IgG-SWITCHED CLL HAS A DISTINCT IMMUNOGENETIC SIGNATURE FROM THE COMMON MD VARIANT: ONTOGENETIC IMPLICATIONS

Anna Vardi¹, Andreas Agathangelidis², Lesley-Ann Sutton³, Maria Chatzouli⁴, Lydia Scarfò², Larry Mansouri³, Vassiliki Douka¹, Achilles Anagnostopoulos¹, Nikos Darzentas⁵,⁶, Richard Rosenquist³, Paolo Ghia², Chrysoula Belessi⁴, Kostas Stamatopoulos¹,³,⁶

1. Hematology Department and HCT Unit, G. Papanicolaou Hospital, Thessaloniki, Greece
2. Division of Molecular Oncology, San Raffaele Scientific Institute, Milan, Italy
3. Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden
4. Hematology Department, Nikea General Hospital, Piraeus, Greece
5. CEITEC - Central European Institute of Technology, Masaryk University, Brno, Czech Republic
6. Institute of Applied Biosciences, CERTH, Thessaloniki, Greece

**Corresponding author:**
Kostas Stamatopoulos
Hematology Department and HCT Unit
G. Papanicolaou Hospital, 57010 Thessaloniki, Greece
Phone: +30.2313.307992/ Fax: +30.2313.307579
e-mail: kostas.stamatopoulos@gmail.com

**CONFLICT-OF-INTEREST DISCLOSURE**
The authors have no relevant conflicts of interest to disclose.
STATEMENT OF TRANSLATIONAL RELEVANCE

The great majority of CLL clones express surface immunoglobulin (IG) of the mu and delta isotypes (IgM+IgD+, MD-CLL). CLL cases where the major clone is isotype-switched are relatively rare and insufficiently characterized. We comprehensively profiled the IG gene repertoires of cases with MD versus G isotypes in the largest series by far studied for this purpose. G-CLL displayed an overall distinct immunogenetic signature from MD-CLL, prompting speculations about distinct ontogenetic derivation and/or distinct immune triggering.

Remarkably, two subsets with quasi-identical (stereotyped) B-cell receptor IGs included only G-CLL cases and accounted for 25% of the respective cohort. Given their markedly different somatic hypermutation status and clinical behavior, these two subsets may be considered paradigmatic for the elucidation of distinct immune pathogenetic pathways and clinical phenotypes in CLL. We argue that this endeavor may prove clinically relevant in light of the increasing use of drugs targeting immune signaling in CLL.
ABSTRACT

Purpose:
IgG-switched chronic lymphocytic leukemia (G-CLL) is a rare variant of CLL, whose origin and ontogenetic relationship to the common IgM/IgD (MD-CLL) variant remains undefined. Here we sought for clues regarding the ontogeny of G-CLL versus MD-CLL by profiling the relevant IG gene repertoires.

Experimental Design:
Using purpose-built bioinformatics methods, we performed detailed immunogenetic profiling of a multinational CLL cohort comprising 1256 cases, of which 1087 and 169 expressed IG mu/delta and gamma heavy chains, respectively.

Results:
G-CLL has a highly skewed IG gene repertoire that is distinct from MD-CLL, especially in terms of: (i) overuse of the IGHV4-34 and IGHV4-39 genes; and, (ii) differential somatic hypermutation (SHM) load. Repertoire differences held also when comparing subgroups with similar SHM status and were mainly attributed to the exclusive representation in G-CLL of two major subsets with quasi-identical (stereotyped) B-cell receptors. These subsets, namely #4 (IGHV4-34/IGKV2-30) and #8 (IGHV4-39/IGKV1(D)-39), were found to display sharply contrasting SHM and clinical behavior.

Conclusions:
G-CLL exhibits an overall distinct immunogenetic signature from MD-CLL, prompting speculations about distinct ontogenetic derivation and/or immune triggering. The reasons underlying the differential regulation of SHM among G-CLL cases remain to be elucidated.
INTRODUCTION

The great majority of chronic lymphocytic leukemia (CLL) cases express surface immunoglobulins (IG) of the mu and delta isotypes (IgM+IgD+, MD-CLL) (1). However, subpopulations of isotype-switched cells often exist within IgM+IgD+ CLL clones (2, 3). Indeed, class switch recombination (CSR) appears to be dissociated from somatic hypermutation (SHM) in CLL, at least in certain cases, and, perhaps paradoxically, in vivo CSR is predominantly identified in CLL clones with unmutated IG receptors (U-CLL) (4, 5).

Activation-induced cytidine deaminase (AID), which is normally required for both SHM and CSR, is expressed in a small proliferative compartment within the CLL clone, irrespective of the immunoglobulin heavy variable (IGHV) gene mutational status, and can be functionally induced in vitro under conditions that mimic T cell help (6). Furthermore, ongoing CSR and high AID expression define a subgroup of U-CLL displaying even worse prognosis, underscoring the importance of intense (micro)environmental stimulation for shaping clonal behavior and eventual clinical outcome (7).

That notwithstanding, the precise signals that induce in vivo CSR in CLL are presently not fully elucidated.

CLL cases in which the major clone is isotype-switched are relatively rare (6-10%) (8). The majority of such cases are IgG-switched (G-CLL), however little published data exists regarding this subgroup. That said, a phenotypic, molecular and functional characterization of 14 unselected G-CLL cases suggested a relationship with CLL bearing mutated IG receptors (M-CLL) and derivation from post-germinal B cells (9). However, the existence of U-CLL with IgG-switched receptors challenges this scenario and warrants the search for alternative possibilities.

Here, we sought evidence concerning the origin of G-CLL by comprehensively profiling the IG gene repertoires in the largest series studied to date for this purpose. Our focus on the immunogenetic characteristics of the clonotypic B-cell receptor (BcR) IGs is justified by both biological and clinical evidence indicating, on one hand, that different types of BcRs convey signals of different intensity and, on the other, that interfering with BcR signaling holds
therapeutic promise in CLL (10-16). We demonstrate that G-CLL exhibits an overall distinct immunogenetic signature from MD-CLL, even when restricting the comparison to cases with mutated IG receptors, thus prompting speculations about distinct ontogenetic derivation and/or distinct immune triggering.
PATIENTS AND METHODS

Patients
We performed detailed immunogenetic profiling of a large multinational CLL cohort (n=1256) from collaborating institutions in Greece, Italy and Sweden. Cases were included in the study if information for the clonotypic heavy chain isotype was available, as determined by either peripheral blood flow cytometry (n=1030) or reverse transcriptase-polymerase chain reaction (RT-PCR) for tumor-specific mu, delta and gamma constant region transcripts (n=226). Within this cohort, 1087 and 169 cases expressed IG mu/delta and gamma heavy chains, respectively. A summary of available demographic and clinical data is provided in Table 1. The diagnosis of CLL was made according to established iwCLL/NCI criteria (17). The study was approved by the local Ethics Review Committee of each participating institution.

PCR amplification and sequence analysis of IGHV-IGHD-IGHJ rearrangements
PCR amplification and sequence analysis of IGHV-IGHD-IGHJ rearrangements were performed as previously described (18). Sequence data were interpreted using our suite of purpose-built bioinformatics methods, the Antigen Receptors Research Tool / ARResT (http://bat.infspire.org/arrest). Stereotyped rearrangements were identified following our previously described criteria (19, 20).
RESULTS

**IG gene repertoire and SHM load in G-CLL versus MD-CLL**

G-CLL was found to exhibit a particularly skewed IG gene repertoire. In particular, IGHV4 was the predominant subgroup (90/169, 53%), followed by IGHV3 (60/169, 35%). Thirty-two functional *IGHV* genes were identified with the *IGHV4-34* gene being by far the most frequent (38%), followed by *IGHV4-39* (9%), *IGHV3-23* (7%) and *IGHV3-30* (5%). Collectively, these four genes accounted for almost 60% of the respective repertoire.

Comparison of G-CLL to MD-CLL revealed marked differences, with the *IGHV4-34* and *IGHV4-39* genes being significantly (p<0.001) overrepresented in the former. This is in contrast to several other genes with significantly (p<0.05) lower frequencies in G-CLL compared to MD-CLL, namely the *IGHV1-69*, *IGHV3-21*, *IGHV1-2* and *IGHV3-48* genes (Figure 1A). The markedly reduced frequency of the *IGHV1-69* gene in G-CLL versus MD-CLL is particularly noteworthy, given that this gene is known to dominate the IG gene repertoire in CLL (20-22).

We next investigated the impact of SHM in G-CLL versus MD-CLL. On the grounds that even a minimal SHM load can have profound functional implications, we subdivided cases into three mutational subgroups, namely (i) truly unmutated i.e. no SHM/100% germline identity (GI); (ii) borderline/minimally mutated (GI=98-99.9%); and, (iii) mutated (GI<98%) (18, 23). We noted a significant (p<0.05) asymmetrical distribution of G-CLL versus MD-CLL cases across these SHM categories. In particular, only 11% (19/169) of G-CLL rearrangements were truly unmutated compared to 32% (347/1087) of MD-CLL rearrangements; a similar result was obtained for borderline/minimally mutated rearrangements (5%, 8/169 for G-CLL versus 12%, 126/1087 for MD-CLL). In sharp contrast, 84% (142/169) of G-CLL rearrangements carried *IGHV* genes with less than 98% GI versus only 56% (614/1087) for MD-CLL (Figure 1B).

On these grounds, we also performed IG repertoire comparisons between G-CLL and MD-CLL for the different SHM subgroups. We noted significant over-representation of (i) the *IGHV4-34* gene among mutated rearrangements in G-
CLL versus MD-CLL (63/142 versus 65/614 cases, respectively; \( p < 0.0001 \)); and, (ii) the \( IGHV4-39 \) gene among truly unmutated rearrangements in G-CLL versus MD-CLL (10/19 versus 23/347 cases, respectively; \( p < 0.0001 \)) (Figure 2). Due to the low number of G-CLL cases (n=8) within the borderline/minimally mutated subgroup, we did not undertake such comparisons.

**BcR stereotypy in G-CLL versus MD-CLL**

Aiming to identify patterns that would explain the IG gene repertoire polarization between G-CLL versus MD-CLL, we next investigated the incidence of BcR IG stereotypy in both subgroups. Cluster analysis of the heavy variable complementarity-determining region 3 (VH CDR3) sequences of our series revealed that the extreme skewing of the G-CLL repertoire was due to the fact that almost one-third of all cases were represented by only three stereotyped subsets, namely: (i) mutated subset #4, defined by stereotyped \( IGHV4-34/IGKV2-30 \) BcRs: 31/169 cases (18%); (ii) unmutated subset #8, defined by stereotyped \( IGHV4-39/IGKV1(D)-39 \) BcRs: 11/169 cases (7%); and, (iii) mutated subset #16, defined by stereotyped \( IGHV4-34/IGKV3-20 \) BcRs: 7/169 cases (4%). Notably, subset #4 and subset #8 cases could be considered as prototypes for mutated G-CLL and truly unmutated G-CLL respectively since they comprised 22% and 42% of the respective categories (Figure 3). The extreme polarization of the G-CLL IG repertoire was also underscored by the fact that all major stereotyped subsets utilizing the \( IGHV1-69, IGHV3-21, IGHV1-2 \) and \( IGHV3-48 \) genes exclusively concerned MD-CLL.

With the exception of subsets #4, #8 and #16, G-CLL BcR IGs were very heterogeneous in terms of \( IGHV \) gene usage and VH CDR3 composition. Interestingly, a group of cases were found to carry co-existing trisomies of chromosomes 12, 18 and 19 (n=8/169, 5% of the G-CLL cohort). These cases carried somatically mutated BcR IGs yet with different \( IGHV \) genes and VH CDR3 features, which is in keeping with a previous study from our group (24).
**Subset #4 and subset #8 display distinct clinico-biological characteristics**

The emergence of subset #4 and subset #8 as the most prominent representatives of G-CLL strikes as odd, given that these two subsets are known to be opposites in terms of clinical presentation and outcome. We therefore looked into the clinico-biological data of cases of the present series assigned to these subsets (summarized in Table 2).

In line with previous studies (25, 26), subset #4 patients were significantly younger at diagnosis compared to subset #8 and presented more frequently with early-stage disease; in addition, with similar follow-up times, patients assigned to subset #4 required treatment significantly less frequently than subset #8. All subset #4 BcR IGs were mutated while subset #8 BcR IGs were unmutated. In addition, subsets #4 and #8 exhibited differential expression of CD38 and ZAP70 (infrequent in subset #4, pronounced in subset #8). Furthermore, they displayed distinct genomic aberration profiles, especially regarding the incidence of trisomy 12 (0% in subset #4 versus 60% in subset #8, p<0.001).
DISCUSSION

We performed a detailed immunogenetic analysis of the largest series of IgG-switched CLL cases studied to date, combined with a search for clinico-biological associations. We demonstrate that this rare CLL subgroup exhibits an immunogenetic signature clearly distinct from non-switched CLL, and remarkably skewed towards the representation of two major CLL stereotyped subsets, namely subset #4 and subset #8.

Subset #4 clones are “born” to be autoreactive, since expression of the *IGHV4-34* gene per se conveys autoreactive potential. In fact, *IGHV4-34*-expressing normal B cells represent an *in vivo* model of autoreactivity, given that they are endowed with the ability to recognize, in a superantigenic fashion, the N-acetyllactosamine antigenic epitope present in both self and exogenous antigens (27). This recognition is mediated through a germline-encoded motif in the heavy variable framework region 1 (VH FR1) of the *IGHV4-34* gene. Importantly, as in previous studies (18), all subset #4 cases of the present series carried an intact VH FR1 motif.

Further molecular hints linking subset #4 with autoreactivity, also evident in the present series, are (i) the predicted high electropositivity of their VH CDR3s, which are enriched in basic and aromatic amino acid residues, reminiscent of pathogenic anti-DNA antibodies; and, (ii) the presence of recurrent SHMs in both the IG variable heavy and kappa (VK) domains, typified by the frequent introduction of acidic residues, similar to edited anti-DNA antibodies (18, 28). Relevant to the latter observation, despite intense ongoing SHM leading to subclone formation and pronounced intraclonal diversification, the IG heavy and light chain sequences of subset #4 cases have been shown to retain the somatically introduced acidic residues, likely due to functional constraints in a context of maintaining tolerance (29-31). This is in line with the previous finding that recombinant monoclonal antibodies (mAbs) from CLL subset #4 cases do not recognize DNA, while their germline revertants regain this capacity (32).

We sought for additional immunogenetic hints into the ontogeny of CLL subset #4, taking advantage of a recent high-throughput sequencing study of the paired IG heavy and light chains of peripheral IgG-switched B cells in
healthy individuals (33). By careful meta-analysis of the respective sequence datasets, we found only one IGHV4-34/IGKV2-30 combination in a total of 1.4x10^5 IGH/IGK pairs, albeit with VH and VK CDR3s drastically distinct from those characterizing subset #4.

Overall, it can be hypothesized that the progenitor of CLL subset #4 might be a very particular, rare type of autoreactive cell that undergoes editing by SHM, thereby alleviating intense self-reactivity and preventing clonal deletion. Questions as to the when, where and under what influence(s) this cell undergoes CSR to become IgG-switched currently remain unresolved and require further study.

At the other end of the G-CLL spectrum, unmutated and clinically aggressive subset #8 exhibits an intriguingly broad antigen reactivity profile (36). It is conceivable that this particularly poly/autoreactive clone does not engage in SHM because the unmutated configuration endows clonal cells with ample opportunities to engage in interactions with a wide range of antigens, thus receiving almost unrestricted pro-survival signals. Altogether, subset #8 can be considered as a paradigmatic example of CLL clones where SHM is dissociated from CSR. What does this tell us about its ontogeny?

Given that CSR is traditionally considered to take place within the germinal centers (GC) of secondary lymphoid organs, this could imply derivation from GC cells and would be plausible for not only subset #8 but also for all CLL clones carrying isotyped-switched, unmutated BcR IGs. However, one cannot exclude the possibility that subset #8 CLL clones originate from IgG-switched memory cells generated by a GC-independent pathway early after antigen encounter (37, 38).

In both subsets, #4 and #8, the available immunogenetic and functional evidence implies that antigenic triggering has been or is still relevant for tumor development and evolution, including CSR induction, through either BcR-dependent or BcR-independent mechanisms (39). A propos of the latter, data suggest that anergic self-reactive B cells may be induced to undergo CSR and produce autoantibodies in vitro through Toll-like receptor (TLR) stimulation or in conditions that mimic T cell help (40). Interestingly, our previous studies documented that subset #4 and subset #8 clones have distinct functional responses to TLR stimulation, with the former exhibiting a
TLR7-tolerant profile and the latter displaying an unrestricted and intense response to multiple TLR ligands, thus differing significantly from other subsets that are far more selective (36, 41). How these functional profiles are linked to the physiology and clonal behavior of subsets #4 and #8, especially with regards to CSR, is currently unknown.

In conclusion, G-CLL exhibits an overall distinct immunogenetic signature from MD-CLL, prompting speculations about distinct ontogenetic derivation and/or immune triggering. Further studies are warranted in order to better understand the differential regulation of SHM among G-CLL cases and how it may affect clonal behavior and eventual patient outcome.
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AUTHOR CONTRIBUTIONS
Anna Vardi performed research, analyzed data and wrote the paper. Andreas Agathangelidis, Lesley-Ann Sutton, Maria Chatzouli and Larry Mansouri performed research. Lydia Scarfò, Vassiliki Douka and Achilles Anagnostopoulos provided samples and associated clinicopathological data. Nikos Darzentas, Richard Rosenquist, Paolo Ghia and Chrysoula Belessi designed the study and supervised research. Kostas Stamatopoulos designed the study, supervised research and wrote the paper.
REFERENCES


FIGURE LEGENDS

Figure 1. IG gene repertoire and somatic hypermutation status in G-CLL versus MD-CLL. A) *IGHV* gene repertoire in G-CLL versus MD-CLL. B) Relative distribution of G-CLL versus MD-CLL into three subgroups defined according to somatic hypermutation load, namely truly unmutated (100% *IGHV* identity to the germline, GI), borderline/minimally mutated (98-99.9% GI), and mutated (<98% GI). Differences at a level of statistical significance, p<0.05 and p<0.001, are marked with [*] and [**], respectively.

Figure 2. *IGHV* gene repertoire comparisons within SHM subgroups. A) Mutated G-CLL versus mutated MD-CLL; B) truly unmutated G-CLL versus truly unmutated MD-CLL. Comparison among borderline/minimally mutated *IGHV* genes in G-CLL versus MD-CLL was not undertaken due to a low number of cases within this subgroup. Differences at a level of statistical significance, p<0.05 and p<0.001, are marked with [*] and [**], respectively.

Figure 3. Schematic representation of the relative frequencies of the two most prominent *IGHV* genes in G-CLL, i.e. *IGHV4-34* and *IGHV4-39*. A) G-CLL versus MD-CLL; B) mutated G-CLL versus mutated MD-CLL; and, C) truly unmutated G-CLL versus truly unmutated MD-CLL. The relative representation of subset #4 and subset #8 among cases expressing mutated *IGHV4-34* and truly unmutated *IGHV4-39* genes, respectively, is also depicted.
### TABLES

#### Table 1. An overview of demographic, clinical and biological characteristics of MD-CLL versus G-CLL cases.

<table>
<thead>
<tr>
<th>COHORT CHARACTERISTICS</th>
<th>MD-CLL</th>
<th>G-CLL</th>
<th>p-value</th>
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</thead>
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<tr>
<td>Age (years, median)</td>
<td>65</td>
<td>61</td>
<td>&lt;0.001</td>
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<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
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<td>Male</td>
<td>581 (65%)</td>
<td>100 (60%)</td>
<td>0.30</td>
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<td>Female</td>
<td>320 (34%)</td>
<td>66 (40%)</td>
<td></td>
</tr>
<tr>
<td>Binet stage B-C</td>
<td>156/744 (21%)</td>
<td>21/132 (26%)</td>
<td>0.18</td>
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<tr>
<td>Surface CD38 &gt;7%</td>
<td>339/832 (41%)</td>
<td>48/148 (32%)</td>
<td>0.06</td>
</tr>
<tr>
<td>Cytoplasmic ZAP-70 &gt;20%</td>
<td>170/460 (37%)</td>
<td>12/67 (18%)</td>
<td>0.002</td>
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<tr>
<td>Mutated IGHV (&lt;98% GI)</td>
<td>614/1087 (56%)</td>
<td>142/169 (84%)</td>
<td>&lt;0.001</td>
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<td>Complex karyotype*</td>
<td>32/362 (9%)</td>
<td>6/62 (10%)</td>
<td>0.83</td>
</tr>
<tr>
<td>Multiple trisomies (+12, +18, +19)**</td>
<td>0/362 (0%)</td>
<td>8/62 (13%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>trisomy 12 (FISH)</td>
<td>45/356 (13%)</td>
<td>14/56 (25%)</td>
<td>0.01</td>
</tr>
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<td>del13q (FISH)</td>
<td>175/362 (48%)</td>
<td>27/57 (47%)</td>
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</tr>
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<td>del11q (FISH)</td>
<td>44/362 (12%)</td>
<td>1/56 (2%)</td>
<td>0.02</td>
</tr>
<tr>
<td>del17p (FISH)</td>
<td>42/369 (11%)</td>
<td>3/59 (5%)</td>
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<tr>
<td>NOTCH1 exon 34 mutation</td>
<td>18/426 (4%)</td>
<td>1/90 (1%)</td>
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<tr>
<td>Need for treatment</td>
<td>327/406 (81%)</td>
<td>60/83 (72%)</td>
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<td>Time to first treatment (months)</td>
<td>23</td>
<td>39.7</td>
<td>0.03</td>
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*Three or more numerical and/or structural aberrations.

**Cases with multiple co-existing trisomies of chromosomes 12, 18 and 19 were analyzed separately from cases with complex karyotype (CK) on the basis of previous reports that they exhibit a particularly indolent clinical course, thus distinguishing them from CK cases (24).
Table 2. Comparison of the demographic, clinical and biological characteristics of subset #4 versus subset #8.

<table>
<thead>
<tr>
<th>COHORT CHARACTERISTICS</th>
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<th>SUBSET #8</th>
<th>p value</th>
</tr>
</thead>
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<td>66</td>
<td>0.02</td>
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<tr>
<td>Gender</td>
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<td></td>
<td></td>
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<tr>
<td>Male</td>
<td>19 (61%)</td>
<td>6 (55%)</td>
<td>0.70</td>
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<tr>
<td>Female</td>
<td>12 (39%)</td>
<td>5 (45%)</td>
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<td>Binet stage B-C</td>
<td>4/26 (15%)</td>
<td>3/6 (50%)</td>
<td>0.06</td>
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<tr>
<td>Surface CD38 &gt;7%</td>
<td>1/27 (4%)</td>
<td>5/6 (83%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cytoplasmic ZAP-70 &gt;20%</td>
<td>1/12 (8%)</td>
<td>2/2 (100%)</td>
<td>0.003</td>
</tr>
<tr>
<td>Mutated IGHV (&lt;98% GI)</td>
<td>31/31 (100%)</td>
<td>0/11 (0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Complex karyotype*</td>
<td>0/12 (0%)</td>
<td>0/3 (0%)</td>
<td></td>
</tr>
<tr>
<td>Multiple trisomies (+12, +18, +19)**</td>
<td>0/12 (0%)</td>
<td>0/3 (0%)</td>
<td></td>
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<td>&lt;0.001</td>
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<td>2/5 (40%)</td>
<td>0.25</td>
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<td>1/16 (6%)</td>
<td>0/5 (0%)</td>
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<td>del17p (FISH)</td>
<td>0/16 (0%)</td>
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<td>Time to first treatment (months)</td>
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<td>50.4</td>
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<td>Richter's transformation</td>
<td>0/31 (0%)</td>
<td>1/11 (9%)</td>
<td>0.09</td>
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</table>

*Three or more numerical and/or structural aberrations.

**Cases with multiple co-existing trisomies of chromosomes 12, 18 and 19 were analyzed separately from cases with complex karyotype (CK) on the basis of previous reports that they exhibit a particularly indolent clinical course, thus distinguishing them from CK cases (24).
Figure 2

A. 

IGHV gene frequency (%)

mutated G-CLL

mutated MD-CLL

IGHV1-2  IGHV1-69  IGHV3-21  IGHV3-23  IGHV3-30  IGHV3-33  IGHV3-48  IGHV3-7  IGHV4-34  IGHV4-39

B. 

IGHV gene frequency (%)

truly unmutated G-CLL

truly unmutated MD-CLL

IGHV1-2  IGHV1-69  IGHV3-21  IGHV3-23  IGHV3-30  IGHV3-33  IGHV3-48  IGHV3-7  IGHV4-34  IGHV4-39

*  **
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