High-density Screening Reveals a Different Spectrum of Genomic Aberrations in Chronic Lymphocytic Leukemia Patients with ‘Stereotyped’ IGHV3-21 and IGHV4-34 B-cell Receptors

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High-density screening reveals a different spectrum of genomic aberrations in chronic lymphocytic leukemia patients with ‘stereotyped’ IGHV3-21 and IGHV4-34 B-cell receptors

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background
The existence of multiple subsets of chronic lymphocytic leukemia expressing ‘stereotyped’ B-cell receptors implies the involvement of antigen(s) in leukemogenesis. Studies also indicate that ‘stereotypy’ may influence the clinical course of patients with chronic lymphocytic leukemia, for example, in subsets with stereotyped IGHV3-21 and IGHV4-34 B-cell receptors; however, little is known regarding the genomic profile of patients in these subsets.

Design and Methods
We applied 250K single nucleotide polymorphism-arrays to study copy-number aberrations and copy-number neutral loss-of-heterozygosity in patients with stereotyped IGHV3-21 (subset #2, n=29), stereotyped IGHV4-34 (subset #4, n=17; subset #16, n=8) and non-subset #2 IGHV3-21 (n=13) and non-subset #4/16 IGHV4-34 (n=34) patients.

Results
Over 90% of patients in subset #2 and non-subset #2 carried copy-number aberrations, whereas 75-76% of patients in subset #4 and subset #16 showed copy-number aberrations. Subset #2 and non-subset #2 patients also displayed a higher average number of aberrations compared to patients in subset #4. Deletion of 13q was the only known recurrent aberration detected in subset #4 (35%); this aberration was even more frequent in subset #2 (79%). del(11q) was more frequent in subset #2 and non-subset #2 (31% and 23%) patients than in subset #4 and non-subset #4/16 patients. Recurrent copy-number neutral loss-of-heterozygosity was mainly detected on chromosome 13q, independently of B-cell receptor stereotypy.

Conclusions
Genomic aberrations were more common in subset #2 and non-subset #2 than in subset #4. The particularly high frequency of del(11q) in subset #2 may be linked to the adverse outcome reported for patients in this subset. Conversely, the lower prevalence of copy-number aberrations and the absence of poor-prognostic aberrations in subset #4 may reflect an inherently low-proliferative disease, which would prevent accumulation of genomic alterations.

Key words: chronic lymphocytic leukemia, stereotyped B-cell receptors, antigens, leukemogenesis.


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Introduction

In chronic lymphocytic leukemia (CLL), the mutation status of the immunoglobulin heavy chain variable (IGHV) genes has emerged as one of the strongest prognostic markers, since it divides patients into two clinical subgroups, those with IGHV-mutated and those with IGHV-unmutated CLL, with different prognoses. Furthermore, CLL displays a remarkably biased IGHV repertoire with over-representation of a limited number of genes, such as IGHV1-69, IGHV4-34, IGHV3-23 and IGHV3-21. Several groups have reported multiple CLL subsets with almost identical, ‘stereotyped’ B-cell receptors (BCR) in up to about 50% of patients. These subsets are defined by certain criteria such as usage of similar IGHV-D-J genes and light-chain genes and an amino acid identity of 60% or more in the heavy-chain complementarity determining region 3 (CDR3), which is the main determinant of antigen specificity. Considering the very low probability of finding two B-cell clones with almost identical BCR by chance alone, these findings have supported the notion that the development of CLL is not stochastic and suggests a potential role for antigens in leukaemogenesis through the recognition of similar epitopes within each subset.

So far, more than 100 different subsets have been defined with stereotyped BCR, with some of the subsets being more frequent than others. Interestingly, CLL subsets expressing a certain stereotyped BCR have also been indicated to share biological and clinical features. For instance, IGHV4-34/IGKV2-30 patients with stereotyped BCR (20 amino acids long, known as subset #4) have an indolent disease course compared to those with non-stereotyped IGHV4-34. Furthermore, subset #4 patients have a low median age at diagnosis, IgG-switched BCR and a potential association with a specific serotype of herpesviruses. Another IGHV4-34 subset, subset #16 (IGHV4-34/IGKV3-20, with a CDR3 that is 24 amino acids long), has been identified, although less is known about the clinical outcome for these patients.

Approximately half of the patients with IGHV3-21 CLL display a stereotyped BCR and have an equal poor overall survival, independently of IGHV mutational status, although stereotypy has been associated with a shorter time to progression and the presence of other markers of a poor prognosis.

Table 1. Clinical data for IGHV3-21 and IGHV4-34 chronic lymphocytic leukemia subgroups included in this study.

<table>
<thead>
<tr>
<th>Samples (n=101)</th>
<th>Subset #2</th>
<th>Non-subset #2</th>
<th>Subset #4</th>
<th>Non-subset #4</th>
<th>Subset #16</th>
<th>Non-subset #16</th>
</tr>
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<td>63 (45-82)</td>
<td>57 (37-72)</td>
<td>68 (52-78)</td>
<td>60 (42-72)</td>
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<td>at diagnosis</td>
<td></td>
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<td>years (range)</td>
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</tr>
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<td>1</td>
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</tbody>
</table>

*Cases with >98% identity to germline were classified as unmutated, whereas cases with <88% identity were considered mutated. Denotes treatment status of patient at sampling. Classified as stereotyped (subset) or non-stereotyped (non-subset) according to previously defined criteria.

Design and Methods

Groups of patients

In total, 101 CLL patients from collaborating institutes in France (n=15), Greece (n=25), Denmark (n=4) and Sweden (n=57) were studied. Most samples were derived from peripheral blood (n=88), but samples from bone marrow (n=8) and spleen (n=5) were also included. All samples were diagnosed according to recently revised criteria for CLL, and showed a typical immunophenotype and 70% or more tumor cells. Clinical data for all patients are summarized in Table 1. Subsets were defined according to Stamatoopoulos et al. and Murray et al. Twenty-nine samples were classified as subset #2 (IGHV3-21/IGKV2-31 usage, CDR3 of 9 amino acids long) and 13 samples as non-subset #2 (IGHV3-21 usage, heterogeneous CDR3 length). Seventeen samples were defined as subset #4 (IGHV4-34/IGKV2-30 usage, CDR3 of 20 amino acids long, IgG-switched), 8 samples as subset #16 (IGHV4-34/IGKV3-20 usage, CDR3 of 24 amino acids long) and 34 as non-subset #4/16 (IGHV4-34 usage, heterogeneous CDR3 lengths).

Single nucleotide polymorphism-array analysis

SNP-array experiments were performed according to the stan-
dard protocols for Affymetrix GeneChip® Mapping NspI-250K arrays (Gene Chip Mapping 500K Assay Manual (P/N 701950 Rev2), Affymetrix Inc., Santa Clara, CA, USA) and the arrays were scanned using the GeneChip® Scanner 3000 7G. Genotype calling and probe level normalization were performed in the Affymetrix GeneChip® Genotyping Analysis Software (GTYPE) 4.1 using the Dynamic Model (DM) algorithm and BRLMM.22 The quality control specifies a neighbor score that represents the average of the Euclidean distances between the logratio of flanking SNP along the chromosomes. Low neighbor scores indicate a low noise level: a neighbor score of 0.4 or less was, therefore, applied as the cut-off for inclusion of samples. In order to produce logratios, copy-num-
ber normalization was performed using the Copy Number Analysis Tool (CNAT) 4.0.1. Normal samples (n=82) analyzed at the Uppsala Array Platform were used as a reference set.

Analysis of Affymetrix data for copy-number alterations
Copy-number analysis using the rank segmentation algorithm and group comparisons were performed employing BioDiscovery Nexus Copy Number 8.0 software (BioDiscovery, El Segundo CA, USA). Copy-number analysis was performed using a significance threshold (P value) of 1×10⁻⁶ and a logratio cut-off at ±0.2 for regions sized 200-500 kbp and ±0.15 for regions longer than 500 kbp. These settings were defined through validation experiments in a previously described sample-set including 203 CLL samples.23 Copy-number variations are genomic variants that exist in the human population and these polymorphic regions can be detected as gains and losses.24 To exclude such polymorphic regions, we compared the regions detected in the copy-number analysis to frequency in a previously described sample-set including 203 CLL samples.23

Copy-number neutral loss-of-heterozygosity analysis
Analysis of CNN-LOH in CLL cells was performed using SNP array-data, taking into account the fraction of normal cells obtained from the flow-cytometry data, as previously described.27 CNN-LOH regions were visualized by mapping them to 200 kb segments beginning at the chromosome start site. Regions containing CNN-LOH larger than 3 Mbp with less than 50% overlap with copy-number variations were considered for further analysis.

Statistical analysis
Statistical analysis was performed using the Statistica Software 8.0 (Stat Soft Inc., Tulsa, OK, USA). A χ² test was applied to determine any statistically significant differences in the frequency of the known recurrent aberrations between the studied groups (subsets/non-subsets and our general Swedish CLL cohort). Similarly, the one-way ANOVA test and t-test were applied to determine any statistically significant differences in the number and size of copy-number aberrations between groups of patients.

Results
We investigated stereotyped and non-stereotyped IGHV3-21 and IGHV4-34 CLL samples using high-density 250K SNP arrays, with particular interest in detecting genomic alterations in these specific subsets. Figure 1 shows the distributions of the number and size of copy-number aberrations in the various subsets, while details concerning the known recurrent aberrations found in these subgroups are provided in Figure 2 and Table 2.

IGHV3-21 chronic lymphocytic leukemia - subset #2 versus non-subset #2
Our copy-number analysis showed that 97% (28/29) of subset #2 and 92% (12/13) of non-subset #2 IGHV3-21 samples carried copy-number aberrations (Figure 1A). Both subgroups displayed a similar average of copy-number aberrations/sample (2.6 vs. 2.3 copy-number aberrations/sample), although non-subset #2 patients carried larger copy-number aberrations compared to subset #2 (median size, 1.8 Mbp versus 1.39 Mbp, P=0.001) (Figure 1B, Online Supplementary Table S1). The known recurrent aberrations were detected in 90% of subset #2 and 54% of non-subset #2 patients (Table 2). The majority of subset #2 samples carried del(13q) (79%), while a smaller proportion of the non-subset #2 samples (31%) displayed this aberration (P=0.0024). A particularly high frequency of del(11q) was detected in subset #2 patients (9/29 samples, 31%) compared with our previous Swedish CLL array-based report (13%) (P=0.015) (Figure 2).26 Also, a relatively high frequen-
copy-number aberrations com pared to sub set #2 (median size, 1.8  Mbp)

IGHV4-34 chronic lymphocytic leukemia - subset #4 versus subset 16 versus non-subset #4/16
Assessment of copy-number aberrations showed that 76% of subset #4 (15/17), 75% of subset #16 (6/8) and 88% of non-subset #4/16 (30/34) IGHV4-34 patients carried such aberrations. The average number of copy-number aberrations/sample for non-subset #4/16, subset #16 and subset #4 cases were 2.1, 1.5 and 1.3, respectively (Online Supplementary Table S1). Subset #16 and non-sub-
set #4/16 carried larger aberrations than subset #4 samples (Figure 1B), although the median size was not statistically different (0.64 Mbp, 0.95 Mbp, 0.74 Mbp, respectively, P=0.24). The known recurrent aberrations were identified in 55% of subset #4, 25% of subset #16 and 59% of non-subset #4/16 cases (Figure 2 and Table 2). The exclusive recurrent aberration in subset #4 cases was deletion of 13q (6/6 cases with known recurrent aberrations). Deletion of 13q was also detected in 44% (15/34) of non-subset #4/16 cases, while this aberration was
absent in subset #16 cases. Deletion of 11q and trisomy 12 were only detected in subset #16 and non-subset #4/16 samples, whereas del(17p) was only observed in non-subset #4/16 samples (Table 2).

Upon removal of known recurrent aberrations from the analysis, losses were more frequent than gains in subset #4, and non-subset #4/16, but not in subset #16 (Online Supplementary Table S2). Furthermore, on examination of alterations greater or less than 1 Mbp, subset #4, subset #16 and non-subset #4/16 patients showed a similar average number of copy-number aberrations/sample and a comparable median length of the aberrations (Online Supplementary Table S4). Among the IGHV4-34 cases three recurrent aberrations were revealed. A deletion on 2q37.3 (5 Mbp), covering the telomeric region, was detected in two subset #4 and two non-subset #4/16 samples. Two samples in the non-subset #4/16 group showed a gain on 7q34 covering 0.3 Mbp, while one subset #4 and one non-subset #4/16 case carried an overlapping gain of 14q23.3 with an average size of 0.6 Mbp. Finally, non-recurring, larger aberrations (>20 Mbp) including trisomy 3, losses of 6q, 14q and gains of 8q, 13q, 22q were only detected in non-subset #4/16 (Online Supplementary Table S3).

**Copy-number neutral loss-of-heterozygosity in IGHV3-21 and IGHV4-34 chronic lymphocytic leukemia**

The evaluation of CNN-LOH, i.e. allelic imbalances without a change in copy-number, revealed that most regions were non-recurrent between samples. For instance, when regions greater than 3 Mbp were evaluated, ten non-overlapping regions were shown in subset #2/non-subset #2 IGHV3-21 samples, whereas 16 individual CNN-LOH regions were detected in subset #4 and non-subset #4/16 IGHV4-34 samples. However, a large recurrent CNN-LOH was detected on chromosome 13q in two subset #2, one subset #4 and two non-subset #4/16 samples, all of which carried a homozygous loss of 13q. Moreover, partially overlapping regions were identified on chromosome 20q11.21-q11.23 (overlapping region: 3.6

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**Figure 1.** Level of genomic complexity and size distribution of copy-number aberrations. The columns represent IGHV4-34 samples; subset #4 (n=17), subset #16 (n=8) and non-subset #4/16 (n=34); IGHV3-21 samples; subset #2 (n=29) and non-subset #2 (n=13) and a ‘general’ CLL cohort (n=203) from the study by Gunnarsson et al. Samples from the current study that overlapped with our recent array-based Swedish CLL cohort study were not removed from analysis, as the overlap between studies was limited. (A) Level of genomic complexity in each group is based on the frequency of samples that carry a certain number of CNAs (0, 1, 2 or ≥3). (B) The size distribution of copy-number aberrations (<1 Mbp, 1-5 Mbp or >5 Mbp) is shown as the frequency of the total number of copy-number aberrations. Note that subset #4 samples do not carry copy-number aberrations greater than 5 Mbp, whereas the non-subset #2 samples show the highest frequency of copy-number aberrations greater than 5 Mbp.

**Figure 2.** Frequency of patients carrying deletions of 11q and 13q individually or in combination. Each pie represents IGHV3-21 samples in subset #2 (n=29) and non-subset #2 (n=13), IGHV4-34 samples in subset #4 (n=17), subset #16 (n=8) and non-subset #4/16 (n=34), and a ‘general’ CLL cohort (n=203) from the study by Gunnarsson et al. Samples that carry ‘Any CNA’ also include del(17p) and trisomy 12, which were infrequently detected in this study, as indicated in Table 2.
Mb) in one subset #2 and one non-subset #2 IGHV3-21 sample. Among the IGHV4-34 patients, two non-subset #4/16 samples had an overlapping CNN-LOH of 3.2 Mb on chromosome 6p22.1.

Discussion

Recently, several groups have reported multiple CLL subsets carrying closely homologous BCR with similar heavy and light-chain CDR3 sequences. These stereotyped BCR suggest a potential role for antigens in leuke- morogenesis, a hypothesis also supported by recent studies detailing the BCR specificity in CLL. Interestingly, patients in CLL subsets expressing a certain stereotyped BCR have been indicated to share biological and clinical features, for instance IGHV3-21 subset #2 patients and IGHV4-34 subset #4 patients. Despite this, limited knowledge exists about the spectrum of genomic aberrations that occur in these different subsets, although such information might give hints of important genetic events occurring during disease development and/or evolution. To gain knowledge on this issue, we used a high-density SNP array to screen for whole-genome copy-number aberrations and CNN-LOH events in stereotyped subset #2 and subset #4/#16 cases and compared them to their non-stereotyped counterparts.

Focusing on IGHV3-21 patients, our analysis revealed that a high frequency of subset #2 and non-subset #2 samples carried copy-number aberrations. In fact, both subset #2 and non-subset #2 displayed a similar average number of copy-number aberrations/sample, which was comparable to the level observed in our recent array-based study on Swedish CLL. Hence, when accounting for all genom ic alterations, IGHV3-21 subset #2 and non-subset #2 cases do not appear to be more complex than other cases of CLL. That notwithstanding, differences were noted in the frequency of the known recurrent alterations, such as a high frequency of del(11q) among subset #2 patients (Figure 2). In fact, the frequency of del(11q) was higher in subset #2 than in either of our Swedish CLL study or other previously reported FISH studies in CLL, although a relatively high frequency was also observed in non-subset #2 patients.

Both ATM and TP53 are important for maintaining genomic stability, with the former acting as a positive regulator of TP53. To assess the possibility that patients carrying a deletion of ATM also have TP53 mutations, which may contribute to an adverse prognosis in CLL, we sequenced all IGHV3-21 patients with del(11q) in search of TP53 mutations (exons 4-8). However, none of the patients displayed any TP53 mutation, which suggests that the deletion of ATM is sufficient to contribute to the adverse outcome observed in IGHV3-21-expressing CLL, particularly in subset #2 patients. Furthermore, the frequency of del(13q) was considerably higher in subset #2 than in non-subset #2 cases or in any other CLL material reported to date. These aberrations may represent important genetic events during the pathogenesis of IGHV3-21 CLL, particularly when considering the frequent finding of concurrent 11q and 13q deletions, especially in subset #2 cases (Figures 2 and 3). Hypothetically, these aberrations may be acquired during the phase of active stimulation by (unknown) antigens, which trigger the IGHV3-21 precursor cell to undergo rapid cell division and hence make it susceptible to clonal alterations.

<table>
<thead>
<tr>
<th>Subgroup:</th>
<th>Known recurrent aberrations in IGHV3-21 and IGHV4-34 subsets compared to in a general chronic lymphocytic leukemia cohort.</th>
<th>General CLL cohort</th>
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<tbody>
<tr>
<td>Subsets: (number)</td>
<td>IGHV3-21</td>
<td>IGHV4-34</td>
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<tr>
<td>Subset #2</td>
<td>Non-subset #2</td>
<td>Subset #4</td>
</tr>
<tr>
<td>Subset #2</td>
<td>(n=29)</td>
<td>(n=13)</td>
</tr>
<tr>
<td>Known recurrent aberrations in total</td>
<td>32</td>
<td>11</td>
</tr>
<tr>
<td>del(11q)</td>
<td>9 (31%)</td>
<td>3 (23%)</td>
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<td>26.1 Mb</td>
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<td>del(13q)</td>
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<td>2 (13%)</td>
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<td>del(17p)</td>
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</table>

Samples from this study that overlapped with our array-based Swedish CLL study were not excluded from the large cohort, since this would have affected the comparison to general CLL material in which IGHV3-21 and IGHV4-34 samples are represented (6.4% and 8.9% of samples, respectively). *Homozygous and heterozygous deletions of 13q were combined to calculate average size (the average size was similar if they were divided into homozygous/heterozygous losses separately).
subset #4 CLL cells may represent a population of cells following these lines of evidence, it seems plausible that in the aforementioned study showed a low percentage of CLL cells (7-15%) carrying del(13q) by applying FISH (K. Stamatopoulos, unpublished data), which would have remained undetected by SNP-array. What could be the reasons why subset #4 cases acquire fewer genomic aberrations than subset #2 cases? We speculate that one important aspect could be the type of antigen stimulation that subset #4 CLL cells experience. Interestingly, IGHV4-34 antibodies are inherently autoreactive due to their recognition of the carbohydrate epitopes on the surface of red blood cells. Sequence alterations by somatic hypermutation may be a means to eliminate or abrogate this autoreactivity, and thus, render IGHV4-34-expressing cells sufficiently safe to enter the peripheral repertoire. In analogy to this observation, it is perhaps relevant to note that (i) IGHV4-34-expressing CLL is most often mutated, and (ii) IGHV-mutated CLL cells have been shown to be less responsive than their unmuted counterparts upon IgM-crosslinking. Hence, subset #4 CLL cells may represent a population of cells which are relatively anergic upon BCR crosslinking. Additionally, we have recently demonstrated that subset #4 CLL cells show extensive intraclonal diversification, possibly due to ongoing antigenic stimulation. Following these lines of evidence, it seems plausible that continuous (super)antigenic interaction may be necessary for IGHV4-34 CLL clones to be maintained, yet the low-proliferative “anergic” state cannot be overcome, thereby preventing the accumulation of additional genomic alterations. This is supported by our findings that subset #4 cases in general showed fewer copy-number aberrations, carried only the good prognostic marker del(13q), and followed a very indolent disease course. Although our high-density SNP-arrays could detect some novel aberrations, few of these were overlapping in subset and non-subset groups. For example, a concurrent gain on chromosome 2q and a loss at 3p were identified in two subset #2 cases. In IGHV4-34 CLL, three recurrent aberrations were observed on chromosomes 2q, 7q and 14q but not in any particular subset. Furthermore, evaluation of CNN-LOH events showed that small non-recurring regions were frequent in all subset and non-subset groups. When focusing on larger regions (>5 Mbp), overlapping CNN-LOH events were detected on chromosome 6 in two non-subset #4/16 patients and on chromosome 20 in one subset #2 and non-subset #2 case. As described in previous CLL studies, we identified five cases with CNN-LOH on chromosome 13q. This recurrent event was detected in both IGHV3-21 and IGHV4-34 CLL with no bias towards any subset/non-subset group. Since CNN-LOH on 13q always harbored a homozygous del(13q), this LOH might be of specific importance in CLL biology although not specifically in relation to stereotypy. In conclusion, high-density screening for genomic aberrations revealed differences in the genomic spectra between subset #4 and subset #2/non-subset #2. As the frequencies of del(13q) and del(11q) were particularly high in subset #2, these deletions probably represent important genetic events during the development of IGHV3-21 CLL. In contrast, subset #4 patients exclusively showed del(13q) and had a lower overall incidence of genomic aberrations. These features suggest that genomic aberrations may not play such a significant role in the pathogenesis of subset #4 CLL cases, which is reflected by their indolent disease course.

Authorship and Disclosures

MiM, NC and RG performed research, analyzed data and wrote the paper. AI, HG and MR performed bioinformatic analyses. MJ and MaM performed laboratory work and analyzed data. FR supervised the research. KK, HOA, JJ, and FD provided samples and associated data. GJ, KS and RR supervised the research and wrote the paper. The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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