Leukemia & Lymphoma, 2013; Early Online: 1–9

ISSN: 1042-8194 print / 1029-2403 online
DOI: 10.3109/10428194.2013.845882

informa
healthcare

ORIGINAL ARTICLE: RESEARCH

Genomic imbalance defines three prognostic groups for risk stratification of patients with chronic lymphocytic leukemia

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Abstract

Array comparative genomic hybridization (aCGH) has yet to be fully leveraged in a prognostic setting in chronic lymphocytic leukemia (CLL). Genomic imbalance was assessed in 288 CLL specimens using a targeted array. Based on 20 aberrations in a hierarchical manner, all 228 treatment-naive specimens were classified into a group with poor outcome (20.6%) exhibiting at least one aberration that was univariately associated with adverse outcome (gain: 2p, 3q, 8q, 17q, loss: 7q, 8p, 11q, 17p, 18p), good outcome (32.5%) showing 13q14 loss without any of the other 10 aberrations (gain: 1p, 7p, 12, 18p, 18q, 19, loss: 4p, 5p, 6q, 7p) or intermediate outcome (remainder). The three groups were significantly separated with respect to time to first treatment and overall survival ($p < 0.001$), and validation of the stratification scheme was performed in two independent datasets. Gain of 3q and 8q, and 17p loss were determined to be independent unfavorable prognostic biomarkers. *TP53*, *NOTCH1* and *SF3B1* mutations correlated with the presence of one poor outcome aCGH marker, at a considerably higher frequency than when only considering poor risk aberrations routinely detected by fluorescence *in situ* hybridization (FISH). These data support genomic imbalance evaluation in CLL by aCGH to assist in risk stratification.

Keywords: Lymphoid leukemia, molecular genetics, prognostication

Introduction

The clinical course of patients with B-cell chronic lymphocytic leukemia (CLL) is highly variable, underscoring the role of risk stratification to guide clinical management [1]. At disease progression, when therapeutic intervention is considered, risk stratification is recommended to include assessment of overall fitness, comorbid conditions and biomarkers, including sequence analysis of the clonally rearranged *IGH-D-J* locus where those with less than 2% variation from germline sequences have been well documented to exhibit a poor outcome [2–4]. Also assessed is the presence of somatic genomic abnormalities by fluorescence *in situ* hybridization (FISH), including loss of 13q14, the *TP53* (17p13) and *ATM* (11q22-q23) loci and trisomy 12 [5]. Currently, this probe combination dichotomizes patients into those carrying del(17p) or del(11q) with a poor prognosis and those who do not. This has reduced prognostic value compared with the original hierarchical model, which also permitted discrimination of patients with a favorable outcome, but failed to classify all specimens [6]. del(17p) is associated with a poor response to chemotherapy, and shorter treatment-free periods and overall survival (OS) [4]. For patients with del(11q), outcomes are more favorable when regimens contain an alkylator [4]. Patients requiring treatment, but who do not carry these two biomarkers, are treated with consideration of age and comorbidities.

Array-based comparative genomic hybridization (aCGH) and, more recently, massively parallel-sequencing technologies have afforded comprehensive evaluations of the CLL genome identifying gain, loss and other mutational events (exemplified by mutations in *TP53*, *NOTCH1*, *SF3B1* and *BIRC3*) that potentially have clinicopathologic relevance [7–17]. While the latter platform has yet to be integrated within diagnostic laboratories, there is precedence for the former. Whole genome aCGH has identified novel recurrent copy number alterations (CNAs) at relatively low frequencies including gain of 2p, 3q and 8q, and loss of 6q and 8p, the clinical relevance of which is for the most part unclear, with only anecdotal reports of associations

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Received 25 March 2013; revised 21 August 2013; accepted 10 September 2013

with clinical endpoints or *IGHV* mutation status [8,9,18–21]. Notwithstanding validation of the prognostic value, routine assessment of these low frequency events by aCGH would increase the potential to identify early those patients with an adverse outcome. Complex patterns of loss at the 13q14 locus have been elucidated by aCGH, where two main types include the *MIR-15A/16.1* locus as the target of the deletion, but exclude (type I) or include (type II) *RB1* [20,22,23]. It has been reported that patients exhibiting type II deletions have poorer outcomes than those exhibiting type I, although not all studies support this finding [20,24–26]. Studies in murine models of CLL with deletions mimicking type I have further suggested an accelerative role for deletion of telomeric sequences (including *DLEU7* and *RNASEH2B*) in the transition of monoclonal B-cell lymphocytosis (MBL) to CLL [27]. Thus, aCGH has contributed to increased understanding of the CLL genome and the etiologic and clinical significance of detected aberrations, but its utility in a clinical diagnostic setting has been limited.

In the present study, aCGH was performed on 288 unselected CLL specimens using a targeted array designed for the detection of genomic imbalance in mature B-cell neoplasms. Recurrent CNAs with clinical significance were identified in treatment-naive specimens that, together with other recurrent CNAs, allowed significant classification of all specimens into one of three groups with poor, intermediate or good outcome. A high correlation was found between the presence of *TP53*, *NOTCH1* and *SF3B1* mutations and one of nine poor aCGH aberrations, beyond those detected routinely by FISH. Overall, the present study shows for the first time how the added genomic information afforded by aCGH can be leveraged for clinical implementation in risk assessment of progression and outcome in CLL.

Materials and methods

CLL patient specimens and DNA extraction

Specimens (blood or bone marrow) were obtained from patients with CLL with informed consent during routine care at the North Shore-LIJ Health System. Dataset 1 (DS1) comprised 119 cryopreserved mononuclear cells (MNCs) isolated from patients with CLL between 1998 and 2009, while dataset 2 (DS2) comprised DNA extracted in the Cancer Genetics, Inc. Clinical Laboratory Improvement Amendments (CLIA)-approved laboratory from 169 blood/bone marrow specimens, consecutively ascertained during 2008 and 2009. Selection of cases was based on classification as CLL according to the World Health Organization (WHO) classification scheme, and availability of a specimen (MNCs or DNA) for study (Supplementary Table I available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882) [28]. Across both datasets, 228 patients were untreated at the time of sampling and 60 were treated. DNA was also extracted from an independent validation dataset of cryopreserved MNCs from 65 similarly selected CLL specimens obtained from patients with consent at the Hackensack University Medical Center (HUMC). For six of these specimens, CD19-immunomagnetic positive selection was performed prior to DNA extraction, on account of low absolute lymphocyte counts. Copy number data assessed using Affymetrix 6.0 SNP arrays were made available for 124 previously untreated prospectively enrolled patients with CLL, performed with consent at the Dana Farber Cancer Institute (DFCI) [18]. All studies were performed with respective Institutional Review Board (IRB) approval.

Custom aCGH

The custom oligonucleotide array was designed within eArray (Agilent Technologies, Inc.) with a 4×44K format comprising 301 features represented five times, 3100 features in duplicate representing the entire genome at an average resolution of 1 Mbp, and 17 348 features in duplicate representing eighty regions of the genome ranging in size from 0.3 Mbp to 21.3 Mbp at an average resolution of 34 kbp (Supplementary Methods available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882). Following aCGH as described in detail in the Supplementary Methods available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882, data extraction was performed (Feature Extraction Version 10.7.3.1; Agilent), duplicate probes averaged and the circular binary segmentation (CBS) method used to define segments ($p = 0.01$) with the DNA copy package in R Bioconductor (Version 2.10). Genomic Identification of Significant Targets In Cancer (GISTIC, Version 0.9.2) was applied after removal of known normal copy number variants (Database of Genomic Variants, http://projects.tcag.ca/variation) with a minimum acceptable segment of eight contiguous probes and an acceptable false discovery rate (FDR) Q-value for significance of 0.25. For manual examination of aberrations in the CBS-segmented profiles, median-normalization was performed. For both GISTIC and manual examination, specimens were scored positive with log ratios ≥ 0.15 for gain and ≤ -0.15 for loss as confirmed by quantitative polymerase chain reaction (QPCR) (Supplementary Methods available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882). Raw data files for DS1 and DS2 have been deposited in Gene Expression Omnibus (GEO, GSE40834). All genomic coordinates are according to the NCBI36/hg18 assembly.

Confirmation of QPCR

QPCR was performed to confirm eight regional aCGH aberrations using the copy number assays given in the Supplementary Methods available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882. In brief, 5 ng DNA per well were amplified in duplicate per gene per DNA, using *TERT* and *RAG2* as control genes. The $\Delta\Delta C_T$ method was calculated using the average of the control genes for two independent MF reference DNA dilutions and then averaged. Specimens with ratios ≥ 1.2 were considered positive for gain, and ≤ 0.8 positive for loss.

TP53, NOTCH1 and SF3B1 mutation analyses

Genomic DNA was submitted to routine bi-directional Sanger sequencing following amplification, using primers

and conditions detailed in the Supplementary Methods available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882. For *TP53*, exons 5–9 were examined, for *NOTCH1*, an 845 bp fragment in exon 34, and for *SF3B1*, exons 14–16. Dilution studies revealed a 20–25% sensitivity of detection of heterozygous mutation.

Clinical correlative analyses

Pairwise comparisons between biomarkers were tested according to Fisher's exact test. For univariate associations between biomarkers and time from diagnosis to first treatment (TTFT) or OS from diagnosis, the Kaplan–Meier method and the log-rank statistic were used. Hazard ratios were calculated using Cox regression. A multivariate Cox regression model was fit using stepwise regression methods. A p-value less than 0.05 was considered significant.

Results

CLL patient datasets

Table I lists characteristics of the 228 unselected treatment-naive patients with CLL in both test datasets used in the present study. Since DS1 was more mature, with a longer median follow-up than DS2, some analyses were independently performed on each dataset. A marginally higher relative proportion of specimens with mutated to unmutated *IGHV* clonal rearrangements was evident in DS2 than in DS1 (61.9% vs. 53.1%), but as expected, those with unmutated *IGHV* significantly exhibited a shorter TTFT and OS in both datasets ($p < 0.001$). An additional 60 specimens sampled from treated patients with CLL were also used in the study (38 for DS1, 22 for DS2). Across all specimens, FISH findings for the four commonly detected aberrations were available for 103 specimens (Table I, Supplementary Table I available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882). Of these, 87 were from treatment-naive patients, where del(17p) significantly correlated with shorter OS ($p = 0.004$) and del(11q) exhibited a trend with shorter OS ($p = 0.086$). These specimens were dichotomized with respect to del(17p) and/or del(11q) versus del(13q), +12 or normal, and the former group were confirmed to exhibit significantly shorter OS ($p = 0.005$), but no significant association was found with TTFT ($p = 0.14$).

Alterations in CLL genome assessed by targeted aCGH

A targeted oligonucleotide array was designed for clinical diagnostic implementation to represent regions commonly exhibiting genomic imbalance and/or reported to have prognostic value in mature B-cell neoplasms. CBS followed by GISTIC was applied to all specimens and each dataset separately where a total of 18 significant CNAs were identified (Table II). As confirmation of the selected cut-off log ratio in GISTIC, treatment-naive specimens in DS1 that scored positive for eight of the significant regions were evaluated by QPCR where, of the total 91 aberrations found, all were confirmed with the exception of one, and for three others where the aberration detected did not include the gene tested by QPCR (Supplementary Table I available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882). Using the 103 specimens with aberrations present in at least 25% of cells by FISH, the sensitivity of detection of aberrations by aCGH was 93.4% and specificity 98.8% for the 76 abnormal and 321 normal FISH results (Supplementary Table I available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882).

Considering the 18 significant CNAs, genomic gain/loss was detected in 91.4% and 72.8% of treatment-naive specimens in each respective dataset (Figure 1). Since the percentage for DS2 was low, the median-normalized log ratios following CBS of the remaining specimens were individually examined for other aberrations that did not solely comprise backbone probes. Overlapping 7q deletions were

Table I. Patient characteristics of CLL datasets 1 and 2.

		Dataset	
		DS1	DS2
Untreated prior to sampling (n)		81	147
Rai stage	0	26 (32.1%)	74 (50.3%)
	I–II	42 (51.9%)	40 (27.2%)
	III–IV	6 (7.4%)	2 (1.4%)
	NA	7 (8.6%)	31 (21.1%)
IGHV mutation status	Unmutated	37 (45.7%)	51 (34.7%)
	Mutated	43 (53.1%)	91 (61.9%)
	Non-clonal	0	5 (3.4%)
	NA	1 (1.2%)	0
Median diagnosis to sampling (months)		57.3	11.1
Median follow-up (months)		147.9	64.8
Treatment events		43	25
Deaths		20	20
Treated prior to sampling (n)		38	22
Total (n)		119	169
FISH aberrations*	Specimens (n)	23	80
	del(11q)	2	6
	+12	4	10
	del(13q)	14	46
	del(17p)	4	12

CLL, chronic lymphocytic leukemia; NA, not available; FISH, fluorescence *in situ* hybridization.
*Specimens with FISH on same sampling date as aCGH.

Table II. Significant regions of gain and loss as identified by CBS and GISTIC.

	Region limits		Peak limits		Dataset 1		Dataset 2	
	Start	End	Start	End	Untreated ($n=81$)	Treated ($n=38$)	Untreated ($n=147$)	Treated ($n=22$)
Gain								
1p36.32	1	3 554 128	1	2 986 575	2 (2.5%)	2 (5.3%)	8 (5.4%)	0 (0.0%)
2p*	1	95 145 278	3 362 198	3 928 623	6 (7.4%)	5 (13.2%)	5 (3.4%)	3 (13.6%)
3q*	119 808 812	197 580 799	166 976 533	167 342 698	2 (2.5%)	2 (5.3%)	4 (2.7%)	2 (9.1%)
7p22.3	1	3 133 906	1	1 658 113	5 (6.2%)	4 (10.5%)	11 (7.5%)	3 (13.6%)
8q*	79 442 457	143 369 815	119 408 616	146 274 826	2 (2.5%)	3 (7.9%)	6 (4.1%)	0 (0.0%)
12*	1	128 433 046	47 778 659	48 150 689	12 (14.8%)	4 (10.5%)	20 (13.6%)	1 (4.5%)
17q*	54 867 627	74 428 327	54 867 627	65 775 886	2 (2.5%)	2 (5.3%)	1 (0.7%)	1 (4.5%)
18p11.32	1	3 269 104	1	762 750	4 (4.9%)	7 (18.4%)	3 (2.0%)	0 (0.0%)
18q*	48 835 323	62 630 655	51 295 555	60 900 467	1 (1.2%)	1 (2.6%)	6 (4.1%)	0 (0.0%)
19	1	63 553 960	1	3 852 455	2 (2.5%)	1 (2.6%)	5 (3.4%)	0 (0.0%)
Loss								
4p15.1	23 583 187	33 122 761	23 583 187	33 122 761	2 (2.5%)	2 (5.3%)	3 (2.0%)	2 (9.1%)
6q21	108 092 926	109 659 755	108.092 926	109 659 755	0 (0.0%)	2 (5.3%)	5 (3.4%)	1 (4.5%)
7p22.3	1	2 005 438	1	1 726 980	7 (8.6%)	1 (2.6%)	6 (4.1%)	1 (4.5%)
8p*	1	24 851 740	10 043 135	10 523 548	3 (3.7%)	2 (5.3%)	5 (3.4%)	1 (4.5%)
11q*	76 932 697	126 037 329	111 800 666	112 203 507	10 (12.3%)	9 (23.7%)	10 (6.8%)	3 (13.6%)
13q*	33 438 491	75 377 448	49 568 035	49 830 378	55 (67.9%)	21 (55.3%)	71 (48.3%)	9 (40.9%)
17p*	1	22 593 075	1 491 587	8 071 136	2 (2.5%)	6 (15.8%)	7 (4.8%)	2 (9.1%)
18p*	1	18 044 710	2 539 225	4 163 324	3 (3.7%)	2 (5.3%)	6 (4.1%)	0 (0.0%)

CBS, circular binary segmentation; GISTIC, Genomic Identification of Significant Targets In Cancer.
*When more than half an arm was involved, the respective arm was listed.

found in two specimens and a 5p15 deletion in another. For 7q, a minimally deleted region of chr7:122 471 896–124 803 693 was found across six specimens in both datasets, and for 5p, across two specimens with a minimally deleted region of chr5:5 460 990–8 079 142 (Figure 1). Since these aberrations were detected in treatment-naive specimens, they were included in clinical correlative analyses when recurrent within a dataset.

Genomic imbalance associated with clinical outcome

All 20 aberrations (18 from GISTIC plus losses of 5p and 7q) were independently tested for association with clinical

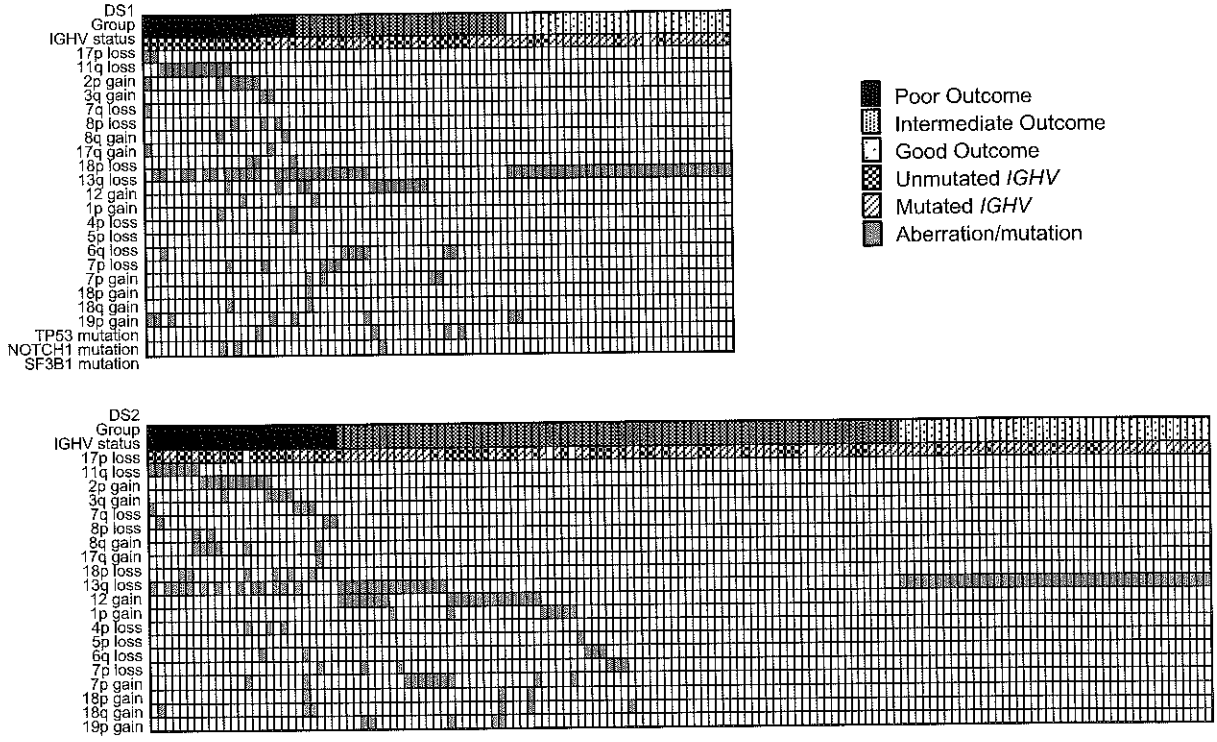

Figure 1. Genomic landscape of the CLL genome. Treatment-naive CLL specimens can be classified into one of three prognostic groups according to genomic imbalance as assessed by targeted aCGH. Each treatment-naive specimen in DS1 ($n = 81$) and DS2 ($n = 147$) is represented as a column. The first row of each provides the prognostic classification group according to presence/absence of the aCGH aberrations recorded in the rows below (total of 155 losses and 109 gains). The mutation status of the *IGHV* clonal rearrangement in both datasets and the presence/absence of *TP53*, *NOTCH1* and *SF3B1* mutations in DS1 are also shown. Full CNA and mutation data for each specimen are provided in Supplementary Table I available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882.

endpoints in untreated specimens of each dataset to capture all clinically relevant aberrations. Ten CNAs significantly correlated with TTFT or OS (some with both endpoints), and with the exception of deletion of 13q14, all were associated with shorter times. Table III lists the 10 CNAs and gives the significance of association with each endpoint for the combined datasets (Kaplan–Meier plots are given for each in Supplementary Figure 1 available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882). Loss of 18p was significantly associated with 17p loss and 2p gain, as was 7q loss with 17p loss, while 8q gain was associated with 11q loss and 8p loss (Supplementary Table II available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882). A multivariate Cox regression analysis incorporating the nine poor outcome aCGH aberrations and *IGHV* status identified 17p loss, 3q and 8q gain and *IGHV* status as independent prognostic markers of OS (Table III). Of note, Rai stage was not entered in the model due to the absence of information for 38 of the 228 specimens. For TTFT, loss of 8p, gain of 3q and *IGHV* mutation status were determined to be independent biomarkers (Table III).

As expected, loss of 17p and 11q were amongst the nine aCGH markers univariately associated with adverse outcome. These aberrations were found in 29 treatment-naive specimens (12.7%) across both datasets. Importantly, an additional 18 specimens (7.9%) bore at least one of the other seven poor aCGH markers: gain of 2p, 3q, 8q or loss of 7q, 8p, 17q, 18p. Combined, these 47 specimens were grouped as having poor outcome. In a hierarchical manner somewhat analogous to the previous stratification scheme based on aberrations detected by FISH [6], a second non-overlapping group of 74 specimens were identified that had 13q14 deletions but no additional aberrations at the 10 other recurrent loci (gain: 1p, 7p, 12, 18p, 18q, 19, loss: 4p, 5p, 6q, 7p). The respective patients with 13q14 loss as a sole abnormality were grouped as having a good outcome, as they exhibited a highly favorable outcome when compared with those with 13q14 deletions plus other aberrations (Supplementary Figure 2 available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882). Lastly, a third group comprised two subsets: 63 that only exhibited any of the 10 recurrent loci used to define 13q14 loss as a sole abnormality (gain: 1p, 7p, 12, 18p, 18q, 19, loss: 4p, 5p, 6q, 7p), and 44 that did not carry any of the total 20 aberrations. Since no difference in TTFT or OS was found between these two subsets ($p = 0.405$, 0.662, respectively) (Supplementary Figure 2 available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882), they were joined into one group (107 specimens) with an intermediate outcome.

Thus, all treatment-naive specimens in DS1 and DS2 were classified into one of three prognostic groups based on the presence/absence of the 20 CNAs (Figure 1, Supplementary Table I available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882). Importantly, highly significant separation was observed between the three groups when tested for association with TTFT and OS [$p < 0.001$, Figures 2(A) and 2(B), respectively]. Pairwise, all showed significant separation except between intermediate and good outcome groups for the TTFT endpoint [Figure 2(A)]. Within the good and intermediate groups, *IGHV* mutation status permitted additional significant stratification of patients for both endpoints (Supplementary Figure 3 available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882). Overall, then, the presence/absence of 20 CNAs as assessed by aCGH permitted classification of all CLL specimens into one of three groups that significantly correlated with time to first treatment and outcome. Validation of the hierarchical

Table III. Association of genomic aberrations with TTFT and OS in 228 treatment-naive CLL specimens.

		Endpoint			
		TTFT		OS	
Dataset		1 + 2*	*p*-Value (HR [95% CI])†	1 + 2*	*p*-Value (HR [95% CI])†
Gain	chr2:1-95 145 278	0.002	NS	< 0.001	NS
	chr3:119 808 812–197 580 799	< 0.001	0.001 (14.28 [2.95–69.24])	< 0.001	< 0.001 (23.08 [6.63–80.31])
	chr8:79 442 457–143 369 815	0.701	NS	0.001	0.001 (6.16 [2.32–16.40])
	chr17:54 867 627–74 428 327	0.004	NS	< 0.001	NS
Loss	chr7:122 471 896–124 803 693	0.886	NS	0.022	NS
	chr8:1–24 851 740	< 0.001	< 0.001 (16.47 [3.41–79.52])	0.041	NS
	chr11:76 932 697–126 037 329	0.015	NS	0.001	NS
	chr13:33 438 491–75 377 448	0.021	Not entered	0.001	Not entered
	chr17:1–22 593 075	< 0.001	NS	< 0.001	0.023 (3.51 [1.20–10.29)]
	chr18:1–18 044 710	0.509	NS	0.009	NS
IGHV mutation status (unmutated)		< 0.001	< 0.001 (5.64 [3.30–9.64])	< 0.001	< 0.001 (6.57 [3.01–14.34])
Dataset		1*		1*	
Mutation‡	*TP53* (all)	0.767	Not entered	0.147	Not entered
	TP53 (excluding *IGHV* mut)	0.001	Not entered	0.007	Not entered
	NOTCH1	0.021	Not entered	0.021	Not entered
	SF3B1	0.004	Not entered	0.238	Not entered

TTFT, time from diagnosis to first treatment; OS, overall survival; CLL, chronic lymphocytic leukemia; NS, not significant; Not entered, variable not entered in multivariate regression analysis.

*Univariate *p*-value from log-rank test, all variables associated with shorter TTFT and/or OS with exception of 13q.

†Multivariate *p*-value after variable selection (hazard ratio [95% confidence interval]).

‡Only performed for DS1.

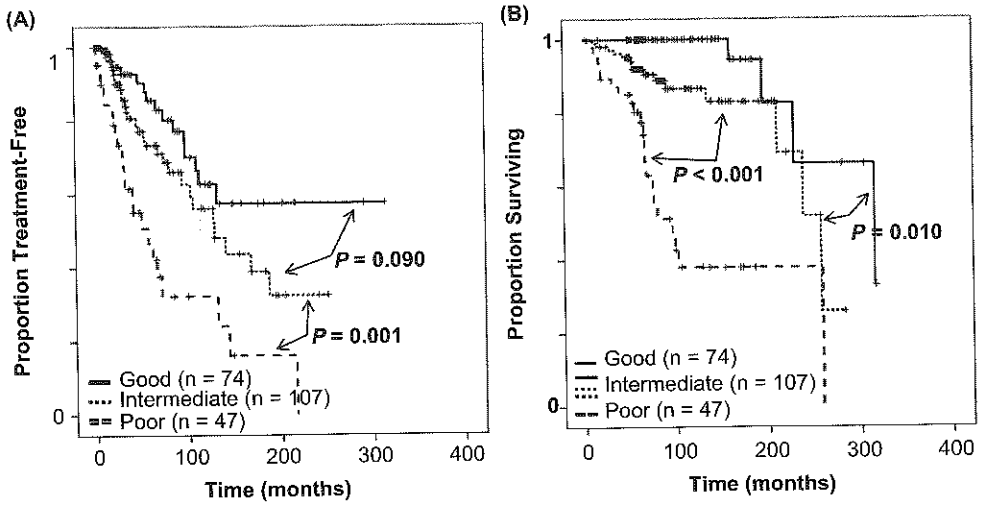

Figure 2. Kaplan–Meier plots of aCGH groups. Kaplan–Meier plots are given for combined DS1 and DS2 treatment-naive specimens ($n = 228$) classified into one of three groups (poor, intermediate, good) based on 20 CNAs in a hierarchical manner. Plots are shown for TTFT (A) and OS (B). The p-values provided are those obtained using the log-rank test between good and intermediate groups and intermediate and poor groups, showing significant separation for OS and between poor and intermediate–good for TTFT. Individual specimen classifications are listed in Supplementary Table I available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882.

classification was performed in two previously untreated CLL datasets from independent institutions (Supplementary Table III available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882). The first (DFCI) comprised 124 specimens submitted to high-resolution single nucleotide polymorphism (SNP) array analysis and the second (HUMC), 65 specimens submitted to targeted aCGH. All specimens were classified into one of three prognostic groups according to the presence/absence of the 20 aberrations, and separately tested for association with TTFT and OS (Supplementary Figure 4 available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882). In the DFCI dataset, association of aCGH outcome group with TTFT was validated ($p < 0.001$), but not for OS ($p = 0.522$), most likely explained by the low number of deaths in this dataset. Significant association of aCGH outcome group with OS was observed for the HUMC dataset ($p = 0.044$), but not TTFT, where the median TTFT was only 17.2 months, being much shorter than expected for an average CLL dataset.

Other studies have reported an association between increased genomic complexity and adverse outcome in CLL, and a similar association was observed for the present CLL datasets ($p < 0.001$), when those exhibiting two or more of the above 20 CNAs (72 of 228 cases) were considered complex. As expected, a higher frequency of genomic complexity was noted within specimens from treated patients (29 of 60).

Impact of other known genome-based markers of outcome in CLL

In order to examine the impact of *TP53*, *NOTCH1* and *SF3B1* mutations on the aCGH classification scheme, genomic DNA from specimens from untreated patients in DS1 was analyzed for *TP53* (exons 5–9), *NOTCH1* (exon 34) and *SF3B1* (exons 14–16) mutations. *TP53* mutations were identified in eight specimens (9.9%) (Figure 1), including two with 17p13 loss and another two with another poor aCGH marker. Three were observed in specimens displaying 13q14 deletions and a mutated *IGHV* clonal rearrangement, in which cases, reportedly, survival is not negatively impacted by the mutation [13]. In the present study, collectively the presence of a *TP53* mutation did not correlate with shorter TTFT or OS, but significantly correlated with adverse outcome and shorter TTFT when the three specimens with mutated *IGHV* were not considered positive (Table III). Four specimens contained *NOTCH1* mutations that correlated with shorter TTFT and OS (Figure 1, Table III) [7,11,12]. All four were in unmutated *IGHV* specimens, of which one carried a poor aCGH marker other than 17p or 11q loss, and one had a gain of chromosome 12. As expected, the most common *NOTCH1* mutation observed was ΔCT7544–7545, in three of the four [7,11,12]. *SF3B1* missense mutations were detected in three specimens, all occurring in unmutated *IGHV* specimens and at previously reported hotspots, but correlated only with shorter TTFT ($p = 0.004$). The presence of either clinically relevant *TP53*, *NOTCH1* or *SF3B1* mutation was in mutually exclusive specimens, and highly correlated with the presence of an unfavorable aCGH marker (66.7%), but less so when only considering aberrations associated with poor outcome and routinely detected by FISH [del(17p), del(11q)] (33.3%).

13q14 deletion type and association with outcome

GISTIC analysis revealed that the peak region of the 13q14 deletion overlapped with the *DLEU2* locus and promoter region. In order to define the 13q14 deletion in the present datasets, samples were recorded according to the CBS segmented, median-normalized log ratios at the *RB1*, *DLEU2*, *DLEU7* and *RNASEH2B* loci [Figures 3(A) and 3(B), Supplementary Table IV available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882). The entire *DLEU2* genic region was deleted in most cases, but partial losses were detected in 22 specimens, all of which included the *MIR-15A/16.1* locus with the

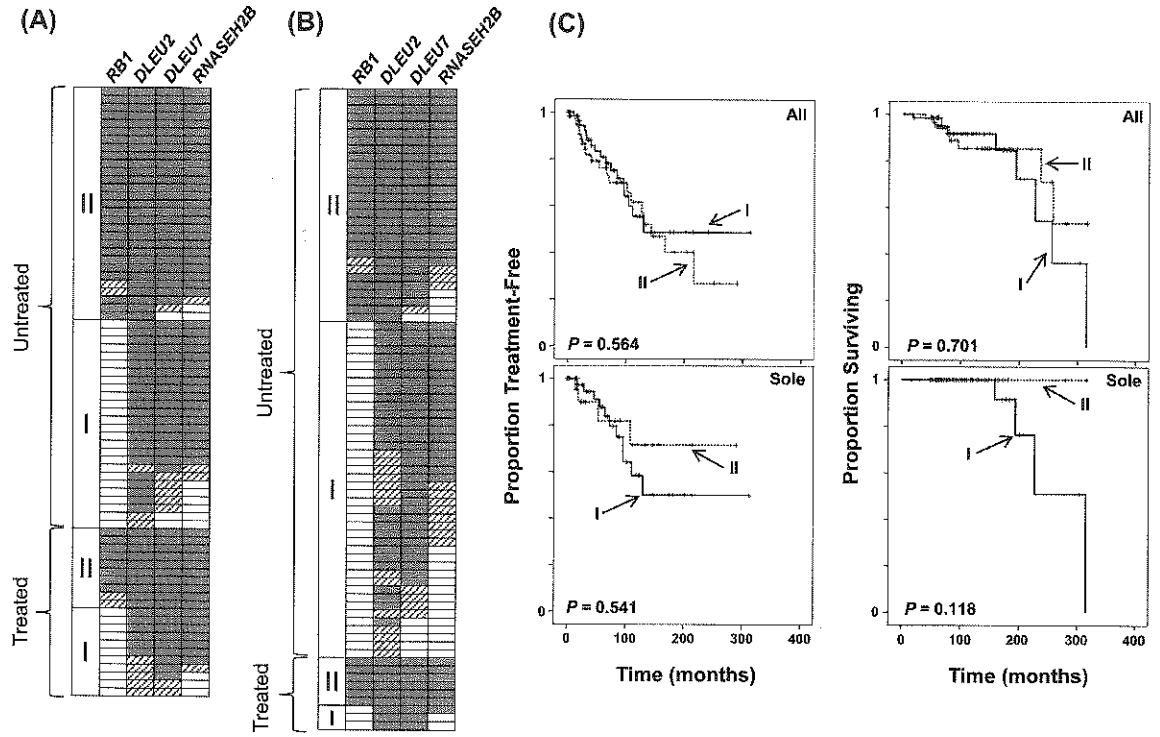

Figure 3. Losses of genes involved in 13q14 deletions. Specimens in DS1 (A) and DS2 (B) with 13q14 deletions were classified as type I or type II based on exclusion or inclusion of or part thereof of *RB1*. The clinical relevance of type I or II deletion was assessed in treatment-naive specimens of DS1 and DS2 combined (C) in all with 13q14 deletions (All) and in those with 13q14 as a sole abnormality (Sole). Individual specimen classifications are listed in Supplementary Table IV available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.845882. Specimens are listed showing all (filled) or partial (hatched) loss of each of the four genic loci: *RB1* (chr13:47 779–47 955 Mbp), *DLEU2* (chr13:49 452–49 599 Mbp), *DLEU7* (chr13:50 187–50 313 Mbp) and *RNASEH2B* (chr13:50 397–50 439 Mbp) loci.

exception of four, for which the telomeric portion of *DLEU2* was deleted along with promoter sequences. The smallest detected partial deletion of *DLEU2* was in case DS2-204 of 366 kbp (chr13:49 464 630–49 830 378). In treatment-naive specimens with 13q14 deletions, 47.3% of DS1 were type I 13q14 deletions, and 59.2% of DS2. When combined and tested for association with clinical endpoints, no significant difference in OS or TTFT was found between deletion types [Figure 3(C)], nor when present as a sole abnormality [Figure 3(C)]. The clinical relevance of the telomeric breakpoint was also examined, where the majority of cases (94.2%) exhibiting loss of *DLEU2* also displayed loss or partial loss of *DLEU7* [Figures 3(A) and 3(B)]. Fewer exhibited concurrent deletion of *RNASEH2B* (80.1%). No significant association with TTFT or OS was found with the extended deletion including one or both telomeric loci, with the exception of longer OS when the deletion extended only to include *DLEU7* ($p = 0.036$).

Discussion

In the present study, a defined panel of genomic CNAs have been identified by aCGH that collectively allow hierarchical classification of all specimens from treatment-naive patients with CLL for risk stratification into one of three groups with poor, intermediate or good outcome. Nine were biomarkers of adverse outcome (gain: 2p, 3q, 8q, 17q, loss: 7q, 8p, 11q, 17p, 18p) and 10 others (gain: 1p, 7p, 12, 18p, 18q, 19, loss: 4p, 5p, 6q, 7p) were used to define loss of 13q14 as a sole abnormality. Prior aCGH studies have reported associations of CNAs with outcome, but none until now have integrated the findings for definitive classification of specimens for clinical utility. Importantly, mutations in the *TP53*, *NOTCH1* and *SF3B1* genes were found to be highly correlated with the presence of a poor aCGH CNA, higher than would have been found based solely on the loss of 17p or 11q, as routinely assessed by FISH. Collectively, these findings support the utility of aCGH to detect genomic imbalance in CLL with prognostic significance in a clinical diagnostic setting.

Nine aCGH aberrations were found to be associated with adverse outcome and shorter time to first treatment, including the well-described losses of 17p and 11q. Much less is known about the low frequency gains of 2p, 3q, 8q and 17q, and loss of 7q, 8p and 18p. These aberrations have been reported in other CLL datasets, often at higher frequencies in progressed and relapsed patients, and sometimes with clinical relevance [8,9,18–21,29–34]. The presences of several of the poor aCGH aberrations were found to be correlated, consistent with increased genomic complexity observed in CLL specimens portending adverse outcome and less durable responses, which was also confirmed in the present study [20,21,30,34,35]. Other studies have implicated *NCOA2*, *ROCK2*, *REL*, *MYCN* (2p), *PIK3CA* (3q), *CAV1* (7q), *TNFSF10A/B* (8p), *MYC* (8q), *ATM* (11q) and *TP53* (17p) as potential target genes for the respective regions based on matched expression and mutation analyses, but their true roles in CLL remain unclear [9,18,32,33,36,37]. Deletion of 6q was

identified in the present study as a recurrent aberration, but did not significantly correlate with disease progression or overall outcome. The clinical relevance of this CNA has been inconsistent across studies, perhaps explained by a minimally deleted region centered at 6q21 that does not include the *MYB* locus, commonly used in FISH for the detection of this abnormality [19,38].

Since the first report of the prognostic relevance of different centromeric breakpoints of deletions at 13q14, there have been other studies with mixed support for the relevance of the two types [22,24–26,39]. In the present study, an association of type with outcome was not confirmed in those having 13q14 deletion, or those detected as a sole abnormality. The significance of the clinical relevance of the telomeric breakpoints is much less known, but murine studies have revealed a role for the *DLEU7/RNASE7H* loci in progression of MBL to CLL, and a germline deletion of this locus has been reported in a family with CLL [40,41]. Most specimens exhibited loss of *DLEU7*, which is perhaps not surprising given that all patients were diagnosed with CLL. Thus, despite the ability of aCGH to accurately define different size deletions at 13q14, the clinical relevance remains unclear.

Currently in CLL, determination of *IGHV* mutation status and detection of genomic imbalance by FISH are recommended as part of risk stratification [4]. Unfortunately, of the four loci evaluated by FISH, no additional outcome stratification is afforded within those patients with CLL who do not bear 17p or 11q loss (up to 85% of unselected patients). Indeed, no difference in OS has been reported for patients with del(13q) as a sole abnormality (based on the four loci) versus those with trisomy 12 or no aberrations [42]. Importantly, the present aCGH study not only identified additional patients with adverse outcome and shorter time to first treatment, other than those with del(17p) or del(11q), it also allowed significant stratification of all remaining specimens into either a good or an intermediate outcome group. The aCGH-based hierarchical scheme is somewhat comparable to that originally proposed using FISH [6], but now all specimens are stratified.

Deep sequencing studies have identified several somatic genic mutations including *NOTCH1*, *SF3B1* and *BIRC3* that are associated with a poor prognosis [7,10–12,34]. Disruption of *BIRC3*, however, is mostly evidenced as bi-allelic deletion or mono-allelic deletion with mutational inactivation of the remaining allele [43]. In the present study, deletion of the *BIRC3* locus without concurrent deletion of *ATM* was rare, and observed in one treated (DS2-235) and one untreated specimen (DS1-1344), which also exhibited gain of 2p. All those with *NOTCH1* mutations in the present study also had unmutated *IGHV*, consistent with other studies, but only one also exhibited trisomy 12 as a sole abnormality [7]. Overrepresentation of *NOTCH1* mutations has been reported in cases with trisomy 12, and it is possible that differences in specimen selection could account for the differences in observed frequency [7]. *SF3B1* mutations occurred at a frequency comparable with other unselected untreated CLL specimen datasets and at similarly reported hotspots [15,16]. In the present study, *NOTCH1*, *TP53* and *SF3B1* mutations were found to occur largely in non-overlapping specimens that bore poor risk aCGH CNAs, often not 11q or 17p loss. This novel finding suggests that in a clinical diagnostic setting, aCGH could be utilized as a stand-alone assay to identify most patients with CLL with an adverse outcome. This represents more patients than those currently identified by FISH alone and also identifies a large proportion of those bearing somatic mutations known to impact survival, thereby reducing the need to perform labor-intensive and costly sequence analysis for each gene for every specimen. While aCGH exhibits reduced sensitivity compared with FISH, it does by virtue of the ability to obtain genomic gain/loss information at more loci provide further risk stratification of patients not bearing any poor aCGH marker, and also allows an evaluation of genomic complexity which, as supported by the present study, correlates with adverse outcome and is mostly observed in specimens bearing poor aCGH markers. In summary, while the CLL genome is on the whole relatively quiet, genomic imbalance as assessed by aCGH in a clinical diagnostic setting may serve as a powerful prognostic tool for risk stratification in patients with CLL.

Acknowledgements

We thank Charles Ma and Ben Ritter for technical assistance.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

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Supplementary material available online

Supplementary Methods, Tables, Figures and showing further results

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