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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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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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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.

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.[1,2] 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*.[2-4] Several groups have reported multiple CLL subsets with almost identical, 'stereotyped' B-cell receptors (BCR) in up to about 30% of patients.[3,5-11] 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.[6,8-10] 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 leukemogenesis 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.[9,10] Interestingly, CLL subsets expressing a certain stereotyped BCR have also been indicated to share biological and clinical features.[9,12] 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*.[9] Furthermore, subset #4 patients have a low median age at diagnosis, IgG-switched BCR[6,9,13] and a potential association with persistent infection by common herpesviruses.[14] 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 (known as subset #2) with a short and highly similar CDR3 (9 amino acids long) and usage of one particular *IG* lambda gene, *IGLV3-21*.[5,12,15] However, it appears that *IGHV3-21* patients with stereotyped and non-stereotyped BCR share an equally poor overall survival, independently of *IGHV* mutational status[5,15,16] although stereotypy has been associated with a shorter time to progression and the presence of other markers of a poor prognosis.[9,17]

No single genetic event has been found in all cases of CLL, although certain recurrent aberrations (e.g. deletions of 13q, 11q, 17p and trisomy 12) are frequently detected and can assist outcome prediction.[18] Patients carrying del(13q) as the sole abnormality have a favorable prognosis,[19] whereas patients with del(11q) (*ATM*) and del(17p) (*TP53*) have an inferior outcome.[18] Until now, few studies have investigated genomic aberrations specifically in relation to CLL subsets with stereotyped BCR. In one study, fluorescence *in situ* hybridization (FISH) analysis indicated a higher frequency of del(11q) in *IGHV3-21* CLL[15] and, in another study, del(13q) was detected more frequently in *IGHV4-34* subset #4 patients than in non-subset patients, who carried more heterogeneous aberrations.[20]

Since genomic profiling of stereotyped subsets might offer clues about the underlying leukemic processes, we here applied 250K single nucleotide polymorphism (SNP)-arrays to investigate the presence of whole-genome copy-number aberrations and copy-number neutral loss of heterozygosity (CNN-LOH) in CLL patients with stereotyped and non-stereotyped *IGHV3-21* and *IGHV4-34* BCR. These relatively common subsets were chosen for analysis as they display distinctive as well as divergent clinical and biological features.

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

Samples (n=101)	Subset #2 (n= 29)	Non-subset #2 (n=13)	Subset #4 (n=17)	Subset #16 (n=8)	Non-subset #4/16 (n=34)
Sex					
Male	19	6	8	3	23
Female	10	7	9	5	1
Median age at diagnosis years (range)	62 (50-86)	63 (45-82)	57 (37-72)	68 (52-78)	60 (42-72)
Binet stage					
A	12	4	14	6	27
B	8	4	2	1	1
C	2	2	0	0	1
Not defined	7	3	1	1	5
***IGHV* mutation status**[a]					
Mutated	20	5	17	8	27
Unmutated	9	8	0	0	7
Treated[b]					
Yes	7	2	2	1	14
No	13	8	14	7	15
Not defined	9	3	1	0	5
Alive					
Yes	13	3	15	6	28
No	14	9	2	1	5
Not defined	2	1	0	1	1

[a]Cases with ≥98% identity to germline were classified as unmutated, whereas cases with <98% identity were considered mutated. [b]Denotes treatment status of patient at sampling. Classified as stereotyped (subset) or non-stereotyped (non-subset) according to previously defined criteria.[9,10]

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,[21] 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 Stamatopoulos *et al.* and Murray *et al.*[9,10] Twenty-nine samples were classified as subset #2 (*IGHV3-21/IGLV3-21* usage, CDR3 of 9 amino acids long) and 13 samples as non-subset #2 (*IGHV3-21* usage, heterogeneous CDR3 lengths). 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 701930 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 log₂ratio 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 log₂ratios, copy-number 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 3.0 software (BioDiscovery, El Segundo CA, USA). Copy-number analysis was performed using a significance threshold (P value) of 1×10^{-6} and a log₂ratio 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 frequent regions of copy-number variations reported by Redon *et al.*[25] and MacCarroll *et al.*[26] Regions that overlapped by less than 50% with copy-number variations were considered as true copy-number aberrations and included in the final analysis, as previously described.[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 χ^2 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=0.013) (Figure 2).[23] Also, a relatively high frequency of del(11q) was found in non-subset #2 cases (23%). Furthermore, of the samples with del(11q), 75% (9/12) also carried del(13q) (Figure 2-3). All 11q deletions were shown to cover the *ATM* gene. del(17p), covering the *TP53* gene, and trisomy 12 were only detected in non-subset #2 patients.

When excluding the known recurrent aberrations, losses were shown to be more common than gains in both subset #2 and non-subset #2 (*Online Supplementary Table S2*). When considering alterations less than or greater than 1 Mbp in size, a similar average of copy-number aberrations/sample was observed within subset #2 and non-subset #2 cases (*Online Supplementary Table S1*). Only two other recurrent alterations were identified on chromosomes 2q and 3p. Specifically, two subset #2 samples (6.9%) displayed a gain at 2q32.1 with an average size of 0.21 Mbp. These samples also demonstrated concurrent deletions of 11q and 13q. Furthermore, two subset #2 samples with 13q deletion demonstrated a loss at 3p21.31-3p21.1 with an average size of 2.3 Mbp. Here, one non-subset #2 patient carried del(17p), trisomy 12, a loss at 7q11.21 (0.7 Mbp) and a large novel loss at 16q11.2-16q21 (13.4 Mbp). In addition, large (>20 Mbp) non-recurring aberrations were identified at 4q, 4p, 5q, 14q and 21q, predominantly within non-subset #2 cases (*Online Supplementary Table S3*).

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 (13/17), 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-subset #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 35% 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 S1*). 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 Mp 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
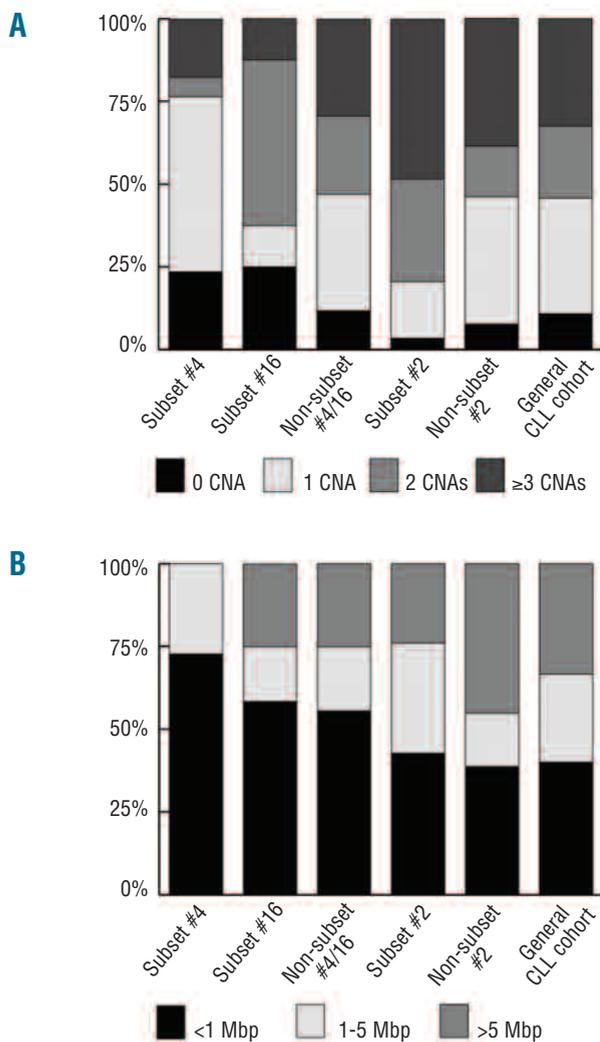
Figure 1. Level of genomic complexity and size distribution of copy-number aberrations. The columns represent *IGHV4-34* samples; subset #4 (n=17), subset #16 (n=8) and non-subset #4/16 (n=34); *IGHV3-21* samples; subset #2 (n=29) and non-subset #2 (n=13) and a 'general' CLL cohort (n=203) from the study by Gunnarsson *et al.*[23] Samples from the current study that overlapped with our recent array-based Swedish CLL cohort study[23] 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.
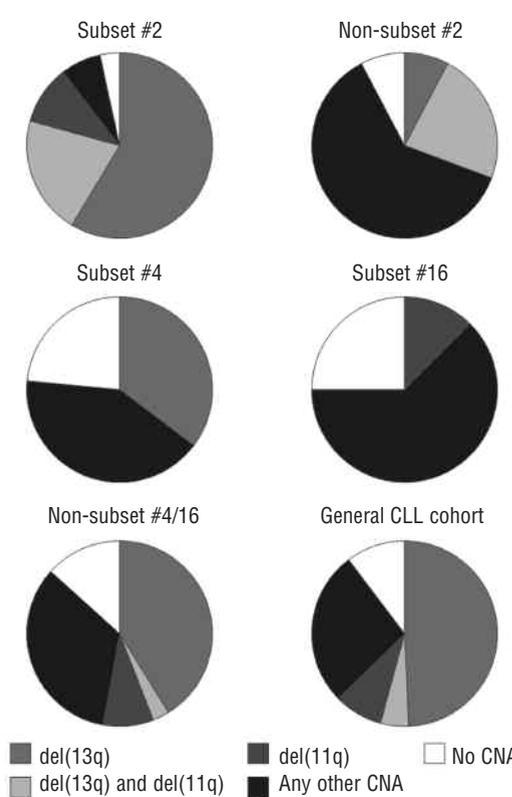
Figure 2. Frequency of patients carrying deletions of 11q and 13q individually or in combination. Each pie represents *IGHV3-21* samples in subset #2 (n=29) and non-subset #2 (n=13), *IGHV4-34* samples in subset #4 (n=17), subset #16 (n=8) and non-subset #4/16 (n=34), and a 'general' CLL cohort (n=203) from the study by Gunnarsson *et al.*[23] Samples that carry 'Any CNA' also include del(17p) and trisomy 12, which were infrequently detected in this study, as indicated in Table 2.

Mbp) 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 Mbp 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.[6-10] These stereotyped BCR suggest a potential role for antigens in leukemogenesis, a hypothesis also supported by recent studies detailing the BCR specificity in CLL.[28-32] 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.[9,12] 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.[23] Hence, when accounting for all genomic 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 our Swedish CLL study or other previously reported FISH studies in CLL,[15,18,23] 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.[18,23] 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.

When investigating subset #4 *IGHV4-34* patients, a group known to have a favorable prognosis with few patients needing treatment, we observed a significantly lower average number of aberrations compared to that in non-subset #4/16, subset #2, non-subset #2 and patients in our recent array-based study (*P*=0.0067, *Online Supplementary Table S1*).[23] This difference was due to the fact that subset #4 carried a higher number of samples with no genomic aberrations compared to subset #2 and non-subset #2 samples (17% *versus* 5%), and that a larger proportion of subset#2/non-subset #2 patients displayed a higher number of copy-number aberrations per sample (Figure 1). Furthermore, the frequency of samples carrying the known recurrent alterations was considerably lower in subset #4 (Table 2). Indeed, the only known recurrent aberration identified in subset #4 was the favorable prognostic marker del(13q) (Figure 2), albeit detected at a lower frequency (35%) compared to a recent report, in which FISH analysis detected del(13q) in 62% of subset #4 cases.[20] However, this apparent discrepancy may be attributed to the fact that many of the subset #4 samples includ-

Table 2. Known recurrent alterations in *IGHV3-21* and *IGHV4-34* subsets compared to in a general chronic lymphocytic leukemia cohort.

Subgroup: Subsets: (number)	*IGHV3-21*			*IGHV4-34*		General CLL cohort
	Subset #2 (n=29)	Non-subset #2 (n=13)	Subset #4 (n=17)	Subset #16 (n=8)	Non-subset #4/16 (n=34)	All (n=203)
Known recurrent aberrations in total	32	11	6	2	22	169
del(11q)	9 (31%)	3 (23%)	0	1 (13%)	4 (12%)	27 (13%)
Average size of del(11q)	24.7 Mbp	26.1 Mbp	-	29.9 Mbp	23.8 Mbp	29.5 Mbp
del(13q) Homozygous del(13q)	23 (79%) 6	4 (31%) 0	6 (35%) 1	0 0	15 (44%) 1	110 (54%)
Average size del(13q) *	5.4 Mbp	13.6 Mbp	1.35 Mbp	-	7.8 Mbp	4.6 Mbp
Combination of del(11q) and del(13q)	6 (21%)	3 (23%)	0	0	1 (3%)	10 (5%)
Trisomy 12	0	3 (23%)	0	1 (13%)	2 (6%)	23 (11%)
del(17p)	0	1 (8%)	0	0	1 (3%)	9 (4%)

Samples from this study that overlapped with our array-based Swedish CLL study[23] 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 heterozygous/homozygous losses separately).*
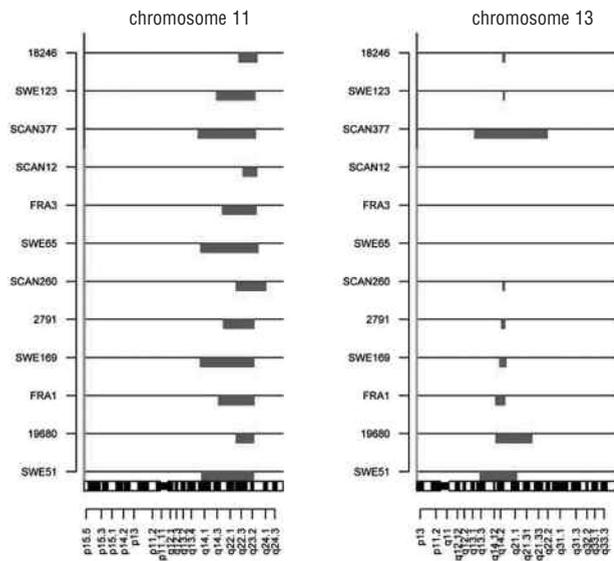
Figure 3. A high frequency of patients with *IGHV3-21* with del(11q) have a concomitant del(13q). All 12 *IGHV3-21* patients who carried del(11q) are displayed. The upper three are non-subset #2 patients and the remaining belong to subset #2.

ed 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.[33-35] 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.[33,36] In analogy to this observation, it is perhaps relevant to note that (i) IGHV4-34-expressing CLL is most often mutated,[10] and (ii) *IGHV*-mutated CLL cells have been shown to be less responsive than their unmutated counterparts upon IgM-crosslinking.[37] Hence, subset #4 CLL cells may represent a population of cells which are relatively anergic upon BCR crosslinking.[13] Additionally, we have recently demonstrated that subset #4 CLL cells show extensive intraclonal diversification, possibly due to ongoing antigenic stimulation.[14,38] 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 few 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 (>3 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.[23,39,40] 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.

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