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REVIEW ARTICLE

Assessing copy number aberrations and copy-neutral loss-of-heterozygosity across the genome as best practice: An evidence-based review from the Cancer Genomics Consortium (CGC) working group for chronic lymphocytic leukemia

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Abstract

The prognostic role of cytogenetic analysis is well-established in B-cell chronic lymphocytic leukemia (CLL). Approximately 80% of patients have a cytogenetic aberration. Interphase FISH panels have been the gold standard for cytogenetic evaluation, but conventional cytogenetics allows detection of additional abnormalities, including translocations, complex karyotypes and multiple clones. Whole genome copy number assessment, currently performed by chromosomal microarray analysis (CMA), is particularly relevant in CLL for the following reasons: (1) copy number alterations (CNAs) represent key events with biologic and prognostic significance; (2) DNA from fresh samples is generally available; and (3) the tumor burden tends to be relatively high in peripheral blood. CMA also identifies novel copy number variants and copy-neutral loss-of-heterozygosity (CN-LOH), and can refine deletion breakpoints. The Cancer Genomics Consortium (CGC) Working Group for CLL has performed an extensive literature review to describe the evidence-based clinical utility of CMA in CLL. We provide suggestions for the integration of CMA into clinical use and list recurrent copy number alterations, regions of CN-LOH and mutated genes to aid in interpretation.

Keywords Chronic lymphocytic leukemia (CLL), Cytogenetics, Fluorescence in situ hybridization (FISH), Chromosomal microarray analysis (CMA), Copy number alterations (CNAs), Copy-neutral loss-of-heterozygosity (CN-LOH).

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Introduction/background

B-cell chronic lymphocytic leukemia (CLL), a mature B cell neoplasm, is the most common adult leukemia in the Western world. In the United States, CLL represents approximately 40% of adult leukemias, with an annual incidence of 2–6 per 100,000 and median age of diagnosis of 70 years of age. Incidence increases with age; however, 30% of patients are younger than 60 at the time of diagnosis and 15% are younger than 50 [1–3]. CLL has the highest genetic predisposition of all hematologic neoplasms; approximately 5–10% of cases have a family history of CLL or other non-Hodgkin lymphoma [4].

CLL exhibits a highly variable clinical course, with life expectancies ranging from a few months to decades. Approximately one-third of patients experience an indolent course with normal survival, one-third experience an initially indolent disease that eventually progresses, and one-third experience an aggressive disease course. Stratifying these patients, particularly in early-stage or asymptomatic disease when most patients are diagnosed, is part of the challenge of this hematologic malignancy. The Rai and Binet clinical staging systems, established in the mid-1970s, remain useful in defining disease extent and prognosis; however, these systems fall short in distinguishing those who will experience an aggressive clinical course, particularly patients with early stage disease.

For the past 25 years, the incorporation of genetic markers has become increasingly important in stratifying patients (reviewed in Zenz [5]), and cytogenetic analysis is well-established as playing a key role in both diagnosis and prognosis. As many as 80% of patients have a cytogenetic aberration. Since the publication of the Dohner hierarchical classification in 2000 [6], interphase fluorescence *in situ* hybridization (FISH) with a four- or five-assay panel has been the gold standard for cytogenetic evaluation [7]; however, metaphase chromosome analysis, which provides a whole genome assessment, allows detection of abnormalities not in the panel, including translocations, complex karyotypes and multiple clones. Chromosomal microarray analysis (CMA) can interrogate the same aberrations as the FISH panel and, like metaphase chromosome analysis, can provide a whole genome assessment, although it will miss balanced translocations and low level clones. In addition, CMA can detect copy-neutral loss-of-heterozygosity (CN-LOH) and chromothripsis, which the other technologies are not able to do. This document focuses on the clinical utility of CMA in CLL based on a review of peer-reviewed publications.

Methods

A systematic literature search was performed for peer-reviewed manuscripts focusing on CNAs and CN-LOH assessment in CLL published between 2000 and 2017. Workgroup members reviewed 72 well-powered studies. The level of evidence for clinical significance of CNAs was assigned as follows: Level 1, present in current WHO classification and/or professional practice guidelines (NCCN); Level 2, recurrent in well-powered studies with suspected clinical significance based upon expert review; and Level 3, recurrent, but uncertain prognostic significance. Single case aberrations were not included. The list of clinically significant

and/or recurrent CNAs selected and evaluated based on this process is provided in Table 1.

Evidence review

Non-Cytogenetic prognostic markers

Although the diagnosis of CLL in most cases can be made relatively easily by morphology and flow cytometry, other information is critical to determine prognosis for the patient. Prognostic indicators have included mutational status of the variable region of the immunoglobulin heavy chain (*IGHV*), expression of biomarkers including ZAP-70 and CD38, and cytogenetic aberrations. Molecular analysis of the immunoglobulin genes indicates that 50–60% of cases exhibit somatic hypermutation (*mutIGHV*, > 2% deviation from germline), while the remainder are unmutated (*unmutIGHV*, > 98% homology with germline). Patients with mutated *IGHV* have a better prognosis than those with unmutated *IGHV*, at least for those with low stage disease [1]. Most recently, an international CLL working group has proposed a prognostic index, CLL-IPI, that includes *TP53* status, *IGHV* mutational status, serum B2-microglobulin concentration, clinical stage, and patient age (Hallek, for the International CLL-IPI working group) [8].

Detection of cytogenetic markers by metaphase chromosome analysis and FISH

Cytogenetic analysis is a key component in diagnosis, prognosis and determination of optimal treatment strategies in CLL. Metaphase chromosome analysis provides a genome-wide view of abnormalities, but historically this method was hampered by poor growth of B-cells in culture. With the publication of the Dohner hierarchical classification in 2000 [6], interphase FISH became the gold-standard test for cytogenetic evaluation in CLL.

With the use of four FISH assays [for the detection of trisomy 12, and deletion of 13q14, 11q22 (*ATM*) and 17p13 (*TP53*)], FISH has an approximately 80% abnormality detection rate in CLL. Deletion of 13q14 is the most common finding, observed in approximately 50% of cases; trisomy 12, *ATM* deletion and *TP53* deletion are seen in about 20%, 15–20% and 5–10%, respectively, of patients, with the distribution of these abnormalities varying with *IGHV* mutational status (WHO 2017, Table 13.01) [1]. The four-probe assay FISH panel also provides useful prognostic information, with deletion of 13q14 as the sole abnormality conferring a favorable prognosis, while trisomy 12 confers an intermediate prognosis and deletion of either *ATM* or *TP53* confers an unfavorable prognosis. Although more than 15 years have passed since the Dohner publication of 2000, the hierarchy has recently been revisited and reaffirmed [9].

Deletion of 6q, seen in approximately 5% of cases, was identified as an unfavorable marker in the original Dohner classification [6]. However, the poor prognostic significance of this aberration has not borne out, and this deletion may be better categorized as an intermediate finding [10–13]. Two deletion regions, 6q12–q23.3 and 6q25–q27, have been observed, only one of which includes the *MYB* gene (6q23.3), the typical locus targeted by commercial FISH assays [11, 14].

Table 1 Regions of recurrent copy number change in CLL.

Chromosome/ region	Abnormality type	Prevalence (%)	Relevant genes	Strength of evidence for gene	Prognostic significance	Strength of evidence for prognosis (Level*)	Comments	References
1p	Gain	?2–5	Unknown	N/A	Favorable	Suspected (2)		[35,82,85]
1q23.2q23.3	Loss	15	Unknown	N/A	Unknown	N/A (3)		[36,86,116]
2p12p25.3	Gain	5–30	<i>ACP1, MYCN, ALK, REL, BCL11A</i>	<i>MYCN</i> (Established), <i>REL, BCL11A</i> (Candidate)	Unfavorable	Established (if <i>MYCN</i> included) (1)		[35,36,40,43,67,82, 83,85–87,117,118]
3p21.31	Loss	1–5	<i>ATRIP, CDC25A</i>	Candidate	Unknown	N/A (3)		[36,39,89]
3q	Gain	2–19	Unknown	N/A	Unfavorable	Suspected (2)	Appears to be particularly prevalent in Japanese	[82,119,120]
4p15.2p16.3	Loss	14	Unknown	N/A	Unfavorable (occurred with del(11q) or del(17p))	Suspected (2)		[48]
6p25.3	Gain	1	Unknown	N/A	Unknown	N/A (3)		[36]
6p22.1	Loss	1	Histone cluster, <i>HFE</i>	Candidate	Unknown	N/A (3)		[36]
6q	Loss	3–6	<i>FOXO3</i>	Candidate	Intermediate	Suspected (2)		[10,13,15,58]
7p	Gain	5–6	Unknown	N/A	Intermediate	Suspected (2)		[82]
7q	Loss	1–2	Unknown	N/A	Unknown	N/A (3)		[67,82]
8p21	Loss	2–5	<i>TRIM35</i>	Candidate	Unfavorable	Suspected (2)	Associated with established unfavorable changes (11q- and 17p-). Not established as an independent prognosticator	[82,121]
8q24.1	Gain	5	<i>MYC</i>	Candidate	Unfavorable	Suspected (2)	Often associated with 11q and 17p deletion; may not be independent [82]	[36,82]
9q13q21.11	Loss	1	Unknown	N/A	Unknown	N/A (3)		[36]
10q24	Loss	2	Unknown	N/A	Unknown	N/A (3)	Clustered around <i>NFKB2</i> gene locus	[36,43,122]
11q22.3	Loss	10–20	<i>ATM, BIRC3, MRE11, H2AFX</i>	<i>ATM</i> established, Others Candidate	Unfavorable	Established (1)		[7]
12	Gain	10–20	Unknown	N/A	Intermediate	Established (1)	Unfavorable if <i>NOTCH1</i> mutation is present	[7]

(continued on next page)

Table 1 (continued)

Chromosome/ region	Abnormality type	Prevalence (%)	Relevant genes	Strength of evidence for gene	Prognostic significance	Strength of evidence for prognosis (Level*)	Comments	References
13q14	Loss	50–60	<i>DLEU2</i> , <i>miR-15a/16-1</i> , <i>DLEU1</i>	Established	Favorable	Established (1)	Co-deletion of <i>RB1</i> may negatively impact time to treatment	[7,123,124]
14q24.1q32.3	Loss	2	Unknown	N/A	Unknown	N/A (3)	Associated with trisomy 12	[36,59,61]
15q15.1	Loss	4	<i>MGA</i>	Candidate	Unknown	N/A (3)		[36,43]
17p13.1	Loss	5–15	<i>TP53</i>	Established	Unfavorable	Established (1)		[7]
17q	Gain	1	Unknown	N/A	Unfavorable	Suspected (2)		[82]
18p	Loss	3	Unknown	N/A	Unfavorable	Suspected (2)		[36,82]
18	Gain	4	Unknown	N/A	Unfavorable	Established (1)	Associated with trisomy 12	[51]
19	Gain	2–5	Unknown	N/A	Unfavorable	Established (1)	Associated with trisomy 12	[36,43,48,51,122]
Genomic complexity	3 or more CNAs	10–15		N/A	Unfavorable	Established (1)		[38,39,43,67]
Chromothripsis	(> 10 copy number states of 2 and 3)	5	<i>SETD2</i> , other markers across genome not defined	Established	Unfavorable	Established (1)		[36,90,124]

* Level 1: present in WHO classification or professional practice guidelines; Level 2: recurrent in well-powered studies with suspected clinical significance; Level 3: recurrent, but uncertain prognostic significance.

A 6q putative tumor suppressor gene has not been identified, although a recent study suggests that *FOXO3* may be involved [15].

With the fairly recent identification of more effective mitogens for CLL cells (CpG oligodeoxynucleotides and CD40 ligands), metaphase chromosome analysis has resurged in the past 5–10 years. Chromosome analysis has the advantage over targeted FISH by providing a whole genome analysis. The detection rate of abnormalities that are not targeted by the FISH panel ranges from 25 to 35% [16–19] and includes the detection of complex karyotypes and multiple clones, both of which are unfavorable prognostic findings that may be missed by FISH alone [20].

The adoption of these new mitogens has also led to the identification of translocations in CLL. Previously not considered to play a significant role in CLL, they are now reported in 30–40% of cases [21–23]. Less favorable prognosis has been associated with translocations; however, studies are limited by small patient numbers and/or an over-representation of patients with advanced disease [21–24]. Thus, the prognostic value is currently uncertain, and the translocations likely represent a diverse group with different implications.

Many apparently balanced translocations are actually unbalanced and have CMA-detectable deletions associated with breakpoints in known regions of genomic imbalance, including 13q14 and 17p13 [25,26]. Another significant percentage of the apparently balanced translocations identified by metaphase chromosome analysis are those involving immunoglobulin genes; these may confer a poor prognosis [27]. The t(14;18)(q32;q21) resulting in *IGH/BCL2* recombination and the variants t(18;22)(q21;q11.2) *BCL2/IGL* and t(2;18)(p12;q21) *IGK/BCL2* are observed as secondary changes in CLL patients, often with trisomy 12, but may also be observed in patients with monoclonal lymphocytosis [28]. The t(14;19)(q32;q13) *IGH/BCL3*, which has been identified in a variety of B-cell neoplasms, is a recurrent translocation in CLL often found with trisomy 12 that may identify a subset of CLL patients with distinctive genetic and pathologic features [29]. Translocations involving *MYC* [t(8;14)(q24.1;q32) *IGH/MYC* as well as the variants t(2;8)(p12;q24.1) *IGK/MYC* and t(8;22)(q24.1;q11.2) *MYC/IGL*] are observed infrequently in CLL and when present, are typically acquired during disease progression [30]. Note that the t(11;14)(q13;q32) *IGH/CCND1* is diagnosed as mantle cell lymphoma according to current diagnostic criteria.

CMA in CLL

CLL is particularly amenable to the detection of CNAs by CMA for the following reasons: (1) genomic gains and losses represent key events with biologic and prognostic significance, with balanced rearrangements being less common and currently of uncertain prognostic value; (2) DNA from fresh samples is generally available, obviating the technical difficulties associated with analysis of DNA from paraffin-embedded tissue; (3) the tumor burden is generally known from flow cytometry studies and can be used to guide the downstream analysis; and (4) the tumor burden tends to be relatively high in peripheral blood. In instances where FISH and CMA data are discrepant, CMA analysis may help to further refine deletion

breakpoints and determine the clinical relevance of atypical deletions.

Clinically validated CMA assays have potential utility for individualized patient risk stratification in CLL. Microarray studies of CLL using bacterial artificial chromosome (BAC), oligonucleotide, targeted oligonucleotide and single nucleotide polymorphism (SNP)-based arrays have been reported. Techniques, technical limitations, clinical applications and challenges have been reviewed by Higgins et al. [25] and Hagenkord and Chang [31]. Comparison of platform performance in CLL has been reviewed by Gunnarsson et al. [32], recognizing that this is a rapidly changing technology. Overall, copy number alterations have been reported in > 90% of cases when CMA assays have been performed for CLL patients, with the number of copy number alterations per patient generally low (0–2). The lower limit of disease involvement required for detection of clonal aberrations varies by report, but likely is around 10–15% with SNP arrays [33]. SNP arrays can additionally detect acquired CN-LOH; studies indicate that CN-LOH occurs in regions of the genome with prognostic relevance in CLL [34,35].

These studies have validated the ability of CMA platforms to detect abnormalities detected by FISH panels as well as to identify novel regions of gain or loss and to identify genomic instability. In general, results of array-based analyses have reported high concordance with FISH results [36–43]. Instances of non-concordance may be due to array resolution, which allows detection of smaller sized abnormalities that cannot be detected with standard commercial FISH probes. Discordance may also be due to cases with low tumor cell percentage or low level subclones, as FISH analyses may be able to identify smaller populations of abnormal cells than can CMA (the lower limit of sensitivity for FISH is typically 5–7% for detection of deletions, while for CMA it may be closer to 10–15%, although several laboratories report sensitivity down to 5%, depending on the size of the aberration and the platform used [33] (Wolff and Chun, personal communication). With the high tumor burden and high intratumoral level of abnormalities in most untreated CLL patients, the sensitivity of CMA is adequate to detect prognostically significant abnormalities. Most often, CMA analysis in CLL is used at time of diagnosis. However, it may also be used on post-treatment specimens, particularly when disease transformation is suspected.

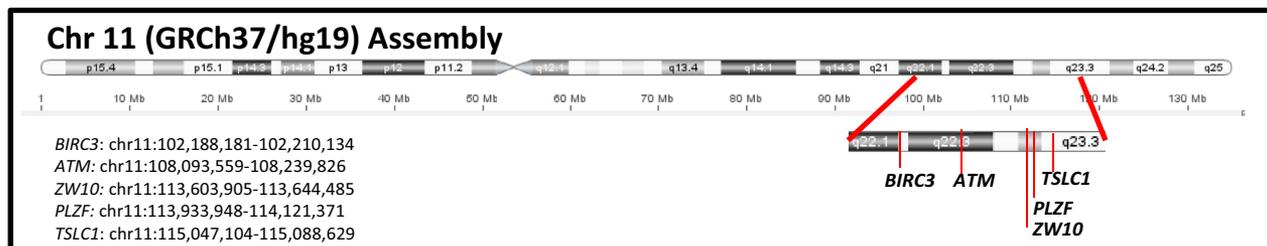
Detection of established prognostic CNVs by CMA

Del(13q)

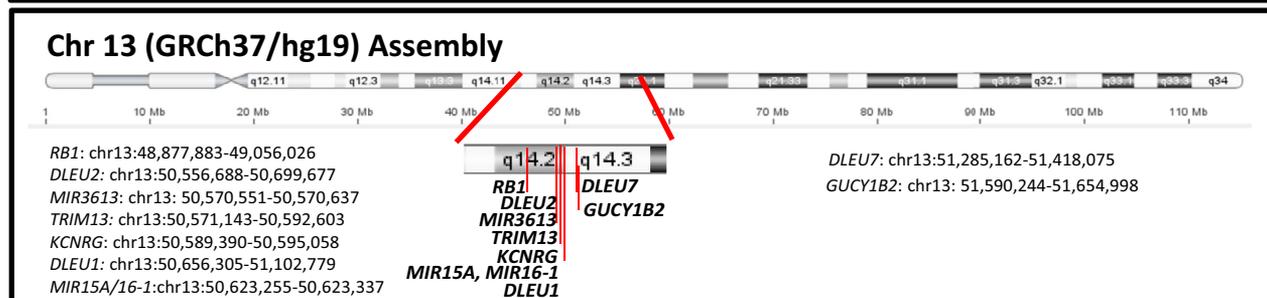
Deletions of 13q14 are the most common genetic change in CLL, usually mono-allelic and occurring more frequently in patients with mut*IGHV*. The mono-allelic deletion is associated with a good prognosis when present as the sole abnormality [6], while bi-allelic loss in a high proportion of cells is associated with a less favorable prognosis [44,45]. Detection of a deletion of 13q, discrimination of mono-allelic and bi-allelic losses, and determination of the extent of the deletion region are readily accomplished by CMA analysis.

Deletion size is heterogeneous across patients. The minimal deletion region contains the *DLEU2* (deleted in lymphocytic leukemia 2) locus, which encodes a long-noncoding RNA (lncRNA), *TRIM13*, *miR-3613*, *KCNRG*, the micro RNA cluster *miR-15a/miR-16-1* and the *DLEU1* lncRNA gene [46]. In

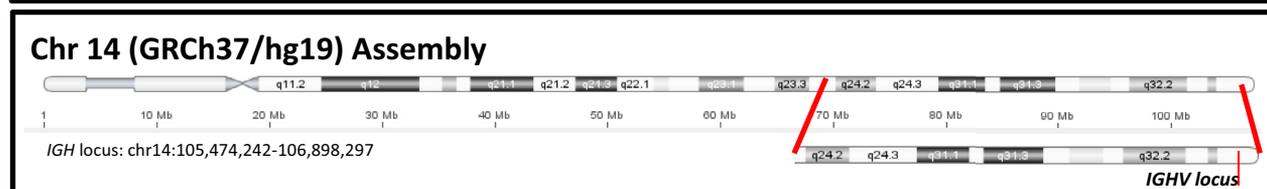
A.



B.



C.



D.

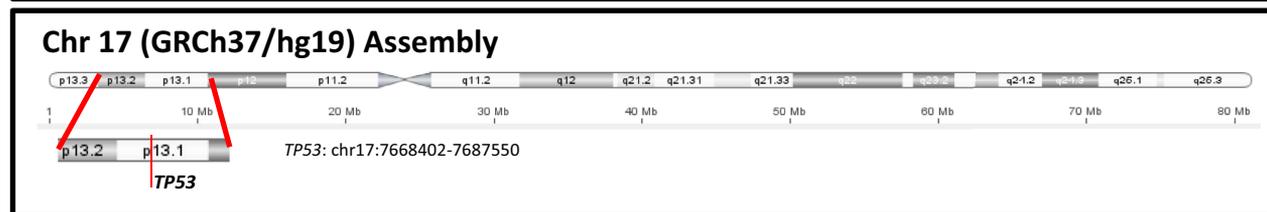


Fig. 1 Chromosomal locations and genomic coordinates of genes in regions with prognostic significance in CLL.

some cases, the deletion includes the *DLEU7* gene. *DLEU7*, which functions as an NF- κ B and NFAT inhibitor, resides within a minimal deletion region for *miR-15a/16-1* [47]. Deletions are seen in two clusters, one of which occurs around *DLEU2* and the second of which is distal to *GUCY1B2*. Several studies have associated larger deletion size with worse prognosis [48,49]. Using the Affymetrix 6.0 array, Mian et al. [50] examined a cohort of 169 patients to further refine 13q deletions. By CLL FISH, the patients had been found to be either normal or to have 13q deletion as the sole abnormality. The 13q deletions were sub-classified into three types. Type 1 deletions were the smallest and encompassed *DLEU2/miR-15a/16-1*. Type 2 deletions were less than 10 Mb in total size and included the Type 1 region as well as *RB1*. Type 3 deletions were greater than 10 Mb and included the deleted regions in Type 2. Type 1 deletions were more often bi-allelic and showed longer time to first treatment (TTFT), while types 2 and 3 experienced a less favorable clinical course, with larger deletions conferring a worse prognosis. A major advantage of CMA versus FISH is the ability to detect and distinguish between the different deletion types in a single assay, as opposed to using multiple FISH probes. See Fig. 1B for region anatomy.

Trisomy 12

Trisomy 12 is considered an intermediate risk prognostic factor, independent of *IGHV* mutational status. Trisomy 12 cases with concurrent *NOTCH1* mutations are associated with a less favorable prognosis. Trisomy 18 and trisomy 19 are not common in CLL, but may be seen together or with trisomy 12 [51,52]. Concurrent trisomy 12 and trisomy 19 have been associated with mut/*IGHV* and with the rare IgG-switched variant of CLL.

Del(11q)

Deletion of the *ATM* gene remains the most important marker of poor outcome in patients with del(11q) [53]. Alternative targets on 11q include *ZW10*, *PLZF* and *TSLC1*, each of which may be co-deleted with *ATM*. Whether the poor prognosis in patients with 11q loss reflects loss of multiple genes remains a question. Atypical 11q deletions or concurrent deletion of additional tumor suppressor gene(s) with *ATM* may contribute to the poor prognosis [54]. *ATM* and *BIRC3* lesions can be found in the same patient. Some 11q deletions include the *BIRC3* gene, but not *ATM*, supporting *BIRC3* as a key player [2,53]. See Fig. 1A for region anatomy.

Del(17p)

The *TP53* deletion/mutation on 17p is considered a highly adverse marker, and its prognostic impact has been discussed extensively [55,56]. Patients with 17p loss identified by FISH were examined using an Affymetrix 50K *Xba* array, which indicated varied breakpoints. These results suggested the loss of multiple tumor suppressor genes in addition to *TP53* and showed that multiple genes may be contributing to the highly adverse prognosis associated with *TP53* loss [2,53,57,58]. Highlighting the importance of identifying these lesions in CLL is the recent FDA premarket approval for the Abbott Molecular/Vysis *TP53* FISH probe as a companion diagnostic for Venclaxta (venetoclax), a *BCL2* inhibitor, to treat *TP53* deletion patients who fail previous therapy. See Fig. 1D for region anatomy.

Note that patients with deletion of both 11q and 17p have an exceptionally poor outcome, significantly worse than either alone [59].

Del(14q)

Deletions of 14q are seen rarely at diagnosis and in ~5% of patients with established CLL; they have been associated with shortened TTFT [60]. FISH studies indicated the presence of deletion of 14q in 1.9% of CLL patients studied, with the deletions observed being of variable size but with breakpoints clustered at the centromeric side in 14q24.1 (~60% of cases) and at the telomeric side within the *IGH* locus at 14q32.3 (45% of cases). In agreement with these results, using FISH and SNP arrays to study 81 CLL patients with del(14q), Cosson et al. demonstrated that while 14q deletion size varies, 48% of patients had the same 14q24.1q32.33 deletion [61]. Del(14q) is associated with several unfavorable markers, including trisomy 12, *NOTCH1* mutations and unmut/*IGHV*. Note that these 14q deletions can involve one-third of chromosome 14 and are much larger than deletions that occur during normal physiological *IGH* gene rearrangement. See Fig. 1D for region anatomy.

Genomic complexity

Increased genomic complexity (widespread gains and losses of chromosomal regions in many chromosomes) reflects genomic instability and is a marker independent of ZAP-70, *IGHV* status and Rai stage for identification of patients with aggressive CLL and a poor outcome [38,62–67]. Genomic complexity was observed for patients both with favorable and with adverse FISH markers [66,68]. Of note, Gunn et al. observed that 21% (37/174) of cases had three or more aberrations not interrogated by the common FISH panel [38]. In another study, Kujawski et al. showed that TTFT was 79 months for non-complex cases and 23 months for complex cases, using their own published complexity algorithm [69]. Greater complexity has also been associated with worse progression-free-survival (PFS) and response to therapy, and patients with *TP53* deletion have been shown to have a higher frequency of large (>5 Mb) aberrations [63,66,70]. Increased genomic complexity appears to be an independent marker for identification of patients with aggressive CLL and shorter survival [62,65]. It should be noted that the phrase “complex karyotype” awaits a formal definition by NCCN, ISCN or another body [71].

Clonal diversity

Clonal diversity, a surrogate marker for clonal evolution, is defined as the presence of two or more clonal populations of cells at different levels of tumor involvement as detected by CMA. For patients with CLL, clonal evolution and an increase in the percentage of cells with CNAs are associated with disease progression [72]. Although both metaphase chromosome analysis and CMA can detect clonal diversity, CMA is more sensitive, has higher resolution and can better define percentages of specific clonal abnormalities [73]. CMA-defined clonal diversity has been associated with progressive disease, relapse, need for therapy and an adverse prognosis [72–74].

Richter transformation

Richter syndrome (RS), which is associated with poor outcome, is the transformation from CLL to a more aggressive lymphoma, most commonly diffuse large B-cell lymphoma (DLBCL). CMA is emerging as a useful methodology both to identify CLL patients at risk for Richter transformation and to offer prognostic information. The most common changes in RS patients are losses at 17p (*TP53*), 13q14.3 (*DLEU2/miR15a/miR16-1*), 9p21 (*CDKN2A*) and trisomy 12. While deletions at 17p and 13q14.3 as well as trisomy 12 are already present during the CLL phase, 9p21 loss is the most frequent lesion acquired during Richter transformation, mostly occurring concomitantly with *TP53* inactivation. *TP53* inactivation and/or 9p21 loss appear to be mutually exclusive to trisomy 12, suggesting that RS may develop through two main genetic pathways [72,75,76]. In addition, it has been well established that one of the most important prognostic factors for Richter transformation is the clonal relationship between the CLL and the lymphoma clones [77]. By assessing the clonal changes by CMA, it is often possible to detect the original CLL clonal changes that reveal a linear progression of the CLL to DLBCL, which has been associated with a less favorable outcome, compared to identification of an apparent new independent clone that would represent non-linear progression and a better overall prognosis [75–79].

Concurrent myelodysplastic syndrome (MDS) related changes

The concurrent presence or development of myeloid disorders (MDS or AML) is relatively uncommon in CLL [80]; however, some CLL chemotherapies are known to increase the risk for dysplasia [81]. MDS-associated cytogenetic abnormalities are readily defined by CMA because, as for CLL, the critical cytogenetic aberrations are copy number changes. Metaphase chromosome analysis also readily identifies MDS-associated abnormalities; CMA has the additional advantage of defining percentages of the abnormal clones, while metaphase chromosome analysis can unambiguously ascribe a particular abnormality to a specific clone (e.g. 13q deletion can be seen in both CLL and MDS). Targeted CLL FISH will not identify MDS-associated abnormalities.

Additional abnormalities identified by CMA analysis

Gain of 2p

Various CMA studies in heterogeneous CLL patient populations have indicated gain of 2p as a recurrent aberration;

BCL11A, *REL* and *MYCN* have been cited as potential targets [35,48,66,67,82–87]. In a recently published study, Cosson et al. [84] identified two minimally gained regions on 2p and implicate *XPO1* as a critical player. Their findings also support earlier publications of the association of 2p gain with unfavorable markers, including del(11q), del(17p) and unmutated status of *IGHV*, and show that 2p gain promotes resistance to several therapeutic drugs. 2p gain also can be present in early stage disease, especially in those patients with other poor prognosis markers.

Chromothripsis

The phenomenon of chromothripsis (which can be detected by CMA, but not by FISH or metaphase chromosome analysis) was first identified following a genome-wide screen of 10 CLL patients [88]. Since that time, two larger scale studies have detected chromothripsis in 4–5% of CLL patients studied by CMA [36,89]. In both of these large-scale studies, chromothripsis was associated with a poor prognosis. In the Edelman study [36], 74% of patients with chromothripsis had unmutated *IGHV* status and 79% had high-risk genomic aberrations, including a *TP53* mutation in 31%, but univariate analysis still showed patients with chromothripsis to have inferior PFS and OS. Specific chromosomes may be preferentially involved; in three of eight cases in the Salaverria series [89], the chromothripsis involved chromosome 5 and resulted in gain of the *TERT* locus. A more recent publication on the role of *SETD2* showed that deletions of this locus were associated with chromosome 3 chromothripsis, as well as *TP53* deletion and genomic complexity [90].

See Table 1 for recurring copy number variations identified by CMA.

CN-LOH

Acquired CN-LOH may be due to mitotic recombination or to segmental deletion with replacement of the deleted region by a copy of the remaining allele during development of the neoplasm. When a deleterious mutation precedes such events, CN-LOH can act as a second hit resulting in mutation of both copies of a tumor suppressor gene. For loss-of-function mutations, this is equivalent to bi-allelic deletion of a gene. SNP microarrays allow detection of CN-LOH that remains undetected by karyotyping or FISH.

CN-LOH in CLL has been reported at variable frequencies in the literature [35,43,91–97]. In untreated CLL patients, two studies reported a frequency of 6–7% [36,91], which is lower than other malignancies [98]. CN-LOH most frequently affects 13q, 17p and 11q [36,46]. Similar to other hematological malignancies, regions affected by CN-LOH encompass genes involved in disease initiation or progression [34], and identification of CN-LOH can help uncover these tumor suppressor genes.

A significant number of CN-LOH could be present as germline variants. In one study that assessed paired CLL tumor and germline samples, 30 of 39 CN-LOH identified regions were germline [36]. The median sizes of tumor-specific and germline CN-LOH were 48.4 Mb and 12.1 Mb, respectively. In the absence of germline testing, strict criteria should be used for identification of tumor-specific CN-LOH. These criteria may include overlap with known deletion regions and whether the region is telomeric, but a minimum cut-off of 10 Mb seems reasonable in the absence of paired analysis.

The most studied CN-LOH in CLL is 13q. CLL patients with 13q CN-LOH have a high frequency (85–100%) of bi-allelic deletion within the CN-LOH region [34,36,46,48,49,91,99]. In one rare case without 13q14 deletion, a homozygous deletion of 1 nucleotide in *miR16-1* was reported [99]. Parker et al. suggested that CN-LOH may result in both bi-allelic deletion of genes as well as homozygosity of a cluster of genes associated with progressive disease [49]. Pfeifer et al. have shown that the 13q14 CN-LOH region ends telomeric to the *miRNA-15a* and *16-1* genes [35] and some authors have suggested that CN-LOH may lead to dysregulation of *miR-15a/16-1* and/or other genes [48].

As in other malignancies, CLL patients with CN-LOH of 17p were frequently associated with homozygous mutations in the *TP53* gene [34,36,43,94]. Less frequent locations include 11q encompassing the *ATM* gene [36]. Pei et al. identified three CLL patients with lymphadenopathy with CN-LOH of 20q, although mutations in the most common gene on 20q implicated in hematological malignancies, *ASXL1*, were absent [93].

See Table 2 for recurrent CN-LOH regions identified by CMA studies.

Gene mutation analysis by next generation sequencing (NGS)

Gene mutations in CLL have been studied by next generation sequencing to determine drivers of tumorigenesis and progression and have been extensively reviewed elsewhere [2,53,58,100,101]. Briefly, the most commonly mutated genes are *TP53*, *ATM*, *NOTCH1*, *SF3B1* and *MYD88*. *DDX3X*, chromatin regulators (*CHD2*, *HIST1C1*), B-cell transcription factors (*EGR2*, *IKZF3*), RNA export factors (*XPO1*, *RANBP2*), ribosomal proteins (*RPS15*), telomere-associated proteins (*POT1*) and signal transducers (*RAS*, *MAP2K1*, *MAP2K3*) have also been implicated [2]. *NOTCH1* mutations are found in approximately 10% of CLL patients at diagnosis, primarily those with unmutated *IGHV* [2]. *NOTCH1* and *SF3B1* mutations appear to be mutually exclusive and are each associated with adverse prognosis [58,100]. The frequency of *BIRC3* mutations in CLL ranges between 0.4% and 14% [102–107]. The prognostic significance of *BIRC3* mutations is unclear at this time. In one study, *BIRC3* lesions (both mutations and deletions) were associated with CLL refractory to fludarabine [108]. In total, over 70% of patients with treatment- (fludarabine) resistant CLL have one or more mutations in the *TP53*, *NOTCH1*, *SF3B1* and *BIRC3* genes, confirming their importance in treatment-resistant CLL pathogenesis [70].

By integrating cytogenetic and mutational data, it has been shown that *TP53* and/or *BIRC3* abnormalities are associated with a high risk, *NOTCH1* and/or *SF3B1* mutations and/or del(11q) are associated with an intermediate risk, trisomy 12 or normal karyotype is associated with a low risk, and del(13q) is associated with a very low risk [2,53,109,110]. Importantly, approximately 20% of patients who would be assigned to low risk categories based solely on FISH prognostic markers would be reclassified to higher risk categories due to the presence of *NOTCH1*, *TP53* or *SF3B1* mutations and *BIRC3* disruption [70].

See Table 3 for gene mutations in CLL.

Table 2 Recurring regions of CN-LOH in CLL.

CN-LOH	Candidate gene	Association	Strength of evidence for prognosis (Level*)	References
13q	<i>miR15a/16-1</i>	Biallelic deletion of 13q	Established (1)	[34–36,46,48,49,91,99]
17p13	<i>TP53</i>	Homozygous <i>TP53</i> mutations	Established (1)	[34,36,43,49,94]
11q13-qter	Includes <i>ATM</i>	Monoallelic <i>ATM</i> deletion	Suspected (2)	[36,49]
20q11	Unknown	None	N/A (3)	[43,93]
1p36	Unknown	None	N/A (3)	[36,97]

* Level 1: present in WHO classification or professional practice guidelines; Level 2: recurrent in well-powered studies with suspected clinical significance; Level 3: recurrent, but uncertain prognostic significance

Technical considerations

For general considerations on the use of CMA in cancer, refer to the American College of Medical Genetics and Genomics guidelines [111]. Additional considerations specific to CLL are discussed below.

CMA design

Laboratories performing CMA analysis should choose a platform with adequate probe coverage for the detection of copy number aberrations associated with CLL [111]. If a targeted platform design is preferred, it should minimally include enough probe coverage to detect deletions at 11q22.3 (*ATM*), 13q14.2q14.3 (*RB1*, *DLEU2* and *DLEU1*), and 17p13.1 (*TP53*), as well as trisomy 12, as these regions correspond to the four prognostic FISH markers, currently the gold standard in CLL analysis. Beyond this, however, a genome-wide platform with SNPs should be designed to detect other copy number changes as well as regions of CN-LOH, which are becoming increasingly important in cancer [35,93,97]. Commercially available platforms such as Affymetrix, Illumina and Agilent have been validated and are commonly used for clinical testing. Tables 1–3 list recurrent copy number abnormalities, regions of CN-LOH and gene mutations identified in CLL.

FISH analysis

In certain cases, concurrent FISH analysis for the *IGH/CCND1* rearrangement should be considered to rule out mantle cell lymphoma. This test would also detect an *IGH* rearrangement with a gene other than *CCND1*. An *IGH* break-apart probe can also be used to detect all *IGH* rearrangements, including fusions with *BCL2* and *BCL3*.

Sample type

A peripheral blood specimen is sufficient for CLL CMA analysis at diagnosis; bone marrow specimens can also be used. DNA can be extracted directly from the specimen, cultured cells or fixed cell pellets. Direct specimen may be preferred over the cultured cells to provide true clonality levels and avoid potential culture bias of a clone; however, for specimens with limited disease content, culture with oligonucleotide mitogen or B-cell enrichment may allow for increased sensitivity. Whichever method is chosen should be made clear to the ordering providers. Additionally, it is important to validate each specimen type on the chosen CMA platform.

Analysis

Size cut-off and backbone threshold parameters should be determined for genome-wide platforms. A .bed file containing

important cancer genes can also be used to highlight copy number changes in these regions. The laboratory should establish methods for the detection of clinically relevant CNVs that fall below the established cut-offs.

Thresholds to identify clinically important regions of homozygosity consistent with CN-LOH should be established. As mentioned above, however, a minimum cut-off of 10 Mb seems reasonable in the absence of paired CLL tumor and germline sample analysis.

Reporting considerations

CMA nomenclature

Current International System for Human Cytogenomic Nomenclature (ISCN) [112] should be used to describe relevant abnormalities in the patient. This is critical for understanding exactly what the abnormality is, not only for the testing laboratory, but for other laboratories who may receive a copy of the original report.

Interpretation

Since ISCN nomenclature is specialized, it is important to describe relevant changes in lay terms in the report. Clinically important genes within the CNVs should be provided. It is also critical to provide prognostic or clinically relevant information on the observed changes to guide the clinician in the management of their patient. References, whenever possible, would be helpful as well.

If a suspected clinically significant constitutional finding is observed, additional studies may be recommended in the report.

Reporting and interpretation should conform to guidelines provided by the College of American Pathologists (CAP) and the American College of Medical Genetics and Genomics (ACMG) [111].

Integration of CMA analysis into clinical use

Per Clinical Laboratory Improvement Amendments of 1988 (CLIA 88) regulations, laboratories using CMA for clinical testing must validate the procedure for the intended use, define specimen acceptability, define cut-off values for determining abnormalities, report results in a meaningful manner including use of ISCN nomenclature, participate in proficiency testing and monitor quality measures. Like any laboratory-developed test, the clinical approach for implementation of CMA for CLL is laboratory-dependent but should include establishing the

Table 3 Recurrent mutated genes in CLL.

Gene	Locus	Function	Mutation type	Prevalence (%)	Prognostic significance	Strength of evidence (Level*)	Comments	References
<i>ATM</i>	11q22.3	DNA repair and cell-cycle control	Missense, nonsense, indel	10–14	Unfavorable	Established (1)	Associated with unmut/ <i>IGHV</i> and 11q-; Candidate driver gene	[7,125]
<i>BIRC3</i>	11q22.2	Apoptosis inhibitor	Frameshift, nonsense, whole gene deletion	1–10 (higher in previously treated patients)	Unfavorable	Established (1)	In ~25% of fludarabine-refractory CLL; Candidate driver gene	[53,100,102,126]
<i>CHD2</i>	15q26.1	Chromatin remodeler	Missense, truncation	5–10	Unknown	N/A (3)		[53,127]
<i>FBXW7</i>	4q31.3	Ubiquitin ligase subunit/targets include <i>NOTCH1</i>	Missense	4	Unknown	N/A (3)	Exclusive to <i>NOTCH1</i> mutation patients; Negatively regulates <i>NOTCH1</i>	[128]
<i>MYD88</i>	3p22.2	Inflammatory pathway signal transducer	Missense	2–10	Favorable/ No effect	Suspected (2)	Candidate driver gene	[102,126,129]
<i>NOTCH1</i>	9q34.3	Intercellular signaling	Missense, nonsense, insertion, duplication, frameshift	4–10 (diagnosis) 12–30 (progression)	Unfavorable	Established (1)	Associated with + 12; Candidate driver gene	[58,125,126,130,131]
<i>POT1</i>	7q31.33	Telomere protector/stabilizer; component of telomerase RNP complex	Missense, frameshift, splicing	5–10	Unfavorable	Suspected (2)	Associated with familial CLL	[132–134]
<i>SF3B1</i>	2q33.1	Spliceosome component	Missense	10 –18	Unfavorable	Established (1)	Enriched in patients with del(11q) and unmut/ <i>IGHV</i> ; Candidate driver gene for disease progression	[102,126,135–137]
<i>TP53</i>	17p13.1	DNA repair and cell-cycle control	Missense	5–10 (higher with progressive disease)	Unfavorable	Established (1)		[7,53,55,56,138]
<i>XPO1</i>	2p15	Exports proteins/RNA fragments from nucleus into cytoplasm	Missense	5–7.5	Unfavorable/ high risk of progression	Suspected (2)	Associated with unmut/ <i>IGHV</i>	[84,129,139]

* Level 1: present in WHO classification or professional practice guidelines; Level 2: recurrent in well-powered studies with suspected clinical significance; Level 3: recurrent, but uncertain prognostic significance

Table 4 Comparison of cytogenetic methods for detecting genetic changes in CLL.

Method	Strengths	Weaknesses
Metaphase chromosome analysis	<ul style="list-style-type: none"> • Whole genome scan. • Detect balanced rearrangements. • Detect clonal evolution. • Discover novel abnormalities. 	<ul style="list-style-type: none"> • Requires cultured cells. • Requires B-cell mitogen (CpG) for increased sensitivity. • Analysis is slow – one case at a time. • Resolution limit ~ 10 Mb. • Exact rearrangements may not be evident by G-banding alone. • Cannot detect regions of homozygosity.
FISH	<ul style="list-style-type: none"> • Sensitivity – detect low level clones. • Batch cases. • Does not require cultured cells. • Can perform on archival specimens (<i>e.g.</i> FFPE). • Detect specific balanced translocations. 	<ul style="list-style-type: none"> • Only detect abnormalities where FISH probes bind. • Multiple FISH probes required to look at different abnormalities. • Clonal evolution may not be apparent. • Cannot detect regions of homozygosity or genomic instability. • Cannot detect genomic complexity.
CMA	<ul style="list-style-type: none"> • Whole genome scan. • Batch cases. • Does not require cultured cells. • Can perform on archival specimens (<i>e.g.</i> FFPE). • Resolution of 50 kb or less depending on platform. • Discover novel imbalances in the genome (<i>e.g.</i> genes involved). • Detect regions of homozygosity. • Detect chromothripsis/genomic instability. 	<ul style="list-style-type: none"> • Cannot detect balanced rearrangements. • Multiple clones not evident. • Clonal evolution may not be apparent. • Less sensitive than FISH. • May require B-cell enrichment if tumor burden is low.

limit of detection, reproducibility, sensitivity and specificity. While each laboratory will need to establish the appropriate approach, one group has proposed integration of CMA for CLL in clinical practice including the use of CMA as a first-line test in patients with > 30% tumor cells as determined by flow cytometry and use of FISH in those with < 30% tumor cells, to assess risk as normal, low or high [25], and for each method to reflex to the other if no abnormalities are found [37]. Using a strategy in which CMA was performed as a first-line test for patients with > 30% tumor cells (as determined by flow cytometry), 89% of cases had abnormalities detected by CMA and were completed without further testing; cases with negative results on CMA were reflexed to FISH. With the combination of CMA and FISH analyses, 96% of CLL cases had clinically significant genomic imbalances [37] and CMA analysis was able to simultaneously reveal prognostic marker status and the level of genomic complexity in > 85% of cases [38, 113, 114]. Typically, disease burden is high enough at time of diagnosis that the percentage of tumor cells is not an issue. If there is a suspicion of mantle cell lymphoma, FISH for *IGH/CCND1* should be considered.

Summary

CLL represents a model hematologic neoplasm for integration of CMA analysis into clinical testing for the following reasons: genetic lesions with known clinical relevance are primarily gains and losses rather than balanced translocations and inversions; DNA from fresh samples is readily available;

tumor burden tends to be relatively high in the peripheral blood and can be assessed by flow cytometry.

Based on the evidence identified through review of the literature, CMA analysis may be sufficient to replace the standard CLL FISH panel and metaphase chromosome analysis in a clinical diagnostic setting (see Table 4 for Comparison of Cytogenetic Technologies). CMA readily detects the gold standard FISH panel abnormalities as well as the genomic imbalances identified by metaphase chromosome analysis. Additionally, CMA has identified the presence of 10–15 second tier abnormalities, present in 1–5% of CLL patients, that are not targeted by FISH panels and may be too small to be seen by metaphase chromosome analysis [115]. CMA is more powerful than either FISH or metaphase chromosome analysis at specifically defining regions of imbalance. It will detect deletions that may be missed by FISH panels, elucidate abnormalities that cannot be characterized by analysis of banded metaphase chromosomes and identify potential imbalances in translocations that appear balanced by metaphase chromosome analysis.

Both metaphase chromosome and CMA analyses can identify genomic complexity, an independent marker for identification of patients with aggressive CLL. However, chromothripsis, another marker of aggressive disease, is detectable only by CMA. Similarly, CMA is the only one of the three technologies that can detect CN-LOH.

Limitations of CMA analysis mainly are decreased performance at low levels of tumor involvement and the inability to detect balanced chromosome rearrangements. Both limitations can be readily overcome in CLL. Flow cytometry informa-

tion is typically available for CLL patients, so the presence of low level disease should be known to the laboratory and CMA analysis should be deferred. Specific translocations of known clinical significance often involve the IGH locus. As such, addition of an *IGH* break-apart probe or an *IGH/CCND1* probe set can be considered to complement CMA analysis in CLL.

Currently, CMA is evolving for clinical application in CLL. Many laboratories are establishing its effectiveness as a stand-alone method that is likely more efficient and cost-effective than the combination of metaphase chromosome analysis and FISH. At this point in time, many clinical trials require FISH for eligibility and those patients with positive CMA results must have redundant FISH analysis performed to be eligible. Widespread acceptance of CMA technology could eliminate the cost of extra testing. Lastly, CMA technology provides the opportunity for discovery of clinically significant genomic alterations that have not been previously identified by other methodologies.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.cancer-gen.2018.07.004](https://doi.org/10.1016/j.cancer-gen.2018.07.004).

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