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The prognostic significance of various 13q14 deletions in chronic lymphocytic leukemia

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Abstract

Purpose—To further our understanding of the biology and prognostic significance of various chromosomal 13q14 deletions in CLL.

Experimental Design—We have analyzed data from SNP 6.0 arrays to define the anatomy of various 13q14 deletions in a cohort of 255 CLL patients and have correlated two subsets of 13q14 deletions (type I: exclusive of *RBI* and type II: inclusive of *RBI*) with patient survival. Further, we have measured the expression of the 13q14-resident microRNAs by Q-PCR in 242 CLL patients and subsequently assessed their prognostic significance. We have sequenced all coding exons of *RBI* in patients with monoallelic *RBI* deletion and have sequenced the 13q14-resident *miR* locus in all patients.

Results—Large 13q14 (type II) deletions were detected in ~20% of all CLL patients and were associated with shortened survival. A strong association between 13q14 type II deletions and elevated genomic complexity, as measured through CLL-FISH or SNP 6.0 array profiling, was identified, suggesting that these lesions may contribute to CLL disease evolution through genomic destabilization. Sequence and copy number analysis of the *RBI* gene identified a small CLL subset that is *RBI* null. Finally, neither the expression levels of the 13q14-resident microRNAs nor the degree of 13q14 deletion, as measured through SNP 6.0 array-based copy number analysis, had significant prognostic importance.

Conclusions—Our data suggest that the clinical course of CLL is accelerated in patients with large (type II) 13q14 deletions that span the *RBI* gene, therefore justifying routine identification of 13q14 subtypes in CLL management.

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Peter Ouillette, Jinghui Li and Sami Malek performed the laboratory research.

Roxane Collins and Sami Malek analyzed clinical data.

Kerby Shedden, Sajid Shakhan and Cheng Li assisted with statistical analysis and software development for data analysis.

Sami Malek conceived the study and supervised the work.

Peter Ouillette, Kerby Shedden and Sami Malek wrote the paper.

Conflict of Interest:

None to declare

Keywords

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INTRODUCTION

CLL has a varied clinical course and genomic aberrations are recognized as important to the diverse biological and clinical phenotypes of CLL(1-4). The most frequent chromosomal abnormality in CLL is commonly referred to as del13q14, which is detectable by FISH or SNP arrays in ~50% of all CLL(5-15). While generally considered to be associated with favorable outcome, clinical experience and published data suggest that for a subset of CLL patients with this deletion outcome is not favorable. The reasons for this observation are not well understood and despite application of currently available measurable CLL risk factors, the reliable identification of the relatively unfavorable 13q14 patient subset is not possible.

While the 13q14 probe used in the CLL FISH panel identifies all typical 13q14 deletions in CLL, regardless of size, recent analyses of CLL genomes using SNP array platforms have resulted in the identification of substantial anatomical heterogeneity of 13q14 deletions in CLL(16-19). This anatomic heterogeneity is reflected in large versus small deletions, monoallelic versus biallelic deletions and various deletion extensions into centromeric and telomeric 13q regions. This heterogeneity provides clues to underlying effects of various 13q14 deletions on CLL biology and clinical behavior and makes it likely that more than one pathobiological mechanism underlies the existence of various 13q14 deletions in CLL.

To advance our knowledge of the effects of various CLL-associated 13q14 deletions on CLL patients, we have investigated prognostic effects of various 13q14 types and associated molecular events, including 13q14-resident miR 15a/16-1 expression levels, in a prospectively collected cohort of 255 CLL patients. In aggregate, our data provide solid support for a clinically meaningful separation of 13q14 deletions into subtypes that are defined as good prognosis type I (exclusive of *RBI*; ~30% of all CLL) and poor prognosis type II (inclusive of *RBI*; ~20% of all CLL) lesions and for routine measurements of these subtypes in CLL clinical management(20-23).

METHODS

Patients

Between January 2005 and September 2009, 266 patients evaluated at the University of Michigan Comprehensive Cancer Center were enrolled onto this study. The trial was approved by the University of Michigan Institutional Review Board (IRBMED #2004-0962) and written informed consent was obtained from all patients prior to enrollment. Data from 255 of these 266 patients were included in this report (5 patients enrolled on the study were excluded due to a diagnosis that was not CLL, and 6 patients had insufficient cryopreserved cells available for the analyses described).

Regardless of whether the subjects were originally diagnosed at our institution or another, we used the same CLL diagnostic criteria, based on the National Cancer Institute-Working Group Guidelines for CLL(24). Eligible patients needed to have an absolute lymphocytosis (greater than 5000 mature lymphocytes per μ l), and lymphocytes needed to express CD19, CD23, sIg (weak) and CD5 in the absence of other pan-T-cell markers.

Time to first therapy (TTFT) and overall survival (OS) were based on the date of trial enrollment (the date of specimen procurement) or alternatively the diagnosis date,

respectively(25). CLL treatment was defined as cytotoxic chemotherapy and/or monoclonal antibody therapy for CLL. Clinical information, including Rai stage and all treatments given, was collected on all patients. Patient samples were characterized for selected CLL-associated chromosomal aberrations as a routine clinical test at the Mayo Clinic/Rochester using FISH. All biomarker assessments were performed on specimens procured at trial enrollment.

Cell Isolation

Flow cytometry sorting of CLL specimens—Cryopreserved PBMCs from CLL blood specimens were prepared for FACS sorting into CD19+ and CD3+ cells as previously described(25).

Preparation of Sample DNA

DNA used for SNP 6.0 profiling was extracted from FACS-sorted CD19+ and CD3+ cells as described(25). For some cases (N=11), paired buccal DNA was used instead of CD3+ cell-derived DNA.

Array Data Analysis

The DNA was prepared for hybridization to SNP 6.0 arrays according to the manufacturers' recommendations. Affymetrix CEL files for each cell sample were analyzed using Genotyping Console software for initial quality control, followed by use of the Affymetrix "Birdseed" algorithm to generate tab-delimited SNP call files in text format. Call rates for the entire group of CD19+ samples included in this report were between 95.06% and 99.63%, with a mean call rate of 98.53%. Corresponding call rates for CD3+ or buccal DNA were between 93.14% and 99.66%, with a mean call rate of 98.49%.

Sample copy number heatmap displays were obtained from CEL files through use of the freely available software dChip(26), adapted to run on a 64-bit computer environment. For genomic copy number analysis, we visually inspected parallel heatmap copy number images of CD19+ and paired CD3+/buccal DNA samples generated through dChipSNP and using the median smoothing functionality. Only those copy number changes detected in CD19+ DNA that were not found at the same position in paired CD3+/buccal DNA were called somatic. This approach followed our previously externally referenced (FISH) SNP 6.0 genomic lesion calling method in AML that resulted in 100% concordance between SNP 6.0 profiling and FISH results (for a total of 56 lesions analyzed)(27). Further, data on sensitivity and specificity of acquired copy number (aCNA) calling calculated based on data for del17p, del11q and del13q14 derived from the clinical CLL-FISH panel are summarized in the Supplementary Methods and Results section. Using this approach, the three shortest identified aCNAs were 0.024, 0.042 and 0.052 Mb in length and were defined by 18, 27 and 19 consecutive SNP positions, respectively, and the vast majority of aCNAs were defined by >100 consecutive SNP positions. In total, we detected 474 subchromosomal losses (size range 0.024-108.73 Mb) and 62 subchromosomal gains (size range 0.114-94.626 Mb; ratio of losses to gains of 7.1 to 1).

The method used to nominate aCNAs using a statistical algorithm (27) is summarized in the Supplementary Methods section.

Exon resequencing of *p53*, *RB1* and the 13q14-resident *miR* genomic locus

Primers to amplify and sequence exons 2-10 of human *p53* and all coding exons of *RB1* and adjacent intronic sequences, including splice junctions, were designed using the primer 3 program (<http://frodo.wi.mit.edu/primer3/>) and sequence information was generated as

described(28). Mutations were confirmed using paired patient CD3+/buccal DNA as templates. The primers to amplify and sequence the 13q14-resident *miR* locus were: F: TTCTAAGCTCTGTTCAAATGCT, R: TTGTGTTTCCTAACCTATAGCACTG and SEQ: TAACAAGATTATCAATAATAC.

Determination of ZAP70 and IgV_H status

Determination of ZAP70 and IgV_H status was performed as described(25).

Measurement of expression of 13q14-resident microRNAs 15a and 16 using normalized Q-PCR

RNA was prepared from $2-4 \times 10^6$ FACS-sorted CD19+ cells using the Trizol reagent and resuspended in 100 μ l DEPC-treated water. Complementary DNA for microRNA reverse transcriptase Q-PCR was made from ~5 ng of RNA using the TaqMan microRNA reverse transcription kit (Applied Biosystems) and RNA specific primers. Primers and TaqMan-based probes were purchased from Applied Biosystems (Primers-on-demand). Primer/probe mixtures included: miR16-1 (TM 391), miR15a (RT 389), RNU43 (TM 1095) and RNU49 (TM 1005). Duplicate amplification reactions for microRNAs included primers/probes, TaqMan® 2x Universal PCR Master Mix, No AmpErase UNG and 1.35 μ l of cDNA in a 20 μ l reaction volume. Reactions were done on an ABI 7900HT machine. Normalization of relative copy number estimates for RNA species of interest was done with the Ct values for the RNU43 or RNU49 as reference (Ct mean gene of interest – Ct mean RNU43 or RNU49). Comparisons between CLL subgroups were performed through subtractions of means of normalized Ct values. Similar efficacies of amplification of cDNA using primers for miR15, miR16-1 and RNU49 had previously been verified using two-fold serial dilutions of cDNA made from cell line-derived RNA over a range of eight two-fold serial dilutions(18).

Statistical Methods

TTFT or overall survival was defined as the time (in months) between CLL diagnosis or CLL trial enrollment and the date (in months) of first treatment or the patient's death. For patients still untreated or alive, the month of censoring was November, 2010. Univariate and bivariate analyses were based on Kaplan-Meier estimates of survivor functions. Median survival times were estimated directly from the survivor function estimates. Significance levels for group-wise comparisons in the univariate and bivariate analyses were based on the log-rank test. Assessment of the relationship between outcomes and various dichotomizations of a continuous risk factor were made by considering all observed values of the risk factor as potential dichotomization thresholds, and using univariate proportional hazards regression to estimate the corresponding hazard ratio.

RESULTS

Patient Characteristics

Characteristics of the 132 CLL patients with typical 13q14 deletions as detected through SNP 6.0 profiling (identified within a prospectively enrolled and profiled cohort of 255 CLL patients) analyzed in this study are summarized in Table 1 stratified by 13q14 subtype and treatment status. Data for 123 CLL patients without typical 13q14 deletions are also summarized (Table 1). Of the 132 CLL patients with typical 13q14 deletions patients, 100 (76%) were untreated (UT) and 32 (24%) relapsed (T) at the time of study enrollment. Within the group of previously untreated patients, the distribution of important biomarkers across type I or II 13q14 lesions was well balanced: Rai stage 0: 45%/41%, Rai stages 1 or 2: 51%/53%, IgV_H unmutated: 32%/29%, ZAP70 positive: 30%/26%, *p53* exons 2-10 mutated: 11%/6%, del17p present: 7%/3% and del11q present: 6%/12%. The median time

from diagnosis to enrollment and from enrollment to data analysis for previously untreated patients is detailed in Table 1. All outcome analyses described below are based on SNP 6.0 array analysis and biomarker measurements that were performed on patient samples procured at study enrollment, thus avoiding confounding effects of longitudinal biomarker instability. Outcome was calculated using either the CLL trial enrollment date or the diagnosis date as the reference dates, as indicated, in order to minimize the effect of lead time biases.

The pathological anatomy of acquired subchromosomal genomic copy number changes spanning 13q14 in CLL as defined through SNP 6.0 array copy number profiling

We catalogued all aCNAs on chromosome 13 in our CLL cohort using visual inspection of simultaneous displays of dChipSNP-based copy number estimates (heatmaps) for CD19+ cells and paired CD3+/buccal DNA (Figure 1). Overall, 51.7% (132/255) of CLL carried a classical 13q14 deletion as detected through SNP 6.0 arrays that included the genomic region recognized in the clinically used CLL FISH panel (range of lesion sizes of 0.198-73.775 Mb; see Supplementary Table 1). In addition, rare atypical 13q deletions were identified. As previously described using lower-resolution SNP array platforms (Affymetrix SNP50k XbaI arrays), 13q14 deletions displayed substantial anatomic heterogeneity(18). Nonetheless, multiple distinct break clusters located close to the 13q14-resident *miR 15a/16-1* loci and, alternatively, close to and inclusive of all or parts of the *retinoblastoma* gene (*RBI*) were identified. A telomeric cluster of breaks was located at ~50.2-50.7 Mb physical position, comprising the vast majority of the breaks of short 13q14 deletions. Of the short and relatively uniform 13q14 deletions, 54% (71/132) were between 0.678 Mb and 1.944 Mb in length, and only 2 lesions were identified that were shorter (CLL145 and CLL97, with lesion lengths of 0.198 and 0.425 Mb, respectively)(29-31).

Using a previously proposed classification schema for typical 13q14 deletions into types I and II (exclusive and inclusive of *RBI*, respectively), we detected 85 type I lesions and 54 type II lesions (including 7 CLL cases in which both lesion types were identified, existing on separate chromosomes) in this cohort. Further, the frequency of 13q14 type I and type II lesions in the entire CLL cohort (N=255) was 33% and 21%, respectively.

Acquired uniparental disomy (aUPD) at 13q was identified in 7 CLL cases as previously described(18). In all these cases the LOH region was very large (Supplementary Table 2). Six out of these seven cases also contained a region of copy loss at 13q14 (typical 13q14 deletions by FISH) while one case was associated with a homozygous *miR16-1-5p* mutation (see below).

A subset of CLL cases display very low *miR16-1/15a* cluster expression

The 13q14-resident microRNAs *miR16-1* and *miR15a*, located at 49,521 Mb physical position, are contributors to the biology of these lesions in CLL cells (18, 32-34). In our cohort of 132 CLL cases with typical 13q14 deletions, we identified 4 cases (CLL # 78, 113, 120 and 214; see Supplementary Figure 1) with breaks occurring in proximity (within 12kb) to the miR locus and with unresolved *miR* gene status, while 134 lesions unequivocally resulted in the removal of one and occasionally (~10%) both copies of these miRs (see below)(16).

We proceeded with an analysis of the expression of *miR16-1-5p* and *miR15a-5p* in 238/255 CLL cases with available intact RNA by extending normalized Q-PCR-based measurements to cases not previously reported(18). Measurements were normalized to the expression of two unrelated microRNAs, *RNU43* and *RNU49*, through simple subtraction of the means of CT values (delta Ct mean = Ctm *miR15a* or 16 minus Ctm *RNU43* or 49; the R²-correlation

for miR measurements using either *RNU43* or *RNU49* was 0.867 [miR16.1 data] and 0.84 [miR15a data], respectively). This resulted in largely negative delta Ct values for miR16-1 and positive values for miR15a; this is due to lower Ct values (higher expression) for miR16-1 relative to the reference microRNAs (Supplementary Table 1).

Subsequently, we ranked all CLL cases according to mean SNP 6.0 array-based chromosomal copy number (CN) estimates for a ~0.669 Mb chromosomal region between rs9535414 (immediately centromeric to the *miR16-1/15a* locus) and rs706593 (~650 Kb telomeric to the *miR16-1/15a* locus) encompassing 216 consecutive CN measurements. Next, we plotted mean delta Ct values for miR15a and 16-1 versus 13q14 genomic CN measurements for all CLL cases (grouped by 13q14 deletion status) and calculated mean normalized expression values for *miR16-1* and *miR15a* for the CLL cases with more extensive chromosomal loss (CN estimates <1) and for the CLL cases with less extensive loss (CN estimates ≥1) or no loss (cases without 13q14 deletions). Data are summarized in Figure 2 A-D with mean miR expression levels indicated with red numbers.

Mean normalized expression values of both *miR16-1* and *miR15a* were substantially lower (miR15a ~8-fold and miR16-1 ~4-fold, respectively) in the CLL subgroup with more extensive del13q14 deletions (CN estimates <1) as opposed to the group with no del13q14. For CLL cases with monoallelic 13q14 deletions and associated CN estimates ≥1 (the majority of such cases had CN estimates of ~1.2) as compared with cases without 13q14 deletions there was evidence for a gene dosage effect for miR15a expression (~1.3-1.9-fold lower) but no such effect for miR16-1 expression.

This combined data suggests that ~12-15% of CLL cases have very low miR16-1/15a levels and that the majority of CLL cases (~85%) display an overlapping range of expression of these miRs, with mild relative reductions in miR15 expression in monoallelically deleted 13q14 cases.

Assessment of elevated hazard ratios for short OS based on analysis of thresholded centromeric or telomeric 13q14 lesion breakpoints

Given various published observations on CLL 13q14 deletion sizes, associated gene deletions and clinical parameters, including our proposal for 13q14 subclassification into types I and II based on *RBI* gene status, we modeled hazard ratios for shortened survival for dichotomized 13q14 groups defined by distinct physical chromosomal break positions. Specifically, various dichotomized 13q14 groups were defined by sliding the separation/break points across all physical positions actually identified in the 13q14 deletions in this cohort (based on the physical positions of either telomeric or centromeric breaks: Supplementary Table 1). Using such an approach, we determined that the location of centromeric breakpoints strongly influenced the risk for short OS in CLL while the telomeric breakpoints had little effect (Supplementary Figure 2).

Specifically, the HR for short OS increased as the centromeric separation break for the two 13q14 groups moved closer to the telomere, with the highest HRs identified for breaks at ~48-48.5 Mb physical position followed by a sharp drop in risk. The *retinoblastoma* gene, with well-documented effects on cell cycle control and chromosomal instability in cancer cells, is located at 47.776 – 47.954 Mb physical position and is very close to the cohort dichotomization breakpoints that are associated with peak HRs for short OS, thus providing justification for a 13q14 classification schema centered on *RBI* (see also *RBI* mutation and expression data below). Additional genes located telomeric to *RBI* and in the area (48-48.5 Mb physical position) demarcated by the highest HRs for short OS included *LPAR6*, *P2RY5*, *RCBTB2* and *CYSLTR2*, but none of these genes have well-established roles in biological processes that are known to affect cancer cells. Finally, only 4 actual 13q14-associated

chromosomal breaks occurred between 48-48.5 Mb physical position, creating modest uncertainty about the actual physical positions associated with peak HRs as estimated by our approach.

Results of univariate outcome analyses of SNP 6.0 array-detectable 13q14 deletions and OS in CLL

We initially determined the prognostic value of SNP 6.0 array-defined 13q14 type I and type II deletions on overall survival in the CLL cohort (UT+T; N=132) using univariate analysis and either the CLL sample procurement/trial enrollment date or the CLL diagnosis date as the reference date. For both analyses, OS was significantly shorter for CLL patients with 13q14 type II lesions as opposed to patients with 13q14 type I lesions (Kaplan–Meier plots for these analyses are displayed in Figure 3A,C). These findings were true in the analyses in which CLL cases that carried both 13q14 lesion types (N=7) were included and hierarchically assigned to type II status (shown) or alternatively, were excluded from analysis. Next, we analyzed the prognostic value of SNP 6.0 array-defined 13q14 type I and type II deletions on OS in the untreated subset of patients (UT; N=100) and again noted shortened survival of CLL patients with 13q14 type II as opposed to type I lesions (Kaplan–Meier plots for these analyses are displayed in Figure 3B,D).

Finally, given that the nature of 13q14 deletion may also influence initial CLL disease progression, we analyzed TTFT estimates for the two 13q14 types but found no strong effects.

Deletion 13q14 type II are associated with a higher incidence of coexisting CLL-FISH-detectable chromosomal abnormalities than 13q14 type I lesions

Next, we determined the frequency of 13q14 deletions of types I and II that are associated with coexisting genomic lesions as detected through i) CLL-FISH-25, or ii) SNP 6.0 array profiling (see below). We defined 13q14 deletions of either types I or II as “FISH-complex” if they were associated with one or more additional abnormalities in the clinically used CLL-FISH panel (and if these abnormalities were detected in $\geq 25\%$ of nuclei analyzed [FISH-25]; this is to remove low percentage lesions of unclear clinical or biological significance). Using these criteria, the following frequencies were measured: 1) 13q14-I (N=85): sole abnormality (74/85=87%), FISH-complex (11/85=13%); 13q14-II (N=54): sole abnormality (38/54=70%), FISH-complex (16/54=30%; [p=0.03, Fisher's exact test]; CLL cases with both lesions types I and II (N=7) were counted as 13q14-I and 13q14-II).

Outcome analysis for del13q14 subtypes and associated CLL FISH lesions

We proceeded with an outcome analysis of the relative contributions of 13q14 subtypes by category (I versus II) versus associated CLL-FISH-25-based genomic complexity. Initially, we focused on bivariate analyses in which 13q14 lesion types were further separated into sole lesions or lesions coexisting with any other CLL-FISH-25 lesion (CLL cases that carried both 13q14 lesion types were included and hierarchically assigned to type II status). Data are summarized in Figure 3E-H. From these analyses the following conclusions can be supported: i) sole 13q14 lesions of either type were associated with a relatively better prognosis than either type existing in the presence of additional FISH findings; ii) trends for shorter OS for 13q14 type II deletions with CLL-FISH-complexity versus 13q14 type I deletions with CLL-FISH-complexity were observed; iii) trends for shorter OS for sole 13q14 type II versus sole 13q14 type I lesions were observed, albeit more pronounced in CLL cases that were relapsed at the time of analysis (UT+T plots); and, iv) 13q14 type II deletions with CLL-FISH-complexity were substantially enriched in relapsed CLL patients (frequency 45%) .

Deletion 13q14 type II are associated with a higher incidence of coexisting SNP 6.0 array-detectable acquired subchromosomal copy number aberrations than 13q14 type I lesions

Next, we determined the frequencies of either 13q14 lesion type that were associated with ≥ 2 additional SNP 6.0 array-based subchromosomal aCNAs (total aCNA complexity ≥ 3). The following frequencies were measured: 1) 13q14-I (N=85): sole abnormality (45/85=53%), 13q14-I plus ≥ 2 aCNA (16/85=19%); 13q14-II (N=54): sole abnormality (20/54=37%), 13q14-II plus ≥ 2 aCNAs (18/54=33%; $p=0.02$, Fisher's exact test; CLL cases with both lesions types I and II were counted as 13q14-I and 13q14-II). For comparison, the frequency of ≥ 3 SNP 6.0 array-based subchromosomal aCNAs in our entire CLL cohort of 255 profiled patients was 20% (35).

Given the known strong association of *p53* mutations with elevated genomic complexity in CLL, we determined *p53* exon 2-10 mutation frequencies in the CLL cases with 13q14 type I or II deletions and ≥ 2 additional SNP 6.0-based aCNAs: There were 16 cases with 13q14-I plus ≥ 2 aCNA, of which 7 (44%) were mutated in *p53* exons 2-10 and there were 18 cases with 13q14-II plus ≥ 2 aCNAs, of which 6 (34%) were mutated in *p53* exons 2-10.

Results of outcome analyses of 13q14 deletions of types I or II stratified by associated SNP 6.0 array profiling-detectable subchromosomal aCNA loads versus OS in CLL

We proceeded with bivariate outcome analysis of 13q14 deletions of types I and II grouped by associated SNP 6.0 array-based subchromosomal aCNA status of ≥ 2 and ≥ 3 , respectively, (CLL cases that carried both 13q14 lesion types were included and hierarchically assigned to type II status). Kaplan-Meier plots for these analyses are displayed in Figure 4A-H. From these analyses the following conclusions are supported: i) 13q14 lesions of either type with elevated associated aCNA counts were associated with a significantly worse prognosis than either type with no or additional aCNAs below indicated thresholds; and ii) 13q14 type II deletions were prognostically more adverse than 13q14 type I deletions, as evidenced by an accelerated disease course in cases with low associated complexity (aCNA ≤ 1 or ≤ 2 , respectively), a finding that was particularly evident in CLL cases that were relapsed at the time of analysis (UT+T plots).

Next, we performed bivariate outcome analysis of 13q14 deletions of types I and II stratified by associated SNP 6.0 array-based subchromosomal aCNA status of ≥ 2 and ≥ 3 , respectively, based on aCNA nominations that had been made using algorithmic aCNA calling methods (see Supplementary Methods). Results were similar to results based on aCNA nominations made through visual heatmap inspection. Kaplan-Meier plots are displayed in Supplementary Figure 3 A-H.

Degree of 13q14 deletions and outcome in CLL

The degree of 13q14 deletions was quantified using the mean of 216 consecutive CN estimates based on probes located within 13q14 deletions and correlated with the outcome measures TTFT and OS for the group of 132 CLL patients with 13q14 deletions. The CLL cohort was dichotomized at every actual CN measurement and hazard ratios for short TTFT or OS were computed. As can be seen in Supplementary Figure 4A-H, a CN estimate of ~ 1 (N=32 for CN <1 and N=100 for CN ≥ 1) and high CN cut-offs (the latter based on small N) optimally separated the cohort, suggesting that the degree of 13q14 deletion could have negative effects on CLL outcome once larger cohorts are studied (for this cohort the 95% confidence bands in grey overlapped a HR of 1)(36).

13q14-resident miR15a/-1 expression and outcome in CLL

The relative expression levels of the 13q14-resident miRs as measured by Q-PCR (see above) were tested as a prognostic factor. The CLL cohort was dichotomized at every actual

normalized miR expression measurement (actual delta CT values) and hazard ratios computed. No clear prognostic effects were identified (Supplementary Figures 5A-H and 6A-H). This was equally true for 13q14-resident miR levels tested as prognostic factors in the entire CLL cohort (N=255; data not shown)

CLL with 13q14 type II are associated with lower *RB1* mRNA expression levels and *RB1* frameshift mutations or biallelic *RB1* deletions in a minority subset of cases

The *RB1* gene coding exons were resequenced in 53 of the CLL with 13q14 type II deletions, resulting in the identification of two cases (CLL# 158 and 173) with somatically acquired truncating frameshift mutations. Together with one case of CLL with biallelic *RB1* deletion (CLL# 13), this indicates that ~5% of CLL with 13q14 type II deletions are *RB1* null.

Next, we measured *RB1* mRNA expression using normalized Q-PCR in the first 160 consecutively enrolled cases with intact RNA and identified significantly lower *RB1* expression in CLL with 13q14 type II deletions as opposed to CLL with 13q14 type I deletions or no 13q14 deletions. Data are summarized in Figure 5.

Identification of rare somatically acquired mutations in 13q14-resident *miR16-3p* and *miR16-5p* in CLL

Sequence analysis of the genomic locus for *miR16* and *15a* and flanking sequences in the entire CLL cohort identified 2 somatically mutated *miR16* cases and no *miR15a* mutations. CLL # 61 harbored a heterozygous 6 bp deletion spanning *miR16-3p*, while CLL # 70 carried a homozygous 1 nucleotide deletion in *miR16-5p* in the setting of chromosome 13 aUPD. The vast majority of CLL cases (99%) carried wildtype *miR 16* and *15a* genes.

DISCUSSION

In this study, we have employed SNP 6.0 arrays to interrogate genomic DNA isolated from FACS-sorted CD19+ cells compared with either paired sorted CD3+ cells (96%) or paired buccal (4%) DNA from 255 CLL patients for acquired chromosomal copy number changes (aCNA) and LOH with a detailed focus on 13q14 deletions. The novel findings from this study are i) that 13q14 deletions have varying effects on CLL clinical behavior, with large 13q14 deletions (type II: inclusive of *RB1*) associated with an inferior prognosis; ii) that 13q14 type II deletions are frequently associated with additional aCNA (a total aCNA count of ≥ 3 was detected in ~35% of cases)(35); iii) that ~10-15% of CLL with biallelic 13q14 deletions display markedly reduced *miR15a/16-1* levels but that the effect of monoallelic *miR* loss on miR expression is subtle and only demonstrable for *miR15a*; iv) that the relative expression levels of the 13q14-resident miRs do not substantially affect CLL prognosis; v) that a few CLL cases with 13q14 type II deletions carry *RB1* frameshift mutations and therefore are *RB1* null, and, vi) that rare CLL cases harbor acquired mutations in *miR16.1*.

This study is characterized by several methodological strengths, including paired (CD19+ versus CD3+) array analysis for all CLL samples, ultra-high-purity (sorted) cell sources as a source of DNA and RNA (for miR expression analysis) and a rigorous and conservative genomic copy number data analysis schema that had previously been externally validated using FISH(27). The analysis of paired samples is of particular importance for the accurate determination of genomic complexity in cancer cell genomes in the setting of ultra-high-resolution SNP arrays, given that the vast majority of CNVs are <1Mb in length. In all, our approach results in very high specificity and sensitivity for aCNA, thus avoiding problems for the precision of genomic clinical correlative analysis resulting from under- or overcalling of genomic lesions.

As part of the work presented here, we provide refined estimates for optimal physical separation cutoffs for 13q14 deletions into prognostically significant subtypes. From a practical perspective, routine inclusion of a probe for *RB1* or genes located within 300-500kb telomeric to *RB1* should reliably identify CLL patients carrying more adverse 13q14 subtypes(17).

Our novel identification of somatically acquired *RB1* null states, including somatically acquired *RB1* mutations, provides evidence that for a subset of 13q14 deletions, *RB1* is a relevant target gene. Furthermore this conclusion is supported by the well-studied effects of *RB1* on cell cycle control and genomic stability(37). However, given the identification of multiple 13q14 deletion types it is likely that additional 13q14-resident genes actively influence CLL biology and clinical phenotypes and that the identification of such genes remains an important research objective.

The identification of rare CLL with *miR16.1* mutations provides additional evidence for a functional role of this microRNA in CLL pathophysiology. Interestingly, one of the two *miR16.1* mutations occurred in *miR16.1-3p*, indicating that further studies of this gene product are warranted. Of note however, monoallelic *miR16.1* loss is insufficient to affect *miR16.1* expression compared with non deleted CLL cases thus providing constraints on models relating to the exact role of this microRNA in 13q14 biology.

This report is based on CLL patients enrolled prospectively at a single center with an outcome focus on overall survival. To minimize the effect of biases introduced through sample collections relative to prior treatments or actual dates of diagnosis, we have analyzed and presented data for the untreated and combined (untreated and relapsed) CLL cohort separately, using either the diagnosis or trial enrollment date as the reference dates. Such an analysis approach seems particularly relevant to biomarkers that change over the course of a patient's illness, or for markers for which such a change is likely but not yet proven through longitudinal analysis (inclusive of the hypothesis that 13q14 type II deletions arise during CLL disease evolution). Regarding the conclusion that 13q14 type II deletions constitute a factor associated with and contributory to more aggressive CLL, we were able to demonstrate that these lesions are disproportionately associated with elevated genomic complexity in CLL: a trait associated with aggressive CLL(25). It is, however, important to note that additional studies with substantially larger patient numbers are needed to be able to measure the relative contribution of 13q14 deletion subtypes versus other factors known to affect CLL clinical behavior.

One of the current, still speculative interpretations of the combined available data on 13q14 deletions in CLL is that short 13q14 deletions affect genes that confer a growth or survival/anti-apoptotic advantage on CLL cells, while longer 13q14 deletions are part of CLL disease evolution and degeneration into more aggressive subtypes; the latter would be due to increased genomic instability (as also seen with 17p and 11q deletions) and an ensuing potential for outgrowth of more aggressive CLL subclones(35, 38) and likely other, as-yet unidentified mechanisms.

In summary, our data demonstrate that 13q14 deletions in CLL are biologically and prognostically distinct entities and that routine clinical identification of 13q14 type I and II deletions would allow for refined CLL patient risk stratification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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TRANSLATIONAL RELEVANCE

Genomic aberrations have important effects on CLL biology and clinical outcome. Recently, a refined description of the substantial anatomic heterogeneity of the most common CLL-associated chromosomal deletion, del13q14, has emerged, and it is increasingly recognized that 13q14 deletions cannot be reduced to a singular pathophysiological mechanism. Furthermore, knowledge is still evolving regarding possible differential prognostic effects imparted on CLL by various 13q14 subtypes. In this report, we provide evidence that large 13q14 deletions that are inclusive of the *retinoblastoma* gene (*RB1*) are prognostically adverse and that these deletions are frequently associated with unstable CLL genomes. This analysis advances our understanding of the clinical relevance of the most common chromosomal deletion type in CLL and informs future refinements in diagnostic approaches to CLL management.

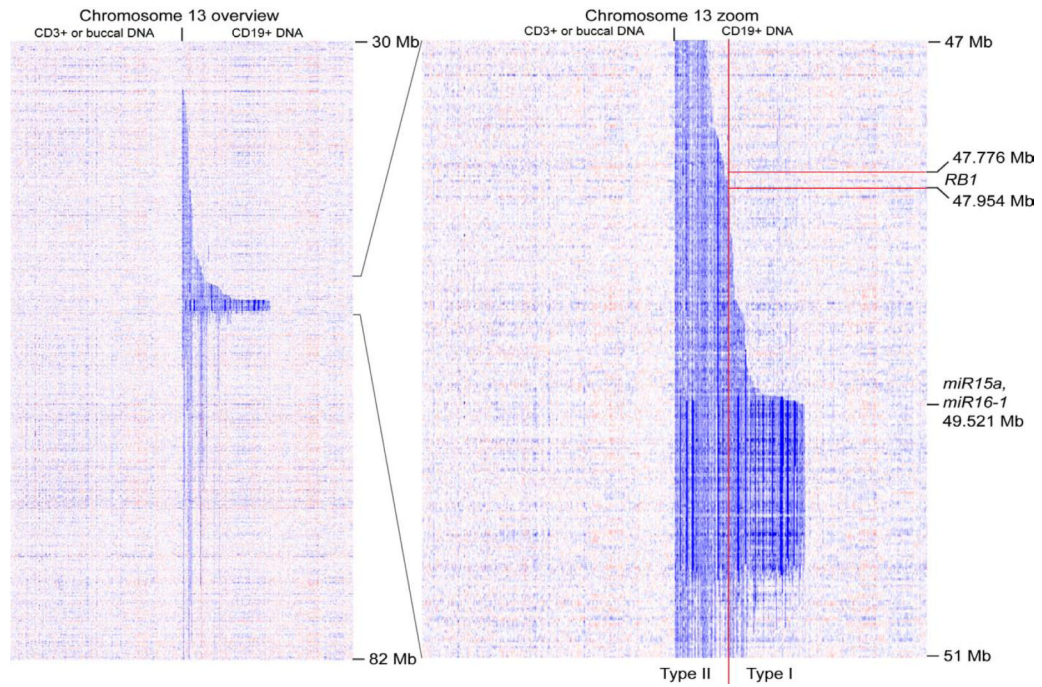


Figure 1. Genomic copy number heatmap display of chromosome 13q of 255 CLL cases ranked by the position of centromeric 13q14 deletion break points: Copy number heatmap displays for paired DNA samples based on SNP 6.0 array profiling were generated using dChipSNP. Left panel: CD3+ or buccal DNA; Right panel: CLL CD19+ DNA. Blue indicates copy loss, red indicates copy gain. Each column represents one patient.

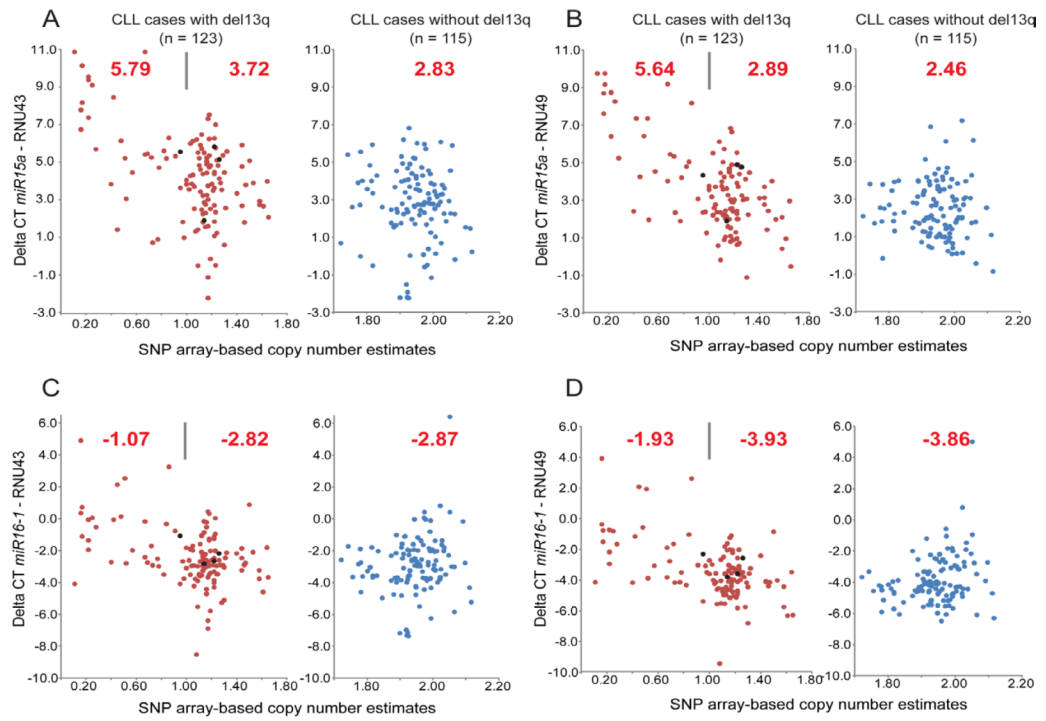


Figure 2A-D.

Normalized expression of miR15a and miR16-1 versus SNP 6.0 array-based copy number (CN) estimates for typical 13q14 deletions: Panels A-D: Displayed are mean delta Ct values (miR15a or 16-1 minus housekeeping microRNAs) as single dots (y-axis) versus SNP 6.0 profiling-based CN estimates for 13q14 deletions (x-axis) grouped by 13q14 deletion status. Red numbers indicate mean delta Ct values for CLL groups with CN<1 or ≥1 and 13q14 deletions or non-del13q14 status. Black dots: CLL cases with unresolved *miR* gene status.

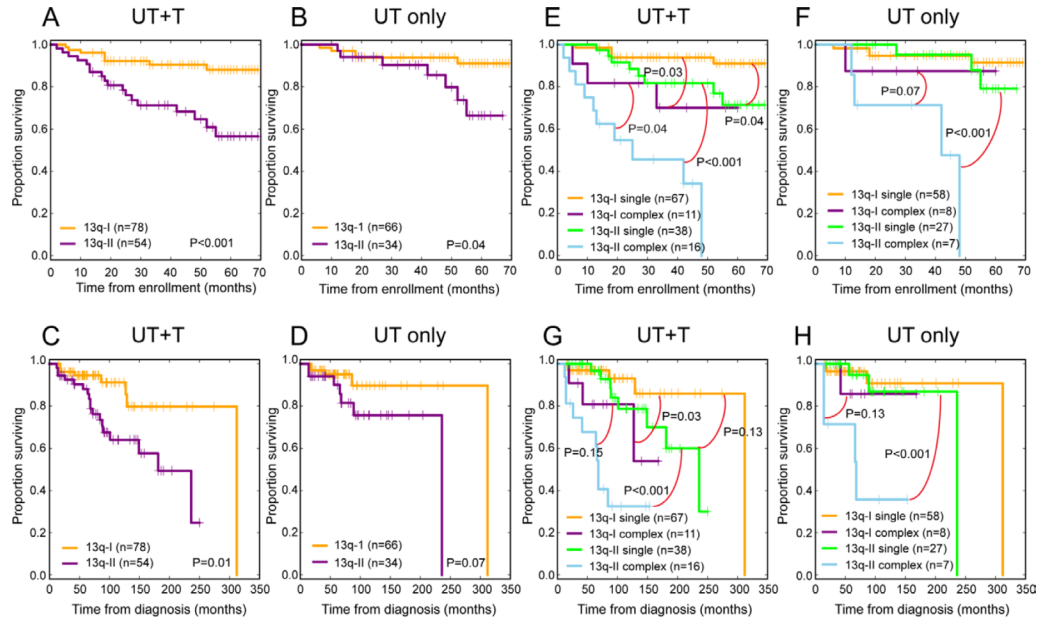


Figure 3A-H.

Deletions 13q14 types I or II and OS in CLL (Kaplan-Meier plots): Panels A-D: Deletion 13q14 types I or II and OS in CLL. UT: untreated at enrollment; UT+T: untreated or relapsed at enrollment. A,B: OS from date of enrollment, C,D: OS from date of diagnosis; Panels E-H: pairwise groupings by 13q14 status (I or II) and any associated FISH lesions. The suffix “complex” indicates a 13q14 deletion with any (≥ 1) coexisting FISH-25 finding.

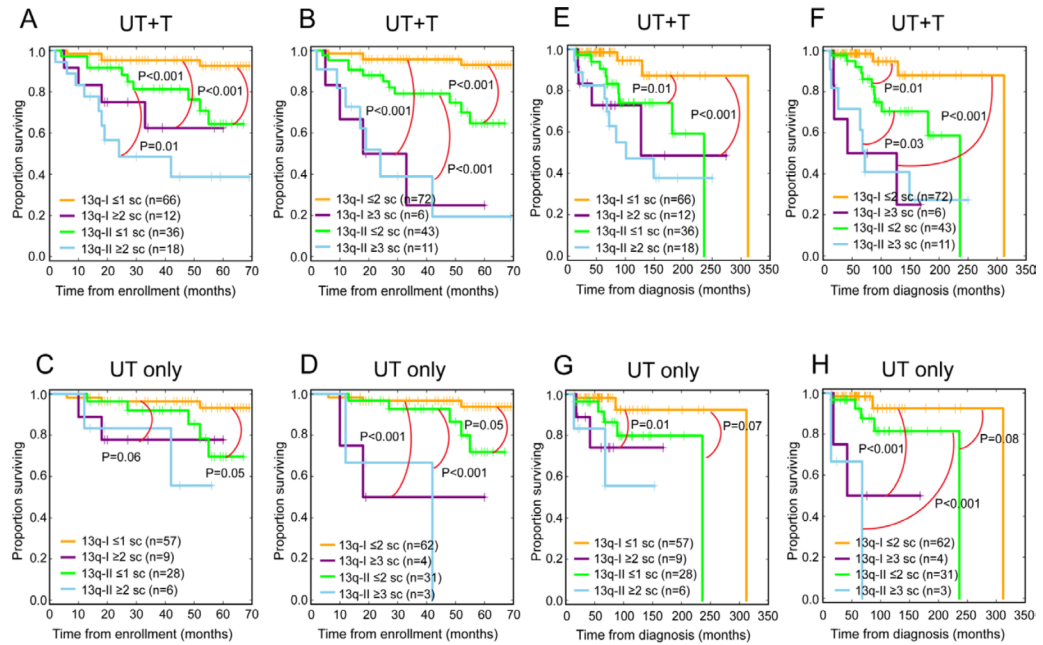


Figure 4A-H.

Deletion 13q14 types I or II and associated SNP 6.0 array profiling-based aCNA and OS in CLL (Kaplan-Meier plots): Panels A-D: OS from date of enrollment. Panels E-H: OS from date of diagnosis. UT: untreated at enrollment; UT+T: untreated or relapsed at enrollment. The suffix “sc” indicates sub-chromosomal aCNA present at indicated thresholds.

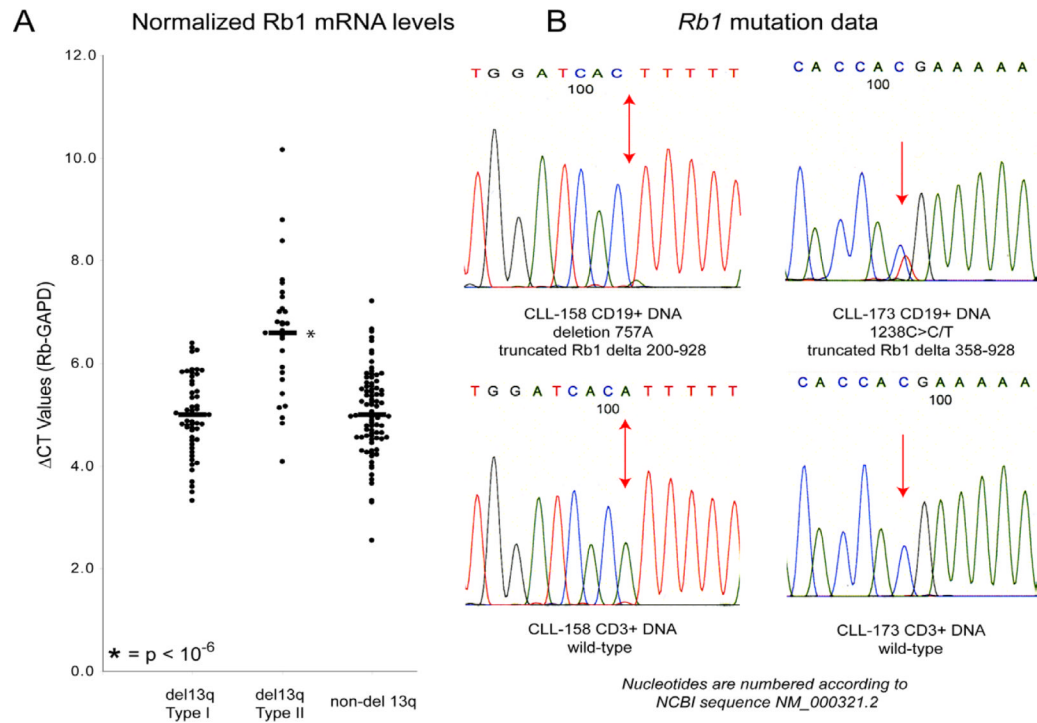


Figure 5A-F. Identification of somatically acquired *RB1* mutations and results of Q-PCR-based *RB1* expression analysis: A: Delta Ct values (C_{tm} *RB1* minus C_{tm} *GAPDH*) with each dot representing the mean of duplicate measurements in individual patients (N=160). Groupings are by 13q14 status. B: *RB1* mutation results in CD19⁺-derived DNA versus CD3⁺- derived DNA.

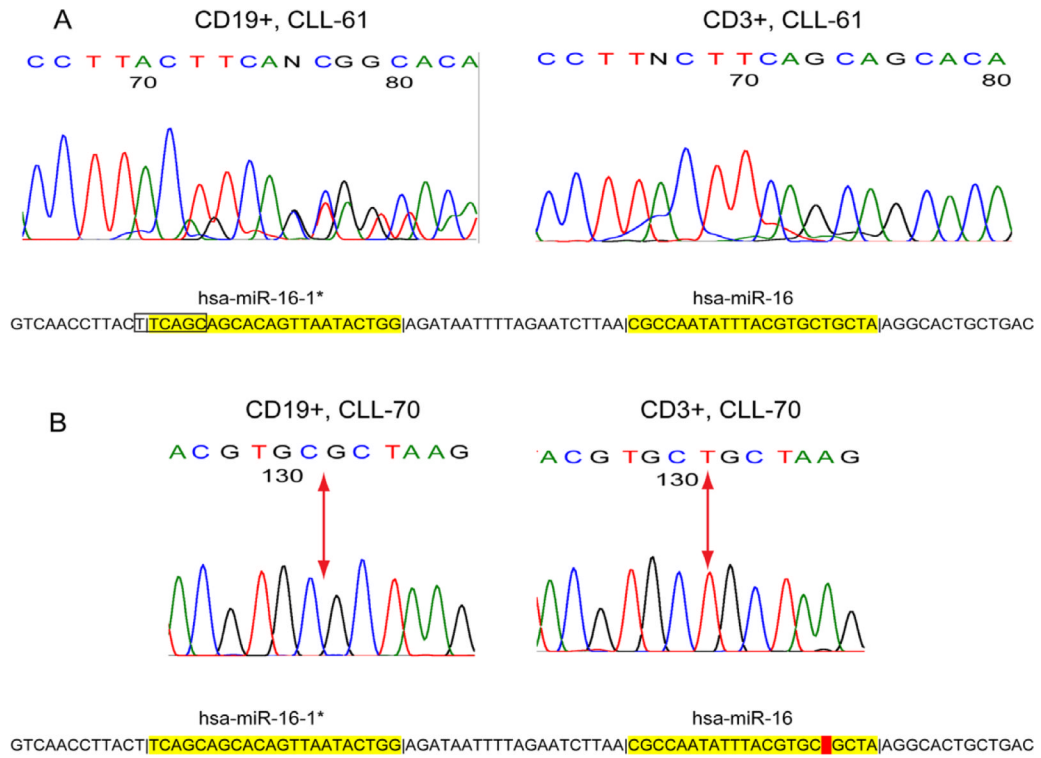


Figure 6A-B. Identification of somatically acquired *miR16.1* mutations: *miR16.1* mutation results in CD19+-derived DNA versus CD3+- derived DNA. The nucleotide sequence of the *miR16.1* gene located within 13q14 is indicated and miR16.1-3p and miR16.1-5p are highlighted in yellow.

Table 1

Patient characteristics.

Patient Characteristics at Enrollment		13q-I	13q-I	13q-II	13q-II	13q-II	Non-13q	Non-13q
		Treatment Naive no. (%)	Relapsed no. (%)	Treatment Naive no. (%)	Relapsed no. (%)	Treatment Naive no. (%)	Treatment Naive no. (%)	Relapsed no. (%)
Sample size N= 255 patients*		71 (27%)	14 (5%)	34 (13%)	20 (8%)	98 (37%)	25 (10%)	
Age, years								
Median		59	63	63	60	62	64	
Range		41 - 84	46 - 77	45 - 85	52 - 76	35 - 96	48 - 82	
Gender								
Female		23 (32%)	4 (29%)	13 (38%)	5 (25%)	36 (37%)	6 (24%)	
Male		48 (68%)	10 (71%)	21 (62%)	15 (75%)	62 (63%)	19 (76%)	
Rai stage								
Low, 0		32 (45%)	6 (43%)	14 (41%)	3 (15%)	49 (50%)	2 (8%)	
Intermediate, I-II		36 (51%)	6 (43%)	18 (53%)	9 (45%)	45 (46%)	16 (64%)	
High, III-IV		3 (4%)	2 (14%)	2 (6%)	8 (40%)	4 (4%)	7 (28%)	
Time from diagnosis to enrollment, months								
Median		6	92	30	76	4	59	
Range		0 - 306	14 - 235	0 - 208	10 - 220	0 - 211	5 - 244	
Time from enrollment to outcome analysis, months								
Median		53	48	52	42	50	47	
Range		14 - 69	16 - 69	14 - 68	14 - 69	14 - 69	15 - 69	
IgV _H mutational status								
Unmutated (≥ 98% homology to germline)		23 (32%)	4 (29%)	10 (29%)	11 (55%)	50 (51%)	19 (76%)	
Mutated (< 98% homology to germline)		46 (65%)	10 (71%)	22 (65%)	7 (35%)	44 (45%)	5 (20%)	
Not Evaluable		2 (3%)	0 (0%)	2 (6%)	2 (10%)	4 (4%)	1 (4%)	
Interphase FISH**								
17p deletion		5 (7%)	3 (21%)	1 (3%)	2 (10%)	11 (11%)	4 (16%)	
11q deletion		4 (6%)	3 (21%)	4 (12%)	8 (40%)	11 (11%)	2 (8%)	
13q deletion		69 (97%)	13 (93%)	32 (94%)	19 (95%)	10 (10%)	4 (16%)	
6q deletion		0 (0%)	0 (0%)	1 (3%)	0 (0%)	3 (3%)	0 (0%)	

Patient Characteristics at Enrollment	13q-I Treatment Naive no. (%)	13q-I Relapsed no. (%)	13q-II Treatment Naive no. (%)	13q-II Relapsed no. (%)	Non-13q Treatment Naive no. (%)	Non-13q Relapsed no. (%)
12q trisomy	2 (3%)	1 (8%)	2 (6%)	2 (10%)	29 (30%)	11 (44%)
Normal karyotype	0 (0%)	0 (0%)	2 (6%)	0 (0%)	40 (41%)	9 (36%)
FISH data not available	1 (1%)	0 (0%)	0 (0%)	0 (0%)	3 (3%)	1 (4%)
Interphase FISH-25 ^{***} (findings in ≥25% of nuclei)						
17p deletion	5 (7%)	3 (21%)	1 (3%)	2 (10%)	10 (10%)	4 (16%)
11q deletion	3 (4%)	2 (14%)	3 (9%)	7 (35%)	8 (8%)	2 (8%)
13q deletion	65 (92%)	13 (93%)	31 (91%)	19 (95%)	0 (0%)	1 (4%)
6q deletion	0 (0%)	0 (0%)	2 (6%)	0 (0%)	2 (2%)	0 (0%)
12q trisomy	1 (1%)	0 (0%)	2 (6%)	1 (5%)	27 (28%)	11 (44%)
Normal karyotype	4 (6%)	0 (0%)	3 (9%)	0 (0%)	54 (55%)	9 (36%)
Fish data not available	1 (1%)	0 (0%)	0 (0%)	0 (0%)	3 (3%)	1 (4%)
Complex interphase FISH-25 (findings >1)	8 (11%)	3 (21%)	7 (21%)	9 (45%)	6 (6%)	3 (12%)
ZAP 70 expression						
Negative (≤ 20%)	50 (70%)	5 (36%)	25 (74%)	8 (40%)	45 (46%)	6 (24%)
Positive (> 20%)	21 (30%)	9 (64%)	9 (26%)	12 (60%)	53 (54%)	19 (76%)
<i>P53</i> exon 2-10 mutations						
Wild type	63 (89%)	11 (79%)	32 (94%)	16 (80%)	84 (86%)	20 (80%)
Mutated	8 (11%)	3 (21%)	2 (6%)	4 (20%)	14 (14%)	5 (20%)
Number of prior therapies						
Median	NA	2	NA	1	NA	1
Range	NA	1 - 5	NA	1 - 5	NA	1 - 7

* 7 patients had both 13q-I and 13q-II lesions

*** Multiple FISH abnormalities per patient were individually counted