Heterogeneity of neoplastic cells in B-cell chronic lymphoproliferative disorders: biclonality versus intraclonal evolution of a single tumor cell clone

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Background and Objectives. B-cell chronic lymphoproliferative disorders (B-CLPD) are usually monoclonal expansions of a single B-cell clone. However in some cases, two unrelated B-cell clones co-exist. Additionally, cases with two B-cell subpopulations displaying a similar phenotype but distinct DNA contents exist, the exact nature of these cases remaining unknown. In order to gain insight into the characteristics of these complex B-CLPD we examined two cohorts of patients.

Design and Methods. One cohort had two phenotypically distinct B-cell populations (group A; n=9) and the other, two B-cell subsets showing different DNA contents and/or light scatter properties, but a similar immunophenotype (group B; n=7).

Results. Fluorescent *in situ* hybridization studies revealed the presence of genetic abnormalities in six cases from group A, either in one (n=5) or the two co-existing B-cell populations (n=1); in all these cases the two B-cell populations showed unrelated IgH gene rearrangements. In all seven cases from group B, the B-cell population showing higher DNA contents had additional chromosomal abnormalities as compared to the other subset; molecular analysis confirmed the monoclonal nature of these cases.

Interpretation and Conclusions. In summary, we show that in group A, two phenotypically/cytogenetically distinct, unrelated B-cell clones co-exist, while the two B-cell populations from group B appear to represent different stages of evolution of a single clone.

Key words: immunophenotype, biclonality, intraclonal evolution, B-cell chronic lymphoproliferative disorders, FISH.

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B-cell chronic lymphoproliferative disorders (B-CLPD) are a heterogeneous group of diseases typically characterized by the clonal expansion and accumulation of mature neoplastic cells derived from one single transformed B cell.[1-6] Despite this, morphologically different populations of neoplastic cells are frequently detected, particularly in some histopathologic and cytologic subtypes such as follicular lymphoma with mixed small and large cells, and prolymphocytic leukemia/chronic lymphocytic leukemia (CLL) variants. Such heterogeneity is usually attributed to intraclonal variation. However, cases have been cited in the literature in which two or more different B-cell clones co-exist,[7-20] at an overall incidence in all B-CLPD of around 5%.[19] Actually, most cases in which more than one immunophenotypically distinct B-cell subset is present in the same sample are considered to correspond to real biclonal B-CLPD,[19] independently of the presence or absence of morphological heterogeneity. Recently, we showed in some cases from a large series of 53 patients displaying two or more phenotypically distinct, genetically unrelated, B-cell clones, that the characteristics of both clones were compatible with the same diagnostic entity; while, in others, biclonality reflected the co-existence of two different diagnostic entities in the same patient.[19] In addition to these patients, there are other B-CLPD cases in which two different B-cell subpopulations can be detected on the basis of either their morphologic heterogeneity, different size or their distinct DNA cell contents, with both groups of cells showing similar phenotypic features. Whether or not these cases correspond to biclonal B-CLPD or to a single disease displaying intraclonal evolution remains unknown. Although immunophenotyping has proven to be a sensitive tool for the rapid screening of biclonal cases,[19] the immunophenotypic patterns associated with intraclonal evolution have not yet been established. In order to gain insight into the characteristics of these complex B-CLPD we compared two cohort of patients: one containing two phenotypically different cell populations and the other showing two B-cell subsets on the basis of the presence of a different DNA cell content and/or different light scattering properties, but with a similar immunophenotype.

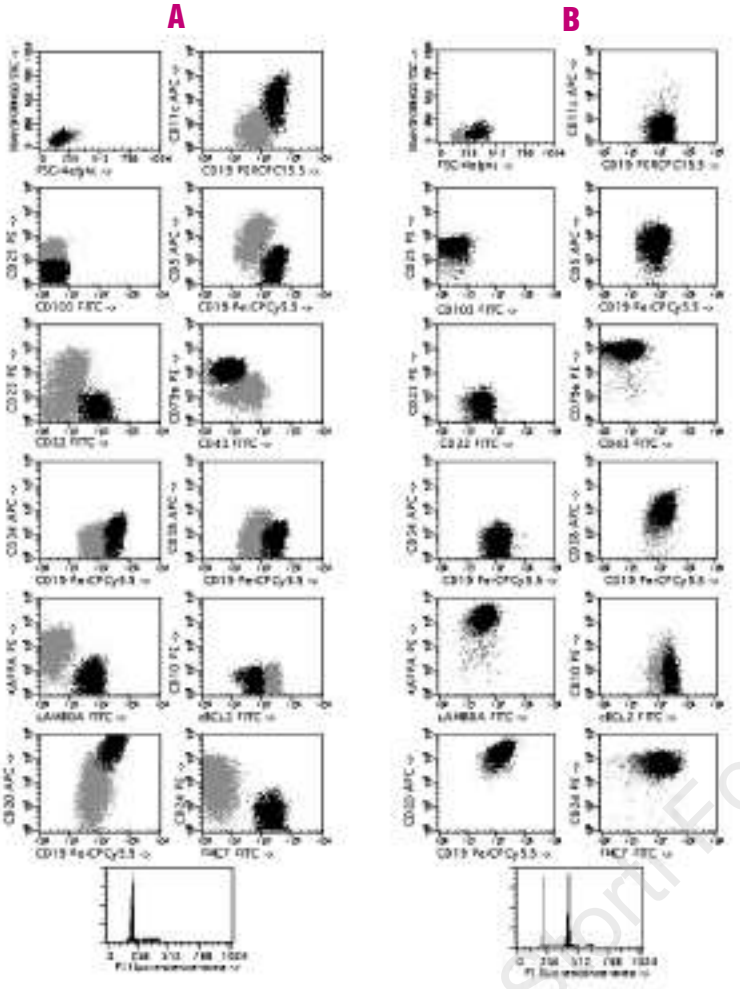

Figure 1. Illustrative bivariate dotplots and histograms corresponding to B-CLPD cases. Panel A. A patient with biclonal B-CLPD patient (case 2 in Table 2) shows two distinct B-cell populations with different phenotypes, corresponding to chronic lymphocytic leukemia and a splenic marginal zone lymphoma. Panel B shows a case corresponding to mantle cell lymphoma with two B-cell populations showing overlapping immunophenotypic characteristics but different light scattering properties, due to progression of a DNA diploid B-cell population into a tetraploid B-cell subset (case 13 in Table 2).

Design and Methods

Patients

A total of 16 patients newly diagnosed with B-CLPD were included in the study. All cases showed co-existence of two phenotypically aberrant B-cell populations. Nine patients (group A) had two B-cell subsets displaying different phenotypic features, each one compatible with a different B-CLPD (Figure 1, panel A). The other seven cases (group B) showed two B-cell subsets with a similar immunophenotype; the presence of two B-cell subpopulations was suspected on the basis of their different light scattering characteristics (n=6) and/or their distinct DNA cell contents (n=7) (Figure 1, panel B). In all cases, diagnosis was based on clinical, morphologic, immunophenotypic, molecular and histologic criteria, according to the WHO classification.[21] The studies described below were performed on peripheral blood (n=7), bone marrow (n=5) and lymph node specimens (n=4). In all cases, samples were obtained after informed consent according to the Ethics Committee of the University Hospital of Salamanca (Salamanca, Spain).

Immunophenotypic analyses

Peripheral blood and bone marrow samples were collected in tubes containing K3 EDTA as anticoagulant; lymph node samples were collected in an isotonic saline buffer containing heparin and gently dispersed into single cell suspensions. All samples were stained using a direct immunofluorescence stain-and-then-lyse technique previously described in detail[22] with the following four-color combinations of antibodies conjugated with fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/peridin clorophyll protein-cyanine 5.5 (PerCPCy5.5)/allophycocyanine (APC): CD22/CD23/CD19/CD20, CD103/CD25/CD19/CD11c,FMC7/CD24/CD19/CD34, CD43/CD79b/CD19/$^{-}$, sIgM/CD27/CD19/$^{-}$, sIgλ/sIgκ/CD19/CD5 and cBCL2/CD10/CD19/CD38. The exact clones and sources of the antibody reagents used are described in Table 1. Data acquisition was performed using the CellQUEST software in a FACScalibur flow cytometer (Becton/Dickinson Biosciences -BDB-, San Jose, CA, USA). In those samples with <20% B-lymphocytes, a selective acquisition of CD19^{+} gated cells was also made as previously described.[22] The Paint-A-Gate PRO software program (BDB) was used for data analysis. B-lymphocytes were identified according to their SSC$^{lo/int}$/CD19^{+} distribution and their percentage was calculated after excluding cell debris and platelets according to conventional procedures.[22]

Table 1. Monoclonal antibodies polyclonal antisera and interphase FISH probes used for the immunophenotypic analysis and cytogenetic characterization of B-CLPD.

Ab conjugates	*Clone*	*Source*	*DNA Probe*	*Recognized chromosome region*	*Source*
CD5-APC	L17F12	Becton Dickinson Biosciences[1]	CEP 1 (Satellite II/III) SO	1q12	Vysis Inc.
CD10-PE	ALB1	Immunotech[2]	CEP 4 SG	4p11-q11	Vysis Inc.
CD11c-APC	S-HCL-3	Becton Dickinson Biosciences[1]	CEP 6 SG	6p11.1-q11.1	Vysis Inc.
CD19-PerCP-Cy5.5	SJ25C1	Becton Dickinson Biosciences[1]	CEP 7 SO	7p11.1-q11.1	Vysis Inc.
CD20-APC	L-27	Becton Dickinson Biosciences[1]	CEP 8 SO	8p11.1-q11.1	Vysis Inc.
CD22-FITC	S-HCL-1	Becton Dickinson Biosciences[1]	CEP 10 SO	10p11.1-q11.1	Vysis Inc.
CD23-PE	MHM-6	Becton Dickinson Biosciences[1]	CEP 12 SO	12p11.1-q11.1	Vysis Inc.
CD24-PE	ALB9	Immunotech[2]	CEP X SO	Xp11.1-q11.1	Vysis Inc.
CD25-PE	2A3	Becton Dickinson Biosciences[1]	CEP Y (Satellite III) SG	Yq12	Vysis Inc.
CD27-PE	L128	Becton Dickinson Biosciences[1]	LSI bcr/abl dual color probe	9q34 and 22q11.1	Vysis Inc.
CD34-APC	8G12	Becton Dickinson Biosciences[1]	LSI PML/RAR-α dual color probe	15q22 and 17q21	Vysis Inc.
CD38-APC	HB-7	Becton Dickinson Biosciences[1]	LSI IgH/CCND1 dual color probe	14q32.3 and 11q13	Vysis Inc.
CD43-FITC	DFT1	Immunotech[2]	LSI IgH/BCL2 dual color probe	14q32.3 and 18q21	Vysis Inc.
CD79b-PE	CB3-1	Immunotech[2]	LSI BCL6 dual color breakapart	3q27	Vysis Inc.
CD103-FITC	Ber-ACT8	Becton Dickinson Biosciences[1]	LSI 13/Rb SO	13q14	Vysis Inc.
BCL2-FITC	124	Coulter Corporation[3]	LSI p53 SO	17p13.1	Vysis Inc.
FMC7-FITC	FMC7	Becton Dickinson Biosciences[1]	LSI IgH/MYC, CEP 8 tri-color dual fusion translocation	14q32/8q24, 8p11.1-q11.1	Vysis Inc.
Anti-Human-IgM-FITC Polyclonal*		DakoCytomation[4]			
Anti-Human-κ-PE Polyclonal°		DakoCytomation[4]			
Anti-Human-λ-FITC Polyclonal#		DakoCytomation[4]			

*FITC: fluorescein isothiocyanate; PE: phycoerythrin; PerCPCy5.5: peridin clorophyll protein-cyanine 5.5 fluorochrome tandem; APC: allophycocyanine. [1]Becton Dickinson Biosciences (San Jose, CA, USA); [2]Immunotech (Marseille, France); [3]Beckman/Coulter (Miami, FL, USA); [4]DakoCytomation (Glostrup, Denmark). *Polyclonal rabbit anti-human IgM serum; °Polyclonal rabbit anti-human kappa Ig light chains serum; #Polyclonal rabbit anti-human lambda Ig light chains serum; SO: spectrum orange™; SG: spectrum green™.*

Analysis of DNA cell contents by flow cytometry

To specifically analyze the DNA content of B cells in each patient, samples were processed using the Cycloscope-NHL kit (Cytognos SL, Salamanca, Spain), strictly following the recommendations of the manufacturer.[23] The DNA index of neoplastic B cells was calculated by dividing the modal propidium iodide (PI)-associated fluorescence intensity (FI) of the G0/G1 peak of the neoplastic B cells by the modal PI-associated FI of normal G0/G1 cells present in the same sample.

Fluorescence activated sorting of B-cell subsets

Specific fluorescence activated cell sorting (FACS) of each B-cell subpopulation was performed on six patients using a FACSVantage flow cytometer (BDB). B-cells were sorted according to their distinct light scattering and/or immunophenotypic characteristics. The purity of the sorted B cell subsets was 96%±3% (range: 93% to 99.5%) with <0.1% contamination by other B cells. Sorted cells were then analyzed using interphase fluorescence *in situ* hybridization (FISH) and/or polymerase chain reaction (PCR) techniques, as described below.

Fluorescence in situ hybridization studies

FISH studies were performed in all cases on slides containing unsorted (n=10) or FACS sorted (n=6) cells fixed in Carnoy´s solution and stored at –20°C, as illustrated in Figure 2. Numerical abnormalities for chromosomes 1, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 22, X and Y as well as structural abnormalities involving IgH/bcl2, IgH/CCND1, bcl6 and c-myc translocations were systematically studied according to previously described methods.[24,25] The panel of Spectrum Orange™ (SO)/ Spectrum Green™ (SG) DNA probes purchased from Vysis Inc. (Downers Grove, IL, USA) and used in double stainings is displayed in Table 1. The number of hybridization spots and their distribution in the nuclei were evaluated using a BX60 fluorescence microscope (Olympus, Hamburg, Germany) equipped with a 100X oil objective according to previously defined criteria.[24-25] The distinction between two different B-cell populations on unsorted samples was based on the interphase FISH patterns observed on nuclei of different size according to the proportion of each B-cell population in the sample, as illustrated in Figure 2, case 13.

Assessment of clonality by molecular biology

Molecular studies were performed in a total of 11 patients of whom five had two phenotypically different B-cell subsets and six had two phenotypically identical subpopulations of B-cells showing different light scattering characteristics and/or DNA cell contents. Genomic DNA preparation, Southern blot analysis, PCR amplification, heteroduplex analysis, sequencing and analysis of Ig genes were performed following protocols which have been previously described in detail.[26,27] Gene-scanning analysis was conducted on the PCR products of all the samples studied. PCR analyses of the VDJ region of the Ig heavy chain (VDJH) Ig H gene rearrangements: VH-JH and DH-JH were performed using approximately 500 ng of genomic DNA. The consensus pairs of primers of the VH or DH and JH segments, as well as the PCR conditions used were as previously described[27] with some modifications: the JH primer was labeled with 6-FAM, the first denaturation step was extended to 15 min at 95° C and the last extension step at 72°C was extended to 45 min.[28] Then, 2 μL of the PCR product were mixed with 0.6 μL of the specific molecular marker (Internal Lane Standard

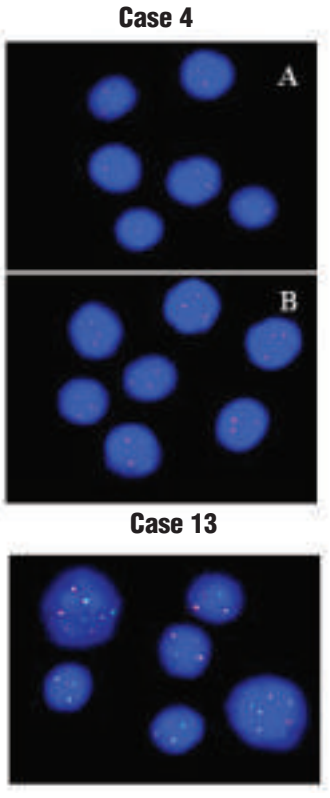

Figure 2. Interphase FISH analyses of nuclei from two peripheral blood samples from different patients with B-CLPD. Panels A and B correspond to purified sIgλ+ (panel A) and sIgλ+ (panel B) clonal B-cells showing unrelated immunoglobulin gene rearrangements and a typical B-cell chronic lymphocytic leukemia phenotype, co-existing in the same patient (case 4); as shown in these two panels the clonal sIgλ+ B-cells displayed trisomy 12 (panel A) whereas the clonal sIgλ+ B-cell population did not (panel B) after hybridization with the CEP 12 DNA probe. Panel C shows the FISH analysis of peripheral blood lymphocytes from a patient (case 13) diagnosed as having follicular lymphoma with a monoclonal pattern of IgH gene rearrangement and t(14;18). Note the typical pattern of IgH/BCL2 gene rearrangement (two fused yellow signals plus one red and one green spot) in the four smaller nuclei while the two large nuclei show an interphase FISH pattern compatible with additional gains of both the rearranged and the normal chromosomes 14 and 18 (four fused yellow signals plus two green and two red spots) for the LSI IgH/BAC-2 dual color probe used.

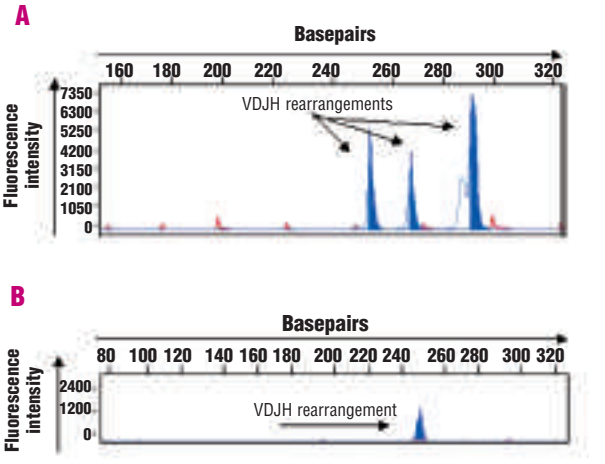

Figure 3. Illustrative histograms corresponding to B-CLPD patients. Panel A. A case displaying three different complete gene rearrangements (blue peaks) indicating the existence of two different B-cell clones (biclonal case). Panel B. Another patient showing only one rearrangement in the FR2 region (blue peaks) and a single B-cell clone (monoclonal case). Red peaks correspond to molecular weight markers (Internal Lane Standard, PROMEGA).

600, Promega, Madison, WI, USA) and 1.5 μL of loading buffer (Blue Dextran Loading Solution, Promega). Afterwards, this mixture was warmed for 3 min at 95°C and loaded onto a 5% polyacrylamide denaturing gel with urea placed in a 377 automated sequencer (Applied Biosystems, Foster City, CA, USA). The electrophoresis was run for 2.5h at 3000V. The fluorescence was quantified and the precise DNA fragment size calculated with GeneScan software (Applied Biosystems). This resulted in either one or two peaks (one of which corresponded to an incomplete VDJ-IgH DH-JH gene rearrangement) depending on the presence of either one type of PCR product (in the case of a monoclonal lymphoproliferation) or more than two (in those cases in which two or more B-cell clones were present) as illustrated in Figure 3. Due to a shortage of sample material, cases 5, 6, 7, 9 and 12 could not be analyzed. PCR products were eluted from the polyacrylamide gels and directly sequenced in an automated ABI 377 DNA sequencer using Big-Dye terminators (Applied Biosystems, Foster City, CA, USA). In order to avoid nucleotide misinterpretations because of *Taq* errors, all products were sequenced at least twice from different PCR reactions using 5´VH-DH and/or 3´JH primers. Germline VH, DH and JH segments from complete VH-JH and DH-JH rearrangements were identified by comparison with the V base[29] and IGMT database[30] using on-line DNAPLOT (MRC Center for Protein Engineering). DJ and JH germline segments from incomplete DJH rearrangements were identified using a BLAST search in the DH-JH germline locus sequence (accession number *EMB/X97051*). Sequences containing more than 2% deviation from the germline sequence were considered as somatically mutated rather than genomic polymorphisms. In such cases, a binomial distribution model was applied in order to discriminate replacement (R) and silent (S) mutations derived from antigen selection or acquired by random choice.

Results

Diagnosis of B-CLPD with two abnormal aberrant B-cell populations

The nine cases showing two phenotypically different B-cell populations (group A) were classified as displayed in Table 2. The remaining seven cases (group B) showed two B-cell subsets with different DNA cell contents and FSC/SSC values but a similar phenotype, and were diagnosed as follicular lymphoma (n=3), mantle cell lymphoma (n=1), lymphoblastic lymphoma (n=1), large diffuse B-cell lymphoma (n=1) and lymphoplasmacytic lymphoma/Waldenström's macroglobulinemia (n=1) (Table 2).

Table 2. Diagnosis, differential immunophenotypic features and molecular characteristics of the two co-existing aberrant B-cell populations present in the B-CLPD cases studied.

Case	*Diagnosis*	*Phenotype of population 1* (% from total leukocytes)*	*Phenotype of population 2* (% from total leukocytes)*	*Pattern of IgH gene rearrangements*
		Cases with two phenotypically different B-cell populations		
1	LDBCL+SLL	FSC/SSC^{hi}, CD5$^{-/+}$, CD19$^{+/++}$, CD20$^{+/++}$,, CD22^{++}, CD23^{-}, CD24^{+}, CD25^{+dim}, CD38^{++}, CD79b^{+}, IgM^{+} (48%)	FSC/SSC^{lo}, CD5^{-}, CD19^{++}, CD20^{+}, CD22^{+dim}, CD23$^{-/+}$, CD24^{-}, CD25^{-}, CD38^{-}, CD79b^{+dim}, IgM^{+dim} (9%)	RR RG
2	CLL+SMZL	FSC/SSC^{lo}, CD5^{+}, CD11c^{-}, CD19^{+}, CD20^{+dim}, CD22^{+dim}, CD2$^{-/+het}$, CD24^{+dim}, CD25^{+dim}, CD27^{+dim}, CD43$^{-/+het}$, CD45^{+dim}, CD79b^{-}, FMC7^{-}, sIgκ^{+} (33%)	FSC/SSC^{hi}, CD5^{-}, CD11c^{+}, CD19^{++}, CD20^{+++}, CD22^{++}, CD23^{-}, CD24^{-}, CD25^{-}, CD27$^{-/+dim}$, CD43^{-}, CD45^{++}, CD79b^{+}, FMC7^{++}, sIgλ^{+} (29%)	RR RG
3	CLL+CLL	sIgκ^{+} (4%)	sIgλ^{+} (4%)	RR RR
4	CLL+CLL	sIgλ^{+} (2%)	sIgκ^{+} (24%)	RR RG
5	FL+LDBCL	FSC/SSC^{lo}, CD10^{+}, CD11c^{-}, CD19^{+}, CD20^{++}, CD22^{+dim}, CD23$^{-/+}$, CD24^{++}, CD25^{+dim}, CD27^{+++}, CD43^{-}, CD79b^{++}, BCL2^{+++}, IgM^{+++} (0.5%)	FSC/SSC^{hi}, CD10^{-}, CD11c$^{+/++}$, CD19^{++}, CD20^{+++}, CD22^{+}, CD23^{-}, CD24$^{-/+}$, CD25^{-}, CD27^{+}, CD43^{+dim}, CD79b^{+dim}, BCL2^{++}, IgM^{++} (0.4%)	NA
6	FL+LL	FSC/SSC^{lo}, CD19^{++}, CD20^{+}, CD23$^{-/+}$, BCL2^{++}, FMC7$^{-/+}$, IgM^{+}, sIgκ^{+} (6%)	FSC/SSC^{hi}, CD19^{+dim}, CD20^{-}, CD23^{-}, BCL2^{+}, FMC7^{-}, IgM^{+}, sIgλ^{+} (2%)	NA
7	unclassNHL +LPL	CD20^{+}, CD23^{++}, CD24^{++}, CD27^{+}, CD38^{+}, CD43^{+dim}, CD79b^{+dim}, IgM^{+}, sIgλ^{+} (1%)	CD20^{++}, CD23^{-}, CD24^{-}, CD27^{-}, CD38^{-}, CD43^{-}, CD79b^{++}, IgM^{++}, sIgκ^{+} (22%)	NA
8	CLL+ unclass NHL	CD5^{+}, CD20^{+d}, CD22-, CD23^{+}, CD27^{+}, CD43^{+}, CD79b^{+d}, BCL2^{++}, FMC7^{-}, IgM^{+d}, sIgκ^{+d} (1.5%)	CD5^{-}, CD20^{++}, CD22^{+}, CD23-, CD27^{-}, CD43^{-}, CD79b^{+}, BCL2^{+}, FMC7^{+}, IgM^{+}, sIgκ^{+} (2.5%)	RR RG
9	CLL+CLL	sIgκ^{+} (30%)	sIgλ^{+} (11%)	NA
		Cases with two phenotypically identical B-cell populations		
10	FL	FSC/SSC^{lo}, DNA aneuploid (11%)	FSC/SSC^{hi}, DNA aneuploid (9%)	RR
11	LL	FSC/SSC^{lo}, DNA diploid (54%)	FSC/SSC^{hi}, DNA aneuploid (44%)	RG
12	LDBCL	DNA diploid (6%)	DNA aneuploid (25%)	NA
13	MCL	FSC/SSC^{lo}, DNA diploid (5%)	FSC/SSC^{hi}, DNA aneuploid (8%)	RG
14	FL	FSC/SSC^{lo}, DNA diploid (62%)	FSC/SSC^{hi}, DNA aneuploid (7%)	RG
15	LPL/WM	FSC/SSC^{lo}, DNA diploid (2%)	FSC/SSC^{hi}, DNA aneuploid (1%)	RR
16	FL	FSC/SSC^{lo}, DNA diploid (23%)	FSC/SSC^{hi}, DNA aneuploid (2%)	RG

**Only light scatter, DNA ploidy and phenotypic differences between co-existing B-cell populations in the same patient/sample are displayed. CLL: chronic lymphocytic leukemia; FL: follicular lymphoma; LDBCL: large diffuse B-cell lymphoma; LL: lymphoblastic lymphoma; LPL/WM: lymphoplasmacytic lymphoma/Waldenström´s macroglobulinemia; MCL: mantle-cell lymphoma; SLL: small lymphocytic lymphoma; SMZL: splenic marginal zone lymphoma; unclassNHL: unclassifiable non-Hodgkin's lymphoma; sIg: surface immunoglobulin; FSC: forward light scatter; SSC: sideward light scatter. R: rearranged allele; G: germinal configuration; NA: not analyzed. FSC/SSC^{lo}: low sideward light scatter; FSC/SSC^{hi}: high forward and sideward light scatter; d: dim antigen expression; ++: strong antigen expression; +++: very strong antigen expression. Het: heterogeneous antigen expression.*

Immunophenotypic features of B-CLPD with two aberrant B-cell populations

Table 2 shows the differences found between the two B-cell aberrant populations detected in each case of group A, as regards their immunophenotype, light scattering properties (FSC/SSC) and DNA cell contents. In three of nine cases in which both subsets showed distinct phenotypic features (cases 3, 4 and 9 in Table 2), the only difference observed was the type of sIg light chain expressed, one B-cell population being sIgκ^{+} and the other one sIgλ^{+}; in these three cases both cell populations showed a typical B-CLL phenotype (CD5^{+}, CD10^{-}, CD11^{chet}, CD20^{+}, CD22d^{dim}, CD23^{+}, CD24^{+}, CD25^{+}, CD27^{+}, CD34^{-}, CD38^{dim}, CD79b^{dim}, CD103-, cytBCL2^{+}, FMC7^{-}, sIgM^{+}). In the remaining six patients belonging to this group, the two B-cell subsets showed various different phenotypic

Table 3. Flow cytometric DNA index and genetic abnormalities differentially expressed by each of the two neoplastic B-cell populations found in each B-CLPD patient as detected by interphase FISH.

	Subpopulation 1				*Subpopulation 2*			
Case	*% aberrant B-cells/sample analyzed by iFISH*	*Genetic abnormalities*	*% of genetic abnormalities*	*FCM DNA index*	*% aberrant B-cells/sample analyzed by iFISH*	*Genetic abnormalities*	*% of genetic abnormalities*	*FCM DNA index*
Cases with two phenotypically different B-cell populations								
1	48%	ND		1.00	9%	ND		1.00
2	33%	ND		1.00	29%	ND		1.00
3	99.5%	+12*	50%	1.00	99.5%	+12*	70%	1.00
4	94%	ND*		1.00	99.7%	+12*	80%	1.00
5	96%	t(14;18)+*	11%	1.00	93%	ND*		1.00
6	6%	t(14;18)+	100%	1.00	2%	ND		1.00
7	93%	ND*		1.00	97%	17p-*	85%	1.00
8	94%	ND*		1.00	95%	+3*	43%	1.00
9	99.4%	ND*		1.00	95%	ND*		1.00
Cases with two phenotypically identical B-cell populations								
10	11%	+18	NAE	1.11	9%	+3, +12, +13, +18	NAE	1.30
11	54%	+12	25%	1.00	44%	+4,+8,+10,+11, +13, +18 ++1,++3,++4, ++6,++7,++9, ++14,++15,++17, ++18,++22,++X ++++12	49%	1.80
12	28%	bcl6+	16%	1.00	10%	bcl6+ +18	11%	1.20
13	9%	t(11;14)+	10%	1.00	14%	t(11;14)+,t(11;14)+ ++1,++3,++4, ++6,+++7,++8, ++9,++10,++12, ++17,++22,++X +++15 ++++18	13%	1.96
14[a]	62%	t(14;18)+	60%	1.00	7%	t(14;18)+,t(14;18)+ ++1,++3,++4, ++6,++7,++8, ++9,++10,++11, ++12,++15,++17, ++22,++X	10%	1.97
15	2%	ND		1.00	1%	+3, +7, +13, +18, +X ++4,++6,++8, ++9,++10,++12, ++22	1%	1.99
16	23%	t(14;18)+	21%	1.00	3%	t(14;18)+,t(14;18)+ ++1,++3,++4, ++6,++7,++8, ++9,++10,++12, ++15,++17,++22	3%	2.00

**Purified populations (purity >93%). ND: no chromosomal abnormalities detected by interphase FISH for the probes studied; -: monosomy; +: trisomy; ++: tetrasomy; +++: pentasomy; ++++: hexasomy; FCM: flow cytometry. NAE: The percentage of B-cells from this subpopulation carrying the corresponding abnormality could not be accurately evaluated on the slides; [a]Genetically, two additional B-cell clones were detected by interphase FISH corresponding to B-cells with t(14;18)+ and 13q- (28% of the cells) and B-cells carrying t(14;18)+, 13q- and trisomy 17 (20% of the cells).*

features; in three of them the expression of sIg light chain was also different in the two B-cell populations (cases 2, 6 and 7 in Table 2) while in the other three both populations expressed the same sIg light chain (cases 1, 5 and 8 in Table 2). It should be noted that in cases 3, 4 and 8, one of the two co-existing aberrant B-cell populations was present at low frequencies, ranging from 1.5% to 4% of all leukocytes in the sample (Table 2). In all except one patient from group B (cases 10 to 16 in table 2), in whom the two B-cell subsets identified showed identical immunophenotypic features, the two B-cell populations could be distinguished on the basis of their different light scatter values as shown in Table 2 and illustrated in Figure 1.

DNA contents of the two aberrant B-cell populations

In group A the two phenotypically different populations from all patients displayed a diploid DNA content; in contrast, in all cases from group B, both B-cell populations were found to have distinct DNA cell contents (Table 3; Figure 1). In all except one case, one of the aberrant B-cell populations was DNA diploid and the other one DNA aneuploid. In the remaining patient, two DNA aneuploid B-cell populations with distinct DNA

cell contents (DNA index of 1.11 and 1.30) were detected (Table 3). Interestingly, in all group B cases, the B-cell population showing higher DNA cell contents corresponded to the FSC/SSC^{hi} cell subset.

Genetic characteristics of B-CLPD with two aberrant B-cell populations

In three out of the nine group A cases, interphase FISH analyses were performed on total nucleated cells. In one of them (case #6 in Table 3), two neoplastic B-cell subpopulations were detected, one showing the t(14;18) translocation, typical of follicular lymphoma, no cytogenetic information being available for the other clone. In the other two cases (cases #1 and 2 in Table 3) no chromosomal abnormalities were detected. In the remaining six cases, interphase FISH studies were performed on FACS purified B-cell subpopulations. Two of them (cases #3 and 9) showed an identical genetic profile in the two co-existing B-cell populations for the FISH probes tested, consisting of trisomy 12 (case #3 in Table 3) and no genetic abnormalities (case #9 from Table 3). The other four cases showed single chromosomal abnormalities, but only in one B-cell clone (Table 3): trisomy 12, t(14;18)+, 17p- and trisomy 3 (cases #4, 5, 7 and 8 in Table 3, respectively); the other B-cell population from these four patients showed no chromosomal abnormalities. As regards group B patients (cases #10 to 16 in Table 3), FISH analyses showed that in most of these cases (cases #10, 11, 12, 13, 14 and 16) the two B-cell subsets displayed at least one genetic abnormality in common, but this was associated with additional chromosomal abnormalities in the B-cell population showing higher DNA cell contents/larger nuclei; in turn, no chromosomal abnormalities were detected by FISH in the smaller sized nuclei (Table 3).

Clonality of B-CLPD with two aberrant B-cell populations

As shown in Table 2, in all patients showing two phenotypically distinct B-cell subsets (group A) in whom molecular analyses of clonality were performed (n=5), more than two IgH rearrangements were detected, suggesting the presence of biclonality. In contrast, all group B patients analyzed, in whom no phenotypic differences between the two B-cell subsets were found (n=6), showed molecular patterns of IgH gene rearrangements compatible with the existence of a single B-cell clone (Table 2). To demonstrate that both rearrangements detected in cases #10 and 15 corresponded to one clone, sequencing analyses were performed which showed the presence of a single functional rearrangement in each case (Table 2); in one patient (case #10) an additional complete VDJH VH-JH rearrangement was detected while in the other case (case #15) together with the functional VDJH VH-JH rearrangement an incomplete DH-JH rearrangement was identified (Figure 4).

Clinico-biological characteristics of B-CLPD with two aberrant B-cell populations

From the clinico-biological point of view, no significant differences were observed between the two groups

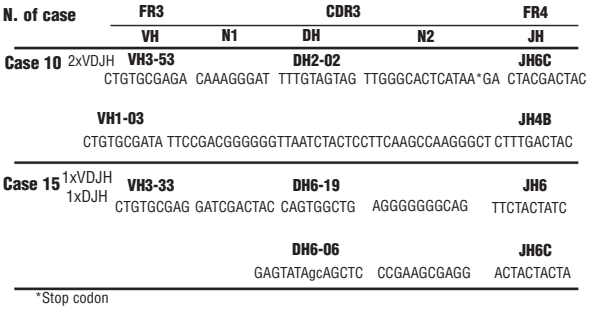

Figure 4. Sequencing of the FR3, CDR3 and FR4 regions of the IgH gene from cases 10 and 15. As shown, two complete rearrangements were detected in case 10, one corresponding to an in-frame rearrangement and the other showing a stop-codon in the nucleotide sequence; in case 15 a complete and functional rearrangement was detected in association with an incomplete rearrangement.

of B-CLPD studied, except for a lower frequency of B-CLL cases among patients showing intraclonal evolution (0% vs 44%, p=0.04), this leading to slightly lower white blood cell (6.8±2.5 vs 13.4±7.8×10^9 leukocytes/L, p=0.06) and lymphocyte counts (2.2±1.7 vs 6.9±5.7×10^9 lymphocytes/L, p=0.09) and a decreased frequency of diffuse infiltration of the bone marrow (0% vs 100%, p=0.18) among these cases. No significant differences were observed in overall survival rates (p=0.44).

Discussion

Immunophenotypic and morphological heterogeneity are relatively common findings in B-CLPD. In a small percentage of cases (around 5%), the presence of phenotypic heterogeneity has been reported to reflect co-existence of two or more unrelated B-cell clones, each probably corresponding to a different B-CLPD.[19] Apart from these cases, it is not unusual to find other B-CLPD patients in whom two or more abnormal B-cell populations showing distinct morphological features, light scattering characteristics and/or DNA cell contents, but an identical phenotype, co-exist; to date, there is no information about their biclonal vs monoclonal nature. In the present study, we show for the first time that these latter cases do correspond to a single disease showing intraclonal evolution, on the basis of their genetic and molecular features. As described above, two groups of B-CLPD showing more than one neoplastic B-cell subpopulation were included in the present study, based on their distinct flow cytometric features. One cohort (group A) was formed of nine patients in whom multicolor flow cytometry showed two B-cell populations with clearly different immunophenotypic features at diagnosis. In these cases, the two B-cell subsets differed for the type of sIg light chain expressed and/or the pattern of expression of other surface markers. Interestingly, in all cases from this group, the two B-cell populations had diploid DNA, independently of their FSC/SSC values. Multicolor interphase FISH analysis, using an adequate combination of DNA probes, is par-

ticularly suited to assessing genetic heterogeneity, thus being an alternative technique to conventional cytogenetics, which has several limitations, including the specific identification of the different subsets of cells present in the sample. In these patients, FISH analyses mostly performed on purified, phenotypically homogeneous B-cell populations, confirmed the presence of different genetic abnormalities in each of the two co-existing cell subsets. Accordingly, in four of these nine cases the FISH analyses revealed the presence of a single chromosomal abnormality in one clone, while no genetic lesions could be identified in the other clone. Interestingly, in these four cases the genetic alterations identified were consistent with the phenotypic profile observed: trisomy 12, del(17p) and t(14;18) in two cases, showing a phenotype compatible with chronic lymphocytic leukemia, lymphoplasmacytic lymphoma, Waldenström's macroglobulinemia and follicular lymphoma, respectively. The other aberrant B-cell populations, which were phenotypically compatible with chronic lymphocytic leukemia, large diffusion B-cell lymphoma, unclassifiable non-Hodgkin's lymphoma and lymphoblastic lymphoma showed no chromosomal abnormalities. However, in case 6 the low percentage of B-lymphocytes present in the sample analyzed by FISH, did not allow a definitive conclusion that this population had a normal karyotype. Similarly, in another case, FISH analyses showed the presence of trisomy 3 in a B-cell clone corresponding to an unclassifiable non-Hodgkin's lymphoma, the B-cell clone from this patient being normal for this chromosome. As expected, molecular analyses, performed in two out of these five cases, showed they had two distinct unrelated B-cell clones. Of the remaining four patients in this group, three showed no chromosomal abnormalities while in the other patient trisomy 12 was found in both B-cell clones. Although the two B-cell populations in this latter patient carried the same single genetic alteration, molecular analyses performed on the same purified B-cell populations as those used for FISH studies showed the unequivocal presence of different IgH gene rearrangements in each of them, confirming the biclonal nature of the disease in this patient and the lack of contamination of each B-cell fraction with cells from the other B-cell clone. In two of the three cases showing non-chromosomal abnormalities by FISH, molecular techniques confirmed the presence of two unrelated B-cell clones; analysis of clonality was not performed in the other patient. Overall, these results confirm previous observations[19] about the biclonal origin of B-CLPD displaying phenotypically different B-cell populations. In addition, we also show for the first time that different genetic profiles are frequently observed in the co-existing phenotypically different B cell clones from these B-CLPD patients. It should be noted that in three cases from this group of patients (cases 3, 4 and 8) low numbers (<5%) of circulating B-cells showing a typical chronic lymphocytic leukemia phenotype were detected. In this regard, using highly-sensitive multiparameter flow cytometric approaches, as in the present study, the presence of low levels of peripheral blood monoclonal B cells has been reported in otherwise healthy individuals.[31-33] Emerging evidence supports the existence of an association between such monoclonal B-cells in healthy individuals and those of patients with B-cell chronic lymphocytic leukemia at both the phenotypic and genotypic levels;[31-33] however, the clinical relevance of this so-called *monoclonal B-cell lymphocytosis* remains unknown. Long-term longitudinal studies are required to confirm the possibility that monoclonal B-cell lymphocytosis evolves into overt B-cell chronic lymphocytic leukemia, in a situation analogous to that of individuals with monoclonal gammopathy of undetermined significance.[34] In the second cohort of B-CLPD studied here (group B) the two B-cell subsets identified could not be distinguished on phenotypic grounds but just showed different light scattering and/or DNA cell contents. FISH analyses confirmed that the two B-cell populations from these patients had at least one chromosomal abnormality in common, frequently corresponding to diagnostic chromosome translocations, i.e., bcl6 gene rearrangements, t(11;14), t(14;18), thus suggesting that both populations could be genetically related. In line with this, in these cases IgH gene rearrangements confirmed the presence of monoclonality. The fact that in each of these cases the B-cell population showing higher DNA cell contents and/or light scatter features had additional genetic abnormalities consisting of chromosome gains, clearly supports the notion that in these patients neoplastic B-cells have undergone clonal evolution, which was frequently associated with chromosomal instability and the emergence of tetraploid or near-tetraploid clones. It has previously been reported that in a significant proportion of B-cell lymphomas, two different histological subtypes, associated with clinical evolution towards a more aggressive disease and shortened survival, are observed in the same patient, either simultaneously or more frequently, at different times during follow-up.[35-36] Evolution and conversion between these histological subtypes of B-cell lymphoma are commonly interpreted as being associated with *dedifferentiation* of B-cells within the original clone and/or emergence of rapidly proliferating tumor cell lines.[35,36] Based on these results, it could be expected that B-CLPD patients showing intraclonal evolution might have a worse prognosis; hence, their early identification, at diagnosis or during follow-up based on the above described criteria, could be of clinical relevance. Further analyses are needed to study the clinical and prognostic impact of intraclonal evolution in larger series of patients. In summary, our results show that those cases of B-CLPD with two B-cell subpopulations displaying different DNA cell contents and/or different light scattering properties but a similar immunophenotype could represent different stages of the evolution of a single B-cell clone in contrast to cases of biclonal B-CLPD, in which two aberrant, phenotypically distinct and unrelated B-cell clones co-exist.

MLS performed the flow cytometry experiments, analyzed the data and wrote the manuscript. JA contributed to the interpretation of data and writing the manuscript; AL provided technical assistance with the flow cytometry experiments; JMS, AR and MDT analyzed the cytogenetic data; EAS, AB and MG developed the molecular assays and interpreted the data obtained; JD-M, CB and AP oversaw patient care, provided patient samples and reviewed the manuscript; JFSM oversaw patient care, provided patient samples, supervised and critically revised the manuscript; AO designed the study and contributed to the interpretation of data and writing the manuscript.

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References

1. Fialkow PJ. Clonal and stem cell origin of blood cell neoplasms. In: Lobue J, Gordon AS, Silber R, Muggia FM, eds. Contemporary Hematology/Oncology. Plenum Publishing Corp., New York, 1980. p. 1-46.
2. Orfao A, Almeida J, ML Sanchez, JF San Miguel. Immunophenotypic diagnosis of leukemic B-cell chronic lymphoproliferative disorders other than CLL. In: GB Faguet, editors: Chronic lymphocytic leukemia: Molecular Genetics, Biology, Diagnosis and Management. Humana Press Inc. Totowa, New Jersey. 2003. p. 173-99.
3. Matutes E, Worner I, Sainati L, de Oliveira MP, Catovsky D. Advances in the lymphoproliferative disorders. Review of our experience in the study of over 1000 cases. Biol Clin Hematol 1989;11:53-62.
4. Matutes E, Owusu-Ankomah K, Morilla R, Garcia-Marco J, Houlihan A, Que TH, et al. The immunological profile of B-cell disorders: A proposal of a scoring system for the diagnosis of CLL. Leukemia 1994;8:1640-5.
5. Catovsky D. Chronic lymphoproliferative disorders. Curr Opin Oncol 1995; 7: 3-11.
6. Montserrat E. Chronic lymphoproliferative disorders. Curr Opin Oncol 1997; 9:34-41.
7. Cleary ML, Sklar J. Lymphoproliferative disorders in cardiac transplant recipients are multiclonal lymphomas. Lancet 1984; 2:489-93.
8. Cleary ML, Warnke R, Sklar J. Monoclonality of lymphoproliferative lesions in cardiac transplant recipients: clonal analysis based on immunoglobulin-gene rearrangements. N Engl J Med 1984;310: 477.
9. Schmitt-Graff A, Hummel M, Anagnostopoulos I, Stoltenburg G, Stein H. Primary brain lymphoma in acquired immunodeficiency syndrome. Immunophenotype and molecular pathologic characterization in stereotactic biopsy, autopsy and cerebrospinal fluid cytology. Pathologe 1995;16:75-80.
10. Armes JE, Angus P, Southey MC, Battaglia SE, Ross BC, Jones RM, et al. Lymphoproliferative disease of donor origin arising in patients after orthotopic liver transplantation. Cancer 1994;74: 2436-41.
11. Sklar J, Cleary ML, Thielemans K, Gralow J, Warnke R, Levy R. Biclonal B-cell lymphoma. N Engl J Med 1984; 311:20-7.
12. Siegelman MH, Cleary ML, Warnke R, Sklar J. Frequent biclonality and Ig gene alterations among B-cell lymphomas that show multiple histologic forms. J Exp Med 1985;161:850-63.
13. Hairman C, Vaeemans E, Carels D, Theunissen J, Van-Camp B, Theilemans K. Isotype switch and idiotype variation in hairy cell leukemia. Leukemia 1990; 4:856-62.
14. Gonzalez-Campos J, Rios-Herranz E, De Blas-Orlando JM, Martin-Noya A, Parody-Ruiz-Berdejo R, Rodriguez-Fernandez JM. Chronic lymphocytic leukemia with two cellular populations: a biphenotypic or biclonal disease. Ann Hematol 1997;74:243-6.
15. Wong KF, So CC. Biclonal B-cell chronic lymphocytic leukemia with inv(14)(q11q32). Cancer Genet Cytogenet 1997; 94:135-7.
16. Fend F, Quintanilla-Martínez L, Kumar S, Beaty MW, Blum L, Sorbara L, et al. Composite low grade B-cell lymphomas with two immunophenotypically distinct cell populations are true biclonal lymphomas. Am J Pathol 1999;154:1857-66.
17. Duque RE, Everett ET, Iturraspe J. Biclonal composite lymphoma. A multiparameter flow cytometric analysis. Arch Pathol Lab Med 1990;114:176-9.
18. Gine E, Bosch F, Villamor N, Rozman M, Colomer D, Lopez-Guillermo A, et al. Simultaneous diagnosis of hairy cell leukemia and chronic lymphocytic leukemia/small lymphocytic lymphoma: a frequent association? Leukemia 2002; 16:1454-9.
19. Sanchez ML, Almeida J, Gonzalez D, Gonzalez M, Garcia-Marcos MA, Balanzategui A, et al. Incidence and clinicobiologic characteristics of leukemic B-cell chronic lymphoproliferative disorders with more than one B-cell clone. Blood 2003;102:2994-02.
20. Cerny J, Slavickova A, Krepelova A, Trneny M, Karban J, Klener P. Biallelic IgH rearrangements in patients with indolent lymphoproliferative disorders: molecular and practical implications. J Cell Physiol 2004;199:217-26.
21. Harris N, Jaffe E, Diebold J, Flandrin G, Muller-Hermelink H, Vardiman J, et al. 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;17:3835-49.
22. Sanchez ML, Almeida J, Vidriales MB, Lopez-Berges MC, Garcia-Marcos MA, Moro MJ, et al. Incidence of phenotypic aberrations in a series of 467 patients with B-chronic lymphoproliferative disorders: basis for the design of specific 4-color stainings to be used for minimal residual disease investigation. Leukemia 2002;16:1460-9.
23. Triolo R, Omede P, Giaretta F, Ravaglia R, Battaglio S, Palumbo A, et al. Cytofluorimetric evaluation of plasma cell proliferative activity in monoclonal gammopathies. Eur J Histochem 1997; 41 Suppl 2:57-8.
24. Tabernero MD, San Miguel JF, Garcia-Sanz R, Najera L, Garcia-Isidoro M, Perez-Simon JA, et al. Incidence of chromosome numerical changes in multiple myeloma: fluorescence in situ hybridization analysis using 15 chromosome-specific probes. Am J Pathol 1996;149:153-61.
25. Sayagues JM, Tabernero MD, Maillo A, Diaz P, Rasillo A, Bortoluci A, et al. Incidence of numerical chromosome aberrations in meningioma tumors as revealed by fluorescence in situ hybridization using 10 chromosome-specific probes. Cytometry 2002;50: 153-9.
26. Gonzalez D, Balanzategui A, Garcia-Sanz R, Gutierrez N, Seabra C, van Dongen JJ, et al. Incomplete DJH rearrangements of the IgH gene are frequent in multiple myeloma patients: immunobiological characteristics and clinical implications. Leukemia 2003; 17:1398-403.
27. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003;17: 2257-317.
28. Lopez-Perez R, Garcia-Sanz R, Gonzalez D, Balanzategui A, Chillon MC, Alaejos I, et al. Gene scanning of VDJH-amplified segments is a clinically relevant technique to detect contaminating tumor cells in the apheresis products of multiple myeloma patients undergoing autologous peripheral blood stem cell transplantation. Bone Marrow Transplant 2001;28:665-72.
29. Cook GP, Tomlinson IM. The human immunoglobulin VH repertoire. Immunol Today 1995;16:237-42.
30. Lefranc MP. IMGT, the international ImMunoGeneTics database. Nucleic Acids Res 2001;29:207-9.
31. Marti GE, Rawstron AC, Ghia P, Hillmen P, Houlston RS, Kay N, et al. Diagnosis criteria for monoclonal B-cell lymphocytosis. Br J Haematol 2005;130:325-32.
32. Marti GE, Carter P, Abbasi F, Washington GC, Jain N, Zenger VE, et al. B-cell monoclonal lymphocytosis and B-cell abnormalities in the setting of familial B-cell chronic lymphocytic leukemia. Cytometry B Clin Cytom 2003;52:1-12.
33. Rawstron AC. Prevalence and characteristics of monoclonal B-cell lymphocytosis (MBL) in healthy individuals and the relationship with clinical disease. J Biol Regul Homeost Agents 2004;18:155-60.
34. Kyle RA, Therneau TM, Rajkumar SV, Offord JR, Larson DR, Plevak MF, et al. Text A long-term study of prognosis in monoclonal gammopathy of undetermined significance. N Engl J Med 2002; 346:564-9.
35. Hubbard SM, Chabner BA, DeVita VT, Simon R Jr, Berard R, Jones B, et al. Histologic progression in non-Hodgkin´s lymphoma. Blood 1982;59:258.
36. Acker B, Hoppe T, Colby TV, Cox RS, Haplan HS, Rosenberg SA. Histologic conversion in the non-Hodgkin´s lymphomas. J Clin Oncol 1983;1:11.