Array-based genomic screening at diagnosis and during follow-up in chronic lymphocytic leukemia

Rebeqa Gunnarsson,[1,2] Larry Mansouri,[2] Anders Isaksson,[3] Hanna Göransson,[3] Nicola Cahill,[2] Mattias Jansson,[2] Markus Rasmussen,[3] Jeanette Lundin,[4] Stefan Norin,[5] Anne Mette Buhl,[6] Karin Ekström Smedby,[7] Henrik Hjalgrim,[8] Karin Karlsson,[1] Jesper Jurlander,[6] Christian Geisler,[6] Gunnar Juliusson,[1] and Richard Rosenquist[2]

[1]Department of Laboratory Medicine, Stem Cell Center, Hematology and Transplantation, Lund University, Lund, Sweden; [2]Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden; [3]Department of Medical Sciences, Cancer Pharmacology and Informatics, Uppsala University, Uppsala, Sweden; [4]Departments of Hematology/Oncology, Karolinska University Hospital, Stockholm, Sweden; [5]Department of Hematology, Karolinska University Hospital, Stockholm, Sweden; [6]Department of Hematology, Leukemia Laboratory, Rigshospitalet, Copenhagen, Denmark; [7]Department of Medicine, Clinical Epidemiology Unit, Karolinska Institutet, Stockholm, Sweden; and [8]Department of Epidemiology Research, Statens Serum Institut, Copenhagen, Denmark

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Correspondence: Rebeqa Gunnarsson, PhD, Department of Immunology, Genetics and Pathology, Rudbeck Laboratory, Uppsala, University, SE-75185 Uppsala, Sweden, Phone: international +46.018.4714852. Fax: international +46.018.559831. E-mail: rebeqa.gunnarsson@igp.uu.se

The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

High-resolution genomic microarrays enable simultaneous detection of copy-number aberrations such as the known recurrent aberrations in chronic lymphocytic leukemia [del(11q), del(13q), del(17p) and trisomy 12], and copy-number neutral loss of heterozygosity. Moreover, comparison of genomic profiles from sequential patients' samples allows detection of clonal evolution.

Design and Methods

We screened samples from 369 patients with newly diagnosed chronic lymphocytic leukemia from a population-based cohort using 250K single nucleotide polymorphism-arrays. Clonal evolution was evaluated in 59 follow-up samples obtained after 5-9 years.

Results

At diagnosis, copy-number aberrations were identified in 90% of patients; 70% carried known recurrent alterations, including del(13q) (55%), trisomy 12 (10.5%), del(11q) (10%), and del(17p) (4%). Additional recurrent aberrations were detected on chromosomes 2 (1.9%), 4 (1.4%), 8 (1.6%) and 14 (1.6%). Thirteen patients (3.5%) displayed recurrent copy-number neutral loss of heterozygosity on 13q, of whom 11 had concurrent homozygous del(13q). Genomic complexity and large 13q deletions correlated with inferior outcome, while the former was linked to poor-prognostic aberrations. In the follow-up study, clonal evolution developed in 8/24 (33%) patients with unmutated *IGHV*, and in 4/25 (16%) *IGHV*-mutated and treated patients. In contrast, untreated patients with mutated *IGHV* (n=10) did not acquire additional aberrations. The most common secondary event, del(13q), was detected in 6/12 (50%) of all patients with acquired alterations. Interestingly, aberrations on, for example, chromosome 6q, 8p, 9p and 10q developed exclusively in patients with unmutated *IGHV*.

Conclusions

Whole-genome screening revealed a high frequency of genomic aberrations in newly diagnosed chronic lymphocytic leukemia. Clonal evolution was associated with other markers of aggressive disease and commonly included the known recurrent aberrations.

Key words: chronic lymphocytic leukemia, chronic lymphocytic leukemia, SNP-array, genomic aberrations, CNN-LOH, clonal evolution.

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Introduction

In chronic lymphocytic leukemia (CLL), recurrent genomic aberrations and molecular markers, such as the mutation status of the immunoglobulin heavy variable (*IGHV*) genes, act as prognostic markers, which are useful for risk assessment.[1-5] The known recurrent genomic aberrations, *i.e.* deletions of 11q, 13q, 17p and trisomy 12, were first described as a result of cytogenetic analyses, but are now routinely identified by fluorescence *in-situ* hybridization (FISH).[2] The 13q deletion is detected in approximately half of CLL patients, and is associated with an indolent disease course when detected as the sole aberration.[2,4] The two micro-RNA, *miR-15a* and *miR-16-1*, which are located within this deletion, have been postulated as "causal genes" in CLL.[6-9] Moreover, the importance of these microRNA in the pathogenesis of CLL was recently illustrated *in vivo*, through generation of a mouse model of CLL that mimics the 13q14 minimally deleted region.[10] Deletion of 11q, which covers the *ATM* gene, and trisomy 12, have been detected in approximately 15-20% of cases of CLL, and are associated with poor and intermediate prognosis, respectively.[2] The deletion of 17p, indicating the worst prognosis in CLL, covers the *TP53* gene and is often accompanied by *TP53* mutations on the remaining allele and a high number of genomic aberrations.[11-13]

The application of microarrays in genomic studies of CLL has provided additional information on genomic alterations such as gain of 2p and 8q,[14-18] and has been useful for frequency and breakpoint analyses of 13q deletions.[19,20] Moreover, this screening method allows investigation of genomic complexity, for instance in samples with 17p deletion, which appears to be related to poor outcome.[12,18] Furthermore, copy-number neutral loss-of-heterozygosity (CNN-LOH) has been identified on chromosomes 11p, 13q, and 17p in single nucleotide polymorphism (SNP) array studies in CLL.[16,8,21]

Clonal evolution in CLL, i.e. the emergence of subclones that have acquired novel aberrations over time, has been investigated almost exclusively by applying FISH or cytogenetics. The follow-up studies have revealed clonal evolution in up to 43% of samples.[22-29] Besides the poor-prognostic deletion of 11q and 17p, deletion of 6q has also been implicated as a marker of recurrent progression.[2,23,29] Moreover, clonal evolution has been associated with unmutated *IGHV* genes, ZAP70 positivity, disease progression and poor outcome.[23,28,29] Nevertheless, since the former follow-up studies were performed with targeted FISH probes or genomic techniques with low resolution, knowledge of clonal evolution in a whole-genome perspective is scarce.

In the present study, we applied a 250K SNP-array (Affymetrix) to screen for genomic aberrations in patients with newly diagnosed CLL (n=369), drawn from a population-based CLL cohort, and for clonal evolution in 59 follow-up samples obtained after 5-9 years.

Design and Methods

Patients

The 369 CLL patients were selected for inclusion in the present study from a Scandinavian population-based case-control study, including patients between 18 and 74 years, called the SCALE (Scandinavian Lymphoma Etiology) study.[30] Data from 203 patients' samples were included in a recent interim analysis and reported in a "Letter to the Editor".[18] Peripheral blood was collected within a median of 3 months from diagnosis during 1999-2001, before FISH analysis was performed routinely. All samples were evaluated according to the recently revised criteria,[31] showing typical immunophenotype and more than 70% tumor cells. The male:female ratio was 2:1, the majority of patients were in Binet stage A (71%) and the median age at diagnosis was 63 years. Clinical data were obtained from medical records and the median follow-up time was 91 months. All patients were untreated at sampling and 145 patients of the 278 patients with known treatment status had received treatment at the last clinical follow-up. During this period most symptomatic patients were treated with fludarabine with or without cyclophosphamide, and some with chlorambucil alone, whereas antibody-containing regimens were rarely used in first-line treatment.

We selected three groups for the investigation of clonal evolution; (i) 24 patients carrying unmutated *IGHV* genes (20 treated), (ii) 25 treated patients with mutated *IGHV* genes, and (iii) 10 patients with mutated *IGHV* genes who remained untreated. The 59 follow-up samples that were included were collected during 2007-2008 at a median time from diagnosis to sampling in follow-up of 80 months (range, 60- 108 months). It is noteworthy that none of the patients with initial 17p deletion was alive at follow-up sampling and could not, therefore, be investigated for clonal evolution.

Informed consent was obtained according to the Helsinki declaration upon inclusion in the founding study, and the study was approved by the Ethics Review Committee at Karolinska Institutet, Stockholm (n. 99-154 and 2006/964-31/2), the Danish Data Protection Agency (2006-41-730) and Scientific Ethics Committee System KF 2006-753. The *IGHV* gene analysis is described in the *Online Supplementary Appendix*.

Microarray single nucleotide polymorphism analysis (250K Affymetrix arrays)

Array experiments were performed according to the standard protocols for Affymetrix GeneChip® Mapping Nsp1-250K arrays (Gene Chip Mapping 500K Assay Manual (P/N 701930 Rev2.), Affymetrix Inc., Santa Clara, CA, USA). Quality control, genotype calling and probe level normalization were performed in the Affymetrix GeneChip® Genotyping Analysis Software (GTYPE) 4.1. The Dynamic Model algorithm was used to perform single sample quality control and genotype calls were made using BRLMM.[32] The applied quality control specifies a neighbor score which is an average of the Euclidean distances between the $\log_2$ratio of five consecutive SNP along chromosomes where low neighbor scores indicate a low level of noise. The neighbor score had to be 0.4 or less for inclusion of samples. Copy number normalization was performed in order to produce $\log_2$ratios using the Copy Number Analysis Tool (CNAT) 4.0.1. Eighty-two normal samples analyzed at the Uppsala Array Platform were used as a reference set. All raw and normalized data from the SNP-arrays can be accessed through Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo*, accession number will be provided soon). Details on copy-number analysis and identification of copy-number neutral loss of heterozygosity are provided in the *Online Supplementary Appendix*.

Statistical analysis

Significance testing for aberrant copy-number (STAC), a method for testing the significance of small copy-number aberrations (CNA) across multiple array experiments, was performed in Nexus (settings $P \leq 0.05$, frequency=1%).[33] Associations between large recurrent and known recurrent CNA were evaluated statisti-

cally by applying the χ^2 test. Receiver operating characteristics (ROC) curve analysis was applied to calculate the optimal cut-off for del(13q) size in patients with sole deletion 13q. The cut-off value predicting survival as above or below the cohort median with the highest sensitivity and specificity was used for further analysis. Kaplan-Meier survival analysis and the log-rank test were applied to calculate the differences in overall survival or time to treatment between different groups of patients, divided according to the presence of various markers. For multivariate analysis, a Cox proportional hazards model was applied to evaluate the possible association between single risk factors and overall survival as well as time to treatment. In the analyses based on known recurrent aberrations, patients who displayed more than one of these aberrations were classified according to the hierarchical model.[2] Overall survival was defined as the time from date of diagnosis until date of last follow-up or death in 364/369 patients. Time to treatment was defined as time from date of diagnosis to date of initiation of first treatment in 278/369 patients with known treatment status, of whom 145 were treated and 133 were untreated. Statistical analyses were performed using Statistica Software 8.0 (Stat Soft Inc., Tulsa, OK, USA).

Results

Overview of copy-number alterations in this cohort of chronic lymphocytic leukemia patients at diagnosis

Of the 369 CLL samples investigated, 333 (90%) carried CNA whereas no aberration was detected in 36 samples (10%). The majority of samples (71%) carried between one and three CNA, 16% showed four to nine aberrations, while only 2% were highly complex (≥10) CNA. Losses were more commonly detected than gains (69% *versus* 31%) with median sizes of 1.4 Mbp for losses and 0.98 Mbp for gains excluding trisomies. A large proportion (41%) of CNA were smaller than 1 Mbp, whereas 29% of CNA ranged between 1-5 Mbp and another 29% were greater than 5 Mbp. The CNAs detected in this CLL cohort at diagnosis are presented in *Online Supplementary Table S1*.

Known recurrent alterations

Deletions of 11q, 13q, 17p and trisomy 12 were detected in 259 samples (70%), constituting approximately one third (31.5%) of the total CNA in this cohort (Table 1). The most frequent aberration was deletion of 13q, detected in 203 CLL samples (55%). The minimally deleted region for deletion of 13q was mapped to 19 kbp and located at 13q14.3 (Figure 1). Among patients with del(13q), 155 (76%) had heterozygous deletions, while 48 (24%) showed homozygous deletions. The homozygous deletions were centered at the 13q14.3 region and had an average size of 1.8 Mbp (size range, 0.4-26 Mbp). Over half (52%) of these homozygous losses were small (<1 Mbp); however, 20 cases were encompassed by larger heterozygous deletions (*Online Supplementary Table S2*). In comparison, the heterozygous losses generally covered larger regions of the 13q-arm and had an average size of 5.5 Mbp (size range, 0.3-76.8 Mbp) (*Online Supplementary Table S2*). Trisomy 12 was the second most common aberration, identified in 39 patients (10.5%), followed by del(11q), which was detected in 37 samples (10%). The majority of the losses on 11q were larger than 20 Mbp, with the average length being 29 Mbp (size range, 9.5-55 Mbp). The minimally deleted region was defined as a 0.68 Mbp segment at 11q22.3 which encompassed the *ATM* encoding region in all cases. This region also includes, among other, the *RAB39*, *CUL5*, *ACAT1*, *NPAT* and *KDELC* genes. Deletion of 17p was noted in 13 samples (3.5%), including two smaller deletions of 10 and 20 Mbp at 17p13.1, covering the TP53 coding region (*Online Supplementary Figure S1*).

Table 1. Description of known recurrent alterations detected in 369 CLL patients.

Variable	del(11q)	del(13q)	del(17p)	trisomy 12
Total number (frequency)	37 (10%)	203 (55%)	13 (3.5%)	39 (10.5%)
Number (frequency) according to hierarchical model	37 (10%)	175 (47%)	13 (4%)	33 (9%)
Size range in Mbp	9.5-55	0.3-76.8	10- whole p-arm	-
Average (median) size in Mbp	29 (30.4)	5.6 (1.46)	-	-
Start and stop positions for MDR[1]	107266185-107946367	49616260-49635447	6946699-7923866	-
Chromosome localization of MDR	del(11)(q22.3)	del(13)(q14.3)	del(17)(p13.1)	-
Size of MDR[1]	0.68 Mbp	19 kbp	10 Mbp	-
Genes in MDR	*RAB39, CUL5, ACAT1, NPAT, ATM, KDELC2, EXPH5*	No genes[2] No genes	*TP53* and >60 other genes	-
Average number of total CNA/sample	4.1	2.2	9.2	2.7
Average number of CNA > 1 Mbp/sample	0.92	1.2	1.5	0.97
Average number of CNA > 5 Mbp/sample	2.2	0.33	4.9	1.5

111 samples did not carry any of the known recurrent alterations. These samples had an average number of 1.3, 1.83 and 0.16 aberrations in total, < 1 Mbp, and > 5 Mbp, respectively. [1]MDR: minimally deleted region. [2]The smallest deletions (n=11) which specified the MDR did not cover miR-15a/16-1 *or* DLEU2.

Detection of other small and large copy-number aberrations

Upon exclusion of known recurrent alterations, genome-wide analysis identified 331 CNA that were less than 1 Mbp (but >200 kbp according to our criteria); the mean size was 0.5 Mbp (median size, 0.46 Mbp). Losses were more commonly detected than gains, representing 58% *versus* 42% of cases, respectively. The majority of the small alterations were non-overlapping and common in all prognostic subsets when grouped according to the known recurrent alterations, as shown in Table 1.

We also evaluated large CNA (>1 Mbp) and, when excluding the known recurrent aberrations, detected 302 such CNA. Losses were over-represented among these aberrations (63% *versus* 37% gains) and the mean size of these CNA was 16.8 Mbp (median size, 4 Mbp). Most CNA larger than 1 Mbp were non-overlapping; however, in contrast to the small aberrations which were mostly detected in individual samples, large regions involving either the p- or q-arm or whole chromosomes were recurrent in some cases. These overlapping CNA included the following regions; amp(2)(p13.2p25.3) in five patients

(1.9%), del(4)(p15.2p16.3) in five patients (1.4%), del(6)(q16.2q27) in two patients (<1%), del(8)(p11.2p23.3) in six patients (1.6%), amp(8)(q21.1q21.3) in six patients (1.6%) and del(14)(q24.1q31.1) in six patients (1.6%) (Table 2). Interestingly, the 2p gains and del(11q) were found to be significantly associated i.e. concomitantly detected in five samples (P<0.001).

Detection of copy-number neutral loss of heterozygosity

CNN-LOH represents homozygous events without a change in copy-number. Evaluation of tumor cell-specific CNN-LOH in the 369 patients revealed recurrent copy number neutral regions covering large parts of chromosome 13q in 13 samples (3.5%) (*Online Supplementary Figure S2*). Among the patients detected with 13q CNN-LOH, 11 patients also carried a homozygous deletion of 13q14. No other recurrent region with CNN-LOH was detected in this cohort of patients.

Genomic complexity and 13q deletion size predicts overall survival and time to treatment

Survival analysis was performed according to the hierarchal model of known recurrent alterations.[2] Although the prognostic impact of 13q and 17p deletion was confirmed (P<0.0001), we observed a similar, more intermediate survival for patients with trisomy 12 or 11q deletions (Figure 2A). In addition, when investigating time to treatment, poor-prognostic aberrations were associated with a shorter time from diagnosis to initiation of treatment (P<0.0001), (Figure 2B). Moreover, in line with previous studies, Binet staging and *IGHV* mutation status were confirmed as significant prognostic factors for both overall survival and time to treatment (P<0.0001 for all analyses) (*Online Supplementary Figure S3*).

Next, patients carrying the del(13q) as the sole known recurrent aberration (n=174) were grouped according to deletion size by applying ROC analysis. Survival analysis of the two resulting groups revealed that patients with larger losses (>1.25 Mbp, n=94) had a worse outcome than patients with small aberrations (<1.25 Mbp, n=81) (P=0.018) (Figure 2C). Moreover, the group of patients with larger del(13q) had a shorter time from diagnosis to initiation of treatment (P<0.01) (Figure 2D).

Various cut-off levels for size of alterations (<1 Mbp, ≥1-5 Mbp and >5 Mbp) were applied to assign patients into particular groups. In keeping with our recent report,[18] we confirmed that a higher genomic complexity of CNA larger than 5 Mbp was strongly correlated with a worse survival (P<0.0001) and a shorter time before initiation of treatment (P<0.0001) (Figure 3A,B) Finally, we applied a multivariate model, including *IGHV* mutation status, known recurrent aberrations, Binet stage and genomic complexity as prognostic markers. In this analysis, genomic complexity failed to predict overall survival or time to treatment, whereas IGHV mutation status (P<0.0001), Binet stage (A *versus* B and C) (P<0.0001) and del(17p)

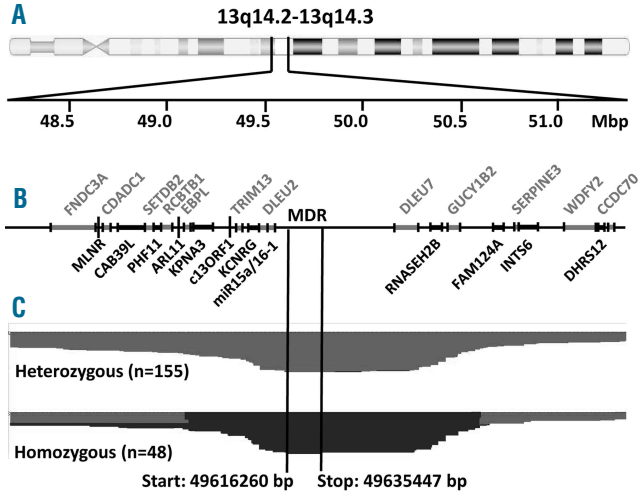

Figure 1. Genomic map of homozygous and heterozygous deletion 13q. (A) Chromosomal bands and location in Mbp, (B) genes and (C) a condensed view of the homozygous (light gray) and heterozygous (dark gray) deletions of the enlarged sub-region of deletion at 13q14.2-14.3. The single nucleotide polymorphisms defining the minimally deleted region (MDR) were rs706612 (49608579-49608580 bp) and rs1359612 (49612082-49612082 bp) for the centromeric breakpoint and rs1750567 (49635023-49635024 bp) and rs1798968 (49635869-49635870 bp) for the telomeric breakpoint.

Table 2. Large recurrent aberrations detected in the 369 CLL patients.

Aberration	Number of patients	Frequency	Start and stop positions of MDR	Overlapping chromosome bands of MDR	Size of overlapping region	Concurrence with known recurrent aberration
Gain of 2p	7	1.9%	0-72949795	amp(2)(p13.2p25.3)	72 Mbp	5 del(11q)* 2 del(13q)
Deletion of 4p	5	1.4%	0-26441706	del(4)(p15.2p16.3)	26.4 Mbp	2 del(11q)* 3 del(17p)*
Deletion of 6q	2	<1%	98910845-170899992	del(6)(q16.2q27)	72 Mbp	1 del(11q)1 del(13q)
Deletion of 8p	6	1.6%	0-38563227	del(8)(p11.2p23.3)	38 Mbp	2 del(11q) 1 del(13q) 2 del(17p)*
Gain 8q	6	1.6%	81588914-146274826	amp(8)(q21.1q21.3)	26.7 Mbp	4 del(11q)* 2 del(17p)*
Deletion 14q	6	1.6%	68379637-80652470	del(14)(q24.1q31.1)	11.8 Mbp	3 trisomy 12*
Trisomy 18	3	0.8%	-	-	whole chromosome	3 trisomy 12*
Trisomy 19	5	1.4%	-	-	whole chromosome	5 trisomy 12*

*Denotes significantly associated large recurrent and known recurrent aberrations according to the χ^2 test. MDR, minimally deleted region.

(P=0.014) were significant independent markers for overall survival. IGHV mutation status (P<0.0001) was also a strong independent marker for time to treatment.

Clonal evolution in chronic lymphocytic leukemia

Fifty-nine follow-up samples were included in the evaluation of clonal evolution in CLL over time and are presented with clinical data in *Online Supplementary Table S3*. Comparison of the SNP-array data from samples taken at diagnosis and follow-up (n=59) showed that 12/59 patients (20%) developed novel aberrations, which are briefly presented in Table 3. We divided the samples into different groups according to *IGHV* mutation status (24 unmutated and 35 mutated), and the group with mutated *IGHV* was further divided into two subgroups according to treatment status (10 untreated and 25 treated). At diagnosis CNA were detected in 20/24 (83%) of the patients with unmutated *IGHV* genes, in 20/25 (80%) of the treated patients with mutated *IGHV* and in 6/10 (60%) of the untreated patients with mutated *IGHV* genes. At the second sampling, 8/24 (33%) of the *IGHV* unmutated patients and 4/25 (16%) of the *IGHV* mutated/treated patients carried newly developed abnormalities (Table 3, *Online Supplementary Table S3*). In contrast, none of the ten untreated patients in with mutated *IGHV* genes acquired new aberrations. Two of the four *IGHV* mutated and treated patients who underwent clonal evolution acquired a del(13q), and a third case involved acquisition of del(17p). Thus, this subgroup only developed novel clones that had acquired known recurrent aberrations. Among the patients with unmutated *IGHV* genes, del(11q), del(13q) and del(17p) were acquired by two, four and one

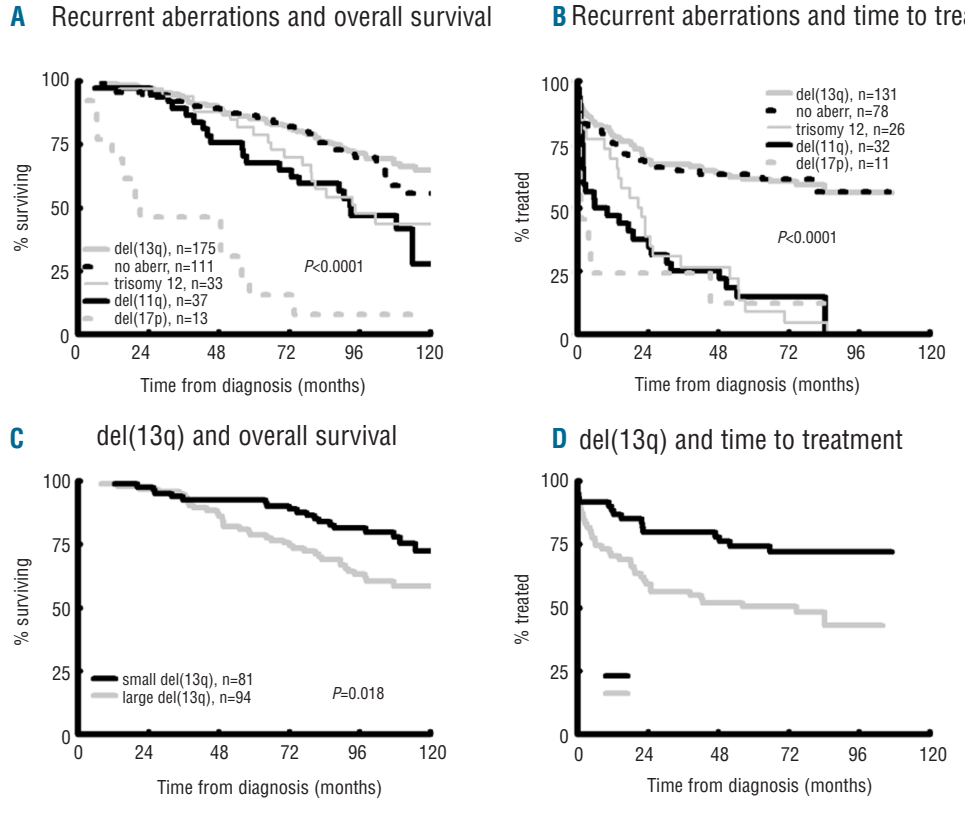

Figure 2. Survival and time to treatment according to known recurrent aberrations and deletion 13q size (Kaplan-Meier plots). (A) Patients with del(17p) had the worst outcome, whereas del(11q) and trisomy 12 indicated similar intermediate survival. Patients with no recurrent aberration and patients with del(13q) showed similar good overall survival (P<0.0001). (B) Patients with poor-prognostic markers had a shorter time to treatment than patients with del(13q) and no recurrent aberration (P<0001). (C-D) Receiver operating characteristics (ROC) curve analysis and overall survival or time to treatment was applied in order to identify the optimal size cut-off for del(13q). Patients with larger del(13q) (>1.25 Mbp) had an inferior prognosis in terms of overall survival (C) (P=0.016), and time to treatment (D) (P<0.01).

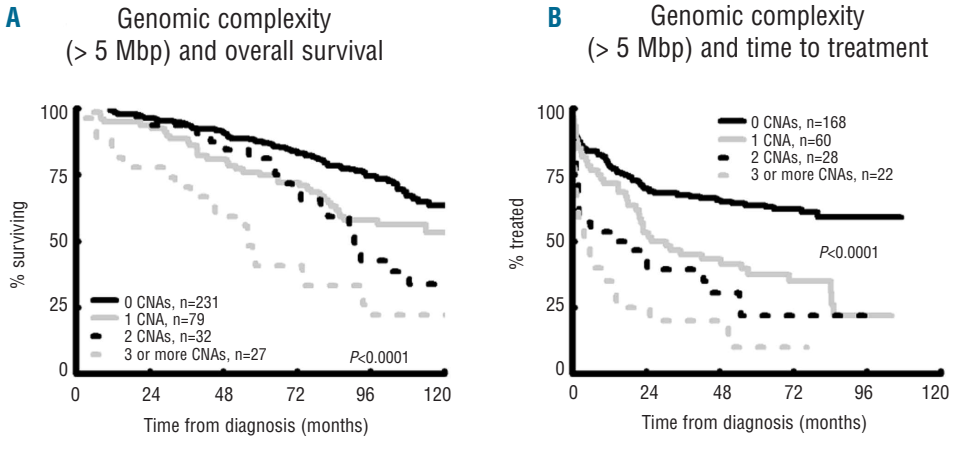

Figure 3. An increased genomic complexity predicts a worse survival and shorter time to treatment (Kaplan-Meier plots). Overall survival of patients was investigated according to the degree of genomic complexity, which showed (A) an inferior survival (P<0.0001) and (B) shorter time to treatment (P<0.0001) for patients carrying an increasing number of copy number aberrations larger than 5 Mbp.

patient, respectively. However, in this group of patients, the acquisition of novel aberrations was not restricted to the known recurrent aberrations, but was also found on other chromosomes, e.g. del(6)(q16.1q22.3), amp(8)(q12.2), del(10)(q23.3q26.1) and del(20)(q13.1) (Table 3, *Online Supplementary Table S3*). The acquisition of a deletion of chromosome 10q is illustrated in Figure 4A. Notably, the patient with unmutated *IGHV* who acquired del(17p) also had a complex genome (7 CNA) (Table 3, Figure 4B).

Discussion

In this study, we investigated genomic aberrations in newly diagnosed CLL patients and follow-up samples from a population-based cohort using high-density genomic SNP-arrays. To our knowledge, this is the largest such study of newly diagnosed unselected CLL patients to date. The evaluation of known recurrent alterations showed that del(13q) was the most common CNA (55%), confirming findings in other studies.[2,16,21,23] In line with a recent study by Mosca *et al.*, we noted that the heterozygous deletions (n=155) of chromosome 13q more frequently covered larger regions compared to the homozygous deletions (n=48), which in general were smaller and focused to 13q14 (Figure 1, *Online Supplementary Table S2*).[19] Accordingly, both the homozygous and heterozygous deletions of 13q covered the *miR-15a/16-1* loci in 94-95% of cases, in comparison to the *Retinoblastoma* (*Rb*) encoding region, which was covered by 10% of the homozygous deletions and 38% of the heterozygous deletions. This finding further supports the belief that *miR-15a/16-1* is involved in the pathogenesis of CLL in patients with 13q14 abnormalities.[6,9,10]

In agreement with a recent report by Strefford *et al.*, our analysis of overall survival and time to initiation of treatment in patients with a sole del(13q) revealed that large

Table 3. Presence of clonal evolution in follow-up samples.

Patient	CNA at diagnosis	CNA during follow up, clonal evolution
	***IGHV* mutated**	
SCAN171	No	CE: del(13)(q14.3)
SCAN174	No	CE: del(13)(q14.2q14.3)
SCAN126	del(13)(q14.1q14.3)	del(13)(q14.12q14.3) CE: del(17)(p13.3q11.1)
SCAN260	del(13)(q14.3) del(11)(q22.3q24.1)	del(13)(q14.3) CE: normal chr 11
	***IGHV* unmutated**	
SCAN392*	Heterozygous del(13)(q14.3)	homozygous del (13)(q14.3) CE: heterozygous del(13)(q13.3q14.3)
SCAN146	No	CE: del(11)(q21q24.2)
SCAN75	No	CE: del(6)(q16.1q22.3) CE: del(8)(p11.2p23.3) CE: amp(8)(q12.2) CE: del(9)(q31.1q32) CE: del(13)(q14.2q14.3) CE: del(17)(p12p13.3) CE: del(18)(p11.2p11.3)
SCAN3	Heterozygous del(11)(q14.1q23.3)	CE: heterozygous del(11)(q14.1q21) homozygous del(11)(q21q23.3)
SCAN279	del(11)(q14.1q23.3)	del(11)(q14.1q23.3) CE: del(13)(q14.1q21.1)
SCAN182	del(15)(q15.1)	del(15)(q15.1) CE: del(10)(q23.3q26.1) CE: del(20)(q13.1)
SCAN204	del(7)(p11.1p12.1) del(13)(q14.3)	del(7)(p11.1p12.1) del(13)(q14.3) CE: del(9)(p21.3)
SCAN6	amp(2)(p11.2p25.3)	amp(2)(p11.2p25.3)
	del(11)(q13.5q23.3) del(20)(p13q11.1)	del(11)(q13.5q23.3) del(20)(p13q11.1) CE: del(13)(q14.1q21.3)

Only patients who developed novel aberrations over time are shown. The patients are grouped according to IGHV *mutation/status and listed according to presence of CNA at diagnosis and during follow-up. All patients were diagnosed in Binet stage A except SCAN204 (Binets stage C) and all patients received the treatment between first and second sampling except SCAN392. Time from first to second sampling ranged from 77 to 96 months for the patients who developed novel aberrations. Acquired aberrations during follow-up are preceeded by clonal evolution (CE) as in clonal evolution.*

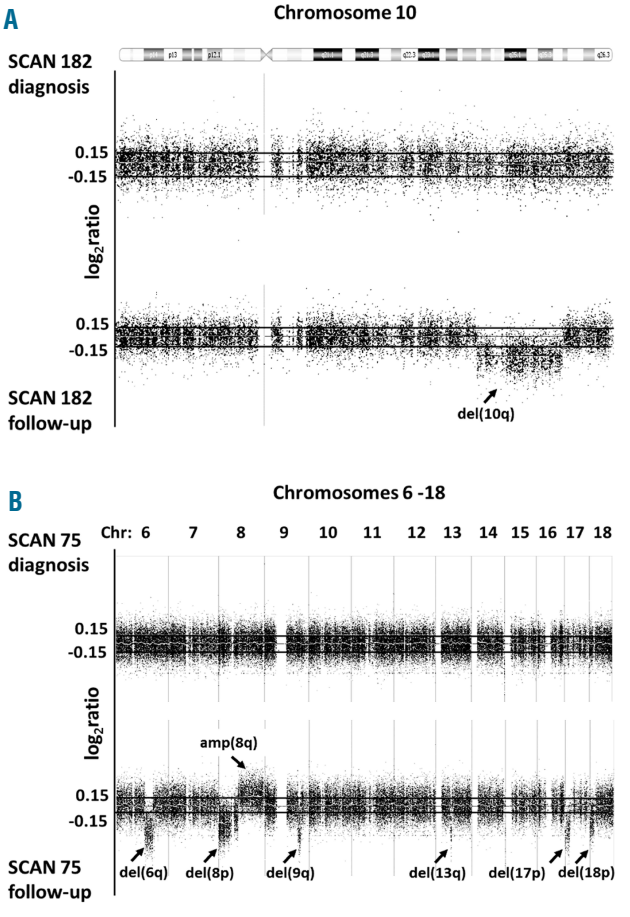

Figure 4. CLL patients with clonal evolution. (A-B) Examples of clonal evolution in two CLL patients for whom the first sample at diagnosis (upper panel) is compared to the follow-up sample in the lower panel of each figure. (A) SCAN 182 (*IGHV* unmutated and treated) had a normal copy-number of chromosome 10 at diagnosis, and developed a loss of the q-arm in the follow-up sample. (B) SCAN 75 (Binet stage A, *IGHV* unmutated) had no aberrations at diagnosis but showed a complex genome with deletions on 6q, 8p, 13q, 17p, 18p and a gain on 8q at follow-up after two lines of treatment with alkylating agents.

deletions of 13q were associated with a worse outcome.[34] This is reflected by the finding that CLL patients clustered according to del(13q) size show specific gene expression profiles.[19] Furthermore, a recent report by Klein *et al.* demonstrated that mice with larger deletions of the minimally deleted region display a more aggressive phenotype than mice with *miR-15a/16-1* deletions, suggesting that additional genetic elements are involved in the tumor suppressive function.[10]

The markers that predict poorer clinical outcome, i.e. del(11q), del(17p) and trisomy 12, were found at lower frequencies than previously reported, which reflects the population-based nature of this newly diagnosed cohort. Furthermore, a deletion on chromosome 6q was detected in only two patients (<1%). The low frequency of del(6q) supports the finding that this aberration is a marker of progression, as reported previously.[23,29] Although the prognostic impact of 13q and 17p deletion was confirmed, we observed a similar, more intermediate survival for patients with trisomy 12 or 11q deletions (Figure 2A).

Large recurrent regions with CNA were also detected on chromosomes 2, 4, 8, and 14 (Table 2). The most recurrent aberration was a gain on chromosome arm 2p (7 samples), which, in most cases, occurred together with a del(11q), as we recently reported.[18] Other recurrent aberrations included loss of a large part of chromosome 4p, and gains and losses of chromosome 8, in three cases through an isochromosome 8q, as both loss of 8p and gain of 8q were seen in these samples. Additionally, six patients displayed a deletion of 14q, an aberration that was reported in previous microarray studies.[16,35] However, in comparison to the findings in the study by Grubor *et al.*, the 14q losses detected in our study were large, ranging between 17.9-41.5 Mbp, thus covering numerous genes in addition to the *IGH* locus.[35] Trisomies were recurrently detected on chromosome 18 and 19, always in combination with trisomy 12. Moreover, trisomy 7, monosomy 9 and loss of chromosome 3p were detected in individual samples.

As indicated in our previous SNP-array study on 203 CLL samples, an increasing genomic complexity of aberrations greater than 5 Mbp was correlated with a shorter survival (Figure 3A).[18] Furthermore, an increasing complexity was also associated with shorter time to treatment when we used this surrogate marker as a clinical end-point (Figure 3B). Moreover, it was noted that large CNA were more prevalent in patients with poor-prognostic aberrations (Table 1). In detail, the patients with genomic complexity of CNA greater than 5 Mbp (n=27) included ten, eight and five patients carrying del(11q), del(17p) and trisomy 12, respectively. The four remaining patients had deletion of 13q (2 patients) or no recurrent aberration (2 patients). Hence, genomic complexity appears to be a marker of progressive disease and inferior survival in newly diagnosed CLL, although the majority of these patients also carried other poor-prognostic aberrations. Consequently, it is likely that the genetic defects resulting from del(11q) and del(17p) promote both an aggressive behavior of the disease and accumulation of a higher number of aberrations. In fact, the close association between genomic complexity and poor-prognostic aberrations is a possible explanation of why genomic complexity was not an independent prognostic marker in the multivariate analysis.

The evaluation of CNN-LOH was performed using a method which accounts for the proportion of tumor cells and excludes LOH-regions which overlap with CNA regions in individual samples, thus detecting tumor-specific CNN-LOH.[36] This evaluation confirmed the finding of CNN-LOH on chromosome 13q as a recurrent event in CLL (*Online Supplementary Figure S2*).[16,18,21] As noted in our interim-analysis, the majority (11/13) of these patients had a concurrent homozygous loss on chromosome 13q14.[18] Given that the CNN-LOH on chromosome 13 is a recurrent finding, we speculate that this type of genomic aberration might be a relatively common and important event during leukemogenesis in CLL, since it may involve an alternative mechanism for deregulation of *miR-15a/16-1* and/or additional genes involved in the pathogenesis of CLL.

Our investigation of clonal evolution included 59 patients from a population-based cohort, from whom samples were taken at diagnosis and between 5 and 9 years later, during follow-up (Table 3, *Online Supplementary Table S3*). The long period between the first and second samples made it impossible to investigate clonal evolution in the patients with the most aggressive disease (e.g. with 17p deletion), since these patients were no longer alive at the time of second sampling. On the other hand, most of these patients showed genomic complexity already at diagnosis, indicating previous acquisition of additional aberrations and clonal evolution. Thus, we evaluated follow-up samples from patients with the expected highest risk of clonal evolution among the survivors [i.e. patients with unmutated *IGHV* (n=24) and patients with mutated *IGHV* who had been treated (n=25)], and included ten 'control' samples from patients with stable untreated disease and an expected low risk of clonal evolution (i.e. untreated patients with mutated *IGHV*). In agreement with the findings of a recent FISH study on clonal evolution by Stilgenbauer *et al.*, we found that acquisition of aberrations was common in the group of patients with unmutated *IGHV* genes, as we detected clonal evolution in 33% of such patients.[29] Interestingly, a notable proportion (16%) of *IGHV* mutated/treated patients developed novel aberrations over time. In contrast, none of the untreated patients with mutated *IGHV* genes acquired novel aberrations during their follow-up. This further supports the hypothesis that an aggressive disease will encourage evolution of subclones, whereas an indolent disease will have a lower risk of acquisition of novel aberrations and clonal expansion. Other studies on clonal evolution in CLL have reported a correlation between unmutated *IGHV* genes, CD38 positivity and/or a high ZAP70 expression alone and clonal evolution, which corroborates the conclusion that patients with a more aggressively progressing disease are more likely to acquire new aberrations.[22,23,28,29]

When evaluating the alterations that were acquired over time, we found that the del(13q) was the most common aberration in the follow-up samples, as 2/4 *IGHV* mutated and treated and 4/8 *IGHV* unmutated cases that underwent clonal evolution acquired this deletion. Thus, del(13q) is not only a common event in early stages of the disease, but can also develop during later stages when the disease progresses. In contrast, novel CNN-LOH was not detected on chromosome 13q during follow-up, indicating that this specific event develops during early stages of the disease. Deletions of 11q (n=3) and 17p (n=2) and 6q (n=1) were also involved in clonal evolution (Table 3, *Online Supplementary Table S3*, Figure 3C). In particular, one patient with unmutated *IGHV* who had a heterozygous

del(11)(q14.1q23.3) at diagnosis developed a homozygous 11q deletion during follow-up.

In contrast to previous follow-up studies on CLL, we applied whole-genome screening for detection of clonal evolution, which showed that the evolution of new CLL cell clones does not only involve the acquisition of the known recurrent aberrations, but also involves other chromosomes such as 6q, 8p, 9p and 10q (Table 3, *Online Supplementary Table S3*). The fact that the acquisition of these aberrations only occurred in patients with unmutated *IGHV* genes is noteworthy. These findings support the advantage of applying whole-genome screening when investigating clonal evolution in CLL. However, the array technique is less sensitive than FISH in detecting small subclones, and we cannot, therefore, differentiate between the development of new subclones and the expansion of pre-existing small subclones. The fact that many of the patients who received treatment also underwent clonal evolution raises the question of whether treatment *per se* could induce genomic instability and lead to the development of novel CNA. However, since we did not detect any specific/recurrent CNA in follow-up - except the known recurrent aberrations - it is unlikely that the therapy given had a direct effect on clonal evolution. However, treatment probably leads to a selective pressure of CLL cells, which may alter the balance between already existing subclones, thus making the expanding subclones detectable during subsequent progression. Prospective studies on clonal evolution in CLL, with samples taken just before and after treatment, may improve our knowledge regarding these aspects.

Unfortunately, in this study we did not have access to paired normal DNA from the CLL patients, which would have simplified identification of copy-number variations in the copy-number analysis. To minimize inclusion of copy-number variations, we therefore excluded regions that had been previously reported as genomic variants, and also adjusted the settings to exclude small patient-specific copy-number variations. However, since the small aberrations were mostly non-overlapping in this study, we do not believe that the lack of constitutional DNA compromised the interpretation of CNA.

In conclusion, whole-genome screening with SNP-arrays revealed a high frequency of known recurrent alterations as well as additional small, non-overlapping and large, but low-frequency, recurrent CNA in patients with newly diagnosed CLL. It is to be hopend that the application of other newly developed techniques, such as next generation sequencing, will reveal novel recurring genetic changes such as mutations of genes involved in important signaling pathways, and thus further increase our understanding of the pathogenesis of CLL. The SNP-technique allowed genomic detection of clonal evolution in *IGHV* unmutated and in *IGHV* mutated/treated CLL-patients. This evaluation revealed that although the known recurrent aberrations were the most commonly acquired CNA during follow-up, additional chromosomal changes developed in patients with other poor prognostic markers.

Authorship and Disclosures

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