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The biology and clinical significance of acquired genomic copy number aberrations and recurrent gene mutations in chronic lymphocytic leukemia

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Abstract

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world and remains incurable with conventional chemotherapy treatment approaches. CLL as a disease entity is defined by a relatively parsimonious set of diagnostic criteria and therefore likely constitutes an umbrella term for multiple related illnesses. Of the enduring fundamental biological processes that affect the biology and clinical behavior of CLL, few are as central to the pathogenesis of CLL as recurrent acquired genomic copy number aberrations (aCNA) and recurrent gene mutations. Here, a state-of-the-art overview of the pathological anatomy of the CLL genome is presented, including detailed descriptions of the anatomy of aCNA and gene mutations. Data from SNP array profiling and large-scale sequencing of large CLL cohorts, as well as stimulated karyotyping, are discussed. This review is organized by discussions of the anatomy, underlying pathomechanisms and clinical significance of individual genomic lesions and recurrent gene mutations. Finally, gaps in knowledge regarding the biological and clinical effects of recurrent genomic aberrations or gene mutations on CLL are outlined to provide critical stimuli for future research.

Keywords

CLL; genomic copy number aberrations; gene mutations

INTRODUCTION

The improvements of technologies to accurately measure genomic changes in cancer cells and application of these to chronic lymphocytic leukemia (CLL) have resulted in detailed knowledge of the pathological anatomy of the CLL genome. Knowledge is most complete for acquired genomic copy number aberrations (aCNA) and gene mutations, and it is complemented with karyotypic information, including descriptions of balanced and unbalanced chromosomal translocations.

The knowledge base of aCNA and loss-of-heterozygosity (LOH) in CLL has been greatly expanded through the use of high-resolution single-nucleotide polymorphism (SNP) array-based genomic copy number assessments that have resulted in precise aCNA demarcations at the kilobase to sub-megabase resolution. The knowledge of recurrent gene mutations in CLL has been aided by the revolution in massively parallel sequencing. Gaps in knowledge

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exist with regard to the types and frequency of genomic changes outside of well-annotated genes, the biological effects of recurrent or rare acquired balanced chromosomal translocations on CLL cells, the stable effects of genomic aberrations on the CLL transcriptome, the mechanistic interplay between aCNA/LOH and gene mutations (and other critical CLL pathways) and the magnitude and importance of epigenetic changes in CLL.

Overall, the CLL genome is characterized by far fewer genomic events than many of the solid tumors and also by fewer events than other B-cell malignancies such as diffuse large B-cell lymphoma, mantle cell lymphoma (MCL) or follicular lymphoma. Within CLL cohorts, however, the range of genomic aberrations is wide, with ~20% of CLL lacking any aCNA (but such cases carry a few gene mutations), whereas others, albeit infrequently, carry >10 such events.

Decade-long research on karyotypic changes in CLL ultimately culminated in the development of a set of fluorescence *in situ* hybridization (FISH) probes and a CLL-FISH panel that has gained widespread clinical use in CLL. This is because subsets of patients with monoallelic loss of *TP53*, as detected by *TP53*-centric FISH probes (as a small part of large deletions on chromosome 17-pcommonly referred to as del17p), or *ATM*, as detected by *ATM*-centric FISH probes (as part of heterogeneous interstitial deletions on chromosome 11q- commonly referred to as del11q), when analyzed as cohorts, have shortened survival relative to comparator cohorts lacking these findings.[1,2] CLL-FISH is therefore an important test in clinical CLL management and has resulted in the realization of risk-adapted therapies for small subsets of CLL patients, including early use of allogeneic transplantations.[3–8]

Research on CLL karyotypes has been revolutionized over the last few years through use of innovative CLL cell stimulants, allowing for successful generation of karyotypes for almost all cases. Results of such analyses are complementary to array-based CLL genomics and have identified a substantial subset of CLL (~20%) with complex aberrant karyotypes and short survival. Stimulated karyotypes have also allowed for the detection of sporadic, as well as recurrent, balanced and unbalanced chromosomal translocations, and the presence of such chromosomal translocations has subsequently been associated with shortened patient survival. Overall, however, it is clear that a dominant high-frequency CLL driver in the form of a recurrent balanced translocation, akin to the Philadelphia chromosome in CML or Ph + ALL, does not exist.

Within the last two years, results from large-scale sequencing studies in CLL have been published that in aggregate are notable for (1) the lack of a dominant high-frequency mutated driver gene in CLL;[9,10] (2) a low gene mutation load per CLL case; (3) the lack of frequent mutations in the clinically important kinase gene families;[11] (4) the identification of novel recurrently mutated genes in CLL, such *NOTCH1*, *SF3B1* and others that, although infrequent, may result in the discovery of novel pathomechanisms in CLL; and, (v) associations of selected gene mutations with specific aCNA, suggesting cooperative effects on the afflicted CLL cell.

In the following paragraphs, a detailed discussion of aCNA, LOH, gene mutations and karyotypes in CLL is presented, and these lesions are reviewed from a biological, diagnostic, prognostic and therapeutic perspective.

ACQUIRED GENOMIC COPY NUMBER ABERRATIONS AND LOSS-OF-HETEROZYGOSITY (LOH) IN CLL

CLL genomic complexity

aCNA and LOH have been measured in paired DNA samples derived from purified CD19 + and CD3 + cells from 255 CLL patients using SNP 6.0 array-based profiling,[12] as well as collectively from hundreds of patients (study sizes ranged from 70 to 369 patients) in DNA of varied purity and/or in tumor DNA only.[13–17] In addition, measurements using array comparative genomic hybridization (CGH) platforms have been reported.[18,19] From this data, a relatively complete view of CLL-associated genomic aCNA has emerged. Overall, CLL is characterized by a relatively stable genome, with the majority of cases displaying between 0–2 aCNA. LOH usually associates with copy loss in CLL, and copy-neutral LOH (cnLOH, also referred to as acquired uniparental disomy (aUPD)) is rare, but when it occurs, for example, in cnLOH-17p, it is of high clinical relevance.[20,21]

Of clinical importance is a subset of CLL with elevated aCNA counts. For instance, aCNA ≥2 is detected in ~35% of all CLL cases and aCNA ≥3 in ~20% of cases, and as such an elevated aCNA count (referred to as elevated genomic complexity) demarcates a CLL subset with progressive and aggressive disease and short survival.[12,14,17,22] In comprehensive bivariate and multivariate analysis, elevated genomic complexity emerged as the dominant predictor of short overall survival (OS), identifying high-risk CLL cases in all CLL cohorts that were previously stratified by other markers. It is thus clear that the inability to maintain genomic stability associates strongly with an aggressive CLL phenotype.

Genomic complexity has also been identified and defined in large-scale CLL karyotyping studies (see below). Approximately 20% of CLL cases were found to carry ≥3 genomic aberrations, and the presence of complex karyotypes in CLL predicted for short OS.[23] It is likely, albeit formally unproven, that both SNP array-based and karyotype-based CLL genome analysis identify largely overlapping subsets of patients, with the former, in addition, detecting cases with microdeletions or cnLOH and the latter detecting cases with chromosomal changes that do not result in genomic copy loss (that is, balanced chromosomal translocations, additions or isochromsomes).

With regard to the likely mechanisms of the strong negative prognostic effects of elevated genomic complexity in CLL, one relates to the association with *TP53* mutations: most *TP53*-mutated CLL is genomically complex.[12] However, approximately two-thirds of CLL with aCNA ≥2 are *TP53* wild type, and despite intense research efforts it is currently unclear as to what molecular aberrations associate with or cause genomic complexity in this CLL subset.

The correlation of known measurable CLL traits with SNP array-based genomic complexity has identified the following traits as independently associated with elevated genomic complexity (in descending order of strength): del17p/*TP53* mutations, del11q and large del13q14 *inclusive* of *RB*.[24] Impaired ATM activation measured following external CLL cell irradiation weakly predicted elevated genomic complexity, but this effect could not be separated from the presence of del11q.[24]

With regard to the underlying reasons why genomically complex CLL is clinically aggressive, three complementary hypotheses are advanced: (i) frequent association with *TP53* mutations/del17p and other defects that impair apoptotic cell responses to genotoxic therapies,[12,24] (ii) ongoing clonal evolution, and (iii) associations with telomere attrition and associated genomic instability. The latter case would place genomic instability as a consequence of telomere dysfunction, which remains an attractive but not definitively

proven concept.[25–29] Furthermore, it remains unclear whether telomere dysfunction can result in interstitial deletions, the most frequent aCNA type in CLL. Ongoing research is directed at the identification of additional molecular aberrations, including gene mutations that are associated with elevated genomic complexity in CLL.

In the following paragraphs, individual high-frequency aCNAs are discussed in detail, followed by a brief summary of novel low-frequency but recurrent aCNAs, for which little detailed information is available at present.

Deletion 13q14

Interstitial deletions of various lengths located at 13q14 are present in ~50% of CLL.[2,30–36] These deletions, commonly referred to as del13q14, display substantial anatomic heterogeneity, and a subset extends telomerically and centromerically for many megabases beyond an approximate anchor point at chromosomal physical position 50 Mb. Approximately 15–20% of CLL cases with del13q14 carry a del13q14 on both chromosomes: either the same lesion or lesions of different lengths. Largely as a result of high-resolution SNP array profiling, the exact anatomy and extent of various del13q14 have been defined.[21] For illustration purposes, a published heatmap and a schema of del13q14, as detected through SNP 6.0 array profiling of 255 CLL cases, is shown in Figure 1.[37] From these studies, a few overriding conclusions can be drawn, including the following: (i) that del13q14 comprises various lesions with likely divergent associated gene deregulations; (ii) previously identified very short lesions used to define a minimal deleted region inclusive of the *miR15/16* locus were not identified in large CLL genomic profiling studies, and instead a relatively uniform lesion type (referred to as type I and present in ~60% of all CLL with del13q14) with a deletion length of ~0.8–1 Mb was identified; (iii) the vast majority (~98%) of del13q14 include the *miR15/16* locus, as well as *DLEU7*, and all intervening genes;[37–39] (iv) some type I del13q14 are biallelic and exist surrounded by large stretches of (many Mb in length) cnLOH, a lesion type that is otherwise rare in CLL; (v) multiple discrete anatomic del13q14 lesion clusters as defined by centromeric break points can be identified, suggesting divergent but largely unknown biological or clinical consequences.

The high frequency of del13q14 in CLL suggests a driver status for this lesion type in CLL. Importantly, however, del13q14 lesions that are indistinguishable from CLL-associated del13q14 have been identified in other cancers, such as MM, diffuse large B-cell lymphoma, AML, prostate cancer and others, and therefore are unlikely to confer CLL-specific biological traits on CLL cells. Furthermore, the frequency of FISH-detectable del13q14 in CD5 + CLL-like monoclonal B-cell lymphocytosis is 50%, but only 1% of these monoclonal B-cell lymphocytosis cases convert to CLL every year, clearly indicating that del13q14 alone is insufficient for the generation of CLL in humans.[40,41]

Despite decades of research, a complete understanding of gene deregulations as part of various del13q14 deletions has not emerged, and none of the genes located within type I 13q14 deletions are recurrently somatically mutated. Nonetheless, important progress has been made in elucidating del13q14 biology. One of the contributing genetic elements to del13q14 is the *miR15a/16.1* locus.[42] Evidence for a role of these miRs in CLL pathogenesis stems from (i) their almost invariate inclusion in all del13q14 lesions;[21] (ii) occasional biallelic deletion status, suggestive of a tumor suppressor gene function (albeit with the caveat that in humans a second locus on chromosome 3 at ~chromosomal position 160.122 Mb encodes for the highly related *miRs 15b* and *16.2* with the potential for compensation of del13q14-resident *miR* loss; (http://www.mirbase.org/index.shtml); (iii) effects of low miR15a/16.1 levels on anti-apoptotic and cell cycle regulatory molecules that are consistent with a role in CLL pathobiology and cancer in general; (iv) occasional epigenetic *miR15a/16.1* downregulation in CLL without del13q14;[43] and (v) evidence from spontaneously

occurring or artificially engineered mice that develop CLL-like illnesses upon ablation of the mouse homologues of hsa-*miR15a/16.1*.[44–46]

Some of the experimental evidence suggesting a less fundamental and possibly just contributory role of 13q14-resident miRs to CLL pathogenesis are (i) that miR expression (in particular miR16.1) levels are not substantially affected by the majority of 13q14 deletions that affect just one chromosome,[37] (ii) the lack of recurrent *miR15a/16.1* mutations and (iii) the low frequency of CLL-like illnesses that occur in mice engineered to carry *miR15a/16.1* deletions alone as opposed to a higher frequency in mice with larger engineered deletions similar to the ones that actually occur in humans.

Recently, interest in del13q14 has shifted to the gene *DLEU7*, which is expressed at subnormal levels in CLL.[47] Effects of DLEU7 on pro-survival cell signaling pathways have been demonstrated in heterologous cell systems, and inherited CNVs spanning this gene have been identified in a family with multiple affected CLL members.[48,49] It is therefore possible that ablation of *miR15a/16.1* together with *DLEU7* and possibly even other genes codetermines 13q14 biology. This research area constitutes work in progress.

Of additional noteworthiness is the fact that an *ad-hoc* summary analysis of recent large-scale sequencing studies detailed below has not identified a frequently somatically mutated gene located within any of the various 13q14 deletions. It is therefore likely that selected genes located within del13q14 are further inactivated through epigenetic regulation or that selected genes display haploinsufficiency.

With regard to the clinical importance of the presence of del13q14 as detected through FISH in CLL, a few considerations are important: (i) the clinically used CLL FISH panel cannot differentiate del13q14 subtypes, as the probe used is located within all 13q14 deletions; (ii) a monoallelic versus biallelic del13q14 status carries no independent prognostic significance; (iii) the expression levels of the del13q14-resident miR15a/16.1 carry no prognostic significance; (iv) the association of a del13q14 of any type with additional FISH abnormalities indicates a more aggressive disease course than the presence of a sole del13q14; (v) large del13q14 (type II and present in ~40% of all CLL with del13q14) that are inclusive of the *Rb* gene (approximate physical location at 48 Mb) demarcate a subset of CLL with del13q14 that is clinically more aggressive. In support of this finding, an association of del13q14 type II deletion with elevated genomic lesion load (genomic complexity) has been detected.[37–39,50–52] It is thus likely that inclusion of a FISH probe centered on *Rb1* will further refine genomic CLL risk stratification.

Deletion 17p

Terminal deletions of the short arm of chromosome 17, commonly referred to as 17p deletions or del17p, are detected by FISH in ~7% of newly diagnosed CLL.[2,53] These deletions are almost always large, measure ~18–22 Mb in length, and are the consequence of various structural changes (deletions, unbalanced translocations and isochromosomes). To assist the reader with the visualization of del17p, LOH and cnLOH at 17p in CLL, a copy number heatmap and LOH analysis and a schema of del17p as detected through SNP 6.0 array profiling are displayed in Figure 2. Del17p invariably results in the deletion of one *TP53* allele with the second retained *TP53* allele reported mutated in 64–100% of cases in various studies.[12,20,54–56] Importantly, these studies differ in various technical aspects of *TP53* mutation analysis and differ substantially in the frequency of mutations in *TP53* exons 4, 9 and 10. Analysis of large unselected CLL cohorts (193, 255 and 400 CLL patients are described in Harferlach *et al.*,[23] Malcikova *et al.*[54] and Ouillette *et al.*[12]) identified between 0–6% of del17p CLL without *TP53* mutations. It reflects the opinion of the author that the vast majority of CLL with del17p carry *TP53* mutations on the retained allele. Small 17p

deletions that are centered on *TP53* have been described as well, albeit at much lower frequencies than the canonical del17p. Most del17p are present in the majority of the CLL clone as assessed by FISH, indicating a strong competitive advantage for growth or survival.

Almost all research on del17p has been focused on the association with *TP53* mutations (see section below). Here the following facts are of clinical importance: (i) all del17p are detectable by the clinically used CLL FISH panel that includes a probe that is centered on the *TP53* gene;[57] (ii) some 17p deletions convert to cnLOH at 17p (cnLOH-17p; unbiased frequencies of aUPD-17p in untreated or relapsed/ refractory CLL patients are not available; three out of 255 such patients were identified in the published cohort by Ouillette *et al.*[12]) that were undetectable by FISH, and as these cases are always associated with homozygous *TP53* mutations they constitute one subset of aggressive CLL that may display falsely reassuring FISH findings;[20] (iii) sporadic, non-del17p-associated or monoallelic *TP53* mutations exist (reported frequencies in large CLL cohorts range from 1–5% and frequencies as high as 18% have been reported in highly selected fludarabine-refractory CLL) independently of del17p (but of unclear relation to cnLOH-17p), and these are also associated with aggressive disease;[54–56,58,59] (iv) the incidence of del17p increases with time from diagnosis and in particular with prior therapies for CLL; (v) presence of del17p is particularly deleterious in the setting of genotoxic therapy (purine analogs and alkylators) for CLL, as these therapies rely on functional TP53 protein for apoptosis induction;[60] (vi) presence of del17p serves as a basis for risk-adapted therapies in CLL, increasingly involving non-genotoxic therapy approaches and reduced-intensity allogeneic stem cell transplantation as consolidation;[3,5,6] (vii) presence of del17p alone should not be used as an indication for initiation of therapy; and (viii) CLL with del17p is clinically heterogeneous and if associated with mutated IgV_H loci or early Rai stage may be clinically stable for years.[61,62] For instance, patients with CLL and del17p that were further analyzed for IgV_H-status and Rai stage demonstrated 3-year OS of 96, 74 and 22% with ≤1, 2 or 3 such factors present.[61]

Somewhat unclear at present are the exact mechanisms contributing to the observed clinical aggressiveness of CLL with del17p. Although it is likely that apoptosis resistance and therefore lack of complete cell kill underlies del17p effects, it is also likely that del17p and associated *TP53* inactivation creates a permissive environment for persistence of new and additional genomic changes, and that some of these changes result in more aggressive CLL subclones. The analysis of clonal evolution in CLL and effects on clinical outcome are a research opportunity in CLL.[1,63–65]

Given the anatomic size of 17p deletions in CLL, it is likely that the full biological impact of this lesion type on CLL cells involves additional gene deregulations. However, very little additional information is available. Genome-wide analysis of gene mutations in CLL did not identify a frequently mutated gene other than *TP53* that is located within del17p. With regard to del17p-associated transcriptome deregulation, unpublished data have identified hundreds of statistically deregulated genes, complicating further in-depth analysis. Associations of del17p with aberrant expression of microRNAs have been reported, and in particular, aberrantly high expression of miR-21 has been proposed to further increase the odds of short survival in CLL.[66] This is an area of future research opportunities.

Deletion 11q

Interstitial loss of chromosomal material located on chromosome 11q, commonly referred to as del11q, is present at diagnosis in ~10% of CLL patients. Most 11q deletions in CLL are large and measure many Mb to tens of Mb in length.[67–69] All 11q deletions that include the *ATM* gene are classical 11q deletions and all involve one chromosome only. Homozygous 11q deletions do not exist, but rare cnLOH at 11q has been described. Atypical small 11q

deletions located closer to the centromere with a minimal deleted region distinct from classical 11q deletions have been identified as well, but nothing is known about their biology or clinical relevance.[12,14]

Despite the substantial size heterogeneity and length of 11q deletions in CLL, much of the research on del11q has focused on *ATM*. Mutations in *ATM* (see section below) in the retained allele have been identified in a minority of 11q-deleted CLL at various frequencies, with the published literature not clearly separating somatically acquired from germline mutations, or even SNPs. In addition, mutations in *BIRC3* have recently been identified, albeit only in a small subset of CLL that had relapsed quickly following fludarabine-based therapy and not in *de novo* CLL.[70] At present, data from large-scale CLL gene sequencing studies do not support the existence of other frequently mutated 11q-resident genes.

Given the absence of high-frequency mutated genes in CLL that are located within the boundaries of del11q, the question arises as to what drives clonal selection of CLL cells with del11q? A second question that may or may not be related to the first centers on the almost complete association of del11q status with unmutated IgV_H status (defined as ≥98% homology to germline), an association that remains fully unexplained at present.

Recently, aberrantly high insulin receptor (INSR) expression was identified in 60–70% of CLL with del11q.[69] Insulin stimulation of CLL with high INSR expression provides anti-apoptotic and pro-growth stimuli to CLL cells, and high INSR expression associates with early CLL disease progression. Currently, it is assumed that one or a few genes located within 11q deletions are negative regulators of INSR expression and that monoallelic deletions of these genes, together with unidentified defects, result in aberrant INSR upregulation. Experiments to identify such INSR regulators are being conducted and should result in greater mechanistic clarity regarding the regulation of INSR expression in CLL.

Summarizing the best available data on the pathophysiology of del11q in CLL, it appears that defects in DNA-double-strand (ds) break and repair responses contribute to the phenotype of CLL with del11q, and such defects are due to (i) occasional *ATM* mutations; (ii) non-mutational ATM dysfunction/hypoactivation owing to unidentified mechanisms; (iii) compound gene deletions involving other genes that are involved in the DNA-ds break and repair responses (for instance, *Mre11a* and *H2AX* together with *ATM*);[24] and (iv) unidentified 11q-associated mechanisms.[71] In addition, pro-survival and pro-growth pathways are activated in CLL cells with del11q, either through aberrant overexpression of the INSR or TCL1, providing credible mechanisms for clonal selective pressure that enriches for the presence of del11q.[72] Finally, the strong association of del11q with elevated genomic complexity in CLL may underlie clonal evolution/diversification in CLL, which is followed ultimately by outgrowth or regrowth of more aggressive clonal variants. It is likely that future research into 11q biology will uncover additional molecular aberrations that are important to 11q subsets.

With regard to the clinical implications of the presence of del11q in CLL, the following conclusions can be supported by current evidence: (i) essentially all CLL with del11q is progressive: long-term disease stability without the need for initial therapy does not exist at appreciable frequencies; (ii) remission durations after frontline therapy for CLL with del11q are shorter than for patients without del11q (and absence of del17p), although recent improvements following the inclusion of cyclophosphamide into the treatment regimens have been noted;[73] (iii) the OS of CLL patient cohorts with del11q is modestly shorter than that for CLL without del11q (and absence of del17p);[2,53] and (iv) CLL with del11q can be associated with disproportionate increases in internal lymph node sizes, a phenomenon that may be related to aberrant high INSR expression.[74]

Trisomy 12

A gain of the entire chromosome 12 (referred to as trisomy 12) is present in ~15–18% of CLL at diagnosis. A few additional CLL cases carry partial gains of chromosome 12, but few, if any, of these gains are recurrent. Despite the substantial fraction of CLL that carries trisomy 12, very little concrete information is available on the molecular mechanisms that are underlying this chromosomal aberration and their possible effects on CLL cells, and patients with trisomy 12 are the least studied of all FISH-defined CLL subsets.

Nonetheless, two recent interesting observations have been reported: (i) the frequent (50%) association of *NOTCH1* exon 34 mutations with trisomy 12 (compared with a frequency of *NOTCH1* exon 34 mutations in unselected *de novo* CLL of ~4–6%);[75–78] and (ii) the heightened dependence of CLL cells with trisomy 12 on hedgehog pathway activation.[79] The latter findings, although still somewhat preliminary, resulted from *ex vivo* studies of inhibitors of the hedgehog pathway in CLL and findings of elevated levels of Desert hedgehog (DHH) and Gli1 in patients with trisomy 12.

Clinically, an isolated trisomy 12 carries no adverse prognostic information. Importantly, patients with isolated trisomy 12 treated with modern chemoimmunotherapy approaches are almost always sensitive to therapy (possibly because of a strong negative association of trisomy 12 with *TP53* mutations or elevated genomic complexity) and often enjoy above-median remission durations.[12,80]

Recurrent aCNAs in CLL with frequencies of 1–5%

Various aCNAs with frequencies of 1–5% have been identified in CLL, largely as a result of high-resolution SNP array-based genomic assessments. Complicating a complete description of the frequency and identity of these second tier aCNAs are various differences in methodologies used for detection and at times lack of analysis of paired normal DNA. The aCNAs as described by Ouillette *et al.*,[12] which are based on direct comparisons of paired DNA samples from 255 CLL patients isolated from FACS-sorted cells, have been tabulated in Table 1 and have been graphically summarized in Figure 3. In addition, recurrent low-frequency aCNAs can be found in various recent publications.[13,15,17,38] Some of these include gains at 3q26 and 8q24 and losses of varying length at 8p. Very little firm knowledge is available about any of these rare recurrent aCNAs. In addition, aCNAs listed in Table 1 often co-occur in the setting of other aCNAs or *TP53* mutations, thus complicating reductionistic research approaches and conclusions.

With regard to the clinical importance of rare but recurrent aCNA, the existing data have to be interpreted with caution, as none are based on large enough CLL cohorts to allow for multivariate analyses and assessments of the independence of effects. Although it is plausible that individual lesions directly affect CLL clinical progression or even chemotherapy resistance, no direct evidence is available to confirm such a hypothesis.

RECURRENTLY MUTATED GENES IN CLL

At present, large-scale sequencing data on > 200 CLL cases have been published.[9–11,81,82] Overall it appears that CLL-associated gene mutations are relatively infrequent and mostly private. One of the important findings from this data in aggregate is the fact that a high-frequency mutated driver gene in CLL has not been identified and likely does not exist. However, CLL subsets that carry recurrent gene mutations in the 1–10% range exist, and knowledge is evolving with regards to the biological and clinical implications of these findings.

Proposals have been advanced to categorize CLL-associated mutations based on gene functions into pathways. Complicating such synthetic approaches is the lack of detailed studies that measure effects of mutations of genes on afflicted CLL pathways. Nonetheless, in addition to the well-known mutations affecting the DNA-ds break response and repair pathway (see below), mutations recurrently affecting innate immune system molecules and RNA splicing have been identified.

In the following paragraphs, selected, recurrently mutated genes in CLL and their biological and clinical effects are discussed in detail. This research area is advancing rapidly and the reader is referred to ongoing research findings for up-to-date information.

TP53 mutations

Somatically acquired mutations in *TP53* are detectable using direct sequencing of PCR amplicons that are templated on genomic DNA in ~10% of newly diagnosed CLL.[54,83,84] The frequency of CLL-associated *TP53* mutations in community-based assessments, however, may be substantially less than 10%.[85] Functional assays for detection of *TP53* mutations have been described as well.[20,71] In CLL, the *TP53* mutation frequency increases slightly with disease duration and is higher in the relapse setting, following the use of genotoxic therapy. Most *TP53* mutations in CLL are missense mutations targeting the DNA-binding domain and most mutations occur in *TP53* exons 5–9. A few additional mutations occur in *TP53* exon 4 and rarely in exon 10.[20,56,58,59] Most missense-mutated TP53 proteins accumulate to high levels in the CLL cells, the pathophysiological relevance of which is unknown. A subset of *TP53* mutations are frameshift mutations because of either indels, nonsense or splice-site mutations, resulting in severely truncated TP53 proteins. It is currently assumed that these are null mutations.

CLL cells with *TP53* mutations are completely resistant to radiation-induced apoptotic cell death, demonstrating the central and absolute requirement for functional TP53 protein in the DNA-ds break-induced apoptotic responses.[24,86] Unclear at present is what other TP53 functions are important contributors to the severe clinical phenotype inflicted by TP53 mutants on CLL cells. It is furthermore unclear if some of the CLL-associated TP53 mutants have additional gain-of-function phenotypes and what the exact phenotype is.

Clinically, CLL with *TP53* mutations is aggressive.[87,88] The OS of patients receiving conventional therapies when assessed from the time of detection of a *TP53* mutation is measured in years, but substantial interpatient variability exists.[89,90] Most of the negative survival impact conferred by *TP53* mutations appears owing to incomplete responses and short response durations to standard therapies, with a subset of patients displaying frank therapy resistance. The latter phenomenon is clinically catastrophic and not well understood in molecular terms, in particular given that the spectrum of clinical responses in *TP53*-mutated CLL can range from CR (albeit rare) to progressive disease.

CLL with *TP53* mutations is currently treated with a variety of risk-adapted therapies and provides the best-studied indication for use of reduced-intensity allogeneic stem cell transplantation (RIC-allo-TX) as consolidation.[91,92]

ATM mutations

The frequency of somatically acquired mutations in *ATM* in unselected CLL cohorts is unknown.[93–95] If extrapolations from large-scale CLL sequencing studies are used, and assuming that all *ATM* exons were adequately covered, it is substantially less than 10%. Within the context of del11q, which invariably results in the removal of one *ATM* allele, the *ATM* mutation frequency estimates range from 8–30%.[96,97] *ATM* mutations comprise missense, nonsense and frameshift mutations, the latter type suggestive of gene product

inactivation. Little information on the function of ATM proteins carrying single amino-acid substitutions is available and it is unclear what fraction of these constitutes complete or partial loss-of-function mutations.

The analysis of ATM protein levels and radiation-induced ATM autophosphorylation in a large CLL cohort of 250 cases has further qualified the incidence of ATM aberrations in CLL.[24,97] From this data, it has become clear that ATM aberrations are infrequent and of modest effect on CLL clinical outcome. Although remission durations of ATM-aberrant CLL are shorter than for various comparator cohorts, effects of ATM aberrations on OS were not detected, suggesting effective salvage of ATM-aberrant CLL in the relapse setting.

Interestingly, while various cell types with ATM inactivation demonstrate a radiation-sensitivity phenotype in clonogeneic plating assays, ATM-aberrant CLL cells are neither resistant nor supersensitive to radiation-induced apoptosis (unpublished observation on the basis of the comparative quantitative analysis of radiation-induced CLL apoptosis and radiation-induced ATM autophosphorylation in purified cells from 210 CLL patients with wild-type *TP53*). This is clearly very distinct from the findings in *TP53*-mutated CLL. Even though ATM functions upstream of TP53 in the DNA-ds break repair and response pathway, a defect in ATM, phenotypically or clinically, does not equal a defect in TP53, suggesting redundant TP53 activation pathways within the CLL cell.

With regard to the clinical importance of *ATM* mutations in CLL, the existing literature is conflicted.[96,98–100] Most importantly, effects of *ATM* mutations and effects of del11q are not separated in published work, and germ-line mutations in *ATM* are not clearly distinguished from somatically acquired mutations. Finally, prospective assessments of the effect of *ATM* mutations on CLL survival in well-defined CLL cohorts using multivariate analysis are not available.

NOTCH1 mutations

The application of massively parallel sequencing to CLL genomes and exomes, and candidate gene resequencing has resulted in the identification of novel recurrent mutations in *NOTCH1* in CLL.[81,82] These mutations cluster in the very large exon 34, disrupt the PEST domain, and have the net effect of stabilization of the NOTCH1 protein. The most commonly identified mutation is a recurrent dinucleotide deletion (NOTCH1 c. 7541_7542het_delCT). Very little biological information of the effect of stabilized NOTCH1 protein on afflicted CLL cells is available, but it is likely that mutated-NOTCH1 protein is still ligand-dependent for full pathway activation and not autonomously activated.

The frequency of *NOTCH1* mutations in large unselected and untreated CLL validation cohorts is ~6%, with higher estimates derived from the original discovery cohorts (mixtures of untreated and relapsed CLL) and higher frequencies in relapsed CLL. It is presently unclear but under active investigation at what frequency *NOTCH1* mutations are acquired during CLL disease evolution. Interestingly, *NOTCH1* mutations are not equally distributed across all CLL but are highly enriched for CLL cases that are ZAP70 + and IgV_H unmutated, therefore displaying strong associations with proliferative and progressive CLL. No information is available that would mechanistically explain this association. In addition, ~50% of CLL with *NOTCH1* mutations also carry trisomy 12, a frequency substantially higher than expected by chance; this again suggests cooperation between these genomic aberrations, but specific information is unavailable.[75,101]

With regard to the clinical implications of *NOTCH1* mutations in CLL, the existing evidence is conflicted.[78] One report, based on large patient numbers and multivariate modeling, suggested a very adverse effect on survival by *NOTCH1* mutations and equated

the negative prognostic impact of the presence of *NOTCH1* mutations with the presence of *TP53* mutations.[102] Data from an independent large CLL cohort employing comprehensive multivariate analyses did not detect such effects.[75] It is thus clear that only carefully conducted research on very large, prospectively collected CLL cohorts can provide definitive answers. In the meantime, various data are somewhat at odds with the postulated strong negative prognostic effects of *NOTCH1* mutations in CLL, including: (i) the strong association with trisomy 12, which usually does not identify aggressive CLL; and, (ii) the lack of associated high-risk genomic features like del17p, del11q, elevated genomic complexity or *TP53* mutations.

Of additional interest is the reported elevated frequency of CLL transformation to large cell lymphoma (Richter's transformation) in CLL with *NOTCH1* mutations.[81,82] Here again, prospectively analyzed large CLL cohorts are needed to prove this hypothesis. If confirmed, CLL with *NOTCH1* mutations may be eligible for novel intervention trials aimed at prevention of such lethal transformation events.

Future work in CLL carrying stabilizing *NOTCH1* mutations will focus on testing the suitability of mutated NOTCH1 as a target for therapy. Such targeted therapy is contingent upon the demonstration that CLL cells rely on NOTCH1 for either survival or proliferation or alternatively, that *NOTCH1* mutations confer chemotherapy resistance on afflicted CLL cells.

SF3B1 mutations

Massively parallel sequencing of CLL exomes and candidate gene resequencing approaches resulted in the unexpected finding of novel missense mutations in the splicing cofactor *SF3B1*.[9,10,103] Mutation frequency estimates based on the original discovery cohorts combined are ~10%, whereas estimates based on large-scale validation studies are closer to 6% (unpublished). Associations with fludarabine resistance, as well as the presence of del11q, have been reported but are in need of validation, especially given the fact that many biomarkers in CLL are enriched in relapsed/refractory CLL without causal link to therapy resistance.

Mutations in *SF3B1* are mono-allelic missense mutations involving recurrently affected codons. Given that SF3B1 is involved in mRNA 3′-splice-site recognition, follow-up studies using RNAseq to detect aberrant mRNA species in cells with *SF3B1* mutations were performed. Indeed, some mRNA species demonstrated aberrant intron retention.[10] It is, however, unclear at present whether aberrant mRNA splicing is indeed the critical pathomechanistic effect of *SF3B1* mutations and if so, what critical mRNA are affected.

With regard to the clinical impact of *SF3B1* mutations in CLL, the data are sparse. In other hematological malignancies which also carry *SF3B1* mutations, effects on prognosis are usually favorable, providing a framework from which to view the effect of *SF3B1* mutations in CLL. Large-scale CLL studies are needed to advance our understanding in this area.

Other recurrently mutated genes in CLL

A number of genes have been found to be recurrently mutated in CLL, albeit at low and largely unknown frequencies in validation cohorts, and the data in this research area are still actively evolving. The best current information can be found in two published large CLL exome sequencing studies and large-scale sequence analysis of kinase genes.[9–11,104] Largely unknown at present is the functional consequence of the known mutations on affected genes, unless genes are mutated through frameshift or nonsense changes, which usually indicate loss of function. A few recurrently mutated genes not discussed above are briefly mentioned here:

Activating *BRAF* mutations occur in 2% of CLL and may lead to the clinical use of BRAF inhibitors for selected cases.[11]

Mutations in *MYD88*, similar to the mutations described in NHL, have been identified in CLL and appear over-represented in CLL with del13q14. MYD88 is involved in TLR signaling and the CLL/NHL-associated mutations are activating and result in NFκB activation.[9,81,105]

XPO1/Exportin mutations (a protein involved in nuclear protein export) have been identified in a few percent of CLL patients.[81]

FBXW7 (a ubiquitin ligase) mutations have been found in sporadic CLL cases and may provide an alternative, albeit infrequent, mechanism for NOTCH1 activation.

POT1 (a member of the shelterin complex) mutations have been detected in CLL and may affect telomere maintenance.[10]

LRP1B (a cell surface receptor) mutations occur in CLL and may affect a generic cancer-relevant pathway, as this gene is one of the most frequently altered genes in all cancer.[10]

Sporadic inactivating *RB1* mutations have been identified in the setting of large 13q14 deletions, resulting in a small CLL subset that is *Rb* null.[37]

THE LANDSCAPE OF aCNA AND CHROMOSOMAL TRANSLOCATIONS IN CLL AS DEFINED THROUGH CLL CELL KARYOTYPING

CLL karyotyping using basic cell culture conditions has been performed for decades and has resulted in valuable information on gross structural aberrations.[106–109] The major limitations of this tool are the inability to generate karyotypes for the majority of patients, as well as the unreliable detection of smaller genomic lesions (sub-megabase to 5 Mb range). Over the last few years, novel CLL cell culture conditions have been established, which now allow for the generation of so-called ‘stimulated karyotypes’ in the vast majority of patients[110] and results from karyotyping efforts of large CLL patient collections have been reported.[111–114] The largest study of CLL genomics based on stimulated karyotypes by Haferlach *et al.*,[23] very nicely summarizes CLL genomics on the basis of karyotyping of 500 CLL; therefore only a few pertinent points are highlighted in the following bullets:[23]

- **i.** Eighty-three percent of analyzed CLL cases carried an aCNA; the median was 1.7 aCNA per case; 2 or more aCNA were detected in ~44% of cases, whereas 3 or more aCNA were detected in ~21%.
- **ii.** Reciprocal translocations, which are understudied in CLL, were detected in ~20% of cases. Of these, translocations involving the Ig loci (heavy- and light-chain loci), as well as cytoband 13q14 were the most frequent. In addition, studies on translocations involving cytoband 14q32 identified BCL2 t(14;18)(q32q21) and BCL3 t(14;19)(q32;q13) as recurrent translocation partners; t(14;19)(q32;q13)/(Ig-BCL3) may be associated with more aggressive CLL, as well as atypical CLL presentations.[115–118]
- **iii.** As expected, karyotyping detected more aCNA than the commonly used CLL-FISH test
- **iv.** aCNAs as detected by karyotyping in CLL with normal FISH are prognostically adverse.[119,120]

v. A complex aberrant karyotype identifies a high-risk subgroup of CLL with short survival; the overlap of CLL cases with a complex aberrant karyotype and presence of either del17p or del11q is partial, and,

vi. The mean number of aCNAs in *TP53*-mutated CLL was five, as compared with a mean of 1.5 in the *TP53* wild-type group.

Some of the caveats regarding the routine use of stimulated karyotypes in CLL management include the following: (i) prospective evaluations of the prognostic value of stimulated karyotyping results in CLL cohorts have not been reported; (ii) as expected, even stimulated karyotyping misses smaller genomic lesions; for instance, short del13q14 as detected through CLL FISH is often not detected and therefore CLL-FISH results are still necessary for proper genomic assessments; (iii) interlaboratory differences in karyotyping results exist; and (iv) differences in the detection rates (frequencies) and types of abnormal karyotypes exist, which is dependent on what specific culture conditions are used.

FUTURE PERSPECTIVES

Despite substantial advances in our knowledge about the biological and clinical implications of aCNA and recurrent gene mutations in CLL, many questions remain. To assist the reader with understanding the limits of current knowledge, as well as with the planning and design of future experimental approaches aimed at improving the understanding of CLL biology and clinical behavior, selected questions have been tabulated in Table 2.

Summarizing briefly the important deficiencies in current knowledge as outlined in Table 2, these center on (1) a more complete molecular characterization of the effects of aCNA on the biology of afflicted CLL cells, including the effects on defined signal transduction pathways and the interplay with pathways known to be central to CLL biology (for instance, the B-cell receptor complex signaling pathway); (ii) an understanding of the effects of recurrent gene mutations on CLL subset biology; (iii) the role of stable transcriptome changes in CLL subsets and the role and effects of epigenetic gene modulation; and (iv) the clinical importance and prognostic effects of rare aCNA and gene mutations—a question that can only be addressed in large-scale CLL collaborations possibly involving thousands of patients.

Given the central importance of aCNA and gene mutations in CLL, a deeper understanding of their mechanistic functions will inform better prognostication and better therapy selection and will ultimately result in further improvements in the outcome for patients with CLL.

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CONFLICT OF INTEREST

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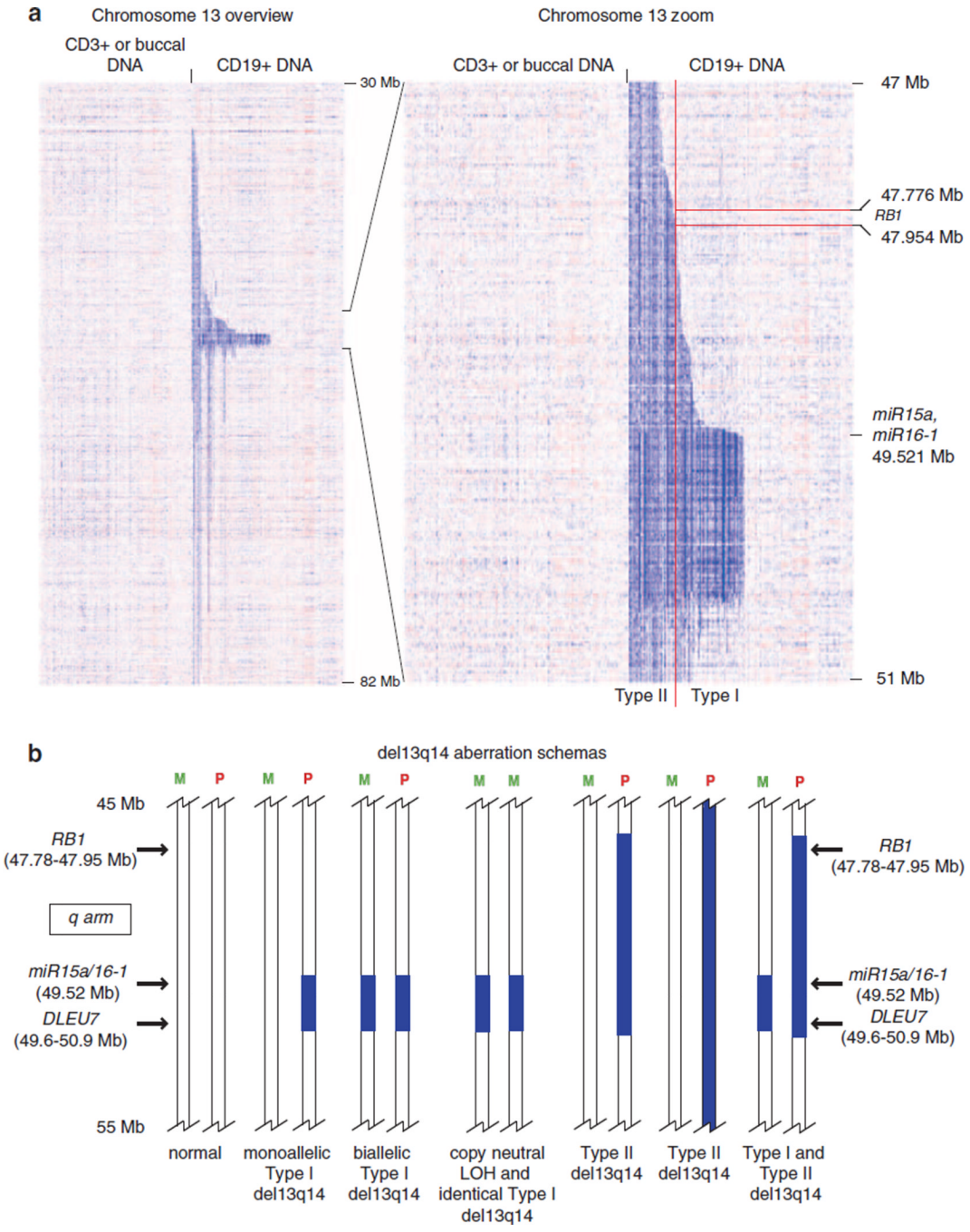

Figure 1.

Genomic copy number heatmap displays of chromosome 13q of 255 CLL cases ranked by the position of centromeric 13q14 deletion break points. (**a**) 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. Each column represents one patient. (**b**) Schemas of various del13q14 in CLL: M, maternal chromosome; P, paternal chromosome. The approximate locations of selected genes (*miR15a/16.1, DLEU7* and *RB*) are indicated. Deletions are indicated by blue bars.

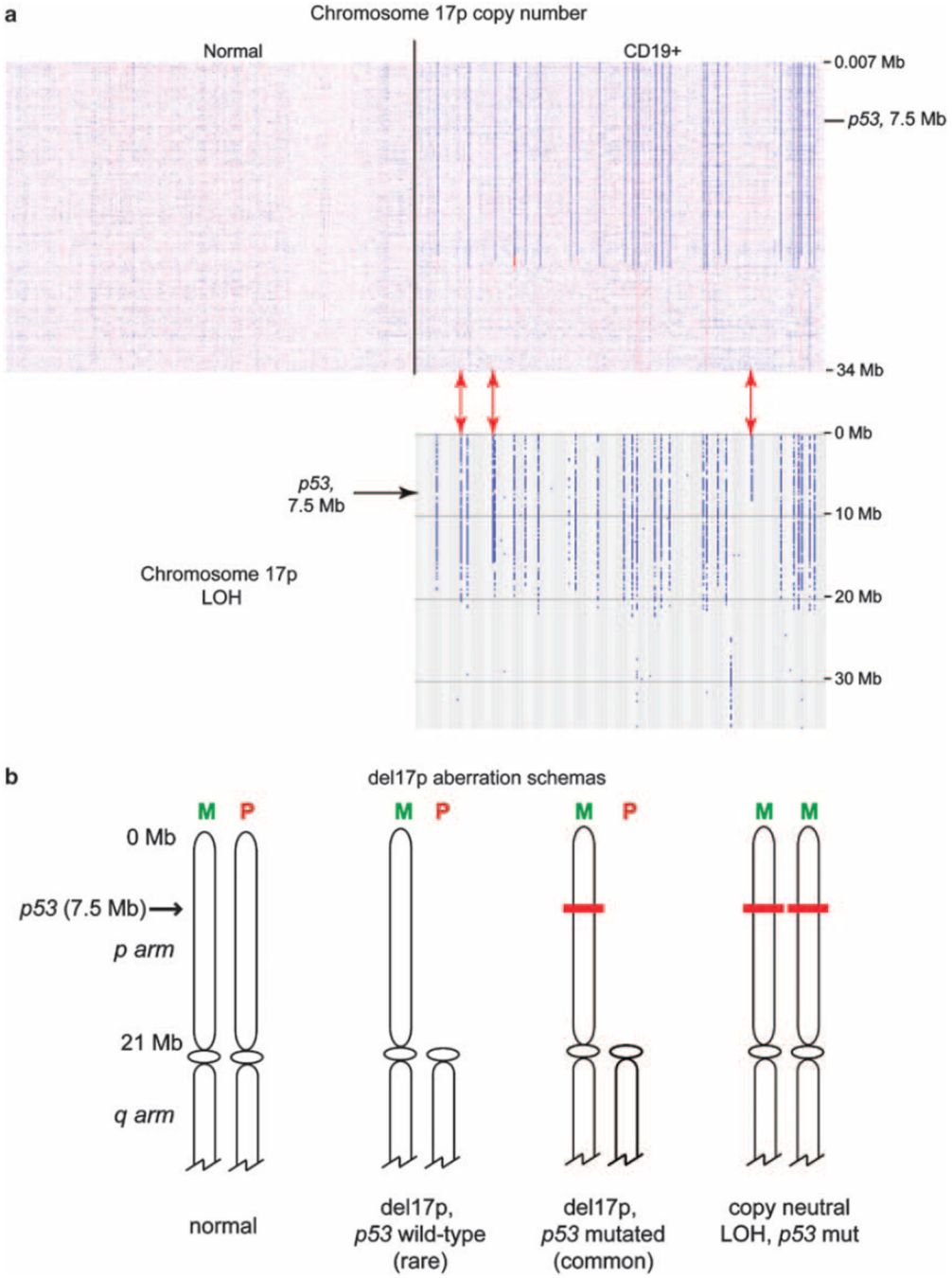

Figure 2.

(**a**) Genomic copy number heatmap displays of chromosome 17p of 255 CLL cases: 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. Lower panel: 17p-LOH; red arrows indicate cases with cnLOH-17p. (**b**) Schemas of del17p in relation to *TP53* mutations and LOH at 17p in CLL: M, maternal chromosome; P, paternal chromosome. The approximate location of *TP53* is indicated. Red bars indicate mutated TP53.

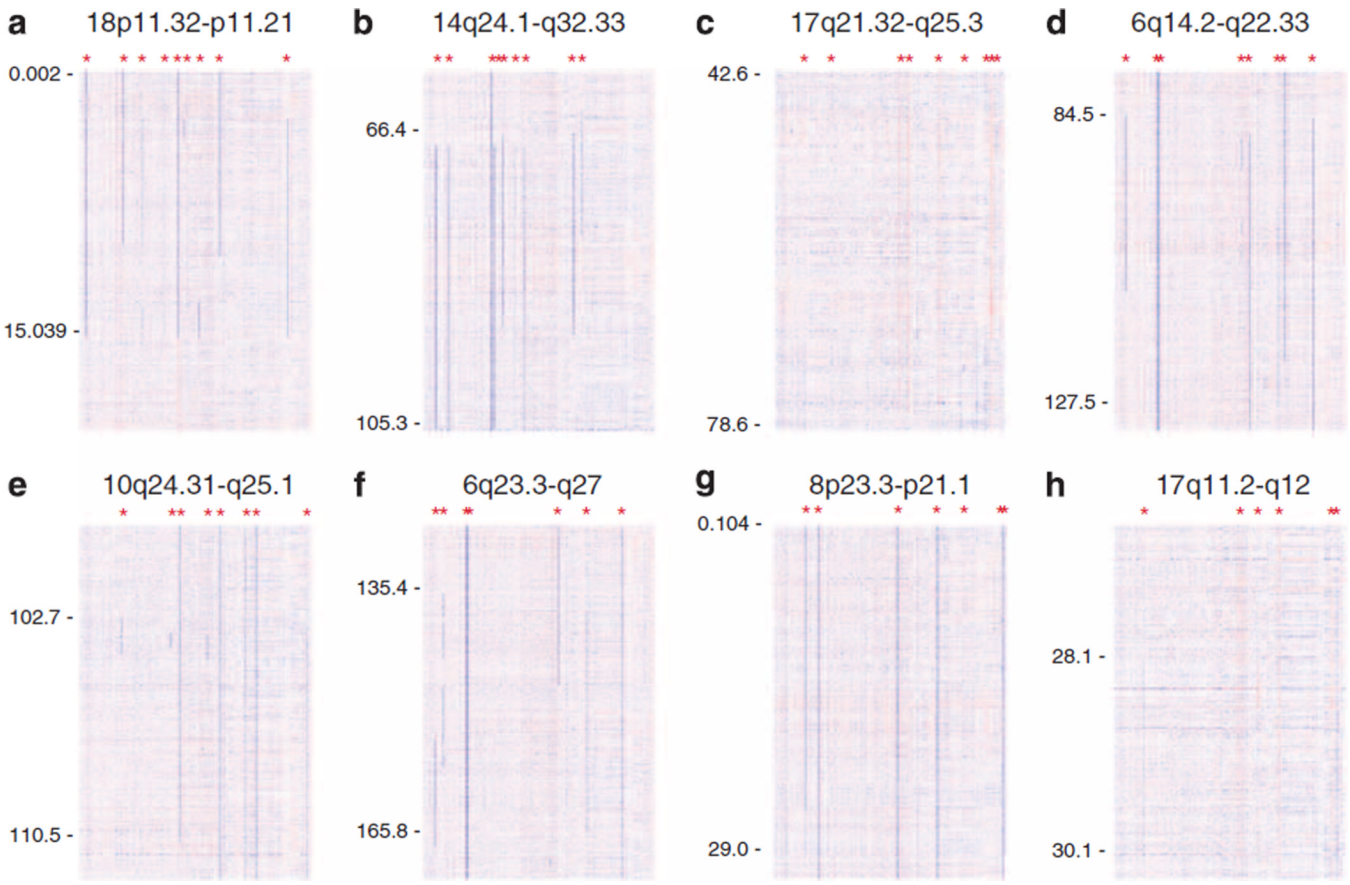

Figure 3.

(**a–h**) Genomic copy number heatmap display of eight distinct recurrent aCNAs based on 255 CLL cases: Blue indicates copy loss, red indicates copy gain. Asterisks indicate an aCNA. Based on highly purified DNA from sorted CD19 + and CD3 + cells as described in Ouillette *et al.* (2011).[12]

Table 1

Acquired genomic copy number aberrations detected in 255 CLL cases using SNP 6.0 profiling. MAR: minimally altered regions (inclusive of gains and losses). Based on highly purified DNA from sorted CD19 + and CD3 + cells of 255 CLL cases as described in Ouillette *et al.* (2011)[12]

Chromosome cytoband	Incidence	MAR start rsID#	MAR start physical position	MAR stop rsID#	MAR stop physical position	MAR length (Mb)	Predominant Lesion Type
13q13.2–q31.1	51.37%	2740536	49.503	201778	49.928	0.425	Loss
11q13.5–q23.3	10.12%	10789636	107.077	4754348	108.087	1.01	Loss
17p13.3–p11.1	9.80%	6565733	0.007	2472708	18.858	18.851	Loss
18p11.32–p11.21	3.92%	646699	2.657	4797120	3.715	1.058	Loss > gain
14q24.1–q32.33	3.53%	1954119	68.841	1955619	79.853	11.012	Loss
17q21.32–q25.3	3.53%	MAR1: 8077375	MAR1: 42.691	MAR1: 16949418	MAR1: 43.346	0.655	Gain
		MAR2: 1486751	MAR2: 47.695	MAR2: 7226166	MAR2: 50.795	3.1	Gain > loss
		MAR3: 9944529	MAR3: 70.793	MAR3: 8067774	MAR3: 71.991	1.198	Gain > loss
		MAR4: 4789911	MAR4: 74.554	MAR4: 12452661	MAR4: 74.852	0.298	Gain > loss
6q14.2–q22.33	3.14%	1474620	97.752	10782162	109.913	12.161	Loss
10q24.31–q25.1	3.14%	2296586	104.236	17113122	104.596	0.36	Loss
6q23.3–q27	2.75%	11966730	138.104	17078283	148.739	10.635	Loss
8p23.3–p21.1	2.75%	13278518	18.508	17468779	18.836	0.328	Loss
17q11.2–q12	2.35%	28921	28.29	16968252	28.717	0.427	Gain > Loss
18q21.33–q23	2.35%	2732226	71.744	4891158	72.18	0.436	Gain > Loss
8q24.21	1.96%	7824785	128.184	16902149	128.476	0.292	Gain
13q31.1	1.96%	4143260	84.332	9555564	85.938	1.606	Loss > gain
17q12	1.96%	2411182	32.134	12449696	32.526	0.392	Gain > loss
17q21.31	1.96%	4793124	40.003	2664008	41.239	1.236	Gain > loss
19p13.3	1.96%	8100066	0.212	10420193	3.478	3.266	Loss > gain
20p13	1.96%	6081944	2.027	7272003	4.261	2.234	Loss
20p12.3–p12.1	1.96%	3897510	6.077	201928	17.473	11.396	Loss
Xp11.4–p11.3	1.96%	5963946	41.042	17214797	42.773	1.731	Loss

Table 2

Selected questions in CLL genomics

A comprehensive molecular understanding of determinants of genomic instability and complexity in CLL.
The relative contributions of individual acquired genomic copy number aberrations and recurrent gene mutations on (i) drug-mediated CLL cell kill versus resistance; (ii) *in vivo* CLL growth kinetics; (iii) CLL clonal diversification; and, (iv) CLL patient survival.
The frequency of all acquired genomic copy number aberrations and recurrent gene mutations in CLL at diagnosis, with focus on minor subclones and possible effects of therapy on clonal selection.[53,63,65]
The direct mutagenic effects of CLL therapy: does CLL therapy cause genomic aberrations, or alternatively, facilitate acquisition of acquired genomic copy number aberrations or gene mutations in CLL through clonal selection?
A complete characterization of acquired genomic copy number aberrations and recurrent gene mutations in relapsed CLL, including CLL with short remission durations following chemoimmunotherapy.
The molecular aberrations associated with del17p other than TP53 mutations that influence CLL prognosis. Understanding why CLL with TP53 mutations/del17p and mutated IgV_H status are clinically less aggressive.
The molecular aberrations associated with del11q subtypes that influence CLL prognosis.
The critical genes and pathways deregulated or altered as part of various 13q14 deletions and del13q14 subtypes.
The genomic aberrations associated with (i) CD5 + monoclonal B-lymphocytosis (MBL) and (ii) with progression (if any) of MBL to CLL.
The effects of second-tier acquired genomic copy number aberrations on CLL biology and clinical outcome: which second-tier acquired genomic copy number aberrations in CLL directly affect CLL prognosis?
The molecular pathways and networks that are stably perturbed by recurrent acquired genomic copy number aberrations, gene mutations or both in CLL subsets.[69,121]
The identification of therapies that are particularly effective in CLL genomic subsets or CLL subsets identified by specific recurrent gene mutations.
The landscape and importance of epigenetic aberrations in CLL.[122]
The effects of *NOTCH1* mutations on CLL biology and patient outcome.
The effects of *SF3B1* mutations on CLL biology and patient outcome.
The effects of individual balanced chromosomal translocations on CLL biology and outcome.
Understanding the clinical importance of acquired genomic copy number aberrations and recurrent gene mutations in the era of targeted therapies.[123–125] For instance, how do CLL patients with elevated genomic complexity, *TP53* mutations, del17p or del11q respond to treatment with novel small molecules (inhibitors of BTK, SYK or PI3Kdelta)?