Molecular and cytogenetic characterization of expanded B-cell clones from multiclonal *versus* monoclonal B-cell chronic lymphoproliferative disorders

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ABSTRACT

Chronic antigen-stimulation has been recurrently involved in the earlier stages of monoclonal B-cell lymphocytosis, chronic lymphocytic leukemia and other B-cell chronic lymphoproliferative disorders. The expansion of two or more B-cell clones has frequently been reported in individuals with these conditions; potentially, such coexisting clones have a greater probability of interaction with common immunological determinants. Here, we analyzed the B-cell receptor repertoire and molecular profile, as well as the phenotypic, cytogenetic and hematologic features, of 228 chronic lymphocytic leukemia-like and non-chronic lymphocytic leukemia-like clones comparing multiclonal (n=85 clones from 41 cases) *versus* monoclonal (n=143 clones) monoclonal B-cell lymphocytosis, chronic lymphocytic leukemia and other B-cell chronic lymphoproliferative disorders. The B-cell receptor of B-cell clones from multiclonal cases showed a slightly higher degree of HCDR3 homology than B-cell clones from monoclonal cases, in association with unique hematologic (e.g. lower B-lymphocyte counts) and cytogenetic (e.g. lower frequency of cytogenetically altered clones) features usually related to earlier stages of the disease. Moreover, a subgroup of coexisting B-cell clones from individual multiclonal cases which were found to be phylogenetically related showed unique molecular and cytogenetic features: they more frequently shared *IGHV3* gene usage, shorter HCDR3 sequences with a greater proportion of *IGHV* mutations and del(13q14.3), than other unrelated B-cell clones. These results would support the antigen-driven nature of such multiclonal B-cell expansions, with potential involvement of multiple antigens/epitopes.

Introduction

B-cell chronic lymphoproliferative disorders (B-CLPD) usually show a monoclonal expansion of a (single) mature-appearing aberrant B-cell clone.[1-4] However, patients diagnosed with composite lymphomas and other B-cell chronic lymphocytic leukemias – e.g. chronic lymphocytic leukemia (CLL) – have been reported in the literature for decades, particularly among immunocompromised subjects.[5-7] Although early reports considered this phenomenon as a rare event, it might have been underestimated because its detection requires sophisticated multidisciplinary approaches encompassing combined histopathology, cytomorphology, immunophenotypic and cytogenetic techniques and/or molecular analyses of purified cell populations.[8] In fact, B-cell neoplasms consisting of two phenotypically distinct populations of clonally unrelated B-lymphocytes coexisting in the same patient (detected either simultaneously or at different time points during follow-up) have been reported in the literature,[9,10] with an estimated overall frequency among B-CLPD patients of around 5%.[11] Recently, it has also been shown that up to 20% of cases of population-based non-CLL and CLL-like low count monoclonal B-cell lymphocytosis (MBL^{lo}) may also carry two different unrelated B-cell clones.[12-14] In addition, data from a small series suggest that the frequency of multiclonality could be particularly high among CLL-like MBL cases in relatives of patients with CLL, (4/6 cases analyzed).[12] Altogether, these results support the existence of multiclonality in a significant proportion of both MBL cases and B-CLPD patients.

Multiclonal MBL and B-CLPD cases may consist of expansions of two or more B-cell clones potentially associated with chronic antigen-driven immune responses.[15-17] In fact, this is particularly frequent in the earlier stages of MBL^{lo}, which would further support the potential reactive nature of MBL among individuals with normal lymphocyte counts, prior to the stepwise acquisition of genetic alterations and progression

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to clinical MBL (MBL^{hi}) and CLL.[13,14,18,19] If this hypothesis holds true, specific antigenic determinants could potentially be more frequently shared between the coexisting B-cell clones of multiclonal cases than between the expanded B cells in different monoclonal MBL and B-CLPD patients, due to a higher probability of interaction with common immunological determinants. This might even be true when the coexisting clones display clearly distinct immunophenotypic and cytogenetic, as well as clinical, features.[20-22]

In order to test this hypothesis, in the present study we compared the B-cell receptor (BCR) repertoire and molecular profile, as well as the phenotypic, cytogenetic and hematologic features of CLL-like and non-CLL-like clones (n=228) from multiclonal (n=41 cases) *versus* monoclonal cases [n=143, including both CLL and CLL-like MBL (n=128), as well as cases of B-CLPD other than CLL and non-CLL-like MBL (n=15)].

Methods

Patients and samples

A total of 184 subjects with one (n=143 monoclonal cases) or two or more (n=41 multiclonal cases) CLL/non-CLL B-CLPD (n=140) and/or CLL-like/non-CLL-like MBL (n=88) B-cell clones, as defined by the World Health Organization 2008 criteria,[23] were included. Binet stage[24] of CLL subjects was retrospectively collected.

From the 41 multiclonal cases, two (5%) corresponded to healthy individuals with CLL-like MBL^{lo}, eight (19.5%) were CLL-like MBL^{hi} cases, 23 (56%) had CLL and eight (19.5%) had B-CLPD other than CLL; four of these latter cases showed coexistence of either one or two CLL-like MBL B-cell population(s). In 3/41 multiclonal cases, three coexisting B-cell populations were detected. From the 143 monoclonal cases, 13 (9%) corresponded to healthy adults with CLL-like MBL^{lo}, 26 (18%) had CLL-like MBL^{hi}, 89 (62%) had CLL, two (1%) had non-CLL-like MBL^{lo}, two (1%) were non-CLL-like MBL^{hi} cases and 11 (8%) had other B-CLPD. The precise criteria used for the classification of MBL^{lo} and MBL^{hi} are detailed in the *Online Supplementary Methods*. The age/gender distribution for each diagnostic group is detailed in *Online Supplementary Table S1*.

Peripheral blood samples were obtained from each subject after written informed consent had been given, and the study was approved by the local ethics committees of the University Hospital of Salamanca and the Blood and Transplantation Center of Coimbra/Portuguese Institute of Blood and Transplantation, in accordance with the Helsinki Declaration of 1975, as revised in 2008.

Immunophenotypic analyses

Immunophenotypic studies to screen for the presence and full characterization of clonal B-cell populations were performed by high-sensitive multiparameter flow cytometry on erythrocyte-lysed peripheral blood samples, according to previously described procedures[18,25-29] which are also detailed in the *Online Supplementary Methods*. All cases showed a clonal (imbalanced SmIgκ:SmIgλ ratio of >3:1 or <1:3) and/or aberrant CD5^{+} B-cell population.

Cytogenetic and molecular studies

Cytogenetic analyses were performed by multicolor interphase fluorescence *in situ* hybridization on slides containing FACS-purified and fixed aberrant B cells, as previously described in detail.[18,30] In parallel, patterns of rearrangement of the immunoglobulin heavy chain variable region genes (*IGHV*) and immunoglobulin κ (*IGKV*) and λ (*IGLV*) light chain genes were analyzed for each FACS-purified B-cell clone[18,31-32] (see the *Online Supplementary Methods* section for detailed descriptions). To investigate the level of phylogenetic relationship among *IGHV* amino acid sequences, a sequence distance tree was built using the neighbor-joining method implemented in the freely available Molecular Evolutionary Genetic Analysis (MEGA) software.[33] Two different, coexisting BCR were considered as being phylogenetically related when their IGHV amino acid sequences, going from framework region 1 to HCDR3 (both regions included), showed an identity ≥ 60%. This "identity" threshold was based on previously published concepts about the phylogeny of human *IGHV* genes based on their amino acid sequences[33], and on the minimum identity percentage observed in colocalized subbranches (presumably with the highest evolutionary relationship[33]) of the sequence distance tree built in this study (see the *Online Supplementary Methods* section for more detailed descriptions). HCDR3-alignments were carried out for each multiclonal case whose coexisting B-cell clones showed HCDR3 regions with identical lengths or lengths differing by one amino acid using the bioinformatic tools available at the web services of the European Bioinformatics Institute (EMBL-EBI Cambridge, UK). Through the EMBL-EBI tools, the identical amino acids or those with analogous side-chain polarity per paired intra-case HCDR3-alignment were highlighted (see the *Online Supplementary Methods* section for more detailed descriptions).

Statistical methods

For all statistical analyses the SPSS software program (SPSS 20.0, IBM SPSS Statistics, IBM, Armonk, NY, USA) was used.

Results

Distribution and immunophenotypic features of B-cell clones

A total of 228 B-cell clones were identified. These corresponded to 143 B-cell clones (89 CLL, 11 non-CLL, 39 CLL-like MBL and 4 non-CLL-like MBL clones) from monoclonal cases and 85 B-cell clones (26 CLL, 14 non-CLL, 40 CLL-like MBL and 5 non-CLL-like MBL clones) from multiclonal cases (*Online Supplementary Table S2*). The complete immunophenotypic and cytogenetic features of the individual clones of multiclonal cases are summarized in *Online Supplementary Table S3*.

In 26/41 multiclonal cases, all coexisting B-cell clones showed a CLL-like phenotype, while in 11 of the remaining 15 cases, at least one CLL-like B-cell population coexisting with another non-CLL aberrant B-cell population was identified. In the remaining four cases, two distinct non-CLL-like B-cell clones were found (*Online Supplementary Table S3*). The distribution of all CLL/non-CLL and CLL-like MBL/non-CLL-like MBL clones analyzed (from all monoclonal and multiclonal cases considered together) in the distinct diagnostic categories was as follows: 27 B-cell clones corresponded to CLL-like MBL^{lo}, 52 to CLL-like MBL^{hi}, 115 to CLL, 5 to non-CLL-like MBL^{lo}, 4 to non-CLL-like MBL^{hi} and 25 to non-CLL B-CLPD (*Online Supplementary Table S2*). The precise diagnoses of the B-cell clones from patients with B-CLPD other than CLL are specified in the footnote to *Online Supplementary Table S2*.

Overall size and B-cell receptor features of B-cell clones from multiclonal versus monoclonal cases of monoclonal B-cell lymphocytosis, chronic lymphocytic leukemia, and other B-cell chronic lymphoproliferative diseases

The relative and absolute median number of peripheral blood clonal B cells was significantly lower in multiclonal than in monoclonal cases (13% *versus* 45% and 2,692 cells/μL *versus* 9,115 cells/μL, respectively; *P*=0.001). Of note, the absolute median numbers of CLL-like MBL^{hi} and CLL B-cell clones were also significantly lower in multiclonal than in monoclonal cases (1,254 *versus* 2,464 cells/μL and 9,113 *versus* 18,600 cells/μL, respectively; *P*=0.004 and *P*=0.02) (*Online Supplementary Figure S1*). In contrast, the absolute median number of peripheral blood CLL-like MBL^{lo} B-cell clones was significantly higher in multiclonal than in monoclonal cases (79 *versus* 1 cells/ μL; *P*=0.002). No significant differences were found in the clone size between non-CLL-like and non-CLL B-cell clones in multiclonal *versus* monoclonal cases (*Online Supplementary Figure S1*). In addition, the frequency of CLL-like MBL B-cell clones was significantly higher in multiclonal than in monoclonal cases (47% *versus* 27%, respectively; *P*=0.002), whereas the frequency of CLL B-cell clones was higher in monoclonal *versus* multiclonal subjects (62% *versus* 31%, respectively; *P*=0.001). CLL B-cell clones from multiclonal and monoclonal CLL patients showed a similar distribution in Binet stage A *versus* Binet stages B/C (*P*>0.05). Of note, non-CLL B-cell clones were present at higher frequencies in multiclonal *versus* monoclonal cases (17% *versus* 8%, respectively; *P*=0.04) (Table 1).

Regarding BCR features, a similar distribution of *IGHV*-mutated and *IGHV*-unmutated B-cell clones was found in multiclonal *versus* monoclonal cases: 51/85 (60%) *versus* 84/139 (60%) and 34/85 (40%) *versus* 55/139 (40%), respectively (Table 1). Despite this, the percentage of alignment of *IGHV* amino acid sequences among B-cell clones from multiclonal cases (n=3,560 two by two comparisons of clonal *IGHV* amino acid sequence) was slightly higher than that obtained among B-cell clones from monoclonal cases (n=8,891 comparisons): median of 52% *versus* 50%, respectively (*P*=0.001; Table 1).

Cytogenetic features of B-cell clones from multiclonal versus monoclonal cases of monoclonal B-cell lymphocytosis and B-cell chronic lymphoproliferative diseases

The frequency of CLL-like MBL and CLL clones from multiclonal cases that showed cytogenetic alterations was significantly lower than that found among CLL-like MBL and CLL clones from monoclonal cases: 27/66 (41%) *versus* 77/128 (60%), respectively (*P*=0.02). Likewise, the proportion of CLL-like B-cell clones showing coexistence of two or more cytogenetic alterations was also significantly lower in multiclonal than in monoclonal cases: 8/66 (12%) *versus* 32/128 (25%; *P*=0.047). This was specially true among B-cell clones from CLL patients: 2/26 (8%) *versus* 29/89 (33%), respectively (*P*=0.03; Table 2).

Regarding each specific cytogenetic alteration, only a decreased frequency of CLL-like B-cell clones with del(13q14) involving the *RB1* gene and a lower percentage of del(13q14)$^+$ cells was found in multiclonal *versus* monoclonal cases: the frequency of del(13q14)$^+$ clones was 5% *versus* 15% with a median of del(13q14)$^+$ cells of 55% *versus* 86%, respectively (*P*=0.01; Table 2). Of note, these differences were mostly due to the lower frequency of B-cell clones with del(13q14) (4% *versus* 19%, *P*=0.01) found among CLL clones from multiclonal *versus* monoclonal cases (Table 2).

Table 1. Peripheral blood B-cell counts and BCR features of multiclonal *vs.* monoclonal B-cell clones from cases of B-cell chronic lymphoproliferative disorders and monoclonal B-cell lymphocytosis.

	Multiclonal B-cells n=85 clones	Monoclonal B-cells n=143 clones	Total n=228 clones
N. of PB clonal B cells (x10^6/L)*	**2,692 (0.6-156,168)[a]**	**9,115 (0.1-369,288)**	5,530 (0.1-369,288)
% of PB clonal B cells from WBC*	**13% (0.1%-89%)[a]**	**45% (0.002%-97%)**	35% (0.001%-97%)
CLL-like MBL^{lo} B-cell clones	14/85 (16%)	13/143 (9%)	27/228 (12%)
CLL-like MBL^{hi} B-cell clones	**26/85 (31%)[a]**	**26/143 (18%)**	52/228 (38%)
CLL B-cell clones	**26/85 (31%)[a]**	**89/143 (62%)**	113/228 (50%)
CLL-stage A clones	12/20 (60%)	53/89 (60%)	65/109 (60%)
CLL-stage B/C clones	8/20 (40%)	36/89 (40%)	44/109 (40%)
Non-CLL-like MBL B-cell clones	5/85 (6%)	4/143 (3%)	9/228 (4%)
Non-CLL B-cell clones	**14/85 (17%)[a]**	**11/143 (8%)**	25/228 (11%)
IGHV mutated CLL-like B-cell clones	40/66 (61%)	76/128 (59%)	116/194 (60%)
IGHV mutated non-CLL-like B-cell clones	11/19 (58%)	8/15 (53%)	19/34 (56%)
% alignment of *IGHV* aa sequences between coexisting B-cell clones*	51% (38%-79%)	NA	51% (38%-79%)
% alignment of *IGHV* aa sequences between each B-cell clone and the other clones*	**52% (31%-100%)[a]**	**50% (29%-100%)**	51% (29%-100%)

*Results expressed as number of B-cell clones and percentage between brackets or as *median value (range). PB: peripheral blood; WBC: white blood cells; CLL: chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL^{lo}: low count monoclonal B-cell lymphocytosis;* IGHV*: immunoglobulin heavy chain variable region genes; MBL^{hi}: clinical monoclonal B-cell lymphocytosis; aa: amino acids. NA: not applicable. [a]Statistically significant differences (P<0.05) found between clones from multiclonal* vs. *monoclonal cases. Information about the parameters included in this table is displayed separately for CLL-like* vs. *non-CLL-like clones in* Online Supplementary Table S7.

No statistically significant differences were observed in the cytogenetic patterns of non-CLL B-cell clones from multiclonal *versus* monoclonal cases, which is probably due to the relatively low number of non-CLL clones included in the study; the precise cytogenetic alterations found in non-CLL/non-CLL-like MBL cases are shown in *Online Supplementary Tables S3* and *S4*. In turn, the overall cytogenetic features of non-CLL like B-cell clones from multiclonal subjects (n=19; 3 non-CLL MBL^{lo}, 2 non-CLL MBL^{hi}, 14 non-CLL B-cell clones) *versus* monoclonal subjects (n=15; 2 non-CLL MBL^{lo}, 2 non-CLL MBL^{hi}, 11 non-CLL B-cell clones) were similar, as regards both the frequency of cytogenetically altered clones (6/19, 32% and 6/15, 40%) and the percentage of cases with two or more genetic alterations: 2/19 (11%) *versus* 2/15 (13%) (*Online Supplementary Tables S3* and *S4*).

Molecular characteristics of the B-cell receptors of B-cell clones from multiclonal versus monoclonal cases of monoclonal B-cell lymphocytosis and B-cell chronic lymphoproliferative diseases

The molecular profile of the BCR of CLL-like MBL^{lo}, MBL^{hi} and CLL B-cell clones and of B-cell clones other than CLL from multiclonal *versus* monoclonal cases was very similar (Table 3 and *Online Supplementary Table S5*). No statistically significant differences in multiclonal *versus* monoclonal *VH* gene usage were found for most groups. Despite this general behavior, CLL-like MBL^{hi} B-cell clones from multiclonal cases less frequently showed usage of the *DH1*, *DH4* and *DH7* gene families than B-cell clones from monoclonal cases; in addition, *JH6* genes were also less frequently used by CLL B-cell clones from multiclonal *versus* monoclonal cases (Table 3). Overall, 33 functional IGHV gene rearrangements were identified from which 12 (*V4-34*, *V3-23*, *V3-48*, *V3-30*, *V1-69*, *V3-21*, *V4-39*, *V3-33*, *V3-11*, *V3-53*, *V1-2*, *V3-7*) were highly represented among the B-cell clones (≥5% of all B-cell clones corresponding to ≥4 and ≥5 B-cell clones sharing the same *IGHV* gene in multiclonal and monoclonal cases, respectively) (Figure 1A). Interestingly, 11 of these *IGHV* genes were found at similar frequencies within the clones of multiclonal *versus* monoclonal cases, while the *V3-33* gene was typically associated with multiclonal cases (6% *versus* 1%, *P*=0.03). Regarding *IGHD* genes, no significant differences were observed between B-cell clones from multiclonal and monoclonal cases, the *D3-3*, *D5-12*, *D3-10*, *D6-19*, *D2-15*, and *D2-2* genes being the most frequently used and shared by both groups of B-cell clones (Figure 1B). Among *IGHJ* genes, significant differences were only observed for the *JH6* gene, which was more frequently used in monoclonal cases (40% *versus* 26%; *P*=0.03) (Figure 1C).

Except for slightly longer LCDR3 sequences of the *IGKV* and *IGLV* genes found among B-cell clones from multiclonal *versus* monoclonal cases, especially among CLL-like MBL^{hi} clones (Table 3), no other significant differ-

Table 2. Cytogenetic features of CLL-like MBL^{lo}, MBL^{hi} and CLL B-cell clones from monoclonal *vs.* multiclonal cases.

Cytogenetic alterations	MBL^{lo} clones		MBL^{hi} clones		CLL clones		Total	
	Multiclonal n=14	Monoclonal n=13	Multiclonal n=26	Monoclonal n=26	Multiclonal n=26	Monoclonal n=89	Multiclonal n=66	Monoclonal n=128
N. of cytogenetically altered clones	2/14 (14%)	6/13 (46%)	13/26 (50%)	13/26 (50%)	12/26 (46%)	58/89 (65%)	**27/66 (41%)[b]**	**77/128 (60%)**
N. of clones with ≥2 alterations	1/14 (7%)	1/13 (8%)	5/26 (19%)	2/26 (8%)	**2/26 (8%)[a]**	**29/89 (33%)**	**8/66 (12%)[b]**	**32/128 (25%)**
Type of cytogenetic changes								
N. of del(13q)$^+$ clones (%) % del(13q)$^+$ cells *	2/14 (14%) 46% (19%-73%)	5/13 (38%) 86% (22%-96%)	7/26 (27%) 74% (15%-98%)	8/26 (31%) 38% (21%-99%)	8/26 (31%) 93% (30%-96%)	36/89 (40%) 80% (47%-99%)	17/66 (26%) 84% (10%-98%)	49/128 (38%) 79% (18%-99%)
N. of del(13q14.3)$^+$ clones (%) % del(13q14.3)$^+$ cells *	2/14 (7%) (19%-73%)	4/13 (31%) 78% (22%-96%)	7/26 (27%) 65% (15%-98%)	8/26 (31%) 38% (21%-99%)	8/26 (31%) 81% (30%-96%)	35/89 (39%) 73% (5%-99%)	17/66 (26%) 80% (15%-98%)	47/128 (37%) 71% (5%-99%)
N. of del(13q14)$^+$ clones (%) % del(13q14)$^+$ cells *	0/14 (0%) -	1/12 (8%) 86% (-)	2/26 (8%) 57% (15%-98%)	1/26 (4%) 96% (-)	**1/26 (4%)[a]** **95% (-)**	**17/89 (19%)** **79% (47%-99%)**	**3/66 (5%)[b]** **55% (10%-98%)[b]**	**19/127 (15%)** **86% (47%-99%)**
N. of trisomy 12$^+$ clones (%) % trisomy 12$^+$ cells *	0/14 (0%) -	1/13 (8%) 59% (-)	6/26 (23%) 87% (19%-95%)	5/26 (19%) 84% (80%-93%)	2/26 (8%) 84% (75%-93%)	17/89 (19%) 76% (33%-97%)	8/66 (12%) 87% (41%-95%)	23/128 (18%) 80% (33%-97%)
N. of t(14q32)$^+$ clones (%) % t(14q32)$^+$ cells *	0/12 (0%) -	0/10 (0%) -	2/26 (8%) 42% (31%-52%)	0/26 (0%) -	1/26 (4%) 98% (-)	10/89 (11%) 82% (18%-94%)	3/64 (5%) 72% (28%-98%)	10/125 (8%) 59% (18%-94%)
N. of del(11q)$^+$ clones (%) % del(11q)$^+$ cells *	0/12 (0%) -	0/11 (0%) -	1/26 (4%) 93% (-)	1/26 (4%) 20% (-)	1/26 (4%) 91% (-)	7/89 (8%) 57% (21%-98%)	2/64 (3%) 92% (91%-93%)	8/126 (6%) 57% (20%-98%)
N. of del(11q22.3)$^+$ clones (%) % del(11q22.3)$^+$ cells *	0/12 (0%) -	0/11 (0%) -	1/26 (4%) 93% (-)	1/26 (4%) 20% (-)	1/26 (4%) 91% (-)	6/89 (7%) 70% (24%-98%)	2/64 (3%) 92% (91%-93%)	7/126 (6%) 68% (20%-98%)
N. of del(11q23)$^+$ clones (%) % del(11q23)$^+$ cells *	0/12 (0%) -	0/11 (0%) -	0/26 (0%) -	0/26 (0%) -	0/26 (0%) -	3/89 (3%) 32% (21%-64%)	0/64 (0%) -	3/126 (2%) 40% (24%-64%)
N. of del(17p13.1)$^+$ clones (%) % del(17p13.1)$^+$ cells *	0/13 (0%) -	0/12 (0%) -	0/26 (0%) -	0/26 (0%) -	0/26 (0%) -	5/89 (6%) 44% (33%-88%)	0/65 (0%) -	5/127 (4%) 44% (33%-88%)

*Results expressed as number of clones with cytogenetic changes from all clones in the corresponding group (percentage) or as *median values of altered cells/clone (range). In seven clones (1 multiclonal MBL^{lo}, 3 monoclonal and 3 multiclonal CLL clones) biallelic del(13q14.3) was detected, and polysomy was found in one multiclonal CLL clone. Statistically significant differences (shown in bold) were found between multiclonal vs. monoclonal B-cell clone groups for [a]CLL clones (P=0.01) and [b]all (total) clones (P=0.01). CLL: chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL: monoclonal B-cell lymphocytosis.*

ences were found in the molecular characteristics of the immunoglobulin light chain genes, either among CLL-like or non-CLL like B-cell clones from multiclonal *versus* monoclonal cases (Table 3 and *Online Supplementary Table S5*). Regarding *IGKV* and *IGLV* genes, only the *VK1-33* gene was associated with multiclonal cases (6% *versus* 0%; *P*=0.02) (Figure 1D)

Molecular features of phylogenetically related B-cell receptors of B-cell clones from multiclonal cases

Thirty-two of the 85 B-cell clones from individual multiclonal cases were phylogenetically closely related and had exactly the same *IGHV* family (*IGHV3* in 28 B-cell clones and *IGHV4* in 4 B-cell clones) (Figure 2). Of note, this subgroup of B-cell clones frequently showed *IGHV3* gene usage (28/85, 33%) and they displayed shorter HCDR3 sequences than other (multiclonal and monoclonal) B-cell clones: 13 (6-25) *versus* 17(9-26) and 16 (8-32) amino acids (*P*=0.001 and *P*=0.004, respectively). In addition, they also showed a higher frequency of del(13q14.3) compared to B-cell clones from multiclonal cases expressing phylogenetically unrelated *IGHV* families (41% *versus* 17%, respectively; *P*=0.05). Moreover, a slightly higher frequency of multiclonal cases whose coexisting clones were cytogenetically altered was found among phylogenetically closely related clones *versus* phylogenetically unrelated clones from multiclonal cases (53% *versus* 34%, respectively; *P*=0.06). Interestingly, a trend towards an increased percentage of *IGHV*-mutated B-cell clones among phylogenetically related B-cell clones from multiclonal cases compared to other B-cell clones from multiclonal cases was also found (70% *versus* 54%, respectively; *P*=0.1). Interestingly, most of the coexisting phylogenetically related clones had a CLL-like phe-

Table 3. Molecular characteristics of the B-cell receptor (BCR) of chronic lymphocytic leukemia (CLL)-like monoclonal B-cell lymphocytosis (MBL)^{lo}, MBL^{hi} and CLL B-cell clones from monoclonal *versus* multiclonal cases.

	MBL^{lo} clones		MBL^{hi} clones		CLL clones		Total	
	Multiclonal n= 14	Monoclonal n= 13	Multiclonal n= 26	Monoclonal n= 25	Multiclonal n= 26	Monoclonal n= 87	Multiclonal n= 66	Monoclonal n= 126
HCDR3 length* (N. of aa)	15 (6-22)	13 (11-20)	16 (8-23)	17 (9-26)	17 (11-26)	18 (8-32)	17 (6-26)	17 (8-32)
VH families								
VH1	2/14 (14%)	2/13 (15%)	3/26 (12%)	4/25 (16%)	4/26 (15%)	25/87 (29%)	9/66 (14%)	31/125 (25%)
VH3	9/14 (65%)	9/13 (69%)	15/26 (58%)	16/25 (64%)	11/26 (42%)	36/87 (41%)	35/66 (53%)	61/125 (49%)
VH4	2/14 (14%)	2/13 (15%)	6/26 (23%)	3/25 (12%)	9/26 (35%)	23/87 (27%)	17/66 (26%)	28/125 (22%)
VH2, VH5, VH6	1/14 (7%)	0/13 (0%)	2/26 (8%)	2/25 (8%)	2/26 (8%)	3/87 (3%)	5/66 (7%)	5/125 (4%)
DH families								
DH1, DH4, DH7	3/14 (21%)	2/13 (15%)	**1/26 (4%)[a]**	**6/24 (25%)**	6/26 (23%)	11/86 (13%)	10/66 (15%)	19/123 (16%)
DH2	2/14 (14%)	3/13 (23%)	5/26 (19%)	7/24 (29%)	4/26 (15%)	15/86 (17%)	11/66 (17%)	25/123 (20%)
DH3	3/14 (21%)	2/13 (15%)	9/26 (35%)	7/24 (29%)	13/26 (50%)	36/86 (42%)	25/66 (38%)	45/123 (37%)
DH5	2/14 (14%)	3/13 (23%)	6/26 (23%)	2/24 (8%)	3/26 (12%)	9/86 (11%)	11/66 (17%)	14/123 (11%)
DH6	4/14 (29%)	3/13 (23%)	5/26 (19%)	2/24 (8%)	0/26 (0%)	15/86 (17%)	9/66 (13%)	20/123 (16%)
JH genes								
JH1, JH2, JH3, JH5	3/14 (21%)	2/13 (15%)	7/26 (27%)	7/24 (29%)	9/26 (35%)	14/86 (16%)	19/66 (29%)	23/123 (19%)
JH4	6/14 (43%)	7/13 (54%)	13/26 (50%)	8/24 (33%)	10/26 (38%)	34/86 (40%)	29/66 (44%)	49/123 (40%)
JH6	5/14 (36%)	4/13 (31%)	6/26 (23%)	9/24 (38%)	**7/26 (27%)[a]**	**38/86 (44%)**	**18/66 (27%)[a]**	**51/123 (41%)**
LCDR3 length* (N. of aa)	9 (8-13)	10 (8-10)	**10 (8-12)[a]**	**9 (7-12)**	10 (8-15)	9 (5-12)	**10 (8-15)[a]**	**9 (5-12)**
VK families								
VK1	0/6 (0%)	1/4 (25%)	6/14 (43%)	3/12 (25%)	6/12 (50%)	23/48 (48%)	12/32 (38%)	27/64 (42%)
VK2, VK5, VK6	1/6 (17%)	0/4 (0%)	1/14 (7%)	3/12 (25%)	2/12 (17%)	8/48 (17%)	4/32 (13%)	11/64 (17%)
VK3, VK4	5/6 (83%)	3/4 (75%)	7/14 (50%)	6/12 (50%)	4/12 (33%)	17/48 (35%)	16/32 (50%)	26/64 (41%)
JK genes								
JK1, JK3, JK5	3/6 (50%)	1/4 (25%)	5/14 (36%)	7/12 (58%)	5/12 (42%)	21/47 (45%)	13/32 (40%)	29/63 (46%)
JK2	2/6 (33%)	2/4 (50%)	8/14 (57%)	3/12 (25%)	4/12 (33%)	10/47 (21%)	14/32 (44%)	15/63 (24%)
JK4	1/6 (17%)	1/4 (25%)	1/14 (7%)	2/12 (17%)	3/12 (25%)	16/47 (34%)	5/32 (16%)	19/63 (30%)
V families								
V 3	2/3 (67%)	NA	1/7 (14%)	0/7 (0%)	2/7 (29%)	8/25 (32%)	5/17 (29%)	8/32 (25%)
Other	1/3 (33%)	NA	6/7 (86%)	7/7 (100%)	5/7 (71%)	17/25 (68%)	12/17 (71%)	24/32 (75%)
J genes								
Jλ1	1/3 (33%)	NA	0/7 (0%)	0/7 (0%)	4/7 (57%)	6/21 (71%)	5/17 (29%)	6/28 (21%)
Other	2/3 (67%)	NA	7/7 (100%)	7/7 (100%)	3/7 (43%)	15/21 (29%)	12/17 (71%)	22/28 (79%)
IGHV mutational status								
Mutated IGHV	9/14 (64%)	8/11 (73%)	17/26 (65%)	20/25 (80%)	14/26 (54%)	46/86 (54%)	40/66 (61%)	74/122 (61%)
Unmutated IGHV	5/14 (36%)	3/11 (27%)	9/26 (35%)	5/25 (20%)	12/26 (46%)	40/86 (47%)	26/66 (39%)	48/122 (39%)

*Results expressed as number of B-cell clones from all clones in the corresponding group (percentage) or as *median (range). CLL: chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL: monoclonal B-cell lymphocytosis; BCR: B-cell receptor; HCDR3: heavy chain complementarity-determining region 3; LCDR3: light chain complementarity-determining region 3; aa: amino acid. NA: not analyzed; [a]statistically significant differences found between groups of clones from multiclonal vs. monoclonal cases (P≤0.03) are shown in bold.*

notype (10/16 cases, identified in *Online Supplementary Table S3* by the ¶ symbol), while in 4/16 multiclonal cases, one CLL-like B- cell clone coexisted with one non-CLL B-cell clone, (2 marginal zone lymphomas, 1 lymphoma of mucosa-associated lymphoid tissue and 1 hairy cell leukemia clones from cases 29, 32, 37 and 38, also identified in *Online Supplementary Table S3* with the ¶ symbol). In a minority of cases (2/16), the two coexisting phylogenetically related clones were both non-CLL-like, their phenotype being consistent with follicular lymphoma and lymphoma of mucosa-associated lymphoid tissue (case 16¶ and case 34¶, respectively, in *Online Supplementary Table S3)*.

Homology of the HCDR3 region between B-cell clones coexisting in multiclonal cases versus non-coexisting (monoclonal) B-cell clones

The HCDR3 amino acid sequence from coexisting B-cell clones had the same length or differed by just one amino acid in 8/41 multiclonal cases analyzed (19%) (Table 4A). The homology of all these case-paired HCDR3 regions was calculated as the number of identical amino acids or amino acids with an analogous side-chain polarity [(excluding the anchor second-CYS104 (C_) and the J-TRP 118 (_W) amino acid positions that delineate the HCDR3 region)] divided by the corresponding HCDR3 length (Table 4A). It is worth noting that the amino acid composition of HCDR3 sequences of the same length (±1 amino acid) that belonged to the same or evolutionary, highly-related *VH* families (e.g. *VH3-48*, *VH3-21*, *VH3-11*)[33] (n=57) from monoclonal cases (*Online Supplementary Table S6*) showed a tendency towards a lower homology than that of multiclonal cases: median of 37% (range: 11% to 71%) *versus* 50% (range: 26% to 64%), respectively; (*P*=0.1). Since stereotyped sequences are widely represented in CLL,[34] we further analyzed the frequency of stereotyped HCDR3 sequences in multiclonal cases (Table 4B) *versus* monoclonal cases (Table 4C). Interestingly, the number of multiclonal cases showing the same or highly similar stereotyped HCDR3 sequences was significantly higher than that of monoclonal cases: 8/41 (19%) *versus* 11/143 (8%), respectively (*P*=0.001). Furthermore, the amino acid composition of HCDR3 sequences from monoclonal cases with stereotyped HCDR3 sequences showed clearly less identical and/or conserved positions than those found among multiclonal cases (underlined amino acids in Tables 4B and 4C).

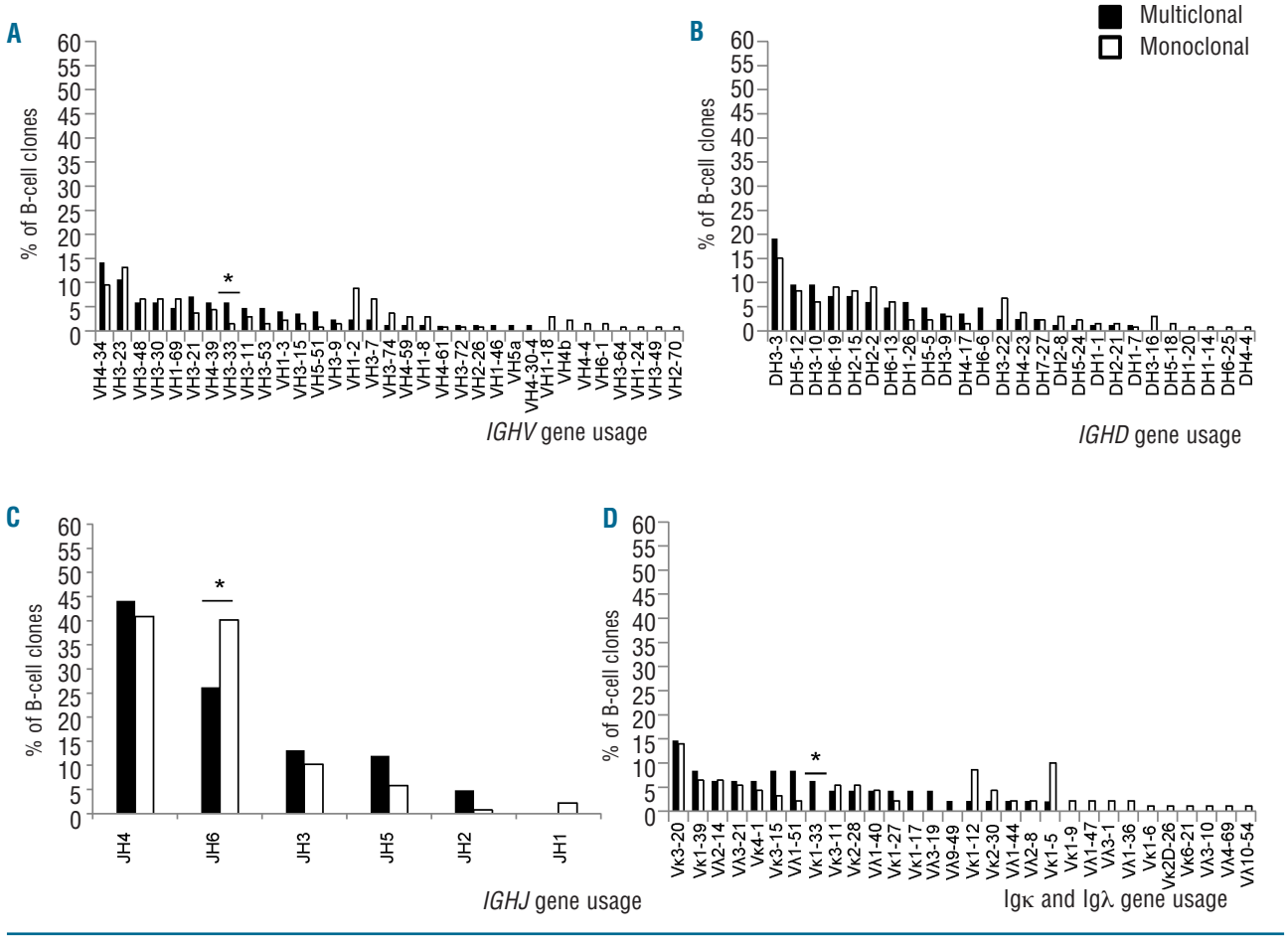

Figure 1. Frequency of *IGHV* (A), *IGHD* (B), *IGHJ* (C) and both *IGKV* and *IGLV* (D) genes in multiclonal *versus* monoclonal CLL and non-CLL-like B-cell clones. Diagrams show the relative frequency of each *IG* gene in multiclonal compared to monoclonal B-cell clones (black and white bars, respectively). *Statistically significant differences were found between the multiclonal *vs.* monoclonal subgroups (*P*<0.05).

Discussion

Multiclonal expansions of phenotypically aberrant B-cell clones (MBL^{lo}) have been reported as frequently present in the general population[35]; of note, multiclonal expansions of immunophenotypically normal B cells can also be found in non-malignant diseases, such as autoimmune disorders and inflammatory responses against several infectious agents (e.g. H*elicobacter pylori*, hepatitis C virus).[36,37] Whether clonal expansions of aberrant B cells found in otherwise healthy individuals (MBL^{lo}) reflect a prominent reactive process against potent antigenic stimuli with unknown clinical relevance, or whether they represent an early (multi)clonal manifestation of a BCR-dependent neoplastic event, still remains to be established. In this regard, it should be noted that between 30% and 40% of such cases show cytogenetic changes shared by clinical MBL and CLL, e.g. del(13q). Of note, among other large structural chromosomal alterations, clonal mosaicism involving del(13q14) has also been recently found in peripheral blood cell populations from otherwise healthy individuals, particularly among subjects with more advanced age (around 2-3% in the elderly), but its potential relationship with MBL and CLL remains unknown.[38,39] Compared to the typical (monoclonal) MBL and B-CLPD, coexisting B-cell clones from multiclonal MBL and B-CLPD may potentially have a greater probability of interacting with common immunological determinants. However, there is still little information about the potential existence of shared BCR features in cases showing two or more coexisting B-cell clones *versus* monoclonal cases.

In the present study, we analyzed for the first time the molecular and cytogenetic features of a large group (n=85) of coexisting, but unrelated, B-cell clones from a series of 41 multiclonal MBL and B-CLPD cases, in comparison to 143 monoclonal cases. Overall, the former clones more

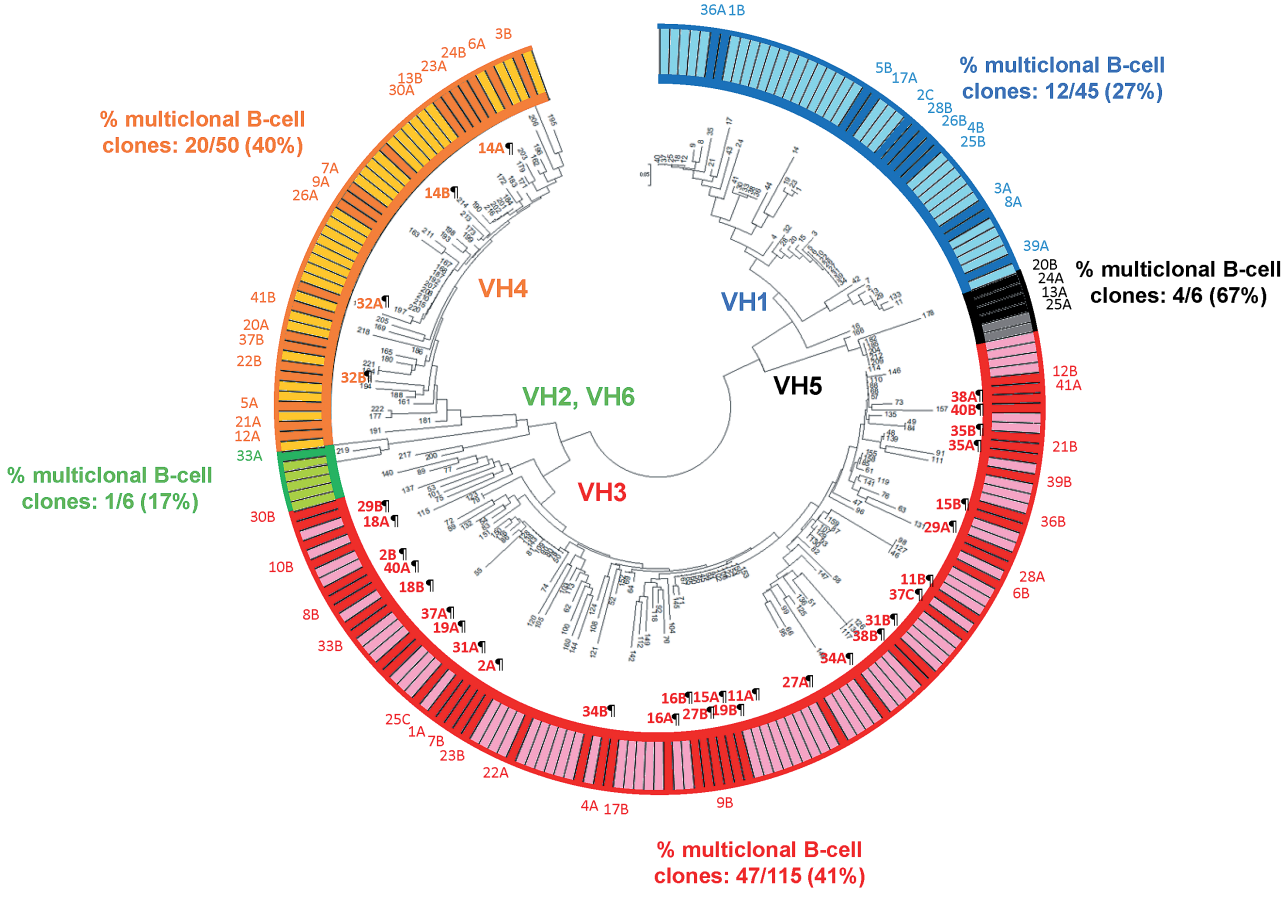

Figure 2. Sequence distance cladogram of *IGHV* gene usage in CLL-like and non-CLL-like B-cell clones from multiclonal (dark colored bars in the outside circle) and monoclonal (light colored bars in the outside circle) cases. Five major branches were found in the sequence distance cladogram (i.e VH1, VH5, VH3, VH2-VH6, VH4). B-cell clones from individual multiclonal cases are represented by numbers; from them, those phylogenetically closely related B-cell clones, which share the same *IGHV* family, are specifically identified by bold numbers in the inner part of the circle and the symbol ¶. Of note, B-cell clones from multiclonal cases 14¶, 16¶ and 35¶ belong to closely located sub-branches of the cladogram, with their *IGHV* sequences having amino acid identity of 79%, 76% and 69%, respectively. In turn, B-cell clones from the multiclonal case 32¶ belong to the VH4 major branch with *IGHV* sequences whose amino acid identity is 69%. Finally, the other B-cell clones from multiclonal cases – cases 2¶, 11¶, 15¶, 18¶, 19¶, 27¶, 29¶, 31¶, 34¶, 37¶, 38¶ and 40¶– belong to the VH3 major branch, having *IGHV* sequences with amino acid identity > 60% (68%, 73%, 73.4%, 61%, 79%, 70%, 63%, 77%, 69.9%, 70%, 72% and 68.4%, respectively).

frequently showed cytogenetic and hematologic features which are typical of the earliest MBL stages and/or initial phases of CLL.[18,36,37] Accordingly, B-cell clones from multiclonal cases more frequently corresponded to MBL cases, whereas B-cell clones from monoclonal cases were more frequently found to correspond to overt CLL. Of note, these findings do not contradict the apparent discrepancy between such associations and our previous observation

Table 4. Multiclonal cases with coexisting B-cell clones sharing HCDR3 features (A). Multiclonal (B) and monoclonal (C) cases with B-cell clones showing stereotyped HCDR3 amino acid sequences.

A. Multiclonal cases with coexisting B-cell clones showing HCDR3 regions of identical length or differing by one amino acid, and analogous composition of amino acids in some parallel positions.

Multiclonal case ID	VH families	AA composition of HCDR3 (length)	% homology*
8A	V1-3	C_*A*RDRVV*I*PDTTT*I*NW*FD*P_W (19)	26
8B	V3-53	C_*A*THPTN*Y*TRWPY*V*SD*MD*V_W (19)	
11A	V3-23	C_ANRGETR*GMDV*_W (11)	54
11B	V3-48	C_*V*RDGFHYY*GFDI*_W (11)	
14A	V4-34	C_*AR*GP*D*R*L*Y*S*G*S*YTRF*D*Y_W (17)	47
14B	V4-34	C_*AR*RE*D*DN*F*W*S*G*F*Y*M*D*V*_W (16)	
22A	V3-74	C_*AR*DL*D*G*S*G*S*G*V*F*DW*_W (14)	64
22B	V4-59	C_*AR*G*W*RS*T*D*S*YY*GMDV*_W (15)	
29A	V3-48	C_*V*RELWF*G*NG*G*D*Y*_W (12)	42
29B	V3-15	C_*A*TA*G*Q*G*SA*D*FL*Y*_W (12)	
31A	V3-33	C_*AR*GE*L*L*H*NW*F*DP_W (12)	58
31B	V3-23	C_*AK*D*GF*PYYG*F*DI_W (12)	
32A	V4-39	C_*AR*QT*GW*LAPS*DY*_W (12)	54
32B	V4-34	C_*AR*RDS*SGW*YYF*DY*_W (13)	
33A	V2-26	C_*A*GTNIPRQFD*F*W*S*G*S*S*P*N*W*F*D*P_W (22)	32
33B	V3-53	C_*A*RAGGYCNSGS*C*R*G*A*P*R*W*Y*F*D*L_W (22)	

*Amino acids with analogous side-chain polarity (highlighted in italics): case 8 (I,V and F,M), case 11 (A,V and M,F), case 14 (L,F), case 22 (L,W; S,T and F,M), case 29 (A,V), case 31 (L,F and H,Y), case 32 (T,S), case 33 (W,C) (EMBL-EBI Cambridge, UK). All cases had coexisting B-cell clones with CLL-like phenotype, except case 29 (one B-cell clone corresponded to a MALT lymphoma-like phenotype), and cases 32 and 33 (both had one B-cell clone with a MZL-like phenotype). AA: amino acid; MALT: lymphoma of mucosa-associated lymphoid tissue; MZL: marginal zone lymphoma. *Number of amino acids with analogous side-chain polarity (excluding the delineating C_ and _W positions)/HCDR3 length*100.*

B. Multiclonal cases with B-cell clones showing stereotyped HCDR3 sequences.

Multiclonal cases ID	V(D)J rearrangement	AA composition of HCDR3
1	V3-30(D3-9)J6	C_AKYGGVKLRYFDWLLYGDYYYGMDV_W
2	V3-30(D3-9)J6	C_AKYGGVKLRYFDWLLYGDYYYGMDV_W
9	V3-23(D5-12)J6	C_ANRGETRGMDV_W
15	V3-23(D3-22)J6	C_ANRGESWGMDV_W
21	V3-21(D2-2)J6	C_ARDANGMDV_W
35	V3-21(D2-2)J6	C_ARDANGMDV_W
22	V3-74(D3-10)J4	C_ARDLDGSGSGVFDW_W
40	V3-21(D4-23)J4	C_ARDLDGGNSVFDC_W

Cases 1 and 2; 9 and 15; 21 and 35; 22 and 40 showed highly similar HCDR3 sequences; the underlined amino acids were different. All the listed B-cell clones had a CLL-like phenotype.

C. Monoclonal cases with B-cell clones showing stereotyped HCDR3 sequences.

Monoclonal Cases ID	V(D)J rearrangement	AA composition of HCDR3
117	V3-7(D3-3)J4	C_VRENELWSGGWGLDG_W
134	V3-7(D3-3)J4	C_VRENEFWSGGWGLDG_W
207	V4-39(D2-2)J6	C_ARHRLGYCSSTSCYYYYYGMDV_W
208	V4-39(D2-2)J6	C_ARHRLGYCSSTSCYYYYYGMDV_W
210	V4-39(D2-2)J6	C_ARDRLGYCSSTSCYYYYYGMDV_W
187	V4-b(-)J4	C_ARSWIQLWSEFDY_W
215	V4-b(D5-5)J4	C_ARAWIQLWSDFDY_W
180	V1-2/D6-19/J4	C_ARLQWLGISHFDY_W
204	V1-2/D6-19/J4	C_ARAQWLVLENFDY_W
196	V4-34/D3-16/J6	C_VRGYPSDYTERRYYYYGLDV_W
198	V4-34/D4-23/J6	C_ARGYGSTGETRRYYYYGMDV_W

Cases 117 and 134; 207, 208 and 210; 187 and 215; 180 and 204; 196 and 198 showed highly similar HCDR3 regions; the underlined amino acids (AA) were different. All the listed B-cell clones had a CLL-like phenotype.

among CLL patients of a worse clinical outcome for multiclonal cases carrying non-CLL clones,[11] as this latter study was restricted to cases of overt CLL. In addition, multiclonal cases were also associated with lower clonal B-cell counts in peripheral blood, a lower number of cytogenetically altered clones, particularly of those carrying del(13q), and a decreased frequency of clones with two or more alterations. Of note, clonal expansions of non-CLL like B-cell clones were also more frequently observed in multiclonal than in monoclonal cases, such expansions corresponding mainly to indolent lymphomas (e.g. marginal zone lymphoma) which have been associated with chronic immune responses.[40,41]

Collectively, these results support the notion that the presence of multiple B-cell clones in the same individual more closely reflects the earlier stages of the disease. If this holds true and chronic antigen stimulation is involved in the onset of MBL and B-CLPD, as has recently been suggested for MBL based on epidemiological studies,[42] it could be hypothesized that B-cell clones coexisting in multiclonal cases would show more closely related BCR features than B-cell clones from monoclonal cases. In this regard, our results point out the existence of a slightly higher level of HCDR3 homology among B-cell clones from multiclonal *versus* monoclonal cases. In fact, in around one fifth of all multiclonal cases, the coexisting B-cell clones showed a high homology in their HCDR3 amino acid sequences; this also held true when we compared the homology of the HCDR3 sequences of these multiclonal cases against those of monoclonal cases whose B-cell receptors were restricted to the same and/or ontogenetically related *IGHV* families. In addition, the frequency of stereotyped HCDR3 was also higher in multiclonal *versus* monoclonal cases. Such more closely related BCR features would be found independently of whether common antigens or superantigens are specifically involved, although the former would potentially lead to a higher HCDR3 homology, whereas superantigens could contribute to a greater frequency of usage of specific *IGHV*, *IGHD* and/or *IGHJ* genes.[13,43]

In the present study, we found a similar frequency of *IGHV* gene usage between coexisting multiclonal and non-coexisting monoclonal B-cell clones in association with a lower frequency of *DH1*, *DH4* and *DH7* as well as *JH6* families in multiclonal *versus* monoclonal B-cell clones. Overall, these results suggest that no single antigen or superantigen is involved in common in MBL and B-CLPD. This is further supported by the relatively low percentage of alignment (≈50%) of the *IGHV* amino acid sequences observed among the different clonal B-cell populations analyzed, since such potential antigens - including superantigens - would require interaction with highly conserved sites at the *IGHV/HCDR3* regions of the BCR.[44] Interestingly however, the higher representation of *DH1*, *DH4*, *DH7* and *JH6 IGH* gene segments in monoclonal *versus* multiclonal B-cell clones, together with the slightly higher levels of HCDR3 homology observed among coexisting (multiclonal) *versus* non-coexisting (monoclonal) B-cell clones from MBL, CLL and other B-CLPD cases, would indicate that still non-random selection of specific HCDR3, *DH* and *JH* segments could exist in the MBL and CLL repertoire of both multiclonal and monoclonal cases, which could reflect antigen-driven selection and expansion of specific B-cell clones, at the MBL and/or CLL stages.[45]

In this regard, based on the phylogenetic proximity of their BCR, we could further identify, within the B-cell clones from multiclonal cases, a considerably represented subgroup of B-cell clones showing preferential usage of *IGHV3* genes and shorter HCDR3 sequences carrying a significantly higher number of *IGHV* mutations *versus* the unrelated clones. These results add support to the involvement of a common antigen, at least in this specific subset of cases.[46] Interestingly, these "phylogenetically-related" B-cell clones coexisting in multiclonal cases showed a significantly higher frequency of del(13q) than B-cell clones expressing other *IGHV* genes. These observations further suggest that the BCR features of this subset of coexisting multiclonal B-cell clones could also contribute to determining the probability and/or type of cytogenetic progression occurring at the earliest stages of the disease, as previously suggested by our group[18] and others.[47,48] Further long-term, longitudinal studies are required to confirm this hypothesis, since multiple productive *IGHV* gene rearrangements may also underlie clonal drift leading to selection for more aggressive clones whose proportions would change over time.[49]

In summary, based on the molecular features of the BCR and the cytogenetic profile of B-cell clones from the multiclonal *versus* monoclonal cases of MBL, CLL and other B-CLPD analyzed here, it may be concluded that multiclonality is typically associated with early stages of B-CLPD; at the same time it appears to reflect more closely an antigen-driven nature of MBL and B-CLPD, with potential involvement of multiple and diverse antigenic determinants.

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