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Diagnostic role and prognostic significance of a simplified immunophenotypic classification of mature B cell chronic lymphoid leukemias

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We verified the diagnostic and prognostic role of a simplified immunophenotypic classification (IC) in a series of 258 patients (M/F: 1.4; median age: 64 years; median follow-up: 64 months; 75 deaths) with mature B cell lymphoid leukemias (MBC-LL) for whom no histopathological diagnosis was available because of minimal or no lymph node involvement. The IC was based on the reactivity of three pivotal immunophenotypic markers: CD5, CD23 and SIg intensity. On the basis of different expression patterns, we identified four diagnostic clusters (C) characterized by distinct clinico-biological features and different prognoses: C1 (149 patients) identified most classical B cell chronic lymphocytic leukemias (CLL-type cluster; $SIg^{dim}/CD5^{+}/CD23^{+}$); C2, 38 patients whose clinico-hematological characteristics were intermediate between C1 and C3 (CLL-variant cluster; $SIg^{bright}/CD5^{+}/CD23^{+/-}$ or $SIg^{dim}/CD5^{-/-}/CD23$ indifferent); C3 (16 patients) most situations consistent with mantle cell lymphoma in leukemic phase (MCL-type cluster; $SIg^{bright}/CD5^{+}/CD23^{-}$); and C4, 55 cases, most of whom were consistent with leukemic phase lymphoplasmacytic/splenic marginal zone lymphomas (LP/S-type cluster; $SIg^{bright}/CD5^{-/+}/CD23$ indifferent). At univariate survival analysis, prognosis worsened from C1 to C4, C2 and C3 ($P = 0.0001$), and this was maintained at multivariate analysis ($P = 0.006$), together with CD11c expression ($P = 0.0043$), age at diagnosis (cut-off 70 years; $P = 0.0008$) and platelet count (cut-off 140 $\times$ 10^9/l; $P = 0.0034$). Besides recognising the two well-known situations of classic B-CLL and MCL, our IC identified situations with distinct prognostic and/or clinical behaviors.
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Introduction

Mature B cell lymphoid leukemias (MBC-LL) are a frequent and biologically heterogeneous group of neoplastic disorders characterized by different clinical behaviors and therapeutic responses, whose first-line definition is based on cell morphology, immunophenotype findings and clinical presentation.[1] Histopathological analysis is fundamental for attributing the leukemic picture to a specific clinico-pathologic entity according to the REAL/WHO classification,[2] but is not always available because of the absence of easily biopsied lymphoid tissue. The development of cytogenetic and molecular studies has allowed the identification of specific markers for only a subset of these diseases, leaving the diagnostic evaluation to flow cytometric analysis. A number of important immunophenotypic diagnostic scoring systems have been proposed, but they were validated on the basis of cytomorphological data and not verified in terms of prognostic impact.[3,4]

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For these reasons, and bearing in mind the relatively high frequency of MBC-LL patients in whom histopathological data cannot be obtained at diagnosis, there is a need for simplified immunophenotypic criteria that could be useful in clinical practice and also applied in centers without highly specialized laboratories. In this study, we evaluated the reactivity of major immunological markers in a series of MBC-LL patients with the aim of investigating their accuracy in distinguishing pathological situations whose biological and clinical characteristics overlap to a certain degree. On the basis of this preliminary analysis, we developed an immunophenotypic classification (IC) involving three pivotal markers (CD5, CD23 and SIg intensity) and tested its role in defining the diagnosis and prognosis of this MBC-LL subset.

Materials and methods

Patients

The study involved 258 patients with mature B cell leukemia and minimal or absent superficial lymph node involvement (152 males and 106 females; ratio = 1.4; median age at diagnosis 64 years, range 30–93) who were evaluated at diagnosis and characterized by means of flow cytometry at the Marcora Center for Blood Diseases between 1993 and 1999. All of them had light chain restricted CD19-positive lymphocytosis ($\geq 4 \times 10^9$/l); those with CD10-positive leukemic phase follicular lymphomas were excluded. The cut-off point for lymphocyte doubling time (LDT) was 12 months, which was not evaluable in 97 patients, six of whom were followed up for less than 12 months and 91 received early treatment because of active disease without any sign of lymphocyte duplication. The Binet staging system could not be used because we selected cases without any significant superficial lymph adenopathies.

At the time of analysis, the median follow-up was 64 months (range 3-96). Seventy-five patients had died: 52 of disease, 17 due to unrelated problems, and six because of second solid tumors. One hundred and seventy patients had received chemotherapy from the start of the study. First-line therapy was relatively homogenous and mainly consisted of alkylating agents.

Morphological analysis

Peripheral blood films stained with May–Grünwald-Giemsa were morphologically evaluated taking into account the FAB criteria[1] and the WHO revised REAL classification.[2] The cases were grouped as follows: (1) typical chronic lymphocytic leukemia (CLL: >90% small lymphocytes); (2) lymphoplasmocytoid lymphoma/immunocytoma (LPIC) in leukemic phase; (3)

splenic lymphoma with villous lymphocytes (SLVL); (4) hairy cell leukemia (HCL); (5) heterogeneous/non-CLL forms. This last group included situations (prolymphocytic leukemia, mixed cell CLL, mantle cell lymphoma (MCL), polymorphic forms) whose morphological discrimination is often quite difficult and poorly reproducible between assessors. The diagnostic definition and morphological classification was supported by bone marrow histology in 218 cases. The histological pattern of bone marrow infiltration (diffuse or not diffuse) was also recorded.

Immunophenotype analysis

For the immunological, molecular and cytogenetic analyses, mononuclear cell suspensions were obtained from heparinized peripheral blood by means of Ficoll–Hypaque gradient centrifugation. The samples underwent direct and indirect immunofluorescence staining with monoclonal antibodies (MoAbs), and the surface immunoglobulins (SIg) were characterized as previously described.[5] The MoAbs used were anti-CALLA FITC for CD10; anti-Leu1-FITC, anti-Leu12-PerCP and anti-Leu20-PE for respectively CD5, CD19 and CD23; anti-IL2-R-FITC for CD25; anti- Leu-M5-PE for CD11c; purified anti-integrin $\alpha 3$ for CD49c (Becton Dickinson, Mountain View, CA, USA); anti-integrin $\alpha 4$-FITC for Cd49d (Immunotech SA, Marseilles, France); anti-human CD1c-FITC (Ancell, Bayport, MN, USA); anti-CD103-FITC (Immuno Quality products, Groningen, The Netherlands); and anti-human FMC7-FITC (DAKO, Glostrup, Denmark). The second-step reagent for indirect immunofluorescence was goat anti-mouse Ig-FITC (Becton Dickinson).

Dual or three-color immunophenotyping was always performed. The controls for these experiments included irrelevant isotype-matched MoAbs and FITC, PE or PerCP mouse Ig. FITC-conjugated $F(ab')_2$ fragments of rabbit Ig (DAKO) were used for SIg analysis. The cells were examined using a FACScan flow cytometer (Becton Dickinson), with the data being acquired and analyzed by means of CELLQuest software (Becton Dickinson). The expression of each marker was calculated in terms of percentage reactivity on $CD19^+$ cells and graded as follows: 0 or – ($<$20% of $CD19^+$ cells), 1 or ± ($\geq$20% and $<$60%) and 2 or + ($\geq$60%). The intensity of SIg and FMC7 was evaluated on the basis of fluorescence intensity and two expression levels were so defined: dim indicated a certain degree of overlapping between the fluorescence histograms of the positive cells and those of the cells used as negative controls (the difference between the median fluorescence channel of the positive and negative cells was between 100 and 390: mean ± s.d. = 292 ± 49); bright indicated that no overlapping was demonstrable (difference between 400 and 750: mean ± s.d. = 514 ± 96). When the fluorescence intensity was evaluated in terms of molecules of equivalent soluble fluorochrome (MESF) (Quantum Kits, Flow Cytometry Standards, San Juan, CA, USA), dim was defined as a fluorescence intensity $\leq 9 \times 10^3$ MESF, and bright one of $>9 \times 10^3$ MESF.

Cytogenetic analysis

The karyotype analyses were performed by means of QFD binding as previously reported,[6] and the chromosomes were classified according to the International System for Human Cytogenetic Nomenclature (ISCN).[7] A clone was defined as two cells having the same structural rearrangement or a gain of the same chromosome, or three cells with the loss of the same chromosome.

Bcl-1 and bcl-6 analyses

Only the patients in clusters C2 and C3 were analyzed for bcl-1 on the grounds of our previous data showing no involvement of this gene in classical CLL,[8] and *bcl-6* was evaluated only in the cases falling into cluster C4 because of the lack of data concerning the frequency of its rearrangement in patients whose phenotype consists of bright SIg and low CD5 and CD23 expression. The analysis was performed as previously reported.[6,9]

Statistical methods

Descriptive statistics were calculated for the quantitative (mean, standard deviation, minimum, maximum and median in the case of asymmetric distributions) and qualitative variables (absolute and relative frequencies) with their pertinent 95% confidence intervals. The diagnostic classes were compared using Kruskal–Wallis non-parametric analysis of variance for quantitative variables, and the chi-square test for qualitative variables. The agreement between the different monoclonal antibody reactivities was calculated according to Cohen's k.[10] The significance level of the multiple comparisons was corrected using Bonferroni's procedure.[11] The follow-up was truncated at 8 years in order to obtain reliable estimates of the cumulative probability of no evolution or survival according to Kaplan and Meier;[12] the differences between the survival curves were evaluated using the log-rank test. The best subset of prognostic factors was obtained using a Cox proportional hazards regression model following a backward procedure; the goodness of fit was checked by means of stratified analysis.[13]

Results

Immunophenotype and diagnostic cluster distribution

The heavy SIg chain was $\mu\delta$ in 147/258 cases (57.0%; 95% confidence intervals, CI: 50.7–63.1%) and μ in 70 (27.1%; 95% CI: 21.8–33.0%); γ, δ, $\mu\gamma$ chain expression was observed in respectively, 18 (7.0%; 95% CI: 4.2–10.8%), 13 (5.0%; 95% CI: 2.7–8.4%) and 10 cases (3.9%; 95% CI: 1.9–7.0%). The proportion of SIg κ cases *vs* SIgλ cases was 1.79. Dim SIg fluorescence was observed in 153 cases (59.3%; 95% CI: 53.0–65.3%). The reactivity of the individual markers is shown in Figure 1. CD5 reactivity = 2 was observed in 75.9% of the cases (95% CI: 70.3–81.0%), and CD5/CD23 co-expression in 187/258 cases (72.5%; 95% CI: 66.6–77.8%), inversely correlated with the intensity of SIg fluorescence (143 of the 163 $CD5^+/CD23^+$ cases were SIg dim; 87.7%). CD49c was highly expressed in 180/245 cases (73.4%; 95% CI: 67.5–78.9%), whereas CD1c reactivity was often = 0 (133/238; 55.9%; 95% CI: 49.3–62.3%). FMC7, CD11c, CD25 and CD49d reactivity = 2 was observed in about 50% of cases. The intensity of FMC7 fluorescence was evaluated in 219 cases: it was dim in 96 (43.8%; 95% CI: 37.2–50.7%) and bright in 123 (56.2%; 95% CI: 49.3–62.8%).

On the basis of the known diagnostic role of CD5

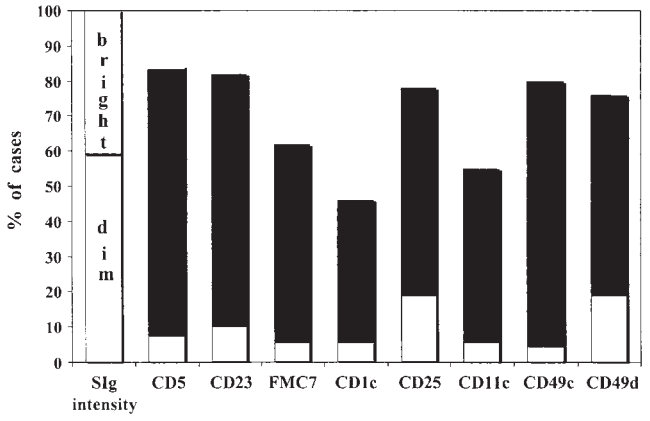

Figure 1 Individual marker reactivities of peripheral blood lymphocytes in 258 cases of mature B cell lymphoid leukemia. Percentage of cases by degree of reactivity: white, reactivity = 1; black, reactivity = 2.

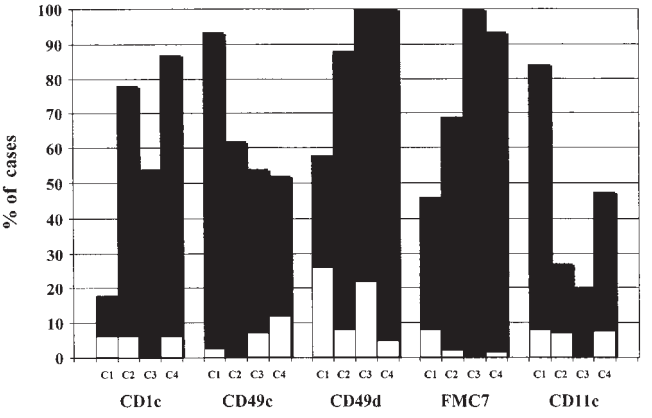

Figure 2 Distribution of individual marker reactivities of peripheral blood lymphocytes in 258 cases of mature B cell lymphoid leukemias on the basis of our diagnostic cluster definition (C1–C4); white, reactivity = 1; black, reactivity = 2.

expression, CD5-CD23 co-expression and SIg reactivity patterns,[14,15] and by analyzing the relative frequencies of the tested MoAb reactivity in our series, we identified four clusters of patients. The two situations consistent with a diagnosis of 'classical' CLL ($SIg^d/CD5^+/CD23^+$) or a leukemic form of MCL ($SIg^b/CD5^+/CD23^+$) were, respectively, classified as CLL-type (C1: 149 patients) and MCL-type clusters (C3: 16 patients). The remaining clusters included less defined situations: a group of 38 patients with a CLL variant pattern because of the persistence of CD5 reactivity = 2 and CD23 reactivity = 1 or 2 in the presence of bright SIg ($SIg^b/CD5^+/CD23^{+/\pm}$), or the persistence of dim SIg in the presence of CD5 reactivity = 0 or 1 ($SIg^d/CD5^{-/\pm}$/CD23 indifferent), was defined as the CLL-variant type (C2); finally, 55 patients with a phenotype characterized by CD5 reactivity = 0 or 1 and bright SIg ($SIg^b/CD5^{-/\pm}$/CD23 indifferent) were considered to be affected by leukemic phase of lymphoplasmocytic/splenic marginal zone lymphomas, and were classified as the LP/S-type (C4).

There were no significant between-cluster differences in terms of SIg classes, but the γ chain was more frequent in C4; the proportion of SIg κ cases *vs* SIgλ cases increased from C1 to C4 (1.73, 2.1, 2.0 and 2.5), but this trend was not significant. CD1c, CD49d and FMC7 were less expressed in C1 than in the other clusters ($P = 0.001$), and the intensity of FMC7 fluorescence in C1-positive cases was always less ($P = 0.0001$). Grade 2 CD49c reactivity was more observed in C1 than in the other clusters ($P = 0.001$), and CD11c reactivity was more frequent in C1 and C4 than in C2 and C3 ($P = 0.001$). There was no significant between-cluster difference in CD25 expression (Figure 2).

Cytomorphology

As shown in Table 1, a typical CLL-like morphology was observed in 155 cases (60.0%; 95% CI 53.8–66.1%), and was prevalent in cluster C1 (80% of the cases, as against 39% in C2, 44% in C3 and 25% in C4); non-CLL forms (103 cases, 40.0%) were significantly more frequent in C4 ($P = 0.0001$). A high number of LPIC and SLVL morphologies were observed in C4; HCL-like forms were observed only in C4.

Clinico-hematological features

The median age of the 258 patients was 64 years (range 30–94), and the male/female ratio 1.43. Table 2 shows their most relevant clinico-hematological characteristics by flow cytometric clusters. There were significant between-cluster differences in splenomegaly, hemoglobin, platelet counts, $\beta 2$ microglobulin and LDH serum levels, the pattern of bone marrow infiltration, patient status and the need for therapy. HCV antibodies were tested in 142 patients at diagnosis: the 11 positive cases (8%) included 5/21 C4 patients.

The median time from diagnosis to therapy was 6 months in the patients as a whole: 11 months in C1, 2 months in C2, 1 month in C3, and 3 months in C4. The relationship between disease-related and overall deaths in the four clusters was 17/29 in C1, 12/16 in C2, 9/9 in C3 and 14/21 in C4. LDT was (12 months in 73.3% (95% CI: 67.8–79.9%) of the patients and (12 months in 26.7% (95% CI: 20.1–34.2%); the cluster distribution is shown in Table 2.

Karyotype

Karyotype abnormalities increased from C1 to C3, but the differences were not significant (Table 3). Chromosome 12 trisomies and partial deletions involving chromosomes 11 and 13 were mainly observed in C1 and C2. Structural abnormalities of chromosomes 14 and 17 were more frequent in C4 than in C1. The most frequent karyotpe abnormalities in C3 were t(11;14) and chromosome 14 alterations, but the limited number of cases does not allow any statistical evaluation.

Molecular findings

Bcl-1 rearrangements were found in C2 (3/33 cases) and mainly in C3 (9/16 cases; $P = 0.001$). In C2, the two bcl-1 rearranged cases were $SIg^{bright}/CD5^+/CD23^{+/\pm}$. Six of the 11 rearranged cases were cytogenetically evaluated: four had the expected t(11;14) translocation, one had a complex karyotype with chromosome 14 abnormalities and one had a normal karyotype (Table 3). None of the C4 patients showed any *bcl-6* rearrangement.

Table 1 Peripheral blood morphology (number of cases and percentages) of the whole series and in relation to the immunophenotype clusters (C1–C4)

	Whole series	*%*	*C1*	*%*	*C2*	*%*	*C3*	*%*	*C4*	*%*
No. of cases	258		149		38		16		55	
CLL	155	60.0	119	79.9	15	39.5	7	43.7	14	25.5
Non-CLL	103	40.0	30	20.1	23	60.5	9	56.3	41	74.5
LPIC	31		6		6		1		18	
SLVL	12		1		5		0		6	
HCL	2		0		0		0		2	
Heterogeneous	58		23		12		8		15	

CLL, typical chronic lymphocytic leukemia; LPIC, lymphoplasmocytoid lymphoma/immunocytoma; SLVL, splenic lymphoma with villous lymphocytes; HCL, hairy cell leukemia.

Table 2 Clinico-hematological data at diagnosis in the whole series and by immunophenotype clusters (C1–C4)

Variables	*Total cases*	*Cluster 1*	*Cluster 2*	*Cluster 3*	*Cluster 4*	*P*
No. of cases	**258**	**149**	**38**	**16**	**55**	
Age <70 years	67 (25.9)	35 (23.5)	9 (23.7)	7 (43.7)	16 (29.1)	NS
Male/Female	1.43	1.36	1.92	1.28	1.39	NS
Splenomegaly[a]	105 (40.6)	31 (20.1)	21 (55.3)	10 (62.5)	43 (78.2)	0.001
Hemoglobin ≤11 g/dl	35 (13.5)	8 (5.4)	6 (15.8)	6 (37.5)	15 (27.3)	0.001
Platelets ≤140 × 10^9/l	72 (27.9)	30 (20.1)	17 (44.7)	7 (43.7)	18 (32.7)	0.006
Serum monoclonal component	63 (24.4)	29 (19.5)	10 (26.3)	4 (25.0)	20 (36.4)	NS
LDH >460 U/l	47 (18.2)	15 (10.1)	7 (18.4)	9 (56.2)	16 (29.1)	0.001
Lymphocytes <20 × 10^9/l	75 (29.0)	43 (28.9)	10 (26.3)	7 (43.7)	15 (27.3)	NS
Cytomorphology						
CLL	155 (60.0)	119 (79.9)	15 (39.4)	7 (43.7)	14 (25.4)	
CLL variant	25 (9.6)	12 (80.5)	7 (18.4)	0 (0)	6 (10.9)	0.001
not CLL	78 (30.2)	18 (12.1)	16 (42.1)	9 (56.2)	35 (63.6)	
Overall deaths	75 (29.0)	29 (19.4)	16 (42.1)	9 (56.2)	21 (38.1)	0.001
Chemotherapy	170 (65.8)	84 (56.4)	25 (65.7)	14 (87.5)	47 (85.4)	0.001
No. of cases	**161**	**111**	**20**	**4**	**26**	
LDT[b] ≤12 months; No. of cases	43 (26.7)	29 (26.1)	5 (25)	2 (50.0)	7 (26.9)	NS
No. of cases	**164**	**101**	**22**	**10**	**31**	
Serum β2 microglobulin >2.6 μg/ml	77 (46.9)	36 (35.6)	14 (63.6)	7 (70)	20 (64.5)	0.003
No. of cases	**218**	**122**	**35**	**14**	**47**	
Diffuse bone marrow histology	74 (33.9)	33 (27.0)	13 (37.1)	9 (64.3)	19 (40.4)	0.025
No. of cases	**220**	**132**	**33**	**8**	**47**	
Abnormal karyotype	75 (34.1)	39 (29.5)	14 (42.4)	5 (62.5)	17 (36.2)	NS
No. of cases	**142**	**95**	**15**	**6**	**21**	
HCV antibody positivity	11 (8.4)	6 (6.3)	0 (0)	0	5 (23.8)	NS

NS, not significant.
[a]Manual and/or ecographic.
[b]LDT, lymphocyte doubling time (not evaluated in 97 patients receiving chemotherapy in first year from diagnosis or with a follow-up of less than 12 months).
Percentages in parentheses.

Survival analysis, prognostic variables and prognostic stratification

Table 4 shows the 5-year cumulative survival probability and 95% CI, and the significance of the variables having a prognostic impact on survival at univariate analysis. In addition to age, the clinico-hematological variables were spleen size, hemoglobin, platelet count, serum LDH, β2 microgobulin level, the bone marrow infiltration pattern and karyotype. There was no statistical significance in terms of gender, the absolute number or morphology of lymphocytes, or the presence of a monoclonal component or HCV antibodies. The probability of survival was highest in the patients with SIg dim and 0 or 1 CD49d/CD1c reactivity, and lowest in those with 0 CD11c/CD49c reactivity, and 0 or 1 CD23 reactivity. No statistically significant association with survival was found in the case of CD25, $CD19^+CD5$ or FMC7 expression. With reference to the immunophenotype diagnostic clusters, survival significantly worsened from C1 to C4, C2 and C3 (Figure 3). The poor prognostic significance of the C2 designation was maintained when the three bcl-1 rearranged cases were excluded from the analysis (data not shown).

Multivariate statistical analyses were performed using Cox's model considering the different clinical occasions on which prognosis could be assessed (Table 5). In our selected series, when the clinico-hematological variables were recorded, age,

Table 3 Peripheral blood cytogenetic findings (number of cases and percentages) by immunophenotype clusters (C1–C4)

	Whole series	*%*	*C1*	*%*	*C2*	*%*	*C3*	*%*	*C4*	*%*
No. of cases	220		132		33		8		47	
Normal	145	65.9	93	70.4	19[b]	57.6	3	37.5	30	63.8
Abnormal	75	34.1	39	29.6	14	42.4	5	62.5	17	36.2
trisomy 12	17	22.6	12	30.7	3	21.4	1	20.0	1	5.9
del (11q)	10	13.3	6	15.3	3	21.4	0	0	1	5.9
del (13q)	4	5.3	4	10.2	0	0	0	0	0	0
t (11;14)	5	6.6	1	2.5	2[a]	14.2	2[a]	40.0	0	0
14 alterations	13	17.3	2	5.1	2	14.2	2[c]	40.0	7	41.1
17 alterations	10	13.3	4	10.2	1	7.1	0	0	5	29.4
Other abnormalities	16	21.3	10	25.6	3	21.4	0	0	3	17.6

[a]bcl-1 rearranged.
[b]One case was bcl-1 rearranged.
[c]One case was bcl-1 rearranged.

platelet count and spleen size made up the most parsimonious subset of variables related to survival. In a sub-sample of 220 patients with available cytogenetic and bone marrow histology patterns, a further model was obtained in which age at diagnosis and spleen size retained a significant independent association with survival (RR 2.11, 95% CI: 1.20–3.69, $P = 0.009$; RR 2.28, 95% CI: 1.18–3.36, $P = 0.003$), together with an abnormal karyotype (RR 1.99, 95% CI: 1.32–3.91, $P = 0.001$). When the immunophenotype features were considered alone, the degree of CD11c expression and the intensity of SIg fluorescence remained independent prognostic factors, but when the immunophenotype clusters were included in the model, SIg fluorescence lost its statistical significance as a result of redundancy. Similar results were obtained using a more parsimonious model that pooled clusters 2 and 3. Finally, in a combined clinico-immunophenotype model, age at diagnosis, platelet count, CD11c expression and cluster classification retained their prognostic significance. On the basis of these variables, the survival analysis of the prognostic score (PS) classes obtained using the Cox models could be clearly differentiated because of the combination of the relative risks. Figure 4 shows the survival curves of the three prognostic classes (PS ≤ 2.3, $>2.3 \leq 3$, $>3 \leq 9$).

Discussion

The REAL/WHO classification has greatly improved the clinico-pathological, immunophenotypic, genetic and molecular characterization of lymphomas and leukemias. However, the integration of these findings with lymphoid tissue analysis is not always possible in the case of MBC-LL for various reasons: (1) the absence of easily accessible pathological tissue; (2) the decision to avoid lymph node biopsy in particular clinical situations (eg elderly patients, comorbidity); and (3) the frequent practice of avoiding invasive diagnostic interventions in cases that are considered to be sufficiently well defined immunocytomorphologically (eg classic CLL). On the basis of these considerations, the immunophenotypic analysis of peripheral or bone marrow cells plays a pivotal diagnostic role. From this point of view, various scoring systems have been proposed over the last few years. The first was suggested by Matutes *et al*,[3] and was based on the reactivity of CD5, CD23, FMC7 and the intensity of SIg and CD22 expression; it correctly diagnosed 87% of CLLs, 89% of other B cell leukemias and 72% of NHLs in leukemic phase in terms of their correspondence with FAB cytomorphology. In 1995, Salomon-Nguyen *et al*[16] proposed another scoring system (based on CD22, CD23 and FMC7 reactivity, and SIg intensity) aimed at distinguishing the rare subsets of $CD5^-$ CLL (less than 10% of CLL) and B-PLL from typical $CD5^+$ CLL. Moreau *et al*[4] have recently proposed a revised version of their scoring system[3] in which CD22 is replaced by CD79b, which proved to be negative in most cases of CLL and positive in most other B cell disorders.[17] Although very important, these studies have only been validated on the basis of morphological data and not extensively verified in a clinical context.

In our study, we made an in-depth analysis of a large number of newly diagnosed MBC-LLs with minimal or absent lymph-node involvement with the aims of: (1) selecting commonly used lymphoid markers to include in a simplified IC that would be useful in identifying in MBC-LL spectrum not only cases consistent with a diagnosis of CLL or MCL, but also other forms possibly characterized by a distinct clinical behavior; and (2) evaluating the prognostic role of the system as an independent or dependent variable when considered together with clinico-hematological prognostic features. Our simplified IC was formulated using the relative frequencies of the reactivity of the tested MoAbs, while taking into account the consistent immunophenotype pattern of typical CLLs and MCLs based on CD5 and CD23 reactivity and the pattern of SIg expression. The combined reactivity of these three markers identified four diagnostic clusters. This phenotypic classification did not include FMC7, CD22 and CD79b reactivity because their reactivity was not negligible in C1 (respectively 40%, 49% and 64%, data not shown), although they were preferentially expressed in C2, C3 and C4. It is interesting to note that the intensity of their fluorescence seemed to be more useful because it was always low in FMC7- and CD79b-positive cases in C1, and there was a close correlation between CD79b and SIg fluorescence intensity (data not shown).

Although our IC is simpler than those previously proposed (in so far as it is based on fewer MoAb markers),[3,4,16] it still seems to be highly capable of dividing the MBC-LL series into different pathological entities characterized by recurrent clinico-hematological features and a different clinical course. C1 (CLL-type cluster) identified classical CLL cases, 80% of which had a CLL morphology. The frequently indolent behavior of CLL was well represented by the favorable clinical characteristics of a large number of patients (little organ involvement, no cytopenia and a long time from diagnosis to therapy), and this cluster also included a larger number of patients with the

Table 4 Univarate survival analysis in patients with mature B cell lymphoid leukemias

Variable	*No. of cases*	*5-year cumulative survival probability (95% CI)*	*P*
Whole series	258	0.73 (0.67–0.69)	
SIg fluorescence intensity:			
Dim	153	0.84 (0.78–0.90)	
Bright	105	0.56 (0.46–0.66)	0.0001
CD11c expression[a]			
1+2	128	0.87 (0.81–0.93)	
0	122	0.58 (0.48–0.68)	0.0001
CD23 expression[a]			
2	185	0.79 (0.73–0.85)	
0+1	73	0.58 (0.44–0.72)	0.0017
CD49d expression[a]			
0+1	113	0.86 (0.79–0.93)	
2	138	0.61 (0.51–0.71)	0.0003
CD49c expression[a]			
1+2	191	0.76 (0.70–0.82)	
0	54	0.58 (0.42–0.74)	0.0143
CD1c expression[a]			
0+1	146	0.79 (0.71–0.87)	
2	92	0.62 (0.50–0.74)	0.0080
Immunophenotypic clusters:			
C1	149	0.84 (0.78–0.90)	
C4	55	0.69 (0.55–0.83)	
C2	38	0.52 (0.32–0.72)	
C3	16	0.22 (0.00–0.56)	0.0001
Age at diagnosis (years)			
≤70	191	0.80 (0.74–0.86)	
>70	67	0.50 (0.36–0.64)	0.0001
Spleen size			
Normal	153	0.84 (0.78–0.90)	
Increased	105	0.56 (0.46–0.66)	0.0001
Hemoglobin (g/dl)			
>11	223	0.77 (0.71–0.83)	
≤11	35	0.47 (0.27–0.67)	0.0011
Platelet count (/(l)			
>140 000	186	0.80 (0.74–0.86)	
≤140 000	72	0.51 (0.37–0.65)	0.0001
Serum L.D.H. (/U.I.)			
≤460	207	0.78 (0.72–0.84)	
>460	47	0.52 (0.36–0.68)	0.0008
Beta-2 microglobulin (μg/dl)			
≤2.6	87	0.98 (0.95–1.00)	
>2.6	77	0.83 (0.74–0.92)	0.0001
Bone marrow infiltration pattern			
Not diffuse	144	0.81 (0.73–0.89)	
Diffuse	74	0.61 (0.52–0.70)	0.0003
Cytogenetic analysis			
Normal karyotype	145	0.82 (0.75–0.92)	
Abnormal karyotype	75	0.60 (0.48–0.72)	0.0008

[a]Monoclonal antibody reactivity: 0: <20% of CD19$^+$ cells; 1: ≥20% and <60%; 2: ≥60%.

karyotype abnormalities most frequently described in CLL (trisomy 12 and partial deletion of chromosome 11). C3 (MCL-type cluster) clearly identified the forms of MCL-related leukemia because the patients in this cluster showed the highest frequency of the karyotypic t(11;14) and genetic alterations (bcl-1 rearrangement) consistent with this type of NHL, and splenomegaly, anemia and thrombocytopenia were particularly frequent. C2 (CLL-variant cluster) identified patients whose immunophenotype has been generically reported as being consistent with a diagnosis of 'variant' CLL. The patients in this cluster more frequently showed splenomegaly, thrombocytopenia, anemia, a shorter time to treatment start, and karyotypic abnormalities and atypical cytomorphologies consistent with a higher degree of malignancy. In this cluster bcl-1 rearrangement was sporadic, confirming the independence of C2 from C3/typical MCL forms. These data support the hypothesis that C2 score identified a 'variant' CLL characterized by a distinctive clinical behavior. C4 (LP/S-type cluster) included a heterogeneous group of cases characterized by bright SIg and little or no CD5 reactivity. Their morphologies were prevalently of the non-CLL type (LPIC and SLVL, even including the only two cases of HCL) and the involvement of chromosome 14 and 17 was remarkably frequent. The frequent splenic involvement (78% of cases) suggested the possibly common splenic origin of these forms, whose subtypes could be better identified using an extended MoAb panel (including CD103 and CyIg).

Our IC also seems to recognize clinical entities with different outcomes as both the univariate and multivariate analyses showed that the patients in C3 and C2 had a significantly

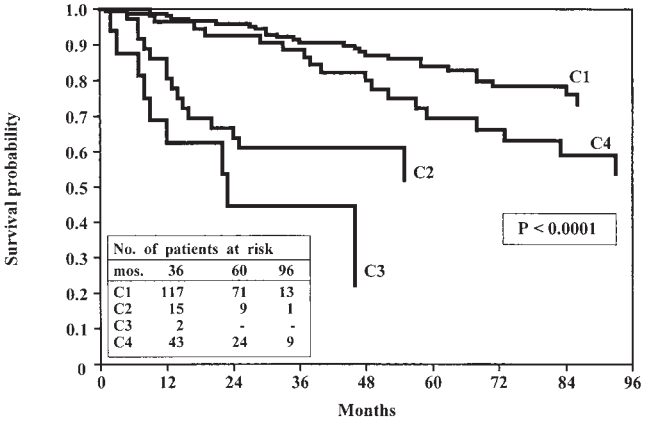

Figure 3 Actuarial survival probability in 258 patients with mature B cell lymphoid leukemias by diagnostic cluster attribution: 149 patients were classified in cluster 1 (C1), 38 in cluster 2 (C2), 16 in cluster 3 (C3) and 55 in cluster 4 (C4).

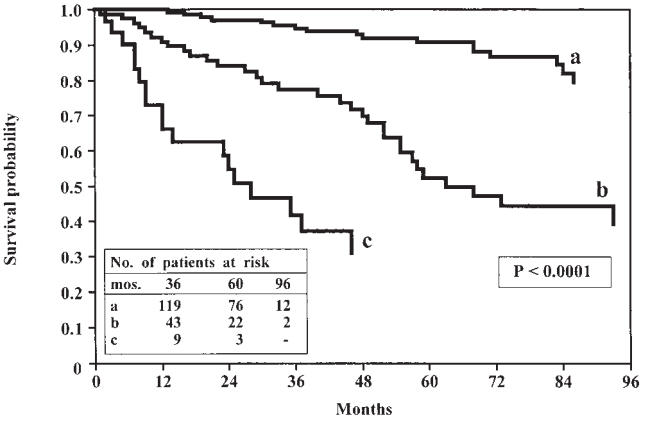

Figure 4 Cumulative survival probability curves obtained using Cox's combined 'clinical and immunophenotype with cluster' model (age >70 years, platelet count ≤140 000/μl, CD11c negativity, cluster 2 or 3). (a) ≤1 unfavorable prognostic factor; (b) two unfavorable prognostic factors; (c) ≥3 unfavorable prognostic factors.

Table 5 Multivariate (Cox) analysis of the parameters statistically significant at univariate analysis

Variables	*Relative risk*	*95% CI*	*P*
Basic clinical model			
Age at diagnosis: >70 *vs* ≤70 years	2.54	1.56–4.14	0.0002
Platelet count: ≤140 000 *vs* >140 000/μl	2.02	1.17–3.51	0.0120
Spleen size: increased *vs* normal	1.77	1.01–3.09	0.0443
Basic immunophenotype model			
CD11c expression: 0 *vs* 1+2[a]	2.56	1.49–4.39	0.0006
SIg fluorescence: bright *vs* dim	2.30	1.39–3.82	0.0013
Immunophenotype with clusters model			
CD11c expression: 0 *vs* 1+2[a]	2.56	1.49–4.39	0.0013
Immunophenotype cluster			0.0001
Cluster 4 *vs* 1	1.55	0.84–2.85	
Cluster 2 *vs* 1	2.75	1.43–5.29	
Cluster 3 *vs* 1	6.10	2.64–14.07	
Combined clinical and immunophenotype model			
Age at diagnosis: >70 *vs* ≤70 years	2.44	1.45–4.11	0.0008
Platelet count: ≤140 000 *vs* >140 000/μl	2.15	1.29–3.59	0.0034
CD11c expression: 0 *vs* 1+2[a]	2.28	1.30–4.01	0.0043
Immunophenotype cluster			0.0060
Cluster 4 *vs* 1	1.20	0.64–2.27	
Cluster 2 *vs* 1	2.46	1.24–4.89	
Cluster 3 *vs* 1	3.63	1.51–8.70	

[a]Monoclonal antibody reactivity: 0: <20% of $CD19^+$ cells; 1: ≥20% and <60%; 2: ≥60%.

worse prognosis than those in C4 and C1. Given the small number of C3 cases, we could not distinguish the prognostic value of a C3 designation *vs* bcl-1 rearrangement or t(11;14), which are generally accepted as defining characteristics of MCL, in establishing the best predictor of a poor outcome. Interestingly, CD11c expression retained its independent prognostic role when cluster attribution was included in the multivariate analysis. This was confirmed by the prognostic scoring analysis of various Cox models, which showed that the combined evaluation of the degree of CD11c expression and cluster attribution was the best immunophenotypical means of prognostic stratification. The prognostic role of CD11c has been previously described, and its greater expression in the clusters with a better prognosis (C1 and C4) not only confirms our previous clinical observations,[5] but is also in line with the results of Eistere *et al*[18] and Sembries *et al*[19] concerning B-CLLs. Our results also confirm the observations of Sembries *et al*[19] regarding the correlation between the presence of the 11q deletion and low CD11c expression: in C1, 11q partial deletions were more frequently observed in CD11c-negative cases (7/27 *vs* 2/40, $P = 0.036$; data not shown). Perhaps CD11c low expression could postulate the presence of karyotypic alterations, one of these is 11q partial deletion, which modifying the expression of different adhesion molecules or tumour-suppressor genes, are important for development and progression of this subset of MBC-LL. In relation to the prognostic role of immunophenotypic markers, such as bright SIg and CD23 reactivity 0 or 1, whose presence negatively influenced survival, our data confirm the previous observations of Geisler *et al*.[20] Moreover, there are interesting similarities in terms of clinical presentation (high frequency of splenomegaly) and prognosis between our LP/S-type cluster and a subset of $SIg^{bright}/CD5^-/CD23^-$ cases reported in the same paper. Cartron *et al*[21] made the same observations, although they did not draw any definite conclusions concerning prognosis. Tefferi *et al*[22] did not confirm the prognostic

role of bright SIg light chain expression, but their series was too small. Recently, in a small series of classic B-CLL patients, Hulkkonen *et al*[23] observed an interesting association between certain phenotypic characteristics and specific karyotypic alterations (eg trisomy 12 and enhanced CD27 and SIgMκ expression, or the deletion of chromosome arm 11q and enhanced expression of CD45RO). We did not measure these markers in our series and thus cannot validate these data. However, they support our findings concerning CD11c expression and the presence of the 11q deletion and suggest a possible relationship between specific karyotypic alterations and the expression of some particular antigens. We finally evaluated the prognostic impact of our IC in relation to the immunophenotype markers and clinico-hematological variables in this selected series of MBC-LL patients. Multivariate analysis showed that only the proposed cluster classification, CD11c expression, age more than 70 years and thrombocytopenia retained their prognostic significance. In this combined prognostic model, although the clinico-hematological features are important because they relate to tumor burden or patient status, our simplified IC seems to be capable of identifying the different entities from a biological point of view.

In conclusion, our immunophenotype classification is not only a simplified and efficacious means of defining different MBC-LL entities, but may also play an important role in terms of prognostic stratification. In particular it identifies not infrequent situations, such as those falling in the CLL-var and LP/S-type clusters (C2 and C4), that have particular prognostic and/or clinical behaviors.

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References

1 Bennet JM, Catovsky D, Daniel M-T, Flandrin G, Galton DAG, Gralnick HR, Sultan C, the French–American–British (FAB) Cooperative Group. Proposals for the classification of chronic (mature) B and T lymphoid leukemias. *J Clin Pathol* 1989; **42**: 567–584.
2 Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, Lister TA, Bloomfield CD**.** World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting, Airlie House, Virginia, November 1997. *J Clin Oncol* 1999; **12**: 3835–3849.
3 Matutes E, Owusu-Ankomah K, Morilla R, Garcia Marco J, Houlihan A, Que TH, Catovsky D. The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. *Leukemia* 1994; **8**: 1640–1645.
4 Moreau EJ, Matutes E, A'Hern RP, Morilla AM, Morilla RM, Owusu-Ankomah KA, Seon BK, Catovsky D. Improvement of the chronic lymphocytic leukemia scoring system with the monoclonal antibody SN8 (CD 79b). *Am J Clin Pathol* 1997; **108**: 378–382.
5 Baldini L, Cro L, Calori R, Nobili L, Silvestris I, Maiolo AT. Differential expression of very late ativation antigen-3 (VLA-3)/VLA4 in B-cell non-Hodgkin lymphoma and B-cell chronic lymphocytic leukemia. *Blood* 1992; **79**: 2688–2693.
6 Baldini L, Fracchiolla NS, Cro LM, Trecca D, Romitti L, Polli E, Maiolo AT, Neri A. Frequent p53 gene involvement in splenic B-cell leukemia/lymphomas of possible marginal zone origin. *Blood* 1994; **84**: 270–278.
7 Mitelman F. *Guidelines for Cancer Cytogenetics. Supplement to an International System for Human Cytogenetic Nomenclature*. Karger: Basel, 1991.
8 Gaidano G, Newcomb EW, Gong JZ, Tassi V, Neri A, Cortelezzi A, Calori R, Baldini L, Dalla-Favera R. Analysis of alterations of oncogenes and tumor suppressor genes in chronic lymphocytic leukemia. *Am J Pathol* 1994; **144**: 1312–1319.
9 Neri A, Fracchiolla NS, Roscetti E, Garatti S, Trecca D, Boletini A, Perletti L, Baldini L, Maiolo AT, Berti E**.** Molecular analysis of cutaneous B and T-cell lymphomas. *Blood* 1995; **86**: 3160–3172.
10 Cohen J. A coefficient of agreement for nominal scales. *Educ Psychol Meas* 1960; **20**: 37–46.
11 Armitage P, Berry G. Statistical methods. In: Armitage P, Berry G (eds). *Medical Research*. Blackwell Scientific Publications: Oxford, 1987, pp 408–420.
12 Kaplan EI, Meier P. Non-parametric evaluation from incomplete observations. *J Am Stat Assoc* 1958; **53**: 457–481.
13 Kalbfleisch JD, Prentice RL. *The Statistical Analysis of Failure Time Data*. John Wiley: New York, 1980.
14 Baldini L, Cro L, Cortelezzi A, Calori R, Nobili L, Maiolo AT. Immunophenotypes in 'classical' B-cell lymphocytic leukemia: correlation with normal cellular counterpart and clinical findings. *Cancer* 1990; **66**: 1738–1742.
15 Cheson BD, Bennett JM, Grever M, Kay N, Keating MJ, O'Brien S, Rai KR. National Cancer Institute-Sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. *Blood* 1996; **87**: 4990–4997.
16 Salomon-Nguyen F, Valensi F, Merle-Beral H, Flandrin G. A scoring system for the classification of $CD5^-$ B CLL versus $CD5^+$ B CLL and B PLL. *Leuk Lymphoma* 1995; **16**: 445–450.
17 Zomas AP, Matutes E, Morilla R, Owusu-Ankomah K, Seon BK, Catovsky D. Expression of the immunoglobulin-associated protein B29 in B-cell disorders with the monoclonal antibody SN8 (CD79b). *Leukemia* 1996; **10**: 1966–1970.
18 Eistere W, Hilbe W, Stauder R, Bechter O, Fend F, Thaler J. An aggressive subtype of B-CLL is characterised by strong CD44 expression and lack of CD11c. *Br J Haematol* 1996; **93**: 661–669.
19 Sembries S, Pahl H, Stilgenbauer S, Döhner H, Schriever F. Reduced expression of adhesion molecules and cell signaling receptors by chronic lymphocytic leukemia cells with 11q deletion. *Blood* 1999; **93**: 624–631.
20 Geisler CH, Larsen JK, Hansen NE, Hansen MM, Christensen BE, Lund B, Nielsen H, Plesner T, Thorling K, Andersen E. Prognostic importance of flow cytometric immunophenotyping of 540 consecutivepatients with B-cell chronic lymphocytic leukemia. *Blood* 1991; **78**: 1795–1802.
21 Cartron G, Linassier C, Bremond JL, Desablens B, Georget MT, Fimbel B, Luthier F, Dutel JL, Lamagnere JP, Colombat P. CD5 negative B-cell chronic lymphocytic leukemia: clinical and biologicalfeatures of 42 cases. *Leuk Lymphoma* 1998; **31**: 209–216.
22 Tefferi A, Bartholmai BJ, Witzig TE, Li CY, Hanson CA, Phyliky RL. Heterogeneity and clinical relevance of the intensity of CD20 and immunoglobulinlight-chain expression in B-cell chronic lymphocytic leukemia. *Am J Clin Pathol* 1996; **106**: 457–461.
23 Hulkkonen J, Vilpo L, Hurme M, Vilpo J. Surface antigen expression in chronic lymphocytic leukemia: clustering analysis, interrelationships and effects of chromosomal abnormalities. *Leukemia* 2002; **16**: 178–185.