of marginal zone resting B lymphocytes, and the more mature plasma cells (PCs) from both RLN ($CD38^{hi}CD20^{-/lo}$) and BM ($CD38^{+++}CD19^{+}$). Overall, these results confirm the occurrence of variations on the proliferative rate of BM, PB, and RLN B cells in association with both the maturation stage and activation status of B cells.[3,69,70]

Previous reports indicate that B-CLPDs displaying a low proliferative rate correspond to relatively indolent, low-grade tumors, while B-CLPDs with a higher proliferative rate typically (> 10%) show an aggressive clinical course.[23,25,28] In line with these observations, we found a clear association between higher percentages of S + G2/M cells and both adverse prognostic features (eg, advanced patient performance status, disease stage, high LDH serum levels, and presence of thrombocytopenia), progressive disease, and a shorter overall survival for the whole B-CLPD group as well as for a specific diagnostic subgroups of the disease such as MCL.

Overall, a relatively high degree of heterogeneity between the different subgroups of B-CLPDs analyzed was also observed. Large cell lymphomas (BL and DLBCL) were those showing the highest percentage of S + G_2/M-phase neoplastic B cells; intermediate proliferative rates were observed for HCL, MCL, MALT-LNH, FL, and LPL/WM, while B-CLL and SMZL patients were those subtypes of B-CLPD showing the lowest proportion of S + G_2/M-phase cells. Upon comparing the proliferative rate of normal versus neoplastic B cells, similar median percentages of S + G2/M cells were found for HCL, SMZL, and MALT-NHL patients. However at present, the normal counterpart for these specific subgroups of B-CLPD—which are presumably derived from either marginal zone B cells or post-CG memory B cells—still remains to be fully identified.[3,71] In contrast, a significantly increased proportion of S + G2/M-phase cells was observed among a relevant fraction of all B-CLL, BL, DLBCL, and MCL cases compared with their normal counterpart ($CD20^{+}CD23^{+}$ PB B lymphocytes, RLN $CD38^{+}CD20^{hi}$, $CD38^{+}CD20^{hi}$, and $CD38^{lo}CD20^{+}$ B cells, respectively). In contrast, most FLs and LPL/WMs displayed a lower proliferative rate than their normal counterpart. Despite this, a more careful analysis of the results shows the existence of an important degree of variability even among individual cases within specific diagnostic subgroups of B-CLPD. Interestingly, significantly higher percentages of S + G2/M-phase cells were found in B-CLL cases in BM versus PB, and in RLN from MCL patients versus both BM and PB. The higher proliferative rate observed in specific tissues supports the existence of a nonrandom distribution of the proliferative compartment of neoplastic B cells in B-CLPD, with specific tissues providing a more favorable microenvironment for the expansion of the neoplastic clones.[3,63,66,68,69,72] In line with this hypothesis, previous studies have shown that the patterns of tissue compartmentalization, intranodal growth, and hematologic spread of malignant cells in B-CLPD are influenced by variations in the adhesion properties of tumor B cells to BM and/or LN stromal cells.[73-78] As an example, low-grade B-NHL typically show stronger expression of CD44 and CD54 in LN than in PB and BM.[79,80] Similarly, among B-CLL cases, a relationship between the expression of CD44 and a diffuse pattern of BM infiltration has been reported,[81] consistent with the finding that CD44 mediates adhesion of maturing B cells to BM stromal cells.[82] In addition, it has also been shown that in individual patients, "pseudofollicular" proliferation centers in tissues involved by B-CLL, including BM on average, display a greater proportion of $CD38^{+}$ cells and higher proliferation, versus neoplastic PB B cells.[83-86] Of note, comparative evaluation of the Ki67 score by immunohistochemistry and the percentage of S + G2/M cells in lymph node biopsies from patients with B-CLPD showed a significant association between both parameters, in line with previous observations.[14,22]

Despite this, variations in cell proliferation of neoplastic B cells within specific subtypes of B-CLPDs could not be fully explained by differences in the tissues analyzed. Accordingly, half of all BL patients displayed aberrantly increased percentages of S + G2/M cells in tumoral LN, compared with GC B lymphocytes. Similarly, one-fourth of all DLBCLs displayed altered (either increased or decreased) cell proliferation in lymphoid tissue samples. These results could suggest that, apart from reflecting the proliferative rate of their normal counterpart, specific genetic abnormalities associated with the different diagnostic subgroups of B-CLPDs analyzed could contribute to modulate the cell-cycling rate of neoplastic B cells.[1] However, upon comparing within individual diagnostic subgroups of patients the proliferative rate of neoplastic B cells from cases carrying different genetic abnormalities, no major differences were observed between MALT, MCL, FL, BL, and DLBCL for the presence versus absence of t(14q32), t(11;14), t(14;18), t(8;14), and *BCL6* rearrangements, respectively. In contrast, in line with previous observations,[55] DNA aneuploid B-CLL cases as well as LPL/WM patients carrying 14q32 gene rearrangements and FL with t(8;14) were associated with an increased cell

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proliferation once compared with DNA diploid B-CLL, 14q32-negative LPL/WM, and t(8;14)-negative FL cases, respectively.

Overall, these findings confirm the notion that among the different genetic abnormalities analyzed, some are more likely related to an inhibitory effect on apoptosis and a prolonged B-cell survival in the absence of an increase in cell cycling (eg, t(14;18) with overexpression of bcl2 in FL[87,88]), while others more frequently lead to an augmented cell proliferation (eg, *C-MYC* gene rearrangements) in BL and FL.[1] Similarly, overexpression of cyclin D1 among MCL patients does not appear to confer a proliferative advantage, in line with previous observations showing a similar gene expression signature and survival rates for typical MCL patients showing overexpression of cyclin D1 and MCL cases that are negative for this cell cycle–associated protein and that have acquired alternative mechanisms to accelerate entry into S-phase, independent of cyclin D1 expression.[89]

In general, low- and high-grade B-NHL are both able to accumulate other alterations in cell-cycle regulators, most frequently involving tumor suppressor genes such as *BCL6, p16*, *p53,* and *p27* that confer an increased aggressiveness and proliferative advantage to tumoral cells.[1,90-92] However, in the present study, neither *p53* deletion in B-CLL nor *BCL6* gene rearrangements in DLBCL and FL were associated with an increased cell proliferation. In line with these observations, previous reports have shown that overexpression of BCL6 is often independent of chromosomal translocations involving the *BCL6* gene, indicating that BCL6 expression might be associated with a high proliferation in both DLBCL and FL cases independently of the occurrence of 3q27 gene rearrangements.[93,94] Similarly, no direct correlation has been observed between the percentage of proliferating Ki67$^+$ B-CLL cells and p53-positive cells.[95]

In summary, our results confirm that the proliferative rate of neoplastic cells from patients with B-CLPD is highly heterogeneous, such variability reflecting their maturation stage and to a certain extent also unique specific underlying genetic abnormalities; at the same time, it is associated with the clinical and biologic behavior of the disease. Further variations in B-CLL and MCL could also be related to the tissue compartment analyzed and the microenvironment of the neoplastic B cells.

Acknowledgments

This work has been partially supported by the following grants: FIS 06/0824, from the Ministerio de Sanidad y Consumo (Madrid, Spain) and RETICC RD06/0020/0035 from the Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo (Madrid, Spain). S.Q. is supported by a grant from COLCIENCIAS (Bogotá, Colombia), J.M.S. is supported by a grant from the Ministerio de Sanidad y Consumo (Madrid, Spain; CP05/00321), A.R. is supported by a grant from the Ministerio de Ciencia y Tecnología (Madrid, Spain) y Fondo Social Europeo, and C.F. is supported by a grant from the Instituto de Salud Carlos III (Madrid, Spain; CM05/00250).

Authorship

Contribution: S.Q. performed research, collected and analyzed data, and wrote the paper; A.L., A.R., S.B., M.L.S., J.F., C.F., and M.C.G. performed research and collected data; J.M.S., C.S.O., N.F., M. González, and P.G. performed research and analyzed data; M. Giralt performed research; M.C.P., J.M.M.-A., O.G., L.P., J.D.M., M.G.S., A.A.R., C.C., J.L.G., and R.B., collected data; J.A. collected and analyzed data, and wrote the paper; A.O. designed research, collected and analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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