Deciphering the molecular landscape in chronic lymphocytic leukemia: time frame of disease evolution

Lesley-Ann Sutton and Richard Rosenquist

Department of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, Sweden

ABSTRACT

Dramatic advances in next generation sequencing technologies have provided a novel opportunity to understand the molecular genetics of chronic lymphocytic leukemia through the comprehensive detection of genetic lesions. While progress is being made in elucidating the clinical significance of recurrently mutated genes, layers of complexity have been added to our understanding of chronic lymphocytic leukemia pathogenesis in the guise of the molecular evolution and (sub)clonal architecture of the disease. As we prepare for an era of tailored therapy, we need to appreciate not only the effect mutations have on drug response but also the impact subclones containing specific mutations have at initial presentation, during therapy and upon relapse. Therefore, although the wealth of emerging genetic data has great potential in helping us devise strategies to improve the therapy and prognosis of patients, focused efforts will be required to follow disease evolution, particularly in the context of novel therapies, in order to translate this knowledge into clinical settings.

Introduction

Chronic lymphocytic leukemia (CLL) is defined as a clonal expansion of small, relatively monomorphic B cells which infiltrate the bone marrow, peripheral blood and often the lymph nodes, and have surface expression of CD5, CD19, CD20 and CD23, and low levels of surface immunoglobulin (IG).[1] Despite this phenotypic homogeneity, the clinical course of CLL is highly variable and ranges from rapid disease progression requiring early and frequent treatment, to survival for decades with minimal or no treatment. Whilst treatment of CLL has advanced significantly over the last decade with the current standard of care for medically fit patients consisting of combination chemoimmunotherapy with fludarabine, cyclophosphamide and rituximab (FCR) and achieving an overall response rate of approximately 90%, the disease ultimately remains incurable, or at best is therapeutically converted into a chronic disease.[2,3] In addition, while it is known that patients harboring abnormalities within the *TP53* gene respond poorly to FCR, accounting for approximately 40% of refractory patients, the molecular basis for chemorefractoriness in the remaining cases remains unexplained.[4] In recent years, genomic approaches have been applied to interrogate the genomic landscape of CLL, and have revealed novel genetic alterations in CLL, most notably *NOTCH1, SF3B1* and *BIRC3* mutations.[5-9] Alterations to these genes occur in 2%-12% of CLL patients at diagnosis; however, their prevalence increases during advanced phases of the disease conferring a poor prognosis.[9-20] Here we aim to review the molecular events and processes that underlie the evolution of CLL, and then, drawing from more recent advances using next generation sequencing (NGS), attempt to trace the evolution of CLL from a pre-leukemic state to overt disease. Although in its infancy, we also describe current knowledge of the subclonal complexity of CLL, and the resulting implications for the progression and treatment of this disease.

Genomic landscape of CLL: how many and what types of mutations drive CLL?

At variance with other lymphoid malignancies, CLL is characterized by a relatively stable genome and is devoid of a common translocation. Nevertheless, at diagnosis more than 80% of cases harbor genomic aberrations and the most frequent chromosomal abnormalities are partial losses of one affected chromosome, such as deletions at 13q (approx. 55%), 11q (12%-18%) or 17p (5%-10%), or gain of a chromosome, e.g. trisomy 12 (approx. 15%).[21,22] The finding of these specific recurrent abnormalities against a backdrop composed of an overall low number of copy-number alterations bodes well for their role in disease pathogenesis, potentially by bestowing the CLL cells containing these lesions with a fitness advantage.[23,24] This line of thinking was subsequently substantiated by the finding that certain cytogenetic abnormalities are associated with a poor clinical outcome, and from these observations stemmed one of the earliest molecular classification schemata in cancer, the Döhner classification, which defined a hierarchy of genetic changes detected by fluorescence *in situ* hybridization (FISH) analysis that could predict disease progression and survival, del(17p) > del(11q) > trisomy 12 > normal FISH analysis > isolated del(13q), and ultimately shape therapeutic strategies.[21] While del(17p) is detrimental due to deletion of the *TP53* gene, and the poor prognosis of patients with del(11q) may relate to the impact of the *ATM* gene on the integrity of the genome, the mechanism by which trisomy 12 contributes to lymphoproliferation remains unknown.[21,23] That said, a distinct, albeit small subgroup of patients with co-existence of trisomy 12 and trisomy 19 has recently been described in CLL, and the emergence of trisomy 18 in 75% of these cases implies that cells harboring both trisomies have a clonal advantage and that the acquisition of trisomy 18 represents a clonal evolution event.[25,26]

To further investigate the dynamics of genomic aberrations and their relation to clinical features, several studies in the pre-

 doi:10.3324/haematol.2014.115923
Manuscript received on August 18, 2014. Manuscript accepted on October 14, 2014.
Correspondence: richard.rosenquist@igp.uu.se

NGS era analyzed longitudinal samples to investigate the issue of clonal evolution.[24,27-30] Although differing in the methodologies utilized (i.e. FISH, single nucleotide polymorphism arrays or array-based comparative genomic hybridization) and overall study design, the overarching consensus was that: i) new aberrations could be acquired over time; ii) the acquisition of new genomic abnormalities coincided with a shorter time-to-first-treatment (TTFT) or overall survival (OS); iii) the nature of the new lesion, according to the Döhner classification, was important, with acquisition of del(17p) and/or del(11q) associated with clinical aggressiveness and a poor response to therapy; and iv) the appearance of new abnormalities was not restricted to cases that had undergone therapy. Owing to these observations, newer guidelines recommend FISH analysis prior to treatment initiation and also following relapse or lack of response to therapy.[31,32]

Despite these findings, the relative paucity of genomic instability in CLL implies that chromosomal aberrations may not be solely responsible for the observed clinical heterogeneity and that additional mechanisms must play a role. Mutations within key tumor suppressor genes such as *TP53* (95% of mutations are localized within the central DNA-binding domain and lead to impaired DNA binding and target gene transactivation) and *ATM* (a mutational hotspot has yet to be defined) have previously been identified in CLL and can affect the response to therapy, and consequently survival.[13,19,20,33-39] While most patients with del(17p) carry mutations within the remaining *TP53* allele (Figure 1), sole *TP53* mutations occur in approximately 3%-6% of patients.[40-42] Such *TP53* mutations have an equally profound impact on outcome and are enriched in patients with both poor prognosis and higher genetic complexity. More specifically, at diagnosis the incidence of *TP53* mutations has been reported to be approximately 4%. However, as the disease progresses, this incidence rises to 10%-12% (first-line treatment) and approximately 40% (refractory CLL).[40,42,43] Due to these observations, the European Research Initiative on CLL (ERIC) recommends *TP53* mutational screening (exons 4-9) for all patients before the administration of any therapy in order to select *TP53*-independent therapy where appropriate.[42] The p53 pathway can also be inhibited independent of TP53 by inactivation of *ATM*, thus it is worthy of note that a proportion of CLL patients (20%-30%) harboring del(11q) carry mutations within *ATM*.[37,44-46] Such patients exhibiting biallelic inactivation of *ATM* were reported to have a poorer prognosis than cases carrying only the deletion.[37,44-46] Due to the size of the *ATM* gene (62 coding exons) and the absence of any well-defined mutational hotspots, screening of *ATM* within a clinical setting has not been feasible. However, this situation is likely to change due to advances in NGS and the availability of custom-designed gene panels that enable sequencing of a large number of genes and patients simultaneously.[47]

Next generation sequencing has proven revolutionary and shifted the paradigm of CLL genomics

The most recent developments in CLL genomics revolve around NGS studies which have effectively clarified the level of genomic complexity in CLL, and revealed that the average number of non-synonymous mutations at diagnosis lies between 10-20, thus rendering CLL with one of the lowest numbers of mutations per case of any adult cancer studied to date.[5-8,48] However, this range does vary widely among individual cases. Another interesting observation arising from these initial screening studies is that CLL does not appear to be defined by a unifying mutation, in the sense that in some hematologic malignancies a common mutation occurs, and is hence thought to be the primary driver mutation, e.g. in hairy cell leukemia (HCL) a mutation (V600E) within the *BRAF* gene is found in all cases, while in Waldenström macroglobulinemia the *MYD88* L265P mutation is found in more than 90% of cases.[49,50] Though CLL may not be associated with a large number of alterations, in addition to mutations within *TP53* and *ATM*, it is characterized by a relatively well-defined set of recurrent mutations which appear to be clinically significant.[5-20]

Among the genes found mutated in CLL, *NOTCH1*, which plays an essential role in hematopoiesis, has emerged as a recurrent target of genetic lesions.[5-8] A 2bp deletion accounts for approximately 90% of all *NOTCH1* mutations and generates a premature stop codon within the PEST domain, resulting in the constitutive activation of NOTCH1 signaling.[5,6,11,14-20,51-53] Mutations within the *NOTCH1* PEST domain have been found in 4%-12% of patients at diagnosis, rising to 21% in patients with chemorefractoriness and to 30% in cases transformed to Richter syndrome (RS).[5,6,11,14-20,51-54] *NOTCH1* mutations are significantly more frequent in advanced *versus* early stage patients and treated *versus* untreated patients, and exhibit a strong positive association with CLL cases expressing unmutated immunoglobulin (IG) genes (U-CLL), stereotyped B cell receptors typical of subset #8, trisomy 12 and an increased risk of RS (Figure 1).[5,11-15,17-20,51-54] Patients with *NOTCH1* mutations have a shorter TTFT and shorter OS independent of other prognostic factors, such as *TP53* abnormalities and IGHV gene mutation status.[11-21,25,26,52-55]

Perhaps one of the most unexpected and intriguing observations arising from initial NGS studies was the finding that *SF3B1*, a ubiquitously expressed component of the spliceosome machinery, was recurrently mutated in CLL (5%-17%), the varying frequencies presumably reflecting the heterogeneous nature of the cohorts studied.[5-8,10,15,19,20] Although the precise functional relevance of *SF3B1* mutations remains unknown, the fact that mutations cluster within evolutionarily conserved hotspots speaks highly for their role in CLL pathogenesis, with *SF3B1* mutations potentially leading to aberrant splicing as a consequence of defective spliceosome complex formation. Investigations into the clinical relevance of *SF3B1* mutations indicate that they have a lower incidence in early stage CLL, are more common in advanced disease, have strong positive associations with del(11q), and are extremely frequent in stereotyped CLL subset #2 (IGHV3-21, variable IG mutational status and very poor clinical outcome) (Figure 1).[7,10,12,13,15,18-20,54,56-61] Taken collectively, these findings strongly suggest the acquisition of *SF3B1* mutations during clonal evolution of the disease.

While mutational analyses were initially performed in heterogeneous retrospective CLL cohorts, the clinical significance of *NOTCH1* and *SF3B1* have since been validated in the context of randomized, prospective clinical trials.[12,19] More specifically, in the UK LRF CLL4 trial (comparison of chlorambucil and fludarabine with and without cyclophosphamide in previously untreated patients), mutations within *NOTCH1* or *SF3B1* were found to identify patients identify patients with an intermediate survival comparable to patients with del(11q).[12] Therefore, although *TP53* aberrations remained the most informative predictor of poor out-

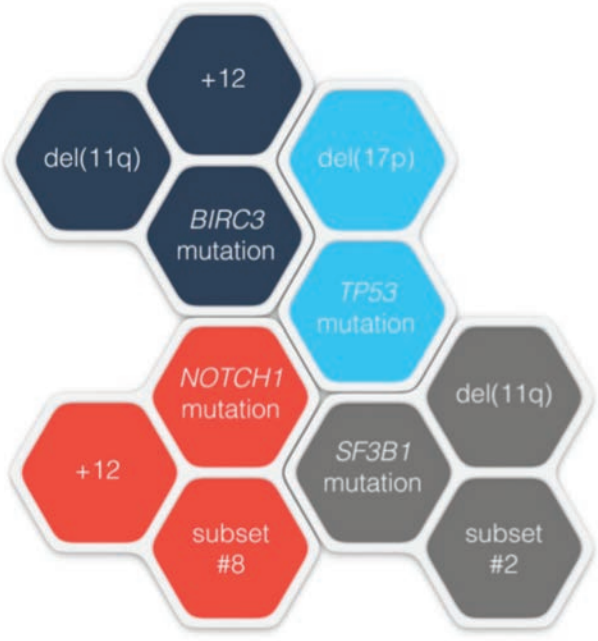

Figure 1. Association between recurrent gene mutations and other genetic/immunogenetic features in CLL. Distinct patterns of associations between recurrent mutations and other molecular features have been evidenced in CLL, suggesting different pathways for clonal evolution. For example, *NOTCH1* mutations exhibit a strong positive association to trisomy 12 while mutations within *SF3B1* often co-occur with del(11q). Although specific patterns of co-occurrence and mutual exclusivity between novel recurrent mutations have been reported, new associations may emerge as larger cohorts are studied.

come within this study, mutations within *NOTCH1* and *SF3B1* were found to be independent prognostic biomarkers and hence deemed to be potentially useful in the risk-adapted stratification of CLL patients. Results from the mutational analysis of patients enrolled in the CLL8 trial (evaluation of first-line therapy with FC or FCR among untreated CLL patients) confirmed *SF3B1* mutations as an independent factor for a more rapid disease progression while *NOTCH1* mutations were found to have a reduced prognostic role.[19] The varying prognostic capacity of *NOTCH1* mutations implies that the influence of gene mutations may differ depending on the type and intensity of the treatment regimen, thus indicating that prognostic markers will need to be reassessed for every novel therapy. That said, although the mutational status of *NOTCH1* was not found to be prognostically informative within this trial, a relationship was identified between *NOTCH1* mutations and rituximab whereby patients with *NOTCH1* mutations did not appear to benefit from its addition. Consequently, *NOTCH1* mutations may prove to be a predictive factor for the reduced benefit from rituximab; however, this finding needs to be confirmed in independent trials before being applied in clinical practice.

Another gene fitting the category of those recurrently mutated in CLL and joining *SF3B1*, *NOTCH1* and *TP53* as mutations that may contribute to disease progression/chemorefractoriness is *BIRC3*.[5-9] Although mutations within *BIRC3* are frequent in chemorefractory patients (24%), they are rare at diagnosis (2%-4%) and have been found to occur mutually exclusive to del(17p). In addition, they are highly enriched in cases carrying del(11q) or trisomy 12 (Figure 1); these latter aberrations were found to be distributed among *BIRC3* mutated cases in a mutually exclusive manner.[9,15,20,39] Such patterns of co-occurrence and mutual exclusivity between specific mutations hint at synergy and redundancy.

Although great attention has been given to identifying individual genes that are recurrently mutated, this gene-centric approach may overlook signaling pathways that are disrupted due to the presence of mutations within several genes, as opposed to harboring recurrent mutations within a single gene. However, this should not automatically render these mutations less important without further investigation. Disruption of such pathways may be critical in a proportion of CLL patients, perhaps providing treatment strategies that have not yet been harnessed. Along these lines, genomic analyses using NGS have identified multiple mutations within the NF-κB signaling pathway. One such example involves the *NFKBIE* gene that is up-regulated following NF-κB activation and whose role involves inhibition of NF-κB-directed transactivation via cytoplasmic retention of REL proteins. In CLL, mutations within the *NFKBIE* gene (nonsense and small deletion), reported to occur in almost 11% of advanced stage patients, result in a truncated protein which fundamentally leads to sustained activation of the NF-κB pathway.[8,62,63]

Next generation sequencing reveals a surprising level of clonal complexity in CLL: the rise and fall of the minority

As detailed above, NGS has greatly increased our ability to characterize the genetic landscape of cancers. This deciphering of the cancer genome has not only identified clonally dominant mutations (i.e. mutations within the majority of tumor cells and potentially representing initiating events), but has also revealed that most cancers, both hematologic and solid, evolve through a complex branching architecture owing to the presence of subclonal mutations, i.e. mutations that only exist in a fraction of tumor cells.[64] While several studies have attempted to unravel the subclonal population structure within hematologic malignancies such as acute myeloid leukemia (AML) or follicular lymphoma (FL), research into intratumoral heterogeneity within CLL remains in its infancy.[65-69] That said, a few key studies utilizing deep sequencing methodologies and analyzing consecutively obtained samples, thus enabling mutation evolutionary hierarchy to be inferred based on temporal changes, have given an insight into the intratumoral structure of mutations and the (sub)clonal behavior that may shape CLL progression in terms of clonal evolution.[70-72] Such studies have revealed significant mutational complexity, indicating that the diversity and relative dominance of subclones varies throughout the disease course, and that the number of genetic abnormalities present within a subclone does not automatically render that subclone numerically dominant, i.e. the major clone. An illustrative example of this phenomenon emanates from a study in which whole genome sequencing was used to track clonal heterogeneity in 3 CLL patients subjected to repeated cycles of therapy.[70] The remarkably different temporal patterns of sub(clonal) repopulation that emerged following treatment varied from a stable equilibrium to dramatic shifts in the clonal dynamics, whereby a minor subclone eventually replaced the dominant clone (Figure 2). Another study, also utilizing ultra-deep sequencing, took a slightly different experimental approach, and by focusing solely on the *TP53* gene in a large cohort of newly diagnosed CLL patients (n=309), they

were able to detect mutations in small CLL subclones (down to 0.3% allele frequency) that went undetected by Sanger sequencing (limit of detection, 15%-20% allele frequency).[72] Until now, the prevailing assumption had been that minor subclones would have limited, if any, clinical impact. However, Rossi *et al.* convincingly demonstrated that, when it comes to *TP53*, response to treatment, and hence survival, will be affected irrespective of the size of the clone.[72] More specifically, they showed that the 6% of patients with subclonal *TP53* mutations exhibited an equally adverse survival as the 9% in whom *TP53* mutations were detected by conventional methods; put simply, size doesn't matter. These minor subclones, or perhaps more aptly microclones, became the predominant population over time and prognosticated chemorefractoriness, underscoring the importance of *TP53* as a driver mutation. Very recently, Malcikova *et al.* also performed a deep sequencing study investigating therapy-driven clonal evolution of *TP53* mutations in CLL.[73] They found that *TP53* mutations within a minor clone had a high risk of being positively selected by therapy, thus corroborating the results by Rossi *et al.*[72] In particular, they not only demonstrated that the majority of minor clones bearing *TP53* mutations expanded to dominant clones under the selective pressure of chemotherapy, but that multiple minor clones with *TP53* mutations are common and may expand simultaneously. A final point worthy of note that stems from such studies relates to the observation that, in some patients, mutations within known driver genes were clonal whereas in other patients such mutations were subclonal.[71] Therefore, even variants with known driver potential can be acquired late in CLL evolution. This implies a striking variability across patients as to which are early molecular events and which arise later during tumor progression.

A recent large-scale study attempted to demystify clonal evolution within CLL by combining exome sequencing and copy number data.[71] By determining what proportion of tumor cells carried a specific mutation they could classify mutations or genetic lesions as either clonal, i.e. occurring in at least 95% of the sample, or subclonal; *MYD88*, del(13q) and trisomy 12 were deemed to be clonal while *SF3B1* and *TP53* were reported as subclonal. Their strategy also enabled them to distinguish earlier events from later events. Findings from the aforementioned studies highlight the intricate 'ecosystem' at play within CLL, where the balance between evolving clones results from the complex interplay between intrinsic and extrinsic/environmental factors.

Clinical implications of clonal evolution and its role in resistance to therapy

The ascent of cancer genomics has not been without formidable challenge and questions, many of which relate to therapy. Can we accurately predict the course of the disease? Can we predict which patients will respond to various therapies? Can we use genomic information to target therapy to the underlying genetic alterations? The list continues, and in order to address such questions and fully integrate patient specific mutations and targeted therapeutic strategies into clinical care, it will be imperative to understand the complex clonal architecture evidenced in CLL cells and distinguish between issues such as driver mutations *versus* passenger mutations, initiation *versus* progression, and dominant clones *versus* subclones.

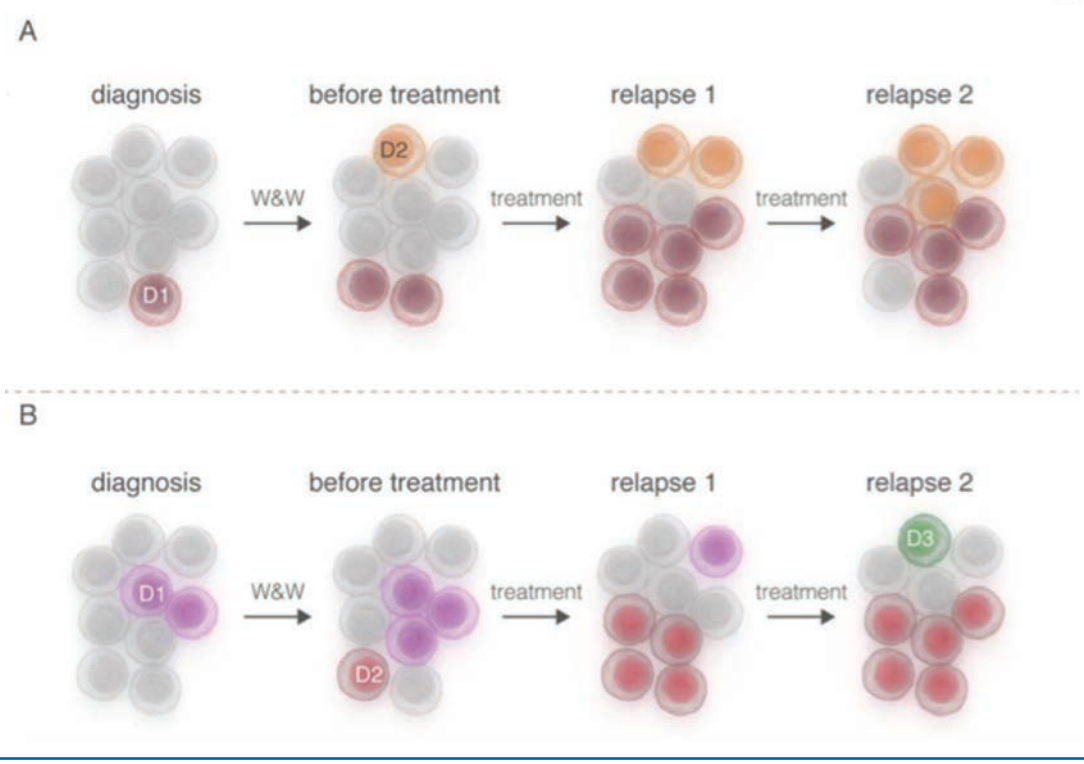

Figure 2. Clonal evolution in CLL. Potential routes by which clonal evolution may proceed following therapy. (A) Therapy has little effect on the major clone while minor subclones begin to expand possibly through the acquisition of new genomic lesions. (B) Therapy is effective in eradicating the major clone. However, minor subclones continue to proliferate with the potential to eventually become the dominant clone. w&w; watch and wait.

Changes imposed on the aforementioned tumor 'ecosystem' after therapy may alter the relative competitiveness of individual subclones. This is exemplified by Schuh *et al.*, whereby a CLL clone containing biallelic *ATM* inactivation due to del(11q) and a nonsense *ATM* mutation was tracked throughout various treatment protocols.[70] Monitoring revealed that following standard FC treatment, this subclone initially gained a proliferative advantage, but was completely abolished after treatment with FCR. An independent subclone was responsible for a subsequent relapse, thereby providing evidence that minor cancer cell populations that survive treatment are capable of regenerating the malignancy and ultimately gaining pre-eminence (Figure 2). Such minor clones may persist due to a differential response among subclones to the therapy administered, i.e. by harboring genetic lesions that render them resistant to a particular therapy, certain subclones may survive and expand uninhibited, since the treatment may have effectively eradicated any susceptible competitor (sub)clones. Thus, if we return to the studies detailing the evolution of clones harboring *TP53* mutations, following the reasoning outlined above, it is not surprising that failure of chemotherapy to completely eradicate CLL cells resulted in subclonal expansion which gave rise to a more resistant, and hence a more 'dangerous' subclone.[72,73]

A similar scenario was observed by Landau *et al.* who reported that patients who received therapy exhibited a greater degree of clonal evolution, and that this increased heterogeneity was linked to a poor clinical outcome.[71] Since selection can stem from the use of chemotherapeutic agents, it is now apparent that it is vitally important to identify low-level molecular lesions that may predict for chemoresistance so that treatment can be tailored appropriately. Although it is difficult to discern whether such minor subclones could have: i) pre-existed at diagnosis but were below the limit of detection; or alternatively ii) whether a cell from the dominant clone randomly survived treatment and subsequently gained a mutation that now conveyed resistance, if one considers that patients harboring new genetic lesions at relapse tend to require further treatment sooner than patients without new mutations, this time frame speaks in favor of the former (Figure 2).

In line with this reasoning, it is worth mentioning that it remains computationally challenging to accurately characterize and resolve genetically distinct microclones within mixed-cell populations, and high resolution or sequencing depth, i.e. the number of times that an individual base or genomic position is sequenced, together with a highly sensitive and specific variant calling method are of paramount importance. Regardless of this, since CLL (or at least some CLL) harbor numerous subclones with a seemingly complex and dynamic nature throughout the course of the disease, it will be necessary to monitor disease progression by re-examining the mutational signature not only prior to initiating any line of treatment, but also upon relapse(s), akin to the monitoring and testing that is currently undertaken for *TP53*. It will also be critical to determine a means of identifying which clones are most biologically relevant to the disease, such as those with genotypes that confer risk of progression or drug resistance.

The time frame of CLL evolution: from a pre-malignant state to overt disease to relapse

i. Monoclonal B-cell lymphocytosis

Based on the information above, it is undeniable that our understanding of the genetic basis, clonal architecture and evolution in CLL pathogenesis has significantly improved (Figure 3). However, a more obscure area of knowledge concerns both the time line and pathway that a normal B lymphocyte follows before transforming to a fully-fledged CLL cell. It has recently been proposed that in CLL, hematopoietic stem cells (HSC) may aberrantly generate monoclonal B cells with CLL-like phenotypes.[74] This scenario reflects the finding of pre-leukemic HSCs in other disease subtypes such as HCL and AML.[75,76] Indeed, Damm *et al.* have recently identified mutations in several well-known CLL oncogenes e.g. *NOTCH1*, *SF3B1* and *TP53*, in the progenitor cells of CLL patients and hypothesized that this may lead to the subsequent transformation of CLL.[63] Although these findings suggest that primary genetic alterations leading to CLL may be acquired at the self-renewing CLL-HSC level, none of the recurrent CLL chromosomal abnormalities were found in the HSC compartment, thus raising the question of how such clones could be selected and subsequently expand into CLL.

That said, the situation is far from bleak, and we now know that CLL is invariantly preceded by a clonal B-cell expansion whose immunophenotype is consistent with that of CLL.[77] This condition, termed monoclonal B-cell lymphocytosis (MBL), is observed in 3.5%-12% of older adults in apparent good health and can be divided into low-count (LC) MBL ($<0.5x10^9$ cells/L) and clinical/high-count (HC) MBL ($>0.5x10^9$ cells/L), with the clinical relevance differing between the two subgroups.[78-86] In the former, the risk of progressing to CLL is negligible and could merely reflect immunosenescence due to aging, and thus be the ultimate biological fate for all humans should they live long enough.[83] A somewhat different scenario is evidenced in HC-MBL with 1%-2% of individuals within this subgroup progressing to CLL requiring therapy per year.[87-92] However, since only a very small proportion of cases evolve into CLL, significant somatic changes must occur within HC-MBL before it emerges as a clinical entity. Therefore, MBL *per se* cannot be considered a surrogate marker for CLL and additional factors must fuel this transition.

What triggers this progression from a 'pre-malignant condition' to full-blown CLL remains unknown; however, thinking logically, if HC-MBL is considered a pre-cursor stage to CLL, then one could assume that they share a genetic profile with CLL. Focusing first on genetic aberrations, perhaps surprisingly, both MBL subgroups were found to harbor the hallmark cytogenetic abnormalities associated with CLL.[81,83,89,93-96] However, this observation may be reconciled with the finding of cells carrying the t(14;18) translocation, the cytogenetic hallmark of FL, in the peripheral blood of more than 50% of healthy individuals; thus underscoring the idea that the presence of these genetic lesions does not inevitably equate with a lymphoma diagnosis, rather it is the frequency of the alteration that may act as a predictive biomarker.[97,98] In addition, since CLL-associated genomic aberrations are also found in LC-MBL, the actual pathogenetic role of these abnormalities should perhaps only be considered in the context of clinical progression. Following this line of thought, the observation that unfavorable cytogenetic lesions, such as del(17p) and del(11q), were rare in Rai 0-CLL (early stage), and even less frequent in HC-MBL, implies that these aberrations are acquired over time and perhaps signify clonal evolution.[91,94] Nevertheless, the finding of del(13q) in approximately 50% of MBL (reflecting the frequency in CLL), infers that this

alteration may represent an early event in CLL pathogenesis.[21,22,95,96]

Moving to gene mutations and recalling that the proportion of CLL cases possessing these alterations is quite low at diagnosis, such genetic lesions are rarely seen in MBL (based on Sanger sequencing data).[94,99-101] In fact, when HC-MBL was compared with Rai 0-CLL, no significant differences regarding the frequency of recurrent aberrations/mutations (*NOTCH1* and *SF3B1*) were observed; however, the proportion of early stage CLL cases exhibiting 3 or more unfavorable markers was larger than that of HC-MBL.[94]

More recently, NGS has been applied to MBL and appears also to be characterized by mutations in putative CLL driver genes e.g. *ATM, SF3B1, BIRC3* and *NOTCH1*. Ojha *et al.* provided insights into the temporal succession of genetic events in MBL and demonstrated the existence of recurrent mutations in small subclones.[102] Mutations remained stable or became more prevalent over time and although the number of cases analyzed was low, several other aspects of clonal evolution were observed, such as alternating clonal dominance and the emergence of novel mutations within genes such as *SF3B1* at subsequent time points. All 4 cases with putative CLL mutations eventually transitioned to overt disease. In a separate study, the *NOTCH1* 2bp deletion was detected in 11% of MBL and 13.4% of CLL Binet stage A patients following deep sequencing.[101] This mutation was frequently observed at a low clonal level, particularly in MBL patients, and sequential analyses demonstrated that the *NOTCH1* mutation generally did not appear during the disease course, and that the mutational load in positive cases remained stable over time. These findings corroborate the recent work by Baliakas *et al.* who reported that among untreated early stage CLL patients, while the incidence of *SF3B1* and *TP53* mutations appears to increase over time, no such increase was observed for *NOTCH1* mutations.[20] Since responsibility for the appearance of MBL and the transformation to CLL does not appear to lie with a single genetic alteration, microenvironmental factors are also considered to play a pivotal role in the evolution of CLL clones. The existence of stereotyped BcRs in approximately 30% of CLL cases, i.e. expression of highly homologous IG heavy and light chain complementarity determining region 3 (CDR3) amino acid sequences, together with the tendency of CLL cells to express poly- and auto-reactive BcRs are indicative of selective pressures, such as autoantigens or pathogens, that favor specific IG gene rearrangements.[58,59,103-105] Such persistent stimulation through the surface IG may

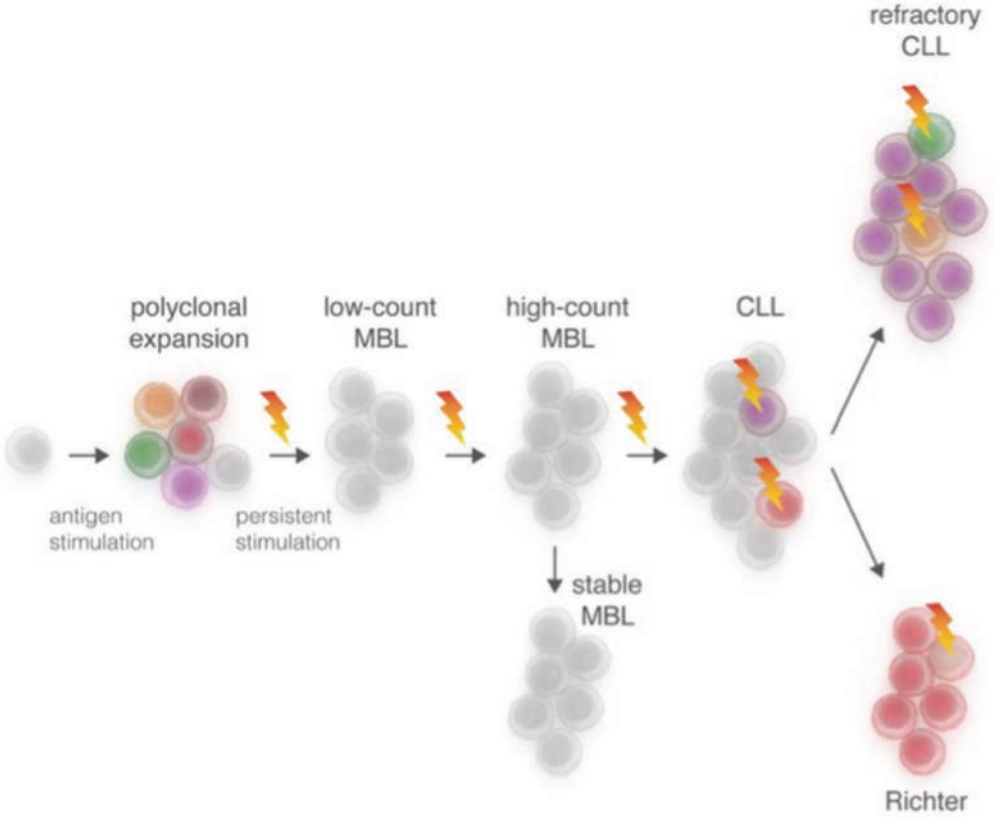

Figure 3. CLL pathobiology: from MBL to Richter syndrome. Although the MBL to CLL conundrum is far from solved, a plausible (although speculative) explanation could be that mechanisms that induce clonal expansion, such as ongoing antigenic stimulation through the B-cell receptor together with accessory cells operating within specific micro-environmental niches, trigger the clonal development of MBLs. Over time, the pressure of stimulation may give rise to enhanced proliferation leading to the acquisition of genetic abnormalities, e.g. del(13q), and gene mutations that appear late in the expanding clone, and eventually some MBLs may progress into overt CLL. Since not all cases of MBL have the potential to progress into overt full-blown CLL, the type of micro-environmental stimulation occurring throughout time as well as the occurrence of distinct genomic aberrations may account for such transformation. Progression toward a more malignant disease may be fueled by the presence of specific genetic lesions, e.g. ***TP53*** abnormalities, ***NOTCH1***, which in turn may lead to resistance to therapy and in rare cases, transformation to Richter syndrome.

be the central event that drives the evolution from a pre-leukemic state to overt leukemia, thereafter chronic BcR engagement may favor the selection of a monoclonal population which subsequently acquires genetic alterations which in some instances may provide a survival and growth advantage.

ii. Refractory CLL

The natural history of many CLLs involves progression toward a more malignant disease and, having transitioned from MBL and now deemed overtly leukemic, the predisposition to clinical progression of CLL appears to be influenced by the acquisition of genetic lesions. *TP53* abnormalities are the best documented predictors of poor survival and refractoriness in CLL and consequently have a well-established clinical relevance.[4,13,15,16,18,20,33-35,40-43] Although the molecular basis by which each of the newly identified genetic lesions may bring about an aggressive phenotype is not fully understood, the observation that these alterations are enriched in patients requiring therapy and refractory to fludarabine (*NOTCH1* mutations: 20%; *SF3B1* mutations: 17%; *BIRC3* disruption: 24%) compared with CLL at diagnosis (*NOTCH1* mutations: 4%-12%; *SF3B1* mutations: 5%-14%; *BIRC3* disruption: 2%-4%) or compared with cases requiring therapy but sensitive to the drug is highly suggestive of a contributing role;[9-20] thus a gradual accrual of genetic lesions may occur as the tumor cells transit through the various disease stages, i.e. pre-malignancy, overt disease and relapse. Nevertheless, much of this reasoning stems from a limited number of studies and the role of novel recurrent mutations in refractory CLL needs to be confirmed by additional studies.

iii. Richter Syndrome (RS)

Whereas MBL mimics what is observed in low-risk early stage CLL, at the other end of the scale we have RS, a highly aggressive form of CLL, which morphologically mimics diffuse large B-cell lymphoma (DLBCL) and typically has a dismal outcome, especially when clonally related to CLL.[1,106-108] Over time, a small fraction of CLL cases (approx. 15%) transform into RS which is characterized by *TP53* disruption (50%-60%), *NOTCH1* activation (30%), and *MYC* abnormalities (30%).[107,108] These lesions are frequently acquired at transformation, underscoring the relevance of these genetic lesions in the emergence and maintenance of the DLBCL phenotype.[5,108] In fact, mutations in *NOTCH1* tend to be mutually exclusive of *TP53* disruption at CLL diagnosis thus differing from what is observed in RS in which mutations of *NOTCH1* associate with *TP53* disruption in 50% of patients, implying that the concomitant occurrence of *NOTCH1* mutations and *TP53* disruption leads to clinical aggressiveness and, potentially, histological transformation to aggressive lymphoma.[5,53] *TP53* disruption and MYC-activating events often co-operate as dual hits in RS reminiscent of what is observed during the evolution of other mature B-cell-derived tumors as they transit from an indolent to an aggressive state.[109,110] A plausible explanation for the exceptionally high frequency of *TP53* disruption observed in RS is that pressure from multiple lines of treatment (at the CLL stage) drove the selection of a clone resistant to therapy. Although the overall genomic complexity appears to differ among transformed cases and no single genetic alteration is common to all RS cases (a third of cases harbor trisomy 12), the fact that the vast majority of cases display alterations to *TP53*, *NOTCH1*, *MYC*, and *CDKN2A/B* (in varying combinations) implies that genes partaking in core cellular functions such as tumor suppression, cell cycle control and proliferation may play a role in transformation from CLL to RS, while B-cell signaling pathways may play a lesser role.[111,112]

Conclusions

Next generation sequencing has been a major success story in CLL genomics and through its application the mechanisms that drive CLL pathogenesis are gradually unfolding. However, while we are certainly moving in a promising direction, the rise of CLL genomics has not been without difficulties and we now need to look not only at the opportunities they present but also at the confounding issues.

We know that CLL is characterized by a relatively well-defined set of recurrent mutations and genetic lesions that appear to be clinically relevant. The clinical implications of these molecular lesions are in some instances well established, as with *TP53* disruption, which is at present the only molecular marker that changes the therapeutic approach, since cases with aberrant *TP53* should be considered for alternative therapies upfront. That said, assessment of novel lesions (e.g. *SF3B1* and *NOTCH1*) may also be prognostically informative and may help to guide treatment decisions in the near future.

From a clinical perspective, attempts to devise a comprehensive prognostic model which would include both chromosomal abnormalities and gene mutations have already begun; however, discrepancies between studies have emerged.[13,18,20] Differences potentially relate to the composition and size of the evaluated cohorts. Nevertheless, regardless of the reason, additional studies are strongly warranted before such schemes can be implemented, ideally within a prospective setting.

In addition, several of the newly identified recurrently mutated genes in CLL, e.g. *BIRC3* and *SF3B1*, represent subclonal driver events that may expand during the disease course. Similar to the study by Rossi *et al.*[72] which assessed the clinical impact of subclonal mutations within *TP53*, deep-sequencing of these lesions at multiple time points, at least before treatment and at progression/relapse, may represent a means to follow disease evolution, in particular in the context of therapy decisions or upon relapse.

Thus, further work is clearly needed to establish: i) whether the extent of clonal diversity could predict for an increased risk of progression, and therefore whether the degree of heterogeneity could serve as a predictive biomarker for clinical progression; ii) whether clonal heterogeneity may be relevant to the response to therapy or indeed whether cancer therapeutics may augment or exacerbate genomic complexity, and thus, there is a need to track the mutational signature of subclones throughout therapy; iii) whether mutations within minor subclones can have an impact on clinical outcome, i.e. whether low-frequency somatic events may drive tumor growth, and whether mutations in subclones may have the same therapeutic relevance as if they were in the dominant clone; and iv) whether different clones can interact and if so does this enhance disease progression or promote resistance to therapy. Each of these aspects of clonal heterogeneity will have consequences for drug discovery and biomarker validation approaches, especially if one bears in mind that the tumor subclone that may ultimately influence therapeutic outcome may evade detection because of its absence or pres-

ence at low frequencies at diagnosis.

The presence of such high variability in the genomic architecture across CLL patients highlights the need for therapeutic interventions directed at multiple targets rather than a single genomic anomaly. Understanding the subclonal architecture of individual patients will be critical to not only interpret but also hopefully predict individual responses to therapy. Through the identification of events that may predispose to therapeutic failure, novel combinatorial therapeutic strategies may be capable of impeding future tumor evolutionary networks and drug resistance mechanisms. Thus although much has to be learned, the likely scenario for a successful targeted therapy will be based on a combination of drugs targeting actionable mutated genes within a heterogeneous setting or dysregulated pathways detected in major or minor clones within the tumor.

Funding

This research was supported by the Swedish Cancer Society, the Swedish Research Council, and the Lion's Cancer Research Foundation, Uppsala.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Dohner H, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood. 2008;111(12): 5446-56.
2. Tam CS, O'Brien S, Wierda W, Kantarjian H, Wen S, Do KA, et al. Long-term results of the fludarabine, cyclophosphamide, and rituximab regimen as initial therapy of chronic lymphocytic leukemia. Blood. 2008;112(4): 975-80.
3. Hallek M, Fischer K, Fingerle-Rowson G, Fink AM, Busch R, Mayer J, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. Lancet. 2010;376(9747):1164-74.
4. Zenz T, Habe S, Denzel T, Mohr J, Winkler D, Buhler A, et al. Detailed analysis of p53 pathway defects in fludarabine-refractory chronic lymphocytic leukemia (CLL): dissecting the contribution of 17p deletion, TP53 mutation, p53-p21 dysfunction, and miR34a in a prospective clinical trial. Blood. 2009;114(13):2589-97.
5. Fabbri G, Rasi S, Rossi D, Trifonov V, Khiabanian H, Ma J, et al. Analysis of the chronic lymphocytic leukemia coding genome: role of NOTCH1 mutational activation. J Exp Med. 2011;208(7):1389-401.
6. Puente XS, Pinyol M, Quesada V, Conde L, Ordonez GR, Villamor N, et al. Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. Nature. 2011;475(7354):101-5.
7. Wang L, Lawrence MS, Wan Y, Stojanov P, Sougnez C, Stevenson K, et al. SF3B1 and other novel cancer genes in chronic lymphocytic leukemia. N Engl J Med. 2011; 365(26):2497-506.
8. Quesada V, Conde L, Villamor N, Ordonez GR, Jares P, Bassaganyas L, et al. Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia. Nat Genet. 2012; 44(1):47-52.
9. Rossi D, Fangazio M, Rasi S, Vaisitti T, Monti S, Cresta S, et al. Disruption of BIRC3 associates with fludarabine chemorefractoriness in TP53 wild-type chronic lymphocytic leukemia. Blood. 2012;119(12):2854-62.
10. Rossi D, Bruscaggin A, Spina V, Rasi S, Khiabanian H, Messina M, et al. Mutations of the SF3B1 splicing factor in chronic lymphocytic leukemia: association with progression and fludarabine-refractoriness. Blood. 2011;118(26):6904-8.
11. Del Giudice I, Rossi D, Chiaretti S, Marinelli M, Tavolaro S, Gabrielli S, et al. NOTCH1 mutations in +12 chronic lymphocytic leukemia (CLL) confer an unfavorable prognosis, induce a distinctive transcriptional profiling and refine the intermediate prognosis of +12 CLL. Haematologica. 2012;97(3):437-41.
12. Oscier DG, Rose-Zerilli MJ, Winkelmann N, Gonzalez de Castro D, Gomez B, Forster J, et al. The clinical significance of NOTCH1 and SF3B1 mutations in the UK LRF CLL4 trial. Blood. 2013;121(3):468-75.
13. Rossi D, Rasi S, Spina V, Bruscaggin A, Monti S, Ciardullo C, et al. Integrated mutational and cytogenetic analysis identifies new prognostic subgroups in chronic lymphocytic leukemia. Blood. 2013;121(8):1403-12.
14. Villamor N, Conde L, Martinez-Trillos A, Cazorla M, Navarro A, Bea S, et al. NOTCH1 mutations identify a genetic subgroup of chronic lymphocytic leukemia patients with high risk of transformation and poor outcome. Leukemia. 2013;27(5):1100-6.
15. Cortese D, Sutton LA, Cahill N, Smedby KE, Geisler C, Gunnarsson R, et al. On the way towards a 'CLL prognostic index': focus on TP53, BIRC3, SF3B1, NOTCH1 and MYD88 in a population-based cohort. Leukemia. 2014;28(3):710-3.
16. Schnaiter A, Paschka P, Rossi M, Zenz T, Buhler A, Winkler D, et al. NOTCH1, SF3B1, and TP53 mutations in fludarabine-refractory CLL patients treated with alemtuzumab: results from the CLL2H trial of the GCLLSG. Blood. 2013;122(7):1266-70.
17. Weissmann S, Roller A, Jeromin S, Hernandez M, Abaigar M, Hernandez-Rivas JM, et al. Prognostic impact and landscape of NOTCH1 mutations in chronic lymphocytic leukemia (CLL): a study on 852 patients. Leukemia. 2013;27(12):2393-6.
18. Jeromin S, Weissmann S, Haferlach C, Dicker F, Bayer K, Grossmann V, et al. SF3B1 mutations correlated to cytogenetics and mutations in NOTCH1, FBXW7, MYD88, XPO1 and TP53 in 1160 untreated CLL patients. Leukemia. 2014;28(1):108-17.
19. Stilgenbauer S, Schnaiter A, Paschka P, Zenz T, Rossi M, Dohner K, et al. Gene mutations and treatment outcome in chronic lymphocytic leukemia: results from the CLL8 trial. Blood. 2014;123(21):3247-54.
20. Baliakas P, Hadzidimitriou A, Sutton LA, Rossi D, Minga E, Villamor N, et al. Recurrent mutations refine prognosis in chronic lymphocytic leukemia. Leukemia. 2014 June 19 [Epub ahead of print].
21. Dohner H, Stilgenbauer S, Benner A, Leupolt E, Krober A, Bullinger L, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. N Engl J Med. 2000;343(26):1910-6.
22. Haferlach C, Dicker F, Schnittger S, Kern W, Haferlach T. Comprehensive genetic characterization of CLL: a study on 506 cases analysed with chromosome banding analysis, interphase FISH, IgV(H) status and immunophenotyping. Leukemia. 2007; 21(12):2442-51.
23. Gunnarsson R, Isaksson A, Mansouri M, Goransson H, Jansson M, Cahill N, et al. Large but not small copy-number alterations correlate to high-risk genomic aberrations and survival in chronic lymphocytic leukemia: a high-resolution genomic screening of newly diagnosed patients. Leukemia. 2010;24(1):211-5.
24. Gunnarsson R, Mansouri L, Isaksson A, Goransson H, Cahill N, Jansson M, et al. Array-based genomic screening at diagnosis and during follow-up in chronic lymphocytic leukemia. Haematologica. 2011;96(8):1161-9.
25. Sellmann L, Gesk S, Walter C, Ritgen M, Harder L, Martin-Subero JI, et al. Trisomy 19 is associated with trisomy 12 and mutated IGHV genes in B-chronic lymphocytic leukaemia. Br J Haematol. 2007;138 (2):217-20.
26. Ibbotson R, Athanasiadou A, Sutton LA, Davis Z, Gardiner A, Baliakas P, et al. Coexistence of trisomies of chromosomes 12 and 19 in chronic lymphocytic leukemia occurs exclusively in the rare IgG-positive variant. Leukemia. 2012;26(1):170-2.
27. Shanafelt TD, Witzig TE, Fink SR, Jenkins RB, Paternoster SF, Smoley SA, et al. Prospective evaluation of clonal evolution during long-term follow-up of patients with untreated early-stage chronic lymphocytic leukemia. J Clin Oncol. 2006; 24(28):4634-41.
28. Stilgenbauer S, Sander S, Bullinger L, Benner A, Leupolt E, Winkler D, et al. Clonal evolution in chronic lymphocytic leukemia: acquisition of high-risk genomic aberrations associated with unmutated VH, resistance to therapy, and short survival. Haematologica. 2007;92(9):1242-5.
29. Berkova A, Zemanova Z, Trneny M, Schwarz J, Karban J, Cmunt E, et al. Clonal evolution in chronic lymphocytic leukemia studied by interphase fluorescence in-situ hybridization. Neoplasma. 2009;56(5):455-8.
30. Braggio E, Kay NE, VanWier S, Tschumper

RC, Smoley S, Eckel-Passow JE, et al. Longitudinal genome-wide analysis of patients with chronic lymphocytic leukemia reveals complex evolution of clonal architecture at disease progression and at the time of relapse. Leukemia. 2012;26(7):1698-701.
31. Eichhorst B, Dreyling M, Robak T, Montserrat E, Hallek M, Group EGW. Chronic lymphocytic leukemia: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2011;22 Suppl 6:vi50-4.
32. Nastoupil LJ, Flowers CR. Management of relapsed chronic lymphocytic leukemia: applying guidelines to practice. Community oncology. 2012;9(12):S85-S92.
33. Zenz T, Krober A, Scherer K, Habe S, Buhler A, Benner A, et al. Monoallelic TP53 inactivation is associated with poor prognosis in chronic lymphocytic leukemia: results from a detailed genetic characterization with long-term follow-up. Blood. 2008;112(8): 3322-9.
34. Malcikova J, Smardova J, Rocnova L, Tichy B, Kuglik P, Vranova V, et al. Monoallelic and biallelic inactivation of TP53 gene in chronic lymphocytic leukemia: selection, impact on survival, and response to DNA damage. Blood. 2009;114(26):5307-14.
35. Dicker F, Herholz H, Schnittger S, Nakao A, Patten N, Wu L, et al. The detection of TP53 mutations in chronic lymphocytic leukemia independently predicts rapid disease progression and is highly correlated with a complex aberrant karyotype. Leukemia. 2009; 23(1):117-24.
36. Guarini A, Marinelli M, Tavolaro S, Bellacchio E, Magliozzi M, Chiaretti S, et al. ATM gene alterations in chronic lymphocytic leukemia patients induce a distinct gene expression profile and predict disease progression. Haematologica. 2012; 97(1):47-55.
37. Ouillette P, Li J, Shaknovich R, Li Y, Melnick A, Shedden K, et al. Incidence and clinical implications of ATM aberrations in chronic lymphocytic leukemia. Genes Chromosomes Cancer. 2012;51(12):1125-32.
38. Stankovic T, Skowronska A. The role of ATM mutations and 11q deletions in disease progression in chronic lymphocytic leukemia. Leuk Lymphoma. 2014; 55(6): 1227-39.
39. Rose-Zerilli MJ, Forster J, Parker H, Parker A, Rodriguez AE, Chaplin T, et al. ATM mutation rather than BIRC3 deletion and/or mutation predicts reduced survival in 11q-deleted chronic lymphocytic leukemia: data from the UK LRF CLL4 trial. Haematologica. 2014;99(4):736-42.
40. Zenz T, Eichhorst B, Busch R, Denzel T, Habe S, Winkler D, et al. TP53 mutation and survival in chronic lymphocytic leukemia. J Clin Oncol. 2010;28(29):4473-9.
41. Gonzalez D, Martinez P, Wade R, Hockley S, Oscier D, Matutes E, et al. Mutational status of the TP53 gene as a predictor of response and survival in patients with chronic lymphocytic leukemia: results from the LRF CLL4 trial. J Clin Oncol. 2011;29(16):2223-9.
42. Pospisilova S, Gonzalez D, Malcikova J, Trbusek M, Rossi D, Kater AP, et al. ERIC recommendations on TP53 mutation analysis in chronic lymphocytic leukemia. Leukemia. 2012;26(7):1458-61.
43. Zainuddin N, Murray F, Kanduri M, Gunnarsson R, Smedby KE, Enblad G, et al. TP53 Mutations are infrequent in newly diagnosed chronic lymphocytic leukemia. Leuk Res. 2011;35(2):272-4.
44. Austen B, Powell JE, Alvi A, Edwards I, Hooper L, Starczynski J, et al. Mutations in the ATM gene lead to impaired overall and treatment-free survival that is independent of IGVH mutation status in patients with B-CLL. Blood. 2005;106(9):3175-82.
45. Austen B, Skowronska A, Baker C, Powell JE, Gardiner A, Oscier D, et al. Mutation status of the residual ATM allele is an important determinant of the cellular response to chemotherapy and survival in patients with chronic lymphocytic leukemia containing an 11q deletion. J Clin Oncol. 2007;25(34):5448-57.
46. Skowronska A, Parker A, Ahmed G, Oldreive C, Davis Z, Richards S, et al. Biallelic ATM inactivation significantly reduces survival in patients treated on the United Kingdom Leukemia Research Fund Chronic Lymphocytic Leukemia 4 trial. J Clin Oncol. 2012;30(36):4524-32.
47. Mansouri L, Sutton LA, Ljungstrom V, Sorqvist EF, Gunnarsson R, Smedby KE, et al. Feasibility of targeted next-generation sequencing of the TP53 and ATM genes in chronic lymphocytic leukemia. Leukemia. 2014;28(3):694-6.
48. Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature. 2013;499(7457):214-8.
49. Tiacci E, Trifonov V, Schiavoni G, Holmes A, Kern W, Martelli MP, et al. BRAF mutations in hairy-cell leukemia. N Engl J Med. 2011;364(24):2305-15.
50. Treon SP, Xu L, Yang G, Zhou Y, Liu X, Cao Y, et al. MYD88 L265P somatic mutation in Waldenstrom's macroglobulinemia. N Engl J Med. 2012;367(9):826-33.
51. Del Poeta G, Dal Bo M, Del Principe MI, Pozzo F, Rossi FM, Zucchetto A, et al. Clinical significance of c.7544-7545 delCT NOTCH1 mutation in chronic lymphocytic leukaemia. Br J Haematol. 2013;160(3): 415-8.
52. Lopez C, Delgado J, Costa D, Conde L, Ghita G, Villamor N, et al. Different distribution of NOTCH1 mutations in chronic lymphocytic leukemia with isolated trisomy 12 or associated with other chromosomal alterations. Genes Chromosomes Cancer. 2012;51(9):881-9.
53. Rossi D, Rasi S, Fabbri G, Spina V, Fangazio M, Forconi F, et al. Mutations of NOTCH1 are an independent predictor of survival in chronic lymphocytic leukemia. Blood. 2012;119(2):521-9.
54. Mansouri L, Cahill N, Gunnarsson R, Smedby KE, Tjonnfjord E, Hjalgrim H, et al. NOTCH1 and SF3B1 mutations can be added to the hierarchical prognostic classification in chronic lymphocytic leukemia. Leukemia. 2013;27(2):512-4.
55. Athanasiadou A, Stamatopoulos K, Tsompanakou A, Gaitatzi M, Kalogiannidis P, Anagnostopoulos A, et al. Clinical, immunophenotypic, and molecular profiling of trisomy 12 in chronic lymphocytic leukemia and comparison with other karyotypic subgroups defined by cytogenetic analysis. Cancer Genet Cytogenet. 2006;168(2):109-19.
56. Ghia P, Stamatopoulos K, Belessi C, Moreno C, Stella S, Guida G, et al. Geographic patterns and pathogenetic implications of IGHV gene usage in chronic lymphocytic leukemia: the lesson of the IGHV3-21 gene. Blood. 2005;105(4):1678-85.
57. Thorselius M, Krober A, Murray F, Thunberg U, Tobin G, Buhler A, et al. Strikingly homologous immunoglobulin gene rearrangements and poor outcome in VH3-21-using chronic lymphocytic leukemia patients independent of geographic origin and mutational status. Blood. 2006;107(7):2889-94.
58. Stamatopoulos K, Belessi C, Moreno C, Boudjograh M, Guida G, Smilevska T, et al. Over 20% of patients with chronic lymphocytic leukemia carry stereotyped receptors: Pathogenetic implications and clinical correlations. Blood. 2007;109(1):259-70.
59. Agathangelidis A, Darzentas N, Hadzidimitriou A, Brochet X, Murray F, Yan XJ, et al. Stereotyped B-cell receptors in one-third of chronic lymphocytic leukemia: a molecular classification with implications for targeted therapies. Blood. 2012; 119(19):4467-75.
60. Strefford JC, Sutton LA, Baliakas P, Agathangelidis A, Malcikova J, Plevova K, et al. Distinct patterns of novel gene mutations in poor-prognostic stereotyped subsets of chronic lymphocytic leukemia: the case of SF3B1 and subset #2. Leukemia. 2013;27 (11):2196-9.
61. Rossi D, Spina V, Bomben R, Rasi S, Dal-Bo M, Bruscaggin A, et al. Association between molecular lesions and specific B-cell receptor subsets in chronic lymphocytic leukemia. Blood. 2013;121(24):4902-5.
62. Domenech E, Gomez-Lopez G, Gzlez-Pena D, Lopez M, Herreros B, Menezes J, et al. New mutations in chronic lymphocytic leukemia identified by target enrichment and deep sequencing. PloS one. 2012;7(6):e38158.
63. Damm F, Mylonas E, Cosson A, Yoshida K, Della Valle V, Mouly E, et al. Acquired Initiating Mutations in Early Hematopoietic Cells of CLL Patients. Cancer Discov. 2014;4(9):1088-101.
64. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144(5):646-74.
65. Ding L, Ley TJ, Larson DE, Miller CA, Koboldt DC, Welch JS, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature. 2012;481(7382):506-10.
66. Sanders MA, Valk PJ. The evolving molecular genetic landscape in acute myeloid leukaemia. Current opinion in hematology. 2013;20(2):79-85.
67. Klco JM, Spencer DH, Miller CA, Griffith M, Lamprecht TL, O'Laughlin M, et al. Functional heterogeneity of genetically defined subclones in acute myeloid leukemia. Cancer cell. 2014;25(3):379-92.
68. Green MR, Gentles AJ, Nair RV, Irish JM, Kihira S, Liu CL, et al. Hierarchy in somatic mutations arising during genomic evolution and progression of follicular lymphoma. Blood. 2013;121(9):1604-11.
69. Okosun J, Bodor C, Wang J, Araf S, Yang CY, Pan C, et al. Integrated genomic analysis identifies recurrent mutations and evolution patterns driving the initiation and progression of follicular lymphoma. Nat Genet. 2014;46(2):176-81.
70. Schuh A, Becq J, Humphray S, Alexa A, Burns A, Clifford R, et al. Monitoring chronic lymphocytic leukemia progression by whole genome sequencing reveals heterogeneous clonal evolution patterns. Blood. 2012;120(20):4191-6.
71. Landau DA, Carter SL, Stojanov P, McKenna A, Stevenson K, Lawrence MS, et al. Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. Cell. 2013;152(4):714-26.
72. Rossi D, Khiabanian H, Spina V, Ciardullo C, Bruscaggin A, Fama R, et al. Clinical impact of small TP53 mutated subclones in chronic lymphocytic leukemia. Blood. 2014;123(14):

2139-47.
73. Malcikova J, Stano-Kozubik K, Tichy B, Kantorova B, Pavlova S, Tom N, et al. Detailed analysis of therapy-driven clonal evolution of TP53 mutations in chronic lymphocytic leukemia. Leukemia. 2014 Oct 28 [Epub ahead of print].
74. Kikushige Y, Ishikawa F, Miyamoto T, Shima T, Urata S, Yoshimoto G, et al. Self-renewing hematopoietic stem cell is the primary target in pathogenesis of human chronic lymphocytic leukemia. Cancer Cell. 2011;20(2):246-59.
75. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3(7):730-7.
76. Chung SS, Kim E, Park JH, Chung YR, Lito P, Teruya-Feldstein J, et al. Hematopoietic stem cell origin of BRAFV600E mutations in hairy cell leukemia. Sci Transl Med. 2014;6(238):238ra71.
77. Landgren O, Albitar M, Ma W, Abbasi F, Hayes RB, Ghia P, et al. B-cell clones as early markers for chronic lymphocytic leukemia. N Engl J Med. 2009;360(7):659-67.
78. Marti GE, Rawstron AC, Ghia P, Hillmen P, Houlston RS, Kay N, et al. Diagnostic criteria for monoclonal B-cell lymphocytosis. Br J Haematol. 2005;130(3):325-32.
79. Rawstron AC, Green MJ, Kuzmicki A, Kennedy B, Fenton JA, Evans PA, et al. Monoclonal B lymphocytes with the characteristics of "indolent" chronic lymphocytic leukemia are present in 3.5% of adults with normal blood counts. Blood. 2002; 100(2):635-9.
80. Ghia P, Prato G, Scielzo C, Stella S, Geuna M, Guida G, et al. Monoclonal CD5+ and CD5- B-lymphocyte expansions are frequent in the peripheral blood of the elderly. Blood. 2004;103(6):2337-42.
81. Nieto WG, Almeida J, Romero A, Teodosio C, Lopez A, Henriques AF, et al. Increased frequency (12%) of circulating chronic lymphocytic leukemia-like B-cell clones in healthy subjects using a highly sensitive multicolor flow cytometry approach. Blood. 2009;114(1):33-7.
82. Scarfo L, Dagklis A, Scielzo C, Fazi C, Ghia P. CLL-like monoclonal B-cell lymphocytosis: are we all bound to have it? Semin Cancer Biol. 2010;20(6):384-90.
83. Fazi C, Scarfo L, Pecciarini L, Cottini F, Dagklis A, Janus A, et al. General population low-count CLL-like MBL persists over time without clinical progression, although carrying the same cytogenetic abnormalities of CLL. Blood. 2011;118(25):6618-25.
84. Almeida J, Nieto WG, Teodosio C, Pedreira CE, Lopez A, Fernandez-Navarro P, et al. CLL-like B-lymphocytes are systematically present at very low numbers in peripheral blood of healthy adults. Leukemia. 2011;25(4):718-22.
85. Rawstron AC, Shanafelt T, Lanasa MC, Landgren O, Hanson C, Orfao A, et al. Different biology and clinical outcome according to the absolute numbers of clonal B-cells in monoclonal B-cell lymphocytosis (MBL). Cytometry B Clin Cytom. 2010;78 Suppl 1:S19-23.
86. Shanafelt TD, Ghia P, Lanasa MC, Landgren O, Rawstron AC. Monoclonal B-cell lymphocytosis (MBL): biology, natural history and clinical management. Leukemia. 2010;24(3):512-20.
87. Fung SS, Hillier KL, Leger CS, Sandhu I, Vickars LM, Galbraith PF, et al. Clinical progression and outcome of patients with monoclonal B-cell lymphocytosis. Leuk Lymphoma. 2007;48(6):1087-91.
88. Mulligan CS, Thomas ME, Mulligan SP. Monoclonal B-cell lymphocytosis and chronic lymphocytic leukemia. N Engl J Med. 2008;359(19):2065-6; author reply 6.
89. Rawstron AC, Bennett FL, O'Connor SJ, Kwok M, Fenton JA, Plummer M, et al. Monoclonal B-cell lymphocytosis and chronic lymphocytic leukemia. N Engl J Med. 2008;359(6):575-83.
90. Shanafelt TD, Kay NE, Jenkins G, Call TG, Zent CS, Jelinek DF, et al. B-cell count and survival: differentiating chronic lymphocytic leukemia from monoclonal B-cell lymphocytosis based on clinical outcome. Blood. 2009;113(18):4188-96.
91. Shanafelt TD, Kay NE, Rabe KG, Call TG, Zent CS, Maddocks K, et al. Brief report: natural history of individuals with clinically recognized monoclonal B-cell lymphocytosis compared with patients with Rai 0 chronic lymphocytic leukemia. J Clin Oncol. 2009;27(24):3959-63.
92. Scarfo L, Zibellini S, Tedeschi A, Maura F, Neri A, Bertazzoni P, et al. Impact of B-cell count and imaging screening in cMBL: any need to revise the current guidelines? Leukemia. 2012;26(7):1703-7.
93. Rossi D, Sozzi E, Puma A, De Paoli L, Rasi S, Spina V, et al. The prognosis of clinical monoclonal B cell lymphocytosis differs from prognosis of Rai 0 chronic lymphocytic leukaemia and is recapitulated by biological risk factors. Br J Haematol. 2009;146(1):64-75.
94. Morabito F, Mosca L, Cutrona G, Agnelli L, Tuana G, Ferracin M, et al. Clinical monoclonal B lymphocytosis versus Rai 0 chronic lymphocytic leukemia: A comparison of cellular, cytogenetic, molecular, and clinical features. Clin Cancer Res. 2013; 19(21):5890-900.
95. Henriques A, Rodriguez-Caballero A, Nieto WG, Langerak AW, Criado I, Lecrevisse Q, et al. Combined patterns of IGHV repertoire and cytogenetic/molecular alterations in monoclonal B lymphocytosis versus chronic lymphocytic leukemia. PloS One. 2013; 8(7):e67751.
96. Henriques A, Rodriguez-Caballero A, Criado I, Langerak AW, Nieto WG, Lecrevisse Q, et al. Molecular and cytogenetic characterization of expanded B-cell clones from multiclonal versus monoclonal B-cell chronic lymphoproliferative disorders. Haematologica. 2014;99(5):897-907.
97. Schuler F, Dolken L, Hirt C, Kiefer T, Berg T, Fusch G, et al. Prevalence and frequency of circulating t(14;18)-MBR translocation carrying cells in healthy individuals. Int J Cancer. 2009;124(4):958-63.
98. Roulland S, Kelly RS, Morgado E, Sungalee S, Solal-Celigny P, Colombat P, et al. t(14;18) Translocation: A predictive blood biomarker for follicular lymphoma. J Clin Oncol. 2014;32(13):1347-55.
99. Rasi S, Monti S, Spina V, Foa R, Gaidano G, Rossi D. Analysis of NOTCH1 mutations in monoclonal B-cell lymphocytosis. Haematologica. 2012;97(1):153-4.
100. Greco M, Capello D, Bruscaggin A, Spina V, Rasi S, Monti S, et al. Analysis of SF3B1 mutations in monoclonal B-cell lymphocytosis. Hematol Oncol. 2013;31(1):54-5.
101. Lionetti M, Fabris S, Cutrona G, Agnelli L, Ciardullo C, Matis S, et al. High-throughput sequencing for the identification of NOTCH1 mutations in early stage chronic lymphocytic leukaemia: biological and clinical implications. Br J Haematol. 2014; 165(5):629-39.
102. Ojha J, Secreto C, Rabe K, da Silva JA, Tschumper R, Van Dyke D, et al. Monoclonal B-cell lymphocytosis is characterized by mutations in CLL putative driver genes and clonal heterogeneity clonal competition many years prior to disease progression. Leukemia. 2014 [Epub ahead of print].
103. Messmer BT, Albesiano E, Efremov DG, Ghiotto F, Allen SL, Kolitz J, et al. Multiple distinct sets of stereotyped antigen receptors indicate a role for antigen in promoting chronic lymphocytic leukemia. J Exp Med. 2004;200(4):519-25.
104. Sutton LA, Agathangelidis A, Belessi C, Darzentas N, Davi F, Ghia P, et al. Antigen selection in B-cell lymphomas--tracing the evidence. Semin Cancer Biol. 2013; 23(6):399-409.
105. Vardi A, Agathangelidis A, Sutton LA, Ghia P, Rosenquist R, Stamatopoulos K. Immunogenetic studies of chronic lymphocytic leukemia: revelations and speculations about ontogeny and clinical evolution. Cancer Res. 2014;74(16):4211-6.
106. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon, France: IARC Press, 2008.
107. Rossi D, Gaidano G. Richter syndrome: molecular insights and clinical perspectives. Hematol Oncol. 2009;27(1):1-10.
108. Rossi D, Spina V, Deambrogi C, Rasi S, Laurenti L, Stamatopoulos K, et al. The genetics of Richter syndrome reveals disease heterogeneity and predicts survival after transformation. Blood. 2011; 117(12):3391-401.
109. Yano T, Jaffe ES, Longo DL, Raffeld M. MYC rearrangements in histologically progressed follicular lymphomas. Blood. 1992; 80(3):758-67.
110. Davies AJ, Rosenwald A, Wright G, Lee A, Last KW, Weisenburger DD, et al. Transformation of follicular lymphoma to diffuse large B-cell lymphoma proceeds by distinct oncogenic mechanisms. Br J Haematol. 2007;136(2):286-93.
111. Chigrinova E, Rinaldi A, Kwee I, Rossi D, Rancoita PM, Strefford JC, et al. Two main genetic pathways lead to the transformation of chronic lymphocytic leukemia to Richter syndrome. Blood. 2013; 122(15):2673-82.
112. Fabbri G, Khiabanian H, Holmes AB, Wang J, Messina M, Mullighan CG, et al. Genetic lesions associated with chronic lymphocytic leukemia transformation to Richter syndrome. J Exp Med. 2013;210(11):2273-88.