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The molecular map of CLL and Richter's syndrome

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ABSTRACT

Clonal expansion of B-cells, from the early stages of monoclonal B-cell lymphocytosis through to chronic lymphocytic leukemia (CLL), and then in some cases to Richter's syndrome (RS) provides a comprehensive model of cancer evolution, notable for the marked morphological transformation and distinct clinical phenotypes. High-throughput sequencing of large cohorts of patients and single-cell studies have generated a molecular map of CLL and more recently, of RS, yielding fundamental insights into these diseases and of clonal evolution. A selection of CLL driver genes have been functionally interrogated to yield novel insights into the biology of CLL. Such findings have the potential to impact patient care through risk stratification, treatment selection and drug discovery. However, this molecular map remains incomplete, with extant questions concerning the origin of the B-cell clone, the role of the TME, inter- and intra-compartmental heterogeneity and of therapeutic resistance mechanisms. Through the application of multi-modal single-cell technologies across tissues, disease states and clinical contexts, these questions can now be addressed with the answers holding great promise of generating translatable knowledge to improve patient care.

Introduction

The natural history of cancer can be understood through the examining drivers of evolution, and the processes of mutation and selection [1,2]. The acquisition of somatic mutations, as a consequence of endogenous or exogenous processes, leads to cellular diversification [3-6]. Selection describes the ability of cells, in the context of their environment, to out-compete relatives as a result of these heritable somatic features [1,7,8]. Cancer is formed of cells referred to as clones, that derive from a common ancestor and have expanded as a result of acquired mutations that confer a survival advantage [9,10]. Genes which harbor such mutations are termed cancer driver genes [10-13]. As well as furthering our knowledge of cancer biology, the study of cancer evolution and clonal selection has implications for our understanding of disease progression, resistance and relapse [14].

The natural history of clonal expansion of B-cells, from the early stages of monoclonal B-cell lymphocytosis (MBL) through to chronic lymphocytic leukemia (CLL) and then to the aggressive lymphoma Richter's syndrome (RS) provides a model of cancer evolution, notable for the marked morphological transformation and distinct clinical phenotypes [15-17]. CLL is the most common leukemia in economically developed countries and over successive decades advances in therapy have resulted in improvements in survival [18]. This contrasts with RS, which occurs in only 2% to 9% of CLL cases, but remains associated with poor prognosis with a median overall survival of less than 1 year [19]. Hence, there is an urgent need to understand the evolutionary processes that drive transformation of CLL to RS to improve patient outcomes.

The long natural history combined with the ease of sampling has allowed CLL to be at the forefront of genomic characterization and evolutionary studies in cancer. A comprehensive analysis of the RS genome however, has only recently been attainable through international initiatives to collect sufficient numbers of appropriate tissue, combined with the development of analytic tools for CLL and RS clonal deconvolution. Here, we provide a unified summary of the molecular and genetic features of CLL and RS including insights into the mechanisms underpinning transformation of CLL to RS and their potential for clinical translation (Fig. 1) [20-23].

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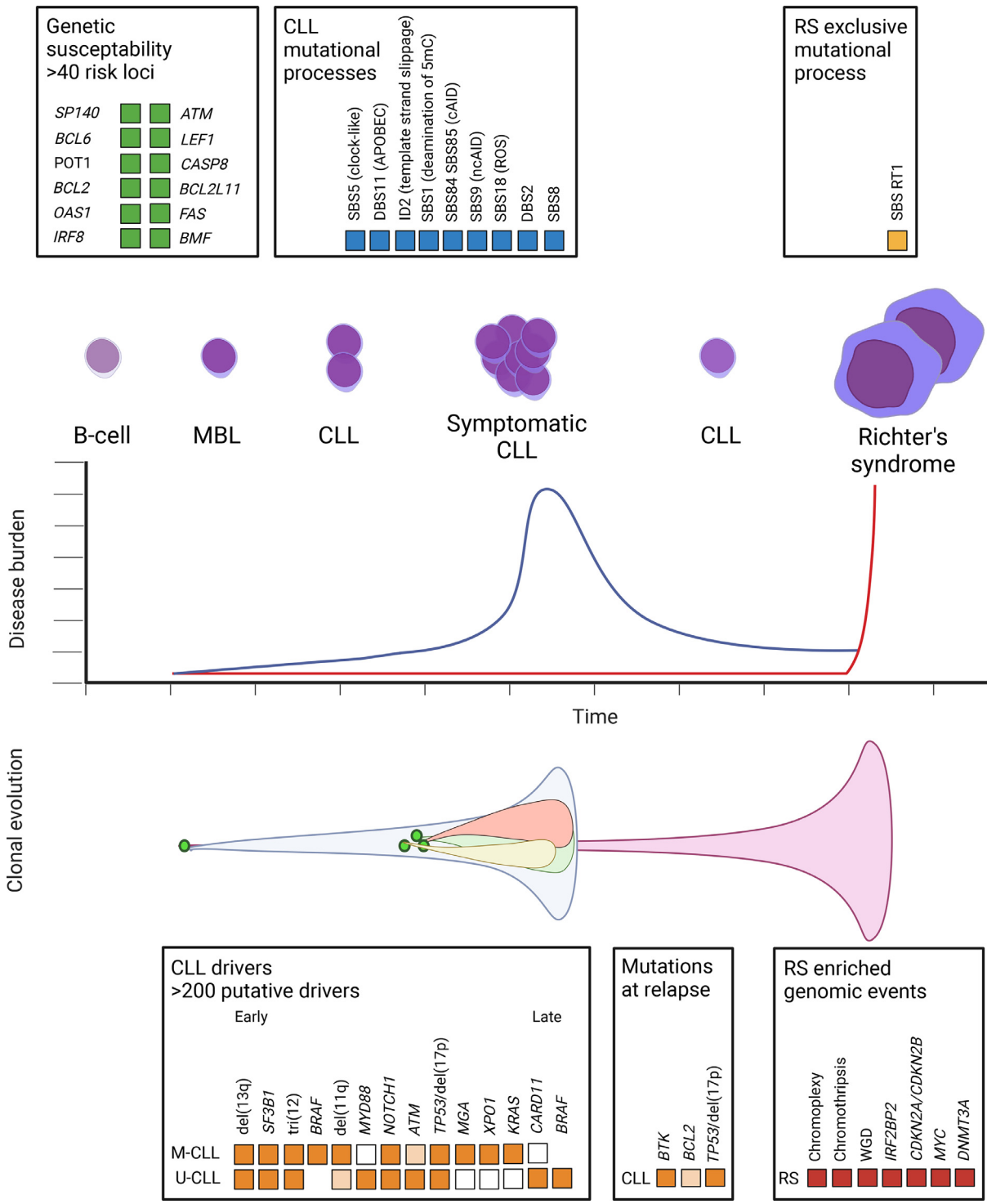

Fig. 1. A schematic for a molecular map of chronic lymphocytic leukemia and Richter's syndrome. MBL, monoclonal B-cell lymphocytosis; CLL, chronic lymphocytic leukemia; RS, Richter's syndrome; M-CLL, mutated chronic lymphocytic leukemia; U-CLL, unmutated chronic lymphoctyic leukemia; 5mC, 5-methylcytosines; cAID, canonical activation-induced cytidine deaminase; ncAID, non-canonical activation-induced cytidine deaminase; ROS, reactive oxygen species; WGD, whole genome duplication.

Germline genetic susceptibility to CLL

Understanding the germline contribution to somatic mutagenesis is informative of the origin of clonal expansion in malignancies such as CLL [24-26]. The germline genetic architecture underscoring the 6-fold elevated familial risk observed in CLL, reflects a range of alleles with varying population frequency and impact [24,27]. Although families segregating CLL provide support for Mendelian susceptibility, only a limited number of rare alleles have been discovered [28-30]. Genome-wide association studies have identified 43 loci, each affording a modest impact on CLL risk (Fig. 1) [31-37]. Elucidating the mechanism that these risk loci, the majority of which map to the non-coding genome, is important for elucidating the biological processes in CLL pathogenesis [24,29]. A number of biological processes have been implicated in CLL predisposition through the integration of these germline ge-

netic variants, gene expression and chromatin structure. These include immune dysfunction (*SP140*, *BCL6*, *OAS1*, and *IRF8*), apoptosis (*BCL2L11*, *CASP8*, *CFLAR*, *FAS*, *BMF*, and *BCL2*), Wnt signaling (*UBR5*, *TLE3*, and *LEF1*), DNA damage (*ATM*) and telomere maintenance (*POT1*, *ACD*) [28,29]. Many of these processes converge with somatic genetic alterations identified through sequencing of CLL cells.

The CLL genome

Whilst the Binet and Rai clinical staging systems are strongly predictive of CLL clinical outcomes, heterogeneity within each group exists. This can be in part explained by the presence of somatic hypermutation of the *IGHV* gene (defined in this context as <98% identity to the germline sequence), which partitions CLL into 2 distinct subsets with different evolutionary histories [38-41]. CLL with an unmutated *IGHV* (U-CLL) is thought to arise from naive B-cells and is associated with an inferior prognosis when compared to CLL with a mutated IGHV (M-CLL), which represents a clonal expansion of a post-germinal center B-cell. Over the past 5 decades, the discovery of somatic mutations in cancer and their direct relevance to biology and therapy, has motivated efforts to catalog recurrent genomic features in CLL [6,42,43].

Hindered by the low mitotic activity of the leukemic cells *in vitro*, fluorescence *in situ* hybridization (FISH) allowed for the detection of chromosomal aberrations not only in dividing cells but also in interphase nuclei [44-46]. Approximately 80% of CLL cases demonstrate a chromosomal aberration, the most common being 13q del (55%), 11q del (18%), tri(12) (16%), and 17p del (7%) [44]. These deletions harbor putative CLL drivers: *ATM* and *BIRC3* (11q), *TP53* (17p), and *miR-15a/16* encoded in an intron of *DLEU2* (13q) [44,45,47-49]. These cytogenetic abnormalities have traditionally been associated with relatively favorable (13q del), unfavorable (del(11q) and tri(12)) and poor outcomes del(17p). Subsequent targeted sequencing studies, informed by biological knowledge, demonstrated recurrent single nucleotide variants (SNVs), insertions or deletions in *TP53*, *ATM* and *NOTCH1* [50-52]. TP53 aberrations were subsequently recognized as being associated with markedly decreased survival and impaired response to chemoimmunotherapy [53].

The advent of high-throughput sequencing (HTS) such as whole-exome sequencing and whole-genome sequencing (WGS) has allowed for unbiased mutation detection, driver gene prediction, mutational signature extraction, identification of clinically relevant biomarkers and modeling of growth kinetics and evolution [54-63]. The overall mutation burden in CLL is low (~1/Mb) when compared to other cancers, with no significant difference between coding mutation rates between M-CLL and U-CLL [61,62]. Mutational signatures in the CLL genome have highlighted biological processes responsible for mutagenesis CLL and include SBS5 (clock-like), DBS11 (APOBEC), ID2 (slippage of template DNA strand during DNA replication), SBS1 (deamination of 5-methylcytosines), SBS84 and SBS85 (canonical activation-induced cytidine deaminase [AID]), SBS9 (non-canonical AID), SBS18 (reactive oxygen species), DBS2 and SBS8 [62-64] (Fig. 1).

Initial HTS studies of CLL, based on cohorts of ~100 CLL cases, confirmed previously observed mutations in *TP53*, *ATM* and *NOTCH1* and identified novel mutated genes in CLL including *SF3B1*, *FBXW7*, *DDX3X*, *MAPK1*, and *ZMYM3* [54–56]. Over the past-decade sample sizes of sequenced cohorts have increased 10-fold which has afforded greater power to detect recurrent coding and non-coding mutations (ability to identify >90% of drivers mutated in 2% of patients) and to associate putative driver mutations with clinical features and outcomes [62]. Moreover, the inclusion of complimentary data such as 3-dimensional protein structure has improved the prediction of driver gene status [10]. To date, >200 putative driver genes of CLL have been identified by HTS (Table 1) [62,63]. Mutations in *SF3B1*, *NOTCH1*, *ATM*, IGLV3-21^{R110}, *TP53*, *POT1*, *CHD2*, and *XPO1* occur in 17.5%, 12.3%, 11.2%, 9.5%, 9.1%, 6.3%, 5.7% and 5% of patients respectively, with the remaining drivers possessing a mutation frequency each of <5%. The genes identified highlight core altered pathways in DNA damage (eg, *TP53* and *ATM*), mRNA processing (eg, *SF3B1, XPO1*), chromatin modification (eg, *HIST1H1E*, *CHD2*, and *ZMYM3*), NOTCH signalling (eg, *NOTCH1*, *FBXW7*), MYC (eg, *MGA*), inflammation (eg, *MYD88*, *BIRC3*) B-cell receptor transcription and signaling (eg, *EGR2* and *BRAF*) and telomere maintenance (eg, *POT1*) (Fig. 2) [62].

Table 1
Cancer driver genes in CLL which are mutated at >5% frequency in CLL, unmutated CLL and mutated CLL. Data from high-throughput sequencing studies of 2 large cohorts.

Study	Disease	Gene
Knisbacher et al. [62]	Mutated CLL	*SF3B1*
		CHD2
		MYD88
		ATM
		KLHL6
Knisbacher et al. [62]	Unmutated CLL	*SF3B1*
		NOTCH1
		ATM
		TP53
		POT1
		XPO1
		MGA
		BRAF
		DDX3X
		EGR2
		RPS15
		ZNF292
Robbe et al. [63]	CLL	*SF3B1*
		TP53
		IGLL5
		NOTCH1
		ATM
		POT1
		BIRC3
		RPS15
		MGA

In addition to single nucleotide variants and small insertions-deletions (generally <50 base pairs) in protein-coding sequences, WGS allows for the detection of recurrent variants outside of the non-coding mutations as well as larger variants such as copy number alterations (CNAs) and structural variants (SVs). >50 recurrent CNAs have been reported in addition to previously identified deletions (13q, 11q, and 17p). Through defining minimally affected regions and the integration of driver gene and data and literature, candidate genes at recurrent CNA sites include *UCP2* and *UCRP3* (del[11q13]), *PCM1* (del[8p]) and *RPS14* and *TCOF1* (del[5q32]) [62,63]. An average of 5 CLL SV breakpoints occur per patient with 46% being identified as clonal, supporting a role for SVs in CLL pathogenesis. Breakpoints frequently involve either the immunoglobulin light chain kappa locus (13%), the immunoglobulin heavy chain locus (13%) or chr13q14.2 (9%) [63]. The most common immunoglobulin translocation partner is *BCL2,* which occurred more frequently in M-CLL [62]. Furthermore, the use of gene expression, chromatin accessibility and 3-dimensional chromatin structure from representative cells has aided in the identification of additional genes affected by somatic non-coding mutations [29,63]. Regulatory elements and genes affected by non-coding variants include the 3′ UTR of NOTCH1, an enhancer centromeric to *PAX5* and *BACH2* promoter mutations [61,63]. Long-read sequencing technology and advances in methods to examine regulatory regions of the genome will expand the catalog of driver mutations in CLL [65-69].

Comparing the mutational profiles of M-CLL and U-CLL in increasingly large datasets has provided further evidence of a dis-

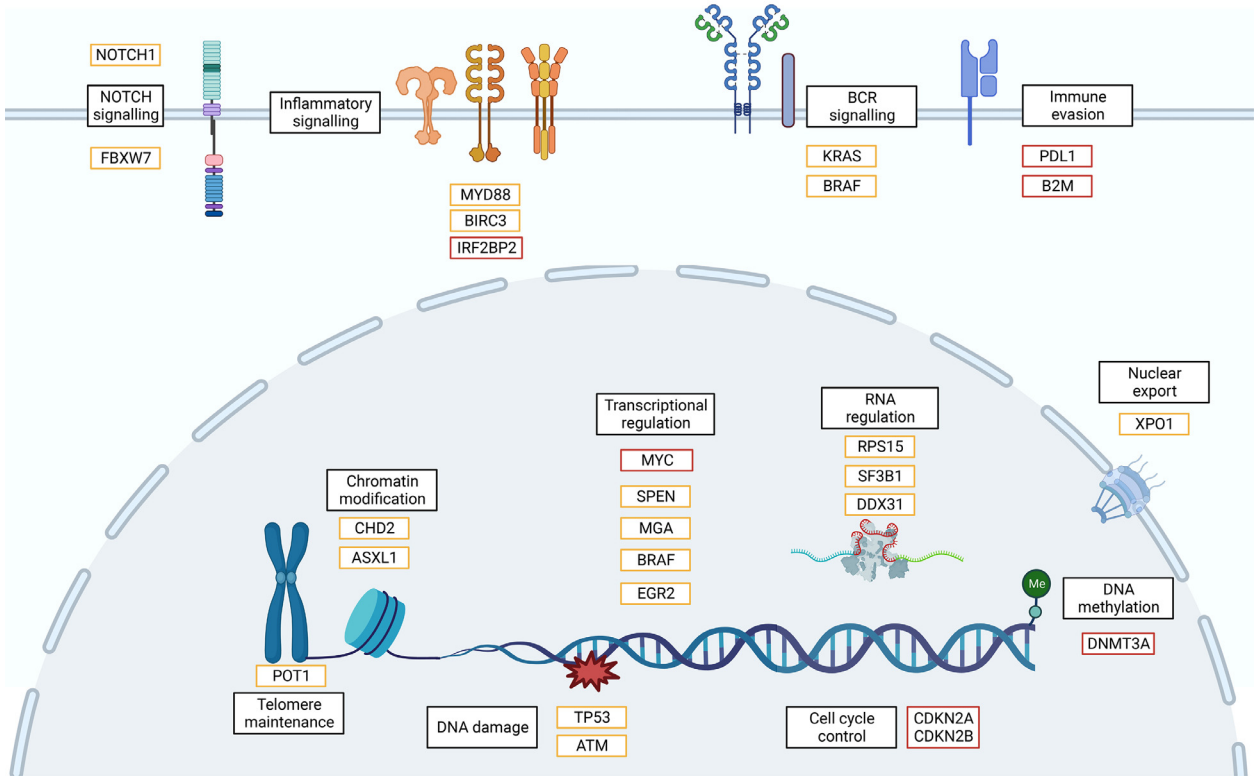

Fig. 2. Biological pathways annotated by driver mutations in chronic lymphocytic leukemia and Richter's syndrome. Red boxes indicate genes where mutations are enriched in Richter's syndrome.

tinct genetic evolutionary history. Likely reflective of the post-germinal center cell of origin, SBS9 and SBS85 mutational signatures (AID) are enriched in M-CLL and SBS9 operates early in disease evolution [62,63,70]. U-CLL possesses a higher number of driver genes, whereas M-CLL has a greater proportion of clonal mutations. Whilst many of the commonly occurring somatic genetic abnormalities occur in both diseases (eg, mutations in *TP53*, *SF3B1* and *POT1*, del(13q), trisomy 12, del(17p)) there are a number that demonstrate specificity. In U-CLL, *KRAS*, *BRCC3*, *BCOR* and *SAMHD1,* del(11q), del(6q) *and* del(2p) appear as exclusive drivers whereas *MYD88*, *ITPKB*, *IGLL5*, *CHEK2* and del(7q36) (*KMT2C*) appear specific to M-CLL [62,63]. Moreover structural variant rearrangement mechanisms differed, with V(D)J recombination driving the *BCL2* events in M-CLL and class-switch recombination facilitating the ZFP36L1-associated deletions in U-CLL [62].

HTS offers the prospect of identifying somatic mutation biomarkers, beyond those previously described such as TP53 aberrations, that are prognostic in CLL. In treatment-naive non-trial patients, examples include mutations in *ZC3H18* and the IGLV3-21^{R110} (failure-free survival, M-CLL) and amp(8q) (overall survival, M-CL2) [62]. In U-CLL, examples include del(7q36), del(1q32) (failure-free survival) and mutations in *ASXL1* and del(8p) (overall survival) [62,63]. Studies of relapsed and refractory CLL have further highlighted increases in the cancer cell fraction of mutations such as *TP53*, as well as increased copy number alterations and distinct clonal evolutionary patterns of CLL at disease progression [60,71,72].

As the therapeutic landscape of CLL has evolved, HTS has also provided novel insights into mechanisms of resistance to targeted therapies [73-76]. In patients treated with first generation BTKi, clinical resistance occurs in 10% to 28% within 2 to 3 years of therapy initiation. The majority of patients relapse with a *BTK* C481S mutation, which reduces the binding affinity of the covalent BTKi [73-78]. Less frequent are gain of function mutations in *PLCG2* which encodes a B-cell receptor (BCR) downstream signaling molecule [73,77,78]. These mutations precede clinical relapse as a result of expansion of a resistant clone [79,80]. A new generation of BTKi which exhibit irreversible BTK binding shows efficacy in patients with the *BTK* C481S mutation, although a wider spectrum of *BTK* mutations resulting in a "dead kinase" are now being described [81,82]. Similarly, in 50% of patients refractory to venetoclax, a CLL clone emerges before clinical relapse with a *BCL2* Gly101Val mutation, albeit with low variant allele frequency (VAF) and a co-occurence of other *BCL2* mutations [83-85]. The Gly101Val mutation reduces the affinity of BCL2 for venetoclax, preventing the drug from displacing pro-apoptotic mediators from BCL2, thereby promoting CLL cell survival [83]. Such a low VAF may represent disease compartment heterogeneity. Notably, not all patients with small-molecule resistant CLL possess a resistance mutation. Selection for 8p deletion and MCL-1 overexpression are thought to contribute to venetoclax resistance [86,87].

Dissecting the evolution of CLL

The use of somatic genomics to infer the evolutionary history of a cancer was first conducted over 35 years ago [88,89]. Sequencing of bulk tumor samples with HTS has enabled the reconstruction of the evolutionary history of diverse tumors, based on a large catalog of somatic mutations [90,91]. The order of genetic events from 'early' to 'late', can be measured by comparing the cancer genome at different temporal stages, and even growth rates can be modeled. Alternatively, the clonal architecture of the tumor sample subjected to bulk HTS can be inferred from the variant allele fraction (VAF) of somatic mutations after accounting for ploidy and tumor purity [92] Mutations common to all sampled tumor cells (clonal) precede mutations present in a fraction of sampled tu-

mor cells (subclonal). Thus, differences in the mutational profiles, or changes in the clonal composition of separate tumor samples, reflect how the cancer develops over time. Using such approaches in CLL, del(13q), del(11q), tri(12), and *MYD88* have been found to occur early in the evolution of CLL whereas *NOTCH1*, *ATM*, *SF3B1*, and *TP53* mutations are later events [59,60,62] (Fig. 1). A notable finding is the observation that *BRAF* mutations occur early in M-CLL but late in U-CLL [62]. Serial measurements of peripheral white blood cell counts in patients have been used to define exponential, indeterminate and logistic growth patterns in naturally progressing CLL [93]. Combining such growth patterns with somatic sequencing data has demonstrated the presence of a higher number of CLL drivers and greater subclonal dynamics with exponential growth. In contrast, logistic growth has been associated with a narrower spectrum of genetic alterations, fewer subclonal drivers, and interclonal stability even in relapse after treatment.

Whilst bulk tumor sequencing can order somatic mutations and infer clonal dynamics, a higher resolution phylogenetic map can be acquired through the application of single-cell technologies. Moreover, single-cell profiling of genomic, transcriptomic, epigenomic, proteomic and other -omic modalities can further our understanding of cancer cell heterogeneity, therapeutic resistance, and mechanisms of tumor-immune interactions [94-108]. Characterizations of single cells from patients with CLL have revealed marked interpatient heterogeneity at the level of the transcriptome, chromatin accessibility, methylome and mitochondrial DNA. This contrasts with non-malignant immune cells, which have greater consistency across patients [109-115]. Moreover, the distinct CLL phenotype observed in each patient is maintained throughout the natural history of MBL to CLL [112].

Prospective lineage tracing through optical or sequencing barcodes has enabled *in vitro* or *in vivo* modeling of tumor evolution [116-122]. Retrospective lineage tracing in primary human tissues, relies on 'native barcodes' such as somatic SNVs, CNAs, methylation and mtDNA mutations [100,111,123,124]. In CLL, the earliest studies to generate a phylogenetic tree used targeted single-cell reverse transcription polymerase chain reactions to detect somatic mutations in hundreds of CLL cells [109]. This approach established a relationship between *SF3B1* mutations and the generation of altered splice transcripts [125]. High throughput methods have now been developed to link genotype with single-cell transcriptomes at scale [126-128]. Using massively parallel single-cell mitochondrial DNA and chromatin profiling in bone marrow-derived mononuclear cells, mtDNA mutations detected in CLL cells can be tracked to early progenitor cells supporting the notion that a CLL clonal mutation may arise earlier in the hematopoietic lineage tree [123,129,130].

As well as assessing native CLL heterogeneity, single-cell technologies have been used to assess the clonal trajectories over time and in response to therapy. Trajectories of CLL clonal evolution have been tracked over time using mtDNA mutations and chromatin accessibility signatures [114]. This has demonstrated clonal persistence over years in the absence of a selective pressure [114]. However, the introduction of a selective pressure such as disease transformation or relapse is associated with changes in CNAs, chromatin accession and gene expression [114]. Another approach used DNA barcoding with single-cell RNA sequencing and clonal isolation to characterize thousands of clones within a heterogeneous cell populations [131]. This functionalized *ex vivo* lineage-tracing system has revealed distinct trajectories of subclones in relation to treatment as well as genomic diversification after chemotherapeutic treatment [131].

A number of studies have assessed the impact of specific therapies on CLL response using single-cell technologies with the aim of elucidating mechanisms of response and resistance. Using single-cell short and long-read RNA sequencing, the complexity of venetoclax resistance in CLL has been further appreciated with NF-κB activation and confirmation of increased *MCL1* expression being a consistent finding [128]. A separate study integrated longitudinal single-cell immunophenotypic, transcriptomic, and chromatin mapping of the molecular and cellular dynamics of CLL and immune cells during ibrutinib treatment [113]. The analysis of the CLL cells revealed reduced NF-κB binding, a reduction of lineage-defining transcription factors, erosion of CLL phenotypic identity and induction of a quiescent state [113]. Finally, a single-cell transcriptomic analysis of CLL following allogeneic stem cell transplantation has demonstrated distinct evolutionary trajectories and insight into the graft vs leukemia effect [132]. Early relapses exhibited genetic and cellular stability over time contrasting with late relapses which displayed notable genetic evolution and evidence of neoantigen depletion [132].

Functional analysis of somatic mutations in CLL and RS

As well as confirming the predicted driver status of genes, functional studies offer an opportunity to directly study the biological consequences of mutations in cancer. *In vitro* model systems that have been utilized include established CLL, isogenic and patient-derived cell lines. Examples of genomic regions and genes that have been interrogated include trisomy 12, *ATM*, *POT1*, *NOTCH1*, *TP53*, *SF3B1*, *RPS15* and *CHD2* [58,133-139]. Such analyses highlight dysfunction of inflammatory and BCR signaling, DNA damage, RNA regulation, chromatin structure and telomeres as being consequences of somatic mutations in CLL (Table 2 and Fig. 2). Through deconvolution of the biological pathways perturbed by somatic mutations in CLL, novel therapeutic vulnerabilities can be identified [133,136,137,140]. Recently developed high-throughput approaches for functional annotation of somatic mutations offers the prospect of testing the functional consequences at scale [141]. Furthermore, murine models allow further functional dissection of involved risk genes [142].

The RS genome

The majority of patients with RS have histology of diffuse large B-cell lymphoma (DLBCL) and it has long been appreciated that RS can arise as either clonally related or unrelated to the CLL, the latter of which has been associated with a more favorable prognosis [143-145]. A number of risk factors for RS have been proposed including, U-CLL, *TP53* mutations and *NOTCH1*, *CDKN2A* or *CDKN2B* loss, stereotyped HCDR3, BCR subset 8 and a complex karyotype [146-151]. Three recent studies, comprising a collective total of >100 individuals, have performed exome- or genome-wide analyses of paired CLL and RS, largely of DLBCL histology [20-22]. Together, these analyses provided a number of insights into the drivers and evolutionary history of CLL transforming to RS. Firstly, through computational deconvolution of clones, the majority of RS indeed is clonally related to the antecedent CLL and is distinct from DLBCL [20,21]. Secondly, transformation to RS is associated with an increase in genomic complexity as evidenced by an increase in somatic mutation burden, CNAs, SVs, kataegis, chromothripsis, chromoplexy, and whole genome duplication (WGD) [20-22]. Thirdly, through identification of coding mutations, CNA and SVs, these studies have expanded the driver genes and pathways involved in transformation to RS (Fig. 2) [20-22,146,148,149,152-158]. Highly prevalent alterations include del(17p), *TP53* and *NOTCH1* mutations were uncovered, consistent with prior study. Taken together the catalog of recurrent candidate driver genes in RS annotate pathways such as DNA damage (*TP53*), cell cycle control (*CDKN2A* and *CDKN2B*), transcriptional regulation in B-cells (*MGA*, *EGR2*, *IRF2BP2*), DNA methylation (*DNMT3A* and *TET2*), RNA splicing (*SF3BA*, *SRSF1*), chromatin

Table 2
Summary of putative role of CLL driver genes which are mutated at >5% frequency in CLL [62,63].

Gene	Putative Role
SF3B1	Defective pre-mRNA splicing [125,139,174-177]
NOTCH1	Constitutive NOTCH1 pathway activation [54,178-183]
ATM	Defective DNA damage response [52,174,184-189]
TP53	Defective DNA damage response [187,190-192]
POT1	Telomere dysfunction [58]
CHD2	Disrupted chromatin states [134]
MYD88	Activation of the Toll-like receptor and IL-1 receptor signaling pathways [54]
KLHL6	Disrupted function of a CULLIN-Ring ubiquitin ligase [193]
XPO1	Disrupted nuclear export cargo [194,195]
MGA	Dysregulation of *MYC* [157,196]
BRAF	Deregulation of BCR signaling [129]
DDX3X	Dysregulation of mRNA translation [197,198]
EGR2	Dysregulation of transcription and BCR signalling [129]
RPS15	Dysregulation of mRNA translation [135,199,200]
ZNF292	Disruption of cellular proliferation and cycling [201]
IGLL5	Disrupted B-cell development [202,203]
BIRC3	Constitutive activation of the NFκB pathway [138,204-206]

structure (*EZH2*), nuclear export (*XPO1*), B-cell receptor signaling (*BRAF*) and immune evasion (B2M) [20,21]. Moreover, mutational signatures that operate in RS independent to those also identified in CLL include SBS44 (defective DNA mismatch repair) and a novel mutational signature [20,22]. Finally, through unsupervised non-negative matrix factorisation on somatic mutation data, RS appears to cluster in 5 distinct groups (RS1-RS5) [21]. Three (RS1, RS3 and RS5) are enriched for *TP53* alterations, display a higher rate of CNAs and genome alterations and are associated with an adverse prognosis. RS1 is marked by WGD, a fractured genomes, *MYC* amplification and del(1p) and del(9p) and an enrichment for transformed M-CLL. RS3 is enriched for *NOTCH1* and *IRF2BP2* mutations as well as CNAs, including del(14q32.11), del(9q), del(15q15.2) (*MGA*), amp(16q23.2) (*IRF8*) and del(2q37.1). RS5 possessed del(16q12.1), del(1p35.2) and amp(7p) CNAs. RS2 is typified by tri(12) co-occurring with *SPEN*/*NOTCH1* and *KRAS* mutations and RS4 is marked by *SF3B1* and *EGR2* mutations on a background of del(13q). The sample sizes used in these studies were modest and expansion of these cohorts along with refinement of methods for clonal deconvolution, will most certainly expand the mutational landscape of RS and increase power to identify novel drivers of histologic transformation.

Given the short median overall survival associated with RS, there is a pressing need to exploit recent molecular insights to improve patient outcomes. Using longitudinal sampling, small subclones possessing genomic, immunogenetic and transcriptomic features of RS were found in 5 of nine CLL patients with available samples and up to 19 years prior to transformation [20]. As well as raising questions regarding the prevalence of RS subclones in CLL patients and the mechanisms that subsequently contribute to overt clinical presentation of transformation, this finding provides preliminary evidence that early detection of RS can be achieved in some patients. Whilst plasma-derived cell-free DNA (cfDNA) in the context of lymphoma diagnosis and monitoring is attractive, it is uniquely challenging in RS due to the presence of circulating CLL cells, which also shed cfDNA [159]. However, using ultra-low-pass whole-genome sequencing, RS specific somatic genomic changes have been detected in plasma, often separate from the circulating CLL. This included 6 of 8 patients at RS diagnosis time and even 2 of 7 patients, months prior to a diagnosis of RS [21]. These studies supports the notion of early non-invasive diagnosis of RS which is valuable when faced with challenges acquiring tissue for a diagnosis. This new understanding of the molecular basis of RS may inform a new generation of therapeutic opportunities. Examples of promising agents In addition to checkpoint blockade include small molecules (eg, inhibition of BTK, BCL2, PI3K and CDK9), bispecific antibodies (eg, CD3/CD20), antibody-drug conjugates (eg, VLS-101, polatuzumab) as well as CAR T-cell therapies [160-167].

Genomics and the microenvironment in CLL and RS

New molecular and genomic approaches allow for the identification of genetically-defined clones and sub-clones of CLL and RS and the interactions of the surrounding cells, thereby adding another layer to the molecular map of CLL. DNA-, RNA-, and protein-based approaches such as multiplexed error-robust fluorescence in situ hybridization [168], protein detection methods [169], and spatial barcoding are now available and are beginning to generate novel biological insights [170]. These technologies are advancing at pace to allow for single-cell resolution and modification to permit the detection of additional molecular features such as T-cell and B-cell receptors [171,172]. Linking CLL and RS genomics to spatial and functional information across tissue compartments and disease stages will generate insights into the organization of clones within the TME, the co-evolution of CLL and TME and ultimately offers the possibility of new disease taxonomy, biomarkers and therapies [173].

Conclusion

The molecular map of CLL and RS to date has generated fundamental knowledge concerning the etiology and evolution of CLL. Such insights are already being exploited to improve patient care through prediction, treatment selection, and ongoing preclinical and clinical investigations. However, this map is only partially complete. Whilst a large component of the mutational landscape of CLL has been described, non-coding mutations in CLL as well as a complete understanding of mutations in RS remain relatively unexplored. Already in progress is the use of model systems to deconvolute the functional consequences of driver mutations which is essential to inform our biological understanding of CLL. Single-cell analyses has enabled high resolution characterization of intratumoral heterogeneity in CLL. However, questions remain regarding the origin of the B-cell clone, the role of the TME and interaction with clonal cells, inter- and intra-compartmental heterogeneity and resistance mechanisms. Through functional studies of mutations and the application of single-cell genomic, transcriptomic, epigenomic and spatial data across tissues, disease states and clinical contexts these questions can be addressed with the promise of generating translatable knowledge to improve patient outcomes.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

A.S. and E.M.P. declares no conflicts of interest. C.J.W receives funding support from Pharmcyclics and holds equity in BioNTech.

CRediT authorship contribution statement

Amit Sud: Writing – review & editing, Writing – original draft, Visualization, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Erin M. Parry:** Writing – review & editing, Writing – original draft, Visualization, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Catherine J. Wu:** Writing – review & editing, Writing – original draft, Visualization, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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