Multiple productive immunoglobulin heavy chain gene rearrangements in chronic lymphocytic leukemia are mostly derived from independent clones

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ABSTRACT

In chronic lymphocytic leukemia, usually a monoclonal disease, multiple productive immunoglobulin heavy chain gene rearrangements are identified sporadically. Prognostication of such cases based on immunoglobulin heavy variable gene mutational status can be problematic, especially if the different rearrangements have discordant mutational status. To gain insight into the possible biological mechanisms underlying the origin of the multiple rearrangements, we performed a comprehensive immunogenetic and immunophenotypic characterization of 31 cases with the multiple rearrangements identified in a cohort of 1147 patients with chronic lymphocytic leukemia. For the majority of cases (25/31), we provide evidence of the co-existence of at least two B lymphocyte clones with a chronic lymphocytic leukemia phenotype. We also identified clonal drifts in serial samples, likely driven by selection forces. More specifically, higher immunoglobulin variable gene identity to germline and longer complementarity determining region 3 were preferred in persistent or newly appearing clones, a phenomenon more pronounced in patients with stereotyped B-cell receptors. Finally, we report that other factors, such as *TP53* gene defects and therapy administration, influence clonal selection. Our findings are relevant to clonal evolution in the context of antigen stimulation and transition of monoclonal B-cell lymphocytosis to chronic lymphocytic leukemia.

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the expression of restricted sets of B-cell receptors (BcR), often with highly similar, stereotyped antigen-binding sites,[1,2] strongly indicating the role of antigen in CLL development.[3-5] Further evidence in support of this notion has been amply provided by the fact that the mutational status of the clonotypic immunoglobulin heavy variable gene (*IGHV*) stratifies CLL patients into two groups with markedly different prognoses.[6,7] This implies that signals conveyed through BcR with distinct molecular structure likely affect the biological behavior of the CLL clone,[8,9] thus contributing to the eventual clinical outcome.

A subgroup of CLL cases carry multiple (mostly double) productive IGH rearrangements (MP-IGH), and pose an exception to the rule 'single clone – single rearrangement'. Such cases have been reported repeatedly and independently by several groups, and they are estimated to account for approximately 2% of all cases of CLL.[10] The true biological and clinical implications are currently unknown, especially when one of the rearrangements is mutated while the other unmutated.

Several mechanisms have been previously linked to the phenomenon of double productive IGH rearrangements. These mechanisms involve two main themes: lack of allelic exclusion at the *IG* loci[11] or the presence of two clonal populations.[12-16]

Allelic exclusion regulates the expression of *IG* genes in any given B lymphocyte so that it may express a single heavy chain and a single light chain. This mechanism is critical for the process of clonal selection and the generation of high-affinity, antigen-specific antibodies. In a minority of B cells, the mechanism can be disrupted, leading to lack of allelic exclusion which is characterized by the production of two functional IGH or IGK/L molecules in a single B lymphocyte.[17]

In CLL, lack of allelic exclusion on heavy chains leading to the presence of double productive IGH rearrangements was first reported in 1997.[11] At roughly the same time, *IGHV* gene replacement was suggested as another molecular mechanism that could lead to the presence of double productive IGH rearrangements in CLL.[18] Whatever the precise molecular mechanism(s) underlying double IGH rearrangements in a single cell/clone, these have been interpreted as evidence for the possible operation of receptor editing in CLL. This is similar to what has been reported for normal B cells, where secondary rearrangements occurring after the expression of a potentially harmful BcR can offer the cell the opportunity to evade apoptotic death.[19]

Double productive IGH rearrangements can also indicate the presence of two clonal populations, each expressing distinct BcR. Several cases with coexistence of two CLL clones have been reported,[12-16] challenging the prevailing notion of CLL being a monoclonal disease. On the other hand, detection of double productive *IGH* gene rearrangements could

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also represent co-existence of CLL with another B lymphoproliferative disorder.[12]

Clonal drift is a phenomenon in lymphoid malignancies with multiple productive antigen receptor gene rearrangements, in particular T-cell large granular lymphocyte leukemia, referring to a dynamic process of alterations in the proportion of the malignant clones.[20] Clonal drift has never been examined in CLL, though potentially relevant given evidence that the proliferation and overall biological behavior of CLL cells may differ between clones with mutated or unmutated *IGHV*.[21,22]

Previous studies analyzing CLL cases with MP-IGH rearrangements lack a detailed genomic analysis of IG light chains and partial IGHD-IGHJ rearrangements that can be extremely informative about the molecular status of the *IG* loci, thus contributing to clarification of the mechanisms involved. In the present study, we performed a comprehensive analysis of IG heavy and light chain gene rearrangements in MP-IGH CLL patients with the aim of obtaining molecular insight into the biological causes of this phenomenon. Also, for the first time, we attempted a systematic study of clonal drift in MP-IGH CLL by tracing each rearrangement at different time-points in the natural history of the disease.

Methods

Study group

MP-IGH cases were sought among 1147 CLL patients tested for *IGHV* gene mutational status at the University Hospital Brno, Czech Republic, from 2003 to 2011. All patients included in this study fulfilled the International Workshop on CLL/National Cancer Institute diagnostic criteria for CLL.[23] Blood samples were taken after written informed consent in accordance with the Declaration of Helsinki under protocols approved by the Ethical Committee of the University Hospital Brno.

Sample processing, nucleic acid isolation and complementary DNA synthesis

B lymphocytes were routinely separated from peripheral blood using RosetteSep kits (StemCell). The purity of enriched B cells and expression of surface markers were evaluated by flow cytometry; all separated samples contained >98% CLL cells. Genomic DNA (gDNA) was isolated using the DNeasy Blood & Tissue Kit (Qiagen). For RNA isolation, either TriReagent (MRC, Inc.) or the RNA mini Kit (Qiagen) was used. Complementary DNA (cDNA) was synthesized using SuperScript II Reverse Transcriptase (Invitrogen) from 500 ng of total cellular RNA.

Polymerase chain reaction amplification of IG gene rearrangements

IGHV-IGHD-IGHJ rearrangements were amplified by reverse transcription polymerase chain reaction (PCR) using primers specific for the leader region of *IGHV* genes along with a consensus primer for the *IGHJ* genes. In cases with multiple amplicons, the PCR for IGHV-IGHD-IGHJ rearrangements was repeated on gDNA with a combination of primers for the framework region 1 (FR1) and a consensus IGHJ primer.[24]

PCR amplification of partial IGHD-IGHJ rearrangements was performed on gDNA utilizing seven subgroup-specific IGHD primers in combination with a consensus IGHJ primer.[24] IGKV-IGKJ and IGLV-IGLJ rearrangements were amplified with primers specific for the FR1 and the *IGKJ* or *IGLJ* genes,[25,26] and/or following the BIOMED-2 protocol,[24] both on cDNA and gDNA.

IG sequence analysis and interpretation

PCR amplicons were subjected to direct sequencing on both strands. If multiple IGHV-IGHD-IGHJ rearrangements were amplified from the same *IGHV*-specific primer, subcloning was performed following recommended strategies.[10] The sequences obtained were analyzed using IMGT® and the IMGT/V-QUEST tool (*http://www.imgt.org*).[27] For partial IGHD-IGHJ rearrangements, sequence analysis was performed by a multistep procedure using BLAST (*http://blast.ncbi.nlm.nih.gov/*), ExPASy (*http://au.expasy.org/*), and IMGT® tools.

Molecular monitoring of B-cell clones over time

For long-term molecular monitoring of clonal dynamics, allele-specific oligonucleotide assays for quantitative real-time PCR (ASO-qPCR) were designed. gDNA was used for quantification of proportions of IG rearrangements. If gDNA was not available in serial samples or the ASO-qPCR assay design was not successful, clonal dynamics was assessed semi-quantitatively based on fragment analysis from cDNA using consensus FR1 and IGHJ primers. In such cases, clone size was estimated according to the size of the area under the curve.

Further details of various aspects of the methods are available in the *Online Supplementary Methods*.

Results

Multiple productive IGHV-IGHD-IGHJ gene rearrangements in chronic lymphocytic leukemia: incidence and overview of IGH gene repertoires

Within a cohort of 1147 CLL patients analyzed for *IGHV* mutational status in this study, 548 (46.3%) sequences carried mutated and 635 (53.7%) unmutated *IGHV*, following the 98% identity cut-off value. The skewing to unmutated cases results from the fact that our Department is a tertiary center to which cases with a less favorable clinical course are referred.

Multiple productive IGHV-IGHD-IGHJ rearrangements (MP-IGH) were identified in 31/1147 cases (2.7%). The patients' characteristics are presented in *Online Supplementary Table S1* and *Online Supplementary Figure S1*. Two or three transcribed productive IGHV-IGHD-IGHJ rearrangements were found in 26 (84%) and 5 (16%) cases, respectively, resulting in a total of 67 sequences. Of these, 29/67 (43%) carried mutated *IGHV*, while the remainder (38/67, 57%) carried unmutated *IGHV*; 27/38 unmutated rearrangements had *IGHV* with 100% identity to germline ('truly unmutated'[3]). Hence, the distribution of the sequences obtained in MP-IGH cases with regards to *IGHV* mutational status was similar to that of the entire cohort.

The *IGHV*, *IGHD*, and *IGHJ* gene repertoires in MP-IGH cases did not differ significantly from those of cases with single productive rearrangements (*Online Supplementary Table S2*). Notably, 15/31 (48%) of MP-IGH cases harbored at least one rearrangement with a stereotyped VH CDR3 region; three of 15 such cases carried two IGHV-IGHD-IGHJ rearrangements assigned to different subsets. From the perspective of individual sequences, 18/67 (26%) of rearrangements from MP-IGH cases were stereotyped.

We compared the *IG* gene repertoire, somatic hypermutation status and CDR3 features between co-existing

rearrangements within each case. Altogether, 41 pairs of rearrangements were analyzed; in the five cases with three rearrangements, all three possible pairs were included in the analysis. The main finding concerning *IGHV* usage was that the predominant pairings were IGHV3+IGHV4 and IGHV1+IGHV3 (8/41 pairs each) whereas the frequency of IGHV3+IGHV3 was low (2/41 pairs). With regards to somatic hypermutation, concordant *IGHV* mutational status was seen in 21/31 cases (67.7%), of which nine (29%) carried only mutated *IGHV* genes while the remaining 12 (38.7%) carried only unmutated *IGHV* genes. Ten of 31 cases (32.3%) were discordant for somatic hypermutation since they carried rearrangements of different mutational status (according to the 98% cut-off value). Detailed results of this assessment are listed in *Online Supplementary Table S3*.

Multiple IGHV-IGHD-IGHJ *gene rearrangements in chronic lymphocytic leukemia: immunophenotypic and molecular hints regarding their origin*

We questioned whether MP-IGH could co-exist in a single clone, alluding to a lack of allelic exclusion, or whether

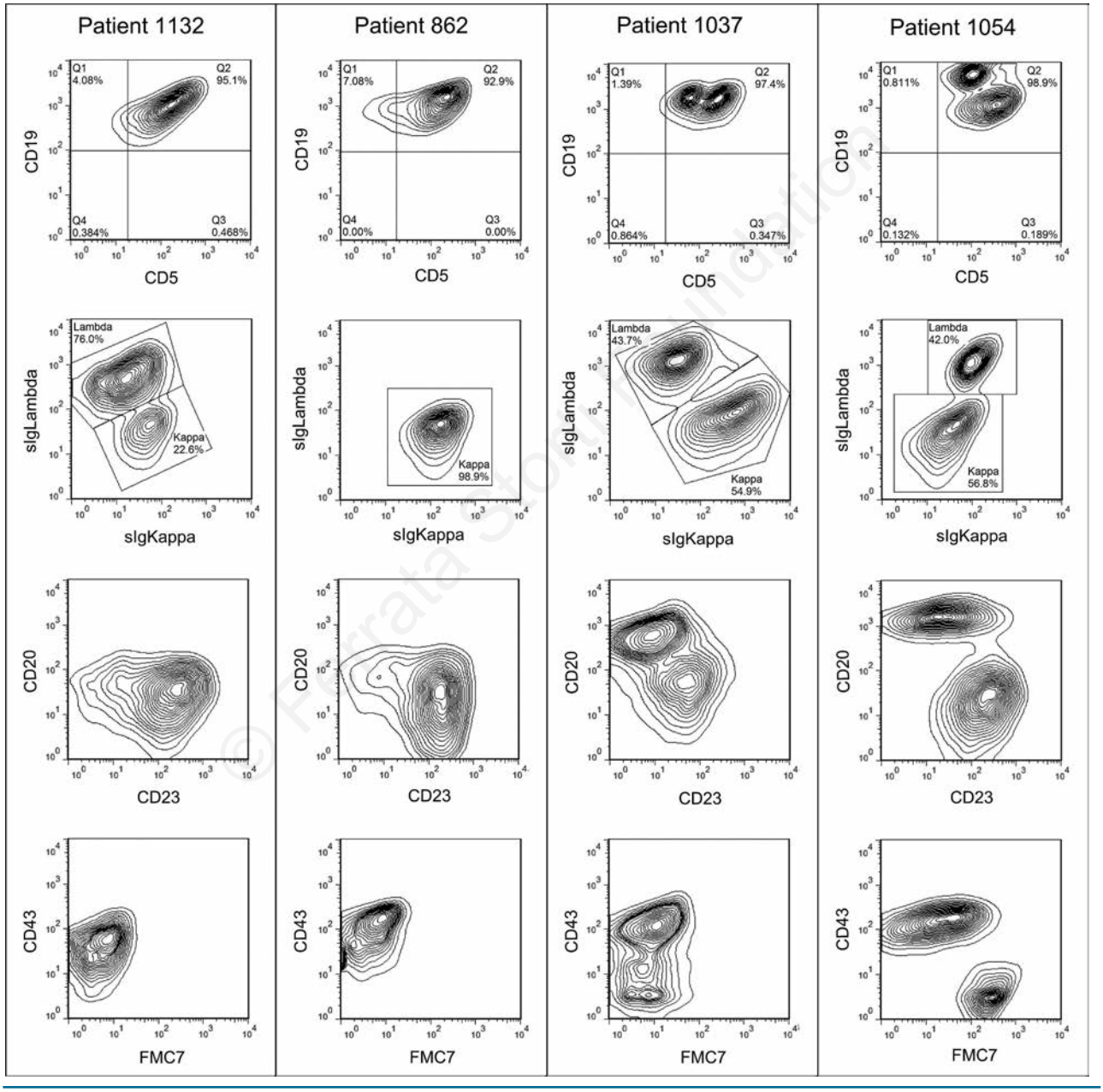


Figure 1. Immunophenotypig of MP-IGH CLL cases. In *Patient 1132*, as an example of typical CLL immunophenotype, both malignant clones differed only in light chain isotype expression. *Patient 862* displayed the typical immunophenotype of monoclonal CLL with IGκ light chain expression. Nevertheless, changes in proportion of the two identified IGH rearrangements were observed (see Figure 2). In *Patient 1037*, the malignant populations displayed distinct immunophenotypic features implying the co-existence of CLL/SLL with atypical CLL or other lymphoproliferative disorder. Mantle cell lymphoma was excluded in this patient because of cyclin D1 negativity, and a diagnosis of SLL was established (bulky disease, absolute B lymphocyte count under 5,000/μL). In *Patient 1054*, two clonal populations were identified; the possibility of another indolent lymphoproliferative disease co-existing with CLL could not be excluded (CLL Matutes score for the atypical clone: 2 – 3).

they derived from multiple co-existing clonal B-cell populations. First, we performed detailed flow cytometry analysis in 22 cases (Figure 1). Twenty of the 22 cases had a homogeneous phenotypic profile suggestive of CLL. Interestingly, 7/20 cases had clear evidence of two co-existing CLL clones with different light chain restriction. In the remaining 2/22 cases, we could document the presence of a CLL population co-existing with another clonal B-cell population with a distinct immunophenotype (cases #1037 and 1054; Figure 1).

We then performed immunogenetic gDNA-based analysis in 26 MP-IGH cases. The reasoning behind this approach is that since a single cell and, by inference, a single clone carries only two IGH alleles, then the expected maximum number of IGH rearrangements per cell/clone is only two. Hence, the detection of partial IGHD-IGHJ (P-DJ) or non-transcribed/unproductive IGHV-IGHD-IGHJ rearrangements in MP-IGH CLL cases might constitute convincing molecular evidence in favor of the existence of multiple clonal B-cell populations, even when displaying a uniform immunophenotype. To exclude coincidental amplification or amplification of germline IGHD7-IGHJ1 region, all PCR products were sequenced and particular *IGHD* and *IGHJ* genes were assigned.

Overall, 20/26 cases were positive for P-DJ, indicating the existence of multiple clones. In 6/26 cases (four of them positive for P-DJ), this presumption was further supported by detection of an additional IGHV-IGHD-IGHJ rearrangement that was either non-transcribed productive (1 case), or unproductive due to an out-of-frame junction (5 cases).

We subsequently extended the immunogenetic analysis to the IG light chains for all 31 MP-IGH cases. Overall, 74 IGKV-IGKJ and IGLV-IGLJ clonal rearrangements were amplified. Twenty-one cases (68%) carried multiple light chain rearrangements; at least two rearrangements were productive in 18/21 cases (86%); multiple productive and transcribed rearrangements were detected in 16/18 cases (52% of all 31 patients).

Additionally, in 26 cases we analyzed rearrangements of the *IGK* loci involving the kappa-deleting element (KDE). Among kappa-expressing cases (14/26), ten had PCR evidence of KDE rearrangements. In the only lambda-expressing case, both IGKV-KDE and IGKJ-C-INTRON-KDE rearrangements were detected. All cases expressing both kappa and lambda light chains (11/26) were positive for KDE rearrangements. In total, all 26 analyzed MP-IGH cases had at least one rearranged IGK allele.

When combining immunophenotypic and molecular results, we could categorize the 31 MP-IGH cases into three groups (Table 1, *Online Supplementary Table S4*, and *Online Supplementary Figure S2)*. Group I, defined by definite co-existence of two clonal B-cell populations, comprised nine cases (29% of MP-IGH cases; 0.79% of the whole CLL cohort), of which seven concerned co-existing CLL populations with different light chain restriction, while the remaining two concerned coexisting CLL + other B-cell clone (cases #1037 and #1054). Group II, defined by highly likely co-existence of at least two clonal populations with CLL-like phenotype, consisted of 16 cases (52% of MP-IGH cases; 1.40% of the whole CLL cohort). Group III, consisting of cases indeterminate as to one or more CLL-like populations, was formed of six cases (19% of MP-IGH cases; 0.52% of the whole CLL cohort) in which we failed to obtain conclusive evidence of more than one clone.

Tumor dynamics: molecular monitoring over time suggesting clonal drift

In 22/31 MP-IGH patients (71%), reverse transcription PCR analysis of IGHV-IGHD-IGHJ rearrangements was performed repeatedly at several time points during the course of the disease (median number of tests, 2.5; range, 2-6). The median interval from the first to the last analysis was 24 months (range, 8-74 months). Additionally, in 11

Table 1. Immunogenetic and immunophenotypic data and resulting category referring to the possibility of co-existence of multiple clones in the respective patient.

Patient	Productive IGHV-IGHD-IGHJ	Partial IGHD-IGHJ	Unproductive IGHV-IGHD-IGHJ	Productive IGKV-IGKJ	Unproductive IGKV-IGKJ	IGKV-KDE	IGKJ-C-INTRON-KDE	Productive IGLV-IGLJ	Unproductive IGLV-IGLJ	Clones by flow-cytometry	Group
261	2			1		1	1	1		2	I
279	2			1			1	1		2	I
319	3	3		3	1	1	1	1	2	2	I
948	2	2		1	1	2		1		2	I
1030	2	1	1	1	1	1		1		2	I
1037	3	1	1	1	2	1	1	1		2	I
1054	2	1		1		1		1		2	I
1072	2	1		1	1		1	1		2	I
1132	2	1		2	2		1	1		2	I
307	2	1		1	1					1	II
511	3		1	1	1				1	NA	II
523	2	1		1		1				1	II
604	3	1			2		1	2	1	1	II
625	3#	1		1	1	1		1		1	II
814	2		1	3						NA	II
846	2	1		1		1				1	II
862	2			3		2				1	II
885	2	1		1		2	1			1	II
892	2	1		1			1			1	II
912	2	1		1						NA	II
923	2	1		1		2				1	II
974	2	1	1	3		2		1		1	II
1029	2	1		2		1				1	II
1049	3	1		2			1			1	II
1087	2	1		1			1			1	II
447	2			2						NA	III
833	2			2						NA	III
845	2			1						NA	III
877	2			1						NA	III
922	2			1						NA	III
1137	2				1			1		NA	III

(I) definite co-existence of two clonal B-cell populations; (II) highly likely co-existence of at least two clonal populations with CLL-like phenotype; (III) indeterminate as to one or more CLL-like populations. NA – not available; #one of the IGH rearrangements non-transcribed.

of the 22 cases with available consecutive gDNA samples, ASO-qPCR assays were designed for all IGHV-IGHD-IGHJ rearrangements to verify the results of PCR analysis and to quantify changes in the relative proportions of the clonal populations over time (Figure 2; *Online Supplementary Table S5*). The observed changes were evaluated through categorizing the detected clonal IGHV-IGHD-IGHJ rearrangements as: (i) diminishing (tendency to decrease in successive samples, detectable → undetectable state, >10% proportion change using ASO-

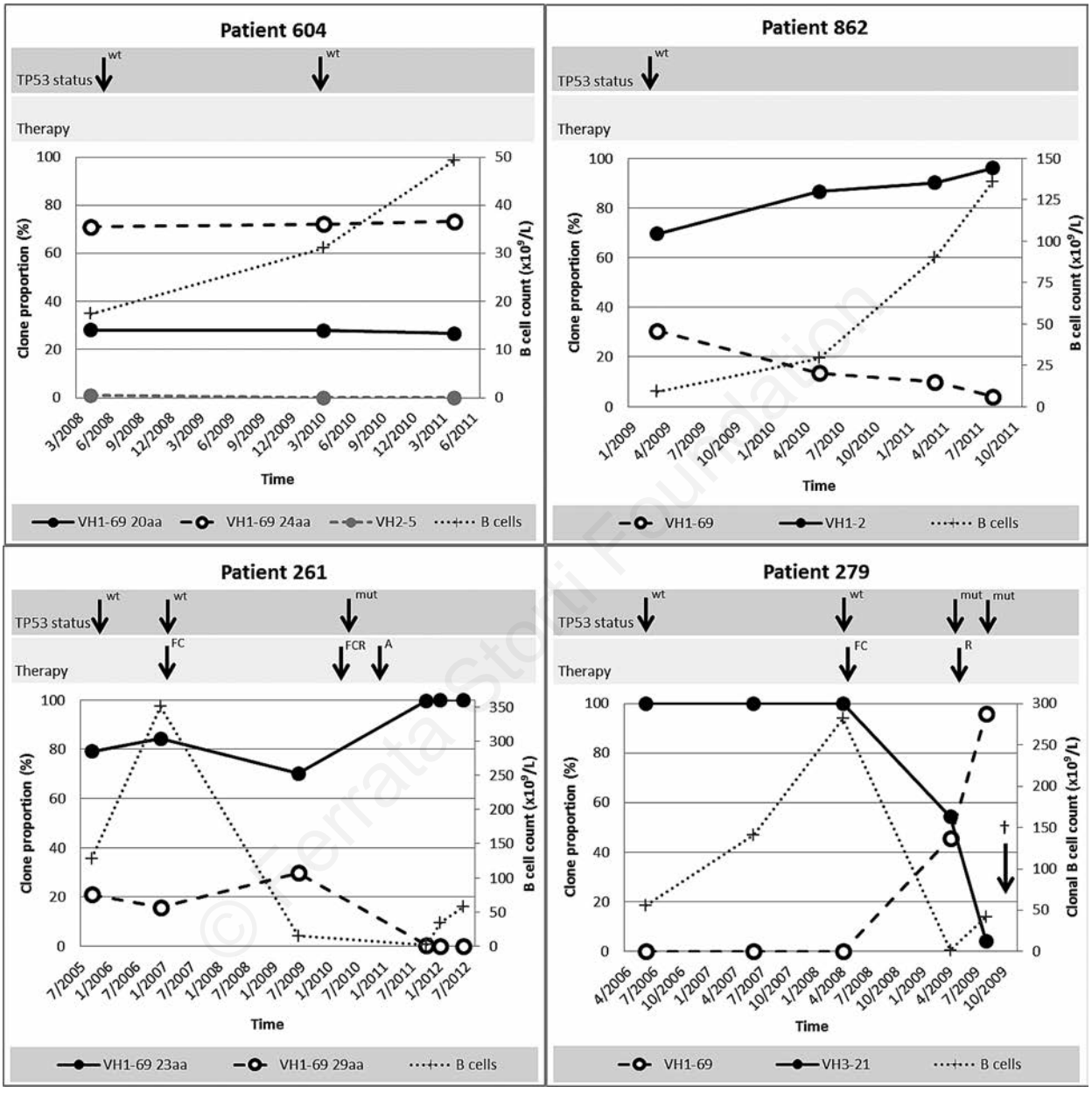


Figure 2. Examples of evolution of biclonal/triclonal CLL. Changes in the relative proportions of the malignant clones assessed using ASO-qPCR assays and flow cytometry were usually accompanied by alterations in absolute count of clonal B lymphocytes (the additional axis "*B cell count*") and accelerated after therapy and/or by co-selection of unfavorable genomic aberrations, such as a *TP53* defect. Time axes are scaled in *month/year*. *Patient 604*: at diagnosis, three rearrangements – two with unmutated *IGHV1-69* (subset 5 with 20 amino acids in VH CDR3; non-stereotyped with VH CDR3 of 24 amino acids) and one with mutated *IGHV2-5* gene (in gray) – were assessed. The *IGHV2-5* gene rearrangement was minor and diminished during the disease course. Both *IGHV1-69* gene rearrangements have persisted over time. The patient did not receive any therapy during the follow-up. *Patient 862*: at diagnosis, two *IGHV-IGHD-IGHJ* gene rearrangements utilizing unmutated *IGHV1-2* and *IGHV1-69* genes were detected. The *IGHV1-69* gene rearrangement was diminishing during the disease course. The patient did not receive any therapy during the follow-up. *Patient 261*: originally biclonal disease, both clones harbored unmutated *IGHV1-69* gene rearrangements (subset 7 with VH CDR3 of 23 amino acids; non-stereotyped with 29 amino acids in VH CDR3). The proportions of the clones changed, especially with therapy administration. After second-line therapy, one clone was diminishing and, at the same time, *de novo TP53* mutation/deletion was detected in the second persisting clone with stereotyped BCR. *Patient 279*: originally monoclonal CLL (unmutated *IGHV3-21*, subset 2); the second CLL clone harboring unmutated *IGHV1-69* (subset 3) and a complex karyotype (including *TP53* defect) appeared in relapse of the disease after the first therapy. Selection of the new malignant clone led to fulminant progression of the disease, and the patient died of infectious complications (†). mut: mutated *TP53* gene; wt: wild-type *TP53* gene, F: fludarabine, C: cyclophosphamide, R: rituximab, A: alemtuzumab.

qPCR), (ii) persistent (present constantly, stable or expanding compared to other persistent or diminishing rearrangement, respectively), or (iii) appearing (originally undetectable, expanding tendency).

Altogether, a clonal drift represented by changes in the proportions of the rearrangements was detected in 19/22 MP-IGH cases (86%). An IGHV-IGHD-IGHJ rearrangement, absent at initial testing, appeared in 3/19 patients besides the original rearrangement(s), whereas in 18/19 patients one of initially multiple detected IGHV-IGHD-IGHJ rearrangements diminished during the disease course (*Online Supplementary Table S1*). Importantly, among the latter cases, diminishing of the IGHV-mutated clone with concurrent persistence of the co-existing IGHV-unmutated clone resulted in re-categorization of seven of ten patients (70%) with originally discordant mutational status to the group with unmutated *IGHV* genes (Table 2).

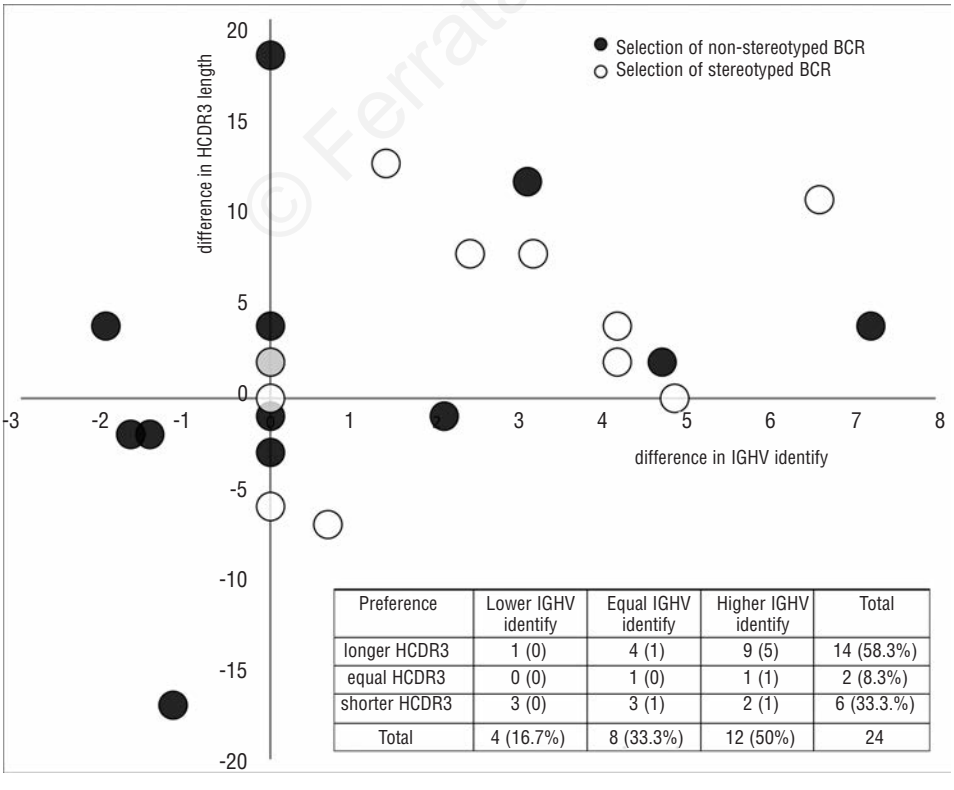
Considering the clinical course of the 19 patients with clonal drift, we noted that the changes in the proportions of clonal populations were frequently related to progressive lymphocytosis or overall disease progression (8 out of 13 cases with progressive disease; 62%). Furthermore, eradication of a clone was observed in relation to therapy administration when one of the clones present before therapy was not detected at disease relapse while the other clone expanded (5 of 8 treated patients; in the remaining 3 patients one clone had diminished before therapy). Altogether, clonal drift was associated with disease progression/therapy in 12 of 13 progressive cases (92%; 63% of all cases with clonal drift).

Since the molecular features of IGHV-IGHD-IGHJ

Table 2. Overview of MP-IGH CLL cases with originally discordant mutational status and the impact of clonal drift on overall *IGHV* mutational status (clones prevailing during the disease course are shown in bold).

Patient	*IGHV* gene	*IGHV* status (identity %)	CDR3 length	Stereotype subset	Over time tendency	Resulting IGHV status
604	***IGHV1-69***	**U (100)**	**24**		→	U
	IGHV1-69	**U (99.3)**	**20**	**5**	→	
	IGHV2-5	M (96.9)	12		↓	
625	***IGHV4-34***	**U (99.3)**	**17**		→	D
	IGHV1-69	**M (93.1)**	**15**		→	
833	***IGHV1-69***	**U (100)**	**21**		→	U
	IGHV2-5	M (92.8)	17		↓	
846	***IGHV1-69***	**U (100)**	**22**	**3**	→	U
	IGHV4-59	M (96.8)	14		↓	
912	***IGHV4-34***	**U (100)**	**23**	**B1**	→	U
	IGHV3-7	M (95.1)	23		↓	
1030	***IGHV1-69***	**U (100)**	**22**		→	U
	IGHV4-4	M (93.4)	11	219	↓	
1037	***IGHV4-39***	**U (99,0)**	**11**		→	D
	IGHV1-8	M (96.9)	12		↓	
	IGHV3-72	**M (94.9)**	**16**		→	
1049	***IGHV4-39***	**U (100)**	**19**	**8**	↑	U
	IGHV1-3	**U (100)**	**17**	**28**	→	
	IGHV3-33	M (95.8)	15		↓	
1087	***IGHV3-43***	**U (100)**	**14**		→	U
	IGHV6-1	M (95.3)	12		↓	
307*	*IGHV1-3*	U (98.6)	13		NA	D
	IGHV3-23	M (89.9)	13		NA	

Mutational IGHV *status: M – mutated, U – unmutated, D - discordant; "↓" – diminishing; "→" – persisting; "↑" – appearing; NA – not available. *Clones were not monitored over time in this case.*



Preference	Lower IGHV identify	Equal IGHV identify	Higher IGHV identify	Total
longer HCDR3	1 (0)	4 (1)	9 (5)	14 (58.3%)
equal HCDR3	0 (0)	1 (0)	1 (1)	2 (8.3%)
shorter HCDR3	3 (0)	3 (1)	2 (1)	6 (33.3.%)
Total	4 (16.7%)	8 (33.3%)	12 (50%)	24

Figure 3. Selection of higher *IGHV* gene identity and/or longer VH CDR3 in patients with clonal drift. Each spot of the plot represents the difference in *IGHV* gene identity and VH CDR3 length in favor of the *IG* gene rearrangement that predominated in an individual patient during the disease course. Specifically, the position of a spot on the x-axis was calculated as the *IGHV* gene identity difference of persistent minus diminishing *IG* gene rearrangement in any individual patient, appearing minus persistent rearrangement, or appearing minus diminishing rearrangement. The position on the y-axis was calculated for VH CDR3 length difference in a similar way. White and black spots represent selection of stereotyped and non-stereotyped BcR IG, respectively. The table lists the numbers of cases (counted per IG pairs) with similar behavior over time in relation to the preference/selection of the monitored immunogenetic features. Counts of cases with selection of a stereotyped BcR IG are shown in brackets.

rearrangements attest to the role of selection by antigen in CLL development,[4,5] they could also be relevant to the emergence, persistence, and drift of individual clones. Thus, in the 19 patients with clonal drift, we compared IGHV-IGHD-IGHJ rearrangements in co-existing pairs (24 in total). Significantly, this analysis was suggestive of selection for clones with: (i) higher *IGHV* gene identity to germline and/or (ii) longer VH CDR3 (cumulative preference in 71% of pairs, P=0.005, χ^2 test; Figure 3).

Moreover, we observed that the IG rearrangement bearing a VH CDR3 assigned to a stereotyped subset was preferred in nine pairs (82% of the pairs with at least one stereotyped BcR; 38% of all pairs with clonal drift) and, strikingly, the aforementioned tendency towards selection for higher *IGHV* identity and longer VH CDR3 was particularly pronounced in this subgroup (P=0.01; χ^2 test) (Figure 3; *Online Supplementary Table S3*).

Genomic background: influence of TP53 *gene defects on clonal drift*

Genomic abnormalities have been documented to affect the survival and time to first therapy of CLL patients,[28] with defects of the *TP53* gene being shown to have the strongest impact on clinical outcome.[29,30] Being interested in whether such defects present in co-existing clones could influence selection of one over the other, we detected *TP53* defects consecutively during the disease course in five patients among the MP-IGH cohort (16%). In these patients, *TP53* mutation and/or 17p deletion and other genomic abnormalities were assigned to individual clones based on: (i) changes in consecutive cytogenetic results that were correlated with changes in the relative proportions of *IG* rearrangements, and (ii) multiplex ligation-dependent probe amplification and *TP53* sequencing of individual FACS-sorted populations. Of significance, in all cases, the *TP53* defective clone expanded to the detriment of the *TP53* unaffected clone (Table 3). In two cases, the selection for the *TP53* defect was therapy-related. Notably, both selected clones harbored stereotyped rearrangements of the *IGHV1-69* gene (subsets 3 and 7)[31] (Figure 2).

Discussion

CLL is usually a monoclonal disease, hence, the entire CLL population constituting the progeny of a single B lymphocyte can be characterized by a unique IGHV-IGHD-IGHJ rearrangement. Significantly, stratification of patients into groups with distinct prognoses is possible through determination of *IGHV* mutational status.[32] In MP-IGH CLL cases, assignment to the *IGHV*-mutated or

Table 3. Comparison of individual B lymphocyte clones in patients with clonal drift and a *TP53* defect. In all cases, a *TP53* defect was related to a distinct B lymphocyte clone whereas another clone harbored intact *TP53* genes.

Patient	Clone with wild-type *TP53*				Clone with mutated *TP53*				
	IGHV and VH CDR3	Karyotype	17p-	Over time tendency	IGHV and VH CDR3	Karyotype	17p-	TP53 mutation	Over time tendency
261	IGHV1-69 (100 %) 29 aa	46,XY	No	↓	IGHV1-69 (100 %) 23 aa Subset 7	45,XY,-17; complex karyotype changes	Yes	p. M133K	→
279	IGHV3-21 (98.61 %) 9 aa Subset 2	46,XY,del(5)(q14.1q21.3)	No	↓	IGHV1-69 (100 %) 22 aa Subset 3	43,X,-Y,t(3;6)(q28;p12), der(4)t(4;21)(p15q11.2), der(5)t(Y;5)(q11.2;q21), t(12;14)(q13;q32.3), -17,-21, der(22)t(17;22)(q13;p11.2)	Yes	p. S215R	↑
319	IGHV3-30 (100 %) 13 aa IGHV4-39 (100 %) 16 aa	46,XX,del(11q), del(13q)	No	→	IGHV3-33 (100 %) 14 aa	46,XX	No	p. Y234C	↑
948	IGHV1-69 (100 %) 22 aa Subset 34	normal	No	↓	IGHV3-11 (100 %) 22 aa	del(13q), del(17p), -12	Yes	c. 782+1G>A	→
1072	IGHV3-21 (99.31 %) 20 aa	46,XX; 46,XX,del(13q)	No	↓	IGHV1-2 (100 %) 13 aa Subset 1	46,XX,del(13q),del(17p); 46,XX,del(17p)	Yes	p. F113V	→

FISH data available in all cases, karyotype data from CpG stimulated metaphase cytogenetics available in all cases but one (#948). Patients 261 and 279: TP53 *gene defect selected in relapse after therapy. In patient 261, subclone with complex karyotype (-17,* TP53 *gene mutation) was selected inside the* IGHV1-69/23aa *clone. Originally major 46,XY and* TP53*-wt fraction of the* IGHV1-69/23aa *clone diminished together with the other clone* IGHV1-69/29aa*. Only subclone I*GHV1-69/23aa *with inactivated TP53 remained. For details see also Figure 2. Patient 319: populations sorted by FACS according to the light chain expression; genomic alterations were assigned to the clones subsequently using multiplex ligation-dependent probe amplification. Not possible to assess whether karyotype 46,XX,del(11q),del(13q) was associated with* IGHV3-30 *or* IGHV4-39 *clone or both, because of uniform lambda light chain expression. "↓" – diminishing; "→" – persisting; "↑" – appearing.*

unmutated category can be difficult, especially when the rearrangements have discordant mutational status, precluding conclusive interpretation.[10] In line with our observations, the incidence of MP-IGH reaches approximately 2% of CLL cases.[10]

We attempted the first systematic assessment of MP-IGH CLL by performing detailed immunophenotypic and molecular profiling with the aim of elucidating the biological cause. Based on the evidence for co-existence of multiple B-cell clones, we assigned these cases into three groups. Group I (29% of MP-IGH cases; 0.79% of the whole CLL cohort) included cases for which we were able to confirm biclonal expansions differing in light chain restriction. The majority displayed typical CLL immunophenotype for both clonal populations. In two cases, the CLL clone co-existed with another B-cell clone expressing an immunophenotype atypical for CLL. Sanchez and colleagues[12] previously reported a higher incidence of CLL cases with two or more phenotypically distinct B-cell clones (~4% when considering typical and atypical CLL together) than was observed in our study. This discrepancy is most likely caused by differences in methodological design. In particular, Sanchez *et al.* investigated the incidence of more B-cell clones in a cohort of patients with leukemic chronic lymphoproliferative disorders characterized by detailed flow cytometry,[12] whereas we undertook a comprehensive molecular immunogenetic profiling complemented by fluorescence *in situ* hybridization (FISH) and flow cytometry studies. Furthermore, we followed a stringent approach for assigning patients to group I, requiring different light chain restriction, because alterations in the expression of other markers could be linked to intraclonal diversification or clonal evolution.

Group II (52% of MP-IGH cases; 1.40% of the whole CLL cohort) consisted of cases with immunogenetic evidence of more than one B-cell clonal population based on the number of detected IG rearrangements exceeding the allele capacity of a single cell/clone, yet in which only one homogeneous population was assessed by flow cytometry. This probably reflected either a very similar immunophenotype of the clones with the same light chain restriction, or a low proportion of one clone in the sample, or both. We were able to document this presumption using ASO-qPCR in five cases. In addition, changes in the relative proportions of the IGH rearrangements were observed over time.

For group III (19% of MP-IGH cases; 0.52% of the whole CLL cohort), we did not obtain definitive evidence mainly due to the lack of available material. Potential explanations could still relate to molecular mechanisms such as BcR editing through *IGHV* gene replacement[18] or lack of allelic exclusion[11] leading to the expression of multiple IGHV-IGHD-IGHJ rearrangements in a single cell. The first possibility (i.e. *IGHV* gene replacement) was effectively ruled out since we did not identify common VH CDR3 motifs co-existing in any patient.[33]

Although the evidence from cases in groups I and II suggests the presence of multiple B-cell clones, alternative options must be considered, including: (i) the presence of extra copies of the IGH locus due to trisomy 14 or amplification of 14q; nonetheless, this possibility is not supported in any case with available cytogenetic data (FISH and/or metaphase cytogenetics in 84% of MP-IGH cases); and, (ii) as already mentioned for group III, lack of allelic exclusion leading to dual IGH-expressing B cells, as reported for autoreactive B cells[17] as well as indirectly for CLL.[11,18] It is worth mentioning that in group II, we identified one case (#974) with dual expression of surface Igκ and Igλ light chains as a possible consequence of receptor editing or allelic inclusion in light chain loci.[34] So far, we have not been able to document whether both IG heavy transcripts detected in this patient were also translated and expressed. Admittedly, however, only analysis at the single cell level could reliably identify the underlying mechanism(s) in the above mentioned case and also confirm the presumption made for the whole group II.

Monoclonal B-cell lymphocytosis (MBL) is regarded as a pre-malignant state of CLL.[35] In contrast to the monoclonal nature of CLL, two or more "low-count" co-existing MBL clones have been documented at a high frequency.[36] Moreover, persistent as well as transient MBL clones have been observed.[37] Thus, CLL cases with MP-IGH gene rearrangements might represent the co-existence of CLL with a CLL-like MBL population, at least for a subset of cases. Following this line of reasoning, our cohort of MP-IGH CLL also featured a number of low-count clones (see group II above), which may signify borderline MBL/CLL clones co-existing for a certain period with the CLL clones eventually prevailing. This hypothesis is also supported by our experience with a case carrying two mutated IGHV-IGHD-IGHJ rearrangements that was originally classified as clinical MBL and, thus, excluded from the cohort studied herein. CLL eventually developed with only one of the original two rearrangements identified at the CLL stage.

Moreover, the idea of multiple B-lymphocyte clones initiating CLL is in the line with the process of antigen stimulation generally considered to contribute to the development of CLL.[3-5,38] Initially, several B lymphocytes with different BcR specificities could target different epitopes of the same (auto)antigen. Later on, only some of these clones eventually gain additional abnormalities driving clonal expansion and profit from favorable interactions within their microenvironment.[39] Thus, although only one clone prevails in the majority of CLL cases, many B lymphocyte clones could be expanded at the beginning. Our present results support this notion since multiple rearrangements were often detected in early stages of the disease at diagnosis (see *Online Supplementary Figure S1* showing a comparison between the MP-IGH cases and cases with only a single productive IGH rearrangement).

Clonal drift as a dynamic process of altering proportions of malignant lymphocyte clones is highly relevant to the understanding of the co-existence of multiple clones and the eventual prevalence of one over the other. It was first described in T-cell large granular lymphocyte leukemia harboring two T-cell receptor beta chain gene rearrangements.[20] Clonal drift had not been referenced in CLL until now. Indeed, a previously published study delineated a relative stability of neoplastic clones.[12] Based on our data, it seems that the emergence of an additional clone is possible but less likely. Similarly, the disappearance of a clone seems to occur more frequently if it co-exists with a more aggressive one. Our results show that higher *IGHV* gene identity and/or longer VH CDR3 regions were preferred over time, a phenomenon more pronounced in patients with stereotyped BcR. Relevant to these observations, telomere length measurements[21] have shown that proliferation of leukemic cells with unmutated *IGHV* is more intense compared to that of cells with mutated *IGHV*. Furthermore, it is worth men-

tioning that longer VH CDR3 are a frequent feature of auto- and multi-reactive cells,[40,41] which is also in line with the reactivity profile of *IGHV*-unmutated CLL clones. This indicates that antigen drive may underline clonal drift leading to selection for more aggressive clones with distinctive molecular features. Overall, based on the results of our study, clonal drift, predominantly leading to shrinkage or disappearance of clones detected at diagnosis, could be one of the factors contributing to the differing proportions of *IGHV*-mutated and *IGHV*-unmutated cases in MBL *versus* CLL.[35,42]

From a different perspective, it is reasonable to presume that genomic abnormalities might also have an impact on clonal drift. We document that the presence of a *TP53* defect in individual clones can also be implicated in clonal drift, further supporting the aggressiveness of p53-defective clones, which may underline selection leading to clonal predominance[29] and monoclonal CLL. In such cases, the p53 defect might represent a stronger phenotype prevailing over recognized unfavorable immunogenetic features. The impact of other genomic abnormalities, such as the 11q deletion, another strong marker of clinical outcome, remains to be elucidated given the low number of cases studied with the respective defects.

Finally, clonal drift might have important implications for decisions related to stratification and clinical management of MP-IGH CLL, especially in cases with discordant *IGHV* mutational status which are inconclusive regarding prognosis.[10] We observed a shift in favor of *IGHV*-unmutated status in the majority of cases with a discordant status. This might be an explanation for recently published data indicating that patients with discordant status have an adverse clinical course similar to that of *IGHV*-unmutated cases.[43] Moreover, based on our observations, the gradual prevailing of one clone over other, also in cases with concordant status, was often accompanied by disease progression. We, therefore, advocate repeated testing of *IGHV* mutational status in MP-IGH CLL since it can indicated changes in disease behavior.

In conclusion, our results suggest that most CLL cases with multiple IGHV-IGHD-IGHJ rearrangements can be accounted for by the presence of multiple B lymphocyte clones with CLL or CLL-like phenotype co-existing within the same patient. Importantly, we document for the first time that their proportions may change over time and that these changes are likely influenced by the molecular features of the BcR, including *IGHV* mutational status and VH CDR3 composition and length, and by the genomic make-up of the clones as well. This is potentially critical for understanding what drives B lymphocyte clonal evolution and could, therefore, provide insights and the means to influence or even halt this process.

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References

1. Messmer BT, Albesiano E, Efremov DG, Ghiotto F, Allen SL, Kolitz J, et al. Multiple distinct sets of stereotyped antigen receptors indicate a role for antigen in promoting chronic lymphocytic leukemia. J Exp Med. 2004;200(4):519-25.
2. Stamatopoulos K, Belessi C, Moreno C, Boudjograh M, Guida G, Smilevska T, et al. Over 20% of patients with chronic lymphocytic leukemia carry stereotyped receptors: Pathogenetic implications and clinical correlations. Blood. 2007;109(1):259-70.
3. Murray F, Darzentas N, Hadzidimitriou A, Tobin G, Boudjogra M, Scielzo C, et al. Stereotyped patterns of somatic hypermutation in subsets of patients with chronic lymphocytic leukemia: implications for the role of antigen selection in leukemogenesis. Blood. 2008;111(3):1524-33.
4. Rosén A, Murray F, Evaldsson C, Rosenquist R. Antigens in chronic lymphocytic leukemia--implications for cell origin and leukemogenesis. Semin Cancer Biol. 2010; 20(6):400-9.
5. Chiorazzi N, Ferrarini M. Cellular origin(s) of chronic lymphocytic leukemia: cautionary notes and additional considerations and possibilities. Blood. 2011;117(6):1781-91.
6. Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood. 1999;94(6):1840-7.
7. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood. 1999;94(6):1848-54.
8. Lanham S, Hamblin T, Oscier D, Ibbotson R, Stevenson F, Packham G. Differential signaling via surface IgM is associated with VH gene mutational status and CD38 expression in chronic lymphocytic leukemia. Blood. 2003;101(3):1087-93.
9. Hervé M, Xu K, Ng YS, Wardemann H, Albesiano E, Messmer BT, et al. Unmutated and mutated chronic lymphocytic leukemias derive from self-reactive B cell precursors despite expressing different antibody reactivity. J Clin Invest. 2005;115 (6):1636-43.
10. Langerak AW, Davi F, Ghia P, Hadzidimitriou A, Murray F, Potter KN, et al. Immunoglobulin sequence analysis and prognostication in CLL: guidelines from the ERIC review board for reliable interpretation of problematic cases. Leukemia. 2011; 25(6):979-84.
11. Rassenti LZ, Kipps TJ. Lack of allelic exclusion in B cell chronic lymphocytic leukemia. J Exp Med. 1997;185(8):1435-45.
12. 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(8):2994-3002.
13. González-Campos J, Ríos-Herranz E, De Blas-Orlando JM, Martín-Noya A, Parody-Ruiz-Berdejo R, Rodríguez-Fernández JM. Chronic lymphocytic leukemia with two cellular populations: a biphenotypic or biclonal disease. Ann Hematol. 1997;74(5): 243-6.
14. Hsi ED, Hoeltge G, Tubbs RR. Biclonal chronic lymphocytic leukemia. Am J Clin Pathol. 2000;113(6):798-804.
15. Chang H, Cerny J. Molecular characterization of chronic lymphocytic leukemia with two distinct cell populations: evidence for separate clonal origins. Am J Clin Pathol. 2006;126(1):23-8.
16. Acker C, Staal A, Aumont C, Didion J, Dorvaux V, Rio Y, et al. [Biclonal or biphenotypic chronic lymphotic leukemia? An answer brought by the treatment]. Ann Biol Clin (Paris). 2011;69(3):325-30.
17. Barreto V, Cumano A. Frequency and characterization of phenotypic Ig heavy chain

allelically included IgM-expressing B cells in mice. J Immunol. 2000;164(2):893-9.
18. Stamatopoulos K, Kosmas C, Stavroyianni N, Loukopoulos D. Evidence for immunoglobulin heavy chain variable region gene replacement in a patient with B cell chronic lymphocytic leukemia. Leukemia. 1996;10(9):1551-6.
19. Wang YH, Diamond B. B cell receptor revision diminishes the autoreactive B cell response after antigen activation in mice. J Clin Invest. 2008;118(8):2896-907.
20. Clemente MJ, Wlodarski MW, Makishima H, Viny AD, Bretschneider I, Shaik M, et al. Clonal drift demonstrates unexpected dynamics of the T-cell repertoire in T-large granular lymphocyte leukemia. Blood. 2011;118(16):4384-93.
21. Damle RN, Batliwalla FM, Ghiotto F, Valetto A, Albesiano E, Sison C, et al. Telomere length and telomerase activity delineate distinctive replicative features of the B-CLL subgroups defined by immunoglobulin V gene mutations. Blood. 2004;103(2):375-82.
22. van Gent R, Kater AP, Otto SA, Jaspers A, Borghans JA, Vrisekoop N, et al. In vivo dynamics of stable chronic lymphocytic leukemia inversely correlate with somatic hypermutation levels and suggest no major leukemic turnover in bone marrow. Cancer Res. 2008;68(24):10137-44.
23. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood. 2008;111(12):5446-56.
24. van Dongen JJ, Langerak AW, Brüggemann 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(12):2257-317.
25. Hadzidimitriou A, Darzentas N, Murray F, Smilevska T, Arvaniti E, Tresoldi C, et al. Evidence for the significant role of immunoglobulin light chains in antigen recognition and selection in chronic lymphocytic leukemia. Blood. 2009;113(2):403-11.
26. Stamatopoulos K, Belessi C, Hadzidimitriou A, Smilevska T, Kalagiakou E, Hatzi K, et al. Immunoglobulin light chain repertoire in chronic lymphocytic leukemia. Blood. 2005;106(10):3575-83.
27. Giudicelli V, Chaume D, Lefranc MP. IMGT/V-QUEST, an integrated software program for immunoglobulin and T cell receptor V-J and V-D-J rearrangement analysis. Nucleic Acids Res. 2004;32(Web Server issue):W435-40.
28. Ouillette P, Collins R, Shakhan S, Li J, Peres E, Kujawski L, et al. Acquired genomic copy number aberrations and survival in chronic lymphocytic leukemia. Blood. 2011;118(11):3051-61.
29. Malcikova J, Smardova J, Rocnova L, Tichy B, Kuglik P, Vranova V, et al. Monoallelic and biallelic inactivation of TP53 gene in chronic lymphocytic leukemia: selection, impact on survival, and response to DNA damage. Blood. 2009;114(26):5307-14.
30. Trbusek M, Smardova J, Malcikova J, Sebejova L, Dobes P, Svitakova M, et al. Missense mutations located in structural p53 DNA-binding motifs are associated with extremely poor survival in chronic lymphocytic leukemia. J Clin Oncol. 2011;29(19):2703-8.
31. Agathangelidis A, Darzentas N, Hadzidimitriou A, Brochet X, Murray F, Yan XJ, et al. Stereotyped B-cell receptors in one-third of chronic lymphocytic leukemia: a molecular classification with implications for targeted therapies. Blood. 2012;119(19):4467-75.
32. Pepper C, Majid A, Lin TT, Hewamana S, Pratt G, Walewska R, et al. Defining the prognosis of early stage chronic lymphocytic leukaemia patients. Br J Haematol. 2012;156(4):499-507
33. Darlow JM, Stott DI. V(H) replacement in rearranged immunoglobulin genes. Immunology. 2005;114(2):155-65.
34. Rezanka LJ, Kenny JJ, Longo DL. Dual isotype expressing B cells [kappa(+)/lambda(+)] arise during the ontogeny of B cells in the bone marrow of normal nontransgenic mice. Cell Immunol. 2005;238(1):38-48.
35. Rawstron AC, Bennett FL, O'Connor SJ, Kwok M, Fenton JA, Plummer M, et al. Monoclonal B-cell lymphocytosis and chronic lymphocytic leukemia. N Engl J Med. 2008;359(6):575-83.
36. Lanasa MC, Allgood SD, Volkheimer AD, Gockerman JP, Whitesides JF, Goodman BK, et al. Single-cell analysis reveals oligoclonality among 'low-count' monoclonal B-cell lymphocytosis. Leukemia. 2010;24(1):133-40.
37. Fazi C, Scarfò L, Pecciarini L, Cottini F, Dagklis A, Janus A, et al. General population low-count CLL-like MBL persists over time without clinical progression, although carrying the same cytogenetic abnormalities of CLL. Blood. 2011;118(25):6618-25.
38. Chiorazzi N, Efremov DG. Chronic lymphocytic leukemia: a tale of one or two signals? Cell Res. 2013;23(2):182-5.
39. Herishanu Y, Pérez-Galán P, Liu D, Biancotto A, Pittaluga S, Vire B, et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. Blood. 2011;117(2):563-74.
40. Almqvist N, Winkler TH, Mårtensson IL. Autoantibodies: focus on anti-DNA antibodies. Self Nonself. 2011;2(1):11-8.
41. Rahman A, Giles I, Haley J, Isenberg D. Systematic analysis of sequences of anti-DNA antibodies--relevance to theories of origin and pathogenicity. Lupus. 2002;11(12):807-23.
42. Landgren O, Albitar M, Ma W, Abbasi F, Hayes RB, Ghia P, et al. B-cell clones as early markers for chronic lymphocytic leukemia. N Engl J Med. 2009;360(7):659-67.
43. Visco C, Moretta F, Falisi E, Facco M, Maura F, Novella E, et al. Double productive immunoglobulin sequence rearrangements in patients with chronic lymphocytic leukemia. Am J Hematol. 2013;88(4):277-82.