Whole-Genome Scanning by Array Comparative Genomic Hybridization as a Clinical Tool for Risk Assessment in Chronic Lymphocytic Leukemia

Shelly R. Gunn,*† Mansoor S. Mohammed,†
Mercedes E. Gorre,† Philip D. Cotter,†
Jaeweon Kim,† David W. Bahler,‡
Sergey N. Preobrazhensky,‡ Russell A. Higgins,*
Aswani R. Bolla,* Sahar H. Ismail,*
Daphne de Jong,§ Eric Eldering,¶
Marinus H.J. van Oers,|| Clemens H.M. Mellink,**
Michael J. Keating,†† Ellen J. Schlette,‡‡
Lynne V. Abruzzo,‡‡ and Ryan S. Robetorye*

From the Department of Pathology,* The University of Texas Health Science Center at San Antonio, San Antonio, Texas; the Departments of Leukemia†† and Hematopathology,‡‡ University of Texas M.D. Anderson Cancer Center, Houston, Texas; Combimatrix Molecular Diagnostics Incorporated,† Irvine, California; the Department of Pathology and ARUP Institute for Clinical and Experimental Pathology,‡ University of Utah, Salt Lake City, Utah; the Department of Pathology,§ The Netherlands Cancer Institute, Amsterdam, The Netherlands; and the Departments of Experimental Immunology,¶ Hematology,|| and Clinical Genetics,** Academic Medical Center, Amsterdam, The Netherlands

Array-based comparative genomic hybridization (array CGH) provides a powerful method for simultaneous genome-wide scanning and prognostic marker assessment in chronic lymphocytic leukemia (CLL). In the current study, commercially available bacterial artificial chromosome and oligonucleotide array CGH platforms were used to identify chromosomal alterations of prognostic significance in 174 CLL cases. Tumor genomes were initially analyzed by bacterial artificial chromosome array CGH followed by confirmation and breakpoint mapping using oligonucleotide arrays. Genomic changes involving loci currently interrogated by fluorescence *in situ* hybridization (FISH) panels were detected in 155 cases (89%) at expected frequencies: 13q14 loss (47%), trisomy 12 (13%), 11q loss (11%), 6q loss (7.5%), and 17p loss (4.6%). Genomic instability was the second most commonly identified alteration of prognostic significance with three or more alterations involving loci not interrogated by FISH panels identified in 37 CLL cases (21%). A subset of 48 CLL cases analyzed by six-probe FISH panels (288 total hybridizations) was concordant with array CGH results for 275 hybridizations (95.5%); 13 hybridizations (4.5%) were discordant because of clonal populations that comprised less

than 30% of the sample. Array CGH is a powerful, cost-effective tool for genome-wide risk assessment in the clinical evaluation of CLL. (J Mol Diagn 2008, 10:442–451; DOI: 10.2353/jmoldx.2008.080033)

Characteristic recurrent unbalanced chromosome aberrations are highly associated with prognosis in chronic lymphocytic leukemia (CLL), and are currently detected by classical cytogenetics and fluorescence *in situ* hybridization (FISH). Although a whole-genome view is afforded by conventional cytogenetics, CLL B-cells do not grow well in culture, and most laboratories report results in only a minority of cases unless special techniques such as stimulation of CLL cells with CD40 ligand are used.¹ Conversely, panels of locus-specific FISH probes show clinically significant results in more than 80% of cases, but provide a very limited view of the genome. With the advent and availability of array-based comparative genomic hybridization (array CGH) technology, high-resolution, locus-specific analysis and genome-wide evaluation can be combined into a single test.^{2–4} A number of array-based studies analyzing copy number imbalances in CLL have been recently reported, including array CGH using bacterial artificial chromosome (BAC) and oligonucleotide microarrays, as well as high-density single nucleotide polymorphism analysis using commercially available arrays.^{5–7} In addition, a small validation study was recently performed in which 31 B-cell-enriched samples were subjected to array CGH analysis using a targeted BAC array for the detection of recurrent chromosomal abnormalities in CLL patients. Copy number changes were detected in 87% of cases and found to be 100% concordant with FISH analysis when chromosomal abnormalities were present in greater than 25% of the cells.⁸ These promising results illustrate the power and limitations of array technology to identify clinically significant copy number changes, and warrant a

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Address reprint requests to Shelly R. Gunn, The University of Texas Health Science Center at San Antonio, Department of Pathology, Mail Code 7750, 7703 Floyd Curl Dr., San Antonio, TX. E-mail: gunn@uthscsa.edu.

larger study to explore the potential usefulness of clinically validated array CGH assays for individualized patient risk stratification in CLL.⁹

In the current study, we describe the rigorous validation and clinical testing of a commercially available BAC CGH array designed to interrogate alterations at loci that have been shown to have a prognostic impact in CLL,^{10,11} as well as genes that are implicated in additional hematological malignancies, including *RB1*, *Leu-1*, *Leu2*, *Leu-5*, *MIR15A/MIR16-1* genes (13q14), *ATM*, *MLL* (11q22-11q23), *MYB* (6q23), *TP53* (17p13), *IGH* (14q32), and *MYCN* (2p24). Array performance was initially tested in an accuracy study that included 20 peripheral blood leukemia samples isolated from patients with CLL, acute lymphoblastic leukemia, and chronic myelogenous leukemia. Clinical testing and validation of the microarray for the identification of CLL prognostic markers was performed using DNA extracted from 174 peripheral blood samples with previous morphological and immunophenotypic diagnoses of B-cell CLL. The validation study compared individual clone performance for CLL prognostic markers included on the BAC array to six-probe FISH panels for CLL markers, and the whole-genome array profiles derived from the 1093 probes contained on the BAC array to those obtained from high-density oligonucleotide array CGH analysis of the CLL tumor genome. Oligonucleotide array CGH was chosen to validate selected results because of the limitations of using a six-probe FISH panel to validate the genome-wide changes detected by an array containing 1093 probes. Oligonucleotide array verification also provided the added advantages of breakpoint definition and accurate mapping of the sizes of deletions and duplications. The CLL prognostic marker array CGH assay showed high accuracy and reproducibility with easily interpretable results suitable for timely incorporation into clinical decision making. In addition, novel and unexpected cryptic recurrent genomic aberrations were identified in 21% of the CLL samples.

Materials and Methods

DNA Samples

De-identified DNA samples extracted from the peripheral blood of 20 patients with diagnoses of CLL, acute lymphoblastic leukemia, and chronic myelogenous leukemia based on standard morphological, immunophenotypic, and/or cytogenetic criteria were used for the initial validation. An additional set of 174 DNA samples previously extracted from patients with morphological and immunophenotypic diagnoses of CLL were used for clinical testing of the assay for CLL prognostic markers. A subset of 48 samples had been previously characterized by a commercially available FISH probe set (Vysis; Abbott Molecular Inc., Des Plaines, IL) for CLL prognostic markers. Pooled male or female human genomic DNA (Promega, Madison, WI) was used as the reference sample for array CGH testing, and sex mismatch served as an internal control. Male reference DNA was used for cases in which the patient sex was unknown.

BAC Array CGH

Diagnostic BAC array CGH analysis of the tumor genomes was performed at the Combimatrix Molecular Diagnostics Laboratory (Irvine, CA). The arrays included 179 clones representing CLL prognostic loci along with 914 additional FISH-mapped clones for generic coverage of the genome at an average resolution of ~2.5 Mb (a complete list of clones can be found in Supplementary Table S1 at <http://jmd.amjpathol.org>). Tumor genomic DNA (test DNA) and reference DNA were differentially labeled with Cy3 and Cy5 dye-containing nucleotides in dye swap reactions using the BioPrime random labeling kit (Invitrogen, Carlsbad, CA) and purified with DNA Clean and Concentrator-5 kits (Zymo Research, Orange, CA). The labeled and purified DNA was combined with 50 µg of human Cot-1 DNA (Invitrogen), precipitated with isopropanol, rinsed in 70% ethanol, and air-dried. DNA pellets were dissolved in 20 µl of Pronto cDNA/Long Oligo hybridization solution (Corning Inc. Life Sciences, Lowell, MA), heated at 95°C for 5 minutes with vigorous vortexing after 2½ minutes, and then co-hybridized overnight (~16 hours) to the Combimatrix Molecular Diagnostics CA1000 microarray at 42°C. Slides were soaked in prewarmed (50°C) 0.1% Igepal/2× standard saline citrate (SSC) for 1 minute to remove the coverslip and then washed at 50°C for 5 minutes (with shaking) in 50% formamide/2× SSC. The wash step was repeated with prewarmed (50°C) 0.1% Igepal/2× SSC for 5 minutes and with 0.2× SSC for 2 minutes at 50°C. The microarrays were briefly rinsed with 0.2× SSC at room temperature for 1 to 2 minutes and then immediately centrifuged for 10 to 20 seconds for drying (with Tabletop Microarray Slide Spinner; ISC BioExpress, Kaysville, UT).

Data Analysis for BAC Arrays

Hybridized microarray slides were scanned with a GenePix 4000B microarray scanner and quantified using GenePix Pro microarray image analysis software (Molecular Devices, Sunnyvale, CA). Cy3 and Cy5 images were scanned independently through two separate channels for both dye-swapped reactions. The quantified data were analyzed using Combimatrix Molecular Diagnostics CGH Analysis Tool software. This software compiles array data into a spreadsheet that links the fluorescence signals obtained from both dye reactions for each clone on the array to a clone name, array position, chromosomal locus, and normalized Cy3 to Cy5 ratio. Normalized Cy3 to Cy5 intensity ratios for each of the two reactions were then plotted together for each of the chromosomes. Ratio plots are assigned such that gains in DNA copy number at particular loci are observed as the simultaneous deviation of the ratio plots from a modal value of 1.0, with a blue ratio plot representing a positive deviation (to the right) and a red ratio plot representing a negative deviation at the same locus (to the left). Conversely, DNA copy number losses show the opposite pattern. The linear order of the BAC clones is reconstituted in the ratio plots as chromosome ideograms, with p termini at the top and q termini at the bottom of each of the ideograms. When the whole genome is viewed, the

loss of a particular clone is manifested as a simultaneous deviation of the ratio plots from a modal value of 1.0, with red ratio plots showing a positive deviation (upward) and blue ratio plots showing a negative deviation (downward) at the same locus. Conversely, DNA copy number gains show the opposite pattern. Mono-allelic and bi-allelic deletion thresholds were defined by comparing apparent mono- and bi-allelic deletions identified by array CGH to those detected by FISH in the same cases. Thresholds were set at forward/reverse reaction ratios less than 3.0 for mono-allelic deletions and greater than or equal to 3.0 for bi-allelic deletions.

Oligonucleotide Array CGH

Thirty-six of the CLL cases were also analyzed using Agilent Human Genome 44K CGH oligonucleotide arrays (Agilent Technologies, Santa Clara, CA) according to the manufacturer's recommendations. Slides were scanned with an Agilent Scanner, and the data were analyzed with Agilent Feature Extraction and CGH Analytics software.

FISH

FISH analysis was performed using standard methods on interphase nuclei. Probes used in the analysis were obtained from Vysis and included LSI BCR/ABL for the initial validation and LSI D13S319, LSI 13RB1, CEP 12, LSI ATM, LSI P53, and LSI IGH for the CLL-specific validation. Nuclei were counterstained with 4,6-diamino-2-phenylindole and scored on an Axioskop microscope (Carl Zeiss, Thornwood, NY) using epifluorescence filters for Spectrum Orange, Spectrum Green, and 4,6-diamino-2-phenylindole. Digital images were captured on a CytoVision workstation (Applied Imaging Corp., Santa Clara, CA). The cut-off value for a positive result was 10%. At

least 200 nuclei were analyzed, and the number of abnormal nuclei was expressed as a percentage of the total number scored.

Results

Assessment of Accuracy and Precision in Heterogeneous Samples

An accuracy and precision study was performed to assess array performance in clinical hematology oncology samples using genomic DNA isolated from 20 leukemic peripheral blood samples with known abnormal results established by cytogenetics, FISH, or commercial research microarrays. Previously identified imbalances were detected by the array in 19 of the 20 samples (the results are summarized in Table 1). Subsets of five samples from the accuracy study were run in duplicate by both the same and different technologists for the assessment of precision and intermediate precision. In both experiments, all of the genomic abnormalities were accurately replicated in all five of the samples.

Clinical Testing of Array CGH for CLL Prognostic Markers

BAC array CGH was evaluated as a clinical test for the detection of recurrent genomic imbalances currently assayed by FISH panels in 174 additional cases of CLL, and significant chromosomal abnormalities were identified in a total of 155 cases (89%). Detection rates of involving losses of 6q, 11q, 13q, 17p, and whole chromosome 12 gain were consistent with previously reported percentages (summarized in Table 2).^{13,14} Losses of 14q32.33 were identified at a higher rate (54%) than has previously been reported. Visual inspection of whole-genome view ratio plots for si-

Table 1. Peripheral Blood Accuracy Study for Leukemia Samples

Type	BAC array	Known chromosomal abnormalities	Concordant
1 CLL	Losses: 13q14, 14q31, Y	Losses: 13q14, 14q32; Y by oligonucleotide array	Yes
2 CLL	Losses: 13q14, 14q32	Losses: 13q14, 14q32 by oligonucleotide array	Yes
3 CLL	Trisomy 12; loss 14q32	Trisomy 12; loss 14q32 by oligonucleotide array	Yes
4 CLL	Losses: 13q14, 14q32	del 13q14, del 14q32 by FISH	Yes
5 CLL	Biallelic loss 13q14; loss 14q32	Biallelic loss 13q14; loss 14q32 by oligonucleotide array	Yes
6 CLL	Loss 11q23	Loss 11q23 by oligonucleotide array	Yes
7 CLL	Trisomy 12	47,XY,+12 by cytogenetics and FISH	Yes
8 CLL	Loss 13q14	Loss 13q14 by oligonucleotide array	Yes
9 CLL	Trisomy 12	+12 by FISH	Yes
10 CLL	Loss 13q14	Loss 13q14 by oligonucleotide array	Yes
11 CML	Balanced genome	Balanced t(9;22)(q34;q11.2) by cytogenetics and FISH	Yes
12 CML	Loss 9q34*	t(9;22)(q34;q11.2) and loss 9q34 by oligonucleotide array	Yes
13 CML	Loss 9q34*	t(9;22)(q34;q11.2) and loss 9q34 by oligonucleotide array	Yes
14 CML	Balanced genome	Balanced t(9;22)(q34;q11.2) by cytogenetics and FISH	Yes
15 ALL	Whole chromosome gains: 5, 6, 8, 21, 21,Y†	52,XY,+5,+6,+8,+21,+21,+mar[8] by cytogenetics	Yes
16 ALL	Balanced genome	Balanced t(6;22)(p21;q11) by cytogenetics	Yes
17 ALL	Whole chromosome gains: 6, 8, 12, 22	50,XX,+6,+8,+12,+22 by cytogenetics	Yes
18 ALL	Whole chromosome loss: 7	45,XY,-7 by cytogenetics	Yes
19 T-ALL	9p loss‡	t(1;14)(p;34;q11) by cytogenetics	No†
20 ALL	Whole chromosome gains: 6, 10, 14, 21	50,XY,+6,+10,+14,+21 by cytogenetics	Yes

*The BAC array showed loss of RP11-88G17 on chromosome 9q34 confirmed by oligonucleotide array. This probe overlaps the derivative chromosome 9 deletion in unbalanced t(9;22).¹²

†Cytogenetics identified a marker chromosome that was found to be gain of Y by BAC array CGH.

‡A cryptic 9p deletion was revealed by BAC array, and a balanced t(1;14) was detected by cytogenetics.

Table 2. Summary of CLL Prognostic Markers Detected by Array CGH

Copy number change	Number of cases	Accompanying prognostic markers
13q14 loss	76 (44%)	6q, 11q, 17p loss; trisomy 12
Complex genome*	37 (21%)	6q, 11q, 13q, 17p loss; trisomy 12
Trisomy 12	23 (13%)	13q14 loss
11q loss	19 (11%)	13q14, 6q loss
17p loss	8 (5%)	13q14, 6q loss
6q loss	7 (5%)	11q, 13q14, 17p loss
14q32 loss†	95 (54%)	6q, 11q, 13q, 17p loss; trisomy 12

*Defined as ≥ 3 significant (nonpolymorphic) genomic changes.

†Losses of 14q32 were detected at a higher than expected frequency because of multiple ($n = 6$) probe coverage of the *IGH* gene region. All of the probes covering the *IGH* gene region on the array have been reported to be polymorphic or overlap regions of genomic variation.

multaneous deviation and forward reaction clone ratio values of ≥ 1.2 (gains) and ≤ 0.8 (losses) was used for the subjective and objective identification of single-clone copy number changes. Bi-allelic deletions were defined by reverse/forward reaction ratios ≥ 3.0 . No gene amplifications were observed in this case series.

Loss of 13q14

Losses of chromosome 13q were present in 76 cases (44%) and represented five recurrent deletion patterns (Figure 1A). The most common pattern involved mono-allelic (41 cases) or bi-allelic (9 cases) deletion of four identical con-

Figure 1. Recurrent patterns of chromosome 13 loss in 76 CLL cases. **A:** The linear positions of eight contiguous clones covering chromosome band 13q14.3 are shown with recurrent deletion patterns observed by BAC array CGH in 76 CLL cases. Sixty-five percent of 13q losses involved mono- or bi-allelic deletion of clones RP11-34F20, RP11-80H2, RP11-48H1, and RP11-1064M14. In six additional cases, these bi-allelic deletions were terminal or interstitial to larger deletions. In 20 mono-allelic cases, deletion breakpoints were outside the 13q14 region. **B–F:** Recurrent deletion patterns are illustrated by ratio plots showing losses of DNA copy number at 13q as the simultaneous deviation of the ratio plots from a modal value of 1.0, with red ratio plots showing a positive deviation (to the right) and blue ratio plots showing a negative deviation at the same locus (to the left). **B:** Mono-allelic, four clones. **C:** Bi-allelic, four clones. **D:** Terminal bi-allelic, four clones. **E:** Interstitial bi-allelic, four clones. **F:** Mono-allelic with breakpoints outside 13q14.3.

tiguous probes in the 13q14.3 region. In six additional cases, this common four-clone bi-allelic deletion was interstitial or terminal to a larger deletion (6 to 26 clones) not confined to the 13q14.3 region. The presence of these mono- and bi-allelic chromosome 13 losses, including homozygous 13q14.3 deletions embedded within larger heterozygous 13q14 deletions, could be objectively determined by the clone ratios as well as subjectively visualized by inspection of the relevant chromosome 13 ratio plots (Figure 1, B–F). 13q14 losses were accompanied by the presence of additional clinically relevant prognostic markers in 22 cases (27%), including 7 cases with loss of 17p, 10 cases with loss of 11q, 5 cases with trisomy 12, and 1 case with loss of 6q. Deletion sizes ranged from complete loss of

chromosome 13 (one case) to less than 1 Mb loss of two clones spanning 13q14.2–q14.3 (one case). A single BAC clone (RP11-34F20) represented the smallest common region of deletion (nucleotides 49478679 to 49633565) in all 76 of the cases that showed deletion of 13q and included the microRNA genes *MIR15A/MIR16-1*.

Trisomy 12

Trisomy 12 was detected in 23 (13%) of the total cases and was accompanied by loss of 13q14 in 5 cases (Figure 2). Trisomy 12 was not detected with losses of 17p, 11q23, or 6q. Ratios for the 37 chromosome 12-specific

Figure 2. CLL with bi-allelic 13q14 deletion and trisomy 12. **A:** FISH analysis shows three copies of CEP 12 with two copies of control probe CEP 7. **B:** A normal nucleus with two signals for D13S319 (red) and control probe CEP 18 (green) contrasted with an abnormal nucleus showing bi-allelic deletion of D13S319. **C** and **D:** An array CGH whole-genome view confirms trisomy 12 with an interstitial bi-allelic 13q14 deletion and reveals losses involving the *IGHV* region on 14q32 and *PRAME* on 22q11.2. This patient also shows a CNV on the X chromosome and loss of Y.

clones showed simultaneous positive deviation on the whole genome and chromosome-specific ratio plots and met numeric criteria for whole chromosome gain in all 23 of the cases. One additional case with a complex karyotype (which included losses of 17p, 11q23, and chromosome 13) showed partial trisomy 12 with gain of 19 clones spanning 12p11.21→q24.33. A single case also showed coexistent trisomy 12 and trisomy 19, which has been previously reported.^{5,15}

Loss of 11q

Loss of 11q was detected in 19 (11%) of the total cases and was present as a single prognostic marker in 8 cases. In 11 cases, 11q23 loss was accompanied either by a 13q14 deletion (10 cases) or a 6q deletion (1 case). No cases in this series showed 11q23 deletion with trisomy 12 or loss of 17p. The largest deletions encompassed 36 of the 46 contiguous clones covering 11q and extended from 11q14.1→q25. The smallest deletions contained 11 clones and extended from 11q22.3→q23.3 (Figure 3A). A minimal region of overlap encompassing the *ATM* gene (Figure 3B) at 11q22.3 included the probes RP11-648J7 to RP11-279M1 (11q22.3→q23.2) and was consistent with a previously described minimal region of deletion on 11q.¹⁶ Breakpoint analysis in seven cases with 11q loss confirmed that the common region of overlap included the *ATM* gene, and showed the size of the most common deletion to be ~8.8 Mb (Figure 3C).

Loss of 17p

Eight cases (5%) showed loss of 17p and were all accompanied by additional genetic alterations, including 13q14 loss in six cases and 6q loss in two cases. Seven of the 17p losses ranged from 18 to 23 probes with centromeric breakpoints localized to probes at 17p11.2 (Figure 4A). These results are consistent with previous reports describing loss of *TP53* in CLL to be attributable to loss of the p-arm rather than monosomy 17 or single copy loss of *TP53*.¹⁷ Interestingly, one case retained five telomeric clones distal to the commonly deleted region seen in the other seven cases and showed a minimal and discontinuous seven-clone deletion that also included the *TP53* gene (Figure 4B). Three cases with 17p loss by the BAC array were confirmed by oligonucleotide array analysis, including a case with a relatively small seven-clone discontinuous loss involving the *TP53* gene (Figure 4C).

Loss of 14q32.33

Ninety-five cases (54%) showed losses of one or more of the six contiguous clones covering the immunoglobulin heavy chain (*IGH*) constant and variable gene regions on 14q32. The increased frequency of 14q32.33 loss observed in this study can be attributed to the increased sensitivity of the BAC array's multiple small (~100 Kb) probes covering the *IGH* gene region compared to the commonly used single large (900 Kb) FISH probe for *IGH*

Figure 4. Patterns of 17p loss observed in eight CLL cases. **A:** All telomeric breakpoints occurred in band p13.3 and extended to pericentromeric band p11.2 in seven cases. **B:** One case showed a discontinuous interstitial deletion involving seven total clones, including RP11-89D11 representing the *TP53* gene. **C:** High-density oligonucleotide array confirmation of the discontinuous interstitial deletion of 17p showed loss of chromosomal material from nucleotides 1963450 to 4290955 and 727151 to 9537360, including *TP53* at oligonucleotides 7512900 to 7530424.

included in most of the clinical panels for CLL. In 10 cases, the 14q32 deletions involved all of the clones in the *IGH* gene region but did not extend proximally beyond 14q32.33. Eight cases showed bi-allelic loss of at least one of the clones from this region (Table 3). All of the contiguous clones on 14q32.33 contained on the BAC array are known to show a variation in phenotypically normal individuals or overlap regions of genomic variation (locus 3878; Database of Genomic Variants; <http://projects.tcag.ca/variation/>), suggesting that at least a small percentage of the single-clone changes observed in this study may represent germ-line polymorphisms.¹⁸ Additional patient material was not available to perform a matched-pair analysis of tumor versus germ-line DNA.

Loss of 6q

Losses of 6q were observed in seven cases ranging in size from five probes to most of the q-arm (19 probes). Proximal breakpoints were observed from band 6q14.1→q22.31, and distal breakpoints were observed from 6q21→q27. No

minimal region of overlap existed between these seven cases, and two cases with five- and six-clone deletions and distal breakpoints at 6q21 and 6q22.31 did not include the *MYB* gene at 6q23.3.¹⁹

Recurrent Gains of 2p Include the *MYCN* Gene Region at 2p24.3

Five cases showed partial or whole-arm gains of chromosome 2p. All of the duplications involved a BAC clone (RP11-355H10) that contained the *MYCN* gene at 2p24.3, including one case that represented the smallest region of overlap (10 interstitial probes from RP11-451A6 at 2p25.2 to RP11-113D22 at 2p22.3). The minimally duplicated case did not include gain of a recently described 3.5-Mb critical region at 2p16 that includes the *REL* and *BCL11A* genes, but this region was duplicated in four cases with proximal breakpoints in bands 2p14 (one case) and 2p21 (three cases).⁷ Concomitant chromosomal losses included 13q (three cases) and 11q (three

Table 3. Deletion Rates for Contiguous Clones Covering the *IGH* Region on 14q32.33 in 95 CLL Cases Showing Loss of 14q32 by BAC Array CGH

Clone	<i>IGH</i> region	Linear center*	Size†	Deletions‡	Biallelic loss
RP11-448N5	Constant	105.35	168,495	<i>n</i> = 60	4 cases
RP11-259B19	Variable	105.56	169,293	<i>n</i> = 81	None
CTD-3039D5	Variable	105.89	71,808	<i>n</i> = 63	4 cases
RP11-76N15	Variable	106.16	131,318	<i>n</i> = 51	1 case
CTD-2011A5	Variable	106.21	184,052	<i>n</i> = 36	None
CTD-2313E3	End of variable	106.27	93,751	<i>n</i> = 36	None

*Linear center is defined as the median distance between the start/end base pair position for each probe.

†Probe sizes are indicated in number of base pairs.

‡Indicates the number of cases of the total 95 showing losses of a particular probe.

cases), as well as whole-arm losses of 18p (one case), 4p (one case), and 20p (one case).

Novel Recurrent Copy Number Aberrations

Novel recurrent genomic imbalances accompanying the known CLL prognostic markers were revealed by array CGH in 13 cases and included losses of 5q (three cases), 2q (two cases), and 8p (two cases), as well as gains of 8q (three cases) and whole-chromosomes 19 (one case) and 22 (three cases). In the three cases showing loss of 5q, the deletions involved the same five clones on 5q14.1→q21.3, an area of the array targeted to detect del(5q)-associated myeloproliferative disorders. 5q losses occurred in two cases with 13q deletion as the only other additional abnormality and in one case with complex genomic changes that also included accompanying losses of 6q, 11q, and 13q. The occurrence of 5q deletions in these cases may be associated with previous cytotoxic therapy or signify the presence of an emerging myeloid clone.²⁰

Concordance with FISH

A subset of 48 CLL samples had previously been analyzed by FISH using commercially available probes. FISH and array CGH results were compared in a blinded manner to assess array performance versus interphase cytogenetics. In a total of 288 hybridizations (six probes per case), 275 hybridizations (95.5%) were concordant with array CGH, and 13 hybridizations (4.5%) were discordant because of clonal cell populations comprising less than 30% of the sample. The discordant results were attributable to sample mosaicism, and all them involved CLL cases with small (~2 Mb) deletions of chromosome 13 interrogated by FISH probes for the 13q14 region (D13S319, LSI 13RB1). Interestingly, two cryptic (~1 Mb) 13q14 deletions detected by the BAC array were missed by both of the 13q14 region FISH probes. When the CLL samples were subjected to analysis using both FISH and array CGH analysis methods, chromosomal aberrations were correctly identified in 96% of the cases.

Confirmation and Breakpoint Mapping by High-Density Oligonucleotide Array CGH

A subset of 36 CLL cases, selected to represent all of the clinically relevant recurrent and novel genomic abnormalities detected by the BAC array, was also analyzed using high-density oligonucleotide array CGH. These arrays were performed to confirm the presence of genetic abnormalities and to define alteration breakpoints. Genomic patterns of gains and losses representative of the BAC array probe sizes (≥150 kb) were compared to those obtained by BAC array CGH and found to be 100% concordant. Results representative of the International System for Human Cytogenetics nomenclature for BAC and oligonucleotide array CGH are summarized in Table 4.

Discussion

We have demonstrated the feasibility of using BAC array CGH as a clinical tool for the evaluation of CLL prognostic markers. A complete analysis of the tumor genome by array CGH can be completed with a reasonable turn-around time (as little as 36 hours per sample) that facilitates the use of this information in clinical decision making for treatment strategies after the pertinent prognostic markers are identified. Array CGH also has the ability to detect genome-wide aberrations indicative of clonal evolution, a distinct advantage over FISH panels, which have recently been shown by Stilgenbauer and colleagues²¹ and Shanafelt and colleagues²² to shorten survival in CLL to a median of 21.7 months. In this study, 21% of patients showed significant genome-wide aberrations involving loci not interrogated by conventional commercially available FISH panels, making genomic complexity the second most common prognostic marker detected, exceeded only by the frequency of 13q14 deletions (Table 2). These results suggest that whole-genome scanning should be incorporated into leukemia workups and combined with other prognostic markers, such as mutation status and CD38 and ZAP-70 expression, to aid in the identification of patients falling into high-risk groups.^{23–25}

Table 4. Examples of ISCN Nomenclature for BAC and Oligonucleotide Array CGH Results

Finding	BAC array CGH	Oligonucleotide array CGH
2p gain	arr cgh 2p25.2p22.3(RP11-451A6→RP11-113D22)x3	arr cgh 2p25.2p22.3(665905-64157367)x3
6q loss	arr cgh 6q14.3q16.3(RP11-386J20→RP11-28K19)x1	arr cgh 6q14.3q16.3(88088740-110588647)x1
11q loss	arr cgh 11q22.3q23.3(RP11-1E8→RP11-648J7)x1	arr cgh 11q22.3q23.3 (106063043-114871570)x1
Trisomy 12	arr cgh 12pter→qter(RP11-283I3→RP11-492L13)x3	arr cgh 12pter→qter(49967-132378836)x3
13q14 mono-allelic loss*	arr cgh 13q14.3(RP11-34F20→RP11-1064M14)x1	arr cgh 13q14.3 (47707348-50535035)x1
13q14 bi-allelic loss*	arr cgh 13q14.3(RP11-34F20→RP11-1064)x0	arr cgh 13q14.3 (47707348-50535035)x0
17p loss	arr cgh 17p13.3p11.2(RP11-411G7→RP11-707H13)x1	arr cgh 17p13.3p11.2(48539-19670088)x1

*Breakpoints mapped by oligonucleotide array CGH in 19 cases with 13q mono-allelic and bi-allelic loss showed the minimal 411-kb region of overlap (nucleotides 49473507 to 49884865) to be consistent with the commonly deleted BAC array probe (RP11-34F20), and included the microRNA genes *MIR15A/MIR16-1*.

Compared to conventional cytogenetics for whole-genome analysis, array CGH is a DNA-based test with the added benefit of simultaneously detecting commonly assayed CLL prognostic markers that exceeds the 80% rate generally attributed to FISH.²⁶ Using a combination of array CGH and FISH analyses, we were able to identify clinically significant genomic imbalances in 96% of the CLL cases, suggesting that using both methods to detect genomic aberrations might be the best approach. However, we use a more cost-effective algorithm in our laboratory in which array CGH is run as a first-line test in cases with more than 30% tumor cells (as determined by flow cytometry), with reflex to FISH in cases with negative results by array CGH (Figure 5). In our experience, ~80% of CLL cases will show genomic aberrations with prognostic significance by array CGH and can be signed out without additional testing. In the 20% of cases found to be normal by array CGH, approximately half of these cases will have clonal cell populations below the 30% detection limit of array CGH that can be identified by FISH analysis.

A consideration when using array CGH whole-genome scanning for the detection of CLL prognostic markers is the incidental finding of single-clone copy number variations (CNVs) that are normally present in all individuals.²⁷ CNVs are readily identified by both oligonucleotide and BAC array CGH at an average of three to five variants/genome, and thus can be expected in any diagnostic evaluation by array CGH.²⁸ CNVs were identified in virtually every case of CLL analyzed in this study. With few exceptions, these changes clearly represented polymorphisms involving single-clone gains or losses overlapping previously reported regions of genomic variation (Database of Genomic Variants; <http://projects.tcag.ca/variation/>). A list of the array probes and percent overlap correlated to CNV

locus IDs obtained from the Database of Genomic Variants can be found in Supplementary Table S1 at <http://jmd.amjpathol.org>. Detection of copy number variants by array CGH is analogous to the finding of heteromorphisms by conventional cytogenetics and should not preclude the use of either of these whole-genome scans in the analysis of inherited or acquired genomic abnormalities. In clinical laboratories where array CGH is now used routinely alongside conventional cytogenetics for the diagnosis of constitutional abnormalities, genomic variants are an expected finding in all comprehensive genomic evaluations.

Deletions involving the *IGH* gene at 14q32.33 were the only instance in which CNVs could not be readily distinguished from clinically relevant genomic changes. The *IGH* gene region is highly polymorphic, making it difficult to distinguish germ-line CNVs from deletions specific to the tumor genome. Losses of 14q32.33 in CLL have been previously reported as incidental findings by FISH, but a recent study hypothesized that these alterations might represent rearrangements of the *IGH* gene in the clonal B-cell population, as opposed to an oncogenic event.²⁹ In the present study, no accompanying duplications of the 14q32.33 clones were identified as would be expected if the observed deletions were germ-line polymorphisms, so it is likely that the majority of these single-clone 14q deletions are markers of B-cell clonality. In addition, 10 CLL cases exhibited larger deletions involving the entire *IGH* gene, possibly representing the presence of unbalanced translocations.¹

In conclusion, clinically relevant copy number aberrations in CLL are readily detected by BAC and high-density oligonucleotide array CGH. BAC arrays were preferred as a clinical genome-scanning tool over oligonucleotide arrays because of the whole-genome ratio plot views, higher signal to noise ratios, and fewer outliers.³⁰ Oligonucleotide arrays were the preferred confirmatory test over FISH because of the ability to confirm genome-wide abnormalities detected by BAC array while simultaneously estimating alteration size and mapping breakpoints. Both platforms show array CGH to be a robust and reliable clinical tool suitable for inclusion in the clinical laboratory test menu for the detection of CLL prognostic markers. Expected and novel genomic aberrations are quickly and accurately identified by this assay and provide readily interpretable results that are suitable for clinical prognostication and treatment planning. Further studies are needed to assess the prognostic significance of the whole-genome patterns identified in this study and to determine whether novel genomic aberrations revealed by the array are markers of clonality, oncogenic events in the pathogenesis of CLL, or both.

Figure 5. Suggested algorithm for integration of array CGH into CLL genomic evaluation. Array CGH is ordered as a first-line test for all CLL peripheral blood samples containing greater than 30% tumor cells by flow cytometry. If prognostic markers are identified, the case is signed out with the recommendation that the patient's tumor genome be monitored periodically for clonal evolution and/or rescanned if there is a significant change in the clinical presentation. Cases with normal results by array CGH are reflexed for FISH analysis and cases with normal results by FISH (even those with <30% tumor cells) are subjected to array CGH analysis. This algorithm has proven to be a cost-effective and nonredundant method to use FISH and array CGH as complementary tests in the clinical laboratory.

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