The mutational signature of chronic lymphocytic leukemia

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Abstract

Advances in next generation sequencing technologies continue to unravel the cancer genome, identifying key biological pathways important for disease pathogenesis and clinically relevant genetic lesions. These studies have provided unprecedented resolution of the cancer genome, facilitating significant advances in the ability to detect many cancers, and predict patients who will develop an aggressive disease or respond poorly to treatment. The mature B-cell neoplasm, chronic lymphocytic leukaemia (CLL) remains at the forefront of these genomic analyses, largely due its protracted natural history and the accessibility to suitable material for study. We now possess a comprehensive view of the genomic copy number mutational landscape of the disease, as well as a detail description clonal evolution, and the molecular mechanisms that drive the acquisition of genomic lesions and more broadly, genomic complexity. Herein, recent genomic insights with associated biological and clinical implications will be reviewed.

Cancer as a genomic disease

Tumourigenesis is a multistep process during which cells acquire a succession of hallmark capabilities including sustained proliferative signalling, evasion of growth suppressors, activation of invasion and metastasis, replicative immortality, induction of angiogenesis, resistance to apoptosis, avoidance of immune destruction and deregulation of cellular energetics [1]. Somatically acquired genomic lesions enable many of these characteristics, presenting as large-scale chromosomal rearrangements, copy number deletions or duplications and sequence base-pair changes. With technological advances over the last 50 years, from molecular cytogenetic techniques to the advent of next generation sequencing (NGS), the scientific community has identifying and characterizing the surfeit of genomic anomalies that contribute to the malignant process, paving the way for improvements in cancer detection, accurate risk-adapted stratification, and the development of targeted therapies for precision medicine.

The mature B-cell neoplasm, chronic lymphocytic leukaemia (CLL) has been a valuable model for the analysis of the cancer genome, placing the disease at the forefront of the genetic analysis of human tumours. This is principally due to its prevalence, its protracted natural history and the accessibility to suitable tumour material for analysis. CLL is the most common haematological malignancy in the Western World [2], predominates in the elderly and is twice as likely to occur in males then females [3]. Patients with CLL have a clonal expansion of mature $CD5^{+}$/ $CD23^{+}$ B-lymphocytes that accumulate in the peripheral blood, bone marrow and infiltrate lymphoid tissue, such as the spleen and lymph nodes [4]. CLL displays extreme clinical heterogeneity, with patients surviving for years with an indolent disease requiring no therapy, whilst others have rapidly progressive disease despite aggressive treatment. This clinical variability is only partially accounted for by clinical staging systems [5, 6]. Two to five percent of patients with CLL will transform to Richters' syndrome (RS), an aggressive B cell lymphoma, with very poor survival and no effective therapeutic strategies for the vast majority of patients. A plethora of biomarkers can help to predict the natural history of the disease. They include the presence of un-mutated Ig heavy-chain variable region genes (*IGHV*) which is associated with an aggressive phenotype compared to patients with mutated-*IGHV* [7] [8], expression of CD38 and ZAP70, and recurrent chromosomal lesions and gene mutations, the relevance of which will be discussed herein. Despite the improved outcomes attributed to risk-adapted patient stratification and the impact of novel therapeutics, CLL remains incurable.

We are now entering a new era in the clinical management of CLL patients, moving from traditional chemotherapy to state-of-the-art targeted treatments. Whilst this shift is the result of greater biological insights, and without reference to advances in genetics, a detailed understanding of the genomic landscape of CLL is very likely to maximize the potential of these new therapeutics. Thirty years of CLL genome research, culminating with recent next generation sequencing (NGS) analysis has provided an accurate and detailed catalogue of the genetic lesions that are prevalent, facilitating a more accurate prediction of the disease

course and identifying underlying genomic lesions that have utility as therapeutic targets. In this review the genomic landscape of CLL will be discussed as well as potential clinical and biological implications.

Somatically acquired copy number abnormalities

Whilst structural chromosomal abnormalities, including balanced translocations and inversions, are the hallmark of many solid cancers and haematological neoplasms, the CLL genome is characterized by copy number changes, particularly the presence of deletion events. The first recurrent cytogenetic abnormality discovered in CLL was trisomy 12 [9, 10], followed by the identification of deletion of 13q14 [11], 11q22 [12], and 17p13 [13]. Twenty years of karyotypic analysis came to fruition with the publication of the 'Döhner' FISH hierarchical prognostic model in 1999, based on the presence of 17p, 11q and 13q deletions, and trisomy 12, with 17p deletion and 13q deletion (as a sole aberration) being the markers associated with the worst and best prognosis, respectively [14]. Whilst this model has been refined in light of gene mutational data, it remains the most accepted and validated genomic prognostic model for patients with CLL.

Deletions of the long arm of chromosome 13 [termed del(13q)] are the most common chromosomal aberrations identified in CLL, observed in 60-80% of patients [11, 15, 16]. The gene content and genomic location of 13q deletions are heterogeneous, though a well-delineated minimally deleted region (MDR), that includes the non-coding genes *DLEU1*, *DLEU2* and the miR-15a/miR-16-1 cluster is consistently lost [17]. The MDR-deleted mouse model developed by Klein et al [18] established a tumour suppressor function for this locus through an important role in controlling B-cell expansion by modulating cell proliferation. However, it is likely that genes beyond the MDR are also important, as larger deletions have been associated with disease progression in murine models and reduced overall survival in patients with larger deletions [15, 16, 19, 20].

Interstitial deletions of the long arm of chromosome 11q23 (termed del(11q)) are found in 10% of patients at diagnosis [14] and approximately 20% of patients requiring treatment [21]. The *ATM* gene is a critical candidate gene on 11q positioned within a well-defined MDR at 11q23. However, it is important to note that deletions are confined to *ATM* or genes within the MDR in only 5% of cases, and the typical 11q deletion results in loss of 100's of genes, suggestive of a pathogenic role for other genes or microRNAs flanking this locus. *BIRC3*, a negative regulator of NF-κB signalling, and the microRNA (miR) cluster that includes miR-34b and miR-34c are likely candidates [22].

Deletions of the short arm of chromosome 17 (termed del(17p)) result in the loss of the crucial tumour suppressor gene, *TP53*. Whilst rare at diagnosis (<5%) [14], more than half of relapse/ refractory patients harbour loss of 17p and it is strongly associated with shorter time to treatment (TTT), overall survival (OS) and resistance to traditional alkylating agents and purine analogues [23]. In addition to deletions, 17p can also be targeted by acquired copy

neutral loss of heterozygosity (cnLOH), that result from a somatic recombination event that duplicated a single mutated *TP53* allele with retained normal diploid copy number [24].

Trisomy 12, the presence of an additional chromosome 12, is detected in 10-15% of cases [9, 25, 26], and whilst the causative genes are still unknown, *MDM2* is over-expressed in these patients [27]. Trisomy 12 preferentially co-exists with additional trisomies [28]; particularly of chromosomes 18 and 19, *NOTCH1* mutations, deletions of 14q, the presence of specific stereotyped immunoglobulin genes and over-expression of key integrins and adhesion molecules [29-35]. Clinically, trisomy 12 confers favourable prognosis, possibly attributable to the infrequent presence of *TP53* deletions or mutations [36].

An abundance of additional recurrent copy number changes have been documented in recent years [37, 38], although their gene targets and clinical relevance are yet to be fully elucidated. The most frequently reported are deletions and duplications of 6q and 2p, respectively. Deletions of 6q have been identified in approximately 5% of patients with CLL [14] but non-overlapping MDRs have made the identification of possible candidate genes quite challenging [39, 40]. Duplication of chromosome 2p also occurs in 5% of early-stage CLL patients [41], although this frequency rises to 28% in later stages of the disease. The two regions most commonly duplicated include the genes *REL, BCL11A, XPO1* and *MYCN* [37, 41]. Other regions targeted by recurrent copy number changes at a low incidence include duplications of 8q24, and deletions of 15q15.1 (4% of cases) and 3p21 (3% of cases), with *c-MYC*, *MGA* and *SETD2* as candidate genes, respectively [40, 42]. Recurrent copy number aberrations in CLL are summarised in **Table 1**.

Genomic complexity and chromothripsis

Genomic complexity, defined by the presence of elevated numbers of chromosomal abnormalities or genomic copy number changes [43, 44], is a feature of a subset of CLL patients' [40, 44-47] with genomic instability. Growing evidence suggests that genomic complexity can predict short overall survival [43, 44], independent of a number of established biomarkers and clinical features [44], including the presence of 17p deletions [48]. Exciting recent evidence suggests that the presence of karyotypic complexity, defined by chromosomal banding analysis, it is a powerful independent predictor of poor response to Ibrutinib [49].

Genomic complexity is often, but not exclusively observed in patients with genomic lesions targeting the *ATM* and *TP53* genes [50]. One hypothesis is that these defects may allow telomeres to shorten below the length at which apoptosis or senescence is normally triggered, thus leading to further telomere attrition and the accumulation of short telomeres [51, 52], enabling uncapped telomeres to fuse, resulting in genomic instability. A number of investigations have shown the impact of telomere length (TL) on the pathophysiology of CLL, including predicted survival, time to treatment and the transformation to Richters' syndrome [53-57]. Evidently, DNA damage can accrue over time, but it is also clear that high-levels of

DNA damage can be acquired rapidly. One example is the catastrophic process termed chromothripsis, evident in approximately 3-5% of human cancers and first identified in a patient with CLL [40, 58]. This process involves genome shattering that occurs during a single mitotic cycle resulting in a pattern of oscillating DNA copy number changes along a single chromosome, or a few chromosomes [58]. Zhang *et al* [59] showed that chromothripsis is the result of a partitioned chromosome(s) in a micronucleus that becomes damaged and is reintegrated into the daughter nuclei [59, 60]. Chromothripsis occurs preferentially in patients with unmutated *IGHV* genes and high-risk genomic aberrations [40], such as mutations in *TP53*, suggesting that a defective DNA damage response is critical to the process of chromothripsis, or the tolerance of the genomic damage [61]. Whilst patients with chromothripsis exhibit both inferior OS and PFS, it is unclear if this is independent of the aforementioned poor-risk genomic lesions [40]. The acquisition of multiple single nucleotide variants can also occur in a single mitotic explosion, termed kataegis [62] has also been observed in the genome of CLL patients. This process drives cytosine-specific mutagenesis, often in regions flanking sites of genomic rearrangement, and can result in up-to several thousand base-pair substitutions occurring rapidly [63].

The mutational landscape of CLL

The development of NGS approaches has provided the opportunity to search the entire cancer genome for sequence alterations with base-pair resolution. To date, information has been amassed on thousands of CLL patients through numerous whole exome and genome sequencing studies [25, 64-71], but it is the seminal studies published in 2015 by Landau *et al* [69] and Puente *et al* (54) that currently provide the most comprehensive depiction of the mutational landscape of CLL. These studies have implicated a number of biological processes in the acquisition of somatic mutations; infidelity of the DNA replication machinery, mutagen exposure, enzymatic modification of DNA or defective DNA damage repair [63]. Both Landau and Puente have identified a number of mutational signatures in CLL; 1) an age-related signature dominated by C>T transitions at CpG sites [69](54); 2) an activation-induced cytidine deaminase (AID) signature detected on *IG* loci (54) and 3) a signature characterised by a high proportion of A>C transitions, specific to *IGHV*-mutated patients [69, 70]. Elevated mutational frequencies are found in *IGHV*-mutated patients as a by-product of normal somatic hypermutation and are likely introduced by error-prone polymerase η [65]. The average mutational burden in CLL of 0.6-0.87 mutations per megabase (Mb) of genomic DNA is low compared to solid tumours, with Landau *et al* and Puente *et al* identifying an average of 15.3 and 26.9 somatic mutations per patient, respectively [25, 70]. Both studies describe a short-list of recurrently mutated genes in CLL and whilst there is significant overlap in the genes identified by these and indeed other NGS studies, there is also a level of discordance, particularly apparent in the rarely mutated genes. For example, of the 36 and 44 recurrently mutated genes identified by Puente and Landau respectively, 22 were implicated in both studies (including *BIRC3*, *CHD2*, *XPO1* and *EGR2*), whilst genes only identified by a single study include *SETD2*, *ARID1A*, *NFKBIE* [70], *KRAS* and *SAMHD1* [69]. These discrepancies are likely

the result of the relatively small size of these patient cohorts and their consequent power to identify rare mutated cancer genes, but could also be attributable to the different cohort composition, different sequencing platforms and bioinformatics pipelines used. Whilst these studies have not identified a unifying mutation shared by all patients, and it is unlikely that such a mutation exists at the genomic level, four genes are recurrently mutated at relatively high frequencies across multiple studies; *TP53*, *ATM*, *NOTCH1* and *SF3B1*. In addition to these more commonly mutated genes, a surfeit of additional mutations are described, most occurring at a frequency of approx. 5%, and lead to the deregulation of eight key cellular pathways; cell cycle regulation, DNA damage response, apoptosis, NOTCH1 signalling, RNA metabolism, NF-kB signalling, chromatin remodelling and BCR signalling (54) **(Figure 1)**. Although it is beyond the scope of this review to detail the plethora of mutations so far detected in CLL, the key recurrently mutated genes with roles in one or more of the above pathways are included herein. It is important to note that whilst mutations in certain genes can be clonal and therefore present in the entire cancer cell population, many of these recurrent mutations are more frequently found in only a small 'sub-clonal' population of cells. As discussed later, the clonality of a mutation can reflect the temporal order of its acquisition in the disease continuum. The application of deep-sequencing approaches has provided an opportunity to detect low-level subclonal mutations present in as little as 0.3% of cancer cells [72] beyond the resolution of standard Sanger sequencing [73].

Cell cycle, Apoptosis and DNA damage.

CLL is a disease characterised by an accumulation of abnormal mature B-cells, caused in part by the deregulation of the carefully balanced processes of cellular proliferation, differentiation and apoptosis in response to DNA damage. Mutations in the *ATM* and *TP53* genes, key components of a number of these of critical pathways are fundamental to CLL pathogenesis. Between 30-40% of del(11q) patients harbour a mutation of the residual *ATM* allele, whilst a further 11% will have an *ATM* mutation without concomitant deletion, making it one of the most frequently mutated genes in CLL [12, 71, 74] **(Figure 1B)**. WES studies of matched pre-treatment and relapse samples show that the deletion event often precedes the mutation, which provides a fitness advantage enabling subsequent growth of co-existing clones harbouring genetic disruption of the remaining allele [69, 75]. *ATM* mutations are associated with reduced survival, a clinical effect that might be accentuated by loss of the wild-type allele; CLL cells with functional loss of *ATM* have defective responses to DNA damage [76] and an associated reduced outcome is seen in patients treated with chemotherapy [71, 74, 77, 78].

Using traditional molecular screening approaches, *TP53* mutations can be identified in approximately 9% of untreated CLL cases, with loss of the second allele seen the majority of mutated cases **(Figure 1B)** [79]. The presence of a *TP53* mutation, thought to be an event preceding clonal evolution [80], is a strong independent marker of adverse survival and a powerful predictor of poor response to chemo- immunotherapy, and therefore has direct implications on treatment decisions [72]. In clinical practice, the analysis of both 17p deletion

and *TP53* mutation status is recommended by a number of international bodies including the European Research Initiative on CLL (ERIC), the British Committee for Standards in Haematology (BCSH), the National Comprehensive Cancer Network (NCCN) and the European Society for Medical Oncology (ESMO). Most *TP53* mutations are missense and located within the DNA binding domain, leading to impaired transcriptional activation of *TP53* response genes, with far-reaching consequences to a diverse number of cellular processes including cell cycle control, DNA damage response and apoptosis. Six sequence hot-spots are mutated in up to 20% of patients [80-85]. Low-level sub-clonal mutations, beyond the resolution of Sanger sequencing (down to 0.3% of the cancer cells) reside in approximately 9% of untreated CLL, mutations that expand to become more clonal in sequential samples from patients that ultimately relapse [72]. Patients with these sub-clonal *TP53* mutations show the same clinical phenotype and poor survival [72, 75] as patients with clonal mutations and carry a higher risk of mutation selection by therapy. Identifying *TP53* defects early in their evolution may enable improved clinical management of high-risk CLL [86]. It is important to mention a rare subset of early-stage CLL patients with mutated *IGHV* genes and *TP53* abnormalities do exhibit a more stable disease course [87].

Other recurrent mutations of note include those targeting *RPS15,* which encodes a component of the 40S ribosomal subunit, recently identified in 20% of CLL patients relapsing post therapy (**Figure 1B**). *RPS15* mutations are early clonal events, associated with reduced survival, the functional consequences of which is defective p53 stability and increased degradation [88]. Mutations in the nuclease *SAMHD1*, occur in 3% of patients at diagnosis [89] (**Figure 1B**), are enriched in therapy-refractory patients and are often concomitant with chromosome 20 cnLOH, the chromosome on which the gene resides. Preliminary data suggests that these mutations may promote leukemogenesis through deregulation of DNA repair [90]. *POT1*, a component of the shelterin complex, plays a critical role in the protection of telomeres. Mutations have been identified at frequencies of approximately 3% at disease presentation [91, 92] (**Figure 1B**) rising to 8.1% in patients receiving chlorambucil based therapy, where they were associated with a shorter survival [93]. In vitro, *POT1*-mutated cell lines [91] have numerous telomeric and chromosomal aberrations suggesting that these mutations maybe promote genomic instability, an observation that has not been corroborated in primary CLL tumours [92].

Notch signalling

The Notch signalling pathway regulates a number of essential cellular processes including proliferation and differentiation. Mutations in *NOTCH1,* encoding a transmembrane receptor protein, were first identified in two patients with a heterozygous 2bp frame-shift deletion (ΔCT7544-7545, P2515Rfs*4) in exon 34. This recurrent 2bp mutation accounts for 85-90% of *NOTCH1* mutations [65] and introduces a premature stop codon within the PEST domain of NOTCH1 [64-67] [94]. This results in increased stability of an activated intracellular Notch1 isoform [65, 95], which confers cell survival and apoptosis resistance [96], in part by sustaining

expression of the anti-apoptotic protein, Mcl-1, and promoting the activity of the key translational regulator, eIF4E [97]. Mutation incidence increases with disease stage; approximately 3% of MBL patients [98], increasing to 10% of CLL's at diagnosis, >20% of patients with chemo-refractory disease [64, 99], and 30% of patients who have transformed to Richters' syndrome [30] (**Figure 1B).** There is a significant enrichment of *NOTCH1* mutations in unmutated *IGHV* patients, where they are associated with increased levels and signalling of surface Immunoglobulin M (sIgM) [100], *ZAP70* expression, trisomy 12 [30] and significant upregulation of Insulin Growth Factor 1 Receptor (*IGF1R*); a gene shown to regulate normal cellular proliferation and contribute to transformation and proliferation of malignant cells [101]. Compared to wild-type cases, *NOTCH1*-mutated cases have progressive disease, significantly shorter survival [30, 75, 102], and demonstrate resistance to the anti-CD20 monoclonal antibody, Rituximab [103], a phenotype thought to be associated with the low CD20 levels and dysregulation of HDAC-mediated epigenetic repression of CD20 expression observed in *NOTCH1* mutated CLL [104]. The mutational frequency of *NOTCH1* is higher in CLL lymph nodes than in matched peripheral CLL B-cells (24%) and the Notch pathway is frequently activated in lymph node cases independently of *NOTCH1* mutational status, suggesting the existence of other initiating mechanisms, such as ligand activation [105]. Mutations in other key proteins in the Notch signalling pathway have also been detected at low frequencies, for example, *FBXW7*, which targets activated *NOTCH1* for degradation, is mutated in ~2.5% patients and may provide another mechanism for activated Notch signalling [106] (**Figure 1B**).

Processing of RNA

Alternative splicing is the cellular mechanism that achieves successful transcription and guarantees the functional diversity of protein species. *SF3B1*, a critical component of the RNA splicing machinery, involved in recognition of the branch point sequence during selection of the 3'splice site, is recurrently mutated in CLL [66, 67, 107]. Whilst analysis of the functional consequences of *SF3B1* mutations is ongoing, it is clear that these mutations do contribute to aberrant splicing [66, 67]. SF3B1 mutants proteins induce anomalous 3' splice site selection resulting in aberrantly spliced transcripts that are sensitive to nonsense mediated decay and therefore downregulation at the mRNA and protein level [108]. Initial studies showed that in *SF3B1* mutated patients, a highly-expressed truncated *FOXP1* transcript, *FOXP1w*, is transcribed that lacks two putative PEST domain sequences involved in protein degradation [67]. Transcriptional profiling with RNA sequencing identified 79 splice junctions that were only present in *SF3B1* mutant patients. Among these, *ATM* was expressed from an alternative 3' splice site specific to *SF3B1* mutants that generated a truncated protein lacking key functional domains. [109]. Functional analyses showed that *SF3B1* mutated samples are associated with increased DNA damage and/or aberrant transcriptional and apoptotic responses to DNA damaging agents, independent of *ATM/TP53* lesions, providing a link between splicing factors and DNA damage response [110]. Other genes with altered splicing in *SF3B1* mutant cells included genes involved in B-cell differentiation, Hippo signalling and

NF-κB activation [109]. *SF3B1* mutations occur in 3-10% of newly diagnosed patients with increasing incidence of up to 20% in relapsed/refractory CLL [106, 111] **(Figure 1B)**. They are associated with advanced stage, *IGHV* un-mutated disease, in cases with no *TP53* abnormality, 11q23 deletions, with the presence of stereotyped *IGHV* usage (subset #2), short TTT and OS independent of other prognostic variables and treatment refractoriness [30, 66, 67, 75, 107, 112, 113]. These observations strongly suggest a role for aberrant spliceosome function in disease evolution and identify the spliceosome as a potential therapeutic target. Indeed, mutations in *SF3B1* have been shown to confer sensitivity to the splicing modulator sudemycin, which produces a collaborative anti-tumour effect with the btk inhibitor, ibrutinib [114], and the SF3B1 inhibitor spliceostatin A has been shown to induce apoptosis in CLL cells via the downregulation of Mcl-1, particularly in combination with Bcl-2/Bcl-XL antagonists [115].

Another gene involved in RNA processing is *XPO1,* which encodes the nuclear exporter Exportin-1, responsible for controlling the directional exodus of over 200 proteins and RNA species from the nucleus to the cytoplasm [116], including tumour suppressor proteins (TSP), cell cycle inhibitors and growth receptor proteins, such as p53 and IkB. Mutations of *XPO1* have been identified in 4.6% of IGHV-unmutated CLL cases [65] **(Figure 1B)** and result in increased levels of Exportin-1 in malignant cells results in the externalisation of key TSP, preventing them from responding to DNA damage and regulating cell cycle, proliferation and apoptosis [116]. Blocking *XPO1* mediated nuclear export of key proteins may restore apoptotic pathways and chemosensitivity, therefore, the development of low-toxicity, small-molecule XPO1 inhibitors may provide a new approach to treating cancer [116].

NFKB signalling

NFκB signalling regulates many cellular processes including cell cycle progression, differentiation and apoptosis [117]. In CLL, recurrently mutated genes have been identified in in both the canonical and non-canonical NFκB signalling pathways, where they can result in activation signalling [118, 119]. One such gene, *BIRC3* cooperates in the protein complex that negatively regulates *MAP3K14*, the central activator of non-canonical NFκB signalling. As *BIRC3* is located at 11q22.2, ~6Mb centromeric to the *ATM* gene locus it is often deleted in 11q deleted patients [74, 119]. Indeed, trying to disentangle the prognostic relevance of *BIRC3* in 11q-deleted CLL is problematic, and current data supports ATM mutational status as the critical driver of the poor prognosis in 11q-deleted CLL [74]. *BIRC3* mutations have been shown to alter protein function by removal of the C-terminal RING domain, which is essential for proteosomal degradation of MAP3K14, and functional analyses show a constitutive non-canonical NF-KB activation in these patients. *BIRC3* mutations are rare at disease presentation (~3% of patients) (**Figure 1B**), but are found in up to 40% of fludarabine-refractory patients [119]. *NFKBIE*, which encodes NFκB-inhibitor epsilon (IKBE), an inhibitor protein that ensures the temporal control of NFκB activation, is another example of a negative regulator of NFκB in B cells that has recently been identified as a target for recurrent mutations in up to 10% of CLL cases [120, 121] **(Figure 1B)**. A 4-bp truncating mutation is frequently observed (6.5% of

cases). Functional studies have shown that patients with this mutation had lower IKBE expression, decreased IKBE-p65 interactions and increased nuclear p65 levels, and constitutive NFκB activity. *NKFBIE* mutations predominate in U-CLL and are also highly enriched in poor prognostic stereotyped subsets, potentially contributing to adverse prognosis [120].

B-Cell Signalling

Signalling through the B-cell receptor (BCR) is critical to controlling survival, proliferation, differentiation and cell death in normal and malignant B lymphocytes [122]. *MYD88* (myeloid differentiation primary response 88), a crucial adaptor of the Toll-like receptor (TLR) complex, is mutated in approximately 3% of CLL cases **(Figure 1B)**. Upon TLR ligand binding, a homo-dimer of MYD88 is recruited to the receptor, forms a complex with IRAK4, activates IRAK1 and 2, and ultimately leads to TRAF6 activation, phosphorylation of IκBα and activation NF-κB [123]. In CLL, the recurrent L265P mutation provides constitutive activation of NF-κB activity, by imposing MYD88-IRAK signalling even in the absence of ligand receptor binding [65]. *MYD88* mutations are exclusive to cases with mutated *IGHV* genes, are enriched in patients with isolated deletion of chromosome 13q [113] and appear to identify a sub-group of patients with low expression of CD38 and ZAP70, and improved survival [124]. However, the clinical impact of *MYD88* mutations remains controversial, as conclusive evidence demonstrating independent prognostic significance is lacking [125]. *EGR2,* a transcription factor activated by B-cell stimulation is mutated in 8% (**Figure 1B**) of advanced stage CLL and is associated with reduced overall survival. *BRAF* mutations may play a significant role in fludarabine-sensitivity and are associated with reduced time to first treatment [70, 121, 126].

Chromatin Modification

The dynamic remodelling of chromatin structure is an essential component of the appropriate regulation of gene transcription as it permits regulatory proteins access to the condensed genomic DNA [127]. The chromatin remodeller, *CHD2*, is recurrently mutated in 5.3% of patients (**Figure 1B**), with mutated individuals more likely to have unmutated *IGHV* genes [128]. Most mutations are truncating or target functional domains supporting a tumour suppressor role for *CHD2* and functional analyses demonstrate that mutations alter the nuclear distribution of *CHD2* and the proteins association with actively transcribed genes [128]. Histone methyltransferases (HMTs) are essential epigenetic regulators of chromatin modification and recurrent mutations targeting such genes have only recently been documented in CLL [70]. *SETD2* is non-redundantly responsible for the trimethylation of lysine 36 on histone 3 (H3K36me3), one of the major chromatin marks associated with active transcription. Rare but recurrent mutations in up to 4% (**Figure 1B**) of patients have recently been identified as early loss-of-function events in CLL pathobiology linked to aggressive disease [42, 69, 70].

Non-coding mutations

In 2015, the CLL Spanish Consortium performed the first sequencing study to provide evidence of the functional and clinical importance of mutations located outside of protein coding sequence in CLL patients. They identified a second hotspot for *NOTCH1* mutations located in the 3'UTR of the gene in 2.5% of patients, resulting in an aberrant splicing event between a cryptic donor site in exon 34 and a newly created acceptor site in the 3'UTR, resulting in loss of the final 158 coding bases [70]. As with mutations within exon 34, NOTCH1 is constitutively activated and patients exhibit similarly reduced survival [70]. In addition, the authors describe a second non-coding mutation, resulting in deregulation of *PAX5*. *PAX5,* which encodes a B-cell lineage-specific activator protein is expressed only in early stages of B-cell differentiation [129]. It is well established as an oncogene and is often the target of somatic hypermutation, deletions, point mutations or gene fusion events in various b-cell malignancies [130]. Mutations located within a telomeric enhancer element, 330Kb from the *PAX5* locus, were identified in numerous B-cell neoplasms, including CLL (10%), diffuse large b-cell lymphomas (29%) and follicular lymphoma (23%). Functionally these mutations were shown to reduce *PAX5* expression, and were the only recurrent mutation in a subset of patients, suggesting that these mutations may contribute to disease pathophysiology [70].

Clonal Evolution

Genomic heterogeneity does not only exist between tumours from different patients (inter-tumour) but also within an individual's cancer (intra-tumour). CLL, like any tumour, can contain many genetically unique subclonal populations of cancer cells that provide the tumour with a reservoir of cells with diverse biological properties. This cellular plasticity is fertile ground for Darwinian selection, driven by the tumour microenvironment and the pressure of therapy, that will select cellular populations with favourable biological traits, thereby fuelling disease relapse. In CLL, FISH analysis and SNP arrays have shown the acquisition of genomic aberrations during disease course, suggesting that the disease at relapse can be genetically distinct from the dominant clonal population at diagnosis [131-135]. However, it is the application of NGS to anatomically or temporally distinct cancer specimens from the same CLL patient that have definitively shown the anatomy of clonal expansion [136]. Two major patterns of clonal evolution which can occur either rapidly or gradually [137] have been identified; 1) linear evolution, which is characterised by the maintenance of a founder clone with successive acquisition of new mutations; and 2) a more complex branching anatomy, defined by competition between different cancer sub-clones that evolve in parallel [138]. In addition, expanding populations, where all mutations are more dominant at later time points [139] and convergent evolution, where independent lesions in the same genes are acquired in different subclones [138], have been described in CLL.

Landau and colleagues [25], proposed accumulation of passenger events prior to the acquisition of recurrent driver mutations (e.g. del(13q), tri12 and *MYD88* mutations) with subsequent malignant transformation. Finally, in the process of disease progression, late sub-

clonal driver mutations expand and target cancer genes including *ATM*, *TP53* and *RAS.* They also showed an elevated rate of clonal evolution in patients receiving treatment, and importantly link the presence of sub-clones to adverse clinical outcome [25]. Subsequent studies extended these findings [139, 140], including evidence of clonal heterogeneity between different anatomical compartments (lymph node and peripheral blood) and proof of principle that mutations in the LN expand in the PB at relapse following positive selection by therapy [141]. A noteworthy case study by Rose-Zerilli and colleagues, described a patient with mutated immunoglobulin genes at diagnosis, who developed a fatal *IGHV*-unmutated CLL clone years later, not evident at diagnosis with traditional molecular approaches, but detectable with modern NGS approaches. These studies support the model that low-level subclonal mutations present in early stage disease can anticipate the evolutionary course of the disease [69], thus impacting on clinical management.

Whilst these studies have provided powerful insights into clonal dynamics, they have focused on the analysis of DNA extracted from bulk tumours, containing millions of cells and a multiplex of sequences derived from all CLL sub-clones. As previously mentioned, by accounting for tumour purity and local copy number changes, computational approaches can only *infer* phylogenetic analysis, and cannot completely resolve the genetic structure of complex sub-clonal populations. This results in simplifying the intra-cellular genomic architecture of a patient's tumour, with limited resolution to identify rare sub-clonal populations. Recent technological developments are beginning to realize the accurate analysis of the cancer genome at the single cell level, but have not been comprehensively applied to the study of CLL to date. These studies are needed to investigate the tumoural sub-clonal architecture in unprecedented depth and will allow tangible links between genetic and disease biology, identifying key clinically-relevant cell populations and pushing forward precision medicine. Single cells can be isolated using modern flow cytometry to distribute into multi-well PCR plates for down-stream analysis, or by the use of microfluidic approaches, which in addition to the cell isolation, permit reaction chambers for further DNA and/or RNA analysis. Both approaches would be straight forward for the analysis of circulating CLL B-cells, but would likely be more problematic for the analysis of cells in the lymph-node or spleen.

Novel genetic mutations in clinical practice

As previously noted in this review, international guidance committees only recommend the analysis of TP53 lesions at a time when a patient requires treatment. However, there is growing interest from clinicians, to screen patients for some of these newer gene mutations, as their clinical importance is now well established. Indeed, international harmonization is underway in this area, such as NGS studies under the direction of the European Research Initiative on CLL (http://www.ericll.org/). The growing acceptance that these mutations have clinical utility comes from the analysis of large retrospective and prospective cohorts. Rossi and colleagues proposed a prognostic algorithm through the integration of gene mutations and chromosomal abnormalities [142]: 1) high-risk patients with either *TP53* defects and/or

BIRC3 disruption; 2) intermediate-risk, harbouring *NOTCH1* and/or *SF3B1* mutations and/or del(11q); 3) low-risk, harbouring trisomy 12 or a normal profile; and perhaps most importantly 4) a very low-risk group with del(13q) only, whose survival did not differ from that of a matched general population. This model adds significantly to the 'Döhner' model [14], due to the co-existence of poor-risk gene mutations in low-risk groups defined purely based on FISH. Subsequent studies have confirmed elements of such a model [106, 143], but further research is required to clearly define the prognostic importance of these lesions, probably through the analysis of many 1000s of cases, spanning the natural history of CLL, coordinated through international collaborative networks. In 2016, Nadeu et al [75] provided further evidence that low-level sub-clonal mutations (down to 0/3% allelic frequency) are associated with reduced survival. They correlated both clonal and subclonal *NOTCH1* mutations with a shorter TTT and a shorter OS in patients with either clonal or subclonal *TP53* mutations. Most *TP53* mutated clones expanded post therapy.

The analysis of randomized clinical trials cohorts, can account for the highly heterogeneous natural history of CLL and the often-serendipitous date of initial diagnosis, and establish the predictive and prognostic relevance of novel biomarkers. For patients receiving first-line therapy, the UK CLL4 trial showed that whilst *TP53* alterations are the most powerful marker of reduced survival, *NOTCH1* and *SF3B1* mutations have added independent prognostic value conferring outcome similar to del(11q) cases [30]. For immune-chemotherapy, the German CLL8 study showed that *NOTCH1* mutations identify patients with decreased benefit from the addition of rituximab to FC [144]. In the CLL2H study of previously treated patients, *NOTCH1* mutations independently identified a group of patients with superior progression free survival [145]. This observation supports the hypothesis that the clinical impact of these gene mutations is likely to be therapy dependant, as a given treatment will exert a specific selective pressure on the malignant clone. For example; *POT1* mutations were associated with reduced OS, and *KRAS* mutations with a poor response to treatment, in patients receiving chlorambucil –based frontline therapy [93]. Furthermore, resistance to the BCR kinase inhibitor ibrutinib is already emerging, particularly in the context of patients with poor-risk cytogenetics, that harbor *BTK* mutations at position 481, abrogating covalent binding of ibrutinib, and activating mutations in a downstream enzyme, PLC**g**2, both found in patients with acquired resistance to ibrutinib [146, 147].

The translation of these recent genomic discoveries into the improvement of patient care must be the ultimate aim. Perhaps re-sequencing panels might be an attractive approach, and several studies have aimed to provide preliminary insights into the application of this technology to improve the management of patients with CLL [148]. A re-sequencing panel is also feasible for the detection of recurrent copy number changes, but would struggle to reliably detect cases with chromothripsis. The detection of low frequency variants in samples with heterogeneous sub clonal populations, low sample purity, or in a background of polymorphisms and random sequencing errors which can vary from ~1% to ~0.05%, will depend on factors such as the read length, use of base-calling algorithms and the type of

variants detected [149]. Increased sequencing depth, advances in base calling, the assignment of unique identifiers to each template molecule [150] and computational algorithms (e.g. THetA2 [151]) will contribute to the faithful detection of low frequency mutations [149, 150]. In addition, management, processing and knowledge extraction of the huge amounts of data generated by current sequencing experiments requires careful consideration, to which the development of standardized protocols, tools, and benchmarks is central [152]. The aforementioned ERIC initiative aim to provide the CLL community with some early guidance on platform choice and approaches to implementation. A re-sequencing platform would provide the capacity for an appropriate number of clinically-relevant genes or genomic regions, and would be an excellent balance between suitable genome coverage, and relatively low financial costs, which would be required for health service implementation whilst providing the deep sequencing capacity for the identification of sub-clonal mutations. Furthermore, this would simplify bioinformatics analysis, reducing the requirements for costly high-performance computing, whilst simplifying interpretations by minimizing incidental genetic findings. It will be important to understand how accurate PCR amplicon-based re-sequencing approaches are at interrogating copy number changes, and perhaps a hybridisation/ capture-based re-sequencing platform will be a more sensitive technique for identifying relevant copy number changes and somatic mutations simultaneously. At disease presentation, the identification of deletions of 13q, 11q, 17p and trisomy 12, with mutations in *NOTCH1, SF3B1, TP53*, *ATM* and *BIRC3* are likely to aid risk-adapted stratification. At a requirement for treatment and during disease monitoring post-therapy, deletions and/or mutations of *ATM*, *TP53* and *NOTCH1*, and genomic lesions in genes and pathways targeted by specific therapies will help guide treatment and monitor response. However, further gene discovery and the development of new single agent or combined treatments, will likely mean that the list of clinically relevant genes will evolve.

Conclusions and future perspectives

Driven by advancing technology, more than three decades of genomic research has resulted in an unbridled view of the CLL genome. However, the next phase of genomic research is just beginning; Significant research is needed that will not only catalogue the complete spectrum of somatically-acquired DNA lesions in CLL, but will realize their clinical and biological importance across the full natural history of the disease. Such approaches are outlined in **Figure 2**. Exactly what will the impact of these observations be for a patient with CLL? Well, these defects may have a rather limited impact on a patient at diagnosis, but will have utility for differential diagnosis of other mature B-cell malignancies. Predicting an aggressive disease course, transformation and poor response to therapy, as well as the molecular monitoring of relapse will have significant implications for clinical management. Indeed, CLL will remain a disease where models of ‘precision medicine’ can be developed, models that are likely relevant for other tumours. In conclusion, the research community must continue in its pursuit for a more detailed understanding of the molecular pathogenesis of CLL that has the potential to highlight new routes to improving patient care.

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HP and JCS wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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Table 1. Recurrent copy number changes in CLL.

Gene name	Prevalence (%)	Principle candidate genes	Other candidate genes	Reference
del(13q)	60-80	*miR-15a/16-1, DLEU2*	*RB1, DLEU7*	[11, 15, 16]
del(11q)	10-20	*ATM*	*BIRC3, MRE11, H2AFX*	[21]
del(17p)	5-50	*TP53*	-	[23]
Trisomy 12	10-15	Unknown	-	[9]
del(6q)	5	Unknown	-	[39]
dup(2p)	5-28	*REL, BCL11A, XPO1*	*MYCN*	[37, 41]
dup(8q)	5	*CMYC*	-	[40]
del(15q)]	4	*MGA*	-	[40, 153]
del(3p)	3	*SETD2*	-	[42]

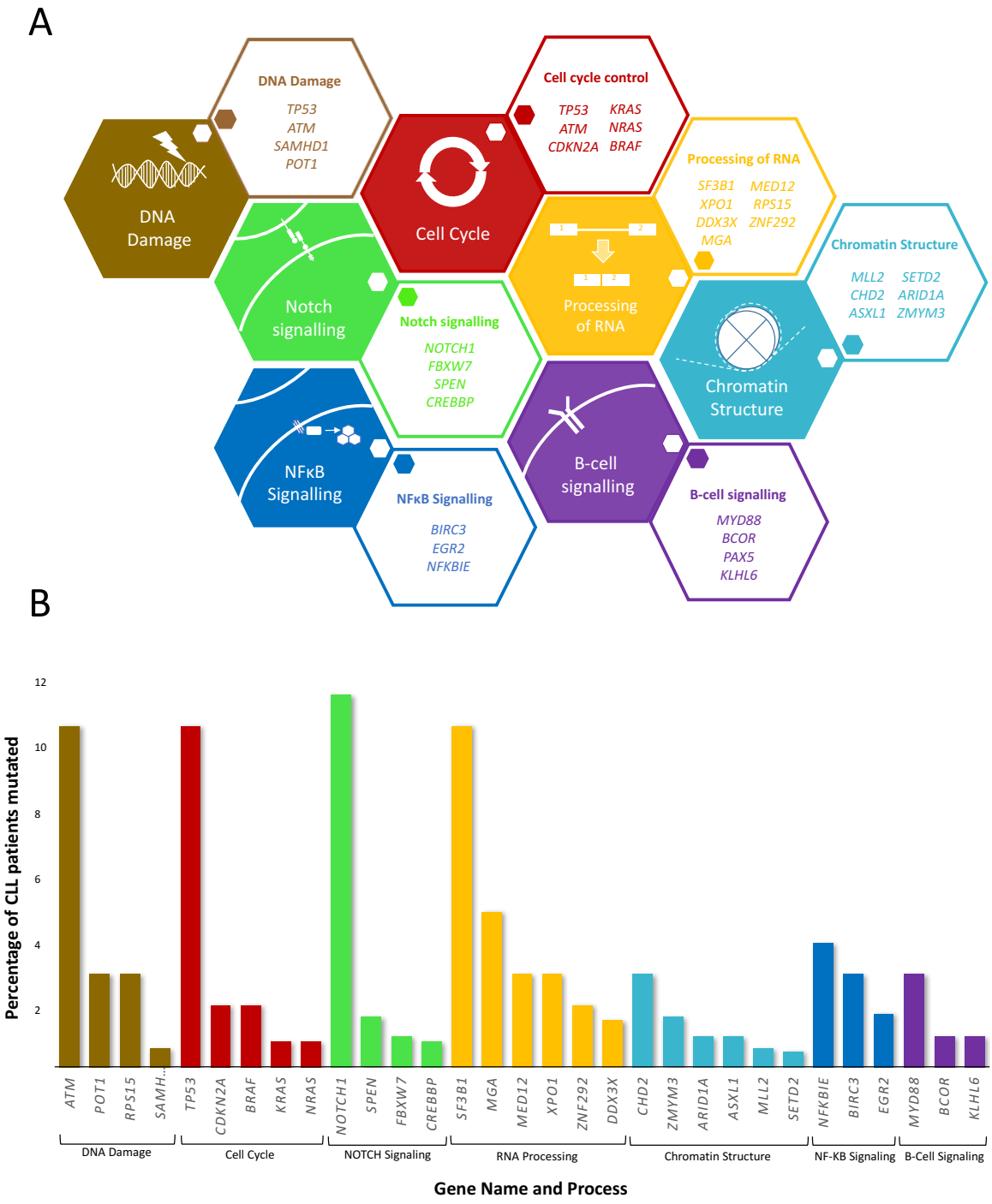

Figure 1. Mutational frequencies in key CLL genes and pathways. (A) The key biologically-relevant genes recurrently mutated in CLL, and the pathways and processes to which they contribute. Genes with functional diversity are represented in the pathway to which they most significantly contribute. (B) Recurrently mutated genes in CLL. The mutation data was obtained from the Sanger Institute Catalogue Of Somatic Mutations In Cancer web site, http://cancer.sanger.ac.uk/cosmic [154]. Each gene is coloured to identify the biological pathway or process to which it contributes, as in (A).

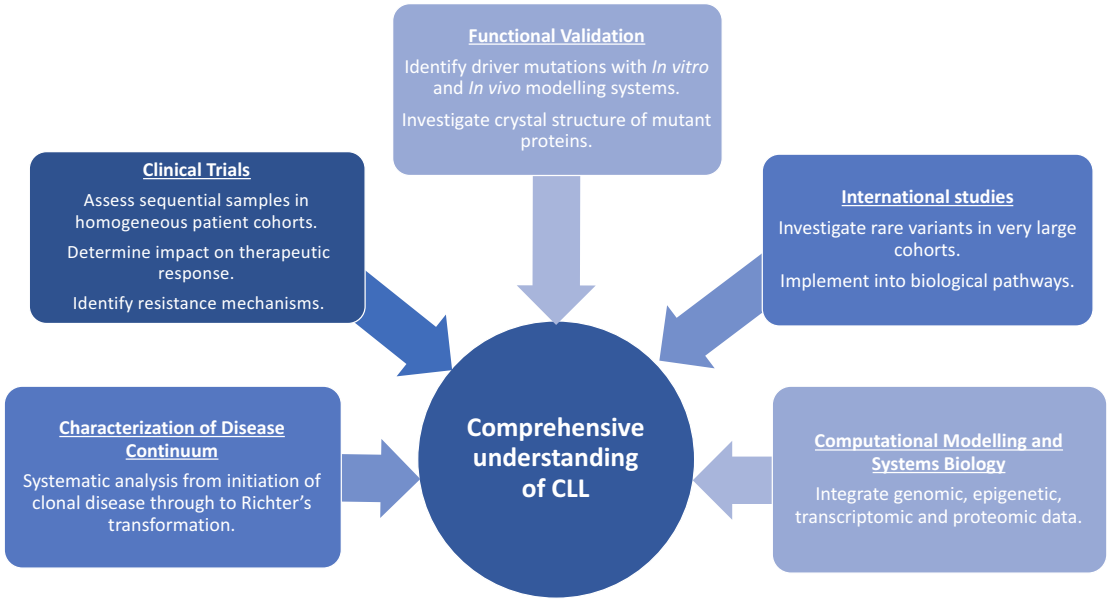

Figure 2. Future perspectives. An overview of the research projects requisite to completely understand biological and clinical relevance of genomic lesions in CLL.