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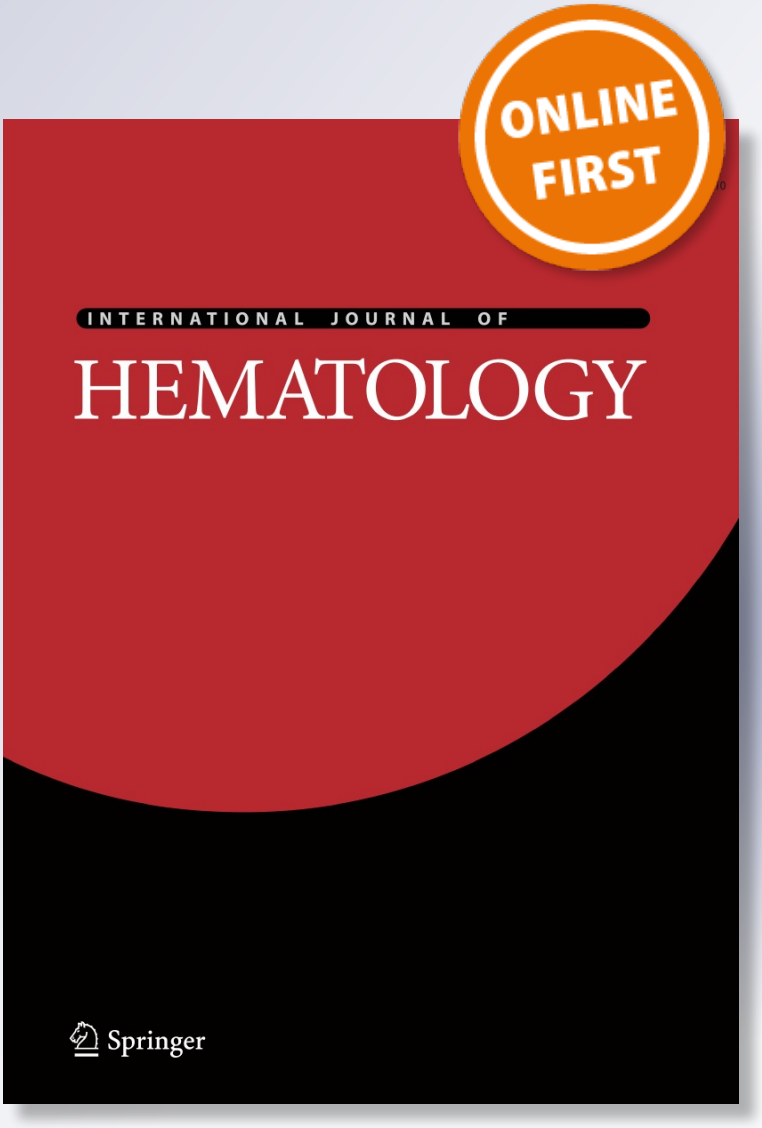

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ORIGINAL ARTICLE

Multiparameter flow cytometry is necessary for detection, characterization and diagnostics of composite mature B-cell lymphoproliferative neoplasms

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Abstract Composite mature B-cell lymphoproliferative neoplasms are rare entities characterized by the simultaneous presence of two or more distinctive B-cell derived monoclonal malignancies. This retrospective study used multiparametric flow cytometric analysis aimed at immunophenotypic profiling of composite mature B-cell lymphoproliferative neoplasms in a cohort of 413 subsequent patients with de novo leukemic B-cell chronic lymphoproliferative disorders diagnosed in our institution during a 30-month period. Biclonality was found in 16 (3.9 %) patients. The vast majority (88 %) of the cases had one of the clones phenotypically corresponding to chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL). Only when composite cases were categorized by phenotype of the non-CLL/SLL malignant population did we find a statistically significant ($P = 0.001$) higher frequency of biclonality among cases with hairy cell leukemia (22 %). Biclonal cases had the overall B-cell membrane κ to λ ratio within the normal range (median, 1.9; reference interval 0.5–4.0), making recognition of malignancy somewhat challenging. Our analysis strategy was therefore based on the detection of aberrant B-cell phenotypes, with subsequent confirmation of the monoclonal nature of neoplastic clones with regards to light chain restriction analysis. Discrimination of the coexisting clones in biclonal cases was possible on the basis of the expression of other antigen(s) (63 %), light scatter properties (44 %), different surface light chain restriction (69 %) and/or pattern of expression (44 %). The most informative cell surface antigens proved to be CD22, CD20, surface IgM, and CD23. In conclusion, historic κ/λ ratio is not a reliable approach and is a poor measurement for the detection of composite lymphomas. More creative analysis techniques should be utilized for this purpose.

Keywords Flow cytometry · Composite lymphoma · Lymphoma, non-Hodgkin · Immunoglobulin light chains · Hematology

S. Perković (✉) · D. Batinić
Clinical Unit for Cellular Immunodiagnostics and In Vitro Procedures, Department of Laboratory Diagnostics, University Hospital Centre Zagreb, School of Medicine, University of Zagreb, Kišpatićeva 12, 10000 Zagreb, Croatia
e-mail: sanja.perkovic@gmail.com

S. Bašić-Kinda · I. Aurer
Division of Hematology, Department of Internal Medicine, University Hospital Centre Zagreb, School of Medicine, University of Zagreb, Kišpatićeva 12, 10000 Zagreb, Croatia

I. Ugrina
Department of Mathematics, Faculty of Natural Sciences, University of Zagreb, Bijenička cesta 30, 10000 Zagreb, Croatia

A. Duletić-Načinović
Department of Hematology, Rheumatology and Clinical Immunology, Clinical Hospital Centre Rijeka, Krešimirova 42, 51000 Rijeka, Croatia

D. Lozić
Department of Hematology, University Hospital Centre Split, Šoltanska 1, 21000 Split, Croatia

D. Batinić
Department of Physiology, School of Medicine, University of Zagreb, Šalata 3, 10000 Zagreb, Croatia

Introduction

Mature B-cell neoplasms are derived from a single transformed B-cell that continuously proliferates resulting in monoclonal expansion [1, 2]. They are a heterogeneous group of diseases that vary in clinical features,

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epidemiology, etiology, morphology, phenotype and sites of involvement [3]. Occasionally, two or more different B-cell clones in the same anatomical site at the same time can be detected [4]. The question arises as to whether these are different maturation stages of the same neoplastic clone or two (or more) independent malignancies differing in morphology, phenotype, molecular and cytogenetic findings [5, 6]. Up to 5 % of all mature B-lymphoproliferations are found to have two or more clones present [7], according to some authors the incidence is higher in HCL [8]. These clones are usually detected by flow cytometry on the basis of different surface immunoglobulin (sIg) light chain restriction or its pattern of expression, expression of other cell surface antigens as well as light scatter properties [9]. Here, we report on our retrospective study aimed at detection, characterization and diagnostics of composite B-cell lymphoproliferations utilizing multiparametric flow cytometry approach in a cohort of 413 subsequent patients with de novo mature B-cell neoplasm with leukemic presentation diagnosed in our institution during a 30-month period.

Materials and methods

Patients

Between August 2009 and January 2012, 413 consecutive adult patients with mature B-cell neoplasm underwent laboratory examination at the Department of Laboratory Diagnostics of the University Hospital Centre Zagreb. Patient's peripheral blood (PB) and/or bone marrow (BM) samples were collected and processed according to the recommendations of the Ethical Committee of the University Hospital Centre Zagreb. Data for this retrospective study were obtained solely by reanalysing pre-existing records. No additional contact with the patients was made and no patient information was revealed in the current study. All patients signed informed consent for diagnostic procedures at the time of obtaining their biological sample. For these reasons, the study was exempted from the need for subsequent informed consent.

Samples were collected in vacutainers containing K_3 EDTA anticoagulant. Red blood cells were lysed with hypotonic ammonium-chloride solution for 10 min. Leukocytes were washed twice and resuspended in phosphate buffered saline (PBS) with 10 % of fetal bovine serum at a concentration of 10^7 cells/mL. Hundred (100) μL of cell suspension was used in a single tube for direct cell staining with specific antibody combinations. Antibodies used for cell staining were conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-cyanin 5 (PE-Cy5) and allophycocyanin (APC). Antibodies were obtained from Becton–Dickinson (CD19-APC, CD22-PE, CD25-APC, CD38-PE), DAKO (CD5-FITC, CD10-PE, CD20-FITC, CD23-PE, CD103-FITC, CD138-PE, anti-κ-FITC/anti-λ-PE/19-PE-Cy5, sIgM-FITC, sIgG-FITC, FMC7-FITC) and Pharmigen (CD38-FITC). Data acquisition and analysis were performed on Becton–Dickinson's FACSCalibur using CellQuestPro software, respectively. Forward/Side scatter (FSC/SSC) gating strategy was used for population detection, antigen expression analysis and debris exclusion. Aberrant phenotypes of B-cells (CD19-positive) were used as a strategy for detection of malignant populations. Aforementioned antigen expression and/or intensity of expression were studied in detail to define aberrant populations. κ to λ ratio was used solely to confirm the monoclonal nature of each clone.

Statistical analysis

R software program (version 2.15.1) was used in all statistical analyses. Descriptive statistical measures, including mean, median and standard deviation were calculated for each categorical and continuous variable. Since the normal distribution could not be inferred, two-sample test for equality of proportions, Fischer's exact test and Mann–Whitney–Wilcoxon test were used to test for differences between groups, with statistical significance set at 0.05 level.

Results

During a 30-month period, a total of 413 patients were diagnosed with de novo mature B-cell neoplasm with a leukemic presentation on the basis of multiparametric flow cytometry immunophenotpying of PB (11 %) or BM samples (89 %). The mean age of patients was 67 years (range, 32–88), 59.8 % of them had surface *kappa* (κ) light chain restriction, 37.8 % *lambda* (λ) light chain restriction, while 2.4 % had both surface light chains present. The slight predominance of males was observed (60 vs. 40 %). Based on flow cytometry findings, all patients were divided into six diagnostic groups. Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) was the most frequent finding—61 %, followed by follicular cell lymphoma (FCL)—7 %, mantle cell lymphoma (MCL)—6 %, and hairy cell leukemia (HCL)—3 %. All other lymphoma types (diffuse large B cell lymphoma, marginal zone lymphoma, Burkitt's lymphoma, Morbus Waldenström, etc.) were assigned to the immunophenotypically unclassified group—19 %. Among 413 patients, there were 16 cases of biclonal mature B-lymphoproliferative disease, for an

overall incidence of 3.9 %. Three of them were woman (19 %) and 13 men (81 %) with a mean age of 67 years (range, 46–81). Two-sample test for equality of proportions with continuity correction revealed statistically significant ($P = 0.006$) tendency of the FCL group having a higher frequency of female patients (17/27, 63 %) in comparison to biclonal group where male patients predominated (13/16, 81 %). However, it is important to stress the fact that this is more likely the feature of FCL group, since statistically significant female predominance in FCL was also found in comparison to CLL/SLL ($P = 0.012$) and HCL ($P = 0.039$). In addition, the biclonal group showed borderline P value ($P = 0.064$) in comparison to unclassified group. Similarly, Mann–Whitney–Wilcoxon rank sum test showed that FCL group comprised of relatively younger patients compared to CLL/SLL, MCL, unclassified and biclonal group, with statistically significant P values equal to 0.001, 0.026, 0.003 and 0.047, respectively. It is important to emphasize that biclonal vs. uniclonal cases did not show any statistically significant difference with respect to patients' age and gender. The median values of κ/λ ratio were determined for each of the six diagnostic groups. In our hands, flow cytometric κ/λ reference interval for healthy people is between 0.5 and 4.0. Among six diagnostic categories, the composite B-cell lymphoproliferative group was the only one with a median value of κ/λ ratio falling within the normal range (1.9). CLL/SLL, MCL, FCL, HCL and unclassified group had median κ/λ ratios of 28.0, 7.0, 9.4, 11.4 and 11.5, respectively. The distribution of κ/λ ratios in different groups is shown in Fig. 1 and frequencies of the affiliation of κ/λ to one of the groups—KL1 (κ/λ <0.5), KL2 (κ/λ between 0.5 and 4) and KL3 (κ/λ >4) in Fig. 2.

Fourteen biclonal cases (88 %) had at least one clone with immunophenotypic characteristics of B-CLL/SLL, while the second neoplastic population varied between cases. Three of them (3/14) had phenotype corresponding to HCL, two (2/14) were MCL, six (6/14) had the second CLL/SLL population differing from the first CLL/SLL clone, one (1/14) was FCL and finally, two (2/14) remained unclassified. Another two cases were combination of MCL + MCL and HCL + unclassified lymphoma (patients 3 and 11, respectively). Clone size was calculated as a percentage of monoclonal B-cells out of all cells present in the sample, excluding cellular debris and erythrocytes based on FSC vs. SSC characteristics. Since multiparameter flow cytometry is a method sensitive enough to detect very small infiltration (10^{-3}) of malignant cells in the sample, we did not set the lower threshold to define a certain population as a clonal mass (Table 1).

To determine which of the diagnostic groups had the highest frequency of biclonality, we assigned each biclonal case to its group according to the phenotype of the non-CLL/SLL clone. Biclonal cases that had one immunophenotypically unclassified clone were assigned to the diagnostic group of the clone that we were able to characterize by flow cytometry. In this way, we determined that HCL had in total frequency of 4/18 (22 %), MCL of 3/27 (11 %), FCL of 1/28 (4 %), while CLL/SLL group had 8/252 (3 %) biclonal B-cell lymphoma cases. Fischer's exact test showed that, when categorized according to the non-CLL/SLL clone, HCL diagnostic group had the higher frequency of composite B-cell lymphoproliferative cases in comparison to CLL/SLL group ($P = 0.001$).

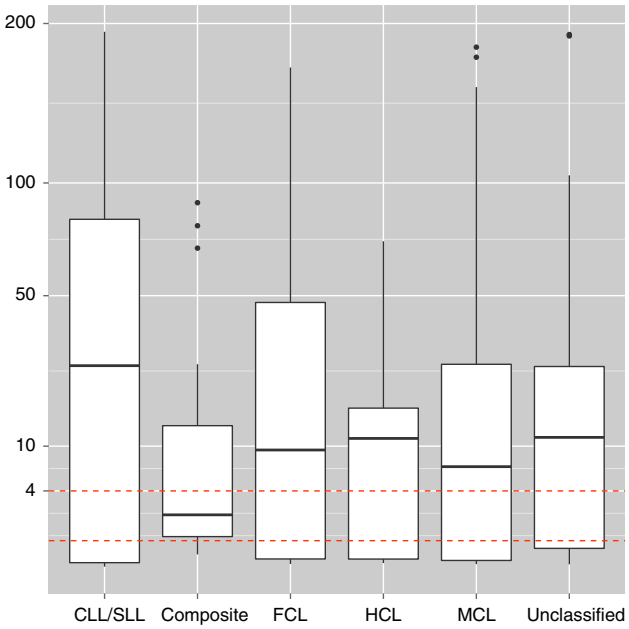

Fig. 1 Boxplots of κ/λ values for different groups of mature B-cell lymphoproliferative neoplasms. Two outliers (485.3, 482.5) for group CLL/SLL are not presented in the figure. *Dashed horizontal lines* mark reference interval for healthy people (0.5–4.0)

Due to thorough analysis of immunophenotype and light scatter features in each of the monoclonal B-cell population in the biclonal group, we assessed the ability of multiparametric flow cytometry not only to detect such cases but also to determine the major markers contributing to differentiation of the two coexisting B-clones within each individual case. We analyzed the light scatter characteristics of cells in the clones (forward-FSC and side-SSC scatter), other cell surface antigen(s) expression, surface Ig light chain isotype expression (*kappa*, κ or l*ambda*, λ) and its membrane intensity (high vs. weak). A total of 7/16 (44 %) of composite B-cell lymphoproliferative neoplasms displayed different light scatter properties between clones, 11/16 (69 %) had different surface Ig κ or λ restriction, while 5/16 (31 %) had the same light chain restriction but could be further identified as separate clones based on the level of the light chain membrane expression in 4/5 cases

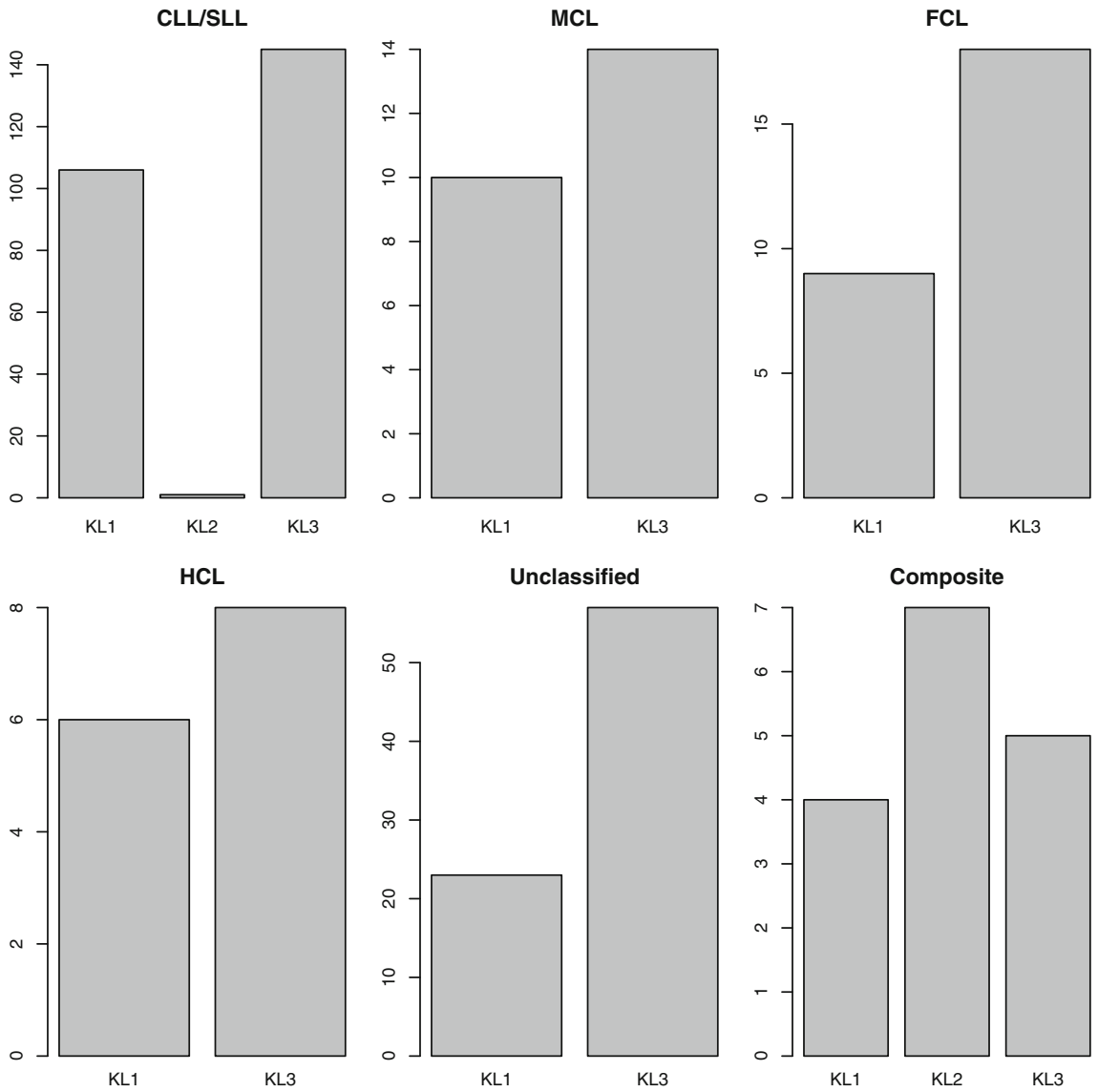

Fig. 2 Frequencies of affiliation to groups KL1 ($0 < \kappa/\lambda < 0.5$), KL2 ($0.5 < \kappa/\lambda < 4$) and KL3 ($\kappa/\lambda > 4$) for different groups of mature B-cell lymphoproliferative neoplasms. *X-axis* indicates the KL1, KL2 and KL3 groups; *Y-axis* indicates the number of patients in each group

Table 1 Immunophenotype and clone sizes in biclonal leukemic B-cell lymphoproliferative disorders

Case no./clone	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Clone 1	CLL	CLL	MCL	CLL	CLL	CLL	CLL	CLL	CLL	CLL	uncl	CLL	CLL	CLL	CLL	CLL
Clone 1 (%)	10	39	10	3	7	8	56	21	1	34	7	43	15	55	30	28
Clone 2	HCL	HCL	MCL	HCL	MCL	MCL	CLL	uncl	uncl	CLL	HCL	CLL	CLL	CLL	FCL	CLL
Clone 2 (%)	18	9	17	6	23	15	19	7	2	15	23	32	9	14	37	12

Clone size is calculated as a percentage of monoclonal B-cells of specific immunophenotype of total cells present in the sample after exclusion of cellular debris and erythrocytes based on FSC vs. SSC parameters

CLL chronic lymphocytic leukemia/small lymphocytic lymphoma, *MCL* mantle cell lymphoma, *FCL* follicular cell lymphoma, *HCL* hairy cell lymphoma, *uncl* unclassified

Table 2 Flow cytometry differentiation of clones in biclonal leukemic B-cell lymphoproliferative disorders

Case no./immunophenotype[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
sIg κλ restriction	+	−	−	−	+	−	+	−	+	+	+	+	+	+	+	+
sIg κλ pattern of expression	−	+	−	+	+	−	−	+	+	−	+	−	−	−	+	−
Other antigen(s) pattern of expression	+	+	+	+	+	+	−	+	+	−	+	−	−	−	+	−
Light scatter properties	+	+	+	−	+	−	−	−	+	−	+	−	−	−	+	−

Case no.[b]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Clone/marker	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2
CD5	+/−	+/−	+/+	+/−	+/+	+/+	+/+	+/−	+/−	+/+	−/−	+/+	+/+	+/+	+/+	+/+
CD10	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	na/na	−/−	na/na	na/na	−/+	−/−
CD19	++/++	++/++	++/++	++/++	++/++	++/++	++/++	++/++	++/+	++/++	++/++	++/++	++/++	++/++	++/++	++/++
CD20	++/+++	+/+++	++/++	+/+++	+/+++	++/++	+/+	+/++	+/++	+/+	++/+++	+/+	+/+	+/+	+/+++	+/+
CD22	+/+++	+/+++	++/++	+/+++	+/++	+/++	+/+	+/++	++/++	+/+	na/na	+/+	na/na	na/na	+/++	na/na
CD23	++/++	++/−	++/−	++/−	++/−	++/−	++/++	++/−	+/−	++/++	−/−	++/++	++/++	++/++	++/++	++/++
CD25	−/++	−/+	na/na	−/−	na/na	na/na	na/na	na/na	na/na	na/na	−/++	na/na	na/na	na/na	na/na	na/na
CD38	−/−	+/−	−/−	−/−	−/+	++/+	−/−	−/−	+/−	−/−	na/na	−/−	−/−	++/++	+/++	−/−
CD103	−/+	−/++	−/−	−/++	−/−	−/−	na/na	−/−	−/−	−/−	−/++	−/−	na/na	na/na	−/−	na/na
CD138	−/−	−/−	−/−	−/−	−/−	−/−	na/na	−/−	−/−	−/−	na/na	−/−	na/na	na/na	−/−	na/na
sIgM	++/−	+/+	++/++	−/+++	+/+++	+/+	−/−	+/++	na/na	+/+	na/na	−/−	na/na	na/na	−/++	na/na
sIgG	−/++	−/−	−/−	−/−	−/−	−/−	−/−	−/−	na/na	−/−	na/na	−/−	na/na	na/na	−/−	na/na
sIgκ	−/++	+/++	++/++	+/+++	+/−	++/++	−/+	+/++	−/++	−/+	++/−	−/+	+/−	+/−	−/++	−/+
sIgλ	++/−	−/−	−/−	−/−	−/+++	−/−	+/−	−/−	+/−	+/−	−/++	+/−	−/+	−/+	+/−	+/−
FMC7	−/+	−/++	++/++	na/na	na/na	na/na	na/na	na/na	na/na	na/na	na/na	−/−	na/na	na/na	++/−	na/na

[a] +, designates characteristic that is able to differentiate two coexisting clones; −, designates the characteristic that is not useful for differentiating two coexisting clones

[b] −, no expression of antigen; +, dim expression; ++, moderate/intermediate expression; +++, bright expression; *na* not assessed

or other cell surface antigen(s) level of expression in 1/5 cases. Finally, in 10/16 (63 %) cases, the clones were differentiated on the basis of other antigen(s) expression characteristics. Two or more distinctive features were present in 9/16 (56 %) samples. Six cases with CLL/SLL + CLL/SLL phenotype had only light chain restriction as differentiating marker between the clones, while light scatter and other antigens did not prove to be useful (Table 2).

Besides κ and λ light chain expression, other cell surface antigens were analyzed to determine the most informative ones in differentiating two concurrent mature B-cell clones by flow cytometry. CD22 (58 %), CD20 (50 %), surface IgM (46 %), CD23 (44 %), CD103 (33 %), CD38 (33 %) and CD5 (31 %) were most frequently found to be differently expressed on coexisting neoplastic populations. Although analyzed in a relatively low number of patients and only when found to be diagnostically necessary, CD25 and FMC7 were found differentially expressed in high percentages (75 and 60 %, respectively) confirming the role of these markers in B-cell leukemia/lymphoma diagnostic set-up (Table 2).

Discussion

Composite B-cell lymphoproliferative neoplasms have been considered to be rare and uncommon in clinical practice [10]. In addition, literature data on this subject are scarce, mostly in the form of case reports [11–16]. However, Sanchez et al. [7] in their comprehensive study reports the overall incidence of composite B-cell neoplasms up to 4.8 % of all newly diagnosed chronic B-cell malignancies, a percentage that cannot be neglected. Methodologies available at this moment have made a great breakthrough in the detection and characterization of such entities, and flow cytometric immunophenotyping provides new possibilities by combining simultaneous detection of leukocyte differentiation antigens with the analysis of clonality.

Here, we report a 3.9 % incidence of biclonal cases within de novo diagnosed mature B-cell neoplasms presenting in PB or BM. Of note, 14/16 (88 %) biclonal cases have at least one monoclonal population phenotypically corresponding to B-CLL/SLL. This observation is likely reflecting the high incidence of CLL/SLL in general, but neglecting this information could be misleading as well. Rather small proportion of biclonality in the CLL/SLL group is easily explainable by the fact that CLL/SLL makes more than 60 % (252 patients) of all de novo diagnosed B-cell lymphoproliferations, resulting in ultimately relatively low percentage of composite B-cell lymphoproliferative neoplasms within the group. Even though, one should acknowledge, and take into consideration while making a diagnosis, the high frequency of clones phenotypically corresponding to CLL/SLL within all biclonal cases. Even the largest study conducted by Sanchez et al. [7] report only 13/53 (25 %) cases in that CLL/SLL is not one of the clones. Only when analyzed as the frequency of biclonality based on the phenotype of the non-CLL/SLL monoclonal B-cell population, HCL is standing out with 22 % biclonal cases of all de novo diagnosed HCL during a 30-month period, followed by MCL (11 %), CLL (3 %) and FCL (4 %). This finding is in concordance with other published data concerning this subject [7, 8]. Taking into account that seven cases in our study have phenotypically similar clones (CLL/SLL + CLL/SLL or MCL + MCL), one could presume they represent intraclonal evolution stages of the same entity as proposed and well argued in the literature [17]. However, it should be emphasized that among these six have different light chain restrictions, making this thesis not applicable. Only one case (patient #3), diagnosed with two clones of MCL having the same light chain restriction could account for the intraclonal evolution. Alternatively, we could state that five of our composite B-cell lymphoproliferation cases had the same light chain restriction and could represent intraclonal evolution. Only one of these cases (patient #3) has two phenotypically resembling clones (MCL + MCL) simultaneously present, thus leaving us with certain doubts and inconclusive finding. Although molecular confirmation of biclonality would be desirable in such cases, unfortunately these data are not available.

To improve diagnostics of composite B-cell lymphoproliferative neoplasms, one should be aware that if two malignant populations have different surface Ig light chain isotype, κ/λ ratio itself might not be indicative of B-cell lymphoma presence [13]. Relying only on historic κ/λ ratio is not sensitive enough to detect these kinds of malignant processes. In addition, the ratio itself is highly dependent on the clones size or level of infiltration. In other words, even cases with one monoclonal population would not be detected by this approach, if the proportion of the malignant clone to polyclonal B-cells in the sample is relatively low. Our results show that the biclonal B-cell lymphoproliferative disorders have a median value of κ/λ ratio within the normal range. This is the result of both surface light chains expressed on the cell membrane of coexisting clones in the majority (11/16 or 69 %) of patients with biclonality. Thus, it is extremely important to primarily analyze other surface markers and physical properties of cells (FSC and SSC, respectively) to establish the correct diagnosis (Fig. 3a). In our hands, CD22, CD20, surface IgM and CD23 expression (positivity/negativity and expression intensity) proved to be informative in differentiating the coexisting clones. Aberrant B-cell phenotype detection

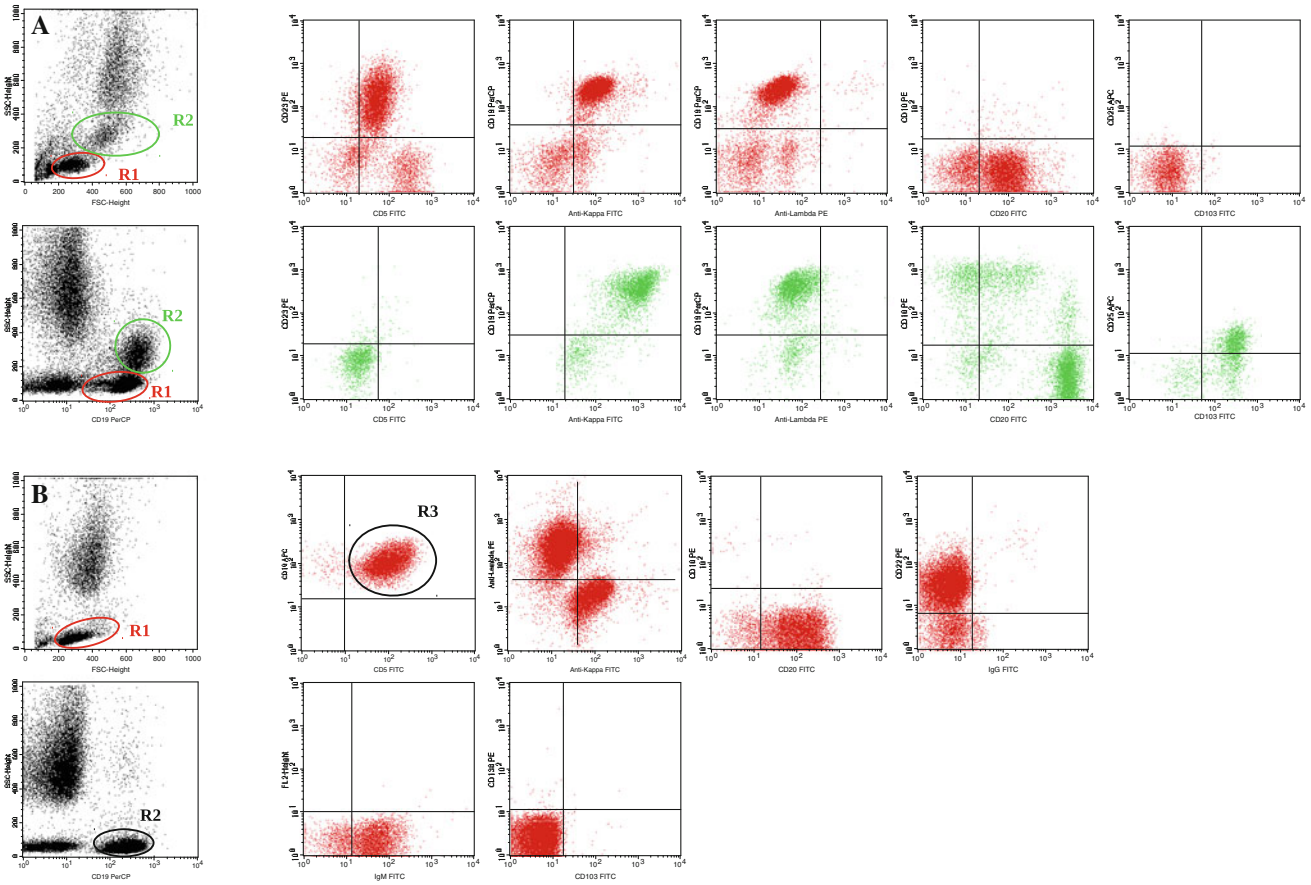

Fig. 3 Flow cytometric detection and characterization of biclonal mature B-cell lymphoproliferative neoplasms. **a** Forward (FSC) and side (SSC) scatter and immunophenotype of CLL/SLL + HCL sample (patient #2). *Upper dot plots* show immunophenotype of cells in *R1* (lymphocytic region), and *lower dot plots* immunophenotype of cells in *R2* (monocytic region). *Plots* (*X axis*/*Y axis*) are as follows (from *left* to *right*): CD5/CD23, surface κ/CD19, surface λ/CD19, CD20/CD10, CD103/CD25, CD19/SSC. **b** Forward (FSC) and side (SSC) scatter and immunophenotype of CLL/SLL + CLL/SLL sample (patient #10). *R1* is lymphocytic region based on FSC/SSC characteristics. *CD19/SSC plot* shows all B-cells in the sample. *CD5/CD19 dot plot* shows immunophenotype of cells in *R2* (B-cell region). *Other plots* (*X axis*/*Y axis*) show phenotype of CD5+CD19+ (region *R3*) cells and are as follows (from *left* to *right*): surface κ/surface λ, CD20/CD10, surface IgG/CD22, surface IgM, CD103/CD138 and are showing immunophenotype of CD5+CD23+ B-cells from *R2* (region of CD5+CD23+ cells). *R1* region one; *R2* region two; *R3* region three

followed by confirmation of monoclonality in κ/λ ratio for each aberrant population is much more sensitive way to detect composite B-cell lymphoproliferations rather than inverse archaic approach. The importance and a necessity of the same analysis strategy are also emerging for those cases with CLL/SLL + CLL/SLL clones. If screened solely for κ/λ ratio, they would be considered polyclonal, whereas, detection of CLL/SLL-associated antigen expression and subsequent light chains expression analysis is of tremendous relevance for establishing a diagnosis of two coexisting CLL/SLL clones (Fig. 3b).

Another important question that needs to be addressed is whether such patients have clinically different disease course or prognosis. This would require long-term monitoring and evaluation of minimal residual disease and response to therapy of both malignant clones. Since the number of patients was limited and follow-up rather short, we cannot draw any conclusions concerning clinical course and outcome.

In conclusion, the multiparametric flow cytometry approach is a very significant mean for detection, characterization and quality diagnosis of patients with composite B-cell lymphoproliferative neoplasms. Not only the markers we use in the diagnostics, but also a sequence and manner of analysis strategy counts. Two major distinctions should be clearly stated. First, to detect composite B-cell lymphoproliferations, it is extremely important to detect the aberrant B-cell phenotypes and define the malignant populations based on the antigens they express. Second, after we have found two coexisting B-cell neoplasms, we can use light chain expression pattern to further differentiate the clones. In this sense, historic κ to λ ratio proved to

be a poor measurement of clonality and is helpful only as an auxiliary tool. As demonstrated, more creative analytical techniques should be utilized in these types of cases, although it is a valid method for detection of monoclonality when only one malignant clone is present.

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Conflict of interest The authors declare that they have no conflict of interest.

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