CHRONIC LYMPHOCYTIC LEUKEMIA: TAKING A BIG LEAP FORWARD

Genomic and epigenomic heterogeneity in chronic lymphocytic leukemia

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Defining features of chronic lymphocytic leukemia (CLL) are not only its immunophenotype of $CD19^+CD5^+CD23^+sIg_{dim}$ expressing clonal mature B cells but also its highly variable clinical course. In recent years, advances in massively parallel sequencing technologies have led to rapid progress in our understanding of the CLL genome and epigenome. Overall, these studies have clearly demarcated not only the vast degree of genetic and epigenetic heterogeneity among individuals with CLL but also even within individual patient leukemias. We herein review the rapidly growing series of studies assessing the genetic and epigenetic features of CLL within clinically defined periods of its growth. These studies strongly suggest an evolving spectrum of lesions over time and that these features may have clinical impact. (*Blood*. 2015;126(4):445-453)

Introduction

Chronic lymphocytic leukemia (CLL) is an initially slow-growing common B-cell malignancy whose hallmark is a highly variable clinical course. For more than a decade, "watch and wait" has been the standard approach for patients without symptomatic disease, with frontline chemotherapy-based therapy as the conventional choice if treatment is required. Over the past 2 years, however, three new drugs have been approved: (1) the novel potent CD20-targeting antibody obinutuzumab,[1] (2) the inhibitor of PI3-kinase idelalisib,[2] and (3) the irreversible inhibitor of Bruton tyrosine kinase ibrutinib.[3] Moreover, several highly active agents, such as the BCL2 inhibitor GDC-0199/ABT-199,[4] are in advanced clinical trials, which further promise to expand treatment options.

With these growing therapeutic possibilities, understanding the heterogeneous features of this disease and how it evolves over time becomes a priority, so that maximum benefit can be gleaned from these diverse therapies. In parallel with the exciting transformation in the therapeutic landscape of CLL, an explosive growth in our understanding CLL genetics has taken place.[5] Several large-scale studies of massively parallel sequencing have defined the mutation spectrum[6-12] and have investigated the altered epigenome of CLL.[13-18] Altogether, these studies have uncovered both the vast genetic and epigenetic heterogeneity among patients, and within individual patient samples. Indeed, recent work has demonstrated intratumoral heterogeneity to impact individual evolutionary trajectories and clinical outcome in CLL.[12,18] These innovations afford the insight that clonal heterogeneity likely fuels clonal evolution and can contribute to the variability in clinical course among CLL patients.

Herein, we review recent progress in our understanding of the complexities of inter- and intratumoral genetic and epigenetic heterogeneity in relationship to evolutionary principles. These new data offer fresh perspectives on our approaches to the prognostication and management of CLL.

The extensive genetic heterogeneity of CLL

Studies of somatic copy number variations using karyotyping, fluorescence in situ hybridization or single nucleotide polymorphism arrays first revealed the high molecular heterogeneity of CLL (extensively reviewed elsewhere).[19,20] The most recurrent lesions identified were deletions of chromosome 13q (55% of cases), 17p (7%), and 11q (6% to 18%); and trisomy 12 (12% to 16%).[21,22] In addition to their prognostic relevance, the minimal deleted region of each of these deletions have been found to contain within them putative CLL drivers: *ATM* and *BIRC3* in 11q, *TP53* in 17p, and miR-15a/16 encoded in an intron of *DLEU2* in 13q.[23]

In recent years, the relative affordability of next-generation sequencing (NGS)-based technologies such as whole-genome and whole-exome sequencing has made it feasible to undertake large-scale efforts in cancer sequencing. A primary goal of the initial forays to dissect the CLL genome was to discover, in an unbiased fashion, new drivers based on statistical modeling. Rapid strides in our genetic understanding were gained because of several salient features of CLL that facilitated genomic investigation; namely, the ready accessibility of purified tumor cells from peripheral blood, its relatively indolent kinetics allowing for repeated sampling over time to study disease evolution and the highly variable clinical courses of patients that provides a strong distinguishing signal from which the impact of novel features can be differentiated.

Collectively, the earliest NGS-based sequencing studies revealed the overall low somatic mutation rate in CLL (~1/Mb), similar to other hematologic malignancies, but at least 10-fold lower than carcinogen- or UV-induced solid tumors (~15/Mb for melanoma).[24] These studies demonstrated the highly heterogeneous genetic nature of CLL, characterized by several "mountains" (ie, significantly recurrent genes) but also "hills" (ie, infrequently recurrent genes), with the lack of any discernible universal genetic event accounting for all cases.[6-11] These

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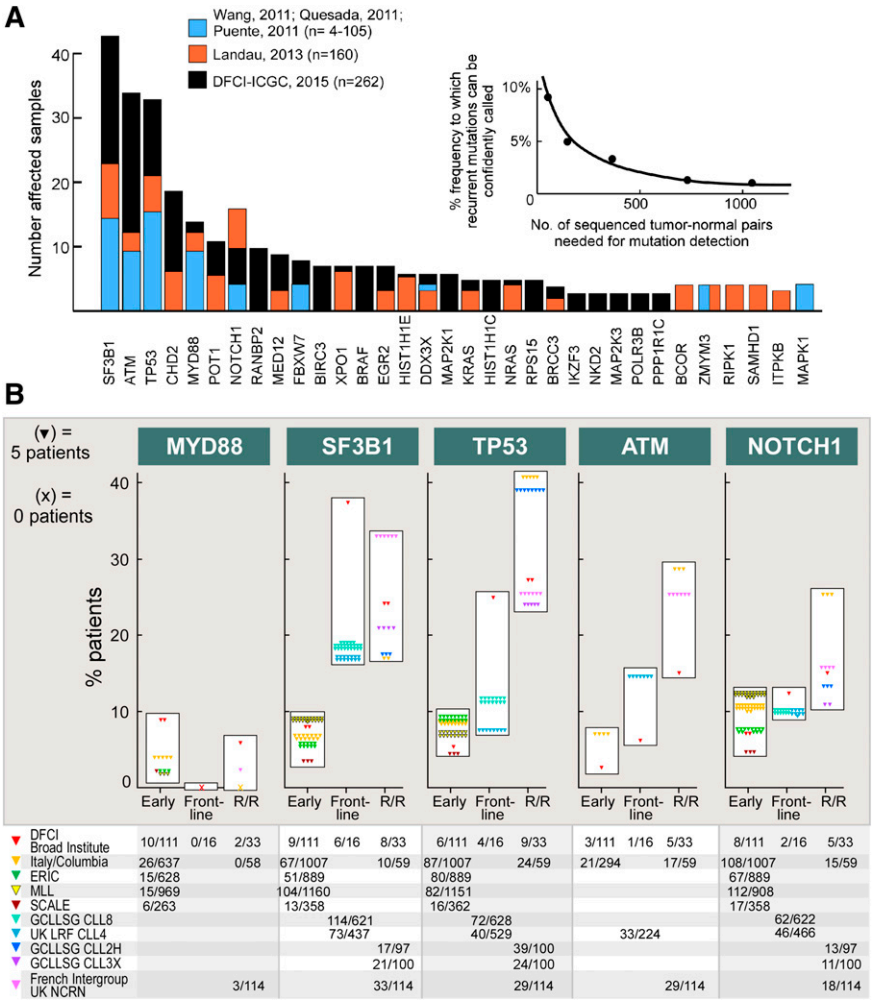

Figure 1. The mutational landscape of CLL. (A) The discovery of recurrently CLL mutated genes has become more sensitive with increased cohort size, with the estimated sensitivity calculated through saturation analysis[6,12,26]; (B) Frequency of gene mutations depending on the course of the disease. "Early" (newly diagnosed and untreated patients); "Frontline" (untreated patients with symptomatic CLL requiring therapy); and "R/R" (relapsing or refractory patients). Unselected cohorts have been included from: DFCI/Broad Institute,[12] Amedeo Avogadro University of Eastern Piedmont, Novara and Sapieza University (Rome, Italy)/Columbia University (New York)[27]; ERIC [28]; MLL[29]; and SCALE.[30] Reported are also series from clinical trials: UK LRF CLL4[31,32]; GCLLSG CLL8,[33] CLL2H,[34] and CLL3X[35]; GCFLLC/MW-GOELAMS ICLL01, and UK NCRN CLL201 and CLL202.[36] DCFI, Dana-Farber Cancer Institute; ERIC, European Research Initiative on CLL; GCFLLC/MW-GOELAMS, French CLL Intergroup; GCLLSG, German CLL Study Group; MLL, Munich Leukemia Laboratory; SCALE, Scandinavian Lymphoma Etiology; UK LRF, United Kingdom Lymphoma Research Foundation; UK NCRN, United Kingdom National Cancer Research Network.

early studies of up to ~100 cases each (with clinically heterogeneous sample cohorts) corroborated known CLL-associated alterations, such as somatic mutations, across the length of the DNA damage response genes *TP53* and *ATM*, consistent with their inactivating effect. Unexpectedly, these studies further uncovered a number of novel frequent somatic changes (likely activating at hot spot locations). Mutations in the PEST domain of the key ligand-activated transcription factor of the NOTCH signaling pathway *NOTCH1* (c.7544_7545fsdel) were among the first novel alterations to be discovered by NGS.[7] Also discovered were the recurrent L265P mutations in the critical adaptor of the toll-like receptor complex MYD88, leading to potential constitutive nuclear factor-κB signaling. Finally, mutations in the essential splicing factor *SF3B1* were identified, localized to evolutionarily conserved hotspots within its carboxyl-terminal repeat HEAT domains (most frequently at K700E).[6,9] SF3B1 is a central component of the U2 spliceosome, which orchestrates the excision of introns from pre-messenger RNA (mRNA) to mature mRNA.[25]

Subsequent to these initial reports, studies with larger-sized cohorts have uncovered additional novel candidate drivers including new chromatin regulators (*CHD2* and *HIST1H1C*), B-cell transcription factors (*EGR2* and *IKZF3*), RNA export factors (*XPO1* and *RANBP2*), ribosomal proteins (*RPS15*), telomere-associated proteins (*POT1*), and signal transducers (*RAS*, *MAP2K1*, and *MAP2K3*) (Figure 1A).[10,12,26] Altogether, these studies underscore the importance of sufficient power for sensitive detection of drivers in this highly genetically heterogeneous disease. Based on a saturation analysis and taking into account the background mutation rate of CLL, it has been estimated that an analysis of ~2000 samples would be sufficient to confidently identify recurrent drivers present in 1% to 2% of the population. Hence, as expected, with the incremental growth in size of the discovery cohort, we have observed a growing "long tail" of significant drivers (Figure 1A).[24,26]

The most commonly mutated genes (*TP53*, *SF3B1*, *MYD88*, *NOTCH1*, and *ATM*) have remained unchanged across studies. However, their reported frequencies across studies have been variable, related to the variable incidence of each particular mutation from the early stages of disease to the time of first therapy, or even at relapse (Figure 1B).[12,27-36] *SF3B1* mutations appear to associate with early progression, with its frequency increasing from 4% to 9% at diagnosis to 17% to 18% or greater by the time of first therapy. By contrast, the frequency of *MYD88* is unchanging across disease stages, whereas *NOTCH1* mutation increases mainly between the time of first therapy (~10%) and relapse (up to 25%). Finally, mutations in *TP53* and *ATM* each rise continuously through the course of disease, from a frequency of <10% in early disease to 25% or greater at relapse. Overall, whereas the variable frequencies of CLL drivers may reflect the association of certain gene mutations to other aggressive CLL features (eg, unmutated immunoglobulin heavy chain variable [*IGHV*] genes), these differing trajectories may also suggest potentially different roles of these drivers over the CLL course.

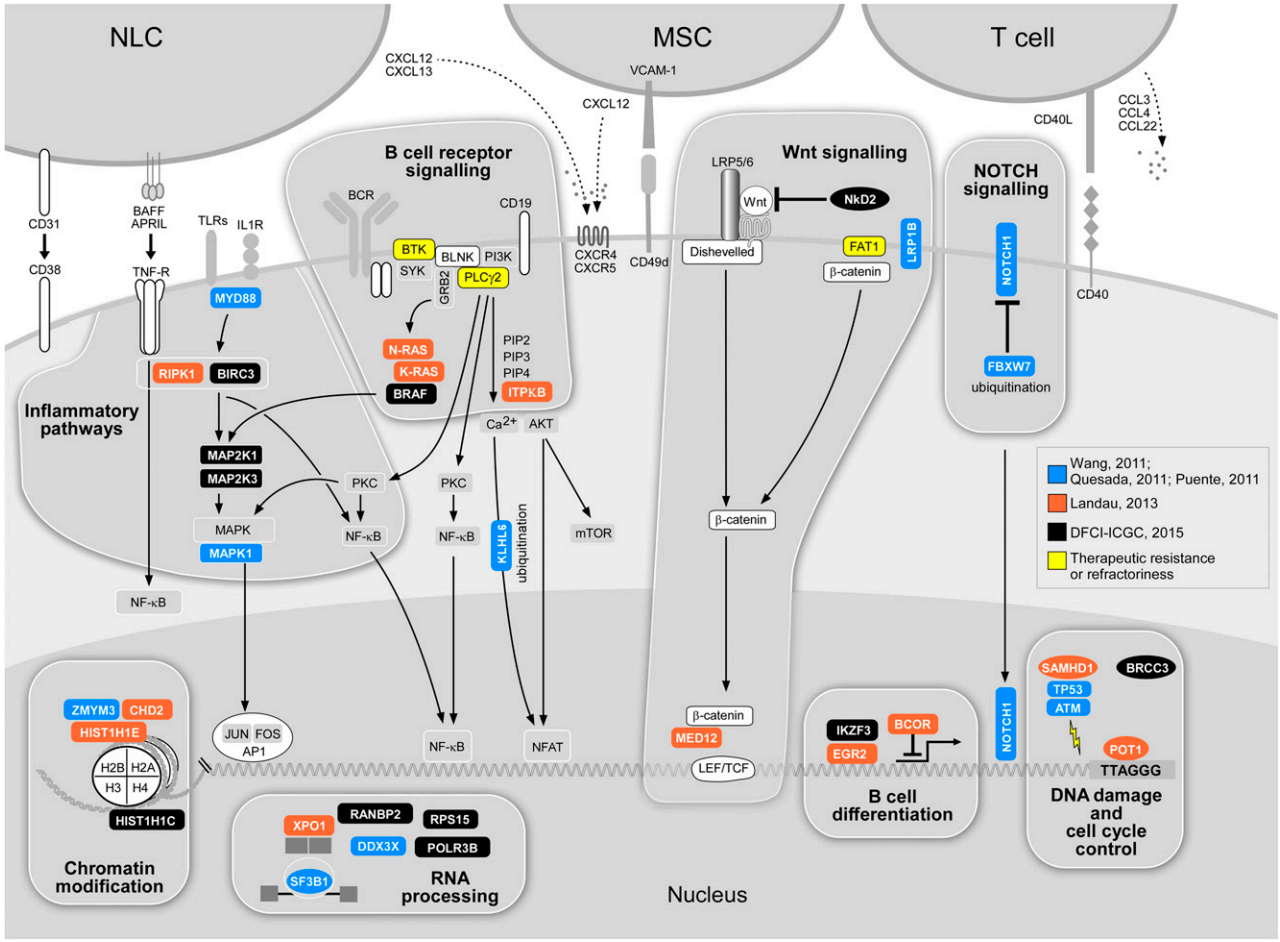

Figure 2. Putative core cellular pathways affected by significantly mutated genes in CLL. Blue-genes identified from CLL series by Wang et al,[6] Puente et al,[8] and Quesada et al[9] (n = 4 to 105 samples); orange-genes identified by Landau et al (n = 160 subjects)[12]; black-genes affected by 262 subjects[23]; yellow gene mutations identified in relationship to drug resistance.[43,44] Several of the affected pathways likely serve as an important bridge with the microenvironment, which is of particular importance in CLL (crucial actors of the CLL microenvironment are represented by NLCs, MSCs, and T cells. NLCs, nurse-like cells; NF-κB, nuclear factor-κB; MSCs, marrow stroma cells; mTOR, mammalian target of rapamycin; TLR, toll-like receptor.

The central roles of the mutated genes in several essential cellular processes pathways have suggested these as core CLL pathways. Indeed, as the experience in characterization of the mutational landscape has expanded, greater resolution for mapping the nodes to which CLL might be sensitive has been achieved. As shown in Figure 2, CLL mutations have been consistently observed to involve pathways in DNA damage (*TP53* and *ATM*), mRNA processing (*SF3B1* and *XPO1*), chromatin modification (*HIST1H1E*, *CHD2*, and *ZMYM3*), Wnt signaling, NOTCH signaling (*NOTCH1*), and inflammation (*MYD88*). More recently, novel drivers further support somatic mutation as a mechanism affecting B-cell–related signaling and transcription (*EGR2* and *BRAF*).[37] The functional role of several novel putative drivers has been confirmed across several studies. For example, through an innovative delivery system for nucleic acids into CLL cells, the silencing of mutated Wnt pathway genes in cells harboring those mutations could be effectively achieved, and demonstrated a loss in the viability of these cells, suggesting dependence of those CLLs on Wnt pathway signaling.[38] Of note, several novel drivers appear to involve the disruption of the DNA damage response. Mutations in *POT1,* involved in the protection of telomeres, were confirmed to prevent its binding to telomeric DNA and to result in the generation of numerous telomeric and chromosomal abnormalities.[10] Mutations in *SF3B1* have been shown to lead to altered splicing,[6,9,39] and was recently linked to an altered DNA damage response.[40] SAMHD1, a regulator of the intracellular dNTP pool, has been demonstrated to be recruited to the site of DNA damage and is likely involved in the response to DNA double-strand breaks.[41] It should be noted that CLL cells are exquisitely sensitive to microenvironmental cues,[42] and hence future studies will undoubtedly evaluate the impact of genetic drivers and pathways on the functional behavior of CLL cells in relationship to immune and stromal cell populations with which they interact as well as treatment received.[43,44]

Intratumoral genetic heterogeneity provides insights into the order of mutation acquisition in CLL

Despite its clonal origin, cancer is characterized by the coexistence of multiple populations within the tumor. Such intratumoral heterogeneity was conceptualized decades ago as an inevitable outcome of the mutational process inherent to cancers[45,46] and could be detected using a variety of experimental methods.[47,48] NGS, however, has been transformative for this effort by providing a comprehensive approach to detect subclones at unprecedented resolution.[49-51] For CLL, the relative

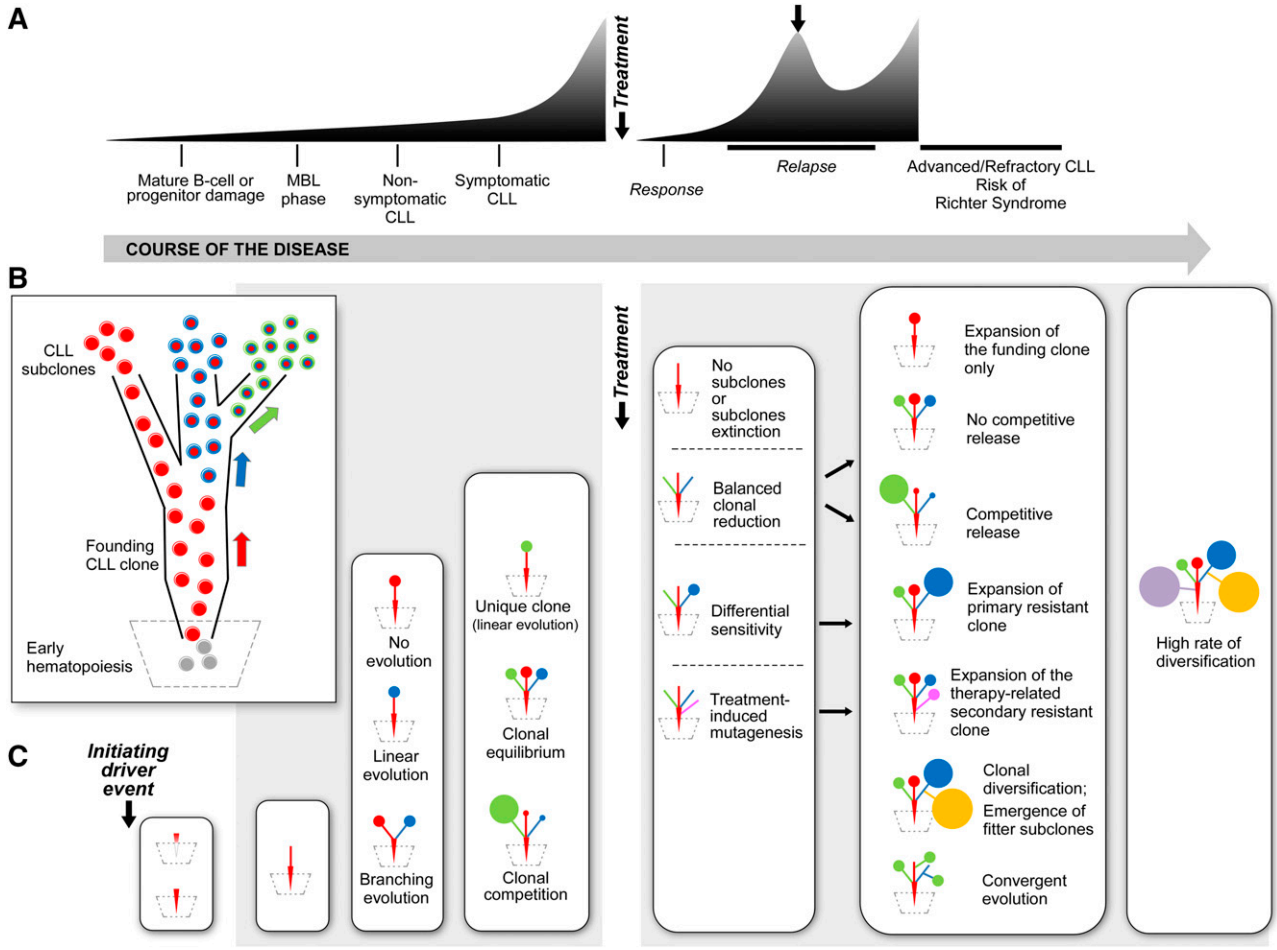

Figure 3. Heterogenous evolutionary trajectories through CLL course and therapeutic intervention. (A) Typical CLL disease course; (B) phylogenetic tree of CLL leukemogenesis (each arrow represents the acquisition of a genetic event); (C) evolution of the CLL phylogenetic tree at various stages of the CLL disease course.[12,48,58-61]

high purity of samples has facilitated the confident detection of rare allelic frequencies of somatic variants, which upon correction for local ploidy and clustering, can lead to the defining of subclonal populations within tumor samples, and hence have led to the understanding of the pervasive extent of intratumoral heterogeneity present in CLL.

The ability to define subclonal architecture in CLL by tumor sequencing has suggested an ability to infer the phylogeny of any case, since the "snapshot" provided by subclonal composition likely results from the stepwise acquisition of mutations across the process of leukemogenesis (Figure 3A-B). Conceptually, the earliest clone-propagating event would have occurred in a single cell, giving rise to a "CLL-founding clone." Thus, within this framework, clonal mutations within a bulk sample represent earlier events, acquired as putative cancer-initiating or driving events or alternatively, as passenger events that were present at the time of transformation. Landau et al found del(13q), trisomy 12, and *MYD88* mutations as the three most significantly clonal lesions; consistent with their acquisition early in the history of individual CLL tumors, these could potentially provide clonal advantage to B cells.[12] Mutations in *NOTCH1* and *SF3B1* were also commonly clonal. Applying machine-learning–based approaches to large cross-sectional CLL datasets, it has been proposed that the acquisition of these early CLL drivers then leads to preferred evolutionary trajectories.[52]

Consistent with the idea that early alterations can provide a clonal advantage to B cells, Klein et al demonstrated that as a lowly penetrant lesion, B cells restricted expression of del(13q) could give rise to histopathologic evidence of CLL in a murine model.[53] Moreover, recent studies have demonstrated that many cancer-driving genetic events were detectable in large population-based cohorts without hematologic malignancies, the so-called "clonal hematopoiesis."[54,55] The frequency of these events increased with age, and although most mutations occurred in *DNMT3A*, *TET2*, and *ASXL1* (associated with myeloid malignancies), significant events were also noted in *TP53* and *SF3B1* as well as rare instances of mutations in *MYD88* and *NOTCH1*. The presence of clonal hematopoiesis was associated with an increased risk of various hematologic cancers including CLL. More directly evaluating early hematopoiesis in CLL, Damm et al applied targeted deep sequencing of candidate early driver mutations on flow-sorted hematopoietic progenitors, and intriguingly, detected the presence of lymphoid oncogenes (ie, mutated *BRAF*, *NOTCH1*, and *SF3B1*) in CD34+ hematopoietic cells.[37] Together with prior murine xenograft studies demonstrating that hematopoietic stem cells (HSCs) from CLL patients can generate clonal B cells with CLL-like phenotype,[56] these data suggest hematopoietic progenitors as cells-of-origin for CLL.

Intratumoral genetic heterogeneity fuels diverse evolution patterns in CLL

Clonal evolution refers to the process in which cancer cells present accumulated genetic (and epigenetic) changes over time giving rise to

new subclones. Cancer is thought to evolve by a process of clonal expansion, diversification, and selection within the tissue ecosystems.[46] This feature appears to be in line with an evolutionary process as those reported by Darwin about species evolution.

A key challenge presented by intratumoral heterogeneity is its capacity to fuel clonal evolution and the generation of therapy resistant subpopulations. Through acquisition of fitter subclones and environmental selective pressures, tumors can evolve with time. Indeed, the presence of subclonal driver mutations as a surrogate of an active evolutionary process driving of clonal diversification was found to be an independent poor prognostic risk factor of more aggressive and/or resistant disease.[12,57]

Genetic changes across the disease course of CLL has been investigated in a growing body of studies, which have used longitudinal follow-up of the subclonal composition by various genomic technologies and have illustrated the patterns of clonal evolution across the natural history of CLL (Figure 3C).[12,48,58-61]

Monoclonal B-cell lymphocytosis (MBL) is thought to be the premalignant lesion of CLL.[62] Although only a small number of MBL cases have been characterized by whole-exome sequencing, a broad heterogeneous spectrum of mutations are clearly present in MBL (including *SF3B1, NOTCH1, FBXW7,* and *DDX3X*), similar to the mutational spectrum of CLL.[63] In the majority of cases reported thus far, trisomy 12 and del(13q) were also detected by fluorescence in situ hybridization, supporting the idea that these are early lesions in CLL.

Overall, two common patterns of clonal evolution in CLL have been observed (Figure 3C).[12,48,58-61] One is that of linear evolution, in which a single clone undergoes successive acquisition of additional driver events over time, that accumulate atop the initial abnormalities. The other is that of branched evolution, in which two or more genetic subclones coexist and evolve in a parallel fashion. In general, inference of subclonal architecture in bulk single time point samples using algorithmic approaches cannot necessarily resolve linear vs branched structures of subclonal populations, although recent in silico models have attempted to explore potential evolutionary paths from early to secondary events.[52] Emerging studies evaluating the role of ultra-deep sequencing on the one hand and novel technologies to dissect the mutational profiles of single cells on the other hand, promise to provide further insights on CLL clonal architecture and evolution.[64-66]

By now, it has become clear that multiple subclones can maintain their relative proportions to each other over years ("clonal equilibrium"), whereas in other cases, individual subclones can emerge as dominant over time, likely because of their relative higher fitness ("clonal competition") (Figure 3C). The proportion of patients in each of these two categories is likely dependent of stage of disease.

The concepts of clonal equilibrium vs competition and evolution have been most evident in longitudinal studies assessing the impact of cytoreductive therapy.[12,48,58-61] Landau et al suggested that the absence of intervening therapy was largely associated with stable subclonal composition over time. In contrast, chemotherapy exposure predominantly resulted in marked clonal evolution.[12] Across studies, the genetic lesions dominating at relapse have been consistently detected in smaller subclones at earlier stages, suggesting the emergence of fitter subclones together with genetic diversification.[52] The most consistent lesion detected in these studies has been preexisting *TP53* mutations, resulting in the outgrowth of highly genomically complex clones at relapse.[48] On one hand, genetic lesions such as mutated *TP53* itself may confer resistance to the therapeutic agent, and a differential sensitivity of the subclone to these drugs may be responsible for its dominance at relapse. Alternatively, the emergence of a dominant subclone could result from a balanced clonal reduction, followed by a competitive release that depends on subclone-related growth properties and mechanisms of tumor progression. Finally, therapy itself could induce de novo mutations conferring major fitness of the related subclones (Figure 3C).[67]

The notion that a coherent pattern of resistance emerging following exposure to therapy has been most clearly demonstrated by recent studies describing resistance developing after exposure to potent inhibition of B-cell–receptor signaling with ibrutinib. Resistance was linked to mutations in Bruton tyrosine kinase (C481S) and/or its immediate downstream partner *PLCγ2* (Figure 2).[43] Remarkably, various individual subjects across studies have been detected to harbor multiple subclones with mutation in PLCγ2, consistent with convergent evolution occurring within these patients.[43,59,61,68] These findings support the idea of individualized evolutionary trajectories taken on by different CLL subclones to functionally circumvent the pathway inhibition imposed by targeted inhibitors.

Profiling CLL epigenetic heterogeneity

In addition to genetic lesions, the disruption of epigenetic mechanisms also plays a role in oncogenesis.[69] DNA methylation, which occurs at the cytosine residue of the CpG dinucleotide, is a crucial facet of epigenetic programming that normally regulates gene transcription and genome stability, and contributes to normal B-cell development.[70] Although potentially reversible, DNA methylation status is considered an inheritable trait. Furthermore, the CLL methylome over time has been characterized as rather stable, with few changes identified between the resting and proliferating compartments of CLL.[16] On the other hand, in striking support for a key role of epigenetics contributing to CLL leukemogenesis, Chen et al reported the detection of aberrant methylation well before disease onset in the Eμ-TCL1 mice, an established murine model of CLL.[71,72] Furthermore, aberrant promoter methylation leading to dysregulated expression of genes such as *TCL1*,[73] DAPK1,[74] or ZAP70,[75] as well as CLL-associated microRNAs[76,77] and long intervening noncoding RNAs[78] have been implicated in CLL pathogenesis.

Comprehensive methylation profiling by genome-wide arrays (high-resolution Illumina 450K) or sequencing (whole-genome bisulfite sequencing) have provided a global picture of methylation changes in CLL compared with normal B cells, and have demonstrated the highly heterogeneous methylation profiles across samples. Similar to other cancers,[79-81] CLL harbors global genome-wide hypomethylation, with localized regions of hypermethylation.[13-18] Among CLL patients, differences in methylation patterns, most of them lying outside CpG islands, have been clearly observed in relation to *IGHV* mutational status.[13,16] In addition, based on similarities in methylation imprint, these systematic investigations have suggested naïve B cells as the putative cell-of-origin of CLLs with unmutated IGHV, and memory B cells for CLLs with mutated IGHV.[15] In multivariate analyses, methylation imprint also influenced time-to-treatment.

More recently, the extensive intratumoral epigenetic heterogeneity in CLL and its impact of clonal evolution was investigated. Oakes et al reported that, unlike normal B cells, CLL cells harbored greater intermediate DNA methylation values, which were attributed to allele-specific methylation.[17] The estimated high level of intratumoral methylation heterogeneity was associated with aggressive features and a shorter time-to-first treatment. Furthermore, evolution of DNA methylation over time, observed in 9 of 28 cases, was linked to a higher level of methylation heterogeneity at the

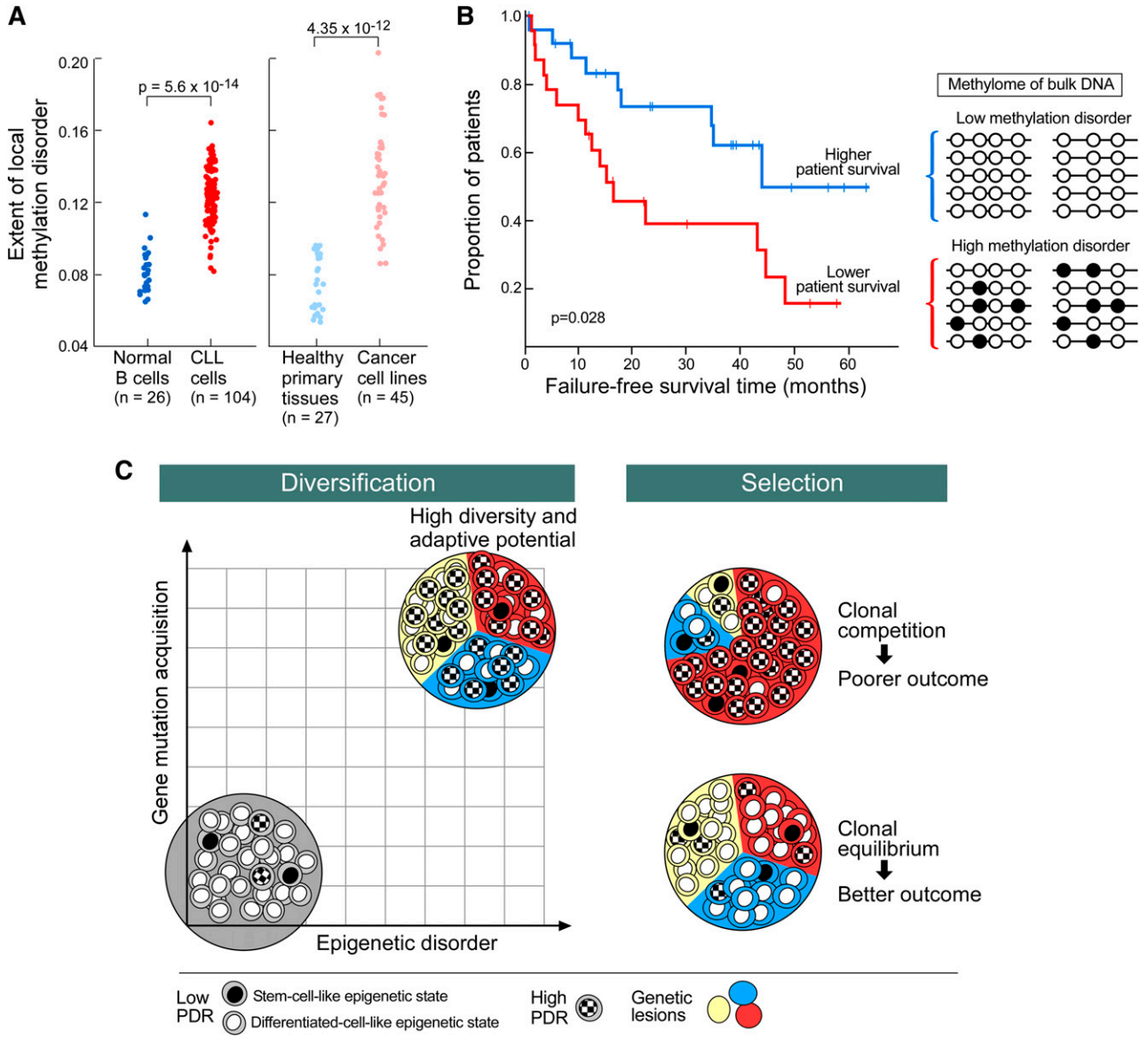

Figure 4. Model of clonal diversification and selection in CLL. (A) Locally disordered DNA methylation is higher in CLL and cancer tissues compared with normal tissues, including B cells (adapted from Landau et al[18]); (B) Adverse impact of locally disordered DNA methylation on failure-free survival[18]; (C) Clonal diversification may result from synergic effects of disordered DNA methylation and genomic instability (left). Clonal selection related to therapeutic and microenvironment pressure shapes the subclonal composition that ranges from clonal equilibrium to clonal competition (right). High level of clonal diversification could lead to the emergence of fitter subclones competing within CLL bulk and is responsible for more aggressive/resistant disease associated with poor survival.

earlier time point and to the presence of subclonal (rather than clonal) genetic events, thus demonstrating a link between genetic and methylation evolution.

Using reduced representation bisulfite sequencing, Landau et al also detected increased intermediate DNA methylation values, but pervasive locally disordered methylation (assessed by proportion of discordant reads [PDR]) throughout the genome was the primary basis of CLL intratumoral methylome heterogeneity.[18] This high level of epigenetic "noise" appeared to arise stochastically and was specific to cancer (rather than a change related to normal tissue differentiation) (Figure 4A).[18,82] Locally disordered methylation impacted the variability of gene expression across and within samples, creating enhanced potential for alternative evolutionary trajectories. In particular, locally disordered methylation preferentially affected genes associated with stem cell biology and hence could provide fuel for the potential subclonal diversification of leukemic cells. High promoter PDR was associated with shorter failure-free survival independently of other risk factors (Figure 4B)[18] and samples with higher promoter PDR were more likely to have a subclonal driver mutation. Hence, methylation disorder may, together with genetic instability, contribute to the clonal diversification process (Figure 4C). Future studies may elucidate the extent to which locally disordered methylation plays a role in CLL initiation, progression, and therapeutic resistance.

Potential prognostic impact of the CLL genomic features

Cytogenetic evaluation remains the gold standard and basis for the long-standing hierarchical classification of CLL.[21] However, given that

several of the novel CLL-associated gene mutations individually have been reported to hold prognostic significance, Rossi et al integrated mutational and cytogenetic information from a heterogeneous series of 637 CLL patients (with further validation using 370 patients), and found that the addition of molecular information improved prognostication of overall survival (OS) compared with cytogenetics alone.[27] Overall, this schema distinguished 4 subgroups: high-risk (*TP53* and/or *BIRC3* abnormalities), intermediate-risk (*NOTCH1* and/or *SF3B1* mutations and/or del[11q]), low-risk (+12 or normal genetics), and very low-risk (del[13q] only). Similarly, Jeromin et al assessed cytogenetic information with a panel of common CLL-associated mutations in 1160 untreated CLL patients (82.6% of cases from diagnosis).[29] These analyses highlight the concept that refinement of prognostic schema is possible with the addition of gene mutation information. In these studies, the presence of *SF3B1* mutations was confirmed as a predictor of significantly shorter time-to-first therapy (3.8 vs 8 years), even within a multivariate model including unmutated *IGHV* and del(11q).[29] Likewise, the European Research Initiative on CLL recently conducted a multivariate analysis including 774 patients evaluable for time-to-first therapy and confirmed the adverse influence on outcome of mutated *SF3B1*, unmutated *IGHV*, and del(11q), in addition to *TP53* disruption.[28] *SF3B1* mutations further retained its poor prognostic impact on OS, in addition to *TP53* disruption.[29]

A growing body of studies have evaluated the impact of a limited number of recurrent CLL-associated gene mutations (*TP53, NOTCH1,* and *SF3B1*) on outcome in the setting of clinical trials.[32-35] The impact of these mutations has been variable across these studies and likely reflects the dependence of the impact of these alterations on when in the course of disease they are evaluated. For example, Stilgenbauer et al evaluated more than 600 of 817 previously untreated patients enrolled on the German CLL Study Group CLL8 trial, and upon multivariate analysis, found that OS was impacted by *TP53* mutation status but not by *SF3B1* or *NOTCH1* mutations, whereas PFS was adversely affected by *SF3B1* and *TP53* mutations.[33,83] Patients with *NOTCH1* mutations did not benefit from rituximab. By contrast, analyses of the United Kingdom Lymphoma Research Foundation CLL4 trial subjects revealed *TP53* disruption, *NOTCH1* mutation, and *SF3B1* mutations as all significantly impacting OS.[32] Two further analyses of clinical trial cohorts (CLL2H–alemtuzumab for fludarabine-refractory patients and CLL3X–reduced-intensity HSC transplantation in poor-risk patients) failed to show any adverse impact of *NOTCH1* or *SF3B1* mutations on response rate, PFS, or OS after multivariate analysis.[34,35] Further research studies will be required to definitively establish the role of individual driver mutations on the response to specific therapies.

Incorporating evolutionary concepts into schema for CLL prognosis, monitoring, and treatment

The tracking of intratumoral heterogeneity over time can provide critical information on clonal dynamics, which could impact the management of patients. Because the dominant subclones at relapse may be present as minor subclones earlier in disease course, it follows that early detection of these subclones of known high fitness could lead to the contraindication of treatment known to select such clones. One striking example is that of somatic mutations disrupting *TP53*. Small *TP53* mutated subclones identified before treatment appear to anticipate the dominant population at relapse.[84] Presence of mutations leading to *TP53* disruption clearly predicts poor response to frontline chemotherapy. Possibly, patients with subclonal TP53 disruption will respond better to novel potent targeted inhibitors (ie, ibrutinib and idelalisib).[85-87] In a separate example, recent mathematical modeling of the kinetics of ibrutinib resistance has suggested that such resistant clones are present at the time of treatment initiation.[88] Hence, high-resolution molecular characterization before and during therapy has the potential to detect disease relapse in advance of full hematologic evidence of resistance.

From the standpoint of therapy, the presence of intratumoral heterogeneity strongly supports on the one hand personalized therapies, and on the other hand, supports the use of therapies that can target multiple vulnerable nodes simultaneously, whether this is through combination therapy that can include chemotherapy and/or combined targeted inhibition, or even immunotherapy.[89]

Because clonal equilibrium has been linked to stable disease, a provocative approach for the monitoring and treatment of CLL could be efforts to lead to a more stable state (as observed in early stages patients). The assessment and monitoring of clonal heterogeneity might be a useful tool to appreciate both the magnitude and the quality of the residual disease. For example, stabilization of the epigenome, which has been recently reported to fuel clonal evolution and diversity in CLL, is now feasible with newly available agents and has not been standardly tested in CLL, and yet may have an impact on limiting the aggressiveness of progressive evolutionary sweeps. Thus, as an "adaptive therapy" strategy,[90] a key goal would be to maintain subclonal relationships such that the emergence of the more resistant clones is prevented. Another potential therapeutic opportunity is presented by the recent data arguing for the occurrence of cancer-driving events at the level of hematopoietic progenitor cells in CLL. Certainly, these findings may explain why, so far, allogeneic HSC transplantation is the sole strategy that has demonstrated curative potential in CLL.[91,92] Furthermore, active immunotherapy, a now maturing field,[93] has the capacity to impose evolutionary pressure distinct from conventional therapies, and which, if applied early enough in disease course, can modify the natural history of this disease.

Conclusion

The advent of genome-wide sequencing technologies has uncovered the tremendous genetic and epigenetic heterogeneity of CLL. The extent to which these features should be taken into account in the management of patients remains to be seen. The identification of novel genetic drivers, which has led to the characterization of interpatient and intratumoral heterogeneity has been shown to clearly impact clinical outcome. Furthermore, a growing series of longitudinal analyses across the stages of CLL disease has delineated not only the heterogeneous trajectories of clonal evolution but also their close link to therapeutic pressure. At the present time, with the ever-increasing availability of effective chemotherapy, immunotherapy, and agents for the targeted inhibition of key CLL pathways, it is evident that each therapeutic modality provides its own unique mode of selective pressure on a population of CLL cells. Further detailed study in each of these areas using existing or novel (including single-cell approaches) genome-wide technologies, together with functional analyses in vitro and in vivo, will certainly inform us of the relationship between the subclonal architecture in CLL and

nodes of therapeutic resistance, and hence provide the critical knowledge gap required for further developing improved individualized and effective therapies for CLL patients.

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References

1. Goede V, Fischer K, Busch R, et al. Obinutuzumab plus chlorambucil in patients with CLL and coexisting conditions. *N Engl J Med.* 2014;370(12):1101-1110.
2. Furman RR, Sharman JP, Coutre SE, et al. Idelalisib and rituximab in relapsed chronic lymphocytic leukemia. *N Engl J Med.* 2014; 370(11):997-1007.
3. Byrd JC, Furman RR, Coutre SE, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N Engl J Med.* 2013;369(1):32-42.
4. Roberts AW, Ma S, Brander DM, et al. Determination of recommended phase 2 dose of ABT-199 (GDC-0199) combined with rituximab (R) in patients with relapsed/refractory (R/R) chronic lymphocytic leukemia (CLL) [abstract]. *Blood.* 2014;124(21). Abstract 325.
5. Gruber M, Wu CJ. Evolving understanding of the CLL genome. *Semin Hematol.* 2014;51(3):177-187.
6. Wang L, Lawrence MS, Wan Y, et al. SF3B1 and other novel cancer genes in chronic lymphocytic leukemia. *N Engl J Med.* 2011;365(26):2497-2506.
7. Fabbri G, Rasi S, Rossi D, et al. Analysis of the chronic lymphocytic leukemia coding genome: role of NOTCH1 mutational activation. *J Exp Med.* 2011;208(7):1389-1401.
8. Puente XS, Pinyol M, Quesada V, et al. Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature.* 2011; 475(7354):101-105.
9. Quesada V, Conde L, Villamor N, et al. Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia. *Nat Genet.* 2011;44(1):47-52.
10. Ramsay AJ, Quesada V, Foronda M, et al. POT1 mutations cause telomere dysfunction in chronic lymphocytic leukemia. *Nat Genet.* 2013;45(5): 526-530.
11. Fabbri G, Khiabanian H, Holmes AB, et al. Genetic lesions associated with chronic lymphocytic leukemia transformation to Richter syndrome. *J Exp Med.* 2013;210(11):2273-2288.
12. Landau DA, Carter SL, Stojanov P, et al. Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. *Cell.* 2013;152(4):714-726.
13. Kanduri M, Cahill N, Göransson H, et al. Differential genome-wide array-based methylation profiles in prognostic subsets of chronic lymphocytic leukemia. *Blood.* 2010;115(2):296-305.
14. Pei L, Choi J-H, Liu J, et al. Genome-wide DNA methylation analysis reveals novel epigenetic changes in chronic lymphocytic leukemia. *Epigenetics.* 2012;7(6):567-578.
15. Kulis M, Heath S, Bibikova M, et al. Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia. *Nat Genet.* 2012;44(11):1236-1242.
16. Cahill N, Bergh A-C, Kanduri M, et al. 450K-array analysis of chronic lymphocytic leukemia cells reveals global DNA methylation to be relatively stable over time and similar in resting and proliferative compartments. *Leukemia.* 2013;27(1):150-158.
17. Oakes CC, Claus R, Gu L, et al. Evolution of DNA methylation is linked to genetic aberrations in chronic lymphocytic leukemia. *Cancer Discov.* 2014;4(3):348-361.
18. Landau DA, Clement K, Ziller MJ, et al. Locally disordered methylation forms the basis of intratumor methylome variation in chronic lymphocytic leukemia. *Cancer Cell.* 2014;26(6):813-825.
19. Zenz T, Mertens D, Küppers R, Döhner H, Stilgenbauer S. From pathogenesis to treatment of chronic lymphocytic leukaemia. *Nat Rev Cancer.* 2010;10(1):37-50.
20. Malek SN. The biology and clinical significance of acquired genomic copy number aberrations and recurrent gene mutations in chronic lymphocytic leukemia. *Oncogene.* 2013;32(23):2805-2817.
21. Döhner H, Stilgenbauer S, Benner A, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med.* 2000; 343(26):1910-1916.
22. Brown JR, Hanna M, Tesar B, et al. Integrative genomic analysis implicates gain of PIK3CA at 3q26 and MYC at 8q24 in chronic lymphocytic leukemia. *Clin Cancer Res.* 2012;18(14):3791-3802.
23. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA.* 2002;99(24):15524-15529.
24. Lawrence MS, Stojanov P, Mermel CH, et al. Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature.* 2014; 505(7484):495-501.
25. Wan Y, Wu CJ. SF3B1 mutations in chronic lymphocytic leukemia. *Blood.* 2013;121(23): 4627-4634.
26. Landau DA, Stewart C, Reiter JG, et al. Novel putative driver gene mutations in chronic lymphocytic leukemia (CLL): results from a combined analysis of whole-exome sequencing of 262 primary CLL samples [abstract]. *Blood.* 2014;124(21). Abstract 1952.
27. Rossi D, Rasi S, Spina V, et al. Integrated mutational and cytogenetic analysis identifies new prognostic subgroups in chronic lymphocytic leukemia. *Blood.* 2013;121(8):1403-1412.
28. Baliakas P, Hadzidimitriou A, Sutton L-A, et al; European Research Initiative on CLL (ERIC). Recurrent mutations refine prognosis in chronic lymphocytic leukemia. *Leukemia.* 2015;29(2): 329-336.
29. Jeromin S, Weissmann S, Haferlach C, 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):108-117.
30. Cortese D, Sutton L-A, Cahill N, 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): 710-713.
31. Skowronska A, Parker A, Ahmed G, 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):4524-4532.
32. Oscier DG, Rose-Zerilli MJJ, Winkelmann N, et al. The clinical significance of NOTCH1 and SF3B1 mutations in the UK LRF CLL4 trial. *Blood.* 2013; 121(3):468-475.
33. Stilgenbauer S, Schnaiter A, Paschka P, et al. Gene mutations and treatment outcome in chronic lymphocytic leukemia: results from the CLL8 trial. *Blood.* 2014;123(21):3247-3254.
34. Schnaiter A, Paschka P, Rossi M, 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):1266-1270.
35. Dreger P, Schnaiter A, Zenz T, et al. TP53, SF3B1, and NOTCH1 mutations and outcome of allotransplantation for chronic lymphocytic leukemia: six-year follow-up of the GCLLSG CLL3X trial. *Blood.* 2013;121(16):3284-3288.
36. Robbe P, Guièze R, Clifford RM, et al. Mutational landscape of 118 relapsed chronic lymphocytic leukemia clinical trial samples; evidence for a multiple-hit profile using targeted next generation sequencing [abstract]. *Blood.* 2014;124(21). Abstract 1974.
37. Damm F, Mylonas E, Cosson A, et al. Acquired initiating mutations in early hematopoietic cells of CLL patients. *Cancer Discov.* 2014;4(9):1088-1101.
38. Wang L, Shalek AK, Lawrence M, et al. Somatic mutation as a mechanism of Wnt/β-catenin pathway activation in CLL. *Blood.* 2014;124(7): 1089-1098.
39. Ferreira PG, Jares P, Rico D, et al. Transcriptome characterization by RNA sequencing identifies a major molecular and clinical subdivision in chronic lymphocytic leukemia. *Genome Res.* 2014;24(2):212-226.
40. Te Raa GD, Derks IA, Navrkalova V, et al. The impact of SF3B1 mutations in CLL on the

DNA-damage response. *Leukemia.* 2015;29(5):1133-1142.

41. Clifford R, Louis T, Robbe P, et al. SAMHD1 is mutated recurrently in chronic lymphocytic leukemia and is involved in response to DNA damage. *Blood.* 2014;123(7):1021-1031.
42. Burger JA. Nurture versus nature: the microenvironment in chronic lymphocytic leukemia. *Hematology Am Soc Hematol Educ Program.* 2011;2011:96-103.
43. Woyach JA, Furman RR, Liu TM, et al. Resistance mechanisms for the Bruton's tyrosine kinase inhibitor ibrutinib. *N Engl J Med.* 2014;370(24):2286-2294.
44. Messina M, Del Giudice I, Khiabanian H, et al. Genetic lesions associated with chronic lymphocytic leukemia chemo-refractoriness. *Blood.* 2014;123(15):2378-2388.
45. Nowell PC. The clonal evolution of tumor cell populations. *Science.* 1976;194(4260):23-28.
46. Greaves M, Maley CC. Clonal evolution in cancer. *Nature.* 2012;481(7381):306-313.
47. Mullighan CG, Phillips LA, Su X, et al. Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia. *Science.* 2008;322(5906):1377-1380.
48. Ouillette P, Saiya-Cork K, Seymour E, Li C, Shedden K, Malek SN. Clonal evolution, genomic drivers, and effects of therapy in chronic lymphocytic leukemia. *Clin Cancer Res.* 2013;19(11):2893-2904.
49. Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med.* 2012;366(10):883-892.
50. Shah SP, Roth A, Goya R, et al. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. *Nature.* 2012;486(7403):395-399.
51. Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature.* 2012;481(7382):506-510.
52. Wang J, Khiabanian H, Rossi D, et al. Tumor evolutionary directed graphs and the history of chronic lymphocytic leukemia. *ELife.* 2014;3.
53. Klein U, Lia M, Crespo M, et al. The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. *Cancer Cell.* 2010;17(1):28-40.
54. Genovese G, Kähler AK, Handsaker RE, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med.* 2014;371(26):2477-2487.
55. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med.* 2014;371(26):2488-2498.
56. Kikushige Y, Ishikawa F, Miyamoto T, et al. Self-renewing hematopoietic stem cell is the primary target in pathogenesis of human chronic lymphocytic leukemia. *Cancer Cell.* 2011;20(2):246-259.
57. Landau DA, Tausch E, Taylor-Weiner AN, et al. Subclonal driver mutations predict shorter progression free survival in chronic lymphocytic leukemia following first-line chemo(immuno) therapy: results from the CLL8 trial [abstract]. *Blood.* 2014;124(21). Abstract 1938.
58. Knight SJL, Yau C, Clifford R, et al. Quantification of subclonal distributions of recurrent genomic aberrations in paired pre-treatment and relapse samples from patients with B-cell chronic lymphocytic leukemia. *Leukemia.* 2012;26(7):1564-1575.
59. Braggio E, Kay NE, VanWier S, 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):1698-1701.
60. Schuh A, Becq J, Humphray S, et al. Monitoring chronic lymphocytic leukemia progression by whole genome sequencing reveals heterogeneous clonal evolution patterns. *Blood.* 2012;120(20):4191-4196.
61. Ojha J, Ayres J, Secreto C, et al. Deep sequencing identifies genetic heterogeneity and recurrent convergent evolution in chronic lymphocytic leukemia. *Blood.* 2015;125(3):492-498.
62. Landgren O, Albitar M, Ma W, et al. B-cell clones as early markers for chronic lymphocytic leukemia. *N Engl J Med.* 2009;360(7):659-667.
63. Ojha J, Secreto C, Rabe K, et al. Monoclonal B-cell lymphocytosis is characterized by mutations in CLL putative driver genes and clonal heterogeneity many years before disease progression. *Leukemia.* 2014;28(12):2395-2398.
64. Eirew P, Steif A, Khattra J, et al. Dynamics of genomic clones in breast cancer patient xenografts at single-cell resolution. *Nature.* 2015;518(7539):422-426.
65. Patel AP, Tirosh I, Trombetta JJ, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science.* 2014;344(6190):1396-1401.
66. Treutlein B, Brownfield DG, Wu AR, et al. Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. *Nature.* 2014;509(7500):371-375.
67. Landau DA, Carter SL, Getz G, Wu CJ. Clonal evolution in hematological malignancies and therapeutic implications. *Leukemia.* 2014;28(1):34-43.
68. Burger JA, Landau D, Hoellenriegel J, et al. Clonal evolution in patients with chronic lymphocytic leukemia (CLL) developing resistance to BTK inhibition [abstract]. *Blood.* 2013;122(21). Abstract 866.
69. Baylin SB, Jones PA. A decade of exploring the cancer epigenome - biological and translational implications. *Nat Rev Cancer.* 2011;11(10):726-734.
70. Cedar H, Bergman Y. Epigenetics of haematopoietic cell development. *Nat Rev Immunol.* 2011;11(7):478-488.
71. Bichi R, Shinton SA, Martin ES, et al. Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. *Proc Natl Acad Sci USA.* 2002;99(10):6955-6960.
72. Chen S-S, Raval A, Johnson AJ, et al. Epigenetic changes during disease progression in a murine model of human chronic lymphocytic leukemia. *Proc Natl Acad Sci USA.* 2009;106(32):13433-13438.
73. Yuille MR, Condie A, Stone EM, et al. TCL1 is activated by chromosomal rearrangement or by hypomethylation. *Genes Chromosomes Cancer.* 2001;30(4):336-341.
74. Raval A, Tanner SM, Byrd JC, et al. Downregulation of death-associated protein kinase 1 (DAPK1) in chronic lymphocytic leukemia. *Cell.* 2007;129(5):879-890.
75. Claus R, Lucas DM, Ruppert AS, et al. Validation of ZAP-70 methylation and its relative significance in predicting outcome in chronic lymphocytic leukemia. *Blood.* 2014;124(1):42-48.
76. Pallasch CP, Patz M, Park YJ, et al. miRNA deregulation by epigenetic silencing disrupts suppression of the oncogene PLAG1 in chronic lymphocytic leukemia. *Blood.* 2009;114(15):3255-3264.
77. Baer C, Claus R, Frenzel LP, et al. Extensive promoter DNA hypermethylation and hypomethylation is associated with aberrant microRNA expression in chronic lymphocytic leukemia. *Cancer Res.* 2012;72(15):3775-3785.
78. Garding A, Bhattacharya N, Claus R, et al. Epigenetic upregulation of lncRNAs at 13q14.3 in leukemia is linked to the In Cis downregulation of a gene cluster that targets NF-kB. *PLoS Genet.* 2013;9(4):e1003373.
79. Berman BP, Weisenberger DJ, Aman JF, et al. Regions of focal DNA hypermethylation and long-range hypomethylation in colorectal cancer coincide with nuclear lamina-associated domains. *Nat Genet.* 2011;44(1):40-46.
80. Ziller MJ, Gu H, Müller F, et al. Charting a dynamic DNA methylation landscape of the human genome. *Nature.* 2013;500(7463):477-481.
81. Shen H, Laird PW. Interplay between the cancer genome and epigenome. *Cell.* 2013;153(1):38-55.
82. Pujadas E, Feinberg AP. Regulated noise in the epigenetic landscape of development and disease. *Cell.* 2012;148(6):1123-1131.
83. Hallek M, Fischer K, Fingerle-Rowson G, et al; International Group of Investigators; German Chronic Lymphocytic Leukaemia Study Group. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. *Lancet.* 2010;376(9747):1164-1174.
84. Rossi D, Khiabanian H, Spina V, et al. Clinical impact of small TP53 mutated subclones in chronic lymphocytic leukemia. *Blood.* 2014;123(14):2139-2147.
85. Zenz T, Gribben JG, Hallek M, Döhner H, Keating MJ, Stilgenbauer S. Risk categories and refractory CLL in the era of chemoimmunotherapy. *Blood.* 2012;119(18):4101-4107.
86. O'Brien S, Jones JA, Coutre S, et al. Efficacy and safety of ibrutinib in patients with relapsed or refractory chronic lymphocytic leukemia or small lymphocytic leukemia with 17p deletion: results from the phase II RESONATE™-17 trial [abstract]. *Blood.* 2014;124(21). Abstract 327.
87. Sharman JP, Coutre SE, Furman RR, et al. Second interim analysis of a phase 3 study of idelalisib (ZYDELIG®) plus rituximab (R) for relapsed chronic lymphocytic leukemia (CLL): efficacy analysis in patient subpopulations with Del(17p) and other adverse prognostic factors [abstract]. *Blood.* 2014;124(21). Abstract 330.
88. Komarova NL, Burger JA, Wodarz D. Evolution of ibrutinib resistance in chronic lymphocytic leukemia (CLL). *Proc Natl Acad Sci USA.* 2014;111(38):13906-13911.
89. Bachireddy P, Burkhardt UE, Rajasagi M, Wu CJ. Haematological malignancies: at the forefront of immunotherapeutic innovation. *Nat Rev Cancer.* 2015;15(4):201-215.
90. Gatenby RA, Silva AS, Gillies RJ, Frieden BR. Adaptive therapy. *Cancer Res.* 2009;69(11):4894-4903.
91. Michallet M, Sobh M, Milligan D, et al; Chronic Leukemia Working Party of the EBMT. The impact of HLA matching on long-term transplant outcome after allogeneic hematopoietic stem cell transplantation for CLL: a retrospective study from the EBMT registry. *Leukemia.* 2010;24(10):1725-1731.
92. Burkhardt UE, Hainz U, Stevenson K, et al. Autologous CLL cell vaccination early after transplant induces leukemia-specific T cells. *J Clin Invest.* 2013;123(9):3756-3765.
93. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med.* 2011;365(8):725-733.