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Biology and treatment of high-risk CLL

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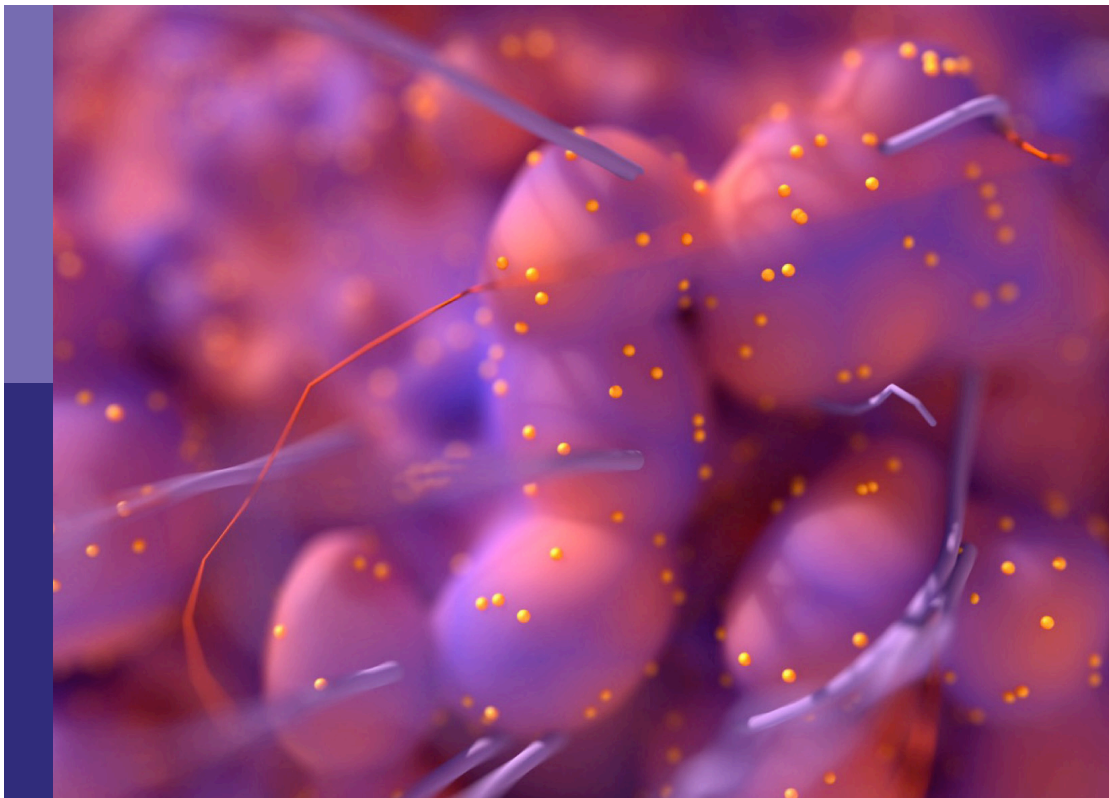

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Biology and treatment of high-risk CLL

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Editorial: Biology and treatment of high-risk CLL

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Editorial on the Research Topic
Biology and treatment of high-risk CLL

In the era of chemoimmunotherapy, high-risk chronic lymphocytic leukaemia (CLL) was defined by the presence of *TP53* loss and/or *TP53* mutation and by refractoriness to purine-analogue based treatment (no remission or remission under 6 months in duration), respectively (1). The advent of chemo-free treatment regimens at all disease stages requires a re-definition of the term "high-risk CLL", but this constitutes a challenging task when considering the rapidly evolving treatment landscape and the increasing knowledge about CLL pathobiology obtained over recent years. While CLL characterization used to focus on clinical parameters and limited genomic analyses (2), samples can nowadays be analysed in a far more comprehensive manner, since "omics" technologies allow an integrative analysis of data obtained at the genomic, epigenomic, transcriptomic, and proteomic level as well as an assessment of spatial tumor heterogeneity and tumor evolution over time. Next to intrinsic CLL characteristics, a growing understanding about the interplay of CLL cells with their microenvironment provides additional aspects to consider for CLL risk stratification. The wealth of information that can in principle be obtained for each CLL case challenges the identification of biomarkers conferring poor prognosis and predicting treatment failure.

From a clinical perspective, high-risk CLL may hitherto best be defined by refractoriness, non-durable response or intolerance towards the two drug classes that have become most relevant for CLL treatment: covalent BTK inhibitors (BTKi) such as ibrutinib and acalabrutinib and BCL2 inhibitors (BCL2i) such as venetoclax (3). However, this clinical definition entails the exhaustion of two treatment lines by the time that high-risk patients become identifiable, a situation in which the current drug approval status has only a limited number of alternative drug classes on offer. To adapt

and personalize CLL therapeutic approaches in a way that enables durable eradication of all CLL clones in high-risk patients in first-line, it will be essential to define biomarkers identifying respective patients before or early after treatment initiation.

Validating the prognostic or predictive impact of potential biomarkers has been complicated by a growing number of targeted compounds, immunotherapeutic agents and products for adoptive cell therapy that need to be tested in prospective clinical trials. The plethora of options for new monotherapies and combination therapies to be evaluated requires large numbers of CLL patients to be enrolled on clinical trials. The distribution of patients across a multitude of therapeutic options makes it difficult to reach the statistical power necessary to validate the impact of each potential biomarker for each individual treatment approach. In keeping with this notion, not even the prognostic impact of *TP53* disruption has yet been conclusively clarified in the setting of BTKi and BCL2i based treatment. This clearly illustrates how difficult it will be to develop multivariate prognostic models that take genetic, epigenetic, transcriptional, phenotypic and clinical parameters into account and permit an individualised treatment recommendation for every CLL patient that considers clinical benefit as well as costs.

The scope of the Research Topic "Biology and Treatment of High-risk CLL" is to provide a state-of-the-art overview of the pathobiological mechanisms underlying high-risk CLL, to list and critically evaluate biomarkers that have been associated with a high-risk disease character and to outline the implications of these biomarkers on response to different treatment approaches including chemoimmunotherapy, targeted therapy and cellular immunotherapy. Another issue discussed within the Research Topic is Richter transformation, which is defined as the development of a high-grade lymphoma in patients with a previous or concurrent diagnosis of CLL or small lymphocytic lymphoma (SLL) and frequently associated with a dismal prognosis.

The Research Topic is opened by a review article in which Kwok and Wu. discuss prognostically relevant biologic alterations in CLL observed at the genetic, transcriptional, epigenetic and microenvironmental level. In case respective data was available, alterations are regarded as dynamic processes throughout which the tumor can evolve towards therapeutic resistance and progression. Building up on this, the authors create a future vision how multidimensional tumor heterogeneity and tumor growth dynamics could become parameters in a prognostic model allowing more personalised treatment choices.

After this introductory review, three articles focus on *TP53* alteration serving as a defining criterion of high-risk CLL and as established predictor for chemoresistance (4). Soussi and Baliakas. first illuminate the pathobiology of *TP53* alterations in CLL as compared to other malignancies and show how to utilize *TP53* locus-specific mutation databases and cancer genome databases for a correct interpretation of *TP53* variants. Lazarian et al. then summarize available data on low-burden *TP53* mutations defined by a variant allele fraction <10% and discuss the open research question as to what extent low-burden *TP53* mutations are clinically relevant. In a Brief Research Report, Catherwood et al. present data on *TP53* alterations obtained from a real-world cohort of 2332 CLL cases analysed by next generation sequencing and fluorescence *in situ* hybridization (FISH).

In the next section of the Research Topic, three additional poor prognostic biomarkers identified at the genetic level are highlighted. First, Chatzikonstantinou et al. present available data on the prognostic and potentially predictive value of karyotype complexity in CLL and discuss the impact of low, intermediate and high genomic complexity against the background of concomitant high-risk biologic features. Subsequently, Zavacka and Plevova. shed light on chromothripsis defined as a genomic event by which a single chromosome or a limited number of chromosomes are shattered into pieces and reassembled in an error-prone process. The authors discuss the potentially underlying biological causes for this event and review the impact of chromothripsis on CLL disease progression and treatment response. Then, Nguyen-Khac. addresses the role of *MYC* rearrangements in CLL with a focus on concurrent loss of the *TP53* gene locus, a constellation termed "double-hit CLL". This latter topic is backed up by an original research article by Ondrouskova et al. describing the frequency of *MYC*-rearranged CLL in a cohort of 303 cases from a single center and dissecting the types of the genomic alteration responsible for *MYC* rearrangement in an extended cohort.

The Research Topic then focusses on two cell signaling pathways shown to influence prognosis. First, B-cell receptor signaling is highlighted as a key survival pathway for CLL cells. Gerousi et al. explain the concept of B-cell receptor stereotypy in CLL and summarise data on CLL subsets defined by stereotyped B-cell receptors and associated with an aggressive clinical course. In a complementary perspective article, Nicolo et al. put emphasis on the fact that alterations in the B-cell receptor immunoglobulin light chain can also drive the development of high-risk CLL. Afterwards, Edelmann. elucidates the impact of *NOTCH1* mutations and further alterations associated with de-regulated NOTCH1 signaling.

Richter transformation is a dreaded complication of CLL evolution with an aggressive clinical course and no established treatment options. Condoluci and Rossi. address the issue of Richter transformation by giving insight into its pathobiology and summarizing data on potential treatment options recently evaluated in clinical trials.

The last section of the Research Topic is dedicated to treatment of high-risk CLL. To systematically approach the question how high-risk CLL behaves under various treatment approaches, Straten et al. select *TP53* alterations, Del(11q), genomic complexity, unmutated IGHV mutation status, stereotyped B-cell receptor subsets, *NOTCH1* mutation and *BIRC3* mutation from the list of poor prognostic factors in CLL and discuss their clinical impact based on available data from clinical trials testing chemotherapy, chemoimmunotherapy, anti-CD20 treatment, BTK inhibition, BCL2 inhibition, Pi3K inhibition and/or allogeneic stem cell transplantation. The authors also comment on the next generation kinase inhibitors zanubrutinib, pirtobrutinib and duvelisib, the anti-CD20 monoclonal antibody ublituximab and on CAR-T-cell and CAR-NK cell therapeutic approaches. To explain the role of allogeneic stem cell transplantation and CAR T-cell therapy in more detail, Barbanti et al. add a review article focussing on the use of these cellular approaches for the treatment of high-risk CLL and Richter transformation.

In a concluding Opinion Article, Edelmann, Malcikova et Riches propose a new definition of high-risk CLL taking disease intrinsic risk factors as well as additional elements with an influence on overall survival into account.

Author contributions

All authors made a substantial, direct and intellectual contribution to the conceptual design of the Research Topic. JE wrote the Editorial. All authors contributed to the article and approved the submitted version. Eugen Tausch, Jitka Malcikova and John Riches contributed equally to the conceptual design of the Research Topic." in Author contributions.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
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Distinctive Signaling Profiles With Distinct Biological and Clinical Implications in Aggressive CLL Subsets With Stereotyped B-Cell Receptor Immunoglobulin

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The ontogeny and evolution of chronic lymphocytic leukemia (CLL) are critically dependent on interactions between leukemic cells and their microenvironment, including antigens, the latter recognized through the clonotypic B-cell receptor immunoglobulin (BcR IG). Antigen selection is key to the pathogenesis of CLL, as evidenced by the remarkable skewing of the BcR IG gene repertoire, culminating in BcR IG stereotypy, referring to the existence of subsets of patients with (quasi)identical BcR IG. Notably, certain of these subsets have been found to display distinct, subset-biased biological background, clinical presentation, and outcome, including the response to treatment. This points to BcR IG centrality while also emphasizing the need to dissect the signaling pathways triggered by the distinctive BcR IG expressed by different subsets, particularly those with aggressive clinical behavior. In this mini-review, we discuss the current knowledge on the implicated signaling pathways as well as the recurrent gene mutations in these pathways that characterize major aggressive stereotyped subsets. Special emphasis is given on the intertwining of BcR IG and Toll-like receptor (TLR) signaling and the molecular characterization of signaling activation, which has revealed novel players implicated in shaping clinical aggressiveness in CLL, e.g., the histone methyltransferase EZH2 and the transcription factor p63.

Keywords: stereotyped subsets, signaling, mutations, expression profiles, high-risk chronic lymphocytic leukemia

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a chronic B-cell malignancy, the most common adult hematologic malignancy in Western countries. CLL displays remarkable clinical heterogeneity regarding both the clinical presentation and the course of the disease, including the response to treatment, likely reflecting the underlying biological diversity (1–4). That notwithstanding, a ubiquitous theme in the natural history of CLL concerns the crosstalk of leukemic cells with the

microenvironment (5), including antigens, thus placing the clonotypic B-cell receptor immunoglobulin (BcR IG) in the spotlight.

The first immunogenetic evidence regarding the involvement of antigens in the pathogenesis of CLL emerged from studies from the 1990s reporting significant biases in the BcR immunoglobulin (IG) gene repertoire, strongly implying a role of antigen selection in disease ontogeny (6). Moreover, it was found that approximately half of CLL patients carried BcR IG with somatic hypermutations (SHM), corroborating the notion of antigen involvement in disease pathogenesis (7, 8).

An in-depth study of SHM mechanism in CLL resulted in the classification of patients in two distinct subgroups based on the SHM imprint within both the rearranged immunoglobulin heavy variable (IGHV) gene and immunoglobulin kappa/lambda variable gene (IGKV/IGLV) of the clonotypic BcR IG. In particular, patients that express rearranged IGHV genes with no or few SHM (≥98% sequence identity between the clonotypic rearranged IGHV gene and its closest germline counterpart; unmutated CLL, U-CLL) generally experience more aggressive disease course with immediate or early need for treatment compared with those with mutated IGHV genes (<98%; mutated CLL, M-CLL) who display a considerably more indolent disease (7, 8). The SHM status of the clonotypic IGHV gene is perhaps the most robust prognostic marker in CLL, independent of the clinical stage or disease evolution (9). Importantly, it is also predictive of the clinical response to therapy (10, 11).

Perhaps the strongest molecular evidence for antigen selection in CLL emerged from the observation that a large proportion of CLL patients carry (quasi)identical, otherwise termed stereotyped, BcR IG (12). The term "stereotyped" is derived from Greek and refers to a form repeated with limited or no variation; hence, it is truly appropriate for describing the remarkable restrictions in the primary amino sequence documented in the clonotypic BcR IG of different patients with CLL. The first striking observation concerned the fact that almost half of CLL patients utilizing the IGHV3-21 gene displayed highly similar variable heavy complementarity determining region 3 (VH CDR3) and, additionally, carried restricted, IGLV3-21-encoded light chains (13, 14). This finding is at odds with classic immunological thinking, whereby the probability of finding identical BcR IG in different B-cell clones is negligible (~10^{-12}–10^{-16}), cementing the concept of antigen selection as a major driver of CLL development.

BcR IG stereotypy is remarkably common in the CLL BcR IG repertoire (15–24), accounting for almost 41% of all CLL, as revealed in our large-scale study comprising ~30,000 patients (25). Based on shared amino acid motifs within the VH CDR3, cases are classified in groups termed "stereotyped subsets" (17, 23, 25): cases belonging to the same subset exhibit several other restricted immunogenetic features besides a highly homologous VH CDR3, extending from the use of phylogenetically related IGHV genes to restricted light chain gene rearrangements (at least for many major subsets), to shared SHM imprints in both the heavy and the light chain variable domains (16, 17, 23, 25, 26). Moreover, accumulating evidence indicates that patients assigned to the same stereotyped subset display consistent antigenic recognition profiles (26) as well as similar landscapes of antigen reactivity (27), BcR IG 3D structure (28), genomic aberrations (29), gene expression (30), epigenetic modifications (31), Toll-like receptor signaling (32, 33), and "classic" (27) and cell-autonomous BcR signaling (34), among others. Moreover, BcR IG stereotypy defines subgroups with shared clinical features and similar outcome (9, 25, 35, 36).

Importantly, certain subsets have also emerged as distinct clinical variants, exemplified by stereotyped subsets #1, #2, #6, and #8, that exhibit particularly aggressive clinical course and outcome (**Figure 1**) (12). On these grounds, BcR IG stereotypy is currently being considered as a means for improved risk stratification of patients with CLL, at least for the best characterized subsets (i.e., subsets #2 and #8) (37).

CLL SUBSET #1

Subset #1 represents almost 2.2% of all CLL and is defined by rearrangements utilizing different yet phylogenetically related IGHV genes belonging to IGHV clan I (IGHV1, IGHV5, IGHV7 subgroups), thus displaying highly similar primary sequences (28). The heavy chain IGHV clan I/IGHD6-19/IGHJ4 gene rearrangements are characterized by the presence of no or little SHM and display a ubiquitous QWL (glutamine-aspartate-leucine) motif within the VH CDR3; furthermore, they are combined with a light chain encoded by an IGKV1(D)-39/IGKJ1-2 gene rearrangement (23, 26). Recently, we documented a close immunogenetic similarity between stereotyped subset #1 and minor subset #99, reflected in highly similar clinical prognosis (25).

Regarding the latter, subset #1 is associated with a poor outcome, displaying shorter time-to-first-treatment (TTFT) and overall survival in comparison to U-CLL with BcR IG using the same IGHV genes albeit in different configurations (38–40). Regarding genomic alterations, a high frequency of *NOTCH1* mutations has been reported (16% to 27% of cases, depending on the series) (35, 41). Moreover, *TP53* mutations (16%) (41) as well as *NFKBIE* aberrations (15%) (42) and del (11q) (35) were all found enriched in subset #1, contributing to the poor prognosis of patients assigned to this subset. *NFKBIE* mutations result in reduced IκBε protein levels, which in turn implies decreased IκBε–p65 interactions, increased p65 phosphorylation, and nuclear translocation, leading ultimately to prolonged CLL cell survival (42).

Regarding signaling pathways, there is significant evidence of distinct expression profiles of TLR pathway-associated genes in subset #1 when compared with other subset or non-subset CLL. More particularly, increased expression of *TLR7* and *NFKBIA* and, in contrast, reduced expression of *CD86* and *TLR4* have been reported in subset #1 versus clinically indolent CLL subset #4 cases (32). These differences are also functionally relevant, considering that TLR stimulation results in distinct regulation of expression of immune-related molecules but also distinct cellular

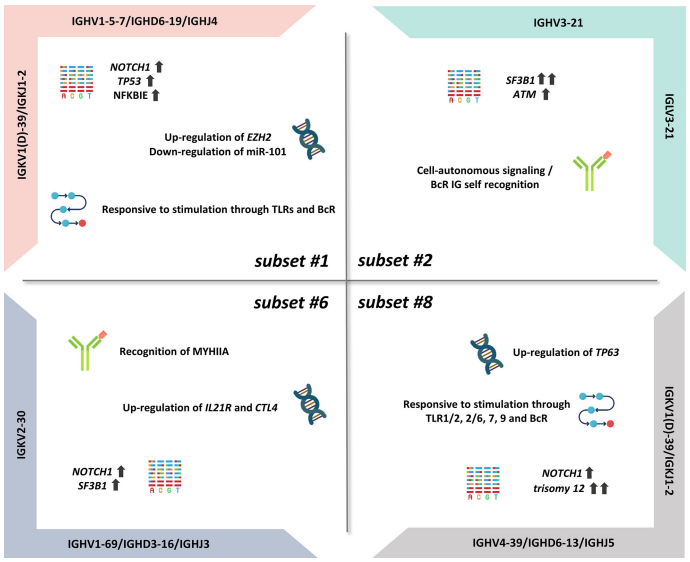

FIGURE 1 | Summary of the biological features of aggressive CLL subsets.

activation outcomes. For example, TLR7 stimulation with imiquimod induces CD25 upregulation in subset #1, albeit not the case in subset #4, whereas TLR9 stimulation leads to antiapoptotic effects preferentially in subset #1 versus all other U-CLL (33).

Subset #1 cases display a unique transcriptional profile even when compared with other CLL cases with concordant SHM status: differentially expressed genes are implicated in apoptosis (e.g., *ATM*, *PARP1*), cell proliferation (e.g., *KRAS*), and oxidative processes favoring the survival of CLL cells (39). In line with these findings, BcR stimulation with anti-IgM led to a higher proliferation rate in both basal state and after 24–48 h of stimulation in subset #1 versus non-subset U-CLL cases (39).

CLL subset #1 is also notable for elevated expression of the histone methyltranferase Enhancer of Zeste Homolog 2 (*EZH2*), the catalytic core protein of the Polycomb Repressive Complex 2 (PRC2) (43). EZH2 represses genes involved in various cellular processes, such us cell cycle regulation and cell differentiation, through trimethylation of histone H3 at lysine 27 (H3K27me3) (43). In a previous study of our group, we showed that EZH2 mRNA levels are increased in subset #1 when compared with indolent subset #4, thus implicating for the first time EZH2 in the pathophysiology of aggressive CLL (43). Of note, EZH2 expression appeared to be partially modulated by miR-101, an "epi-miRNA" that inhibits the function of EZH2 and was found downregulated in subset #1, inversely correlating with EZH2 protein and mRNA levels; this conclusion was supported by the fact that forced overexpression or downregulation of miR-101 in primary cells of subset #1 cases affected EZH2 protein levels in the exact reverse way (43).

Prompted by these observations, we next investigated at the preclinical level the impact of EZH2 inhibition in aggressive CLL cases, particularly subset #1. We found that combined inhibition of EZH2 activity and BcR signaling had synergistic antitumor effects while EZH2 inhibitors exhibited *ex vivo* efficacy in CLL cases unresponsive to signaling inhibitors (44). These results should be interpreted clinically considering that EZH2 was also found to regulate the PI3K/AKT prosurvival pathway in a PRC2-independent, non-canonical way by directly binding to the *IGF1R* promoter (45). On these grounds, EZH2 emerges as a potential therapeutic target in CLL, warranting further preclinical and clinical investigation.

CLL SUBSET #2

Subset #2 represents the largest stereotyped subset in CLL, accounting for ~2.5%–3% of all patients and ~5.5% of patients requiring treatment (9, 25, 40). The particular BcR IG of subset #2 is composed of heavy and light chains encoded by the IGHV3-21 and the IGLV3-21 genes, respectively. The clonotypic IGHV3-21 genes bear a variable SHM load, with most cases (~60%–65%) classified as M-CLL (23, 25). The SHM patterns in both the heavy and light chains of subset #2 supported antigen pressure, with some SHMs revealed as critical for self-association leading to cell-autonomous signaling (36, 46). Relevant to mention, we recently demonstrated that stereotyped subset #169, a minor CLL subset (~0.2% of all CLL), bears striking immunogenetic but also biological and clinical similarities to subset #2 (25).

Independent of the SHM status, subset #2 cases have a particularly dismal clinical outcome (9, 40, 47) similar to that of patients with *TP53* aberrations, although they very rarely harbor such aberrations (29, 40, 41, 47–51). Instead, subset #2 and subset #169 display a remarkably high frequency of mutations in *SF3B1*, which encodes a splicing factor with a

crucial role in the spliceosome machinery (52). Indeed, approximately half of the subset #2 patients carry *SF3B1* mutations (41, 48, 49), in contrast with patients belonging to other aggressive CLL subsets, namely #1 and #8 (4.6% and 0%, respectively) or non-subset CLL, where such mutations are present in 5%–8% of cases (48). The exact functional role of spliceosome deregulation in subset #2 remains to be fully elucidated. *ATM* mutations and del(11q) are also significantly enriched in subset #2 cases (40, 51). ATM disruption is associated with short telomeres which in turn correlates with reduced TTFT and overall survival (OS) in subset #2 (51).

Uniquely among B-cell malignancies, CLL has been found to display an alternative mode of cell activation that is independent of antigen and results from homotypic interactions between two different BcR IG molecules (34). Studies from our group have dissected the molecular basis of cell-autonomous signaling in CLL, revealing distinct modes of homotypic interactions in different CLL subsets (36, 46). Particularly for subsets #2 and #169, it has been demonstrated that BcR–BcR interactions critically rely on light chain-mediated contacts, with a specific mutation from the germline sequence in the linker region between the variable and the constant domain of the light chains, namely, the substitution of arginine for glycine (termed R110) in the clonotypic light chain encoded by the IGLV3-21*01 allele (IGLV3-21^{R110}), identified as key to the capacity for homodimerization underlying cell-autonomous signaling (36, 46).

More recently, the expression of IGLV3-21^{R110} immunoglobulin light chains was documented in CLL cases beyond subsets #2 and #169 (53, 54). Such cases have been reported to be associated with a distinct gene expression profile and aggressive clinical courses, regardless of IGHV gene usage, SHM status, and classic cytogenetic abnormalities (53, 54). Altogether, these findings highlight the critical role of IG light chains in shaping the functional status and, eventually, the clinical behavior of CLL clones, while also pointing to another form of stereotypy, mainly defined by IG light chain restrictions.

CLL SUBSET #6

Subset #6 is another well-characterized clinically aggressive CLL subgroup (0.8% of all CLL), concerning cases bearing unmutated BcR IG (25). The clonotypic IGHV1-69/IGHD3-16/IGHJ3 gene rearrangements are combined with restricted IGKV2-30 gene light chain rearrangements (20).

An integrated epigenomic and transcriptomic comparison of subset #6 versus subset #8, another well-characterized U-CLL subset (see next paragraph), has revealed that *IL21R* and *CTLA4* are hypomethylated in both groups, however showing increased mRNA expression in subset #6 versus subset #8 (55). These findings are relevant, considering that the interleukin-21 receptor (IL-21R) is upregulated by CD40 stimulation and mediates proapoptotic signaling in CLL (56), while *CTLA4* augmented expression results in decreased proliferation and cell survival (57, 58). Moreover, these results appear to be in line with the more indolent disease course of subset #6 compared with subset #8 (55).

Regarding the genetic landscape, CLL cases assigned to stereotyped #6 display low frequency of *TP53* mutations (4%), low-to-intermediate frequency of *SF3B1* mutations (13%) and, in contrast, high frequency of *NOTCH1* mutations (22%) which, interestingly, was not accompanied by trisomy 12 in almost none of the cases (41). Moreover, there is a strong evidence for selection by a common antigen in subset #6: in fact, it has been conclusively demonstrated that subset #6 BcR IG recognizes non-muscle myosin heavy chain IIA (MYHIIA), which appears on the surface of cells undergoing stress or apoptosis, with this recognition driving CLL cell survival and proliferation (59).

CLL SUBSET #8

Subset #8 accounts for approximately 0.5% of all CLL and includes cases bearing unmutated IGHV4-39/IGHD6-13/IGHJ5 gene rearrangements paired with IGKV1(D)-39/IGKJ2 gene rearrangements (17, 60). Notably, the stereotyped heavy chains of subset #8 are IgG-switched, itself a rarity in CLL (61). From a clinical perspective, subset #8 has emerged as a prototype of clinical aggressiveness as it displays the highest risk for Richter's transformation among all CLL (35).

Subset #8 cases exhibit a unique constellation of genomic abnormalities including high frequency of trisomy 12 (63%–87%) (40, 49) as well as *NOTCH1* mutations (from 14% to 62%, depending on the studied cohort) (41, 48, 49). From a different perspective, subset #8 cases display excessive (promiscuous) antigen reactivity as the corresponding BcR IG, expressed as recombinant monoclonal antibodies (rmAbs), bound a plethora of antigens, including autoantigens and neo-epitopes, in contrast with other aggressive CLL subsets, namely #1 and #2, that did not exhibit such polyreactivity (27).

Probably as a result of the broad antigen reactivity, subset #8 CLL cells also displayed pronounced signaling capacity responding to triggering through both adaptive and innate immunity receptors. In particular, BcR and TLR stimulation induced a significant increase in the phosphorylation of ERK and PLCγ2 in subset #8 compared with subsets #1 and #2 (27). These results are in keeping with our observation that subset #8 exhibits intense responses to TLR1/2, 2/6, 7, and 9 stimulation, including upregulation of the costimulatory molecules CD25 and CD86 (33). On these grounds, we propose that the transformation propensity of subset #8 CLL clones may be linked to both the extreme antigen polyreactivity of the clonotypic BcR IG and the excessive signaling capacity of the malignant cells.

Cases assigned to subset #8 exhibit distinct epigenetic profiles compared with other subset and non-subset U-CLL cases (55). In fact, comparison of the DNA methylation profiles between subsets #8 and #6 revealed mainly hypomethylated sites in the former, particularly in gene bodies and promoters of genes implicated in several pathways including cancer cell signaling (55). Integrated transcriptome and methylation analysis of these two subsets highlighted the *TP63* gene as hypomethylated and

overexpressed in subset #8 versus subset #6 cases (55). p63, the protein encoded by the *TP63* gene, is a transcription factor of the p53–p63–p73 family which regulates several cellular processes, e.g., apoptosis, proliferation, cell adhesion, and differentiation (62). mRNA and protein expression analysis confirmed that subset #8 cases displayed the highest *TP63* expression among all CLL cases examined (55). Of note, p63 expression was found to be modulated by immune signaling through the BcR with differential effects between subsets. In more detail, BcR stimulation resulted in significant upregulation of p63 levels and cell viability in subset #8 cases, while it did not affect the corresponding expression levels in subset #6 cases (55). Confirmation of the prosurvival role of p63 was achieved by RNA silencing of the *TP63* gene which led to notable downregulation of p63 levels and decrease of the number of viable cells providing evidence for the contribution of p63 in clinical aggressiveness of CLL subset #8 cases (55).

CONCLUSIONS

BcR IG stereotypy allows the subdivision of CLL patients into subsets with homogeneous profiles, allowing to consider targeted therapeutic approaches tailored to each subset. This is clinically relevant, given that CLL remains incurable despite major therapeutic advances achieved in recent years thanks to the introduction of signaling and BCL2 inhibitors in the clinical practice. This highlights the urgent need to further dissect the heterogeneity of CLL toward identifying additional mechanisms of resistance: arguably, zooming on subsets is a plausible strategy toward this aim.

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Chromothripsis in Chronic Lymphocytic Leukemia: A Driving Force of Genome Instability

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Chromothripsis represents a mechanism of massive chromosome shattering and reassembly leading to the formation of derivative chromosomes with abnormal functions and expression. It has been observed in many cancer types, importantly, including chronic lymphocytic leukemia (CLL). Due to the associated chromosomal rearrangements, it has a significant impact on the pathophysiology of the disease. Recent studies have suggested that chromothripsis may be more common than initially inferred, especially in CLL cases with adverse clinical outcome. Here, we review the main features of chromothripsis, the challenges of its assessment, and the potential benefit of its detection. We summarize recent findings of chromothripsis occurrence across hematological malignancies and address its causes and consequences in the context of CLL clinical features, as well as chromothripsis-related molecular abnormalities described in published CLL studies. Furthermore, we discuss the use of the current knowledge about genome functions associated with chromothripsis in the optimization of treatment strategies in CLL.

Keywords: chromothripsis, chronic lymphocytic leukemia, complex chromosomal rearrangements, copy number alterations, genomic array, paired-end sequencing, oncogene amplification, tumor suppressor inactivation

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in Western countries with a highly variable clinical course. Several recurrent chromosomal alterations have been associated with prognosis and may guide risk-adapted therapy. Besides deletions on chromosomes 11, 13, 17, and trisomy 12, high genomic complexity (high-GC) has also been recognized as a feature with prognostic value (1, 2) and is associated with poor clinical outcome (3, 4). Cytogenetics and array-based methods define high-GC as five or more chromosomal defects (1, 2). In many instances, highly complex karyotypes can be caused by chromothripsis (cth) (5), a genomic event by which a single or a limited number of chromosomes are shattered into pieces, followed by error-prone reassembly (6–9).

Among all cancers, it was CLL where the evidence of cth was reported for the first time. This finding was made already a decade ago *via* the whole-genome sequencing screening of 10 CLL

patients (5). In a sample from a 62-year-old woman without any previous CLL treatment, a massive rearrangement of chromosomal arm 4q and focal alterations on chromosomes 1, 12, and 15 were found, showing striking patterns. It was proved that this complex genomic remodeling had occurred before the diagnosis and persisted until the rapid disease relapse after alemtuzumab treatment without further evolution. The phenomenon was termed chromothripsis (from Greek; chromos for chromosome, thripsis for shattering into pieces) and was subsequently observed in many other tumor types (5, 10–15).

In contrast to the traditional view of tumorigenesis as the multi-step accumulation of mutations, cth arises *via* a single devastating event. Within a single cell division, tens to hundreds of DNA double-strand breaks are generated and imperfectly assembled into derivative chromosomes, most often *via* non-homologous end joining (NHEJ), whereas some fragments can be lost (**Figure 1A**). The massively rearranged genomes of the cells that survive such an event propagate in daughter clones and are likely to have gained a strong selection advantage, as cth could disrupt the functions of tumor suppressors, support the oncogene amplification, and/or give rise to pathogenic gene fusions. Thus, cth is a potential driving force of malignant transformation and tumor progression.

DETECTION OF CHROMOTHRIPSIS-LIKE PATTERNS

Cth is characterized by several hallmarks that set it apart from other complex genomic changes: (a) occurrence of tens to hundreds of chromosomal rearrangements with pronounced clustering, (b) random orientation of rearrangements resulting in equal representation of deletions, inversions, and tandem duplications, (c) copy-number alterations (CNAs) oscillating between two (occasionally three) copy-number states, (d) alterations of segments that retained heterozygosity and segments with loss-of-heterozygosity (LOH), (e) structural rearrangements displaying a bias toward occurring on a single chromosome homolog, and (f) presence of double-minute chromosomes (5, 15). The evidence of different cth patterns in various cancer types and among individual cases (21) suggests different mechanisms of its origin. The mechanisms, presumed most frequently to cause cth, include asynchronous DNA replication in abnormal nuclear structures called micronuclei (6, 22, 22) (**Figure 1B**) and the fragmentation of dicentric chromosomes resulting from the telomere crisis due to their extreme shortening (23) (**Figure 1C**).

Since the genomic profile originating in cth could be similar to stepwise processes, the detection of cth is often challenging. Therefore, a set of criteria was generated for accurate and reproducible cth inference (7). Most of these criteria take into account the entire set of structural rearrangements that occurred on a chromosome, including the relative order and orientation of rearranged segments. They are typically detected using whole-genome paired-end DNA sequencing. Copy-number states can also be analyzed by array-based comparative genomic hybridization (aCGH) or single nucleotide polymorphism (SNP) arrays. However, for the most accurate detection of cth, it is desirable to use a complex approach that combines sequencing genomic methods with molecular cytogenetics and other complementary methods (24). A conventional karyotyping of metaphases can be useful to identify numerical and structural chromosomal abnormalities. Various fluorescence *in situ* hybridization (FISH) techniques may aid the identification of interacting chromosome partners and localization of breakpoints. Spectral karyotyping in combination with fluorescent locus-specific probes can effectively detect the double-minute chromosomes (5). Above that, RNA-Seq can assist in revealing abnormalities at the transcriptional level such as *de novo* fusion transcripts or abnormal gene expression, both of which can be revealed with advanced analytical methods (25).

CHROMOTHRIPSIS IN HEMATOLOGICAL MALIGNANCIES

Cth has been observed in primary tumors of various histological types, including hematological malignancies, such as lymphomas (19, 21, 26), multiple myeloma (11, 26, 27), myelodysplastic syndrome (26, 28, 29), and leukemias (2, 3, 5, 15–21, 24, 30–39). The prevalence of cth across cancer types ranges from units to tens of percent with the highest proportions in sarcomas – up to 100% (5, 21, 40). However, the comparison of published studies provides only rough estimation due to different methodologies and definitions used for cth scoring. The cth frequencies observed in hematological malignancies are summarized in **Table 1**.

For most hematological diseases, cth provides independent prognostic information and is associated with adverse clinical outcome. In myelodysplastic syndrome, the complex chromosomal rearrangements caused by cth are related to advanced disease stages prone to transform to acute myeloid leukemia (AML); as a consequence, they recurrently involve 5q deletions (28). Similarly, AML patients with cth have a high recurrence of 5q losses, and also *TP53* dysregulation and the presence of marker chromosomes (30–32). Besides that, cth appears to be mutually exclusive with *FLT3* and *NPM1* mutations (30, 32). In acute lymphoblastic leukemia (ALL), cth occurs predominantly in specific subgroups, such as early T cell precursor ALL (35, 37), iAMP21 B-ALL (36), and ataxia-telangiectasia-related T-ALL (37).

The evidence of cth cases described in CLL indicates that this phenomenon is a recurrent event. By exploring larger cohorts of CLL patients, cth was observed with frequencies from 1.2 to 10% (2, 3, 5, 18, 21, 24, 38, 39). Although the reported prevalence is relatively small, the analysis of cth-like patterns may be beneficial for clinical decision-making and precision medicine, as cth represents a driving force of genome evolution in CLL (5, 16, 17, 20).

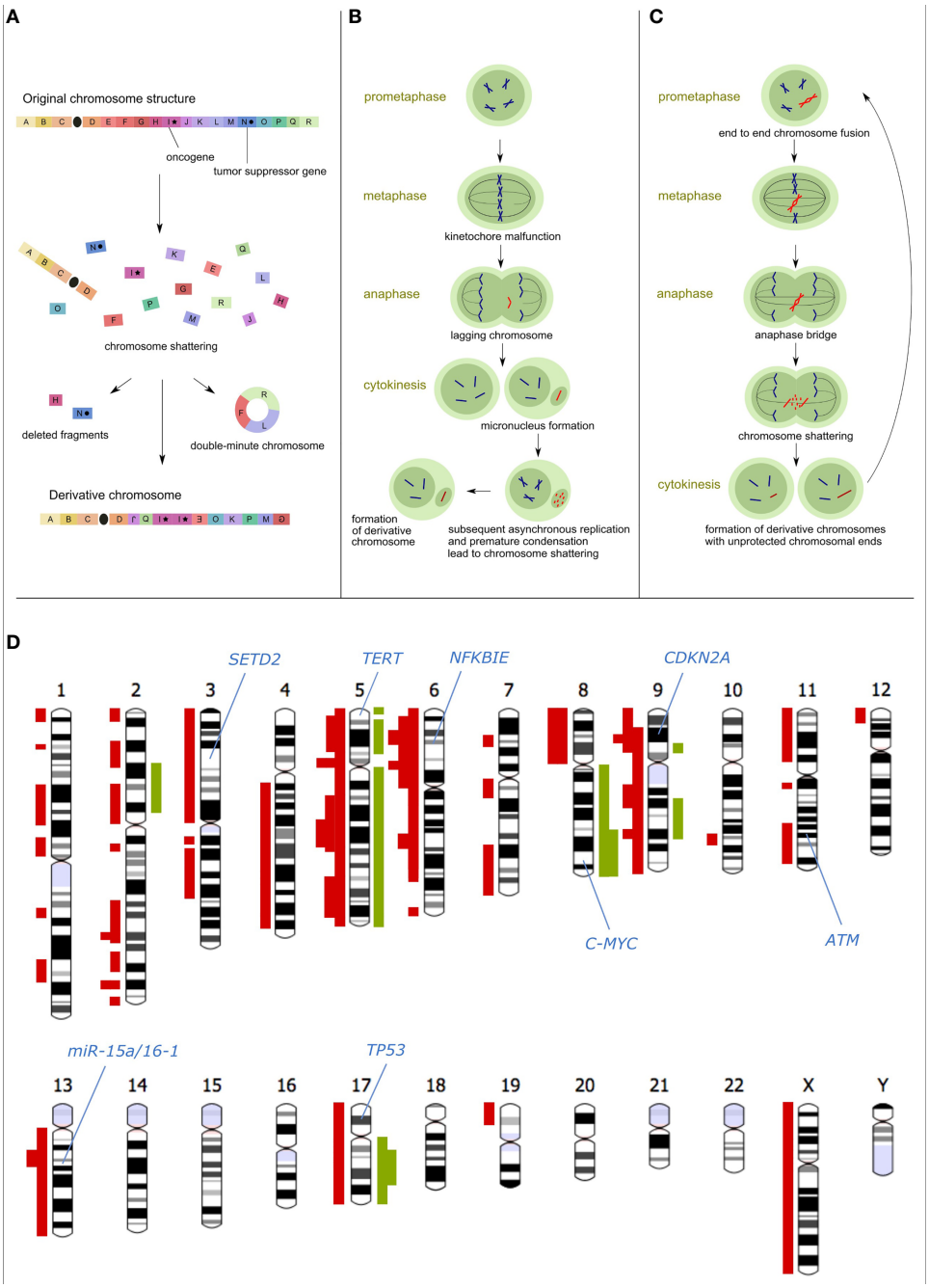

FIGURE 1 | Causes and consequences of chromothripsis. **(A)** Schematic model of chromosome shattering and reassembly *via* cth: After chromosome fragmentation, some regions are incorporated (possibly in multiple copies) into a derivative chromosome, whereas other regions can be lost or fused to episomal structures called double-minute chromosomes. **(B)** The micronuclei hypothesis of the cth origin: Chromosomes that are missegregated during cell division are entrapped in the micronucleus, followed by asynchronous replication compared to the main nucleus. This leads to premature chromosome condensation and shattering. Rejoining of fragments gives rise to the derivative chromosome which can subsequently be reincorporated into the main nucleus. **(C)** The origin of cth due to breakage-fusion-bridge (BFB) cycles and telomere crisis: Chromosome ends that become unprotected due to telomere shortening are fused into a dicentric chromosome containing two centromeres. In the subsequent cell cycle, this unstable structure is pulled to opposite spindle poles forming an anaphase bridge between the two daughter cells. The rupturing bridge generates two new unprotected chromosomal ends and initiates a new round of the BFB cycle. This repeats until the derivative chromosome becomes stable. **(D)** Chromosomal ideograms with cth-derived gains (green) and losses (red) observed in the following CLL studies: Stephens et al., 2011 (5); Edelmann et al., 2012 (3); Pei et al., 2012 (16), Bassaganyas et al., 2013 (17); Salaverria et al., 2013 (18); Tan et al., 2015 (19); Parker et al., 2016 (20); Leeksma et al., 2021 (2). The thickness of the highlighted loci corresponds to the number of studies referring to the respective regions affected by cth. Only studies mentioning specific affected areas and distinguishing individual patients were compiled.

TABLE 1 | Prevalence of chromothripsis in CLL and other hematological malignancies.

Reference	Clinical characterization of the cohort	Clinical characterization of cth cases	n/N	Cth prevalence	Method
Chronic lymphocytic leukemia					
Stephens et al., 2011 (5)	not specified	rapid relapse after alemtuzumab	1/10	**10%**	WGS
Edelmann et al., 2012 (3)	treatment-naïve; samples from the GCLLSG CLL8 trial	poor survival; 74% with unmutated IGHV; 32% with mutated *TP53*	19/353	**5.4%**	SNP array
Salaverria et al., 2015 (18)	26% treatment-naïve	poor survival; 75% with *TP53* abnormality (mutation and/or deletion)	8/180	**4.4%**	aCGH
Puente et al., 2015 (38)	treatment-naïve	26% with mutated *TP53*; 26% with inactivated *SETD2*, 25% with loss of *mir-15a/mir-16*	15/452	**3.3%**	SNP array, WGS
Parker et al., 2016 (20)	93% treatment-naïve; 84% of samples from the ADMIRE, ARCTIC, UK CLL4, GCLLSG CLL8, and SCSG CLL2O trials	poor outcome; 26% with *SETD2* deletion	27/1,006	**2.7%**	SNP array
Burns et al., 2018 (39)	52% treatment-naïve	with *TP53* deletion	1/46	**2.2%**	WGS
Cortés-Ciriano et al., 2020 (21)	data from the PCAWG Consortium (41)	not specified	1/86	**1.2%**	WGS
Leeksma et al., 2021 (2)	86% treatment-naïve; samples from 13 CLL diagnostic centers participating in ERIC	poor survival; all with *TP53* abnormality (mutation and/or deletion) and del(11q)	32/2,293	**1.4%**	SNP array, aCGH
Ramos-Campoy et al., 2021 (24)	treatment-naïve; 47% with complex karyotypes	poor outcome, 73% with *TP53* abnormality	30/340	**8.8%**	SNP array, aCGH
Acute myeloid leukemia					
Rausch et al., 2012 (15)	non-M3 AML; treatment-naïve; adults	poor survival, 89% with mutated *TP53*	9/108	**8.3%**	SNP array
Fontana et al., 2018 (32)	82% *de novo* AML, 12% AML secondary to myelodysplastic syndrome, 1% AML secondary to myeloid neoplasms, 5% therapy-related AML; mostly adults (median age 59.35)	poor outcome; 70% of cases treated with chemotherapy did not respond; 88% with *TP53* abnormality (mutation and/or deletion)	26/395	**6.6%**	SNP array
Myelodysplastic syndrome					
Kim et al., 2013 (26)	data from the GEO database (42)	not specified	7/393	**1.8%**	aCGH
Zemanova et al., 2014 (28)	treatment-naïve; with complex chromosomal rearrangements (≥3 aberrations)	not specified	77/157	**49%**	SNP array
Abáigar et al., 2016 (29)	treatment-naïve	high-risk MDS; all died within one year; all with mutated *TP53*	3/240	**1.3%**	aCGH
Acute lymphoblastic leukemia					
Zhang et al., 2012 (35)	childhood early T cell precursor ALL	2 cases relapsed 8 and 13 months after diagnosis, 1 case underwent bone marrow transplantation; all died	3/12	**25%**	WGS
Li et al., 2014 (36)	childhood ALL; 56% with sporadic iAMP21, 44% with rob(15;21)c-associated iAMP21	not specified	8/9	**89%**	WGS
Ratnaparkhe et al., 2017 (37)	childhood ataxia-telangiectasia-related T-ALL	1 case died 2 years after diagnosis, 1 case died from toxicity, 3 cases still alive (2/3 in remission)	5/7	**71%**	WGS
Ratnaparkhe et al., 2017 (37) *	sporadic childhood T-ALL	not specified	4/92	**4.3%**	WGS
Multiple myeloma					
Magrangeas et al., 2011 (11)	treatment-naïve	50% with rapid relapse	10/764	**1.3%**	SNP array
Stevens-Kroef et al., 2012 (27)	82% treatment-naïve	not specified	1/28	**3.6%**	SNP array
Kim et al., 2013 (26)	data from the GEO database (42)	not specified	8/391	**2%**	aCGH
Voronina et al., 2020 (43)	data from the NCT/DKTK-MASTER platform (44)	not specified	2/6	**33%**	WGS
Lymphoma					
Cortés-Ciriano et al., 2020 (21)	mature B cell non-Hodgkin lymphoma; data from the PCAWG Consortium (41)	not specified	19/105	**18%**	WGS

**refers to unpublished data discussed with Meijerink et al., partly published in Li et al., 2016 (45).*

n, the number of cth cases; N, the total number of cases analyzed in the respective study; GCLLSG, German CLL Study Group; PCAWG, Pan-Cancer Analysis of Whole Genomes; ERIC, European Research Initiative on CLL; GEO, Gene Expression Omnibus; NCT/DKTK-MASTER, National Center for Tumor Diseases/German Cancer Consortium-Molecularly Aided Stratification for Tumor Eradication; WGS, whole-genome sequencing; SNP array, single-nucleotide polymorphism array; aCGH, array comparative genomic hybridization.

IMPACT OF CHROMOTHRIPSIS ON CLL ONSET AND PROGRESSION

CLL patients with cth (cth-CLL) were shown to have inferior time to first treatment (24), progression-free survival (3), and overall survival (2, 3, 18, 38). The overall survival of cth-CLL cases was even worse than of cases with *TP53* abnormality or del(11q) without cth (2). The majority of cth-CLL cases have unmutated IGHV (3, 17, 24, 38, 39). Two studies concluded that cth is more frequent in the IGHV-unmutated group with statistical significance (3, 38). There is also a strong link between the presence of cth and high-risk genomic aberrations like del(11q) and del(17p) (2, 3, 16, 17, 19, 20, 24, 39).

Some studies reported that cth occurs before the CLL diagnosis indicating that the complex genomic remodeling could be a CLL-initiating event (5, 16) or one of the earliest events in the CLL pathogenesis (20). On the contrary, a case study from 2013 showed that cth is not necessarily triggering the CLL onset. In this case, cth was a consequence of previous alterations accumulated since the time of diagnosis and contributed to the increase of CLL aggressiveness, as a subclone carrying complex structural variants expanded and outbalanced the predominant tumor population before the first treatment (17). Interestingly, the cth-subclone was eradicated by chemotherapy and did not reappear throughout a 10-year follow-up period. This observation contrasts other data strongly associating cth-clones with chemotherapy resistance and/or poor clinical outcome (2, 3, 5). That points to the substantial need for larger cohorts of cth cases to be analyzed to better understand the dynamics of cth in CLL.

GENOMIC REGIONS ASSOCIATED WITH CHROMOTHRIPSIS IN CLL

Chromosomes 2, 3, 6, 8, 9, 11, 13, and 17 were impacted by cth in CLL most frequently (2, 3, 16, 17, 19, 20, 39) (**Figure 1D**). Many cth-CLL cases harbor del(17p) (2, 3, 16, 19, 20, 24, 39) spanning the *TP53* gene, the most important predictor of disease and treatment outcome (46–51). Alterations in *TP53* are the most common changes associated with cth in medulloblastoma (15), acute myeloid leukemia (15), pediatric cancers (52), and CLL (2, 3, 20, 24, 38). *TP53* is responsible for cell cycle control, genome maintenance, and apoptosis (53, 54), confirming its plausible involvement in genome instability preceding cth. The frequent co-occurrence of *TP53* alterations and cth in CLL supports both possibilities of their relation, i.e. cth resulting from *TP53* disruption as well as cth leading to *TP53* abnormalities and therefore more aggressive disease. Alterations in *ATM* including del(11q) and gene mutations can also explain the rise of cth considering its role in the regulation of the DNA damage response (DDR) and were observed in patients with cth (2, 3, 20, 39). In this context, Bassaganyas et al. (17) observed the ATM^{R1897T} mutation in the CLL patient two years before cth detection.

Moreover, *SETD2* deletions have been associated with the loss of *TP53*, genomic complexity, and cth and define a subgroup of patients with poor outcome (20). The published data highlight *SETD2* aberrations as a recurrent, clonal, early loss-of-function event in CLL pathobiology that appears to be the result of cth and linked to aggressive disease. In this comprehensive study, 26% CLL cases with *SETD2* deletions showed evidence of cth on chromosome 3, constituting predominantly cases with ultra-high-risk CLL. Another study also proved that *SETD2* inactivation is more frequent in CLL cases with cth than in non-cth cases (26% versus 1.4%) (38).

In the case study by Bassaganyas et al. (17), the authors found cth-derived deletion of 6q21 spanning the *NFKBIE* gene. In general, del(6q) is known to be present in 6% of CLL and linked to shorter progression-free survival (3, 46, 55). In the reported case, the concurrent $NFKBIE^{E285X}$ mutation on the other allele led to the absence of a functional *NFKBIE* in cth-subclone. Moreover, del(10q24) involving *NFKB2*, a subunit of NF-κB transcription factor complex regulating the *NFKBIE* transcription, was observed (17).

Although seen with low frequency, there were observations of the cth-related gain of 8q (the *C-MYC* gene) (19), loss of chromosome 13 (mir-15a/mir-16) (5, 38), and loss of 14q (16), which are recurrently detected in CLL. Loss of 8p, associated with a higher number of CNAs in CLL (56, 57), was also observed in cth-CLL (39). In addition, RNA-Seq revealed a fusion transcript of *UBR2-SPATS1* in one case (17) potentially contributing to disease aggressiveness, as the *UBR2* gene is involved in the cell growth controlling (58) and could have been deregulated or have gained a new function due to premature truncation and fusion with the second partner.

ASSOCIATIONS OF TELOMERE BIOLOGY AND CHROMOTHRIPSIS IN CLL

Telomere dysfunction is known to have a dynamic role in shaping a disease course in CLL (59, 60). Physiological telomere shortening corresponding to the number of divisions a cell goes through leads to gradual uncapping of the chromosome ends. At a certain critical point, telomeres are recognized as DNA double-strand breaks and trigger the DDR. As a consequence, the senescence and/or apoptosis checkpoints are activated to prevent neoplastic transformation (61). If protective mechanisms are compromised, cells may continue to proliferate, which results in genomic instability (62). Studies have shown that CLL cells have a close inverse correlation between telomere length and telomerase activity compared to healthy cells (63–66). This could be explained by the theory that the genomic instability associated with shorter telomeres promotes the selection of fit CLL clones that overcome senescence and sustain cell survival due to the maintenance of minimal telomere length by telomerase. It was shown that the tumor microenvironment-mediated signaling, such as BCR or PI3K signaling, contributes to telomerase activation (67).

The dysfunctional telomeres often induce intra- or inter-chromosomal end fusions that can occur as clonal events. Their frequency was found to increase with the advancing disease stage in CLL (68). Such telomere fusions result in the formation of dicentric chromosomes that undergo breakage at the anaphase. This phenomenon is known as the breakage-fusion-bridge (BFB) cycle (69) and can be a precursor to genomic complexity such as cth (23, 70) (**Figure 1C**). Studies described the association of short telomeres with complex karyotypes (64, 71, 72) or with a higher number of CNAs (73, 74) in CLL. Unlike other tumor entities (e.g. central nervous system tumors), CLL cells were shown to have shorter telomeres in the cases with cth as compared to the cases without cth (70).

In general, the telomere length has been proposed to be an independent prognostic factor in CLL, with short telomeres being associated with adverse outcome (63–66, 73–76), the presence of del(11q) and del(17p) (64, 72–75, 77), as well as mutations in *ATM* and *TP53* (72–74, 76–78) both of which serve as critical checkpoint genes activated upon telomere shortening. However, the association between telomere dysfunction and cth was confirmed to be independent of the *TP53* mutation status in CLL (70). It has been supposed that in cases where no somatic *TP53* mutation was detected, other aberrations affecting the DDR and/or potentially inducing p53 dysfunction likely allow the cell to avoid apoptosis despite telomere dysfunction. On the other hand, del(17p) treatment-naïve CLL patients with cth have significantly shorter telomeres compared to those without cth (79). Moreover, loss of *SMC5*, which is involved in maintaining genomic stability and plays a role in telomere-related functions, might favor cth, especially when co-occurring with short telomeres and *TP53* defects (79). In addition, certain CLL cases with cth-like patterns in the 5p region were discovered, including gains of *TERT*, which encodes the telomerase reverse transcriptase (18).

It is presumed that the derivative chromosomes resulting from cth are likely stabilized hindering further progressive chromosomal cataclysm that would be incompatible with cell survival. From longitudinal observations, the chromothriptic patterns in CLL patients are either stable, in which case the relapse specimens show similar aberrations to the primary samples (5, 70), or they are lost by clonal selection in the relapse (17). Thus, telomere stabilization mechanisms are likely activated after the occurrence of cth to prevent continuing (and presumably lethal) genome-wide disruption.

All the mentioned findings confirm that the telomere attrition followed by end-to-end chromosome fusion and subsequent breakage leads to cth in CLL. This is followed by the establishment of telomere maintenance mechanisms that "lock-in" these alterations and prevent further lethal events. It, therefore, highlights the importance of detecting cth in the context of telomere length for risk stratification as well as for monitoring and early identification of clonal changes. Similarly, telomere maintenance mechanisms may represent a target for therapeutic intervention in cth-positive cases.

CHROMOTHRIPSIS IN CLL DIAGNOSTICS AND TREATMENT

The available data suggest the potential of cth detection for better stratification of CLL patients by recognizing cases with highly complex karyotypes and thus adverse prognosis. Studies showed that cth-CLL patients show adverse clinical course and demand an early therapeutic intervention (2, 3, 5, 18, 19), often even evolving refractory disease (3, 5, 20).

As follows from the information above, cth is a consequence of genomic instability and is associated with aberrations in specific molecular pathways (15, 22, 80). In these cases (presumably more than in others) the cell signaling inhibitors should provide a promising outcome, similarly to the cases with defective *TP53*. However, no studies are available yet.

In general, the detection of cth-associated abnormalities could serve for the identification of molecular therapeutic targets. For instance, targeting oncogenes amplified *via* cth might provide a therapeutic benefit. Additionally, leukemic cells with cth could successfully respond to immune checkpoint blockade due to potential neoantigens generated from genomic rearrangements (81). The neoantigens were proven to bind patient-specific major histocompatibility complex molecules and to expand tumor-infiltrating T cell clones (82). These findings might be exploited for the development of novel immunotherapeutic approaches as well as the selection of patients to be administered immunotherapies. This strategy has already been suggested for a subset of AML patients with a high burden of alterations (32). Similarly, cth-derived fusion genes can help to increase the sensitivity of cancer cells to certain types of agents. An example is a patient with myelodysplastic syndrome, where several cryptic fusions, including *ETV6–PDGFRB*, were found (83). This is underlain by the fact that the myeloid neoplasms associated with *PDGFRB* rearrangement represent a specific entity sensitive to tyrosine kinase inhibitors (84).

Besides that, a synthetic lethality approach (85) is also an option for cth-CLL treatment. This approach is based on targeting a synthetic-lethal partner of a gene that is already mutated or overexpressed – that means targeting a gene that is compensating for the loss of activity of the dysfunctional one. Simultaneous inactivation of such gene pair results in cell death (85). As the defects in the DDR mechanism are frequently associated with cth, the cells have an increased level of DNA damage and evolve new mechanisms to resist endogenous and exogenous stress. The strategy of synthetic lethality in such cases could combine current treatment modalities with drugs targeting residual DNA repair pathways that such cells are dependent on (86).

CONCLUSIONS

Based on the available data, cth is a recurrent event in CLL and could have a strong prognostic value. Although there is rapid progress in understanding molecular processes behind cth, current studies have important limitations. The biggest

drawback is a relatively small number of CLL patients that have been analyzed so far which hampers the reproducibility of published results. Another issue is missing longitudinal observations. Most studies focus on a single time point of the disease, usually treatment-naïve. However, the information about the dynamics of the cth and the changes accompanying this event is lacking. It would be of interest to elucidate which changes precede the development of cth and which, in contrast, are more frequently its consequence. These findings would facilitate a better understanding of CLL clonal evolution and its driving forces and could reveal recurrently altered molecular pathways with different prognostic impacts.

The genomic landscape induced by cth is complex and linking cth to specific clinical outcomes is not always straightforward. The genes and genomic regions affected by cth appear to be the most important factors for the disease phenotype, not the occurrence of cth itself. This highlights the growing need for personalized medicine to be implemented into CLL treatment. Analyzing tumor samples at different time points should also be a part of the clinical program to elucidate clonal genotypes that could be therapy-resistant, which might help in therapeutic decisions along the disease course.

AUTHOR CONTRIBUTIONS

KZ drafted the manuscript and created figures. KP proposed the structure and supervised manuscript preparation, both authors performed the literature search and contributed to manuscript writing. Both authors contributed to the article and approved the submitted version.

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Biology and Treatment of High-Risk CLL: Significance of Complex Karyotype

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Several reports highlight the clinical significance of cytogenetic complexity, namely, complex karyotype (CK) identified though the performance of chromosome banding analysis (CBA) in chronic lymphocytic leukemia. Indeed, apart from a number of studies underscoring the prognostic and predictive value of CK in the chemo(immune)therapy era, mounting evidence suggests that CK could serve as an independent prognosticator and predictor even in patients treated with novel agents. In the present review, we provide an overview of the current knowledge regarding the clinical impact of CK in CLL, touching upon open issues related to the incorporation of CK in the clinical setting.

Keywords: CLL (chronic lymphocytic leukemia), prognosis, prediction, high-risk, complex karyotype (CK), cytogenetic complexity

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common hematological malignancy among the elderly in the western world, characterized by clonal growth of mature, CD5+ B lymphocytes in the bone marrow, peripheral blood, and secondary lymphoid organs (1). The clinical course of CLL is extremely variable, ranging from asymptomatic to highly aggressive, which likely reflects the underlying biological heterogeneity (2). A great number of clonal- and patient-related features with prognostic and/or predictive value have been identified over the last decades, in an effort to optimize the management of CLL (3–9).

Aberrations detected with Fluorescence *in situ* hybridization (FISH), namely the Döhner hierarchical model, have for the last 20 years served as the backbone of CLL diagnostics, dictating the treatment choice, together with the mutation status of *TP53* gene and also the segregation into mutated- and unmutated-CLL (M-CLL and U-CLL respectively) (10, 11). Nevertheless, FISH analysis cannot provide a comprehensive overview of the genomic background of the clone, something that can be accomplished with other methodologies, such as chromosome-banding analysis (CBA), chromosome microarray analysis (CMA) or genome-wide analysis (12–14). A major advantage of these methods, especially CBA, is the identification of complex karyotype (CK) which according to recent reports is a significant prognostic feature with the potential of becoming also a novel predictive biomarker (4, 15). A number of methodological issues, mainly the obtainment of adequate number of representative metaphases, have been overcome with the application of specific culture protocols, making CBA a robust and reproducible method (16–18).

In CLL similarly to other hematological malignancies, CK is defined by the presence of ≥3 numerical or structural abnormalities in ≥2 metaphases in the same clone. CK is reported in 10–20% of untreated patients with CLL and in 8% of patients with monoclonal B lymphocytosis (MBL) (4, 15, 18, 19). The association of CK with advanced-stage disease, U-CLL, *TP53* mutations, adverse FISH abnormalities (del(17p) and/or del(11q)), and telomere dysfunction (15, 20), suggests a potential role of chromosomal aberrations early in the disease development. CK development has been proposed to arise in a 4-phase process in U-CLL: enhanced response of lymphocytes to antigens leading to stimulation of intracellular B-cell-receptor (BCR) signaling and proliferation; telomere shortening during each cell division to a critical point; inactivation of genes implicated in DNA repair (e.g., TP53, ATM), ubiquitin-mediated degradation of oncoproteins (e.g., FBXW7), and to the inflammatory pathway (e.g., MYD88) and; increased genomic instability and risk of chromosome break events (21).

In the present review, we focus on the prognostic and the predictive value of CK in CLL, while we further discuss future perspectives regarding the potential role of CK in the management of CLL as well as issues related to the applied methodology and interpretation of eventual findings.

PROGNOSTIC SIGNIFICANCE OF CK IN CLL

The prognostic value of CK in CLL has been mainly assessed in retrospective studies of both untreated and treated patients, mostly in the era of chemo(immune)therapy. Juliusson et al. first reported that patients with indolent lymphomas (including CLL) and three or more cytogenetic aberrations exhibited dismal clinical outcome compared to patients with normal karyotype (22). In a later CLL-specific cohort, it was reported that three or more aberrations, were linked to shorter overall survival (OS), while specific structural abnormalities, such as aberrations involving 14q32 and trisomy 12 correlated with worse prognosis (23, 24). The advent of specific culture protocols with the addition of mitogens, namely CD40 ligand (CD40L) or CpG-oligonucleotide DSP30 plus interleukin-2 (IL-2) allowed the performance of large cytogenetic studies that further highlighted the association between CK and inferior clinical outcome in CLL. Mayr et al. reported that patients with CLL and CK had a shorter treatment free survival (TFS) and OS compared with patients with fewer aberrations, especially if accompanied by the presence of translocations (25). In a seminal study including 506 patients with CLL, CK was detected in 16% of the cohort, while interestingly paired analysis with FISH indicated that the two methods complement each other and that their combination provides a far more comprehensive genetic characterization than each assay alone (26).

Despite not being part of the standard diagnostic algorithm in CLL, several institutions included CBA in the CLL work up. This approach allowed the performance of the first large scale retrospective study with the inclusion of 1,001 patients with CLL, where CK was reported in 16% of the cohort at the time of CLL diagnosis (15). Furthermore, CK was, as in previous studies, associated with U-CLL, high expression of CD38, del (11q) and del(17p), while it was also an independent prognosticator for shorter time to first treatment (TTFT) and OS. In addition, it was suggested that the presence of ≥5 aberrations correlated with an even worse clinical outcome, however, the number of the patients in that subgroup was relatively low precluding from definite conclusions. Interestingly, the impact of CK was not affected by the presence of translocations, neither balanced nor unbalanced.

In a cohort with fewer patients, Rigolin et al. reported that the co-existence of CK and unbalanced rearrangements was associated with inferior TTFT and OS. In that study though, cases with CK and unbalanced rearrangements were enriched for TP53 aberrations (deletion and/or mutation). That said the negative impact of unbalanced rearrangements was independent of TP53 status (27). The association of TP53 status and CK was further evaluated by Puiggros et al. (28), who within a cohort of 1,045 patients with CLL detected 99 (10%) with CK. Once more, CK was associated with del (17p) and del(11q) [del(17p) and del(11q) defined as high-risk (HR) FISH], as well as with shorter TTFT and OS. Patients with CK and HR FISH exhibited the worst overall prognosis. However, the clinical outcome of patients with CK was not affected by the presence of HR-FISH, suggesting that other clinicobiological features than TP53 status may also contribute to the poor prognosis of CLL with CK.

Further insight on the prognostic value of CK in CLL and the impact of TP53 aberrations within this group was obtained in a large multi-institutional retrospective study, performed by the European Research Initiative on CLL (ERIC), which included more than 5,000 patients (4). The major conclusion of this study was that not all CKs in CLL are equivalent. CK segregated in three subgroups: low-CK (three aberrations), intermediate-CK (four aberrations) and high-CK (≥5 aberrations). Only patients with high-CK exhibited uniformly dismal clinical outcome irrespectively of other features including TP53 status. In contrast, low-CK and intermediate-CK was associated with unfavorable prognosis only in the presence of TP53 aberrations. Interestingly, 20% of patients with high-CK were lacking TP53 aberrations even when evaluated with sensitive high-throughput sequencing. Furthermore, among patients with CK, those carrying +12,+19 exhibited indolent clinical courses, confirming previous reports which suggested that CLL with +12,+19 represents a distinct subset with unique clinicobiological features (29, 30). In conclusion, the largest study thus far on CK in CLL highlights once more the heterogeneity of CLL even within this subgroup of patients, which for decades was considered as homogeneous both biologically and clinically.

PREDICTIVE SIGNIFICANCE CK IN CLL

It is strongly suggested that CK is an unfavorable predictive marker among patients with CLL treated with chemo(immune) therapy (31–33). It should be however noted that this issue has not been addressed properly, since for many years CBA was not included in the standard work-up of clinical trials in CLL. Thus,

it is still unclear if CK is indeed an independent predictor for patients treated with chemo(immune)therapy or the reported effect of CK is the result of a joint impact of CK and other unfavorable biomarkers, namely TP53 aberrations and U-CLL.

The predictive value of CK in CLL becomes even more relevant as the treatment of CLL is shifting drastically towards novel agents, i.e., B cell signaling kinase inhibitors and the Bcl-2 inhibitor venetoclax. Several studies have attempted to address this issue in the context of clinical trials and assess the potential utility of CBA as a predictive factor. Interestingly, cytogenetic complexity has been even assessed with the advent of CMA. In the great majority of these studies, CK was considered as one homogeneous group, defined by the presence of ≥3 cytogenetic abnormalities without taking into account the differentiation to low-CK, intermediate-CK and high-CK (**Table 1**).

Ibrutinib-monotherapy, both in treatment-naïve and relapsed/refractory (R/R) CLL patients, demonstrated an overall response rate of approximately 90% (39). The presence of CK was associated with shorter duration of response [DOR, 31 months *vs*. not reached (NR)], PFS (31 months *vs*. NR), and median OS (54 months *vs*. NR) compared to patients with ≤2 cytogenetic aberrations (non-CK) (34, 39). That said, survival in R/R patients with CK appeared to be significantly influenced by the coexistence of del(17p) which was associated with decreased overall response rate (ORR, 82% *vs*. 100%; DOR, 23 *vs*. 53 months), PFS (25 *vs*. 55 months), and OS (32 months *vs*. NR).

Interestingly, a follow-up of the RESONATE study, a randomized comparison of ibrutinib to ofatumumab in previously treated CLL patients, demonstrated similar ORR (90% *vs*. 89%) and PFS (72% *vs*. 80%, log-rank p-value = 0.25) in ibrutinib patients with CK compared to those with non-CK (34). In striking contrast, patients enrolled on the ofatumumab arm carrying CK had a significantly lower ORR (6% *vs*. 33%, p <0.05) and PFS (0% *vs*. 10%) compared to the non-CK ones. The impact of ibrutinib on CLL with CK was further evaluated in treatment-naïve individuals within the Alliance A041202 study (35). Of note, the presence of baseline complexity did not portend a higher risk of progression or death in ibrutinib treated patients, raising questions whether baseline CK is biologically equivalent to CK due to clonal selection acquired after the administration of chemotherapy.

A randomized, double-blind, phase 3 study assessing the efficacy of idelalisib in combination with rituximab (IR) in patients with relapsed CLL and significant comorbidities, showed that IR provided ORR of 81 and 89% in CK and non-CK groups, respectively (odds ratio 0.5, p = 0.3509) (40). An extended study was designed to elucidate further the efficacy of IR in the presence of CK. Around 60% of patients with CK carried also TP53 aberrations compared to 43% of patients without CK. The median OS was prolonged in the CK-group treated with IR [median: 28.3 (range 16.6, NR) months), compared to patients with CK who received placebo/rituximab [median: 9.2 (range: 2.0, 53.5) months]. Of note, co-existence of CK and TP53 aberrations or del(11q) did not significantly affect survival in patients who received IR. That said, solid conclusion cannot be drawn since the sample size was small while the methodology for the detection of CK was not uniform (36).

Regarding venetoclax-based regimens, a 4-year clinical follow-up of MURANO study explored the predictive value of cytogenetic complexity in R/R CLL patients treated with

TABLE 1 | Randomized control trials that assessed the impact of complex karyotype in chronic lymphocytic leukemia.

Study	Number of patients	Type of treatment	Line of treatment	Definition of CK	PFS/OS	Comment
Brown et al. (34)	Total: 195 CK: 39	Ibrutinib	≥ 2nd line	≥ 3 cytogenetic abnormalities	**18-mo PFS**: 72%	PFS with Ibrutinib was similar regardless of the
	Total: 196 CK: 33	Ofatumumab			**18-mo PFS:** 0%	presence of CK.
Woyach et al. (35)	Total:333 CK: 99	Ibrutinib ± Rituximab	1st line	≥ 3 cytogenetic abnormalities	NA	CK did not influence Ibrutinib-induced PFS in
	Total: 166 CK: 44	Bendamustine plus Rituximab				1st line, HR = 1.01 (95% CI 0.68-1.51, p = 0.95)
Kreuzer et al. (36)	Total: 127 CK: 50	Idelalisib plus Rituximab	≥ 2nd line	≥ 3 cytogenetic abnormalities	**Median OS:** 28.3 [16.6, NR] months **Median PFS**:20.9 (8.5, NR) months	In the Idelalisib plus Rituximab arm, no significant difference in OS was noted between patients with or without CK.
		Rituximab plus placebo			**Median OS:** 9.2 [16.6, NR] months	
Kater et al. (37)	Total: 288 low-GC: 63 high-GC: 31	Venetoclax plus Rituximab Bendamustine plus Rituximab	≥ 2nd line	low-GC: ≥ 3 genomic abnormalities high-GC: ≥ 5 genomic abnormalities	NA	Venetoclax plus Rituximab was superior in each CK category. GC had a major influence on clinical outcome.
Al-Sawaf et al. (38)	Total: 397 CK: 64	Venetoclax plus Obinutuzumab Chlorambucil plus Obinutuzumab	1st line	≥ 3 cytogenetic abnormalities	**2 year-PFS:** 78.9% **2-year-OS:** 88.2% **2 year-PFS:** 36.6% **2-year-OS:** 82.7%	Venetoclax plus Obinutuzumab showed similar PFS and OS rates in patients with and without CK

CK, complex karyotype; GC, genomic complexity; OS, overall survival; PFS, progression free survival.

venetoclax–rituximab (VenR) or bendamustine–rituximab (BR) (37). The authors followed the segregation in low-, intermediate- and high-CK, with the advent of MCA. Patients with non-CK demonstrated better PFS than those with either low-CK or high-CK status (HR, 2.0; 95% CI, 1.4 to 6.3; p = 0.025 and HR, 2.9; 95% CI, 1.1 to 3.6; p = 0.0057, respectively). Additionally, patients with high-CK showed a trend towards worse PFS *versus* those with fewer abnormalities (HR, 1.5; 95% CI, 0.7 to 3.4; p = 0.29).

he role of CK in venetoclax-based combination was also assessed in treatment-naïve patients following therapy with venetoclax–obinutuzumab (VenG) or cholambucil–obinutuzumab (ClbG) within the CLL14 trial (38). Not surprisingly, 32% of patients with CK carried also TP53 aberrations contrasting non-CK patients, (8%). U-CLL was present in similar proportions of patients with CK and non-CK. VenG was associated with significantly better responses independently of the presence of CK with ORRs of 82.4 and 87.3% (p= 0.42) for patients with CK and non-CK respectively. The complete eradication of the leukemic cells is a desired endpoint in CLL management usually translating in a better outcome (41–43). In the VenG arm, the rates of undetectable MRD (uMRD) were similar between patients with CK and non-CK in the peripheral blood (79.4% *vs* 77.1%; p = 1.0) and in the bone marrow (58.8% *vs*. 57.8%, p = 1.0). These high uMRD-rates were reflected into non-statistically significant differences between those groups in PFS (median, NR; 2-year-PFS rate, 78.9 and 91.1%, respectively; HR, 1.909; 95% CI, 0.806–4.520) and OS (median, NR; 2-year-OS rate, 88.2 and 93.2%; HR, 1.511; 95% CI, 0.496–4.600). Interestingly, no difference was neither observed within the CK cohort when the level of cytogenetic complexity was taken into consideration, with the number of aberrations (≥ or <5) not having any impact on clinical outcome. That said the number of patients with ≥5 aberrations was extremely low. Finally, TP53 aberrations did not have any impact on the outcome of patients with CK treated with VenG.

DISCUSSION

As the concept of precision medicine becomes part of the routine-management of CLL, the need for identifications of biomarkers, which may guide treatment choices, is imperative. These markers should be solid, reproducible and highly specific to the available treatment alternatives. Until today, TP53 aberrations and the somatic hypermutation status of the immunoglobulin heavy variable genes (IGHV) are the main disease-related features that shape the treatment algorithm in CLL. Several other genetic abnormalities have been associated with distinct clinical outcomes without however being incorporated in the clinical praxis. CK is a novel candidate biomarker that seems to be of significance regarding prognosis, but more importantly prediction, even in the era of the novel agents. However, several issues need to be further addressed before CK can be integrated in the clinical setting.

For many years, there were concerns regarding the applied methodology for the detection of CK. Today there is no doubt that with the advent of specific culture protocols, i.e., the addition of mitogens, CBA is a robust and reproducible methodology in CLL, and therefore, the obtained karyotypes are fully trustworthy and representative of the CLL clone. However, it is still unclear whether CMA can replace CBA and serve as a surrogate method for the detection of cytogenetic complexity. Indeed, CMA can be informative regarding the grade of complexity but adoption of definitions based on CBA, namely, low-CK *vs* high-CK should be followed with caution as the two methodologies have a number of differences that may affect their output.

When it comes to prognosis in cancer, it is highly common to apply prognostic indices, which are generated after the performance of statistical analysis taking into account a number of clinical and biological features. CLL is no exception (6). Over the years, numerous prognostic indices have been proposed. Nevertheless, their clinical utility has been questioned, since their actual applicability is limited (44, 45). CK has not thus far been assessed within the context of a prognostic index, mainly due to missing cytogenetic data for the great majority of the patients that have been included in such studies. Whether, the implementation of CK in the generation of prognostic indices has the potential to improve their applicability is still unknown.

Another issue that remains open is whether CK is indeed independent of other high-risk features mainly TP53 aberrations and U-CLL. Undoubtedly, patients with CK are enriched for TP53 aberrations and U-CLL with that enrichment reaching higher rates as the number of cytogenetic aberrations increases. That said, high-CK is even present in M-CLL as well as in patients without TP53 aberrations retaining its prognostic value. Therefore, the traditional claim that CK and TP53 aberrations always co-exist seems not to be true. Of note, in a meaningful proportion of CLL with CK, the presence of TP53 aberrations has been excluded even with the performance of highly sensitive methodologies that allow the detection of even small TP53 clones. One could therefore suggest that at least within a number of patients with CK, genomic instability could be independent of p53 biology. Coming to the interaction of CK and U-CLL, there are still many unaddressed issues. In general, U-CL is associated with high-risk genetic features with CK being no exception. However, CK has been reported even within M-CLL where it seems to be associated with worse clinical courses. This notion however does apply in all M-CLL since specific cytogenetic aberrations seem to overcome the negative impact of cytogenetic complexity. A typical example is M-CLL with CK carrying +12,+19, a subgroup accounting for 1-2% of all CLL that exhibits extremely indolent clinical behavior irrespectively of the grade of cytogenetic complexity (4).

In the context of clinical trials, the data regarding the impact of CK are still scarce, since the number of included cases is extremely low and therefore any extrapolation is quite uncertain. Therefore, more studies are needed in order to reach solid conclusions regarding the predictive value of CK. Nevertheless, the low number of high-CK (5%) at least at front-line treatment underscores the need for large population-studies, since it will be difficult to recruit the required number of patients with high-CK

in the context of a clinical trial in order to reach statistical significance. Following this approach, it was recently reported that increasing cytogenetic complexity was an independent predictor of shorter PFS [HR1.07 (95% CI 1.04–1.10), p <0.0001] and overall survival [HR 1.09 (95% CI 1.05–1.12), p <0.0001] for patients treated with ibrutinib (46).

Taking into consideration the available data it is obvious that the heterogeneity of CLL extends even within the CK group with not all CKs being equivalent. The number of aberrations, the type of aberrations as well as the impact of clonal selection due to treatment are only few of the parameters that seem to impact on the clinical significance of CK in CLL. Therefore, it is highly urgent to obtain concrete guidelines for the interpretation of CBA and CMA findings in the clinical practice in order to reach consensus on the potential role of CK in the management of CLL.

In conclusion, CK is a strong prognostic marker in CLL, while its predictive value remains unclear, especially in the era of novel agents.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Treatment Approaches to Chronic Lymphocytic Leukemia With High-Risk Molecular Features

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The clinical course of chronic lymphocytic leukemia (CLL) is highly variable. Over the past decades, several cytogenetic, immunogenetic and molecular features have emerged that identify patients suffering from CLL with high-risk molecular features. These biomarkers can clearly aid prognostication, but may also be capable of predicting the efficacy of various treatment strategies in subgroups of patients. In this narrative review, we discuss treatment approaches to CLL with high-risk molecular features. Specifically, we review and provide a comprehensive overview of clinical trials evaluating the efficacy of chemotherapy, chemoimmunotherapy and novel agent-based treatments in CLL patients with *TP53* aberrations, deletion of the long arm of chromosome 11, complex karyotype, unmutated IGHV, B cell receptor stereotypy, and mutations in *NOTCH1* or *BIRC3*. Furthermore, we discuss future pharmaceutical and immunotherapeutic perspectives for CLL with high-risk molecular features, focusing on agents currently under investigation in clinical trials.

Keywords: chronic lymphocytic leukemia, high-risk, treatment, *TP53*, del(11q), complex karyotype, unmutated IGHV, *NOTCH1*

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most prevalent form of leukemia in the Western world with an age-standardized incidence rate of 4-5 cases per 100,000 persons per year (1–5). The clinical course of CLL is characterized by marked heterogeneity, with some patients surviving for more than 10 years without treatment, whereas others suffer rapid disease progression and poor outcome, in spite of the availability of effective treatment regimens. Historically, prognostication in CLL has relied on clinical staging: the Rai and Binet staging systems, developed approximately 40 years ago, are still frequently used in clinical practice (6, 7). However, as advanced molecular techniques such as fluorescence *in situ* hybridization (FISH), next-generation sequencing (NGS) and microarray-based genomic profiling have provided greater insight into the biology of CLL cells, the prognostication paradigm has shifted towards a perspective that not only relies on clinical

features, but also incorporates high-risk genetic and molecular biomarkers. Indeed, the CLL international prognostic index (CLL-IPI) incorporates cytogenetic, immunogenetic and molecular features to predict the survival of CLL patients (8). In addition to prognostication, the presence of certain cytogenetic, immunogenetic and molecular features can predict differential responses to treatment. Several of the more newly discovered biomarkers are not routinely measured in clinical research and patient care, despite evidence of their predictive impact. Conversely, some routinely measured biomarkers that were historically associated with poor prognosis in the chemo (immuno)therapy era may have lost their predictive value in the context of novel agent-based therapies. Presently, the international workshop on CLL (iwCLL) guidelines recommend assessment of immunoglobulin heavy chain gene (IGHV) mutational status and *TP53* aberrations in every CLL patient before the initiation of treatment (9). *TP53* aberrations should be assessed by both targeted FISH and either Sanger sequencing or NGS. Whereas the IGHV mutational status is stable over time, additional (cyto) genetic abnormalities with therapeutic implications may be acquired over the course of the disease, necessitating reassessment at every subsequent line of therapy (9).

This narrative review discusses such therapeutic implications of CLL with high-risk molecular features. Specifically, we review the predictive impact, if any, of *TP53* aberrations, deletion of the long arm of chromosome 11, complex karyotype or genomic complexity, unmutated IGHV, B cell receptor stereotypy, mutated *NOTCH1* and mutated *BIRC3*. Furthermore, we discuss future perspectives for CLL with high-risk molecular features, focusing on upcoming agents in the therapeutic armamentarium of CLL.

TP53 ABERRATIONS

Among the numerous prognostic and predictive biomarkers that have been identified over the previous decades, *TP53* aberrations indisputably remain the single most impactful genetic lesion in CLL. The *TP53* locus encodes the tumor-suppressor protein p53, which plays a key role in cell division, apoptosis and genomic stability. *TP53* signaling can be impaired through deletion of the *TP53* gene in the chromosomal locus 17p13.1, i.e. del(17p), or through genetic lesions, including missense and nonsense mutations, deletions, insertions or splice-site mutations. Cumulatively, these aberrations are present in 4-8% of all CLL patients at diagnosis, 10% at start of first-line therapy and 30-40% in relapsed or refractory (R/R) CLL patients who were previously treated with chemoimmunotherapy (10). In CLL patients, impaired *TP53* signaling is associated with a poor response to chemotherapy and chemoimmunotherapy. Chemotherapeutic agents exert their cytotoxic effects by causing DNA damage. In *TP53* wildtype cells, such irreparable damage results in apoptotic cell death. However, if *TP53* is not expressed or not functional, chemotherapy-induced DNA damage does not lead to apoptosis and as consequence, administration of these drugs leads to accumulation of detrimental genomic mutations while failing to induce cell death, possibly worsening disease prognosis.

First-Line Setting

Indeed, after first-line treatment with fludarabine monotherapy (F) or fludarabine and cyclophosphamide (FC) in the CLL4 trial, conducted by the German CLL study group (GCLLSG), the overall survival (OS) of CLL patients was markedly shorter in patients harboring *TP53* mutations, compared with those without (median OS, 23.3 *versus* 62.2 months) (11, 12). The comparable E2997 (US) and LRF CLL4 (UK) trials yielded similar results (13, 14). In the phase III GCLLSG CLL8 trial, first-line treatment with FC was compared with fludarabine, cyclophosphamide and rituximab (FCR) (15, 16). While patients with del(17p) had relatively short PFS in both arms, median PFS in the FCR-arm was superior (FCR, 11.2 months *versus* FC, 9.1 months; hazard ratio [HR], 0.49; 95% CI, 0.25-0.93; *P*=0.02). Although FCR was the first treatment regimen that prolonged OS in CLL patients, the OS of patients with del(17p) was not significantly different between both arms (HR 0.66; 95%CI 0.33-1.31), underscoring the poor response in the *TP53* aberrant group. Indeed, multivariable analysis identified *TP53* aberrations as the strongest predictor of inferior PFS (HR 2.92; 1.78-4.78) and OS (HR 2.72, 1.60-4.60). Similarly, in a large Italian retrospective cohort study, patients with del(17p) had worse prognosis after FCR (median PFS, 22.5 months, compared with 58.9 months for patients without del(17p), HR 3.72; 95%CI, 2.42-5.72) and a high probability (19%) of developing secondary malignancies (17).

Based on the evidence from the trials discussed above, chemoimmunotherapy is no longer considered an acceptable first-line treatment for patients with *TP53* aberrations. Initially, the anti-CD52 monoclonal antibody (mAb) alemtuzumab, with or without methylprednisolone, was considered the only effective pharmaceutical treatment in patients with *TP53* aberrations, despite limited efficacy (overall response rate [ORR], 82%, median PFS 11.8 months, median OS 23 months) and an unfavorable toxicity profile (18). In addition, an allogeneic hematopoietic stem cell transplantation (alloHSCT) was considered as a first-line consolidation treatment for patients with *TP53* aberrations (19–21). The GCLLSG CLL3X trial demonstrated that the 6-year OS rate after alloHSCT was approximately 60%, irrespective of *TP53* aberrations (19). Although potentially curative, alloHSCT is only available for a highly selected young and fit CLL patient population, given the high risk of transplant-related morbidity and mortality. Thus, historically, treatment of patient with *TP53* aberrations has been challenging. Fortunately, alemtuzumab and alloHSCT have been replaced as treatment of choice for these patients by newer agents with greatly improved efficacy.

Advanced insight in the pathophysiology of CLL have led to the development of small molecule inhibitors that have revolutionized the treatment of patients with *TP53* aberrations. Ibrutinib is an irreversible inhibitor of Bruton's tyrosine kinase (Btk), a signaling molecule downstream of the B cell receptor (BCR) (22–24). In the phase III RESONATE-2 trial, the efficacy of ibrutinib was compared with chlorambucil monotherapy

(Clb) in unfit, treatment-naïve CLL patients (25–27). Although CLL patients with del(17p) were excluded from this trial, twelve ibrutinib-treated patients had mutated *TP53*. Their outcome following ibrutinib treatment was comparable to patients without *TP53* mutations (5-year PFS rate 56% *versus* 73%, HR 0.87; 95%CI, 0.26-2.85). Furthermore, in a phase II trial conducted by Farooqui et al., median PFS was not reached in *TP53* mutated CLL patients treated with ibrutinib monotherapy after a median follow-up of 35 months (28, 29). In the phase III ALLIANCE trial, previously untreated unfit CLL patients were treated with bendamustine rituximab (BR), ibrutinib alone or ibrutinib plus rituximab (R-ibrutinib). PFS was superior in all patients in both ibrutinib-based arms, compared with the BR arm, but this difference was most pronounced in patients with del(17p) (median PFS not reached [NR] after 38 months for both ibrutinib and R-ibrutinib *versus* 7 months for BR) (30). There was no difference in PFS for patients with del(17p) between the ibrutinib and R-ibrutinib arms (HR 1.57; 95%CI, 0.80-3.09). In the first-line phase III iLLUMINATE CLL trial, obinutuzumab plus ibrutinib (O-ibrutinib) was compared with obinutuzumab plus chlorambucil (O-Clb). In CLL patients with *TP53* aberrations, median PFS after O-ibrutinib was superior, compared with O-Clb (NR after 31 months *versus* 15.2 months, HR 0.14; 95%CI, 0.04-0.51) (31). Based on the evidence presented above, ibrutinib has thus become the gold standard for first-line therapy in patients with *TP53* aberrations. Interestingly, Brieghel et al. demonstrated that in ibrutinib-treated patients, the co-occurrence of multiple *TP53* aberrations (multi-hit) was associated with an inferior PFS and time-to-progression as compared with those with a single-hit (HR for PFS 14.1; 95%CI, 1.60-1849) (32). This observation has not yet been validated in larger cohorts, or in different treatment settings.

Importantly, ibrutinib monotherapy needs continuation until progression and is associated with adverse events such as fatigue, diarrhea, nausea, bleeding complications, cardiac arrhythmias and, rarely, sudden death (in 1% of treated patients) (33). In addition, in some population-based studies, adverse events were more severe resulting in a higher discontinuation rate, compared with those in clinical trials (34–36). The second-generation Btk-inhibitor acalabrutinib has a more selective binding profile and could therefore potentially overcome ibrutinib-associated toxicities. In the phase III ELEVATE-TN study, acalabrutinib and acalabrutinib plus obinutuzumab (O-acalabrutinib) were compared with O-Clb in unfit, treatment-naïve CLL patients (37). The estimated median 24-month PFS rate in patients with del(17p) was longer following treatment with O-acalabrutinib, compared with O-Clb (88%; 95%CI, 61–97% *versus* 22%; 95%CI, 5–45%). Similar results were obtained in patients with mutated *TP53* (24-month PFS rate 95%; 95%CI 70-99% *versus* 19%; 95% CI 5-41%).

Another small molecule inhibitor is the Bcl2-inhibitor venetoclax. Although this compound mainly acts through induction of apoptosis, it does so in a p53-independent manner (38). In the phase III GCLLSG CLL14 study, time-limited venetoclax plus obinutuzumab (Ven-O) was compared with O-Clb in previously untreated, unfit CLL patients. In patients with *TP53* aberrations, response and survival outcomes were better for Ven-O treated patients, compared with O-Clb treated patients (ORR 81% *versus* 36%, median PFS approximately 35 months *versus* 17 months, 24-month PFS rate approximately 96% versus 77%) (39–41). However, in the Ven-O arm, PFS for patients with *TP53* aberrancies was still inferior, compared with patients with intact *TP53* (HR 1.96; 95%CI, 0.92-4.17). Of note, none of the patients with *TP53* aberrations had progressive disease while receiving a therapeutic dose of venetoclax (39).

R/R Setting

In the R/R setting, not surprisingly, chemoimmunotherapy yields disappointing results in patients with *TP53* aberrations. Badoux et al. reported poor response (ORR, 35%) and survival (median PFS 5 months and median OS 10.5 months) in R/R CLL patients with *TP53* aberrations after treatment with FCR (42). Consequently, R/R CLL patients with *TP53* aberrations require treatment with novel agent-based regimens.

In the R/R setting, Zelenetz et al. evaluated the efficacy of idelalisib (IDELA), a small molecule inhibitor of phosphoinositide 3-kinase (PI3K) in combination with BR (BR-IDELA) to treatment with BR alone. In patients with aberrant *TP53* signaling, ORR and PFS were superior after treatment with BR-IDELA, compared with BR alone (ORR: 58% vs 23%, median PFS 11.3 months *versus* 8.3 months, HR 0.47; 95%CI, 0.31-0.72) (43). However, when comparing patients with either del(17p) and/or *TP53* to patients with neither, median PFS after BR-IDELA remained poorer in patients with *TP53* aberrations (11.3 months *versus* 24.5 months) (43). Notably, in a R/R CLL cohort treated with rituximab and IDELA, followed by open-label, single-agent IDELA, the presence of *TP53* aberrations did not influence PFS (*TP53* aberrations: median PFS 20.8 months *versus* wildtype: 18.8 months, HR 1.03; 95%CI, 0.62-1.72) (44).

Several trials have demonstrated the impressive efficacy of ibrutinib in R/R CLL with *TP53* aberrations. In the phase III RESONATE trial, ibrutinib was compared with ofatumumab monotherapy in patients with R/R CLL (45–47). The 6-months PFS rate was 83% and 49% for patients with *TP53* aberrations treated with ibrutinib and ofatumumab, respectively (45). After six years of follow-up, ibrutinib-treated patients without *TP53* aberrations had a median PFS of 56.9 months (95%CI, 36.4-NR), compared to 40.7 months (95%CI, 25.4-57.3) for patients with either del(17p) or *TP53* mutations (48). Similarly, O'Brien et al. evaluated the efficacy of ibrutinib in R/R CLL patients with del (17p) in the RESONATE-17 trial (49). At 24 months, the ORR was 83% (95%CI, 76%-88%), with 63% of patients remaining progression-free (95%CI, 54%-70%). More recently, in the phase III ASCEND trial, acalabrutinib was compared with BR or R-IDELA in fit, R/R CLL patients (50). After a median follow-up period of 16.1 months, median PFS was NR and 16.5 months (95%CI, 14.0-17.1) for acalabrutinib and the investigators choice, respectively (HR 0.31; 95%CI, 0.20-0.49). Interestingly, very recently, the first head-to-head comparison of ibrutinib and acalabrutinib was performed (51). In this trial, Byrd et al. compared the efficacy of ibrutinib and acalabrutinib in R/R

CLL patients. Acalabrutinib was non-inferior compared with ibrutinib in patients with del(17p): median PFS was 32.9 months (95%CI, 25.2-38.4) after treatment with acalabrutinib, compared with 27.6 months (95%CI, 21.8-28.5) for ibrutinib (HR 1.00; 95% CI, 0.73-1.38) (51).

Venetoclax has demonstrated comparable efficacy in R/R CLL with *TP53* aberrations. In the phase II Pivotal trial, venetoclax monotherapy was evaluated in R/R CLL patients with del(17p). The ORR was 77% for the overall cohort. The estimated 24-months PFS and OS were 66% (95%CI, 55%-74%) and 73% (95% CI, 65%-79%). In the phase III MURANO trial, venetoclax plus rituximab (Ven-R) was compared with BR in physically fit, R/R CLL patients (52–54). Outcome with Ven-R was superior in terms of median PFS as compared with BR in patients with del (17p) (NR after 48 months *versus* 15.4 months, respectively). However, in a pooled analysis of four early-stage trials, patients with either del(17p) or *TP53* mutations remained at higher hazard of relapse, following venetoclax-based treatment (HR 1.7; 95%CI, 1.2-2.4) (55).

Taken together, given the availability of more efficacious drugs, chemoimmunotherapy is no longer a suitable treatment option for CLL patients with *TP53* aberrations, both in first-line and in R/R settings. As a consequence, ideally every CLL patient with an indication for treatment should undergo cytogenetic and molecular testing for *TP53* disruption. At present, patients with *TP53* aberrations qualify for treatment with novel agents such as ibrutinib, acalabrutinib or venetoclax. Still, even after novel agent-based treatment, patients with *TP53* disruption seem to have inferior outcome, compared with patients with intact *TP53*, although these differences are not statistically significant in every trial. At present, there is no preference for either ibrutinib, acalabrutinib or venetoclax if considering treatment for patients with *TP53* aberrations. As such, the choice should be determined by the physician and the patient jointly, taking into account treatment duration, side effects, comorbidities and previous lines of therapy. A complete overview of clinical trials comparing treatment regimens in previously untreated or R/R CLL patients with *TP53* aberrations is given in **Table 1** and **Table 2**, respectively.

DELETION OF THE LONG ARM OF CHROMOSOME 11

Deletion of the long arm of chromosome 11, i.e. del(11q), is one of the most common structural chromosomal aberrations in CLL. At diagnosis, del(11q) is present in 10% of patients with early-stage and 25% of patients with advanced stage, treatment-naïve CLL (82, 83). Patients carrying a del(11q) characteristically have bulky disease, rapid progression and a shorter OS (82). The minimally deleted region in del(11q) encompasses several tumor suppressor genes such as *ATM, FDX, MLL* and *RDX*. The tumor suppressor gene *ATM* encodes the serine-threonine kinase ATM, which is important in the repair of double-strand DNA breaks. Deleterious *ATM* mutations in the residual allele can be found in 36% of the CLL patients with a del(11q) and are associated with a poorer prognosis, compared with patients with del(11q) alone (84–86). Since ATM functions as a positive upstream regulator of p53, loss of ATM could interfere with chemotherapy-induced apoptosis.

First-Line Setting

Concordantly, in the chemotherapy era, prognosis for CLL patients with del(11q) was poor (83). In the US E2997 trial, first-line treatment with FC did not improve PFS in patients with del(11q), compared with F alone (median PFS 25.2 and 16 months, respectively). In the FC arm, PFS was 25.2 months and NR after a follow-up of 60 months for patients with del(11q) and those without, respectively. Moreover, in a multivariable analysis, del(11q) was strongly associated with reduced PFS (HR 1.904; P=0.006) (13, 87). Similarly, in the CLL8 trial, first-line treatment with FC resulted in a complete response (CR) rate of only 19% in del(11q) patients (15, 16). In contrast, in this trial, patients with del(11q) responded very well to treatment with FCR (CR rate 71%). In addition, the 5-year PFS rate for patients with del(11q) was 11.4% after FC, compared with 31.4% after FCR (HR 0.47; 95%CI, 0.32-0.68). Similarly, the 5-year OS rate of patients with del(11q) was superior after treatment with FCR, compared with FC (HR 0.35; 95%CI, 0.20-0.61). Indeed, subgroup analysis revealed that patients with del(11q) and mutated IGHV genes responded very well to FCR, with outcomes similar to patients without del(17p) and del(11q) (88). In a retrospective observational cohort study by Rossi et al., physically fit, treatment-naïve CLL patients were treated with FCR (17). After a median follow-up of 70 months, median PFS was 43.5 months (95%CI, 32.2-54.7) for patients with del(11q) and 56.9 months (95%CI, 47.1-66.6) for patients without del (11q) (P=0.01). In a multivariable analysis, del(11q) was identified as an independent predictor of PFS (HR 1.67; 95% CI, 1.13-2.46). Furthermore, in the GCLLSG CLL10 trial, a phase III non-inferiority trial comparing FCR to BR in physically fit, treatment-naïve CLL, patients with del(11q) had shorter PFS in the BR arm (HR 2.33; 95%CI, 1.47-3.67) (89). In summary, although the introduction of chemoimmunotherapy, especially FCR, has significantly improved the outcome of patients with del (11q), their prognosis after chemoimmunotherapy is still inferior, compared with patients without del(11q).

Treatment with ibrutinib has proven very effective in patients with del(11q), yielding outcomes similar to patients without del (11q) (25–27, 30, 90). For example, after first-line treatment with ibrutinib alone in the RESONATE-2 trial, the 60-month PFS rate was 79% in patients with del(11q), compared with 67% for patients without del(11q) (25–27). Surprisingly, pooled data from three phase III ibrutinib trials (RESONATE, RESONATE-2 and HELIOS) demonstrated that ibrutinib-treated patients with del(11q) had slightly longer PFS, compared with ibrutinib-treated patients without del(11q) (42-month PFS rate, 70% *versus* 65%, P=0.02) (91). In the trial by Shanafelt et al., PFS was superior for ibrutinib-R compared with FCR in treatment-naïve CLL patients with del(11q) (HR 0.24; 95%CI, 0.10-0.62) (90). Likewise, Woyach et al. demonstrated that in patients with del(11q) PFS was comparable after treatment with ibrutinib (median PFS NR) or ibrutinib-R (median PFS NR), but inferior after treatment with BR

TABLE 1 | An overview of clinical trials comparing first-line treatment regimens for patients with *TP53* aberrations.

Ref.	Trial	Authors	Year	Median FU	Treatment	Deletion 17p				No deletion 17p			
						N	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)	N	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)
(11, 12)	CLL4	Eichhorst et al.	2006	52.8	F or FC	28	–	23.3	29.2	261	–	62.2	84.6
(13)	E2997	Grever et al.	2007	–	F	9	–	8.9	–	20	–	14.1	–
					FC	10	–	11.9	–	17	–	NR	–
(14, 56, 57)	LRF CLL4	Catovsky et al.	2007	120	F, FC or Clb	55	27%	6	31.8	444	78%	26	73.8
(15, 16)	CLL8	Hallek et al.	2010	70	FC	29	34%	9.1	23	58	–	–	–
					FCR	22	68%	11.2	33.1	80	–	–	NR
(58)		Wierda et al.	2011	8	OFA-FC	8	83%	–	–	7	100%	–	–
(18)	CLL206	Pettitt et al.	2012	–	Alemtuzumab-MP	41	82%	11.8 (6.5-18)	23 (16.4-NR)	–	–	–	–
(59)		Strati et al.	2014	33	FCR-, R-, Len-based	63	63%	14 (10–18)	63 (43–83)	–	–	–	–
(25–27)	RESONATE-2	Burger et al.	2015	60	Clb	12	–	NR	–	112	–	NR	–
					OFA	3	–	–	–	91	–	–	–
(28, 29)		Farooqui et al.	2015	57	Ibrutinib	35	97% (86%-100%)	NR	NR	–	–	–	–
(17)		Rossi et al.	2015	70	FCR	30	–	22.5 (8.5-36.4)	–	48	–	58.9 (49.3-68.4)	–
(60, 61)	COMPLEMENT1	Hillman et al.	2015	28.9	Clb	39	–	3.7	18	343	–	12.9	NR
					OFA-Clb		–	12.8	NR		–	24	NR
(62)		O'Brien et al.	2015	22.4	R-IDELA	9	100%	NR	NR	52	96%	NR	NR
(63)		Le Bris et al.	2017	56.5	FCR	10	–	12	–	100	–	55	–
(64)		Mato et al.	2017	17	Ibrutinib	440	71%	36	–	181	–	NR	–
					IDELA	54	85%	12	–	8	–	16	–
(65)		Mato et al.	2018	17	Ven	56	71.4%	14		69	72%	NR	–
(66)		Takahashi et al.	2018	32.9	Len	9	–	6	34.6	89	–	55.9	98.2
(30)	ALLIANCE 041202	Woyach et al.	2018	38	BR	14	–	7	–	29	–	50	–
					Ibrutinib	9	–	NR	–	32	–	–	–
					R-Ibrutinib	11	–	NR	–	29	–	–	–
(39–41)	CLL14	Fischer et al.	2019	28.1	O-Clb	14	36%	15.1	NR	42	–	NR	NR
					O-Ven	17	81%	29	NR	50	–	NR	NR
(67)		Burger et al.	2019	36	Ibrutinib	41	80%	NR	–	35	74%	NR	
					R-Ibrutinib	36	97%	NR	–	49	86%	NR	
(31)	iLLUMINATE	Moreno et al.	2019	31.3	O-Ibrutinib	18	90%	NR	–	95	–	–	–
					O-Clb	23	68%	11.3	–	93	–	–	–
(68)	ELEVATE-TN	Sharman et al.	2020	28.3	O-Acalabrutinib	3	–	NR	–	11	–	NR	–
					Acalabrutinib	6	–	NR	–	20	–	NR	–
					O-Clb	16	–	13	–	77	–	23	–
(69)	GREEN	Stilgenbauer et al.	2021	43.7	O-mono	2	50%	15	NR	10	70%	35	NR
					O-Cbl	7	71.4%	20	30	10	80%	26	NR
					O-B	20	65%	22	45	61	86.9%	NR	NR
					O-FC	5	20%	10	30	20	100%	NR	NR

All follow-up is reported in months. 95%CI, 95% confidence interval; B, bendamustine; BR, bendamustine-rituximab; Clb, chlorambucil; F, fludarabine; FC, fludarabine; cyclophosphamide; FCR, fludarabine; cyclophosphamide and rituximab; FU, follow-up; IDELA, idelalisib; Len, lenalidomide; MP, methylprednisolone; mOS, median overall survival; mPFS, median progression-free survival; NR, not reached; OFA, ofatumumab; O, obinutuzumab; ORR, overall response rate; R, rituximab; Ven, venetoclax.

(median PFS 41 months; 95%CI, 36-NR) (30). In the ILLUMINATE trial, treatment-naïve CLL patients with del (11q) had significantly better PFS after treatment with ibrutinib (median PFS NR (95%CI, 17.4-NR), compared with O-Clb (median PFS 15.2 months (95%CI, 14.1-20.8). The ELEVATE-TN trial has demonstrated that after first-line treatment with acalabrutinib, PFS is comparable for patients with and without del(11q) (68). A complete overview of clinical trials comparing first-line treatment regimens for patients with del(11q) is provided in **Table 3**.

TABLE 2 | An overview of clinical trials comparing treatment regimens for relapsed or refractory CLL patients with *TP53* aberrations.

Ref.	Trial	Authors	Year	Median FU	Treatment	Deletion 17p				No deletion 17p			
						N	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)	N	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)
(70)	CLLH	Stilgenbauer et al.	2009	37.9	Alemtuzumab	31	39%	5.8	18.3	18	50%	6.5	15.5
(42)		Badoux et al.	2011	43	FCR	20	35%	5	10.5	–	–	–	–
(45–47)	RESONATE	Byrd et al.	2014	74	Ibrutinib	63	90%	40.6 (25.4-44.6)	61.8 (38.7-NR)	132	91%	42.5 (31.7-56.2)	–
					OFA	64	12%	6	–	132	27%	9	–
(44, 71)		Furman et al.	2014	18	R-IDELA	46	–	18.7 (16.6-32.4)	–	64	–	20.8 (16.4-28.9)	–
					R	49	–	4.0 (3.7-5.7)	–	61	–	8.1 (5.1-8.2)	–
(72)	PCYC-1102	Byrd et al.	2015	32.5	Ibrutinib	34	79%	28.1	–	91	95%	NR	–
(73)		Thompson et al.	2015	28	Ibrutinib-based	43	94.1%	32	33	24	100%	NR	NR
(28)		Farooqui et al.	2015	57	Ibrutinib	16	80%	36	60	–	–	–	–
(74)		Jaglowski	2015	16.4	OFA-Ibrutinib	31	74.2% (55%-88%)	NR	–	39	87.2% (73%-96%)	–	–
(75)	CLL9	Bühler et al.	2016	24	Len	23	21.7%	4.9	18.9	70	47.1%	11	34.9
(76)		Roberts et al.	2016	17	Ven	31	71% (52%-86%)	16 (11–25)	–	60	80% (68%-89%)	25	–
(77)		Byrd et al.	2016	14.3	Acalabrutinib	18	100%	NR	–	43	–	–	–
(49)	RESONATE-17	O'Brien et al.	2016	27.6	Ibrutinib	144	83.3% (76.2-89.0)	NR (27.7-NR)	NR (29.5-NR)	–	–	–	–
(78)	M13-982	Stilgenbauer et al.	2016	12	Ven	107	74%	NR	NR	–	–	–	–
(43)		Zelenetz et al.	2017	14	BR + IDELA	69	58%	11.3	–	138	73%	24.6 (19.5-30.3)	–
					BR	68	23%	8.3	–	141	50%	11.2 (11.1-13.6)	–
(66)		Takahashi et al.	2018	32.9	Len	33	–	8.5	29	138	–	63.9	63.9
(79)		O'Brien et al.	2018	60	Ibrutinib	34	79%	26	57	92	–	NR	NR
(52–54)	MURANO	Seymour et al.	2018	48	Ven-R	46	–	NR	–	127	–	NR	–
					BR	46	–	15.4	–	123	–	21.4	–
(80)	Pivotal	Stilgenbauer et al.	2018	23.1	Ven	153	77%	27.2 (21.9-NR)	38.8	–	–	–	–
(81)		Bryd et al.	2020	41	Acalabrutinib	27	93% (76%-99%)	36 (21-NR)	–	–	–		
(50)	ASCEND	Ghia et al.	2020	16.1	Acalabrutinib	22	–	NR	–	132	–	NR	–
					BR or R-IDELA	13	–	13.8 (6.4-16.7)	–	141	–	16.9	–
(51)		Byrd et al.	2021	40.9	Acalabrutinib	121	–	32.9 (25.2-38.4)	–	147	–	–	–
					Ibrutinib	120	–	27.6(21.8-38.5)	–	145	–	–	–

All follow-up is reported in months. 95%CI, 95% confidence interval; BR, bendamustine-rituximab; FCR, fludarabine; cyclophosphamide and rituximab; FU, follow-up; IDELA, Idelalisib; Len, lenalidomide; mOS, median overall survival; mPFS, median progression-free survival; NR, not reached; OFA, ofatumumab; O, obinutuzumab; ORR, overall response rate; R, rituximab; Ven, venetoclax.

R/R Setting

Treatment with chemoimmunotherapy in patients with R/R CLL with del(11q) is generally ineffective: in a study by Badoux et al. in the R/R setting, patients with del(11q) treated with FCR had a median PFS of only 12 months (42).Similar to the first-line setting, in R/R CLL with del(11q) treatment with novel agents has impressive efficacy. In the ASCEND trial, the 12-month PFS rate of patients with del(11q) after acalabrutinib was approximately 90%, compared with approximately 60% in patients with del(11q) after treatment with BR or R-IDELA (50). In the recent head-to-head trial in the R/R setting by Byrd et al., acalabrutinib was demonstrated to be non-inferior to ibrutinib in patients with del(11q) (51). In the CLL14 study, the presence of a del(11q) was associated with adverse PFS in the context of O-Clb (HR 3.44; 95%CI, 1.80-6.60), but not in the context of Ven-O (HR 0.94; 95%CI, 0.29-3.05) (39–41). Likewise, treatment with Ven-R in the MURANO trial resulted in a comparable PFS for R/R CLL patients with del(11q) (median

TABLE 3 | An overview of clinical trials comparing first-line treatment regimens for patients with deletion 11q.

Ref.	Trial	Authors	Year	Median FU	Treatment	Deletion 11q				No deletion 11q			
						N	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)	N	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)
(13)	E2997	Grever et al.	2007	–	F	16	–	14.9	–	20	–	14.1	–
					FC	24	–	25.9	–	17	–	NR	–
(15, 16)	CLL8	Hallek et al.	2010	70	FC	69	87%	26	64	58	91%	–	–
					FCR	84	93%	50	NR	80	89%	–	–
(14, 56)	LRF CLL4	Catovsky et al.	2014	120	F, FC or Clb	71	–	16.8	57.6	374	–	–	–
(17)		Rossi et al.	2015	70	FCR	61	–	43.5 (32.2-54.7)	–	256	–	56.9 (47.1-66.6)	–
(25–27)	RESONATE-2	Burger et al.	2015	60	Ibrutinib	29	100%	NR	–	101	90%	NR	–
					Clb	25	–	9	–	108		18	–
(89)	CLL10	Eichhorst et al.	2016	37.1	FCR	68	99%	43.1	–	68	94%	–	–
					BR	63	90%	NR	–	76	97%	–	–
(30)	ALLIANCE 041202	Woyach et al.	2018	38	BR	33	–	41	–	134	–	50	–
					Ibrutinib	35	–	NR	–	137	–	NR	–
					R-Ibrutinib	37	–	NR	–	132	–	NR	–
(67)		Burger et al.	2019	36	Ibrutinib	27	89%	NR	NR	75	79%	NR	NR
					R-ibrutinib	15	87%	NR	NR	86	90%	NR	NR
(90)	ECOG-1912	Shanafelt et al.	2019	33.6	R-Ibrutinib	78	–	NR	–	69	–	NR	–
					FCR	39	–	NR	–	37	–	NR	–
(31)	iLLUMINATE	Moreno et al.	2019	31.3	O-ibrutinib	13	90%	NR	–	100	–	–	–
					O-Clb	22	68%	15.2	–	94	–	–	–
(39–41)	CLL14	Fischer et al.	2019	28.1	O-Clb	38	58%	18	–	42	80%	NR	
					O-Ven	36	81%	NR	–	50	84%	NR	
(68)	ELEVATE-TN	Sharman et al.	2020	28.3	O-Acalabrutinib	31	–	NR	–	148	–	NR	–
					Acalabrutinib	31	–	NR	–	148	–	NR	–
					R-Clb	33	–	17	–	144	–	28	–

All follow-up is reported in months. 95%CI, 95% confidence interval; BR, bendamustine-rituximab; Clb, chlorambucil; F, fludarabine; FC, fludarabine; cyclophosphamide; FCR, fludarabine; cyclophosphamide and rituximab; FU, follow-up; mOS, median overall survival; mPFS, median progression-free survival; NR, not reached; O, obinutuzumab; ORR, overall response rate; R, rituximab; Ven, venetoclax.

PFS 48 months) and without del(11q) (median PFS 49 months) (52–54). A complete overview of all trials evaluating regimens for R/R CLL patients with del(11q) is provided in **Table 4**.

In summary, in the historical context of chemotherapy, del (11q) was considered a marker of poor prognosis. Treatment with chemoimmunotherapy, specifically FCR, largely mitigates the deleterious outcome associated with del(11q), although survival of patients without del(11q) after FCR remains somewhat superior. Treatment with ibrutinib, acalabrutinib or venetoclax yields equal outcomes in patients with and without del(11q).

COMPLEX KARYOTYPE/GENOMIC COMPLEXITY

The prognostic and predictive value of complex karyotype (CK) or genomic complexity (GC) in CLL has been reported in an increasing number of studies over the past decade. CK is traditionally defined as CLL with 3 or more cytogenetic aberrations, as measured by chromosomal banding analysis (CBA) or interphase FISH. When chromosomal aberrations are detected using chromosomal microarray analysis (CMA), the presence of 3 or more aberrations it is generally referred to as genomic complexity (GC). CBA and interphase FISH are complementary techniques (94). More specifically, CBA can detect abnormalities that cannot be found by a limited panel of FISH probes and FISH can detect abnormalities that cannot be found by CBA due to an inherently lower resolution (95–98). As such, performing CBA in combination with interphase FISH results in a more sensitive measurement of CK. Alternatively, CMA, often referred to as digital karyotyping, allows for screening the entire genome, and therefore has a higher detection rate of cytogenetic abnormalities, but may fail to detect cytogenetic aberrations without copy number aberrations, such as balanced translocations. Another advantage of CMA over CBA is that it is a DNA-based analysis and therefore culturing of B-cells in combination with mitogen stimulation is not needed to generate chromosome metaphases (99–101).

CK is associated with poor OS in CLL patients (98, 102–113). Indeed, in a large multicenter cohort study, Baliakas et al. confirmed that the presence of CK was associated with a shorter time-to-first-treatment (TTFT), compared with CLL patients with a normal karyotype (P=0.01) (114). Furthermore, TTFT was even shorter in patients harboring ≥5 chromosomal aberrations (P<0.001). The latter was confirmed in 2019, when the same research group proposed a hierarchical model

TABLE 4 | An overview of clinical trials comparing treatment regimens for relapsed or refractory CLL patients with deletion 11q.

Ref.	Trial	Authors	Year	Median FU	Treatment	Deletion 11q				No deletion 11q			
						N	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)	N	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)
(70)	CLLH	Stilgenbauer et al.	2009	37.9	Alemtuzumab	20	30%	9	22.7	18	50%	6.5	15.5
(42)		Badoux et al.	2011	43	FCR	13	69%	12	33	–	–	–	–
(45–47)	RESONATE	Byrd et al.	2014	74	Ibrutinib	63	90%	60.7(36.4-NR)	–	132	90%	42.5 (31.7-56.2)	–
					OFA	59	12%	–	–	132	32%	–	–
(72)	PCYC-1102	Byrd et al.	2015	32.5	Ibrutinib	35	97%	38.7 (31.2-NR)	NR (41.2-NR)	66	85%	NR (NR-NR)	NR (NR-NR)
(76)		Roberts et al.	2016	17	Ven	28	82%	–	–	62	76%	–	–
(75)	CLL9	Bühler et al.	2011	24	Len	28	–	7.3	21.3	65	–	17.6	35.4
(92, 93)	HELIOS	Chanan-Khan et al.	2016	34.8	Ibrutinib-BR	23	–	NR	–	65	–	NR	–
					BR	55	–	11.73	–	172	–	16.36	–
(79)		O'Brien et al.	2018	60	Ibrutinib	36	–	51	NR	96	–	NR	NR
(52–54)	MURANO	Seymour et al.	2018	48	Ven-R	45	–	48	–	97	–	49	–
					BR	47	–	16	–	99	–	20	–
(50)	ASCEND	Ghia et al.	2020	16.1	Acalabrutinib	39	–	NR	–	116	–	NR	–
					BR or R-IDELA	44	–	17	–	110	–	28	–
(81)		Byrd et al.	2020	41	Acalabrutinib	21	95%	NR	–	107	95%	NR	–
(51)		Byrd et al.	2021	40.9	Acalabrutinib	167	–	38.4 (33.0-44.0)	–	101	–	–	–
					Ibrutinib	175	–	41.6 (38.0-44.8)	–	90	–	–	–

All follow-up is reported in months. 95%CI, 95% confidence interval; BR, bendamustine-rituximab; FCR, fludarabine; cyclophosphamide and rituximab; FU, follow-up; mOS, median overall survival; IDELA, Idelalisib; Len, lenalidomide; mPFS, median progression-free survival; NR, not reached; OFA, ofatumumab; O, obinutuzumab; ORR, overall response rate; R, rituximab; Ven, venetoclax.

for CK in which high CK (≥5 aberrations) exhibits the worst prognosis (median OS 3.1 years), compared with intermediate CK (4 aberrations, median OS 7.25 years) and low CK (3 chromosomal aberrations, median OS 12.3 years) (115). In that study, high CK emerged as a powerful prognostically adverse biomarker, independent of well-established prognostic indices. An exception to the former is posed by CLL patients that either have two translocations or trisomy 12 and trisomy 19, in combination with another cytogenetic lesion, which have a remarkably indolent disease course. The median survival of these patients was not reached after a median follow-up time of 7.1 years, and was superior compared with patients with no CK (median OS, 6.2 years, $P<0.001$) and patients with normal karyotype (median OS, 11.1, $P<0.001$). Although the impact of CK in CLL has been examined in many trials, there is no official consensus on the definition of CK. Consequently, it is challenging to evaluate the clinical significance of CK in the context of treatment. For example, several trials do not report on the minimum number of cytogenetic abnormalities required to define CK. Moreover, measurement techniques have not been standardized and it is often unclear whether only abnormalities that are present in a single clone are counted, or whether cytogenetic abnormalities in unrelated clones can also contribute to CK definition (116). Finally, patients with two translocations or trisomy 12 and 19 and another aberration, which have a more favorable prognosis, are rarely classified separately.

First-Line Setting

Several trials indicate that CLL with CK (defined as ≥3 chromosomal aberrations) respond poorly to chemo-immunotherapy. In a study by Le Bris et al., in patients with previously untreated CLL treated with FCR, the presence of CK was associated with a significantly shorter median PFS (21 *versus* 55 months) and an inferior 5-year OS rate (72.4% vs 85.8%), compared with CLL patients without CK (63). In the R/R setting, Badoux et al. reported an ORR of 64% in patients with CK after treatment with FCR, with significantly shorter median PFS and OS compared with patients without CK (median PFS: 9 months *versus* 20.9 months, $P<0.001$, median OS: 26 months *versus* 46.7 months, $P<0.001$) (42). In a multivariable analysis, CK was associated with poorer PFS and OS independently of the presence of del(17p) (PFS: HR 2.6; 95%CI, 1.5-2.4 and OS: HR 1.9; 95%CI, 1.1-3.2). In the phase III GCLLSG CLL11 trial, the efficacy of Clb alone was compared with treatment with rituximab and chlorambucil (R-Clb) and O-Clb (117). In a subgroup analysis, Herling et al. reported on the impact of CK and coexisting mutations on the survival outcome of patients treated in this trial (118). Patients with CK pooled over all treatment arms, here defined as ≥3 aberrations by karyotyping, had inferior median OS, compared with patients without CK (37 months *versus* 60 months, $P<0.001$). This deleterious effect was largely dependent on patients with both CK and *TP53* aberrations, as their median OS was markedly poorer, compared with patients with CK and intact *TP53* (26 months *versus* 50 months). In a multivariable analysis, CK was a

predictor for inferior OS, independent of well-established prognostic markers such as advanced clinical stage, unmutated IGHV and elevated β-2-microglobulin (HR 2.7; 95%CI, 1.4-5.3). The adverse impact of CK was retained when the model was limited to patients treated with chemoimmunotherapy (HR 2.6; 95%CI, 1.2-5.7) (118). In the CLL14 trial, first-line treatment with O-Clb resulted in significantly shorter PFS and OS in patients with CK, compared with those without (2-year PFS rate, 36.6% *versus* 69.6%, OS: HR 3.76; 95%CI, 1.36;10.29) (40). Contrastingly, in this trial PFS and OS were similar between patients with and without CK after treatment with Ven-O (2-year PFS rate: 78.9% *versus* 91.1%, HR. 1.91; 95%CI, 0.81-4.52 and 2-year OS rate: 88.2% and 93.2%; HR 1.51, 95%CI, 0.50-4.60) (40). Moreover, the presence of del(17p) and/or *TP53* mutation in patients with CK was associated with inferior PFS in the O-Clb arm (HR 2.10; 95%CI, 0.80-5.57), but not in the Ven-O arm (HR 1.42; 95%CI, 0.32-6.35).

R/R Setting

Kreuzer et al. assessed the prognostic value of CK in R/R patients treated with R-IDELA (119). Interestingly, there was no impact of the presence of CK on ORR (80.8% *versus* 89.2%) and median PFS (20.9 *versus* 19.4 months; HR 1.22, 95%CI; 0.60-2.47). Similarly, Mato et al. found that R/R CLL patients with CK treated with IDELA had similar PFS to patients without CK (median PFS 9 months *versus* 12 months) (64).

Thompson et al. were the first to report on the impact of CK in the context of ibrutinib-based therapy (73). Although R/R CLL patients with and without CK had high ORR (90.5% *versus* 97.1%), median event-free survival (EFS) was significantly worse in patients with CK (19 *versus* 38 months; $P<0.001$). Importantly, in this trial, almost all patients with CK had an additional del (17p) (81%). Comparing patients with CK including or excluding del(17p) yielded a strong trend towards inferior median EFS in those with an additional del(17p) (22 months *versus* 34 months; $P=0.056$). OS was inferior in patients with CK, independently of the presence of del(17p) (HR 5.9; 95%CI,1.6-22.2). Likewise, in the RESONATE trial in R/R CLL, median PFS after treatment with ibrutinib was shorter in patients with CK, compared with those without (40.8 months *versus* 44.6 months, HR 1.292; 95%CI, 0.770-2.168) (45). Furthermore, with a median follow-up of 60 months, O'Brien et al. also demonstrated that R/R CLL patients with CK have inferior PFS and OS after treatment with ibrutinib, compared with patients without CK (median PFS: 31 months *versus* NR, median OS: 54 months *versus* NR) (49). In contrast, in a pooled analysis of three phase III ibrutinib trials for both treatment-naïve and previously treated CLL patients (the RESONATE-2, RESONATE and HELIOS trials), no effect of the presence of CK on PFS could be demonstrated (91). Specifically, the 42-month PFS rate was 63% in patients with CK, compared with 69% in those without (HR 1.02; $P=0.95$). Of note, the RESONATE-2 and HELIOS trials excluded patients with del (17p) (25, 92). Likewise, the ALLIANCE trial did not substantiate inferior survival in patients with CK after first line treatment with BR, ibrutinib and R-ibrutinib, compared with those without CK (HR 1.01; 95%CI, 0.68-1.51) (30). The impact of CK on treatment with acalabrutinib has only been evaluated in a single phase II trial in R/R CLL patients (81). PFS was shorter for patients with CK, compared with the overall cohort (33 months *versus* NR after 41 months).

The MURANO trial analyzed the impact of GC (≥3 cytogenetic aberrations, as measured by CMA) after treatment with Ven-R (53). In this trial, GC was stratified into low GC (3-4 aberrations) and high GC (≥5 aberrations). After treatment with Ven-R, patients without GC had superior PFS, compared with patients with low- and high GC (HR 2.9; 95%CI, 1.1-3.6). In addition, the co-occurrence of *TP53* aberrations only negatively affected PFS in patients with high CK, but not low CK. In concordance, in a pooled analysis of three phase I trials, Anderson et al. showed that the presence of CK was associated with shorter PFS in R/R CLL patients treated with venetoclax monotherapy (HR 6.61; 95%CI, 1.47-29.75) (120).

Altogether, the predictive impact of CK is challenging to disentangle, mostly due to inconsistent reporting and co-occurrence of *TP53* aberrations. While patients with CK seem to have a high chance of relapse, even in the absence of *TP53* aberrations, after treatment with chemoimmunotherapy, evidence of the impact of CK on the efficacy of novel agents such as ibrutinib and venetoclax is contradictory. Consequently, further research into the impact of CK is warranted, including clear reporting on the definition of CK and multivariable analysis to correct for the co-occurrence of *TP53* aberrations. For an overview of clinical trials comparing treatment regimens in CLL patients with and without CK, see **Table 5**.

UNMUTATED IGHV

The prognostic and predictive impact of the somatic hypermutation (SHM) imprint on the IGHV gene of the leukemia-specific BCR rearrangement has been recognized over the past two decades (121, 122). Whereas CLL with abundant SHM (IGHV germline identity <98%, 'IGHV mutated', or M-CLL) generally has an indolent disease course, a paucity of SHM (IGHV germline identity ≥98%, 'IGHV unmutated', or U-CLL) is a biomarker of high-risk disease and is associated with a shorter TTFT and OS, compared to M-CLL patients (121–123). Consequently, in early stage CLL, the proportion of patients with unmutated IGHV is around 50%, whereas this prevalence enriches to approximately 60% at first-line therapy and up to 80% in R/R CLL.

First-Line Setting

After first-line treatment with FCR, a significant proportion of patients with M-CLL experience durable remission, whereas almost all patients with U-CLL eventually relapse (88, 124). For example, in the CLL8 trial U-CLL patients had significantly shorter PFS and OS following first-line treatment with FCR, compared with M-CLL patients (PFS: 5-year rate 33.1% *versus* 66.6%, OS: 5-year rate ~73% *versus* 83.6%, both $P<0.001$) (15, 16, 89). Similarly, in a trial by the MD Anderson Cancer Center the median PFS and OS of U-CLL patients after first-line FCR was 50.4 and 112.8 months, respectively, whereas median PFS and OS of M-CLL patients were NR after 12.8 years of follow-up (125). Additionally, the CLL10 trial demonstrated that the

TABLE 5 | An overview of clinical trials comparing treatment regimens for patients with complex karyotype defined as ≥3 chromosomal aberrations.

Ref.	Trial	Authors	Year	Median FU	Treatment	Complex karyotype				Normal karyotype			
						N	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)	N	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)
(42)		Badoux et al.	2011	43	FCR	22	64%	9	26	–	–	–	–
(117, 118)	CLL11	Goede et al.	2014	40.9	Clb, R-Clb, O-Clb	30	–	–	37	124	–	–	60
(59)		Strati et al.	2014	33	FCR-, R- orLen-based	29	–	13	–	25	–	20	–
(45–48)	RESONATE	Byrd et al.	2014	74	Ibrutinib	39	90%	40.8 (22.5-44.6)	–	114	89%	44.6 (37.9-61.0)	–
					Ofatumumab	33	6%	–	–	114	33%	–	–
(73)		Thompson et al.	2015	28	Ibrutinib-based	21	90.5%	19	25	35	97.1	NR	NR
(63)		Le Bris et al.	2017	56.5	FCR	38	–	21	–	72	–	55	–
(120)		Anderson et al.	2017	23	Ven	11	–	16	–	19	–	NR	–
(64)		Mato et al.	2017	17	Ibrutinib	96	–	29	–	179		NR	
					IDELA	12	–	9	–	37		12	
(52, 53)	MURANO	Seymour et al.	2018	48	Ven-R	48	–	42	–	94	–	55	–
					BR	47	–	15	–	100	–	22	–
(65)		Mato et al.	2018	7	Ibrutinib	52	–	–	–	89	–	–	–
(79)		O'Brien et al.	2018	60	Ibrutinib	37	89%	31	54	64	–	NR	NR
(30)	ALLIANCE 041202	Woyach et al.	2018	38	BR	44	–	–	–	122	–	–	–
					Ibrutinib	39	–	–	–	126	–	–	–
					R-ibrutinib	60	–	–	–	108	–	–	–
(66)		Takahasi et al.	2018	32.9	Len-based	37	–	7.6	23	94	–	20.2	62.8
(119)		Kreuzer et al.	2019	29.2	R-IDELA	26	80.8% (60.6-93.4)	20.9 (8.5-NR)	28.3 (16.6-NR)	37	89.2% (74.6-97.0)	19.4 (16.4-28.9)	49.7 (25.5-NR)
					R-placebo	24	–	–	9.2 (2.0-53.5)	33	–	–	37.3 (16.0-NR)
(39–41)	CLL14	Fischer et al.	2019	28.1	O-Clb	30	50%	19.4		167	77.8%	NR	NR
					Ven-O	34	82.4%	NR		166	87.3%	NR	NR
(81)		Byrd et al.	2020	41	Acalabrutinib	20	90% (68%-99%)	33 (17-NR)	–	114	–	–	–

All follow-up is reported in months. 95%CI, 95% confidence interval; BR, bendamustine-rituximab; Clb, chlorambucil; fludarabine; cyclophosphamide and rituximab; IDELA, Idelalisib; FU, follow-up; mOS, median overall survival; Len, lenalidomide; mPFS, median progression-free survival; NR, not reached; O, Obinutuzumab; ORR, overall response rate; R, rituximab; Ven, venetoclax.

median PFS of U-CLL patients after treatment with FCR or BR was markedly shorter, compared with the median PFS of M-CLL patients (FCR: 42.7 months (95%CI,36.2-55.2) and BR: (95%CI, 33.6 months (30.3-38.4) in U-CLL, *versus* NR for both in M-CLL, $P<0.001$) (89).

Controversy remains as to whether chemoimmunotherapy or novel agent-based regimens are more appropriate as first-line treatment for patients with U-CLL. Head-to-head comparisons in U-CLL patients have demonstrated that, compared with chemoimmunotherapy, treatment with ibrutinib results in longer PFS. In the ALLIANCE trial, while U-CLL patients in both ibrutinib-based arms had similar median PFS (NR after 38 months), median PFS in the BR arm was markedly shorter (39 months, 95%CI,32-NR) (30). Likewise, the ECOG-1912 phase III trial compared the efficacy of R-ibrutinib to FCR in previously untreated CLL (90). In this trial, the 3-year PFS rate after FCR was 62.5% in patients with U-CLL, compared with 90.7% after R-ibrutinib (HR 0.26; 95%CI, 0.14-0.50). However, an OS benefit for patients with U-CLL after first-line treatment with ibrutinib-based regimens, compared with chemoimmunotherapy, has not been conclusively demonstrated. Consequently, guidelines differ on the most appropriate first-line regimen for U-CLL.

There is similar ambiguity regarding the most appropriate first-line treatment for unfit U-CLL patients, in which treatment with FCR or BR is contraindicated. In the CLL11 trial, first-line treatment with O-Clb resulted in significantly longer PFS, both compared with Clb alone (HR 0.23; 95%CI, 0.13-0.42) and R-Clb (HR 0.39; 95%CI, 0.29-0.53) in U-CLL (117). In the RESONATE-2 trial, the 18-month PFS of U-CLL patients was 89% after treatment with ibrutinib, compared with 47% after treatment with Clb (25). As both these trials demonstrated an OS benefit over Clb alone, treatment with O-Clb, R-Clb or ibrutinib has become the cornerstone of first-line therapy in unfit CLL patients. In a similar setting, U-CLL patients in the iLLUMINATE trial treated with O-ibrutinib had longer median PFS, compared with O-Clb (NR after 31.3 months

versus 14.6 months, HR 0.15, 95%CI,0.08-0.27) (31). Newer agents have also been evaluated as first-line treatment for unfit CLL patients. In the CLL14 trial, treatment with Ven-O resulted in a superior 24-months PFS rate in U-CLL patients, compared with O-Clb (89.4% *versus* 51%, HR 0.22, 95%CI, 0.12-0.38) (40). Additionally, in the ELEVATE-TN trial, first-line treatment with O-acalabrutinib resulted in a 24-month PFS rate in U-CLL patients of 91% (95%CI, 83%-95%), compared with 31% (95% CI, 22%-40%) after O-Clb (68). However, none of these trials have so far demonstrated an OS benefit over O-Clb (51). Moreover, ibrutinib, venetoclax and acalabrutinib have not been compared in a head-to-head fashion in first-line setting. For these reasons, similarly to fit U-CLL patients, the most appropriate first-line treatment for unfit CLL patients remains controversial. In any case, all available options should be carefully discussed with the patient, taking into account the efficacy, contraindications, treatment duration and any side effects. For an overview of clinical trials comparing first-line treatment regimens in U-CLL, see **Table 6**.

R/R Setting

In U-CLL patients with early relapse or refractory disease, chemoimmunotherapy usually yields disappointing results. In a trial conducted by Fischer et al., median PFS and OS in R/R U-CLL patients after treatment with BR were 13.8 and 25.6 months, respectively (127). Consequently, patients with R/R U-CLL usually require treatment with novel agent-based regimens. Two trials have demonstrated the efficacy of IDELA-based regimens in R/R U-CLL. In the trial operated by Furman et al., treatment with R-IDELA achieved a median PFS of 19.4 months in R/R U-CLL patients, compared with 5.6 months after rituximab alone (44, 71). In a similar setting, Zelenetz et al. demonstrated that R/R U-CLL patients have superior ORR, PFS and OS after treatment with BR-IDELA, compared with BR

TABLE 6 | An overview of clinical trials comparing first-line treatment regimens for patients with U-CLL.

Ref.	Trial	Authors	Year	Median FU	Treatment	Unmutated IGHV				Mutated IGHV			
						n	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)	n	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)
(13)	E2997	Grever et al.	2007	–	F	53	–	15.0	–	60	–	23.4	–
					FC	57	–	31.4	–	65	–	NR	–
(41, 124)		Tam et al.	2008	153.6	FCR	126		50.4	112.8	88	–	NR	NR
(15, 16, 124, 126)	CLL8	Hallek et al.	2010	70.8	FC	194	76%	32.0	72.0	166	84%	41.9	NR
					FCR	196	91%	41.9	84.0		93%	NR	NR
(62)	101-08	O'Brien et al.	2015	36.4	IDELA	37	97.3%	NR	NR	37	95.7%	NR	NR
(72)	PCYC-1102	Byrd et al.	2015	35.2	Ibrutinib	15	87%	–	–	16	81%	–	–
(17)		Rossi et al.	2015	70	FCR	216	–	48.2 (43.7-52.7)	–	120	–	NR	–
(25–27)	RESONATE-2	Burger et al.	2015	60	Ibrutinib	42	95%	NR	NR	40	88%	NR	NR
					Clb	60	–	9	NR	42	–	17	NR
(89)	CLL10	Eichhorst et al.	2016	37.4	FCR	155	95%	42.7 (36.2-55.2)	–	196	95%	NR	–
				36.0	BR	108	95%	33.6 (30.3-38.4)	–	86	97%	55.4	–
(63)		Le Bris et al.	2017	56.5	FCR	77	–	36	NR	24	–	92	NR
(30)	ALLIANCE 041202	Woyach et al.	2018	38	BR	71	–	39 (32-NR)	NR	52	–	51 (51-NR)	NR
					Ibrutinib	77	–	NR	NR	45	–	NR	NR
					R-Ibrutinib	70	–	NR (48-NR)	NR	45	–	NR	NR
(90)	ECOG-1912	Shanafelt et al.	2019	33.6	R-Ibrutinib	210	–	NR	–	70	–	NR	–
					FCR	71	–	NR	–	44	–	NR	–
(31)	iLLUMINATE	Moreno et al.	2019	31.3	O-Ibrutinib	66	–	NR	–	41	–	–	–
					O-Clb	57	–	14.6 (11.1-15.1)	–	50	–	–	–
(39–41)	CLL14	Fisher et al.	2019	28.1	Ven-O	121	84%	NR	NR	76	85%	NR	NR
					O-Clb	123	63%	25.6	NR	83	85%	NR	NR
(68)	ELEVATE-TN	Sharman et al.	2020	28.3	O-Acalabrutinib	103	–	NR	–	76	–	NR	–
					Acalabrutinib	119	–	NR	–	60	–	NR	–
					O-Clb	116	–	20	–	61	–	NR	–
(69)	GREEN	Stilgenbauer et al.	2021	43.7	O-mono	28	71.4%	20	NR	23	69.6%	NR	NR
					O-Cbl	33	75.8%	26	NR	20	90%	34	NR
					O-B	180	82.2%	40	NR	107	71.3%	NR	NR
					O-FC	86	87.2	NR	NR	42	95.2%	NR	NR

All follow-up is reported in months. 95%CI, 95% confidence interval; B, bendamustine; BR, bendamustine-rituximab; Clb, chlorambucil; F, fludarabine; FC, fludarabine and cyclophosphamide; FCR, fludarabine; cyclophosphamide and rituximab; IDELA, Idelalisib; FU, follow-up; mOS, median overall survival; mPFS, median progression-free survival; NR, not reached; O, Obinutuzumab; ORR, overall response rate; R, rituximab; Ven, venetoclax.

alone (ORR: 71% (95%CI, 63%-77%) *versus* 43% (95%CI, 35%-51%), median PFS: 19.5 months (95%CI, 16.1-24.6) *versus* 10.9 months (95%CI, 8.6-11.1), median OS: NR *versus* 31.6 months (95%CI, 22.2-NR) (43). However, due to the introduction of newer agents with better tolerability, IDELA is now used less frequently.

Several trials have demonstrated the impressive efficacy of ibrutinib in R/R U-CLL. In the RESONATE trial, the median PFS of U-CLL patients in the ibrutinib arm was 49.7 months (45–47). In the HELIOS trial, treatment with ibrutinib-BR for R/R U-CLL resulted in longer PFS, compared with BR (median PFS NR *versus* 13.8 months, HR 0.16; 95%CI, 0.11-0.21) (92, 93). Similarly, in the ASCEND trial, treatment with acalabrutinib alone achieved longer PFS in R/R U-CLL patients (median NR after 16 months), compared with BR (median 16.9 months; 95% CI, 11.6-NR) or R-IDELA (median 15.8 months; 95%CI, 13.9-17.1 months) (50). Recently, a head-to-head trial demonstrated that acalabrutinib and ibrutinib achieved similar PFS in R/R U-CLL patients (HR 1.09; 95%CI, 0.85-1.40) (51). Finally, venetoclax-based regimens are an efficacious option for R/R U-CLL. In the MURANO trial, R/R U-CLL patients achieved longer PFS after treatment with Ven-R, compared with BR (median PFS NR after 48 months *versus* 15.7 months, HR 0.16 (95%CI, 0.10-0.26)) (52). In R/R CLL, venetoclax, ibrutinib and acalabrutinib have not been compared in a head-to-head fashion.

In summary, for refractory or early relapsed U-CLL, novel agent-based regimens, either ibrutinib-, acalabrutinib- or venetoclax-based, are appropriate treatment options. For an overview of clinical trials comparing treatment regimens in R/R U-CLL, see **Table 7**.

BCR STEREOTYPED SUBSETS

The molecular composition of the leukemia-specific BCR rearrangement has importance beyond its mutational status. Despite the immense theoretical variety of the BCR repertoire, subgroups of unrelated CLL patients express (quasi)identical leukemia-specific BCRs, a phenomenon known as BCR stereotypy. Although cumulatively, BCR stereotyped subsets are common, encompassing up to 41% of all CLL, the prevalence of each individual subset is low: the largest subset, subset #2 (defined as patients with a BCR comprised of IGHV3-21, IGLV3-21, with a short, stereotypic heavy-chain complementarity-determining region of 9 amino acids), represents around 2.5% of all patients (128). Certain

TABLE 7 | An overview of clinical trials comparing treatment regimens for patients with relapsed or refractory U-CLL.

Ref.	Trial	Authors	Year	Median FU	Treatment	Unmutated IGHV				Mutated IGHV			
						n	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)	n	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)
(70)	CLL2H	Stilgenbauer et al.	2009	37.9	Alemtuzumab	71	37%	8.0	18.6	22	32%	5.8	22.7
(42)		Badoux et al.	2011	43	FCR	59	96%	28	50	27	78%	44	NR
(15)		Fischer et al.	2011	24	BR	51	58.7%	13.8	25.6	25	78.2%	13.8	NR
(45–48)	RESONATE	Byrd et al.	2014	74	Ibrutinib	98	92%	49.7 (40.2-NR)	NR	36	89%	48.4 (35.6-60.8)	NR
					Ofatumumab	83	27%	–	NR	49	24%	–	NR
(44, 71)		Furman et al.	2014	18	R-IDELA	65	–	19.4	–	10	–	22.1	–
					R	75	–	5.6	–	13	–	8.5	–
(72, 79)	PCYC-1102	Byrd et al.	2015	61.5	Ibrutinib	79	91%	43	NR	16	81%	63	NR
(75)	CLL9	Bühler et al.	2016	24	Lenalidomide	69	39.7%	10.4	NR	20	45%	6.5	31.9
(76)		Roberts et al.	2016	17	Venetoclax	46	76%	–	–	17	94%	–	–
(92, 93)	HELIOS	Chanan-Khan et al.	2016	34.8	Ibrutinib-BR	67	–	NR	–	11	–	NR	–
					BR	178	–	13.8	–	28	–	24.6	–
(43)		Zelenetz et al.	2017	14	IDELA-BR	75	71% (63–77)	19.5 (16.1-24.6)	NR (26.8-NR)	9	68% (50–83)	26.4 (19.3-NR)	NR
					BR	127	43% (35–51)	10.9 (8.6-11.1)	31.6 (22.2-NR)	22	56% (38–72)	13.7 (8.3-18.5)	NR (15.2-NR)
(52–54)	MURANO	Seymour et al.	2018	48	Ven-R	123	–	NR	NR	53	–	NR	NR
					BR	123	–	15.7	NR	51	–	22.9	NR
(67)		Burger et al.	2019	36	Ibrutinib	61	92%	–	–	43	88%	–	–
					R-Ibrutinib	62	94%	–	–	42	95%	–	–
(50)	ASCEND	Ghia et al.	2020	16.1	Acalabrutinib	118	–	NR	–	33	–	NR	–
					BR or R-IDELA	125	–	16.2 (13.9-17.1)	–	26	–	18.3 (11.2-NR)	–
(81)		Byrd et al.	2020	41	Acalabrutinib	81	95% (88–99)	NR	–	30	–	NR	–

All follow-up is reported in months. 95%CI, 95% confidence interval; BR, bendamustine-rituximab; FCR, fludarabine; cyclophosphamide and rituximab; FU, follow-up; IDELA, Idelalisib; mOS, median overall survival; mPFS, median progression-free survival; NR, not reached; O, Obinutuzumab; ORR, overall response rate; R, rituximab; Ven, venetoclax.

stereotyped subsets have been associated with distinct clinico-biological profiles. For example, expression of a subset #2 stereotyped BCR is associated with poor prognosis, irrespective of IGHV mutational status, whereas patients from subset #8 have a very high risk of developing Richter's syndrome (5-year risk: 68.7%) (129, 130).

Due to their low individual prevalence, little is known about the predictive impact of BCR stereotyped subsets. Jaramillo et al. analyzed the pooled results from the CLL8, CLL10 and CLL11 trials, which evaluated the efficacy of chemoimmunotherapy as first-line treatment for CLL (129). In these trials, compared with all other patients with mutated IGHV, patients with subset #2 and mutated IGHV had significantly shorter time to next treatment (TTNT) (HR 2.01; 95%CI, 1.23-3.28), numerically comparable to U-CLL patients. As of yet, there is no available data regarding the predictive impact of BCR stereotypy in the context of novel agent-based treatment regimens. Consequently, the therapeutic consequences of BCR stereotypy remain undefined, and testing for stereotypy has thus far not been embedded in regular CLL care.

NOTCH1 MUTATED CLL

Activating mutations in *NOTCH1*, most often located in the PEST-domain or 3'-untranslated region (3'-UTR), are present in around 6-12% CLL patients at diagnosis and in 15-20% of patients with relapsed or refractory disease (131). CLL patients harboring a mutation in *NOTCH1* (*NOTCH1*-mut) have shorter OS, compared with their *NOTCH1*-wildtype (-wt) peers (132–134). Interestingly, some trials have provided evidence that *NOTCH1*-mut CLL patients may not benefit from treatment with an anti-CD20 mAb. In the CLL8 trial, *NOTCH1*-wt patients benefited significantly from inclusion of rituximab in their treatment regimen (FC: median PFS 32.8 months *versus* FCR: median PFS 57.3 months, $P<0.001$) (16). Contrastingly, the median PFS of *NOTCH1*-mut patients did not improve significantly upon the addition of rituximab (FC: 33.9 months *versus* FCR: 34.2 months, $p=0.9$). Multivariable survival analysis yielded a statistically significant regression coefficient for the interaction term between *NOTCH1* status and treatment arm (HR 1.65; 95%CI, 1.076-2.535), thereby satisfying the formal criterion for a predictive variable (16). Concordantly, Dal Bo et al. demonstrated that *NOTCH1*-mut patients, in contrast to *NOTCH1*-wt patients, do not benefit from rituximab consolidation following treatment with fludarabine and rituximab (135). Finally, in the phase III COMPLEMENT1 study, which evaluated the efficacy of ofatumumab-Clb compared with Clb alone in unfit, treatment-naïve CLL patients, *NOTCH1*-mut patients did not benefit from the incorporation of ofatumumab in their treatment regimen (60). More specifically, the median PFS of *NOTCH*-wt patients treated with ofatumumab-Clb was longer, compared with those treated with Clb alone (23.8 months *versus* 13.3 months, HR 0.50; 95% CI, 0.39-0.63), whereas *NOTCH1*-mut patients had similar PFS, regardless of the treatment arm (17.2 months *versus* 13.1 months, HR 0.81; 95%CI,0.50-1.31). Again, a statistically significant interaction term between *NOTCH1* status and treatment arm provided evidence of the predictive impact ($P=0.05$) (60). Similar research for obinutuzumab, a type II anti-CD20 mAb, has not been performed and is warranted. PFS of *NOTCH1*-mut patients was similar to that of *NOTCH1*-wt patients in the RESONATE, CLL14 and MURANO trials, suggesting that *NOTCH1* status is not predictive in the context of novel agents (39, 46, 53). An overview of all clinical trials evaluating *NOTCH1* mutations in the context of therapy is given in **Table 8**.

BIRC3 MUTATED CLL

Deleterious mutations in *BIRC3*, a negative regulator of non-canonical NF-κB signaling, are present in 3-5% of newly diagnosed CLL patients (84). Though relatively rare, *BIRC3* mutations are associated with poor outcome, with a 10-year OS rate in the chemoimmunotherapy era of just 29%, which was comparable to the OS of patients with *TP53* aberrations (137). Evidence from several trials suggests that patients with a *BIRC3* mutation (*BIRC3*-mut) respond poorly to chemoimmunotherapy. Firstly, *BIRC3* mutations are enriched in patients with fludarabine-refractory disease, with a prevalence of up to 24% (84). Furthermore, Diop et al. demonstrated that after treatment with FCR, *BIRC3*-mut patients had significantly shorter PFS, compared with patients without *BIRC3* mutations (*BIRC3*-wt) (median PFS 26.4 months *versus* ~54 months, $P<0.001$) (138). Moreover, in the CLL14 trial, the 24-month PFS rate of *BIRC3*-mut patients after treatment with O-Clb was considerably lower compared with *BIRC3*-wt patients, and similar to that of patients with del(17p) (*BIRC3*-mut: 14.3%, del(17p): 23.1%, all patients: 64.1%) (39, 40). Indeed, the ORR of *BIRC3*-mut patients after O-Clb was 38%, considerably lower than the ORR of the overall O-Clb arm (71%, $P<0.05$). In contrast, the presence of a *BIRC3* mutation does not seem to associate with inferior response to novel agents. In the Ven-O arm of the CLL14 trial, the 24-month PFS rate of *BIRC3*-mut patients was similar to the overall 24-month PFS rate in that arm (85.7% *versus* 88.2%). Similarly, in the RESONATE trial, PFS after treatment with ibrutinib for *BIRC3*-mut and *BIRC3*-wt patients was not significantly different (46).

Interestingly, *BIRC3* is located on chromosome 11q22, and is co-deleted together with *ATM* in 80% of CLL cases with del(11q) (85). In addition, genomic *BIRC3* defects mainly cluster in del (11q) patients, leading to biallelic loss of *BIRC3* (85). As such, the clinical significance of trials that report on the impact of mutated *BIRC3* need to be interpreted with caution, and ideally stratified for the presence of del(11q) to avoid confounding effects. Monoallelic loss of *BIRC3* did not influence survival after chemotherapy in the LRF CLL4 trial, whereas biallelic loss of *BIRC3* was associated with shorter OS (median OS 3.3 years *versus* 4.8 years, $P=0.03$) (56). Additional research on the predictive impact of monoallelic versus biallelic defects in *BIRC3* is warranted. An overview of all clinical trials evaluating *BIRC3* in the context of therapy is given in **Table 9**.

TABLE 8 | An overview of clinical trials comparing treatment regimens in patients with *NOTCH1* mutated CLL.

Ref.	Trial	Authors	Year	Median FU	Treatment	*NOTCH1* mutated				*NOTCH1* wildtype			
						n	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)	n	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)
(14, 56, 136)	LRF CLL4	Catosky et al.	2007	120	F, FC or Clb	46	–	22.0 (17.2-26.9)	54.8 (31.0-78.5)	420	–	26.4 (23.6-29.3)	74.6 (68.4-80.9)
(15, 16)	CLL8	Hallek et al.	2010	70.8	FC	62	87.1%	33.9	85.9	560	88.1%	32.8	83.7
					FCR		90.0%	34.2	79.2		96.6%	57.3	NR
(135)		Dal Bo et al.	2014	55	FR +/- R maint	20	90%	24	72	103	97%	88	126
(45, 46)	RESONATE	Byrd et al.	2014	74	Ibrutinib	43	–	NR	–	111	–	NR	–
(33, 90)	COMPLEMENT1	Hillmen et al.	2015	28.9	Clb	65	–	13.1	NR	318	–	13.3	NR
					Ofa-Clb		–	17	NR		–	23.8	NR
(63)		Le Bris et al.	2017	56.5	FCR	19		42	48	91		55	NR
(52, 53)	MURANO	Seymour et al.	2018	48	Ven-R	19	–	43		175	–	50	–
					BR	27	–	23	–	168	–	16	–
(39, 40)	CLL14	Fischer et al.	2019	28.1	Ven-O	47	78%	NR	–	164	–	NR	–
					O-Clb	48	62%	23.4	–	162	–	NR	–
(135)		Del Bo et al.	2020	25	Ibrutinib	65	–	26	38	115	–	NR	NR

All follow-up is reported in months. 95%CI, 95% confidence interval; BR, bendamustine-rituximab; Clb, chlorambucil; F, fludarabine; FC, fludarabine and cyclophosphamide; FR, fludarabine and rituximab; FCR, fludarabine; cyclophosphamide and rituximab; FU, follow-up; maint, maintenance; mOS, median overall survival; mPFS, median progression-free survival; NR, not reached; O, Obinutuzumab; Ofa, ofatumumab; ORR, overall response rate; R, rituximab; Ven, venetoclax.

TABLE 9 | An overview of clinical trials comparing treatment regimens in patients with *BIRC3* mutated CLL.

Ref.	Trial	Authors	Year	Median FU	Treatment	*BIRC3*-mutated				*BIRC3*-wildtype			
						n	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)	n	ORR (95%CI)	mPFS (95%CI)	mOS (95%CI)
(14, 56)	LRF CLL4	Catovsky et al.	2007	120	F, FC or Clb	28		20	72			24	72
(45–47)	RESONATE	Byrd et al.	2014	74	Ibrutinib	21	–	NR	–	133	–	NR	–
(39, 40)	CLL14	Fischer et al.	2019	28.1	Ven-O	7	82%	NR	–	204	–	NR	–
					O-Clb	9	38%	16.8	–	201	–	NR	–
(138)		Diop et al.	2020	81.6	FCR	9	–	26.4	–	278	–	54	–

All follow-up is reported in months. 95%CI, 95% confidence interval; Clb, chlorambucil; F, fludarabine; FC, fludarabine and cyclophosphamide; FCR, fludarabine; cyclophosphamide and rituximab; FU, follow-up; mOS, median overall survival; mPFS, median progression-free survival; NR, not reached; O, Obinutuzumab; ORR, overall response rate; Ven, venetoclax.

NEW PHARMACEUTICAL PERSPECTIVES FOR CLL PATIENTS WITH HIGH-RISK MOLECULAR FEATURES

Notwithstanding the significant advances in the field of CLL therapy, as detailed above, a proportion of CLL patients will exhaust all currently approved treatment options. In general, these are patients suffering from CLL with high-risk molecular features, such as aberrant *TP53* signaling, presence of CK/GC, or both. For these patients, a number of experimental treatments are currently under development (see **Table 10**).

Zanubrutinib is a second-generation Btk inhibitor, which, compared with ibrutinib and acalabrutinib, has fewer off-target effects and a longer half-life. In a phase I trial in previously untreated or R/R CLL patients, zanubrutinib monotherapy resulted in an ORR in the overall cohort of 96.2%, and an ORR of 100% in patients with del(17p) (140). Moreover, in another phase I trial, the efficacy and safety of zanubrutinib plus obinutuzumab (O-zanubrutinib) was evaluated (143). This combination yielded an ORR of 100% in previously untreated CLL patients and 92% in R/R CLL patients. The ORR was 100% and 80% in treatment-naïve and R/R CLL patients with del(17p), respectively. The currently ongoing phase III SEQUOIA trial will evaluate the efficacy of zanubrutinib compared with BR in treatment-naïve CLL patients (148). Considering the poor outcome associated with any chemoimmunotherapy in patients with del(17p), those patients were not randomized in this trial but assigned to receive single-agent zanubrutinib, analyzed separately. Tam et al. published the primary report on safety and efficacy of the latter cohort (141). The ORR was 94.5%, with estimated 18-months PFS and OS of 88.6% (95%CI, 79%-94%) and 95.1% (95%CI, 88%-94%), respectively. The currently ongoing, phase III ALPINE trial will evaluate whether in R/R CLL, the efficacy of zanubrutinib is non-inferior, compared with ibrutinib (149).

To overcome treatment resistance against covalent Btk inhibitors, pirtobrutinib, previously known as LOXO-305, a non-covalent Btk inhibitor, has been developed. In the phase I/II

TABLE 10 | An overview of phase 1 and 2 trials of new drugs according to high-risk CLL subgroups.

Ref.	Authors	Year	Treatment	Setting	*TP53* aberrations			11q deletion			IGHV-unmutated		
					N	ORR (%)	CR (%)	N	ORR (%)	CR (%)	N	ORR (95% CI)	CR (%)
(139)	Sharman et al.	2017	Ublituximab + Ibrutinib	R/R	12	95	–	12	95	–	–	–	–
(140)	Tam et al.	2020	Zanubrutinib	TN and R/R	18	100		–	–	–	–	–	–
(141)	Tam et al.	2020	Zanubrutinib	TN with del (17p)	109	94.5	2.8	–	–	–	–	–	–
(142)	Xu et al.	2020	Zanubrutinib	R/R	22	86	–	20	82	–	51	82	–
(143)	Tam et al.	2020	Zanubrutinib + O	TN and R/R	16	88	–	10	–	–	19	–	–
(144, 145)	Davids et al.	2020	Duvelisib	TN and R/R	26	77	12	20	–	–	65	–	–
(146)	Davids et al.	2021	Duvelisib + FCR	TN	3	66	0	8	–	–	18	–	56
(147)	Mato et al.	2021	Pirtobrutinib	R/R	28	79	0	15	60	0	71	68	0
(139)	Sharman et al.	2021	Ublituximab + Ibrutinib	R/R	30	–	–	30	–	–	53	–	–

CR, complete response rate; del(17p), deletion of the short arm of chromosome 17; FCR, fludarabine; cyclophosphamide; rituximab; ORR, overall response rate; O, obinutuzumab; R/R, relapsed or refractory. TN, treatment naïve.

BRUIN study, the safety and efficacy of pirtobrutinib were evaluated in R/R B-cell malignancies, including CLL (147). The ORR was 63% (95%CI, 55%-71%) for patients with CLL or SLL, irrespective of whether patients previously discontinued a covalent BTK inhibitor for progression, toxicity or other reasons. In patients with del(17p) and/or *TP53* mutation the ORR was 79%, compared with 60% in patients with del(11q) and 68% in patients with U-CLL. The currently ongoing phase III BRUIN CLL-321 trial will compare the efficacy of pirtobrutinib to either R-IDELA or BR in R/R-CLL patients that have been previously treated with a covalent Btk inhibitor (150).

Whereas IDELA only targets the delta subunit of PI3K, the novel agent duvelisib is a dual inhibitor of both the delta and gamma isoforms of PI3K. In the phase III DUO trial, duvelisib treatment was evaluated in patients who were progressive on ofatumumab (144, 145). Duvelisib treatment resulted in an ORR of 77%, with and median PFS of 15.7 months. Comparable responses were seen in patients with del(17p) and/or TP53 mutation (ORR, 77%, median PFS 14.7 months). Based on these results, the Food and Drug Administration (FDA) approved duvelisib for the treatment of R/R CLL and SLL in September 2018 (151). Duvelisib in combination with FCR was recently evaluated in young treatment-naïve CLL patients. The overall response was 88%. Hematological toxicity and infectious complications were common. Only three patients with *TP53* aberrations were identified, of whom two responded to the treatment (146).

Ublituximab is a next-generation, glyco-engineered, type I, anti-CD20 mAb that binds to a unique CD20 epitope which is different from the target site of rituximab, obinutuzumab and ofatumumab. In a phase II trial, ublituximab plus ibrutinib yielded an ORR of 88% in all R/R patients and 95% of patients with either *TP53* aberrations or del(11q) (139). In a recent, multicenter, phase III trial, ublituximab plus ibrutinib was compared with ibrutinib alone in R/R CLL patients (139). In the overall cohort, the ORR was 83% for ublituximab plus ibrutinib and 65% for ibrutinib alone (P=0.02). PFS was significantly longer in patients treated with ublituximab plus ibrutinib (HR 0.46; 95%CI, 0.24-0.87). In a subgroup analysis, PFS benefit was retained in patients with aberrant *TP53* signaling (HR 0.25; 95%CI, 0.10-0.65), but not in patients with del(11q) (HR 0.97; 95%CI, 0.36-2.61).

CELLULAR IMMUNOTHERAPY AS NEW PERSPECTIVE FOR CLL PATIENTS WITH HIGH-RISK MOLECULAR FEATURES

Although novel-based agents have revolutionized CLL therapy, they remain incapable of complete disease eradication. To this day, alloHSCT remains, in the context of CLL, the sole treatment with curative intent. Notwithstanding the availability of highly effective, highly tolerable agents, alloHSCT remains a relevant treatment option in several specified situations (152, 153). Currently, alloHSCT can be considered for patients with a relapse after chemoimmunotherapy, either in the presence of *TP53* aberrations (high-risk category 1) or with additional failure to BTK inhibitors and/or BCL2 inhibitors, irrespective of the presence of *TP53* aberrations (high-risk category 2) (153–155). In the high-risk category 1, the long-term benefits and risks of alloHSCT should be carefully balanced on an individualized basis. Specifically, a younger patient age (<65 years), absence of comorbidities and the availability of a suitable stem cell donor would argue in favor of an alloHSCT, whereas in the converse situation, novel-based agents would be more suitable. Contrastingly, alloHSCT is a more proportional treatment option for patients in high-risk category 2, considering their markedly poor prognosis, due to the limited availability of alternative options (154).

However, the field of immunotherapy has in recent years been revolutionized by the generation of chimeric antigen receptor (CAR) cytotoxic cells. These cells, most often T cells, are molecularly modified to express a single-chain antibody-variable fragment (scFab) with specificity for a marker that is ubiquitously expressed on the malignant target cells, fused to an intracellular CD3ζ domain. CAR efficacy has been further enhanced by the

inclusion of a costimulatory domain, most often either CD28 or 4-1BB. CAR-T cells have been approved for the treatment of acute lymphoblastic leukemia, certain types of large B-cell lymphoma and multiple myeloma (156–158). Several investigators have evaluated the efficacy of CAR-T cells, most often directed against CD19, in the setting of R/R CLL (see **Table 11**). While the reported efficacy differs from study to study, the ORR, CR and median PFS reported in the larger studies (n≥10) are markedly lower (ORR: weighted mean 53%, range 38-71%, CR: weighted mean 26%, range 21%-29%, median PFS 3.1-7 months), compared with the impressive efficacy of CAR-T cell treatment in other lymphatic cancers (165, 169, 171, 174). One possible explanation for the unexpectedly low efficacy or CAR-T cell treatment in CLL is T cell exhaustion. In CLL patients, T cells express markers associated with T cell exhaustion, and the CAR-T cells generated from these source cells may have an impaired ability to kill malignant cells (177). Interestingly, ibrutinib has been found to boost T cell numbers and function in CLL, possibly through off-target effects on interleukin-2 inducible T cell kinase (ITK) or ZAP70, providing a rationale for concurrent treatment with CAR-T cells and ibrutinib (178). Indeed, Gauthier et al. treated 19 heavily-pretreated R/R CLL patients with anti-CD19 CAR-T cells and ibrutinib, achieving an ORR and CR of 83% and 22%, resulting in 1-year PFS and OS rate of 59% and 86%, respectively (172). An alternative approach to circumvent the problem posed by T cell exhaustion is using CAR-transduced natural killer (NK) cells. Allogeneic NK cells can be safely transfused irrespective of a full human leukocyte antigen (HLA) match, allowing the generation of an off-the-shelf, non-patient-specific CAR-construct generated from healthy donor cord blood. In a pilot study, Liu et al. treated 5 R/R CLL patients with anti-CD19 CAR-NK cells from HLA-mismatched donors (173). Of these five patients, three had a complete response, one had a partial response, and one patient did not respond and went on to

TABLE 11 | An overview trials evaluating the efficacy CAR-T/NK cell trials in patients with CLL.

Ref.	Authors	Year	N	Setting	Ag Target	Costim. molecules	Lymphodepletion	Source	Concomitant drugs	CR (%)	ORR (%)	mPFS	mOS
(159)	Porter et al.	2011	1	R/R, TP53 ab	CD19	4-1BB	PC	Autologous	none	100%	100%	–	–
(160)	Brentjens et al.	2011	8	Chemorefractory	CD19	CD28	C (n=3)	Autologous	none	0%	0%	–	–
(161)	Kalos et al.	2011	3	R/R	CD19	4-1BB	PC or BR	Autologous	none	67%	100%	–	–
(162)	Kochenderfer et al.	2012	4	R/R	CD19	CD28	FC	Autologous	interleukin-2	25%	75%	–	–
(163)	Cruz et al.	2013	4	Relapse after HSCT	CD19	CD28	none	Allogeneic	none	0%	25%	–	–
(164)	Kochenderfer et al.	2015	5	R/R	CD19	CD28	FC	Autologous	none	60%	100%	–	–
(165)	Porter et al.	2015	15	R/R	CD19	4-1BB	B, FC or PC	Autologous	none	29%	57%	7	29
(166)	Fraietta et al.	2016	3	R/R	CD19	4-1BB	none	Autologous	Ibrutinib	33%	100%	–	–
(167)	Brudno et al.	2016	5	Relapse after HSCT	CD19	CD28	none	Allogeneic	none	20%	40%	3	–
(168)	Ramos et al.	2016	2	R/R	Igκ	CD28	BR or FR	Autologous	none	0%	0%	–	–
(169)	Turtle et al.	2017	24	R/R	CD19	4-1BB	F, C or FC	Autologous	none	21%	71%	–	–
(170)	Geyer et al.	2018	8	PR after first line CIT	CD19	CD28	C	Autologous	none	38%	25%	13.6	–
(171)	Geyer et al.	2019	16	R/R	CD19	CD28	B or C	Autologous	Ibrutinib (n=5)	25%	38%	3.1	17.1
(172)	Gauthier et al.	2020	19	R/R, Ibrutinib failure	CD19	4-1BB	FC	Autologous	Ibrutinib	21%	83%	–	–
(173)	Liu et al.	2020	5	R/R, CAR-NK cells	CD19	CD28	FC	Allogeneic	none	60%	80%	–	–
(174)	Frey et al.	2020	32	R/R	CD19	4-1BB	B, FC, PC, OFAO or GEMOX	Autologous	none	28%	44%	1	64
(175)	Shah et al.	2020	3	R/R	CD19 +CD20	4-1BB	FC	Autologous	none	67%	100%	–	–
(176)	Cappell et al.	2020	7	R/R	CD19	CD28	FC	Autologous	none	63%	88%	40.5	–

All follow-up is reported in months. ab, aberrations; Ag, antigen; B, bendamustine; BR, bendamustine-rituximab; C, cyclophosphamide; CIT, chemoimmunotherapy; costim, costimulatory; CR, complete response rate; F; fludarabine; FC, fludarabine and cyclophosphamide; FR, fludarabine and rituximab; GEMOX, gemcitabine and oxaliplatin; HSCT, hematopoietic stem cell transplantation; mOS, median overall survival; mPFS, median progression-free survival; NK, natural killer; OFAO, oxaliplatin; fludarabine; cytarabine and ofatumumab; ORR, overall response rate; PC, pentostatin and cyclophosphamide; R/R, relapsed or refractory.

receive a stem-cell transplantation. Although these results seem promising, both CAR-T cell therapy combined with ibrutinib treatment and CAR-NK cell therapy require more comprehensive evaluation in a larger cohort.

MEASURING PREDICTIVE BIOMARKERS IN CLINICAL CARE AND RESEARCH: AUTHOR RECOMMENDATIONS

In this paragraph, based on the evidence presented above and summarized in the provided tables, we outline recommendations regarding when to measure the previously discussed predictive biomarkers. The powerful predictive impact of *TP53* aberrations is universally recognized and is presently incorporated in treatment decision algorithms in routine care. Consequently, *TP53* status should be both cytogenetically and molecularly assessed in all clinical trials and in all CLL patients with an indication for treatment. Although ambiguity remains whether the IGHV mutational status should influence the choice of first-line therapy, it can differentiate between patients with potential long-term remission and patients with a risk of earlier relapse after a time-limited highly effective first-line treatment regimen such as FCR. As such, we recommend assessment of the IGHV mutational status in all clinical trials and in all CLL patients with active disease. Accumulating evidence suggests that CK/GC could function as a predictive marker and is associated with poorer prognosis after chemoimmunotherapy. Although routine measurement of CK/GC may be desirable in clinical care, more evidence is required of the impact of CK in the context of novel agents before it can be incorporated in therapeutic decision-making. Consequently, we strongly recommend to perform CBA or CMA in all clinical trials, especially in trials evaluating novel agents. Additionally, measurement by CBA/CMA will detect the presence of more classical cytogenetic abnormalities, including del(17p) and del(11q), although the predictive relevance of the latter has significantly diminished since the introduction of chemoimmunotherapy and novel agents. The proposed resistance of *NOTCH1*-mutated CLL to treatment with rituximab and ofatumumab is intriguing and requires further research, especially focusing on the predictive impact of *NOTCH1* mutations in the context of regimens containing obininuzumab or ublituximab. Although the incorporation of *NOTCH1*-mutations in clinical care is not yet warranted, we recommend the assessment of *NOTCH1* mutational status in all trials evaluating regimens that include anti-CD20 mAbs. The predictive impact of BCR stereotypy and *BIRC3* mutations is currently unclear. As such, we do not recommend their routine assessment in clinical research, nor in patient care. As the prevalence of individual stereotyped subsets and *BIRC3* mutations is relatively low, the predictive impact of these biomarkers should be assessed either retrospectively in several pooled trials, or prospectively in specialized trials that specifically recruit patients with the molecular features of interest.

CONCLUDING REMARKS

In this review, we have discussed treatment approaches to CLL with high-risk molecular features, providing a comprehensive overview of trials on this topic. Catalyzed by the advent of more advanced molecular techniques, our understanding of the pathophysiology of CLL has deepened over the years, leading to the identification of several cytogenetic, immunogenetic and molecular features that can differentiate between patients with low- and high-risk disease. Some of these, most notably *TP53* aberrations, have clear predictive impact and are presently incorporated in decision algorithms in routine care. Their presence is strongly associated with inferior response to chemoimmunotherapy and necessitates the use of novel agents. The predictive capability of other molecular features is less clear, especially in the context of novel-based treatment. The predictive importance of del(11q) has significantly diminished since the advent of chemoimmunotherapy and novel agents and is redundant in therapeutic decision-making. Despite accumulating evidence of a predictive impact from clinical trials, the lack of consistent reporting and standardization prohibits the current use of CK/GC in therapeutic decision-making. In fit patients, stratification by IGHV mutational status identifies patients who benefit markedly from treatment with first-line FCR, but whether U-CLL always warrants first-line treatment with novel agents remains controversial. Interestingly, some aberrations may be predictive in certain specific contexts only, such as the proposed resistance of *NOTCH1*-mutated CLL to treatment with rituximab and ofatumumab, but this observation requires further validation before *NOTCH1* status should be used to guide treatment choice. The data concerning the predictive impact of BCR stereotypy and *BIRC3* mutations are currently immature, and treatment choice should not dependent on the presence of these features. The place of second-generation novel agents and cellular immunotherapy in the treatment of CLL with high risk features is still elusive, but forthcoming data from early-stage trials is promising, necessitating further study. Of note, the vast majority of data concerning the predictive impact of the biomarkers discussed above has been obtained through prespecified or *post-hoc* subgroup analysis. While informative, these trials have not necessarily been powered to answer such questions, especially in the case of rare features. As such, there is an unmet need for randomized trials that evaluate the efficacy of treatments, especially of novel agent-based regimens, in cohorts of patients with pre-specified high-risk molecular features, to move further towards patient-tailored treatment strategies.

AUTHOR CONTRIBUTIONS

LvdS and PJH performed the collection and interpretations of all relevant literature. LvdS and PJH write the manuscript. APK, AWL and M-DL critically read and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Clonal Evolution of High-Risk Chronic Lymphocytic Leukemia: A Contemporary Perspective

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Clonal evolution represents the natural process through which cancer cells continuously search for phenotypic advantages that enable them to develop and expand within microenvironmental constraints. In chronic lymphocytic leukemia (CLL), clonal evolution underpins leukemic progression and therapeutic resistance, with differences in clonal evolutionary dynamics accounting for its characteristically diverse clinical course. The past few years have witnessed profound changes in our understanding of CLL clonal evolution, facilitated by a maturing definition of high-risk CLL and an increasing sophistication of next-generation sequencing technology. In this review, we offer a modern perspective on clonal evolution of high-risk CLL, highlighting recent discoveries, paradigm shifts and unresolved questions. We appraise recent advances in our understanding of the molecular basis of CLL clonal evolution, focusing on the genetic and non-genetic sources of intratumoral heterogeneity, as well as tumor-immune dynamics. We review the technological innovations, particularly in single-cell technology, which have fostered these advances and represent essential tools for future discoveries. In addition, we discuss clonal evolution within several contexts of particular relevance to contemporary clinical practice, including the settings of therapeutic resistance to CLL targeted therapy and immunotherapy, as well as Richter transformation of CLL to high-grade lymphoma.

Keywords: chronic lymphocytic leukemia, clonal evolution, intratumoral heterogeneity, single-cell analysis, Richter syndrome

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INTRODUCTION

Clonal heterogeneity and evolution are among the most fundamental properties of cancer. Through a reiterative process of clonal proliferation, diversification and Darwinian selection, cancers continually adapt within the host microenvironment, progressively acquiring and accumulating enabling attributes that allow them to develop and expand (1). Intratumoral heterogeneity fuels this evolutionary process

by providing a diverse pool of candidates from which the fittest parental tumor subclone is selected and propagated to the subsequent generation. Intratumoral heterogeneity is underpinned by genetic heterogeneity with different tumor subclones each harboring a unique constellation of genetic aberrations. In addition to genetic diversity, intratumoral heterogeneity also manifests in other dimensions. These include variation in transcriptional cell states, epigenetic programs, and tumor-immune interactions among different cancer cell populations within the tumor ecosystem (2, 3). Studying clonal evolution thus allows the capture of this dynamic, iterative process that results in tumor initiation and progression, and that dictates subsequent treatment response and relapse.

Chronic lymphocytic leukemia (CLL), a malignancy of $CD19^+$ $CD5^+$ B lymphocytes, offers an informative disease model to study cancer evolution. First, CLL is characterized by clinical heterogeneity that encompasses a range of disease trajectories including rapid progression, treatment refractoriness and high-grade transformation at one end of the spectrum (4, 5), to a highly stable clinical course or even spontaneous disease regression at the opposite end (6, 7). This allows tumor evolution to be studied across a range of differing clinical contexts. Second, the typically protracted disease course in CLL allows clonal evolution to be deciphered through frequent longitudinal sampling over a period of many years, thereby providing a wealth of data on evolutionary dynamics at high temporal resolution. Third, CLL cells circulate continuously between peripheral blood and the lymph node and bone marrow compartments (8, 9). Tumor samples of high purity and quantity can thus be readily obtained from peripheral blood. In addition, lymph node and bone marrow specimens can also be accessed with relative ease complementing peripheral blood samples to allow the comprehensive study of tumor co-evolution with the immune microenvironment (10).

The clinical heterogeneity of CLL necessitates identification of biological correlates of high-risk CLL, in order to define the patient population most at risk of CLL progression that merits close monitoring and focused study. Over the past two decades, biomarkers of high-risk CLL have evolved with our increasingly sophisticated understanding of CLL biology. Concurrently, advances in bulk sequencing and more recently integrative single-cell sequencing technology have facilitated the longitudinal study of CLL clonal evolution in this patient group. These studies have illuminated our understanding of CLL clonal architecture and the complex clonal evolutionary dynamics that give rise to CLL progression, resistance to different CLL treatments and high-grade transformation, revealing diverse biological processes and novel mechanisms. In this review, we will appraise recent advances in our understanding of intratumoral heterogeneity and clonal evolution in patients with high-risk CLL, and discuss the technological innovations that have facilitated this understanding. We will focus particularly on clonal evolution within several topical contexts, including the settings of therapeutic resistance to CLL targeted therapy and immunotherapy, as well as Richter transformation of CLL to high-grade lymphoma.

THE EVOLVING DEFINITION OF HIGH-RISK CLL

Our understanding of what constitutes high-risk CLL has evolved considerably over recent years (**Figure 1A**). Conventional definitions of high-risk CLL are based on clinical information supplemented by a limited number of adverse genetic and flow cytometry-based biomarkers. Novel definitions of high-risk CLL, on the other hand, reflect the integration of a multitude of biological information pertaining to the tumor that serves to enhance prognostic stratification. We discuss herein the various definitions of high-risk CLL which provide an important basis for the study of CLL clonal evolution.

Conventional Definitions of High-Risk CLL

Historically, high-risk CLL was defined solely on the basis of clinical features, with the presence of cytopenias being surrogates of CLL risk, as reflected in the Rai and Binet staging systems (11, 12). With the ubiquitous use of flow cytometry for CLL diagnosis, subsequent developments have linked several CLL cell-surface proteins, such as a high level of CD38, ZAP-70 and/or CD49d expression, to adverse prognosis (13–15). At the same time, our increasing appreciation of the role of B-cell receptor (BCR) signaling as a major driver of CLL proliferation has led to the identification of two distinct biological subtypes of CLL distinguished by the status of somatic hypermutation within the immunoglobulin heavy chain variable region (IGHV). IGHV-unmutated CLL (U-CLL) is associated with a higher progression risk compared to its IGHV-mutated counterpart (M-CLL) (13, 16), relating in part to higher capacity for BCR signaling in the former (17, 18). BCR stereotypy is a feature of CLL, and specific stereotyped subsets, such as subset #2 characterized by IGHV3-21/IGLV3-21 gene usage, as well as subsets #1 and #8, confer increased disease aggressiveness (19–22). Notably, subset #2 is linked to an aggressive CLL clinical course independent of IGHV mutational status (19). Recent work has shown that subset #2 CLL uniformly harbors the $IGLV3\text{-}21^{R110}$ mutation (23). Moreover, non-stereotyped CLL possessing this mutation exhibits similar adverse biological and clinical characteristics to stereotyped subset #2 CLL, suggesting that this subset could be defined by the $IGLV3\text{-}21^{R110}$ mutation.

In addition to IGHV and cell-surface biomarkers, fluorescence *in-situ* hybridization (FISH) has enabled CLL risk stratification into distinct cytogenetic risk categories with del (17p) carrying the highest risk, del(11q) and trisomy 12/normal FISH conferring high and intermediate risk respectively, and isolated del(13q14) being associated with lower risk (24). Moreover, early studies into CLL molecular genetics have established the adverse prognostic impact of somatic mutations involving *TP53* and *ATM* (25–27). These prospectively validated, conventional biomarkers of high-risk CLL, particularly del(17p) as well as IGHV and *TP53* mutational status, continue to find relevance in contemporary clinical practice, and are featured within widely used prognostic indices such as the CLL International Prognostic Index (CLL-IPI) (28). Finally, the importance of complex karyotype identified by chromosome

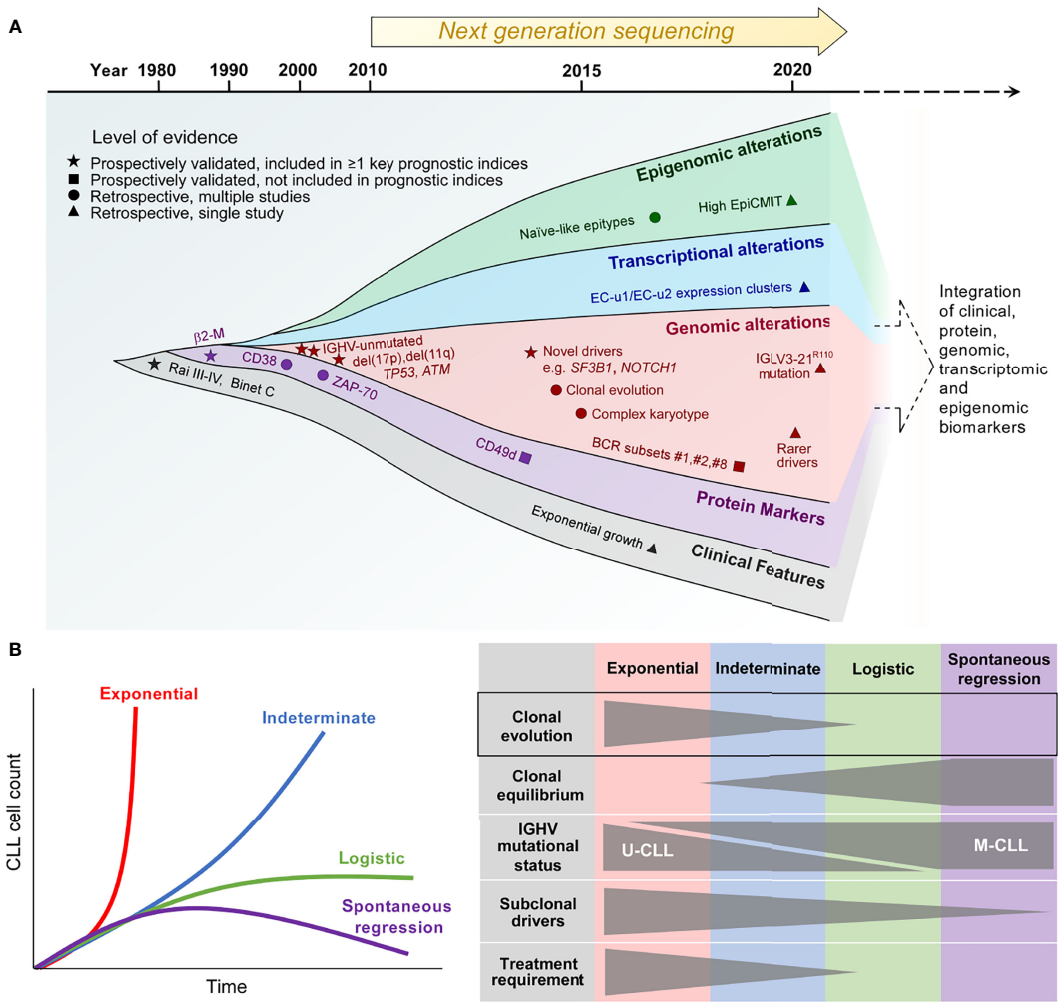

FIGURE 1 | The biological traits of high-risk CLL. **(A)** The definition of high-risk CLL evolving from the traditional reliance on a single or several discrete biomarkers towards the multimodal integration of multiple biomarkers that reflect the clinical, phenotypic, genetic, transcriptional and epigenetic properties of high-risk CLL. **(B)** Patterns of CLL growth dynamics, highlighting the importance of clonal evolution for exponential growth and CLL progression. β2-M, β2-microglobulin; IGHV, immunoglobulin heavy chain variable region; IGLV, immunoglobulin light chain variable region; EpiCMIT, epigenetically-determined cumulative mitoses; U-CLL, IGHV unmutated CLL; M-CLL, IGHV mutated CLL.

banding analysis and/or genomic microarrays has recently arisen, with complex karyotype conferring inferior outcome independently of the CLL-IPI (29–31).

Re-Defining High-Risk CLL Through Multimodal Integration of Biological Traits

The advent of next-generation sequencing technology in the past decade heralded an expansion in our knowledge of the CLL genome, epigenome and transcriptome (32, 33). In line with this, additional biomarkers of high-risk CLL have emerged. First, bulk whole exome sequencing (WES) and whole genome sequencing (WGS) efforts in large patient cohorts have provided comprehensive atlases of recurring CLL genomic alterations with putative functional significance encompassing both single-nucleotide variations (SNVs) and copy-number alterations (CNAs), revealing hitherto unknown genomic CLL drivers (34, 35). Some of these novel drivers, such as *SF3B1* and *NOTCH1* mutations, identified patients at higher risk of disease progression as well as disease recurrence after chemotherapy-based CLL therapy (34, 36, 37). Second, genome-wide methylation studies have identified three CLL epigenetic subtypes differentiated on the basis of their methylation profiles (38–40). These distinct epitypes reflect the developmental maturation state of the putative normal B-cell counterpart from which the different CLL subtypes are derived. Of these epitypes, naïve-like CLL, which is less epigenetically mature than the other epitypes (i.e. intermediate and memory-like CLL) and possesses ability for further epigenetic programming, is associated with higher progression risk. Furthermore, within individual epitypes, a higher

epigenetically-determined cumulative mitoses (epiCMIT) score, which reflects more extensive CLL proliferation history, correlates with adverse prognosis (41).

A recent large-scale analysis of the CLL transcriptome has yielded 8 gene expression clusters (ECs) with prognostic significance, each corresponding to a distinct transcriptional profile that reflects a unique CLL phenotypic state (42). On this basis, U-CLL clusters into two subtypes (EC-u1, EC-u2), whereas M-CLL can be clustered into four subtypes (EC-m1, EC-m2, EC-m3 and EC-m4). The remaining two clusters are EC-i and EC-o respectively, the former closely associates with the intermediate CLL epitype, while the latter does not correlate with any previously defined CLL group. The two EC-u clusters confer adverse risk, with EC-u1 and EC-u2 having similarly short progression-free and overall survival.

Some of these newer biological correlates of high-risk CLL, such as adverse epitypes and ECs as well as high epiCMIT, require further prospective validation. Nevertheless, their characterization has offered opportunity for the integration of genetic, transcriptional, epigenetic, phenotypic and clinical parameters to refine prognostic stratification, thereby providing a more accurate definition of high-risk CLL. Indeed, a recent multicenter effort by Knisbacher and colleagues utilizing data acquired from hundreds of patient samples have generated multivariate prognostic models that incorporate these different parameters (42). While much of our current understanding of CLL clonal evolution is derived from studies based on conventional definitions of high-risk CLL, as well as other adverse clinical features such as therapeutic resistance and high-grade transformation, contemporary prognostic classification constructed upon the basis of biomarker integration provides a useful foundation for the future study of evolutionary dynamics in high-risk CLL.

Clonal Evolution as a Determinant of High-Risk CLL

The clinical heterogeneity of CLL is quite evident in highly disparate clinical trajectories that can be observed amongst patients. An indolent or slowly progressing clinical course is observed in the majority of CLL patients, with rapid disease progression and spontaneous regression at the two extremes. These different clinical trajectories are mirrored by differences in clonal growth dynamics (43). Growth patterns that have been demonstrated in CLL include exponential unbounded growth, as well as logistic growth that stabilizes at a specific carrying capacity and plateaus over time, with exponential growth being considered higher risk as evidenced by a shorter time to treatment compared to logistic growth (**Figure 1B**, left panel). In addition, an indeterminate category falls between these two clearly defined growth patterns.

Through the analysis of serial samples from CLL patients, Gruber and colleagues linked differences in CLL growth trajectories to variations in clonal genetic composition as well as the extent of clonal evolution (43). Compared to patients exhibiting logistic CLL growth, patients who exhibit exponential growth are more likely to have U-CLL, harbor greater number of CLL driver mutations, and display more extensive clonal evolution marked by more profound shifts in subclonal proportions over time. In contrast, clonal equilibrium, wherein subclonal proportions remain stable over time, is more commonly observed in patients with logistic growth (**Figure 1B**, right panel). Clonal equilibrium associated with a relative paucity of subclonal genomic drivers also appears to be the norm among the rare cases of spontaneously regressing M-CLL, as reported recently by Kwok et al. (7). These findings corroborate earlier work that has established a correlation between CLL clonal evolution and adverse prognosis (34, 44). Together, these studies highlight the role of clonal evolution in shaping the natural history of individuals with high-risk CLL.

HARNESSING TECHNOLOGICAL ADVANCES TO INTERROGATE CLL CLONAL EVOLUTION

The study of clonal evolution in cancer is reliant on data generation and analytical platforms that are capable of delineating clonal architecture and subclonal phylogenetic relationships from longitudinal patient samples (3, 45). In CLL, these are usually peripheral blood samples, occasionally complemented by bone marrow and lymph node specimens to allow the study of CLL tumor-immune co-evolution within important microenvironmental niches (**Figure 2**, upper panel). Bulk sequencing analysis of these samples have facilitated much of our current understanding of CLL clonal evolution, including in high-risk patients. On the other hand, recent advances in single-cell sequencing technology provide opportunities for the interrogation of CLL clonal evolution at unprecedented resolution, which will likely transform our understanding of evolutionary mechanisms in high-risk CLL (**Figure 2**, middle and lower panel). These two approaches will be discussed in turn.

Bulk Analysis of CLL Clonal Evolution

Computational analysis of bulk WES and WGS data allows identification of genomic drivers that are more recurrent than expected by chance, and are inferred to increase clonal fitness and drive oncogenesis, distinguishing them from passenger somatic alterations that are co-incidental and do not confer growth advantage or directly drive initiation or progression. Moreover, the computational integration of read depth and variant allelic frequencies of somatic mutations permits estimation of the cancer cell fraction (CCF) of each driver that corrects for CLL sample purity and chromosomal copy number alteration (34, 44). Analysis of longitudinal samples from individual patients using bulk WES or WGS thus permits inference of CLL clonal architecture and subclonal phylogenetic relationships from the coordinated patterns of temporal fluctuations in CCF. CLL genomic drivers that are consistently clonal (i.e. present at CCF >0.95), such as *MYD88* mutation, trisomy 12 and del(13q14), reflect early genomic events that mediate CLL development. On the other hand, other driver mutations that are typically present in only a

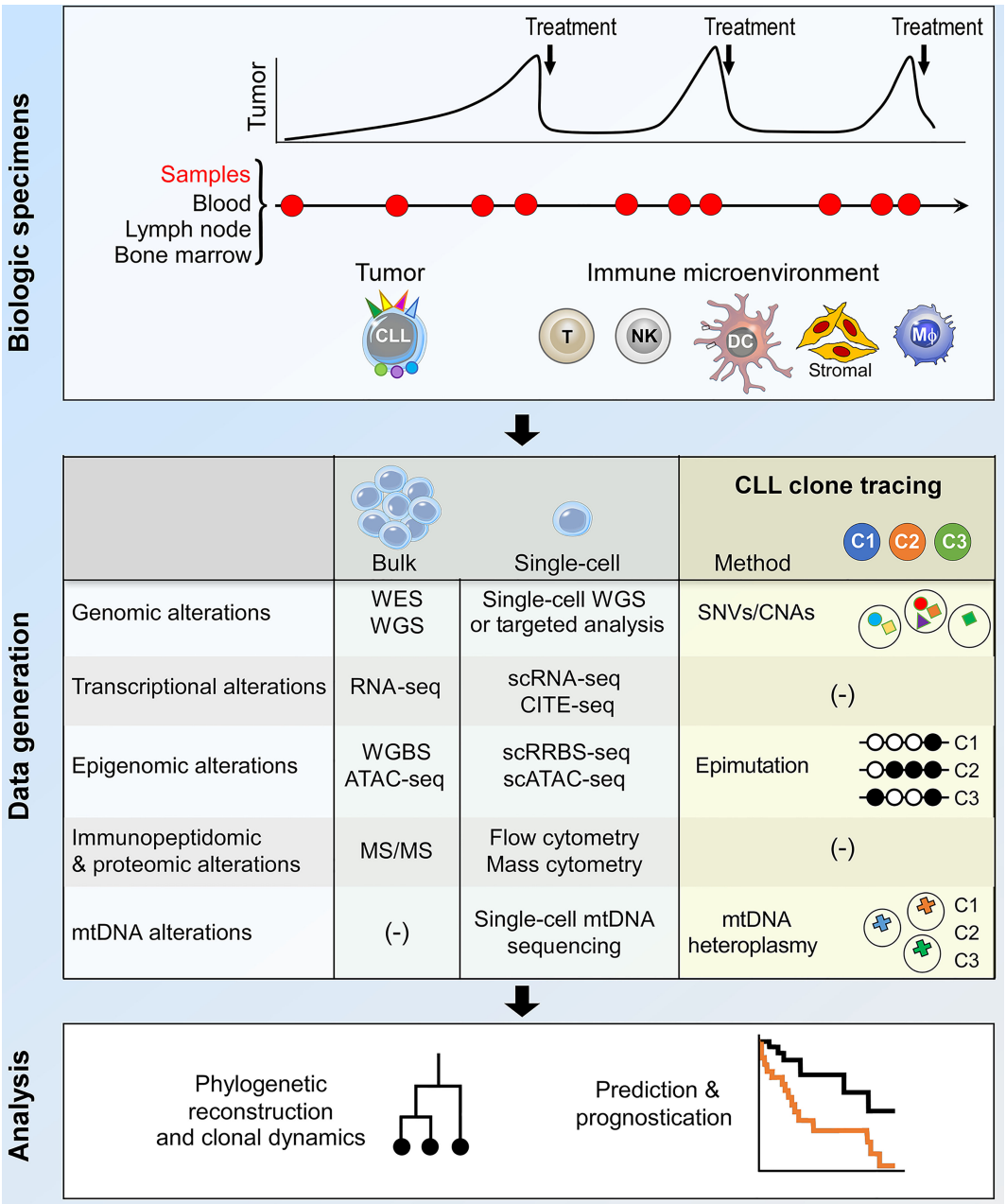

FIGURE 2 | Integrative bulk and single-cell analysis of CLL clonal dynamics. The current approach to the study of CLL clonal evolution is summarized, highlighting the use of biological specimens (top panel) and methodologies for data generation and analysis (middle and bottom panels respectively). Longitudinal samples collected at various time points during the clinical course of a patient allow analysis of both the tumor and the immune microenvironment. Various bulk and single-cell data generation approaches can be used to interrogate biological alterations that underpin clonal evolution. Within the context of single-cell analysis, lineage tracing techniques facilitate the identification of CLL subclones and the integration of multimodal data pertaining to individual subclones. The data thus generated can be used for phylogenetic reconstruction, analysis of subclonal dynamics and clinical prognostication. DC, dendritic cell; Mφ, macrophage; WES, whole exome sequencing; WGS, whole genome sequencing; SNV, single-nucleotide variation; CNA, copy number alteration; mtDNA, mitochondrial DNA; scRNA-seq, single-cell RNA-seq; CITE-seq, cellular indexing of transcriptomes and epitopes by sequencing; WGBS, whole genome bisulfite sequencing; scRRBS-seq, single-cell reduced representation bisulfite sequencing; scATAC-seq, single-cell sequencing assay for transposase-accessible chromatin; MS/MS, tandem mass spectrometry.

fraction of CLL cells are subclonal and likely represent later events that arise from subclonal selection. Subclonal mutations are thought to confer enhanced clonal fitness and drive CLL progression (34, 44). Examples of subclonal CLL drivers include mutations within *ATM*, *SF3B1*, *TP53* and *BIRC3*. In addition to genomic analyses, transcriptome profiling studies (e.g. bulk RNA-seq) demonstrate global transcriptional changes that often accompany genetic clonal evolution (46–48), while

epigenomic studies such as genome-scale DNA methylation analyses on bulk CLL cell populations reveal remarkable intratumoral epigenetic heterogeneity that fuels clonal evolution (49).

Despite their proven utility, a fundamental limitation of clonal evolution studies based on bulk sequencing methodologies lies in their inability to resolve with precision subclonal phylogenetic relationships at low CCFs, because the capacity to detect rare subclonal genomic events is often limited by sequencing read depth. Moreover, bulk analyses do not readily permit an integrative analysis of the genetic, epigenetic and transcriptional dynamics of individual CLL subclones that is essential to understand complex evolutionary mechanisms. These limitations can be addressed through contemporary approaches that leverage multidimensional single-cell sequencing technology.

Single-Cell Analysis of CLL Clonal Evolution

Single-cell analysis, by definition, allows high-resolution reconstruction of clonal phylogenetic architecture, as well as the determination of cell state dynamics in relation to genetic lineage history, through the integration of multiple strata of biological information across longitudinal time points at single-cell resolution (3, 45). The latter is achieved through coupling single-cell RNA-seq (scRNA-seq) with single-cell reduced representation bisulfite sequencing (scRRBS-seq), single-cell chromatin accessibility assays [e.g. single-cell sequencing assay for transposase-accessible chromatin (scATAC-seq)] and/or single-cell WGS or genotyping, performed simultaneously on RNA and DNA extracted from the same cells, thereby linking cellular transcriptional states with gene regulatory networks and genomic aberrations. The deconvolution of CLL subclonal dynamics through an integration of multimodal single-cell biological data necessitates the deployment of robust methodologies to track individual subclones over time, a process known as lineage tracing. The use of synthetic sequencing barcodes enables prospective lineage tracing within *in vitro* and *in vivo* CLL models (50), and is being increasingly explored across cancer systems. With the currently available tools, however, such a strategy is largely unfeasible to use in primary biospecimens from CLL patients given the well-known obstacles to efficiently introduce such barcodes into primary B cells. Instead, retrospective approaches for lineage tracing that exploit heritable native barcodes such as SNVs/CNAs, mitochondrial DNA (mtDNA) heteroplasmy or epimutations have been used to identify and mark each individual subclone (50–57). These approaches will each be elaborated in more detail.

Novel Approaches for Lineage Tracing in CLL at Single Cell Resolution

An established method for lineage tracing involves the tracking of SNVs and/or CNAs that are present within individual subclones. Such an approach utilizes experimental platforms that integrate the sequencing of single-cell genomes and transcriptomes (e.g. G&T-seq, sci-L3-RNA/DNA) (51, 52), and others that incorporate single-cell somatic genotyping within scRNA-seq [e.g. targeted RNA qPCR, Genotyping of Transcriptomes (GoT)] (53, 54). However, a major limitation of this approach is the fact that SNVs and CNAs can be infrequent in certain CLL subclones. They are also vulnerable to selection pressure during the course of clonal evolution, therefore lacking the required stability and consistency for a lineage marker. Moreover, single-cell WGS has limited scalability with allelic dropout issues, while GoT is challenging to use in the context of lowly expressed genes. Alternative lineage markers, such as mtDNA heteroplasmy and epimutations, provide opportunities to overcome these barriers.

Mitochondrial DNA heteroplasmy are naturally occurring, stochastic mtDNA mutations that can serve to identify individual tumor subclones (55). Such mutations are particularly attractive for lineage tracing owing to their consistent and stable propagation within a specific subclonal lineage from one generation to the next. In a proof-of-concept study, Penter and colleagues applied mitochondrial scATAC-seq (mtscATAC-seq), which provides conjoint readout of mtDNA mutations and chromatin accessibility information, to the analysis of clonal dynamics in patients with high-risk CLL (56). This study confirms the ability of distinct mtDNA mutations to stably mark separate CLL subclones with different chromatin states. Moreover, the use of mtDNA mutations as lineage markers allow efficient tracking of the varied temporal dynamics of CLL subclones in response to different treatment modalities and during Richter transformation. Notwithstanding uncertainties surrounding the role of mtDNA heteroplasmy in CLL pathogenesis, and our as yet nascent understanding of mtDNA dynamics, mtDNA-based lineage tracing represents a major technical advance for the single-cell analysis of CLL clonal evolution.

Similar to mtDNA mutations, stochastic DNA methylation changes known as epimutations are heritable marks that can be adopted for lineage tracing (57). Epimutations that lead to the random site-specific gain or loss of DNA methylation are accumulated during DNA replication and cell division, reflect cellular proliferation history, and can serve as epigenetic molecular clocks. Landau and colleagues showed that epimutations are ubiquitous features of CLL cells, readily detectable by RRBS-seq across large swathes of the CLL genome (49). Applying scRRBS-seq and scRNA-seq to longitudinal CLL samples, Gaiti et al. demonstrated the capability of epimutation information to identify individual CLL subclones with distinct genetic and/or transcriptional profiles, thereby enable accurate reconstruction of clonal phylogenies and characterization of CLL subclones with differential treatment response (57).

Finally, recent innovations in synthetic barcode technology for single-cell sequencing promise to revolutionize the use of *in vitro* and *in vivo* models to interrogate CLL clonal evolution. An example of this is ClonMapper that enforces expression of unique single-guide RNA (sgRNA) barcodes within single cells (50). These barcodes can be captured subsequently during

scRNA-seq, thereby coupling clonal identity with single-cell transcriptomics, and allowing for the isolation of subclones of interest for further integrative multiomics study. To illustrate the applicability of this technology for modelling clonal evolution in high-risk CLL, Gutierrez and colleagues implemented this platform to monitor subclonal diversification in a CLL cell line in response to treatment, uncovering a host of genomic and transcriptomic cell state changes, and unique subclonal dynamics (50). Although this study was carried out *in vitro*, one can envisage the use of similar barcode technology within various murine models of CLL, including Eμ-TCL1, CLL patient-derived xenografts and newer CRISPR/Cas9-engineered transgenic models (58–61), opening up unprecedented opportunities for the prospective investigation of clonal evolution under experimental CLL therapies or therapeutic combinations.

Analysis of Tumor-Immune Co-Evolution

Tumor cells reside within microenvironmental niches where they constantly interact with immune cells. These tumor-immune interactions contribute to shaping clonal evolution, and our appreciation of their importance have led to a growing impetus for the study of tumor-immune dynamics which is dependent upon both intrinsic tumor immunogenicity and the extrinsic immune microenvironment. With regard to tumor immunogenicity which in turn is determined by its antigenic landscape, mass spectrometric analysis of CLL major histocompatibility complex (MHC) class I and II immunopeptidomes, complemented by computational analysis of genomic and transcriptomic data (62, 63), enables characterization of the CLL antigenic landscape and its evolution over time. In relation to the extrinsic immune microenvironment, single-cell transcriptomics and epigenomics, applied across longitudinal patient samples or within *in vivo* models, enable accurate delineation of different immune populations as well as the characterization of dynamic immune cell states and tumor-immune interactions.

Altogether, these exciting new technological innovations will undoubtedly further advance our understanding of clonal evolution in high-risk CLL. In order to appreciate the context upon which future discoveries can be made, our current understanding of the molecular basis of intratumoral heterogeneity and clonal evolution in CLL is reviewed in the next sections.

THE MOLECULAR BASIS OF CLL INTRATUMORAL HETEROGENEITY AND EVOLUTION

Intratumoral heterogeneity in CLL is commonly understood to stem from genetic heterogeneity as a consequence of mutations and other genetic alterations. However, possession of genetic drivers is not a prerequisite for CLL progression. Indeed, sequencing efforts have failed to identify such drivers in some instances of CLL progression and relapse (43). Conversely, the presence of genetic drivers does not inevitably result in disease progression (64), as evidenced by patients harboring these drivers who remain at the monoclonal B-cell lymphocytosis (MBL) or indolent CLL stage for many years (65–67). Patients with CLL who spontaneously regress despite harboring *TP53* mutations in their CLL clone provide further testament to the notion that the mere presence of CLL genetic drivers is insufficient to drive clonal evolution or disease progression (7). Multiple lines of evidence now support an interplay between genetic, epigenetic and transcriptional cell states, as well as microenvironmental and immune factors in contributing to CLL intratumoral heterogeneity and clonal evolution (49, 57, 68–70). These various sources of CLL heterogeneity will be discussed below, highlighting their role in driving clonal evolution (**Figure 3**).

The Genetic Basis of CLL Intratumoral Heterogeneity

Genetic variation underpins much of the clonal heterogeneity in CLL, with an average mutation rate of 0.6 to 1.1 per megabase but with a wide variation across individuals (range, 0.03 to 2.3) (34, 35, 42, 44, 71). In addition to SNVs and CNAs, more profound genomic disruptions such as kataegis (localized hypermutation hotspots), chromothripsis (localized clusters of hundreds to thousands of chromosomal rearrangements within a single or several chromosomes) and chromoplexy (complex chromosomal rearrangements involving multiple chromosomes) have been described in CLL (35, 71, 72). Analyses of CLL mutational signatures have revealed several mutagenic processes as likely contributors to its genetic heterogeneity. These include age-related mutagenesis reflecting a predilection of CLL for the elderly population, as well as activation-induced cytidine deaminase (AID) and APOBEC-related mutagenic processes that reflect CLL as a mature B cell malignancy putatively derived from antigen-experienced B cells with capacity for AID/APOBEC-mediated somatic hypermutation (42, 71, 73). Recent analyses of CLL WGS data have additionally identified mutational signatures arising from oxidative stress (42), as well as from DNA polymerase activity, replication slippage and defective DNA repair (74), the latter reflecting replication errors as sources of genomic alteration. Genomic instability in CLL may be further promoted by permissive genetic contexts resulting from loss of cell cycle control (e.g. *TP53* or *CDKN2A/B* mutation), DNA damage response (DDR; e.g. *ATM* or *SAMHD1* mutation) (75) or telomere maintenance (e.g. *POT1* mutation) (76), giving rise to additional genetic heterogeneity.

Genetic Evolution in CLL Development and Natural Progression

Clonal evolution studies based on the analysis of WES or WGS data have enabled the characterization of a founder clone in CLL patients wherein somatic mutations are present (44, 64). These clonal mutations likely include initial leukemogenic drivers contributing to malignant transformation. On the other hand, subclonal mutations that subsequently emerge

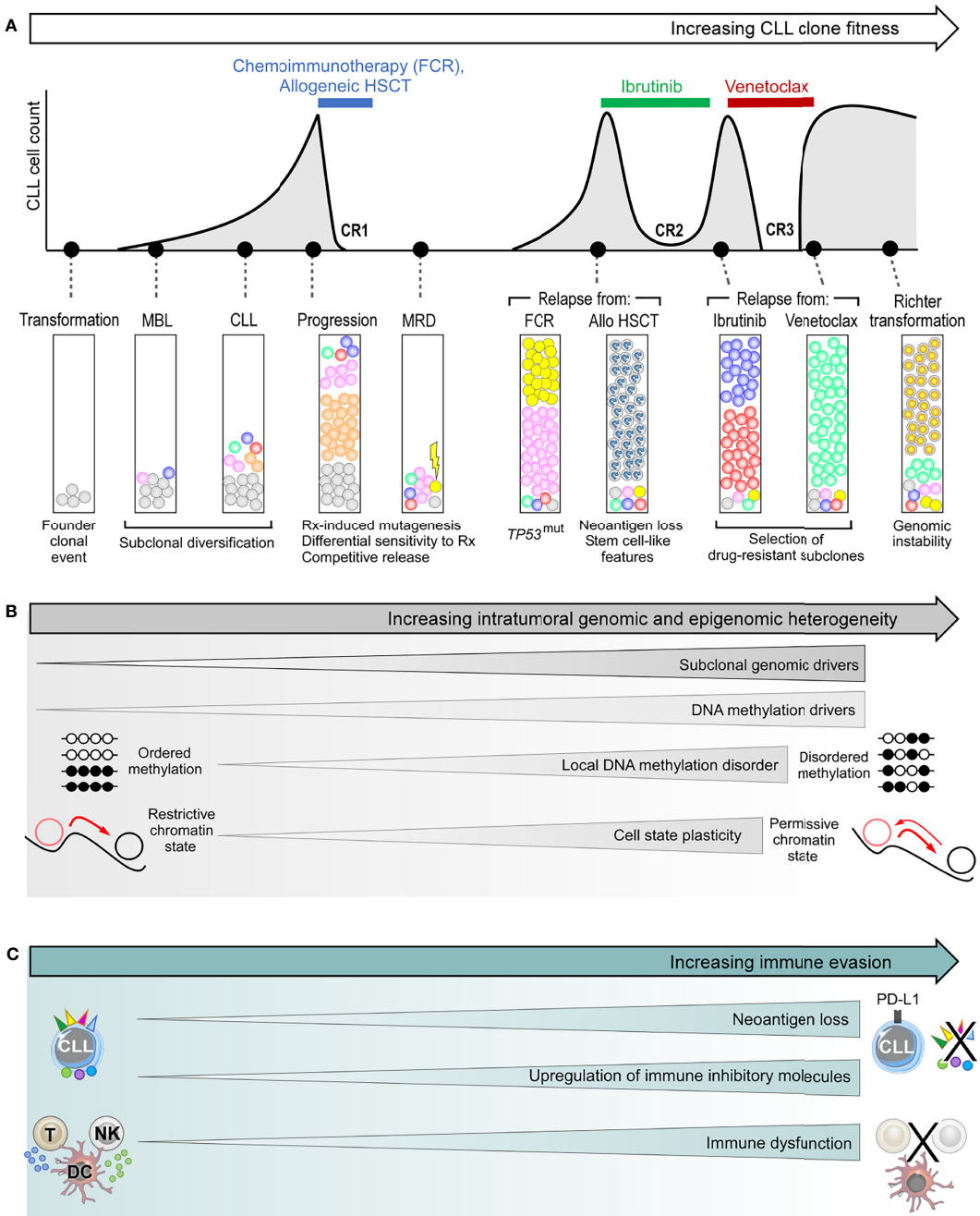

FIGURE 3 | The genetic and non-genetic basis of intraclonal heterogeneity and clonal evolution in CLL. **(A)** Clonal evolution during the clinical course of a typical patient with high-risk CLL, illustrating subclonal dynamics during natural progression, as well as different patterns of subclonal selection that accompany treatment with chemoimmunotherapy, allogeneic HSCT (allo-HSCT), ibrutinib and venetoclax. **(B)** The various sources of intratumoral genetic and epigenetic heterogeneity that underpin clonal evolution in CLL. **(C)** The mechanisms of immune evasion that can facilitate clonal evolution. FCR, fludarabine, cyclophosphamide and rituximab combination; HSCT, hematopoietic stem cell transplantation; CR, complete response; MBL, monoclonal B-cell lymphocytosis; MRD, measurable residual disease.

drive CLL clonal evolution. Underlining the importance of the latter, Landau et al. showed that many subclonal drivers expand towards clonality concomitantly with disease progression, and that the presence of such drivers confers adverse prognosis (34, 44). Murine models also support the role of specific mutations as drivers of CLL progression, either individually or in combination (61, 77–81).

The corresponding stages of normal B cell maturation during which founder mutations begin to emerge and subclonal genomic diversification commences is a matter of considerable

contention. For patients with MBL who subsequently progress to CLL, several studies have demonstrated that the mutational burden as well as the clonotypic and genomic landscapes of MBL and CLL are largely similar (66, 67, 82, 83), indicating that the process of subclonal genomic diversification that drives leukemic progression is likely to have been established during or prior to the MBL stage. Provocatively, CLL driver mutations have also been reported within hematopoietic stem cells (HSCs) as well as myeloid and lymphoid progenitor cells in some patient with CLL, albeit at low variant allelic frequencies (84, 85), akin to age-related clonal hematopoiesis (55, 86–88). The potential role of HSCs in the pathogenesis of CLL has been corroborated by experiments which demonstrated the ability of HSCs from CLL patients to produce *de novo* CLL-like disease with distinct IGHV rearrangements upon xenotransplantation into immunodeficient mice (89). The occurrence of phylogenetically unrelated oligoclonal IGHV rearrangements in a substantial proportion of patients also supports the proposition that CLL-initiating events could predate somatic V(D)J recombination (90). On the other hand, clonally-related IGHV variants could reflect subclonal diversification events occurring downstream of the CLL founder clone, highlighting the importance of IGHV-D-J sequencing to track clonal evolution in CLL (91). If indeed the initiating genomic alteration in some patients occurs at the level of HSCs or progenitor cells, it is conceivable that further genomic events occur downstream of this, possibly after commitment to the B-cell lineage, and prior to establishment of the founder CLL clone.

The subsequent clinical course following CLL establishment is highly divergent and is underpinned by heterogeneous evolutionary dynamics. While clonal equilibrium features in the majority of patients within the treatment-naïve setting, clonal evolution is observed in others **(Figure 3**) (44, 64, 92). Gruber et al. showed that even in patients exhibiting clonal equilibrium that typically manifests in logistic growth, there is a varying degree of subclonal competition resulting in mild fluctuations in subclonal proportions, and such complex intraclonal dynamics can result in net carrying capacity (43). In some cases, certain subclones can acquire exponential growth which could be a harbinger for subsequent disease acceleration. Moreover, as recently suggested by *in vitro* models, the persistence of clonal equilibrium may also be predicated upon dynamic intracellular interactions between subclonal CLL populations, the disruption of which may perturb this delicate balance resulting in specific subclonal outgrowth (50).

Clonal evolution, on the other hand, can be considered from a purely genetic standpoint as the natural consequence of differential growth accelerations conferred by the different genetic drivers within competing subclones. In CLL, the strongest growth accelerations appear to be associated with tumor suppressor genes such as *TP53* or *ATM*, with other drivers such as *ASXL1*, *GNB1*, *XPO1*, and *KRAS* also conferring substantial growth acceleration (43). Differential growth rates conferred by different drivers are also evident from a CLL cell line model engineered to express different driver mutations (93). The heterogeneous growth rates of competing subclones result in linear or branching patterns of clonal evolution, both of which are frequently observed in CLL. Linear evolution is characterized by the sequential acquisition of advantageous alterations within a subclonal lineage that allows it to outcompete and dominate all antecedent subclones *via* successive selective sweeps. Branching evolution, on the other hand, is characterized by multiple subclones co-evolving in parallel, either mutating the same driver ('convergent evolution') or different drivers ('divergent evolution') to compete for dominance.

Genetic Evolution in Response to CLL Treatment

CLL treatment has consistently been shown to fuel clonal evolution, resulting in fitter subclones with higher growth rates at the time of relapse. The resultant evolutionary pattern can be seen as a function of the pre-existing subclonal landscape at the time of treatment, and the nature of that CLL treatment. The importance of the former is evidenced from observations that a CLL harboring a wider spectrum of genetic alterations, more subclonal drivers, more complex subclonal landscapes, and exponential growth at the time of treatment is likely to experience more profound clonal shifts in response to treatment (43, 44). This can be understood from the availability of a wider pool of genetically fitter subclones from which therapeutic resistance could develop. Indeed, in many instances the dominant CLL subclone at the time of relapse is already pre-existing as a minor subclone at the time of treatment (34, 44, 64, 92), reflecting treatment-induced selection of resistant subclones. In many cases, these subclones with selective advantage are also those possessing higher levels of genomic instability that predispose them to additional genomic alterations, resulting in higher potential for further genetic diversification.

With regards to the nature of the CLL treatment, different treatment modalities exert differing selective pressures, the consequence being that subclones harboring distinct genetic drivers may have different strengths of fitness advantage under different treatments. For instance, *TP53*-mutant and/or del(17p) CLL subclones are almost invariably selected under chemotherapy-based treatments (34, 94, 95), whereas this is often not the case with targeted therapy (47, 96). In addition, chemotherapy-induced mutagenesis can contribute to genetic diversification, a process not seen with targeted therapy. Nevertheless, all CLL treatments regardless of modality create some manner of an evolutionary bottleneck (44, 97). This alters the subclonal landscape by removing incumbent subclones in favor of those with diminished sensitivity to treatment or enhanced survival and growth advantage that are 'competitively released' into the tumor ecosystem following treatment cessation.

The Epigenetic Basis of CLL Cell State Heterogeneity

Notwithstanding the unequivocal impact of genomic aberrations, clonal fitness is not exclusively determined by genetic features. Indeed, there are documented examples of

CLL subclones with exceptional growth rates but no identifiable genetic drivers (43). Moreover, CLL progression or relapse following treatment does not invariably coincide with the expansion of subclones harboring specific genetic alterations (64). These observations imply the existence of other factors influencing clonal fitness. In recent years, studies harnessing scRNA-seq technology have demonstrated substantial CLL transcriptional changes accompanying disease evolution, with transcriptional features evolving alongside genetic features (53, 98). Moreover, apparently divergent patterns of genetic evolution could potentially mask consistent transcriptional changes occurring in genetically distinct subclones, resulting in convergent evolution at the transcriptional level that could foster gene expression cohesiveness among CLL cells (53). Recent work has also revealed profound epigenetic cell state heterogeneity in CLL that influences clonal fitness and underpins clonal heterogeneity at the genetic, transcriptional and phenotypic levels (49, 57, 69).

Our current understanding of the epigenetic landscape in CLL is derived, to a large extent, from DNA methylation studies (38–40). These studies have shown that the CLL methylome is characterized by global gene body hypomethylation, accompanied by focal hypermethylation at promoters of tumor suppressor genes. The latter results in functional inactivation of these genes, thus representing a non-genetic mechanism through which CLL subclones could acquire fitness advantage. Tumor suppressor genes that are inactivated through this mechanism are known as DNA methylation drivers. A recent study by Pan and colleagues reported 122 putative DNA methylation drivers in CLL, of which 3 were functionally validated (70). These were *DUSP22*, *RPRM* and *SASH1*, which impact upon diverse leukemogenic processes including oncogenic signaling, cell cycle dysregulation and CLL migration. Analysis within patient cohorts showed that the presence of such drivers confers adverse prognosis. Moreover, longitudinal study revealed the emergence of novel DNA methylation drivers at the time of CLL relapse, thus substantiating their role as drivers of clonal evolution and disease progression.

DNA methylation studies also revealed that the inherited DNA methylome of a CLL represents an epigenetic imprint of both its putative cell of origin and proliferation history (38–41). In most patients, global patterns of CLL DNA methylation exhibit longitudinal stability (99), with evolution from the ancestral methylome being reported only in some individuals during CLL progression accompanying genetic evolution (68). Nevertheless, CLL cells display local methylation disorder arising from epimutations that were discussed earlier (49, 57). These epimutational changes are reminiscent of the process of genetic diversification, resulting in cell-to-cell variability in local DNA methylation patterns. Furthermore, recent integrative epigenomic analysis incorporating histone modifications and DNA methylation demonstrated corrupted coherence across different strata of the CLL epigenome (69). As reported by Pastore et al, this manifests in specific chromatin regions simultaneously acquiring activating acetylation marks as well as repressing methylation marks, modifications that are normally mutually exclusive.

Local DNA methylation disorder, together with corrupted coordination of epigenetic modifications, thus generate enormous intraclonal epigenetic diversity in CLL. Such epigenetic heterogeneity has several consequences. First, the resultant assortment of different chromatin states within individual genomic loci leads to decreased epigenetic-transcriptional coordination, thereby introducing greater cell-to-cell discordance in gene expression (49, 69). This gives rise to enhanced CLL intraclonal transcriptional heterogeneity. Second, increased transcriptional heterogeneity inevitably translates into greater phenotypic variability. This results in a permissive CLL cell state that confers a higher level of plasticity wherein an admixture of cells with different epigenetic identities lowers the barrier for transition between cell states (3, 69). Third, a more permissive epigenetic landscape could also promote genetic clonal evolution by supporting the propagation of newly acquired genetic alterations to progeny CLL cells (49). Thus, epigenetic heterogeneity underpins intratumoral heterogeneity at multiple levels, together fueling CLL clonal evolution. Although unproven, one can reason that the level of epigenetic heterogeneity would increase in tandem with genetic evolution and clinical progression. Moreover, enhanced cell state plasticity that could corrupt differentiation programs and undermine differentiation hierarchies may hold particular relevance for Richter transformation.

Microenvironmental Heterogeneity and Tumor-Immune Co-Evolution

Our understanding of clonal evolution in CLL is incomplete without considering the evolutionary dynamics of the myriad of interactions between CLL cells and their surrounding tumor immune microenvironment. The impact of the tumor microenvironment is evident from the tumor-supporting interactions upon which CLL cells depend for survival and proliferation. Notable examples of such interactions within lymph nodes include the CD40-CD40L interaction with follicular T cells (100), and the BAFF/APRIL-tumor necrosis factor receptor (TNF-R) interaction with nurse-like cells (101), as well as antigen-BCR interactions (102). On the other hand, the interactions that underpin anti-CLL immunity are less well characterized, but there is emerging *in vitro* and *in vivo* evidence for their existence (103–106). Capitalizing on advances in proteogenomic platforms and computational algorithms (62, 63), studies in recent years have begun to elucidate the repertoire of CLL tumor-associated antigens and neoantigens (103, 104, 107, 108). These studies revealed diverse neoantigen sources including somatic mutations (104), small insertions or deletions (indels) (107), splice variants and novel unannotated open-reading frames (nuORFs) (108), all capable of eliciting potent antitumor immune response. Recent work has also begun to unravel the immune cellular populations and tumor-immune interactions that may be important for antitumor immunity, notably within murine models of CLL (105, 106).

Cancer cells subvert antitumor immunity both by evolving strategies to evade immune detection and by suppressing the function of immune cells, leading to attenuated antitumor response. Tumor cells, for instance, can evade immune detection by downregulating tumor expression of MHC or otherwise interfering with the process of antigen processing or presentation, leading to loss of tumor antigen expression (10). These mechanisms have been suggested in CLL models (109), but have yet to be convincingly demonstrated in patients with CLL. However, CLL cells are known to upregulate immune inhibitory molecules such as PD-L1 (110). Moreover, CLL cells exert direct inhibitory effect on cytotoxic T cell function, resulting in impaired motility, immune synapse formation and cytotoxicity (111, 112). Chronic antigenic stimulation also renders T cells anergic, contributing to an exhaustion phenotype associated with functional impairment (113). In addition, natural killer (NK) cells, dendritic cells and monocytes are also functionally impaired in CLL, and the number of myeloid-derived suppressor cells (MDSCs), particularly of the polymorphonuclear MDSC subtype, are increased, which contribute to immune escape (114, 115). On the other hand, established and experimental CLL treatments such as ibrutinib, lenalidomide/avadomide, immune checkpoint inhibitors, and hematopoietic stem cell transplantation (HSCT) can reverse CLL-induced immune dysfunction (112, 116–122). Therefore, CLL-induced immune evasion and immunomodulatory treatments can be viewed as two opposing forces shaping the dynamic antitumor immune landscape.

The contribution of antitumor immunity to molding diverse CLL clinical trajectories is currently unclear. One can envision that whereas CLL progression coincide with immune escape, long-term disease stability as seen in patients with a highly indolent or spontaneously regressing clinical course, or in the majority of individuals with MBL who never progress to CLL, may be dependent to varying degree on immune control. This process is commonly known as cancer immunosurveillance (123). In relation to the latter, a recent study found differences in the inflammatory signatures between MBL and CLL (124). Likewise, sustained CLL remissions, particularly with novel therapeutic agents that modulate the immune system, may be contingent upon antitumor immunity, with immune escape presaging disease relapse. The evolving immune selection pressures and immunoediting processes, as well as the dynamics of immune cell states, tumor antigenic landscapes and tumor-immune interactions that govern CLL immune control and escape have not been fully characterized, particularly at the single-cell level, and represent important areas for future investigation.

PATTERNS OF TREATMENT-INDUCED CLONAL EVOLUTION IN CLL

All too often, high-risk CLL progresses quickly to a clinical stage where treatment is required. With our expanding understanding of CLL pathobiology comes a paradigm shift in the therapeutic management of CLL, in which targeted therapies such as BCR signaling inhibitors (e.g. ibrutinib) and BCL-2 inhibitors (e.g. venetoclax) are increasingly replacing conventional chemotherapy-based treatments (125–127). Although the use of allogeneic HSCT (allo-HSCT) is also decreasing with the expanding availability of targeted agents, allo-HSCT remains an important therapeutic modality in multiply relapsed or refractory CLL (128–130). Moreover, immunotherapy is a burgeoning area of CLL research, as apparent from the flurry of recent research activity on chimeric antigen receptor (CAR) T and NK cells (131–133). Despite the transformative nature of novel therapies, therapeutic resistance inevitably arises. Of particular clinical relevance is how CLL cells evolve to become resistant to these new treatments. To illustrate the clonal evolutionary mechanisms that accompany resistance to targeted therapy and immunotherapy, we herein review recent work on ibrutinib, venetoclax and allo-HSCT.

Convergent Evolution Leading to Resistance to CLL Targeted Therapy

The mechanism of resistance to targeted therapies frequently involves interfering with drug target binding or circumventing the target. The most comprehensively documented mechanism of acquired resistance to ibrutinib is mutations of the Bruton tyrosine kinase (*BTK*) or *PLCG2* genes (134, 135), reflecting ibrutinib as a BTK inhibitor that suppresses BCR signaling. *BTK* mutations confer ibrutinib resistance by prohibiting irreversible drug binding, with the *BTK-C481S* mutation as the most predominant. On the other hand, *PLCG2* gain-of-function mutations promote BTK-independent BCR signaling. With respect to venetoclax, *BCL2* mutations represent the most common resistance mechanism, with multiple studies reporting the detection of the *BCL2-G101V* mutation in association with clinical relapse (136–138). *BCL2* mutations result in diminished venetoclax binding to BCL-2, thus conferring drug resistance. Intriguingly, the *BTK*, *PLCG2* and *BCL2* mutations reported thus far have predominantly been subclonal (135, 136, 138, 139).

Longitudinal studies have provided insight into the evolutionary features associated with the acquisition of these mutations. While different evolutionary patterns have been observed, convergent evolution appears to be particularly common. For ibrutinib, this involves several different *BTK* or *PLCG2* mutations evolving in parallel (**Figure 4A**) (47, 139–141). For venetoclax, multiple *BCL2* mutations are likewise often seen to coexist (**Figure 4B**) (136–138). The CCFs of these variants within the same patient typically differ, suggesting that they arise within different subclones evolving independently, although definitive evidence will come from future single-cell analysis. These subclones may display different growth rates. For example, in the case of ibrutinib, Ahn et al. reported that *BTK*-mutated subclones often expand more rapidly than their *PLCG2*-mutated counterparts (139). Thus, one subclone may have growth advantage over other subclones, intrinsically or through the acquisition of additional alterations, and could achieve dominance over time.

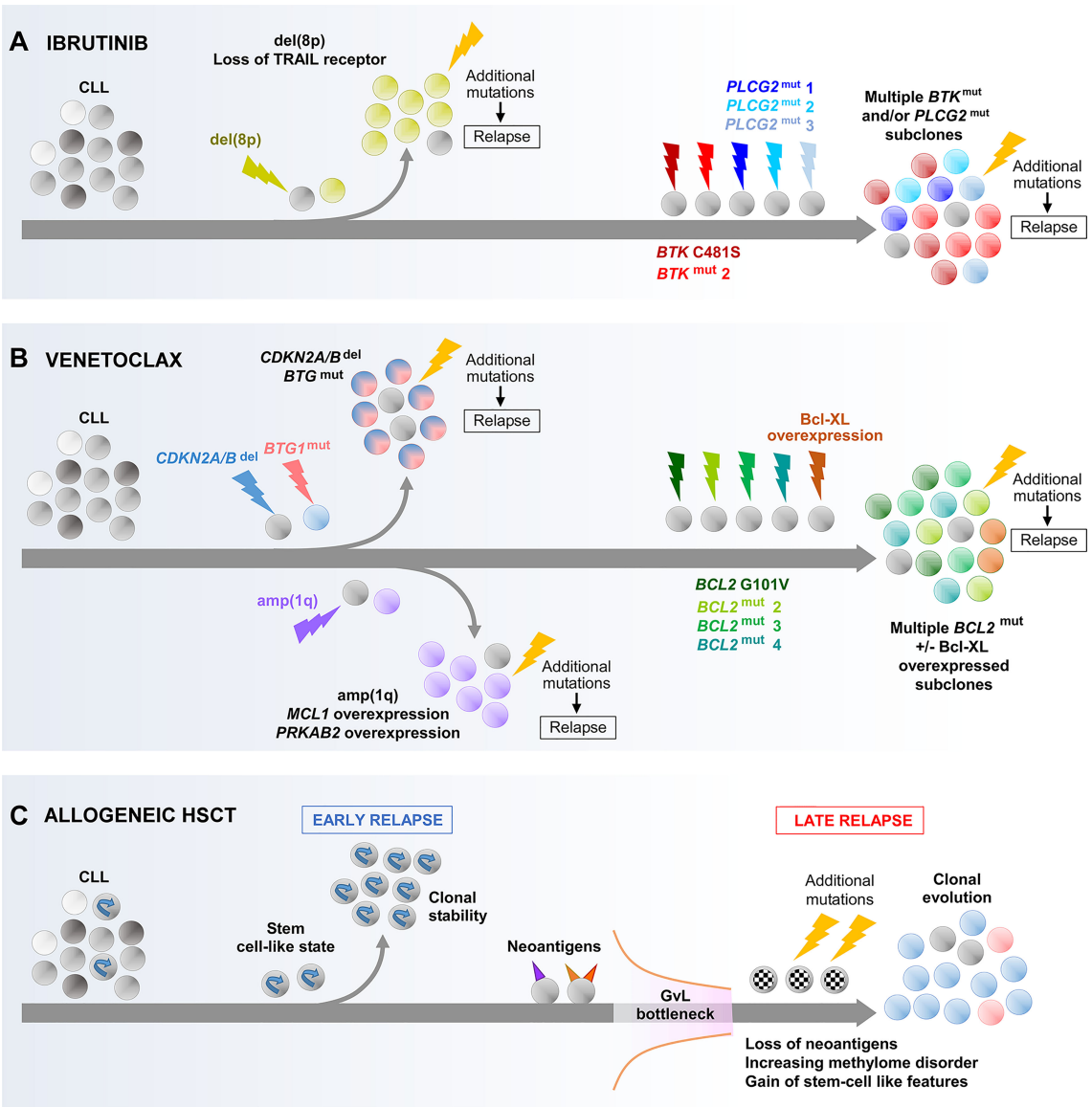

FIGURE 4 | Main evolutionary paths towards the development of CLL resistance to ibrutinib **(A)**, venetoclax **(B)** and allogeneic HSCT **(C)**. As illustrated, multiple different routes can be undertaken by CLL that result in resistance to these treatments. In the case of resistance to ibrutinib or venetoclax through acquired *BTK*, *PLCG2* or *BCL2* mutations, the co-evolution of multiple subclones harboring different mutations of the same gene is shown which illustrates the concept of convergence evolution. *BTK*, Bruton tyrosine kinase; GvL, graft-versus-leukemia effect.

Whether these mutations arise *de novo* during treatment or have pre-existed before treatment continues to be a matter of debate. Computational models support the existence of resistant subclones prior to treatment initiation (142), which has also been experimentally demonstrated in pre-treatment patient samples (141). Although in a proportion of patients the analysis of samples obtained near the start of treatment failed to detect resistance mutations that were identified in later samples (139), the detection of pre-existing mutations at low CCFs would likely have been limited by assay sensitivity. In any case, the simultaneous presence of multiple evolving subclones harboring alterations affecting identical genes demonstrates the enormous selection pressure that likely takes place during treatment, wherein CLL cells, through extensive trial and error, adapt by eventually evolving similar traits within distinct branches to create a cohesive resistant phenotype.

Heterogeneous Evolutionary Paths to Ibrutinib and Venetoclax Resistance

Although the evolution of *BTK*/*PLCG2* or *BCL2* mutations represents a common mechanism of resistance to ibrutinib and venetoclax respectively, these mutations are not universally

detected in therapy-resistant patients. Indeed, acquired *BTK* or *PLCG2* mutations are typically present in only 80% of individuals who relapse from ibrutinib (135). Likewise, acquired *BCL2* mutations were reported in only 7 of 15 patients (47%) relapsing from venetoclax in a recent study by Blombery and colleagues (136). Moreover, in some reported cases *BTK*/*PLCG2* or *BCL2* mutations remain at very low CCFs (<0.1) at the time of relapse (135, 136, 138, 139). This indicates that in some patients, other mechanisms are likely involved in mediating resistance to ibrutinib or venetoclax.

Two studies have analyzed WES data from longitudinal CLL samples to infer additional evolutionary routes to ibrutinib resistance (**Figure 4A**). In one study, Burger and colleagues identified the expansion of del(8p) subclones as a recurrent mechanism (141), occurring principally in patients without acquired *BTK* or *PLCG2* mutations within their CLL clones, which was corroborated by a separate study from Landau et al. (47). The deleted region in del(8p) involves the TRAIL receptor, a TNF-family extrinsic apoptotic receptor, the loss of which can be expected to enhance apoptotic resistance. Importantly, these studies showed that the fitness advantage conferred by del(8p) is conditional upon the acquisition of further genomic aberrations, without which the del(8p) subclone is not selected.

In the case of venetoclax resistance (**Figure 4B**), Blombery et al. showed that subclones with Bcl-xL overexpression and wild-type *BCL2* could coevolve alongside those harboring *BCL2* mutation (136). In addition, Herling and colleagues reported a recurring resistance mechanism in *BCL2* wild-type patients, characterized by the emergence and selection of CLL subclones harboring *CDKN2A/B* deletions and/or *BTG1* mutations (143), the former likely undermining cell cycle regulation, whereas the latter could contribute to apoptotic resistance and enhanced proliferation downstream of *BCL2* and *CDKN2A/B*. Similar to the evolution of del(8p) in ibrutinib-resistant cases, the presence of additional alterations appears to be a prerequisite for the selection of subclones harboring *CDKN2A/B* and *BTG1* defects with venetoclax. Finally, Guièze and colleagues reported amp(1q) as a recurrent lesion in a subset of venetoclax-resistant patients with wild-type *BCL2* (144). The amplified region contains *MCL1* and *PRKAB2*. As a gene encoding an anti-apoptotic protein with recognized roles in CLL, *MCL1* upregulation enhances apoptotic resistance (145). On the other hand, overexpression of *PRKAB2* was shown to confer metabolic advantage by increasing the capacity of CLL cells for oxidative phosphorylation, mediated through its regulatory role within the protein kinase A/AMP-activated protein kinase (PKA/AMPK) pathway (144).

As depicted by the examples of ibrutinib and venetoclax, there are multiple evolutionary paths of acquired resistance to targeted CLL therapies. Even accounting for the discovery of the aforementioned additional mechanisms, we would not have identified all the disparate genetic changes that CLL cells can accumulate on their road to therapeutic resistance. The heterogeneity of evolutionary mechanisms underpinning ibrutinib or venetoclax resistance, especially in patients without identifiable *BTK, PLCG2* or *BCL2* mutations, can potentially complicate further therapeutic targeting. On the other hand, the evolutionary dynamics at the transcriptional, epigenetic and phenotypic levels that accompany genetic evolution leading to ibrutinib or venetoclax resistance remain largely unexplored. In this respect, the work of Guièze et al. which applies genome-wide CRISPR screens and RNA-seq to cell line models has offered critical insight into important phenotypic mechanisms, for instance identifying metabolic dysregulation as a resistance mechanism to venetoclax (144). However, the application of integrative multidimensional single-cell analysis to longitudinal primary samples within uniformly treated patient cohorts, ideally within the setting of a clinical trial, will greatly enrich our understanding of the evolutionary processes underpinning resistance to targeted agents, potentially identifying common mechanisms and novel therapeutic targets.

Tumor-Immune Dynamics in Response and Resistance to Targeted Therapy

In contrast to chemotherapy-based treatments, multiple studies have demonstrated the ability of ibrutinib to reverse T cell dysfunction in CLL (116–119). In particular, recent work by Baptista and colleagues showed that clinical response to ibrutinib is accompanied by the oligoclonal expansion of cytotoxic $CD8^+$ T cells, which is reversed upon subsequent CLL progression (119). Moreover, these oligoclonal T cell populations were capable of eliciting potent anti-CLL cytotoxicity. Notwithstanding the impact of ibrutinib on CLL tumor burden that could confound their interpretation, these results suggest that tumor-immune co-evolutionary dynamics could potentially determine response and resistance to ibrutinib.

To assess the changes in CLL and immune cells that accompany response to ibrutinib, Rendeiro et al. recently carried out an integrative immunophenotypic, single-cell transcriptomic and chromatin mapping study on longitudinal peripheral blood samples from patients receiving ibrutinib therapy (146). Analysis of CLL cells revealed reduced NF-κB binding, curtailed activity of lineage-defining transcription factors, erosion of CLL cell identity, and the acquisition of quiescence-like transcriptome signature as features that characterize CLL response to ibrutinib. Peripheral blood T cells exhibited a quiescence-like gene signature, whereas monocytes and macrophages displayed an upregulation of inflammatory genes, both associated with defined chromatin accessibility changes. However, the assessment of tumor-immune dynamics in this study was hampered by a lack of lymph node and bone marrow samples. Moreover, the absence of CLL cases progressing on ibrutinib did not allow a comparison of response versus resistance to identify determinants of clinical outcome. Nevertheless, this study provides a relevant foundation upon which future studies can build. To comprehensively assess tumor-immune dynamics, longitudinal studies should ideally incorporate the analysis of tumor antigenic landscapes and tumor-immune interactions as well as immune cell states within the different microenvironmental compartments in which CLL cells reside.

In CLL, measurable residual disease (MRD) is predictive of long-term treatment outcome within various therapeutic settings

(147–149), and is widely adopted as a surrogate endpoint as well as a guide to treatment duration within clinical trials. The study of tumor-immune co-evolution may be of particular relevance within MRD-adaptive treatment settings where a targeted therapy or therapeutic combination (e.g. ibrutinib plus venetoclax) is administered until MRD negativity is attained (150). It will be of importance, for instance, to ascertain whether differing tumor-immune dynamics impact upon MRD response kinetics. For example, more profound anti-CLL immune activity may characterize rapid responders and those individuals achieving sustained MRD negativity, compared to slow responders and others with short-lived treatment response. The findings of such an investigation could inform the potential use of immunomodulatory treatments to preempt CLL relapse.

Clonal Evolution Underpinning CLL Relapse Following Allogeneic HSCT

Allogeneic HSCT exemplifies the power of harnessing antitumor immunity for cancer treatment, but relapses nevertheless occur. Recently, Bachireddy and colleagues investigated the evolutionary dynamics underlying resistance to graft-versus-leukemia (GvL) effect, uncovering mechanisms that may have potential broader relevance for CLL immunotherapy (151). Through longitudinal analysis integrating genetic, transcriptomic and epigenetic analyses, Bachireddy et al. identified two distinct evolutionary paths that give rise to early and late relapses respectively post allo-HSCT (**Figure 4C**). Early relapses are characterized by clonal equilibrium, and are underpinned by a pre-existing stem-like transcriptional state that confers resistance to GvL. In contrast, late relapses are characterized by clonal evolution secondary to a GvL selection pressure ('GvL bottleneck'), wherein the more immunogenic subclones expressing neoantigens with stronger predicted MHC binding affinity, and hence likely to have attracted stronger GvL response, are selectively depleted. Late relapses are therefore mediated by CLL subclones with neoantigen loss and poor immunogenicity. Moreover, these subclones acquire a stem-like state through upregulating stem cell pathways. The latter is underscored by enhanced local DNA methylation disorder, particularly within the promoters of stem cell pathway genes. Thus, stem cell properties are important determinants of CLL relapse post allo-HSCT, with these properties having pre-existed within the CLL clone in instances of early relapse, and acquired in the case of late relapse.

As apparent from the above, the pattern of clonal evolution leading to relapse post allo-HSCT differs markedly from the evolutionary dynamics that mediate resistance to chemotherapy or targeted therapy. This serves to illustrate the vastly different selection pressures that are imposed by different treatment modalities. The work of Bachireddy et al. also exemplifies the use of integrative multidimensional approaches to dissect clonal evolution which allow us to appreciate the non-genetic and genetic determinants of clonal dynamics, a comprehensive understanding of which will enable us to devise better treatments to manage CLL relapse.

CLONAL EVOLUTION LEADING TO RICHTER TRANSFORMATION

Richter Syndrome (RS), characterized by the high-grade transformation of CLL to diffuse large B cell lymphoma (DLBCL) and less commonly to Hodgkin lymphoma, represents a catastrophic clinical sequel of high-risk CLL. RS occurs in an estimated 2% to 10% of CLL patients (5). Individuals with RS respond poorly to currently available treatments, with dismal overall survival typically in the range of 3 to 6 months (152, 153). Richter transformation therefore represents the greatest current unmet need in CLL. There is compelling rationale to understand clonal evolution within this context, as an essential foundation for the development of improved therapeutics. On the basis of the clonal relationship between the antecedent CLL and the transformed lymphoma, RS can be classified as either clonally-related or unrelated, with identical or distinct IGHV rearrangements respectively (5). In the final part of our review, we will discuss our current understanding of the evolutionary processes underpinning these two types of RS (**Figure 5**), highlighting important areas for future investigation.

Clonally-Related Richter Transformation

Two earlier studies have identified common genetic evolutionary routes which CLL cells undertake during the course of clonally-related Richter transformation. In the first study, Chigrinova et al. analyzed CLL and DLBCL samples from a series of patients who have undergone transformation to RS, identifying genetic lesions that are likely acquired prior to and during transformation using SNP array and targeted gene sequencing (154). This study revealed two genetic pathways leading to RS, the first involving *TP53* inactivation and/or *CDKN2A/B* loss, alongside *MYC* activation, and the second involving trisomy 12. In addition, *NOTCH1* mutations were found frequently in patients with RS, particularly among individuals also harboring trisomy 12. The second study by Fabbri et al. (155), which employed WES to interrogate CLL-RS pairs and additional Richter-transformed lymphomas, confirmed the prevalence of genetic lesions identified in Chigrinova et al. Moreover, this study revealed a predominantly linear evolutionary trajectory that accompanies Richter transformation, wherein the majority of CLL phase genetic alterations are maintained in the Richter-transformed lymphoma, with the acquisition of additional heterogeneous lesions at the time of transformation.

The role of the most commonly encountered genetic alterations in Richter transformation was explored in recent studies utilizing mouse models of CLL. Chakraborty and colleagues showed that the concurrent loss of *TP53* and *CDKN2A/B* led to an abolition of cell cycle control, allowing unrestrained BCR signaling-driven clonal proliferation that obviates the need for co-stimulatory signals (156). This is evidenced by the induction of a Richter transformation-like process within the Eμ-TCL1 model upon combined deletion of these two genes. In a separate study, Kohlhaas et al. showed that constitutive activation of Notch1 within Eμ-TCL1 mice recapitulated the Richter phenotype (157), thus substantiating

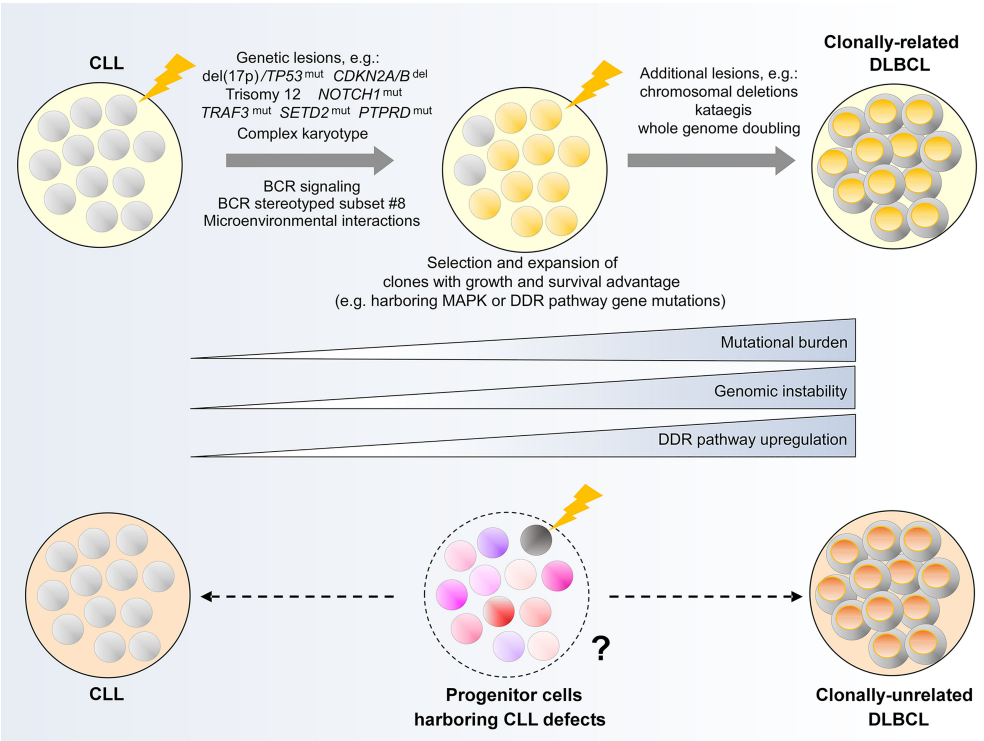

FIGURE 5 | Clonal evolution underpinning Richter transformation of CLL to DLBCL. (Upper panel) Genetic alterations and biological processes implicated in the development of clonally-related Richter syndrome. (Lower panel) Hypothetical model illustrating a possible evolutionary path underlying clonally-unrelated Richter syndrome. MAPK, mitogen activated protein kinase; DDR, DNA damage response.

the role of gain-of-function *NOTCH1* mutations in RS. Alternatively, at least within the Eμ-TCL1 model, it appears that constitutive activation of AKT, a component of the BCR signaling pathway, could drive Richter transformation through Notch1 activation in the absence of *NOTCH1* mutations. The latter seems to be mediated through an AKT-induced expansion of $CD4^+$ T cells within the tumor microenvironment that express the Notch1 ligand DLL1 (157), indicating that genetic and non-genetic evolutionary mechanisms could converge on the same phenotypic outcome. Indeed, certain stereotyped BCR subsets, particularly subset #8, have been shown to confer increased risk of RS (20, 158). These findings highlight the need for multimodal clonal evolution studies on patients with RS that consider genetic and non-genetic mechanisms in equal measures.

In this context, Klintman and colleagues recently performed an integrative analysis of CLL-RS pairs in patients with RS that included transcriptomic analysis in addition to WGS, uncovering important biological processes during the evolution of CLL to high-grade lymphoma (48). First, RS is accompanied by an increase in mutational burden affecting large numbers of genes not previously implicated in CLL. These include recurrent mutations in *TRAF3*, *SETD2* and *PTPRD*, as well as genes with important roles in DDR or MAPK-RAS-ERK signaling. Preferential selection of subclones harboring these alterations was consistently observed. Second, in association with genetic evolution, transcriptome analysis revealed differential regulation of DDR genes. Coupled with prominent DDR-related mutational signatures, Klintman et al. highlighted the contribution of a corrupted DDR to genomic instability in RS, with genomic instability providing a permissive condition for the acquisition of further genomic events in the evolutionary process. These include CNAs and whole genome doubling, described recently by Parry et al. (159), as well as kataegis, reported by Klintman et al. (48). Recent work also demonstrates complex karyotype as a risk factor for the development of RS (31), with complex karyotype representing another consequence of genomic instability.

Taken together, these reports underscore multiple tumor-intrinsic and extrinsic factors that likely influence clonal dynamics in clonally-related RS. Further work should harness integrative single-cell technology to interrogate clonal dynamics and to resolve the interaction among genomic, transcriptomic, epigenomic and microenvironmental determinants of clonally-related RS. Further work should also apply the same technology to study clonal evolution in Richter transformation occurring during treatment with targeted agents such as ibrutinib or venetoclax. Studies thus far revealed diverse genetic features in these patients, with *BTK, PLCG2* or *BCL2* mutations being absent in many instances (96, 141, 143, 160, 161). Integrative analysis within larger cohorts will shed light on potential shared mechanisms in these individuals.

Clonally-Unrelated Richter Transformation

In contrast to clonally-related Richter transformation, there has been minimal substantive work on the evolution of clonally-unrelated Richter transformation. A previous study by Lucas and colleagues showed that crossing Eμ-TCL1 mice with Eμ-Myc mice generated coexisting CLL and clonally-unrelated Richter-like lymphoma (162), suggesting that clonally-unrelated RS could potentially be MYC-driven. In addition, it would not be unreasonable to speculate that clonally-unrelated RS could originate from the evolution and subsequent transformation of HSCs or progenitor cells that harbor CLL/lymphoma genomic alterations ('CLL/lymphoma reservoirs'), rather than from the transformation of the established CLL clone. This remains unproven and further work on longitudinal patient samples and animal models will undoubtedly enlighten our understanding of clonal evolution in this uncharted area.

CONCLUSIONS AND PERSPECTIVES

In this review, we provided a contemporaneous account of clonal evolution as it relates to high-risk CLL, highlighting recent discoveries that have offered novel insight. The past several years have seen profound shifts in our understanding of clonal evolution underpinned by a maturing definition of high-risk CLL and an increasing sophistication of next-generation sequencing technology. We have begun to understand the non-genetic sources of clonal heterogeneity, and the relevance of tumor-immune dynamics. We have also come to appreciate that with each therapeutic innovation comes the inevitable problem of therapeutic resistance, which can only be tackled through an exhaustive understanding of clonal evolution.

Amidst the seemingly diverse CLL evolutionary landscape, we have begun to identify some recurring patterns and commonalities; the convergent evolutionary patterns mediating resistance to both ibrutinib and venetoclax is a case in point. At the same time we recognize that many aspects of CLL clonal evolution remain unresolved. Why do some MBLs and CLLs progress, while others harboring similar genetic abnormalities remain stable for decades, or even spontaneously regress? In patients developing resistance to treatment, how do genetic, transcriptional, epigenetic, tumor antigenic and immune microenvironmental alterations converge to produce a shared resistant phenotype? Furthermore, what evolutionary mechanisms underpin Richter transformation, particularly in clonally-unrelated cases? The application of integrative single-cell technology within well-characterized patient cohorts and relevant disease models will spearhead advances in these areas and address these fundamental questions in the years to come.

An equally important consideration is how we can utilize and translate this newfound knowledge of CLL clonal evolution for the betterment of our patients. Already we know that intratumoral genetic heterogeneity and clonal evolution predict for shortened time to CLL progression (34, 44). On the other hand, how heterogeneity at the transcriptional, epigenetic and microenvironmental levels interacts with genetic heterogeneity in influencing CLL prognostication remains to be ascertained. Current prognostic models are constructed based on data derived from a single timepoint, for instance at diagnosis or prior to treatment, which provide only a limited snapshot of the individual CLL within its longitudinal evolutionary history. Intratumoral heterogeneity, subclonal architecture, growth dynamics and evolutionary trajectories, in contrast, are arguably more tangible measures of past behavior and potentially more reliable predictors of future outcome. These parameters may therefore have a place within future prognostic models so long as they can be easily assessed and quantified. Future translational efforts should therefore be directed at converting highly granular, genome-scale assessments of clonal evolution, which are laborious, expensive and generate enormous quantities of data, into assays that are equally informative but are also adequately scalable, reproducible and quantifiable to be used in the diagnostic setting and longitudinally for routine disease monitoring.

Routine monitoring of clonal evolution, in turn, may potentially open up a future 'brave new world' of personalized CLL medicine in which treatments are adapted according to subclonal dynamics and initiated preemptively to target subclonal outgrowth. Indeed, rising mutant CCFs of *BTK*, *PLCG2* or *BCL2* frequently anticipates disease progression and may signal the need for preemptive salvage treatment (135, 136, 139). The argument of whether therapeutic targeting should focus on clonal (truncal) or subclonal (branch) alterations has been extensively addressed within previous reviews (97, 163), and may be reconciled in the ideal scenario by the simultaneous targeting of truncal and branch lesions through rational treatment combinations. Targeting truncal lesions could eliminate the majority of the tumor load, while targeting branch lesions that confer the greatest survival and growth advantage may potentially avert selection of the most aggressive subclones. Our increasing knowledge of the non-genetic as well as genetic determinants of intratumoral heterogeneity lends itself to the future expansion of our therapeutic armamentarium to include novel treatments that target cellular dependencies unique to specific CLL subclones. These dependencies may arise from distinct tumor-immune interactions or from specific genetic, epigenetic or transcriptional alterations. Indeed, as alluded to in our review, immunotherapy is a rapidly developing area in CLL, and experimental investigations of epigenetic therapies, such as BET bromodomain inhibitors (164), are also emerging. It is therefore possible to envision a future in which the timing and choice of CLL treatment are guided by longitudinal monitoring of subclonal dynamics.

Finally, the concepts of evolutionary herding and clonal homogenization are gaining traction and may become feasible in the future world of evolution-adapted treatments. These proactive therapeutic strategies aim to maintain clonal equilibrium and reduce subclonal diversity, thereby impeding CLL progression and preventing relapse. Targeting concurrently trunk and branch lesions represents one way of achieving this, as is the simultaneous targeting of trunk lesions and any anticipated

escape mechanisms or backup pathways pertaining to the truncal target. Evolutionary herding and clonal homogenization could also be achieved by therapeutically inhibiting the genetic or epigenetic mechanisms underpinning subclonal diversification, or by targeting subclones with the highest level of epigenetic plasticity or genomic instability which are most likely to further diversify and evolve. In relation to the latter, synthetically lethal strategies that target cellular dependencies specific to the most genetically unstable CLL subclones are being investigated (165); e.g. ATR pathway targeting of *TP53*-mutant subclones (166). Single-cell technology such as ClonMapper, which facilitates subclonal tracking and integrative analysis within *in vitro* and *in vivo* CLL models (50), will be hugely invaluable to this endeavor.

In conclusion, we have made massive strides in advancing our understanding of CLL clonal evolution over the past decade. Further research effort harnessing technological innovations will undoubtedly address current knowledge gaps and unanswered questions. Moreover, clinical translation of these advances has enormous potential to revolutionize prognostication and treatment of patients with CLL, bringing us closer to the 'brave new world' of tomorrow.

AUTHOR CONTRIBUTIONS

MK and CW conceptualized the manuscript. MK drafted the manuscript. MK and CW edited and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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The Determinants of B Cell Receptor Signaling as Prototype Molecular Biomarkers of Leukemia

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Advanced genome-wide association studies (GWAS) identified several transforming mutations in susceptible loci which are recognized as valuable prognostic markers in chronic lymphocytic leukemia (CLL) and B cell lymphoma (BCL). Alongside, robust genetic manipulations facilitated the generation of preclinical mouse models to validate mutations associated with poor prognosis and refractory B cell malignancies. Taken together, these studies identified new prognostic markers that could achieve characteristics of precision biomarkers for molecular diagnosis. On the contrary, the idea of augmented B cell antigen receptor (BCR) signaling as a transforming cue has somewhat receded despite the efficacy of Btk and Syk inhibitors. Recent studies from several research groups pointed out that acquired mutations in BCR components serve as faithful biomarkers, which become important for precision diagnostics and therapy, due to their relevant role in augmented BCR signaling and CLL pathogenesis. For example, we showed that expression of a single point mutated immunoglobulin light chain (LC) recombined through the variable gene segment IGLV3-21, named IGLV3-21^{R110}, marks severe CLL cases. In this perspective, we summarize the molecular mechanisms fine-tuning B cell transformation, focusing on immunoglobulin point mutations and recurrent mutations in tumor suppressors. We present a stochastic model for gain-of-autonomous BCR signaling and subsequent neoplastic transformation. Of note, additional mutational analyses on immunoglobulin heavy chain (HC) derived from non-subset #2 CLL IGLV3-21^{R110} cases endorses our perspective. Altogether, we propose a model of malignant transformation in which the augmented BCR signaling creates a conducive platform for the appearance of transforming mutations.

Keywords: BCR signaling, CLL, biomarkers, transformation, immunoglobulin genes

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INTRODUCTION

The B cell antigen receptor (BCR) signaling is the key survival and growth promoter for both normal and malignant B cells, controlling important cell fate decisions including proliferation and differentiation (1). Depending on quality, capacity and relevance, three different types of BCR signaling were described: cell-autonomous, tonic and standard ligand-dependent signaling (**Figure 1A**). B cells are engineered to control the BCR signaling strength and type during their

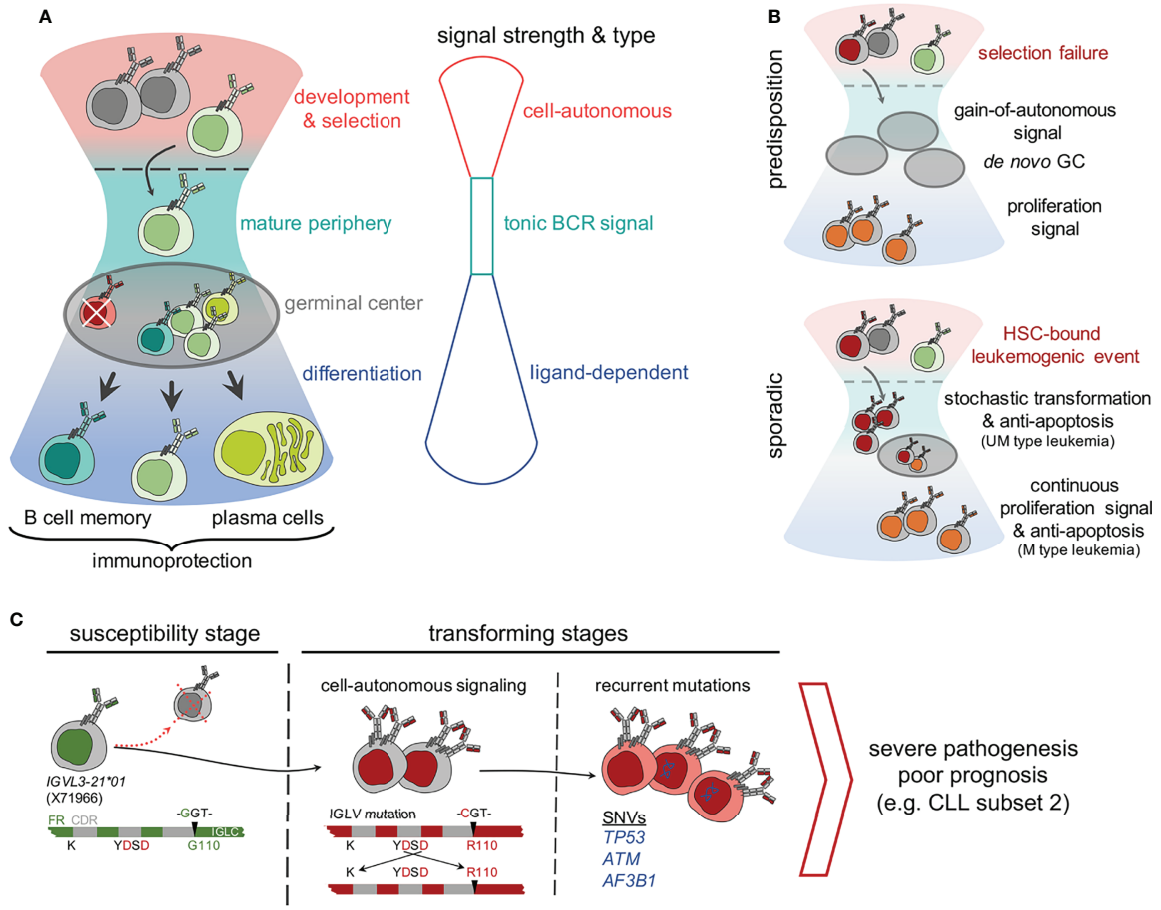

FIGURE 1 | Compounding effect of molecular determinants of BCR signaling and recurrent mutations in genetically predisposed and sporadic origin CLL. **(A)** A schematic hourglass portrays the course of B cell development, selection, peripheral maintenance, activation and differentiation regulated through tight controls and varying strength of BCR signaling. As shown, each stage represents a predominant type of BCR (or pre-BCR) signaling, which controls the outcome as follows, 1) cell-autonomous signaling at bone marrow dwelled development and selection, 2) tonic signal at the mature peripheral compartments, and 3) antigen responsiveness or ligand-dependent activation signal for memory and plasma cell differentiation during the germinal center reaction. **(B)** Mutations that supersede the balance between BCR signal strength and developmental stages are a threat for genetic predisposition of leukemia. For example, a selection failure or gain-of-autonomous signaling mutation result in persisting autonomously active B clones in the peripheral compartment that promote proliferative boosts and prime *de novo* germinal center (GC) formations. In contrast, sporadic mutations have diverse origins. For example, a leukemogenic event occurring at haemopoietic stem cells (HSC) undergoing stochastic transformation might lead to anti-apoptosis and proliferation boost. **(C)** A stochastic model of neoplastic transformation through acquisition of biomarkers on susceptible genetic background exemplified by the acquired IGLV3-21^{R110} mutated CLL pathogenesis. As shown, individuals carrying the allele *IGLV3-21*01* are predisposed to gain-of-autonomous BCR signaling through homotypic BCR : BCR interaction stabilized by IGLV3-21^{R110}. Although mostly eliminated and undetected in the peripheral blood of a healthy *IGLV3-21*01* carrier, a single point G→C mutation, possibly induced by activation-induced cytidine deaminase (AICDA) activity, converts a Glycine (G) to Arginine at 110th residue (R110) and initiates the neoplastic transformation. The gain-of-autonomous BCR signaling and enduring survival advocate the persistent single nucleotide variations (SNV's) stochastically in *TP53*, *ATM*, splicing factor *SF3B1* and in epigenetic modifiers to culminate the leukemogenesis and develop severe CLL.

development step-by-step (1–3). First, the autoreactive clones are self-eliminated by altered BCR activation threshold in the bone marrow compartment. Thereafter, tonic BCR signaling controls cell survival and proliferation, relying on the crosstalks integrating microenvironmental signals and cytoskeletal remodeling (1, 4). Finally, the antigen stimulated BCR signal promotes clonal expansion and differentiation during the germinal center (GC) reaction. A dysregulation of BCR signaling at any of the aforesaid levels, such as a gain-of-function mutation in signaling components, results in altered B cell survival, most often allowing escape from self-elimination process and resulting in primary immunodeficiencies, autoimmune diseases and B cell malignancies (**Figure 1B**) (5, 6). In particular, mutations or differential membrane organization of receptors which lead to the constitutive activation of the BCR are mainly associated with B cell malignancies, such as CLL and activated B cell-like diffuse large BCL (ABC DLBCL) (1, 2, 7, 8). Although sporadic and non-familial pathogenesis predominates in B cell neoplastic diseases, recent sequencing approaches and GWAS studies

provided evidence of an inherited predisposition by identifying risk-associated loci in CLL and DLBCL (9–13). Recurrent mutations are mostly located in the non-coding regions of the genome, affecting active promoters or enhancers (12, 14). Very often, these recurrent genomic aberrations are functionally associated to genomic-instability, anti-apoptosis, and abnormal lymphoproliferation signaling networks (**Figure 1B**) (9, 11, 15, 16). Notably, the dysregulation of the Bcl-2 family of anti-apoptotic proteins, including Bcl-2, Bcl-xL and Mcl-1, permits escape from intrinsic and extrinsic apoptosis (17). Targeting this anti-apoptosis pathway using Bcl-2 inhibitors such as venetoclax, is a faithful alternative for treatment of resistant and refractory CLL as well as other B cell malignancies (18, 19). Along with identifying reliable signature mutations, continuous efforts have been made to analyze the leukemogenic potential of these sporadic mutations in genetically modified animals (20–23). For example, the *TP53* and *ATM* mutations worsen the CLL prognosis and B lineage specific deletions of these genes induce early disease onset and aggressive leukemic features in Eμ-TCL1 mice model (22, 23). Similarly, gain-of-function mutation in the splicing factor SF3B, when combined with *ATM* deletion, presents CLL-like B cells in mouse models (23). These results strongly imply the role of prognostically adverse mutations and chromosomal anomalies in CLL pathogenesis but are unable to define the role of BCR signaling in the transforming process.

Importantly, CLL prognosis is still strongly relying on the BCR and associated features, in particular on the mutational status, rearranged immunoglobulin genes and complementary determining region 3 (CDR3) (24, 25). These molecular determinants are potential functionally relevant biomarkers. Here, we review the current understanding of BCR associated biomarker exemplifying a novel IGL mutation, termed IGLV3-21^{R110} (where a glycine is replaced by arginine at position 110), in severe pathogenesis including stereotypic CLL subset #2 (26–28). Additionally, we show that the gain-of-autonomous BCR signaling requires point mutations in both IGLV and IGHV genes derived from a non-subset #2 CLL IGLV3-21^{R110} case. Thus, we postulate that the gain-of-autonomous BCR signaling and a convoluted signaling crosstalk might favor the recurrent mutations, thus contributing to genomic-instability and anti-apoptosis. Taken together, we endorse the use of BCR associated molecular biomarkers as a novel tool for an easy and comprehensive characterization of CLL.

IDENTIFICATION OF IGLV3-21^{R110} AS A MODEL OF MOLECULAR BIOMARKER FOR RAPID PROGNOSIS OF SEVERE CLL

The clinical course of CLL varies widely, ranging from patients with stable, asymptomatic disease without need of therapeutic intervention to patients suffering from a progressive disease requiring immediate treatment after diagnosis (15, 24, 25, 29). CLL diagnosis is established by routine laboratory tests such as blood counts, blood smear, immunophenotyping, and assessment of the IGHV mutational status or chromatin aberration by FISH (15, 24). Notably, the IGHV mutational status is a first-line molecular determinant or biomarker for prognosis and classification of CLL with highly significant differences in clinical behavior (30, 31). Notably, a biomarker indicates the disease states or medical signs of patients. The first working definition of biomarker from the U.S. National Institutes of Health (NIH) and the World Health Organization (WHO) delineated the objective characteristics, i.e., accurate and reproducible indicators of pathogenic processes or responses to a therapeutic intervention (32, 33). Moreover, there are at least two subclasses of biomarkers, namely prognostic and predictive, based on interaction and differential response to treatment (34, 35). In the last twenty years, this broad objective definition was extended, diversified, and subclassified by several authors (36). Nevertheless, the improvements in precision medicine compel the search for new biomarkers. More recently, we focus on the mechanism-centric approaches for functionally meaningful biomarker selection (36). Unfortunately, identification of a functionally relevant biomarker, specifically for B cell malignancies, requires multifaceted validations, including the generation of mouse models, which makes it meticulous and challenging (20, 21, 37).

For many years, classical staging systems of Rai and Binet defined three major prognostic groups based on clinical parameters derived from physical examination and the aforementioned standard laboratory tests. However, with recent developments in CLL therapy and the sequencing techniques, a number of novel potential prognostic markers has been identified. Thus, the classical staging systems have become insufficient to distinguish prognostic groups and predict early disease progression (24, 38). To overcome these limits, a novel comprehensive prognostic score, the CLL international prognostic index (CLL-IPI) was created. The CLL-IPI combines biochemical and clinical parameters (age, clinical staging, serum ß-2 microglobulin) with cellular and genetic features such as *TP53* and IGHV mutational status, to provide a more advanced risk stratification (24, 39). Of the five prognostic factors used by CLL-IPI, *TP53* and IGHV mutation status have been described as particularly important in determining patient outcomes (15, 24, 39). In parallel, prognostic groups of stereotyped CLL subsets have also been defined according to the distinctive amino acid pattern within the IGHV CDR3 region (25, 40, 41). Nevertheless, not all CLL cases belong to stereotyped subsets, with roughly 60% of the CLL BCRs remaining unclassified (25, 41). Notably, neither the CLL-IPI grading system nor the subset classification considers the mutations in immunoglobulin light chain (LC), which are known to have direct impact on BCR signaling and prognostic relevance (26–28, 42). Intriguingly, most stereotypic BCR consist of an unique pair of HC and LC recombined through specific V(D)J segments (41, 43). Yet, the role of the LC is frequently underestimated. This is best exemplified by the light chain derived from variable gene segment IGLV3-21, associated with poor prognosis, independently of IGHV mutational status and stereotype (27, 28).

In particular, we identified the single point mutated IGLV3-21^{R110} as a novel molecular biomarker directly linked to BCR signaling and survival of CLL cells. Notably, crystallographic

analyses identified the crucial residues of IGLV3-21^{R110} required for homotypic BCR-BCR interaction leading to autonomous signaling in CLL subset #2 (**Figure 1C**) (27, 44). Critical residues include a non-synonymous mutation in the IGLJ segment of the IGLV3-21 derived LC, introducing an indispensable arginin at the position 110 (R110). Of note, IGLV3-21 is the only LC variable gene segment which encodes the required D residues within the CDR2 region, for the homotypic interaction (**Figure 1C**). Among the three major IMGT annotated alleles of IGLV3-21 locus, only allele *IGLV3-21*01* encodes the prerequisite K16 and YDSD motif in CDR2. Notably, a recent update in IMGT reference directory (release 202018-4) includes *IGLV3-21*04*, which also fulfills these requirements. Thus, the allelic variants *IGLV3-21*01* or *IGLV3-21*04* have intrinsic potential for the generation of autonomously active BCRs. Especially, B cells expressing the autonomously signaling IGLV3-21^{R110} variant are counter-selected in healthy donors (HDs) (27). Although IGLV3-21 is commonly found in association with its heavy chain counterpart IGHV3-21 in stereotyped subset #2 CLL, recent studies confirmed the prognostic value of IGLV3-21^{R110} beyond the IGHV identity and epigenetic classifications of CLL (26, 27, 42, 44). This prompted the idea of a novel subgroup of aggressive CLL expressing the mutated IGLV3-21^{R110}, demonstrating the importance of the light chain as an inclusive criteria for CLL prognosis (26, 27). Notably, the recent finding of higher-order correlation between several major and minor CLL subsets are in line with the IGLV3-21^{R110} based broader classification which is best exemplified by CLL subset #2 and #169 (27, 41). Both stereotyped subsets employ the IGLV3-21^{R110} light chain and display structural and immunogenetic similarities with severe clinical courses suggesting a common antigen selection process in their pathogenesis (45). Alternatively, the homotypic BCR-BCR interaction caused by IGLV3-21^{R110} and subsequent autonomous signaling promote the clonal expansion (27). Given the important role of IGLV3-21^{R110}, we developed two monoclonal antibodies, one to detect the expression of the functional biomarker IGLV3-21^{R110} and one to distinguish it from the unmutated founder LC. Indeed, the monoclonal anti-wild-type IGLV3-21 antibody can recognize the expression of the susceptible allele *IGLV3-21*01* in HDs which is regarded as a risk index for CLL development. Altogether, the discovery of IGLV3-21^{R110} in severe CLL cases, the related mechanism of B cell activation and the development of antibody-based immunophenotyping tools established IGLV3-21^{R110} as functionally linked biomarker for CLL pathogenesis.

THE STOCHASTIC PROCESS OF NEOPLASTIC B CELL TRANSFORMATION AND GAIN-OF-AUTONOMOUS SIGNALING THROUGH IMMUNOGLOBULIN MUTATIONS

The process of neoplastic transformation in CLL and other B cell malignancies is controversial. Recent evidence proved the existence of susceptible alleles and mutations which might act as the main drivers of neoplastic transformation (46, 47). Conversely, clonal tracking of leukemic cells indicate that mutations arise at different stages prior and during the course of the disease leading to different cell fates depending on the affected genes (16, 48, 49). Moreover, clonal diversity analyses of IGHV-IGHD-IGHJ sequences revealed the continuous occurrence of mutations regardless of the initial mutation load in the parental (clinically dominant) leukemic clone (50). In short, the rate of intraclonal diversification is similar in UM- and M-CLL. Although this particular model suggests an AID-independent, non-canonical mode of somatic hypermutation, we and others found AID overexpression in M-CLL cases and in IGLV3-21^{R110} cases (27, 51, 52). The off-target effect of such non-canonical SHM results in non-IG genomic mutations (53, 54). When combined with the strength of BCR and other survival signals, the newly induced non-IG genomic mutations persist and clonally propagate. Alternatively, genomic mutations themselves deliver survival advantage in combination with structurally altered FRs of mutated BCRs allowing sustained signaling. In this context, we demonstrate that the survival signal is sustained by the gain-of-autonomous BCR signaling attained through point mutations in IGLV and IGHV genes (**Figure 2**) (27). Thus, we propose a stochastic transformation process, which continuously evokes new mutations that break-out the growth and survival dependencies and overrule the intrinsic DNA damage repair system (**Figure 1C**) (55, 56).

As described before, IGLV3-21^{R110} is originally identified in stereotyped CLL subset #2, which represents a BCR isotype IgM/λ encoded by the IGHV3-21/IGLV3-21 variable gene segments displaying distinctive SHMs (44). Nevertheless, we showed that IGLV3-21^{R110} LCs are compatible with a range of mutational status and different IGHV identities including IGHV3-48 derived IgM-BCRs (26, 27). Notably, stereotyped CLL subset #2 has eight related immunogenetic subsets, defined as satellite subsets, with the most frequent being CLL subset #169 (41). Intriguingly, multiple features of CLL subset #2, including IGHV mutational status, CDR3 length and mutated IGLV3-21^{R110} association, are adopted in the major satellite subset #169, e.g., case P6540 (45). Remarkably, P6540 derived IGHV sequence annotates to mutated IGHV3-48*02 among 4 major IMGT IGHV3-48 alleles (**Figure 2A**). Apart from the acquired LC mutation resulting in IGLV3-21^{R110}, the IGHV sequence introduces three critical acidic D residues in each of CDR1, CDR2 and framework region 3 (FR3) regions. Considering P6540 as a model, we examined the combined effect of IGHV and IGLV mutations on autonomous BCR signaling. Using a bifluorescent expression system, the original P6540 derived IgM-BCR and its IGHV mutants were expressed and analyzed for Ca^{2+} influx in triple knockout (TKO) cell system, as described before (57). Interestingly, complete reversion of the IGHV3-48 segment to its germline version ceases the autonomous signaling, despite the use of the IGLV3-21^{R110} light chain (**Figures 2B, C**). Similar result is found in double mutant D35/53S replacing each of CDR1 and CDR2-bound aspartic acid (D) residues with serine (S). Therefore, the acquired point mutations in the CDR1 and CDR2 regions of the IGHV3-48 segment are crucial for the autonomous signaling of

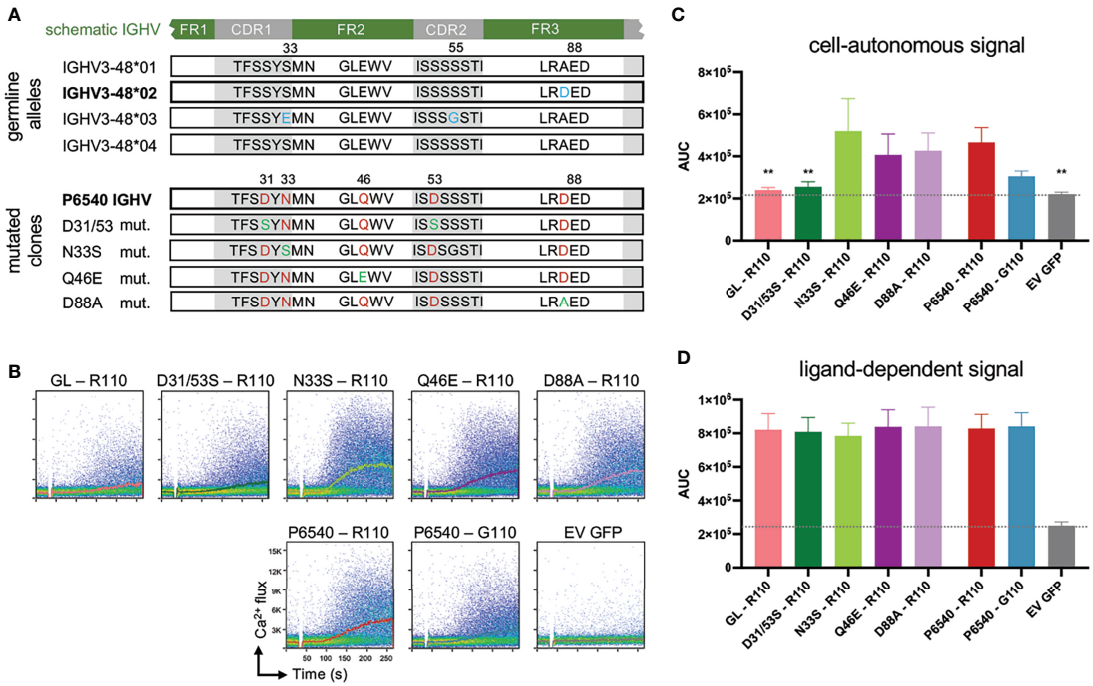

FIGURE 2 | Specific IGHV associated point mutations of autonomous active BCR affect cell-autonomous signal. **(A)** In the upper part sequence alignment of 4 identified germline alleles of IGHV3-48 are shown, differences are marked in blue. Allele 01 and 04 are identical. In the lower part sequence alignment of CLL-patient P6540 derived IGHV is displayed, which is related to *IGHV3-48*02* allele (Mutations marked in red). Several single point-mutations were reverted to their germline version, depicted in green. **(B-D)** IGHV and IGLV sequences obtained from the CLL patient P6540 were cloned into retroviral expression vectors for human µHC and λLC and expressed in the TKO system (see **Supplementary Material and Method**). Proper BCR expression on the TKO cells was assessed *via* flow cytometry (data not shown). Germline *IGHV3-48*02* (GL) was included for comparison. IGHVs were co-expressed with autonomously active subset #2 IGLV3-21^{R110} LC variant. As control, P6540 IGHV was expressed with IGLV3-21^{G110}. Indo-1 staining was performed to analyze intracellular calcium release. In particular, cell-autonomous signaling was assessed *via* calcium mobilization after 4-Hydroxytamoxifen administration, **(B)** shown in representative dot plots with kinetics and **(C)** statistically summarized as area under the curve of the kinetic (AUC). **(D)** As control B-cell receptors were additionally stimulated with anti-LC antibody to mimic ligand-dependent signal. One-Way ANOVA (Bonferroni Correction), significance of mean to mean of P6540 R110 is shown, (**p < 0,01) N = 5.

the P6540 BCR (**Figures 2B, C**), but irrelevant for ligand-dependent signaling (**Figure 2D**).

Interestingly, both subset #2 and its satellite subset #169, as IGLV3-21^{R110} CLL expressing cases, exhibit high frequency genomic aberrations in regulators of DNA damage response, including splicing factor *SF3B1* (41, 49). Furthermore, we found that in non-subset #169 IGHV3-48 associated cases mutations also accumulate in *TP53*, *ATM*, chromatin modifying *EP300*, and PI3K signaling associated phosphatase *PTEN* (**Figure 1C**) (27). These recurrent mutations often arise in chemotherapy resistant patients (9, 16, 22, 58). Specifically, *TP53* and *ATM* mutations are associated with poor outcome in CLL. Indeed, B lineage specific deletion of *TP53* and *ATM* in Eμ-TCL-1 mice model results in earlier disease onset and more aggressive leukemic features compared to wild-type littermates (20–23). Therefore, recurrent mutations rejuvenate the malignant growth and contribute to the neoplastic transformation but may not resemble the primary transforming factor. On the contrary, B cell specific double transgenic mice overexpressing anti-apoptotic Bcl-2 and dominant negative form Traf2 adapter develop CLL/SLL-like phenotype with restricted IGHV usage mimicking CLL BCR stereotypes (59). This model suggests a conducive role of anti-apoptosis, allowing either selection of BCR clones from an indolent pool or escape of autoreactive clones from the self-elimination process. Thus, either aberrant BCR signaling or the compounding effect of *ad hoc* signaling crosstalk are the foremost transforming factor. On top, the appearance of further recurrent mutations in genes such as *SF3B1* and *TP53* accelerate and exacerbate the disease when combined with permissive BCR signaling or other conducive features - resembling a stochastic process.

DISCUSSION

With the advent of GWAS and other NGS approaches, we witnessed the expansion of the previously used criteria for CLL prognosis and the introduction of new CLL-IPI classification (24, 25). Nevertheless, the CLL-IPI classification requires extensive genetic and molecular characterization (*TP53* and IGHV) limiting a quick assessment. In contrast, novel diagnostic tools exploiting the existence of functionally relevant

biomarkers delivers more quicker and easier assessment. To this aim, we developed monoclonal antibodies for specific detection of the point-mutated light chain IGLV3-21^{R110}, which with appropriate and extensive validation could represent an easy and quick diagnostic tool for severe CLL cases. The presence of IGLV3-21^{R110} positive B cells or the expression of unmutated IGLV3-21 from susceptible allele *IGLV3-21*01* in healthy donors (HD) is regarded as a risk index for CLL development (26–28). For early diagnosis and preemptive therapy, IGLV3-21^{R110} is a gem and we foresee future development of antibody-based direct cell-depletion or drug delivery interface. Additionally, quick immune-detection of unmutated IGLV3-21 LC expression combined with genetic analyses of IGLV3-21 allele in HD will predict the susceptibility to severe CLL.

Innovative genomic studies pointing out the existence of risk-associated loci and novel murine models of genomic alteration such as *Sf3b* mutation and *ATM* deletion, prompted the idea of a stochastic process of malignant transformation where the expression of a susceptible allele or mutations are regarded as the driver event for future development of neoplastic diseases. The existence of risk-associated loci also highlights the dynamics of familiar CLL, confirming that relatives of CLL patients have an increased risk of developing the disease (60, 61). In particular, CLL cases within the same family presented uniform BCR IG and similar genomic profiles, highlighting the correlation between germline predisposition and immunogenetic markers and pointing towards a shared mechanism of CLL development (62). Of note, recurrent unfavorable mutations are often associated with survival and anti-apoptotic pathways or with BCR signaling pathway. It is important to understand the role of these mutations as prognostic indicators, not only to improve diagnosis, but also to develop suitable preclinical models, which comprehend the heterogeneity of CLL.

So far, the majority of CLL mouse models mimic genetic aberrations or deregulated gene expression in CLL without taking into account the clonality of the BCR (63). Nevertheless, in a stochastic process of malignant transformation, cells accumulate numerous mutations which do not proliferate until the occurrence of additional events that overcome the checkpoints and drive the leukemogenesis. Thus, the generation of an inducible monoclonal CLL-mouse model to study CLL development in a stepwise manner would be of particular relevance to clarify the role of aberrant BCR signaling in the development of CLL. We propose that the use of a CLL-derived autonomous BCR, e.g. the aggressive subset #2 will help to elucidate the course of CLL. In fact, it is interesting to follow the growth of subset #2 BCR expressing B cells, preferably in inducible manner, *in-vivo* and to screen for acquired mutations. Moreover, subsequent changes in anti-apoptotic and pro-survival properties of these autonomously active B cells are of immense relevance for both indolent and aggressive outcome. Thus, an inclusion of functionally relevant prognostic parameters, even for indolent cases, can only be beneficial. Of course, further and larger independent studies must be carried out in order to validate these parameters.

Characterization of the molecular prognostic parameters and genomic landscape predict primary response but not relapse to treatment. Often, the underrated interactions between molecular cytogenetics features and the microenvironment specific survival signals mediated by direct contacts, adhesion molecules, chemokines and ligand-receptor interactions are of great prognostic value (15, 64–66). The kinases of BCR signaling pathway such as PI3K, BTK and SYK, are also involved in receptor mediated crosstalk (67). For example, *ibrutinib*, the BTK inhibitor, interferes with the homeostasis of the leukemic cells in the survival niches, thus demonstrating efficacy in high-risk CLL (22, 64, 68–70). Moreover, tissue infiltrating and colonizing ability of leukemic cells alters growth rate, metabolic dependency and proliferation of CLL cells (71). Recent studies have highlighted the importance of analyzing CLL cells in a physiologically relevant environment recreating the interactions between leukemic cells and their milieu (64). Such 3D-cell culture and bioreactor models analyze the role of the microenvironment comprehensively. This is of great resonance to characterize tumor cells in their milieu providing novel *in vitro* strategies to test new therapeutic agents and assess their effects under more *in vivo*-like conditions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

PCM designed the study, led the experiments and interpreted the results together with HJ. AN and ATL performed the experiments, wrote the MS with PCM, and all authors reviewed, commented on, and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.771669/full#supplementary-material

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"Double-Hit" Chronic Lymphocytic Leukemia, Involving the *TP53* and *MYC* Genes

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Although the 17p deletion [del(17p)] is rare in cases of treatment-naive chronic lymphocytic leukemia (CLL), its frequency is higher in refractory/relapsed CLL – particularly in patients undergoing chemo(immuno)therapy. *TP53* disruption (deletion and/or mutation) is the strongest prognostic factor for refractoriness to chemotherapy; the use of Bruton tyrosine kinase inhibitors and BCL2 inhibitors is then indicated. Rare cases of CLL can also harbor translocation or gain of the *MYC* oncogene. "Double-hit CLL" (with del(17p) and *MYC* gain) is associated with a very poor prognosis. The prognostic impact of *TP53* disruption with *MYC* aberrations in patients receiving targeted therapies must now be evaluated.

Keywords: TP53, MYC, 17p deletion, chronic lymphocytic leukemia, MYC gain, MYC translocation

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INTRODUCTION

Loss of the short arm of chromosome 17 [del(17p)] results from various chromosomal abnormalities, including deletions, translocations, isochromosomes, and ring chromosomes. All these chromosomal abnormalities lead to the loss of one copy of the *TP53* gene (located at 17p13) in patients with chronic lymphocytic leukemia (CLL), and the remaining allele is mutated in more than 90% of cases. del(17p) is often associated with a complex karyotype (three or more chromosomal abnormalities) (1). Rare CLL cases can also harbor translocation or gain of the *MYC* gene, independently or in association with del(17p) (1–3).

MYC Translocation

The *MYC* oncogene (located at 8q24) is a transcription factor involved in many biological mechanisms, including as cell cycle control, apoptosis, cell growth, and cell differentiation. The translocation t(8;14)(q24;q32) and its variants t(8;22)(q24;q11) and t(2;8)(p11;q24) are typically associated with Burkitt lymphoma; *MYC* then comes under the control of an immunoglobulin heavy chain enhancer, a lambda light chain enhancer or a kappa light chain enhancer, respectively. *MYC* also has non-immunoglobulin gene partners. These translocations can be observed in other B cell neoplasms, such as diffuse large B-cell lymphoma (DLBCL), B-prolymphocytic leukemia (B-PLL) and CLL (4, 5). The World Health Organization's classification of large B-cell lymphomas now includes a new entity called "double hit high-grade B cell lymphoma" (HGBL), in which *MYC* rearrangement is combined with a *BCL2* and/or

BCL6 rearrangement (6). This category of double- or triple-hit lymphomas only comprises translocations involving *MYC* and the two other genes; hence, lymphomas expressing *MYC* with *BCL2* and/or *BCL6* (according to immunochemical assessments) but that lack translocations are not encompassed by the definition (7).

MYC and Transformed Indolent B Cell Malignancies

MYC is often involved in transformed indolent mature B neoplasms, such as the transformations of follicular lymphoma (FL) to DLBCL and CLL to Richter syndrome (8). Transformation of FL occurs in 25-35% of cases. A very small proportion of cases of FL (<0.5%) harbor a t(*MYC*), and a progression to a HGBL double hit may occur in cases with both t(14;18) and t(*MYC*) (6). Extra copies of *MYC* can also be observed in FL but (unlike t(*MYC*)) do not appear to be associated with a risk of transformation (9). Although *MYC* translocation/activation is rare in FL, up to 75% of cases of transformed FL show a gain in MYC activity (8). With regard to DLBCL-type Richter syndrome, the MYC pathway is deregulated in about 70% of cases, and somatic structural *MYC* alterations are present in 30% of cases. *MYC* deregulation is often acquired upon transformation (10).

del(17p) and *MYC* Aberrations in B-Prolymphocytic Leukemia

MYC translocations (t(*MYC*)) are frequent in B-PLL (4). In a recent study, we found that 21 of the 34 cases (62%) of B-PLL had a t(*MYC*). Furthermore, the translocated *MYC* gene was mutated in 3 of the 10 tested cases (30%). *MYC* gain was also observed in this disease, albeit at a lower frequency (5 out of 34, 15%) than t(*MYC*). Interestingly, t(*MYC*) and *MYC* gain were mutually exclusive; t(*MYC*) was present in the major clone, and *MYC* gain was mainly subclonal. It is noteworthy that *MYC* gain was associated with a highly complex karyotype, with five or more chromosomal abnormalities. We have shown that B-PLL patients with an MYC aberration (translocation or gain) and a del(17p) had the worse prognosis. In all evaluable del(17p) B-PLL cases, the remaining *TP53* allele was mutated. However, the small sample size prevented a statistical analysis of *TP53* mutational status and *MYC* aberration. Thus the combination of *MYC* and a *TP53* aberration is associated with a very high-risk form of B-PLL (4).

del(17p) and *MYC* Aberrations in CLL

In contrast to B-PLL, translocations involving *MYC* are very infrequent (<0.5%) in CLL (2, 11). t(MYC) is often a secondary event in the course of the disease and is associated with a complex karyotype, an elevated prolymphocyte count, and an aggressive form of CLL (2). The *MYC* gene is also involved in 8q24 gain, which is detected in less than 0.5% or 3-4% of cases of CLL (using chromosome banding and microarrays analyses, respectively) (11–14). Gain of 8q can occur early in the course of CLL (15). It has been linked to a complex karyotype, a shorter overall survival time, and a shorter time to first treatment (11–14). Overall, *MYC* abnormalities – whether translocations or gains – are associated with a poor prognosis in CLL.

Harbel et al. showed that del(17p) occurred with a more than 3-fold increase in a cohort of 33 t(*MYC*) CLL compared to general CLL (3). The frequency of *MYC* gain is higher in CLL with del(17p) (ranging from 9% to 44%) (13, 14, 16–18), and we have demonstrated that the del(17p) + 8q24 gain combination (involving *TP53* and *MYC* respectively) was associated with a very poor outcome within the del(17p) CLL. The remaining *TP53* allele was mutated in 55 (92%) of the 60 evaluable del(17p) patients. The small number of cases prevented a statistical analysis of *TP53* mutational status and *MYC* gain. It should be noted that there were not t(*MYC*) cases in our del(17p) CLL series (n=195). By analogy with double-hit HGBL, we identified double-hit CLL as an aggressive form of the disease (**Figure 1**) (1, 6).

Cooperation Between *MYC* and *TP53* Defects

It has been shown that *MYC* and *TP53* defects cooperate in MYC-induced murine lymphomas. In Eμ-MYC transgenic mice, MYC activation strongly selected for surviving cells, with

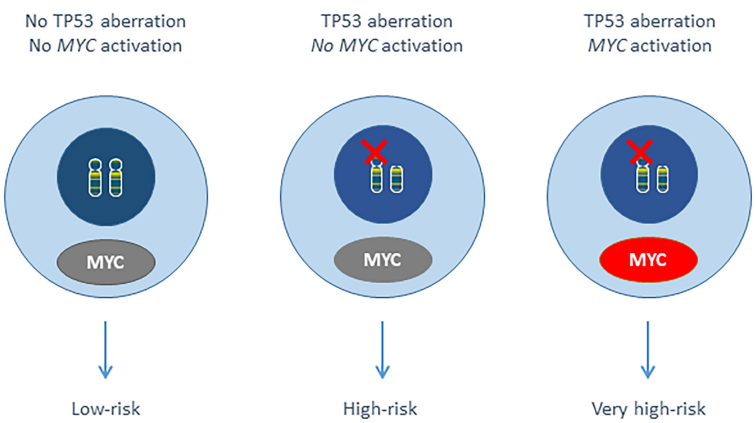

FIGURE 1 | Putative scheme of double-hit CLL.

inactivation of the ARF-Mdm2-p53 pathway (19). Thus lymphomagenesis in MYC mouse models requires additional genetic alterations - such as loss of p53 (20). In our del(17p) CLL series, *MYC* gain and del(17p) were in the same clone in 8 (62%) of the 13 evaluable cases, *MYC* was gained before the del(17p) in 3 cases (23%), and *MYC* was gained after the del(17p) in 2 cases (15%). It is noteworthy that 6 of the 13 (46%) cases carried the der(17)t(8;17) abnormality, with an unbalanced translocation between the short arm of chromosome 17 and the long arm of chromosome 8; this results in both *MYC* gain and del(17p) (1). Regarding t(*MYC*) in CLL, Put et al. described a case with t (*MYC*) before del(17p) and a case with t(*MYC*) and del(17p) in the same clone. In B-PLL, the majority of the 7 evaluable cases with t(*MYC*) and del(17p) in the literature had both abnormalities in the same clone (6/7); the last case had the del (17p) before t(*MYC*) (2, 4). There were two B-PLL cases with both *MYC* gain and del(17p): *MYC* gain and del(17p) were present in the same clone for one patient, and *MYC* was gained after del(17p) in the other patient (4). Overall, it is difficult to draw conclusions about the order of appearance of these two abnormalities, except in cases with the der(17)t(8;17). The two types of longitudinal event (*MYC* followed by *TP53* aberrations, and *TP53* followed by *MYC* aberrations) may exist.

BCR Signaling

Given that TP53 downregulates BCR signaling, and MYC represses downregulators of BCR signaling, both *TP53* and *MYC* aberrations might results in elevated FOXP1 levels. One can reasonably hypothesize that a combination of an *MYC*-activating aberration (repressing miR-150 and miR-34a) and *TP53* deletion/mutation (further repressing miR-34a) can lead to very prominent activation of FOXP1 and then the BCR. Both miR-150 and miR-34a target FOXP1, albeit at different positions (21–24).

It would be interesting to evaluate the response to Bruton tyrosine kinase inhibitors (BTKi) in patients with double-hit CLL. As MYC acts as a key downstream BCR effector, its overexpression is known to rescue the absence of BCR activity in some B cells (8, 25). Indeed, upregulation of *MYC* has been observed in ibrutinib-resistant mantle cell lymphoma cell lines (26). Treating CLL with *TP53* and *MYC* aberrations might be challenging. Intriguingly, it has been shown that in a context of chemotherapy in B-cell lymphoma with inactive p53, MYC gain can be used to over-activate cells and induce apoptosis (27).

"Double-Hit" CLL

The concept of a double hit involving the *MYC* gene in HGBL could be thus extended to other B cell malignancies in general and B-PLL and CLL in particular. When combined with del(17p) in B-PLL and CLL, *MYC* aberrations (translocations or gains) appeared to be associated with a very poor prognosis. However, only retrospective cohorts have been studied to date, and most patients were undergoing chemo(immuno)therapy. Moreover, *TP53* mutational status must be further evaluated, in order to confirm that the combination of a *TP53* mutation [and not only del(17p)] with a *MYC* aberration results in a poor prognosis. Given the low frequency of CLL cases with *MYC* aberrations, and the low proportion of cells with *MYC* aberrations (in case of a subclonal abnormality) and thus the requirement for systematic screening with a fluorescent *in situ* hybridization (FISH) probe, it will be challenging to evaluate the prognostic impact of these two abnormalities in prospective trials of targeted therapies (e.g. BTKi and BCl2 inhibitors). However, understand the mechanisms of resistance to new drugs is essential, and any aggressive abnormalities must be carefully analyzed. Although t (*MYC*) is easy to observe by karyotype, the *MYC* gain might be difficult to detect. In CLL, we recommend karyotyping and systematic FISH analysis with *TP53* and *MYC* probes prior to the initiation of each line of treatment. It is noteworthy that *MYC* and *TP53* aberrations can be present in a subclone and so might be overlooked by techniques like chromosomal microarrays, multiplex ligation-dependent probe amplification, massively parallel sequencing, and optical genome mapping. FISH is still the most sensitive technique for detecting chromosomal gains and losses. Of course, *TP53* mutation analyses should (in addition to FISH) be performed in CLL (28).

In conclusion, the results of a retrospective study showed that del(17p) and 8q gain (involving *TP53* and *MYC*, respectively) are associated with a very poor prognosis in CLL. This very high risk of double-hit CLL must now be confirmed (including the impact of *TP53* mutation status and rare translocations involving *MYC*) for the targeted therapies (e.g. BTKi and BCL2 inhibitors) now used as first-line treatments.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Impact of Low-Burden *TP53* Mutations in the Management of CLL

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In chronic lymphocytic leukemia (CLL), *TP53* abnormalities are associated with reduced survival and resistance to chemoimmunotherapy (CIT). The recommended threshold to clinically report *TP53* mutations is a matter of debate given that next-generation sequencing technologies can detect mutations with a limit of detection of approximately 1% with high confidence. However, the clinical impact of low-burden *TP53* mutations with a variant allele frequency (VAF) of less than 10% remains unclear. Longitudinal analysis before and after fludarabine based on NGS sequencing demonstrated that low-burden *TP53* mutations were present before the onset of treatment and expanded at relapse to become the predominant clone. Most studies evaluating the prognostic or predictive impact of low-burden *TP53* mutations in untreated patients show that low-burden *TP53* mutations have the same unfavorable prognostic impact as clonal defects. Moreover, studies designed to assess the predictive impact of low-burden *TP53* mutations showed that *TP53* mutations, irrespective of mutation burden, have an inferior impact on overall survival for CIT-treated patients. As low-burden and high-burden *TP53* mutations have comparable clinical impacts, redefining the VAF threshold may have important implications for the clinical management of CLL.

Keywords: CLL (Chronic Lymphocytic Leukemia), *TP53*, NGS (next-generation sequencing), clinical impact, minor clone

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INTRODUCTION

The heterogeneous clinical course of chronic lymphocytic leukemia has highlighted the need to define prognostic and predictive markers to improve the management of patients (1). On one hand, prognostic markers reflect the underlying biology and natural history of CLL and are informative for untreated patients or those requiring treatment (2, 3). On the other hand, predictive markers provide information on the likely benefits or contraindications of a given treatment. *TP53* abnormalities, namely, both deletion of the 17p chromosome and mutations at *TP53* loci, are one of the gold standards of high risk in CLL because these abnormalities indicate both an adverse prognosis and predict chemoresistance (4, 5). In the past decade, the therapeutic landscape of CLL has considerably improved, offering the possibility for patients with *TP53* defects to benefit from targeted therapy with BcR pathway or bcl2 inhibitors (6–8). Although the first-line treatment strategy may differ among countries, assessment of *TP53* status has become essential, as it serves as a contraindication for the use of chemoimmunotherapy (CIT) (9). Hence, in daily clinical practice, the use of *TP53* status as a predictive marker is mandatory for treatment decisions before the addition of each new line of treatment (10, 11).

The implementation of NGS sequencing technologies with high sensitivity has facilitated the detection of *TP53* mutations with the possibility of detecting variants with allelic fractions (VAFs) below the current conventional threshold of 10% published by the European Research Initiative on Chronic Lymphocytic Leukemia (ERIC) in 2018 (12), above which *TP53* mutations should be clinically reported. Nevertheless, the clinical and biological relevance of these minor clones is debated.

The definition of minor clones and their biological and clinical significance have been discussed in numerous studies. However, contradictory results are often reported that might be in part attributed to different cohort compositions and variable low-burden threshold definitions. To clarify the clinical role of low-burden *TP53* mutations in CLL, the prognostic and predictive impact of *TP53* mutations were analyzed in different cohorts. The results and conclusions are discussed in this review.

WHAT IS A LOW-BURDEN *TP53* MUTATION OR MINOR CLONE?

Del(17p) associated with *TP53* mutations is the most common abnormality affecting the *TP53* gene in CLL, accounting for approximately two-thirds of cases. The remaining cases either exclusively harbor *TP53* gene mutation(s) or rarely a 17p deletion. Moreover, *TP53* mutation can be accompanied by the mutation of the second allele or a copy number neutral loss of heterozygosity (13).

Historically, *TP53* abnormalities were first analyzed by conventional karyotyping combined with Fluorescence *In Situ* Hybridization (FISH), which allowed the detection of cells carrying a deletion of chromosome 17p13.1 (TP53) with a sensitivity of >5% positive cells (14). Despite a relatively good sensitivity of detection, cytogenetic techniques failed to detect approximately 30–40% of patients carrying only mutations in the gene. Later, *TP53* mutation screening relied on Sanger sequencing covering exons 4 to 9 of the gene with a sensitivity of approximately 10–20%. Hence, combining FISH analysis and sequencing substantially improved the detection of *TP53* aberrations. The advent of NGS technologies next provided the opportunity to reduce the threshold of detection of *TP53* mutations and to deeply examine the clonal heterogeneity of CLL. In a retrospective analysis of newly diagnosed patient samples, NGS sequencing could detect low-burden *TP53* mutations previously identified as unmutated by Sanger sequencing due to their low abundance in the tumor cell population (15). Altogether, Sanger sequencing led to misclassification of approximately 6% of newly diagnosed and untreated patients harboring low-burden *TP53* mutations with a VAF ranging from 0.3 to 11% (15–19). Of note, a fraction of patients harbored low-burden mutations associated with high-burden mutations, revealing the intratumoral heterogeneity of these mutations and the complexity of the *TP53* clonal architecture.

The definition of minor clones often relies on the VAF threshold used to detect mutant alleles by Sanger sequencing, which is typically approximately 10–12%. This conventional threshold corresponds to the current recommendations published by ERIC in 2018, above which *TP53* mutations should be clinically reported. Mutations with VAFs below the threshold are considered low allele frequency, whereas VAFs above the threshold are of a high allele frequency. This recommendation is still currently applied due to technical difficulties in detecting low-burden mutations. However, with the wide generalization and feasibility of NGS sequencing on a routine basis, the threshold to report *TP53* mutations and hence to define minor clones is debated.

Indeed, below this arbitrary threshold of 10%, a wide range of *TP53* variants can be detected by NGS sequencing with high confidence until reaching a limit of detection as low as 0.3% VAF (corresponding to three mutant alleles in a background of 1,000 wild-type alleles) while respecting specific procedures and quality criteria. First, CLL lymphocyte population purity greater than 80% reduces the possibility of dilution in nontumoral DNA that could underestimate a very low-burden mutation. Second, sufficient DNA corresponding to >6,000 diploid genomes and a third high target read depth is required to detect a very low-burden mutation with VAF<1% (20). Finally, robust bioinformatic workflows were developed to call true variants distinguished from background error noise. However, despite the very high confidence of *TP53* variant detection by NGS sequencing, the limit of detection of these ultrasensitive technologies needs to be evaluated to distinguish true *TP53* variants from background sequencing noise to avoid misdiagnosing *TP53* unmutated patients as mutated. The sequencing background depends on sequencing technologies and library preparation, which differ in capture and amplicon-based processes (21, 22).

CLONAL EVOLUTION OF LOW-BURDEN *TP53* MUTATION AFTER CHEMOTHERAPY

While *TP53* abnormalities account for approximately 10% of naïve-treatment patients, these abnormalities are found in greater than 40% of patients with fludarabine-refractory CLL, which highlights the phenomenon of clonal evolution of *TP53* mutation induced by chemotherapy (13). Despite the current recommendations that consider <10% of minor clones to be of uncertain significance, accumulating evidence based on longitudinal studies argues for the clinical relevance to report *TP53* minor clones (15, 18, 20, 23–25). NGS sequencing of serial samples before and after treatment has allowed characterization of the dynamics of the minor clones under treatment and demonstrated their biological and clinical relevance.

Longitudinal retrospective studies based on NGS sequencing of fludarabine relapsed/refractory *TP53* mutated patient samples showed that low-burden *TP53* mutations were detected early in

the disease course and before the onset of chemotherapy. These pre-treatment samples were initially screened using Sanger sequencing, and mutations were missed due to the lack of sensitivity of the technique. Interestingly, longitudinal analysis indicated that the acquisition of *TP53* mutations clearly preceded karyotype evolution, which highlights the genetic instability related to the presence of a *TP53* mutation and its likely role in the development of a complex karyotype (24). It is widely accepted that chemotherapy plays a key role in driving the selection of clones carrying *TP53* mutations (26). Fludarabine is a purine analog that inhibits DNA synthesis in tumor cells. In the case of defects in the *TP53* pathway, CLL cells lose their capacity to stop cell division and to trigger apoptosis in response to chemotherapy. As a result, the mutation induces a fitness effect by conferring a growth and survival advantage to the low-burden *TP53* mutation, which expands under the selection pressure of chemotherapy (27). The fact that a given low-burden *TP53* variant detected at the time of treatment initiation is found at relapse after a fludarabine-based regimen clearly demonstrates that these minor clones are not sequencing artifacts and highlights the need to redefine this threshold for optimal clinical practice.

Finally, relative stability in the *TP53* variant allele frequency is observed in some patients as long as they are not treated with chemotherapy. This notion is particularly true for *IGHV*-mutated patients, which have a more indolent disease course and can show the persistence of the mutated clone for years (28–30). On the other hand, given the natural clonal evolution of the disease with time, *TP53* minor clones can also be acquired during the disease course, independent of any pressure of selection induced by chemotherapy. This finding justifies early and iterative screening for *TP53* abnormalities during follow-up and before each new line of treatment with a sensitive sequencing technique.

IMPACT OF TARGETED AGENTS ON LOW-BURDEN *TP53* MUTATIONS

Given that *TP53*-mutated patients can benefit from targeted therapies with improved remission duration, there is a need to evaluate the impact of these therapies on the evolution of the *TP53*-mutated clone. Data on the clonal evolution of low-burden *TP53* mutations upon targeted treatment are limited (23, 31). Malcikova et al. showed that upon the use of BcR or bcl2 inhibitors as a second line of treatment, the percentage of VAF in the residual lymphocytosis remains stable, which reflects the efficacy of these treatments on the mutated clones (23). Indeed, BcR and bcl2 inhibitors target the BcR signaling pathway and apoptosis, respectively, and therefore overcome the p53 pathway. However, the persistence of *TP53*-mutated clones after treatment shows the failure to eradicate the disease (32). In some progressive patients treated with targeted therapies, the major *TP53* mutated clone becomes minor. However, in these cases, mutations that confer resistance to ibrutinib (i.e., *BTK* mutation) or Venetoclax (i.e., *BCL2* mutations) are frequently found. In another longitudinal study including treatment-naïve and relapsed/refractory patients treated with BcR inhibitors, the dynamics of *TP53* mutated clones were complex. Most of the *TP53* mutations decreased or were undetectable, but one-third remained stable with no differences noted between low- or high-VAF clones. A small proportion of *TP53* mutations increased. After a prolonged follow-up of greater than 2 years, the overall stability of low-burden *TP53* mutations was noted, supporting the notion of the lack of specific positive selection of *TP53* mutations under conditions of ibrutinib treatment (31). Nevertheless, all these observations need to be confirmed in a cohort of patients treated with novel agents in the frontline setting. To date, this has not been explored within clinical studies, and data are preliminary, especially for bcl2 inhibitors.

LOW- AND HIGH-BURDEN *TP53* MUTATIONS HAVE THE SAME UNFAVORABLE PROGNOSTIC IMPACT

In most studies focusing on the clinical impact of *TP53* minor clones, an arbitrary threshold of 10–12% VAF was chosen to define patients with low- or high-burden *TP53*-mutated clones. Most studies conducted in untreated patients (15, 18, 20) showed that low-burden *TP53* mutations significantly reduced the OS compared to cases with unmutated *TP53* genes. Moreover, the impact on OS was the same for patients harboring minor clones or high-burden *TP53* mutations (**Table 1**). The clinical consequence of *TP53* mutations was similar when patients with low VAF were stratified into subclasses <1%, between 1% and 5% or 5% and 10%. Shorter OS was also confirmed when separately considering patients with single or multiple mutations classified as high VAF or low VAF (15, 20).

The presence of del(17p) and/or *TP53* mutations are parameters of the CLL-International Prognostic Index (CLL-IPI), which combines five parameters (age, clinical stage, *TP53*, *IGHV* mutational status, serum β2-microglobulin) to predict survival and time-to-first-treatment (TTFT) in CLL patients. However, the value of the VAF threshold used to consider *TP53* mutated considerably impacted this score. Indeed, revisited CLL-IPI combining both high- and low-VAF *TP53* mutations significantly better discriminated high-risk patients than standard CLL-IPI, which exclusively considered high-VAF *TP53* mutations (20, 23, 35). Therefore, minor clones should be considered to refine prognostication models.

Most studies evaluating the predictive impact of *TP53* mutations showed significantly reduced survival in CIT-treated patients harboring either low- or high-burden *TP53* mutations (15, 20, 23, 33). Clonal expansion is likely the main factor contributing to the inferior survival of CIT-treated patients with low-burden *TP53* mutations, as demonstrated by longitudinal studies comparing pre- and post-treatment samples showing that the mutation burden consistently increases at relapse (18, 20, 23). Furthermore, the risk of *TP53*

TABLE 1 | Summary of the prognostic and predictive impact of *TP53* mutations evaluated in 6 studies in CLL.

Cohort		Total patients/ patients treated during follow up	*TP53* mutated patients		OS (months)			Low burden threshold	Prognostic impact of low burden	Predictive impact of low burden
			High burden *TP53* mutations	Solely low burden *TP53* mutations	*TP53* wild type	*TP53* Mutated high burden	*TP53* Mutated low burden			
Untreated patients										
	Rossi 2014 (15)	309	28	15	75.1%*	34.6%*	46.3%	0.3–10%	p 0.0042	
	Nadeu 2016 (18)	405/208	28	16	82%*	54%*	64%*	0.3–12%	p 0.011	
	Bomben 2021 (20)	1,220	92	76	NR	60	80	0.4–10%	P <0.0001	
	Brieghel 2019 (33)	290/97	20	25	NR	60	NR	0.2–10%	NS	
At the time of treatment										
First line (CIT)	Rossi 2014 (15)	53	11	6	54.3%*	12.1%*	0%*	0.3–10%		p 0.017
	Bomben 2021 (20)	544	61	42	NR	47	62	0.4–10%		p <0.0001
	Brieghel 2019 (33)	61	7	10	72	26	14	0.2–10%		p 0.002
	Blakemore 2020 (34)	499	43	16	73	26.1	50.5	<12%		NS
	Malcikova 2021 (23)	511	59	82	68.4	21.6	40.8**	0.1–10%		p 0.0004
2nd line Targeted Treatment	Malcikova 2021 (23)	159	57	48	51.6	36	NR	0.1–10%		NS

**5 year OS.*

***not receiving targeted Treatment.*

NS, non-significative.

The overall survival (OS) in subgroups of patients with TP53 wild type, low-burden, or high-burden TP53 mutations is indicated in months, or the 5 years OS rate is reported. P value corresponds to a comparison of OS of TP53 low-burden mutated patients vs TP53 wild-type patients. NR, not reached; NS, not significant.*

mutation expansion beyond the current threshold of 10% in the first relapse was significantly higher for patients carrying mutations with VAF >1% than for those with VAF <1% (23). Additionally, very low clonal abundance cell populations (as low as 0.3%) are clinically relevant, as they are resistant to CIT, are positively selected and may become the dominant leukemic population at the time of relapse. Blakemore et al.'s (34) LRF CLL4 clinical trial could not demonstrate inferior survival associated with cases harboring <12% VAF *TP53* mutations but rather an intermediate-risk group, revealing heterogeneity among studies based on the patients included, the duration of follow-up, and the thresholds used.

Therefore, these observations strengthen the need to redefine the clinically relevant threshold of VAF, which better discriminates *TP53*-mutated patients who will benefit from a targeted therapy (15, 26, 36–38).

The literature on the impact of *TP53* minor clones on targeted therapies is less abundant. One study (23) showed that in a cohort of relapsed/refractory patients entering treatment with BcR and bcl2 inhibitors, OS in response to targeted treatment in *TP53*-mutated patients did not significantly differ from that of *TP53* wild type patients irrespective of VAF.

DISCUSSION

The main focus of this review was to demonstrate that low-burden *TP53* mutations have an impact on CLL survival. This review analyzing different retrospective and prospective CLL cohorts highlights the need to detect *TP53* mutations with highly sensitive NGS technology in a routine setting due to the clonal expansion of minor clones after CIT. NGS sequencing technology can detect low-burden *TP53* mutations that are as low as 0.3% over the background noise using specific bioinformatics pipelines. The clinical relevance of these low-burden mutations is evaluated as prognostic or predictive markers, and most of the studies identified that cases bearing low-burden *TP53* mutations (VAF <10%) experienced shorter OS similarly to cases with high-burden *TP53* mutations (VAF >10%) compared to patients harboring wild type *TP53*. These concordant observations highlight the need to redefine the threshold used to identify *TP53*-mutated cases, as these findings may have important implications in the setting of CLL treatment.

Low-VAF mutations showed the same molecular characteristics and distribution as high-VAF mutations, confirming that they are

not sequencing artifacts. Moreover, the pathogenicity of these mutations was confirmed using different databases (IARC *TP53*, UMD database) (39, 40). Accordingly, in longitudinal studies, sequential samples from CIT-treated patients showed that minor clones were positively selected and became dominant at relapse, confirming that these low-burden mutations that initially occur in a minority of cells are true mutations that expand under selective pressure (26).

Focusing on studies designed to assess overall survival (OS) between cases harboring the wild-type *TP53* gene versus cases with low-burden *TP53* variant (15, 18, 20, 35), the frequencies of *TP53* mutation ranged from 10.6 to 27.5%, of which 26.8 to 45.2% cases exclusively harbored low-burden *TP53* mutations depending on the threshold used to discriminate between low- and high-VAF *TP53* mutations. Blakemore et al. failed to demonstrate a clinical impact of low-burden *TP53* mutations but identified an intermediate-risk group. These findings were probably due to the choice of an arbitrary threshold of 12% for discriminating low- and high-burden *TP53* mutations and a minimum VAF >1% (34).

The impact of *TP53* mutations on OS also depended on the composition of the cohort with different proportions of patients carrying mutated *IGHV* or 17p deletion or variable times to diagnosis. Indeed, newly diagnosed patients often harbor mutated *IGHV*, and *TP53* abnormalities may not have a negative impact on the indolent disease course (23, 28–30, 35). These observations suggest that *TP53* mutation testing should be performed exclusively before treatment. Conversely, Brieghel et al. demonstrated that neither high nor low burden *TP53* mutations at the time of CLL diagnosis influenced OS independently (35). Surprisingly, patients with 17p deletion had an inferior outcome, and only the subgroup of patients with high-burden *TP53* mutations and unmutated *IGHV* demonstrated an inferior OS. This discrepancy may be explained by the composition of the cohort and the more indolent nature of the disease for the patients included. The frequency of 17p deletion was only 2.4%, whereas *TP53* mutations without 17p deletions were more frequent (10.7%). Furthermore, the proportion of newly diagnosed *TP53*-mutated patients with unmutated *IGHV* genes was low (32%) as compared to 57% (18) and 35.5% (15).

Given that NGS technology can detect low-burden *TP53* mutations at levels as low as 0.3%, should this limit of detection be used as a threshold to identified patients with *TP53* mutations? One study further stratified patients based on a 5% VAF threshold and observed shortened survival only for mutations with 5–10% VAF but not for mutations with 1–5% VAF. Interestingly, the subgroup carrying mutations with <1% VAF showed significantly shortened OS. In addition, the risk of a rapid expansion of the clone to greater than 10% in the first relapse after CIT treatment was higher for patients carrying mutations with >1% VAF than for those with <1% VAF (23). These results suggest that a >1% VAF threshold could be clinically relevant.

Further standardization (41) and bioinformatics development (42) may be necessary to identify the background noise at each position of the *TP53* gene to validate very low-burden mutations (as low as 0.3%).

Hence, there is a need to harmonize the methodologies used to detect minor clones and minimal requirements for the standardized assessment of such clones. An ERIC (European research initiative on CLL http://www.ericll.org/) multicenter study on the prognostic and predictive impact of low-burden *TP53* mutations is in progress with three phases: 1) compare results among laboratories performing NGS analysis of *TP53* mutations in CLL with a detection limit of ≤1% VAF, 2) assess the prognostic and predictive impact of low-VAF *TP53* variants in patients entering first-line treatment, and 3) re-evaluate the cut-off for reporting of *TP53* variants in CLL and, if needed, to update recommendations on minor *TP53* variant detection, validation, and reporting. Forty-one laboratories participated in the 1st phase of the study and analyzed the same samples with low-VAF *TP53* mutations. The collected results show that the 2% VAF cut-off could be reproducibly applied for the planned multicenter study on the clinical significance of low-VAF *TP53* variants (43). The collection of clinical and biological data from a consecutive cohort of patients, namely, both wild-type and mutated *TP53* CLL entering 1st-line therapy, is currently in progress to re-evaluate the cut-off for reporting *TP53* variants.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Landscape of TP53 Alterations in Chronic Lymphocytic Leukemia *via* Data Mining Mutation Databases

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Locus-specific databases are invaluable tools for both basic and clinical research. The extensive information they contain is gathered from the literature and manually curated by experts. Cancer genome sequencing projects generate an immense amount of data, which are stored directly in large repositories (cancer genome databases). The presence of a *TP53* defect (17p deletion and/or *TP53* mutations) is an independent prognostic factor in chronic lymphocytic leukemia (CLL) and *TP53* status analysis has been adopted in routine clinical practice. For that reason, *TP53* mutation databases have become essential for the validation of the plethora of *TP53* variants detected in tumor samples. *TP53* profiles in CLL are characterized by a great number of subclonal *TP53* mutations with low variant allelic frequencies and the presence of multiple minor subclones harboring different *TP53* mutations. In this review, we describe the various characteristics of the multiple levels of heterogeneity of *TP53* variants in CLL through the analysis of *TP53* mutation databases and the utility of their diagnosis in the clinic.

Keywords: mutation database, TP53 mutation, chronic lymphocytic leukemia, variant classification guidelines, genetic analysis model

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INTRODUCTION

In 1956, Ingram used protein sequencing to provide the first demonstration of a severe disease (human sickle-cell anemia in that work) resulting from a single amino acid substitution (1). Since then, it has been largely demonstrated that gene mutations are the basis for most genetic diseases. The development of DNA sequencing and molecular cloning technologies in the late 1970s contributed greatly to the identification of genes involved in both monogenic and polygenic disorders, including complex diseases like cancer (2). The alterations occurring in those genes are numerous and variable in nature, ranging from point mutations to large deletions or translocations. Moreover, the task of reporting, storing, classifying and analyzing them has been a major challenge (3). To provide a pertinent response to this latter, locus-specific databases (LSDBs) have been developed (**Figure 1**). Although intended for single genes, LSDBs do offer great accuracy as they are curated manually by experts in the field (4, 5). They also provide information that can be used for large-scale analyses and often include structural, functional or evolutionary data (6).

For constitutional mutations associated with a genetic syndrome, several LSDBs also include phenotypic data useful for the study of genotype-phenotype correlation (7).

Genomic studies of tumor samples in the pre-genomic era were focused either on a small number of genes analyzed in large patient cohorts, or on a more significant number of genes but in only a few tumors. Indeed, large-scale analyses combining a multitude of genes and tumors represented a Herculean and costly task. The development of high-throughput methodologies capable of sequencing an entire genome in only a few days (next generation sequencing, NGS) has radically changed the entire field of cancer biology. In the present post-genomic era, whole genome sequencing in a multitude of tumors can be performed in a matter of days. The International Cancer Genome Consortium (ICGC, http://dcc.icgc.org/), the Cancer Genome Atlas Project (TCGA, http://cancergenome.nih.gov/) and the Sanger Institute (http://www.sanger.ac.uk/) have undertaken large-scale cancer genome analyses in different types and subtypes of cancer. That work has led to the creation of large data repositories (cancer genome databases, CGDs) freely available to the entire scientific community (8–10). Both LSDBs and CGDs can be considered as central hubs linking clinical and basic research (**Figure 1**). They all make important contributions to our knowledge of the intricate pathways regulating cell fate, and our ability to identify new clinical biomarkers and develop novel therapeutic molecules.

TP53 mutation databases are the perfect example of the successful use of these compilations of cancer associated alterations. Indeed, the *TP53* suppressor gene is the most frequently mutated gene in human cancer and analyses of these alterations have fueled basic and clinical research, leading in turn to a number of novel therapeutics currently in phase III trials (11).

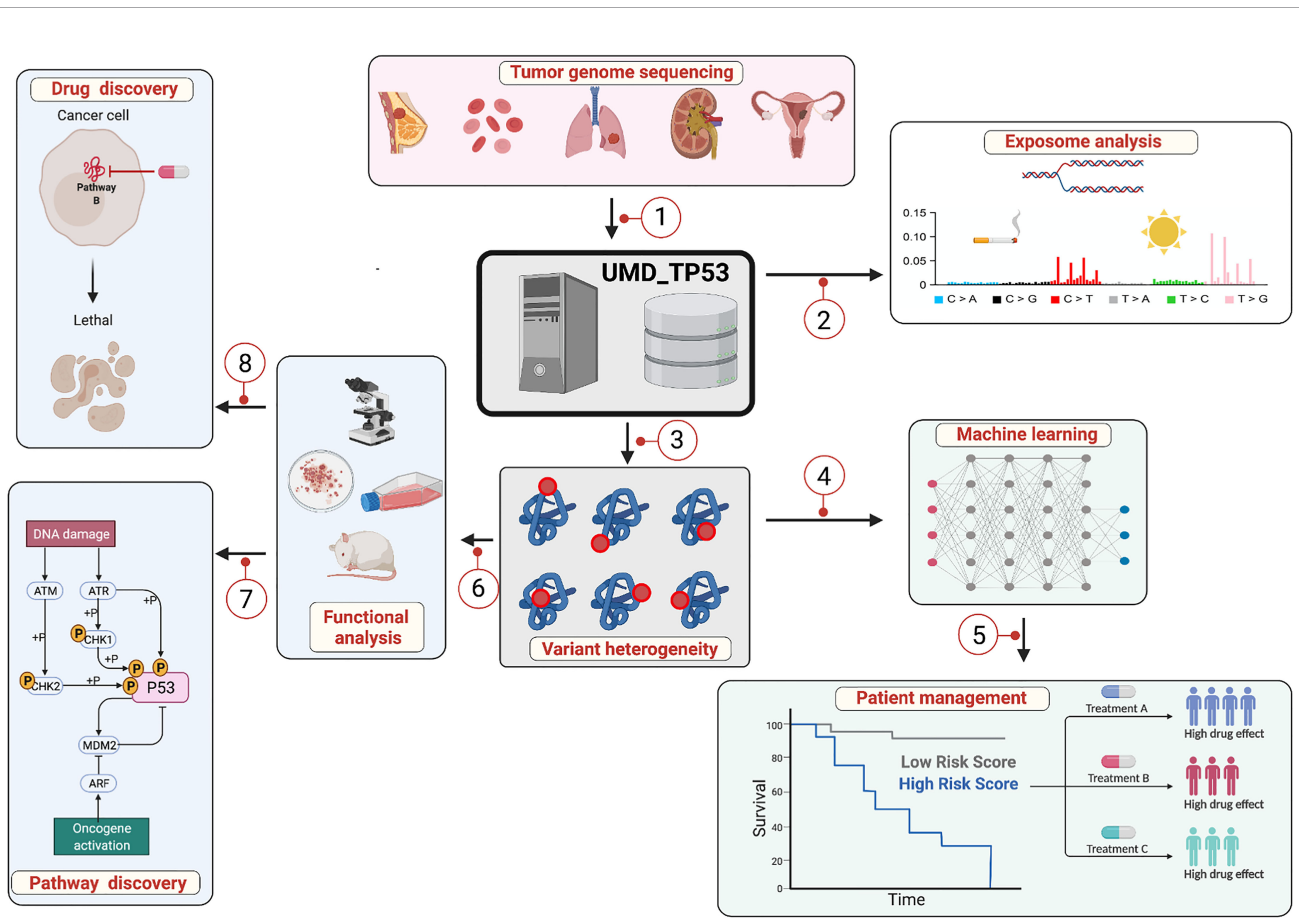

FIGURE 1 | The locus-specific database UMD_*TP53*: a central hub for multifactorial analysis. 1: *TP53* variants and patient information are collected and stored in a relational database specifically developed for the storage and the analysis of genetic variants. 2: Exposome analysis: influence of the external and internal environment on the landscape of mutational events to identify the links between exposure to various types of carcinogens, specific mutational events in the *TP53* gene and the development of specific cancers. 3: More than 7,000 different *TP53* variants have been discovered in various types of cancer with heterogeneous LOF and GOF. 4: Multiple bioinformatics tools, including machine learning, have been developed to predict and classify *TP53* variants. 5: Genome-based prognostic biomarkers can be used for several cancer types for potential incorporation into clinical prognostic staging systems or practice guidelines such as *TP53* and CLL. 6: Analysis of *TP53* variants points to the various functional domains of the protein essential for tumor suppression. 7: Functional analysis has led to the identification of the multiple pathways regulated by *TP53*. 8: Small molecules have been developed that specifically target missense *TP53* variants and restore p53 transcriptional activity, thereby enabling tumor regression. Although this figure describes the *TP53* database, the various aspects can be applied to other genes as well.

TP53 DATABASES AND REPOSITORIES

Although multiple *TP53* LSDBs have been created, only two, UMD_*TP53* (Universal Mutation_Database, developed by the present team) and IARC, count 30 years of *TP53* mutation analyses in various types of cancer (**Table 1**) (12, 13). Both have been regularly updated with both *TP53* variants and new tools to classify them. The IARC database was updated for the last time in 2019 and is currently awaiting transfer to a new host. The next update to the UMD_*TP53* will be performed in March 2022. It will bring a new innovative system to classify *TP53* variant pathogenicity and a new version of Seshat to analyze variants (14).

The number of CLL-related *TP53* mutations in the various databases is quite low except in UMD_*TP53* (**Table 1**). Because of the clinical importance of *TP53* mutations in CLL, a curated subset for that pathology, called UMD_CLL, has been added to the UMD_*TP53* database (**Figure 3A**). The latest version of UMD_CLL includes 4,698 mutations, corresponding to 3,419 samples, as patients with multiple *TP53* mutations are frequent in this disease. The characteristics of these variants are discussed in the following sections of this review.

As early as 2005, in collaboration with C. Ishioka's group, UMD_*TP53* was updated with *TP53* functional data to improve the curation of the database and develop the first tools to assess *TP53* variant loss of function (LOF) (15, 16). These tools have shown tremendous value for distinguishing true oncogenic *TP53* variants from passenger or artifactual mutations. Data from two recent large-scale studies analyzing *TP53* LOF *via* multiple assays in mammalian cells have also been included in UMD_*TP53* to refine *TP53* variant classification (17–19). Version 1 of Seshat was released in 2018. Seshat is a web service for annotating *TP53* information derived from sequencing data. It allows the use of mutation annotation format (MAF) or variant call format (VCF) files. Seshat performs accurate variant annotations using the nomenclature of the Human Genome Variation Society and the stable *TP53* genomic reference provided by Locus Reference Genomic (14).

Several single nucleotide polymorphisms (SNPs) in the coding region of the *TP53* gene have been identified and extensively characterized. Among the missense SNPs, rs1042522 (p.Pro72Arg) is common in all populations across the globe. Contrastingly, rs1800371 (p.Pro47Ser) has been shown to be specific to the African population (20). Both SNPs are

TABLE 1 | TP53 mutation databases.

	UMD[1] LSDB	IARC[2] LSDB	LOVD[3] LSDB	COSMIC[4] CGD	TCGA[5] CGD	ICGC[6] CGD	MSKSCC[7] CGD	GENIE[8] CGD
Version	2021R1	R20, July 2019	*TP53*:210617	v94	NA	v28	V10	V10
Creation date	1991	1991	2013	2004	2008	2013	2016	2016
Last update	2021	2019	Jun-21	May-21	Jun-21	Mar-21	Jun-21	Jun-21
Number of entries	170,428	29,891	676[9]	47,788	4,250	6557	3,249	4,813
Unique variant	8,046	4,526	400	5,705		1,961	1031	11,30
Cell lines data	Yes	Yes	No	Yes	No	No	No	No
Curated publications	6,704	2,273	6	4,129	32 studies	86 projects	NR	NR
Online search	Yes	Yes	No	Yes	Yes	Yes	No	Yes*
Publication warning[10]	Yes	No	No	No	No	No	No	No
Sex/Age/Ethnicity	No	Yes	No	Partial	Yes	Yes	Yes	Yes
Curation for duplicate publications[11]	Yes	Unknown	Unknown	Unknown	NR	NR	NR	NR
Sample duplications	No	No	No	Yes	NR	NR	NR	NR
SNP curation	Yes	Partial	No	Partial	Partial	Partial	Partial	Partial
Availability of functional data	Yes	Yes	No	No	No	No	No	No
Availability of predictive data	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
ACMG criteria	Yes	No	No	No	No	No	No	No
Data accuracy	Yes	Yes	unknown	Yes	Yes	Yes	Yes	Yes
Germline mutation	Yes	Yes	Yes	Yes	No	No	No	No
Familial data	No	Yes	No	No	No	No	No	No
Availability for Download	Yes	Yes	Yes	Yes	Yes	No*	Yes	Yes
Submission for analysis	No	No	Yes	No	No	No	No	No
Current status	Alive	on hold	Unknown	Alive	Alive	Alive	Alive	Alive
CLL publications/cases	179	31	0	412	0	0	6 CLL cases	235 CLL cases
Number of *TP53* variants in CLL	4,698	187	0	40	0	0	0	13

[1]*http://p53.fr/tp53-database/mutation-database.*
[2]*https://p53.iarc.fr/.*
[3]*https://databases.lovd.nl/shared/genes/TP53; LOVD database includes mostly non-pathogenic SNPs reported in population studies.*
[4]*https://cancer.sanger.ac.uk/cosmic.*
[5]*Only the 32 PAN cancer studies (10,967 samples) are included here.*
[6]*https://www.cbioportal.org/.*
[7]*https://www.synapse.org/#!Synapse:syn7222066/wiki/405659; MSKSCC data were extracted from GENIE V10.0.*
[8]*All GENIE data except MSKSCC study.*
[9]*LOVD database includes mostly non-pathogenic SNPs reported in population studies.*
[10]*Manuscript known to includes spurious data are flagged.*
[11]*Multiple publications report genetic information for the same patient.*
**Only via https://genie.cbioportal.org/.*

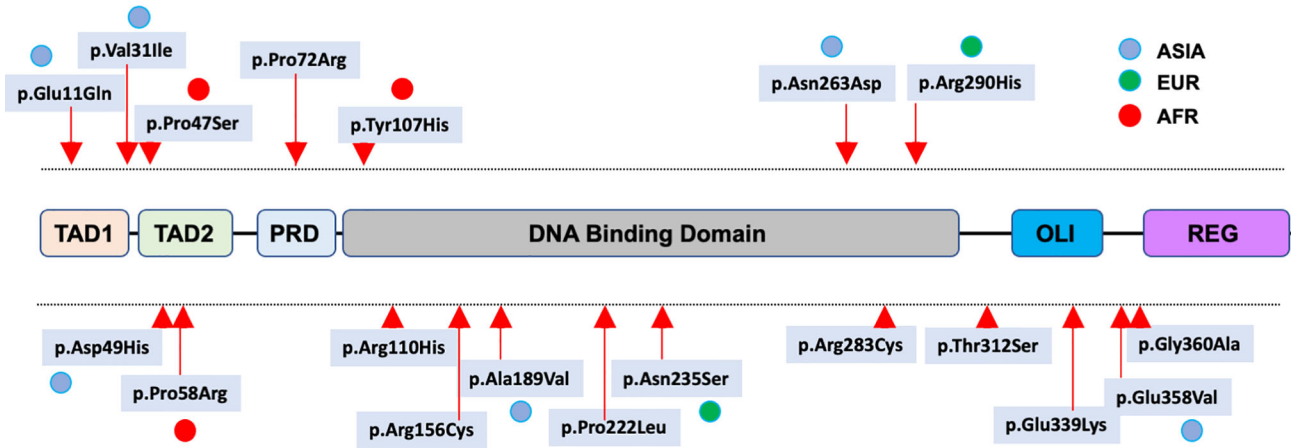

FIGURE 2 | Distribution of benign missense *TP53* SNPs in the p53 protein. SNPs specific for an ethnic population are indicated by colored dots.

included in ClinVar and considered benign according to American College of Medical Genetics and Genomics (ACMG) criteria. In a recent survey, new *TP53* missense SNPs, including five variants specific to the Asian population, were identified and characterized (**Figure 2**) (21). None of these variants were found to display LOF compared to the normal *TP53* gene (**Figure 3B**) and they are now defined as bona fide benign SNPs (21). In UMD_*TP53*, these variants are specifically flagged as germline SNPs. However, other LSDBs and CGDs define several of them as somatic and potentially pathogenic variants.

The three major CGDs (ICGC, TCGA and GENIE) include data from both whole exome and whole genome sequencing of multiple tumors (**Table 1**). CGDs list fewer *TP53* variants than LSDBs do. However, the former are able to show the full pattern of mutations in a single tumor, which enables analyses that are not possible with the latter. For example, CGDs enable the identification of mutual exclusivity of genomic alterations to identify genes belonging to a same functional pathway, as they do not mutate simultaneously in a same patient (22).

SHAPING THE LANDSCAPE OF *TP53* MUTATIONS IN CLL

Although most DNA damage resulting from endogenous and/or exogenous insults is successfully managed by the various DNA repair mechanisms, some does escape those processes and transform into stable mutations. Of these latter, only a few will target cancer genes and thus confer a growth advantage (driver mutations). The remaining mutations will be co-selected during the neoplastic process (passenger mutations). The number of driver mutations is very low (less than 20). However, that of passenger mutations is several orders of magnitude higher, ranging from 0.8 substitutions per megabase for hematological neoplasms such as CLL to 9 or 11 for lung cancer or melanoma respectively (23).

As a result, due to the specificity of the damage caused by such insults and the specific repair mechanisms used by the cell to correct the damage, mutagenic processes generate characteristic point mutation rate spectra, which are called mutational signatures. These signatures point to the mutagenic processes active in a tumor and reveal the high tissue specificity of these mutagenic mechanisms. For these analyses, passenger mutations are preferred as they are not subject to any selective process. In contrast, mutations in driver genes are highly biased as only those able to drive a cancer phenotype will be selected, whether it is *via* the LOF of a tumor suppressor gene or the gain of function of an oncogene. For the latter, mutations (predominantly missense variants) are restricted to a few codons in the gene targeting key functional residues. For tumor suppressor genes, mutations (predominantly nonsense or frameshift) will lead to a null phenotype or the synthesis of an inactive truncated protein. The mode of inactivation of *TP53* is unique compared to other tumor suppressor genes, with more than 80% of somatic and germline *TP53* alterations being missense mutations that lead to the synthesis of a stable mutant protein that accumulates in the nucleus of tumor cells (24). The classification of *TP53* as a tumor suppressor gene led to a general belief wherein the loss of *TP53* function is the sole mechanism associated with *TP53* mutations. In fact, this strong selection to maintain expression of mutants in tumors is known to have a vital role in transformation, including dominant activity (DN) and/or a gain of function (GOF), making *TP53* variants oncogenic. The distribution of mutations in the p53 protein is also unique among oncogenes and tumor suppressor genes as nearly all of the protein's 393 amino acid residues have been the target of at least one mutation in human cancer. Each residue in the core domain (containing the DNA-binding region) has been found to be mutated at least five times in independent tumors, and up to 6,000 times for hotspot mutants.

Nevertheless, the distribution of these mutations, and therefore the landscape of *TP53* variants observed in a number of types of cancer, is very heterogenous. This aspect may result

from the specificity of the insults that generate the mutations (5-methylcytosine deamination at CpG dinucleotide, UV, tobacco carcinogens or chemotherapy) or from the tissue-specific selection of *TP53* variants with a special growth advantage (25). At first glance, an analysis of the 3,914 cases of CLL in UMD_*TP53* shows a mutation profile similar to those of other cancers (**Figures 3C, D**), with 76% of missense mutations mostly localized in the DNA-binding domain of *TP53*.

The unusual feature of *TP53* mutation in CLL is the presence of a specific hot spot variant: a deletion of two nucleotides at codon 209 (c.626_627del) leading to premature termination (p.Arg209LysfsTer6) (**Figure 3E**). Frameshift variants are found all along the *TP53* gene in every type of cancer, but variant c.626_627del is highly predominant in CLL (15% of frameshift mutations in CLL compared to 1 to 2% in other cancer types). The sequence around codon 209 contains an inverted repeat that could explain its specific mutability. Furthermore, the observation of this variant in both untreated and treated patients indicates that it originates from an unknown endogenous mechanism. Although frameshift variants are usually not expressed due to NMD (nonsense-mediated mRNA decay) and protein instability, a specific selection for a truncated *TP53* cannot be formally excluded.

SUBCLONALITY OF *TP53* MUTATIONS

Whole exome and whole genome sequencing have provided new insights into the heterogeneity and evolution of tumors, with, importantly, the detection of a high number of subclones in a single tumor (26, 27). This knowledge on the subclonality of *TP53* mutations is likely to have implications for biomarker discovery and/or cancer therapy, particularly in the era of targeted treatments. Furthermore, indications of a relationship between this heterogeneity and clinical outcomes are emerging.

TP53 mutated subclones with variant allele frequencies (VAFs) lower than 10% (range 0.3% to 10%, depending on the study), undetectable by conventional Sanger sequencing, have been reported in multiple studies (28–33). Subclonal *TP53* variants and high VAF variants have the same profile, including similar hot spot variants. Longitudinal studies have shown that some of these clones can become more prevalent during the development of the tumor, regardless of whether the patient was treated or not. These small mutated subclones have been shown to be associated with unfavorable prognoses in some studies. However, this issue remains controversial, and there is currently no use of mutated subclones in the clinic. *TP53* classifications and the methods and cut-offs used to define low VAF clones must be harmonized to enable consensus.

Another characteristic is the high number of CLL patients with multiple *TP53* variants (**Figure 3F**). This feature appears specific to CLL; it has not been observed in other types of cancer (22). Bi-allelic *TP53* inactivation could explain two *TP53* variants but not a higher number of them (range 3 to 10) (34). This high intratumor heterogeneity has been detected in multiple independent studies and validated by specific analyses such as FASAY (functional analysis of separated alleles in yeast) and SMRT (single-molecule real-time sequencing) that confirm different allelic locations for these *TP53* variants. Like for minor subclones, most *TP53* variants identified in tumors bearing multiple *TP53* variants are truly pathogenic. The basis of this specific selection for multiple *TP53* variants during the course of CLL is currently unknown.

TP53 MUTATION HETEROGENEITY AND PATHOGENICITY

As early as the nineties, it was obvious that *TP53* mutant LOF was heterogenous. Variants were classified as "contact" or "structural," depending on whether the substituted amino-acid acted directly on DNA interactions (p.Arg273His) or caused a general effect on the protein structure (p.Arg175His). Several classifications for variants based on *TP53* aspects have been suggested to stratify patients with *TP53* mutations but none have reached the clinic due to the high heterogeneity of the variants and the specificity of the variants among cancer types. A number of predictive tools have been developed, exploiting such information as sequence phylogenetic conservation, amino acid physicochemical properties, functional domains and structural attributes. Commonly used variant effect prediction methods include SIFT (35, 36), PolyPhen (37), GERP++ (38), Condel (39), CADD (40), fathmm (41), MutationTaster (42), MutationAssessor (43), GESPA (44) and, more recently, REVEL (45) and ENVISION (46). Several of these methods, such as fathmm, Condel, CADD and REVEL, integrate data from multiple tools to improve classification accuracy. Recent methods have used machine learning processes. Their training and validation were conducted using datasets of classified variants taken from either pathogenic (COSMIC, TCGA, GENIE, HGMD) or benign (dbSNP, gnomAD or Clinvar) variant databases. Nonetheless, for *TP53* and other genes, these various classifiers have heterogenous outcomes and no consensus for their use has been reached. GENIE uses SIFT and PolyPhen, whereas TCGA uses SIFT, PolyPhen and MutationAssessor, and COSMIC uses fathmm. When employing predicting methods based on phylogenetic conservation, tools based on amino acid physicochemical properties such as SIFT or PolyPhen should be used with great precaution as the relation of the deleteriousness of the protein predicted by these tools and any association with disease is far from being straightforward.

To solve this issue, the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) have published standards and guidelines for the interpretation of sequence variants (47). These guidelines describe a proposition for classifying variants as "pathogenic," "likely pathogenic", "uncertain significance", "likely benign" or "benign" according to a series of criteria with levels of evidence defined as "very strong", "strong", "moderate" or "supporting". They have been widely adopted by clinical laboratories around the world. However, these recommendations were primarily designed for constitutional variants. Thus, their use for somatic

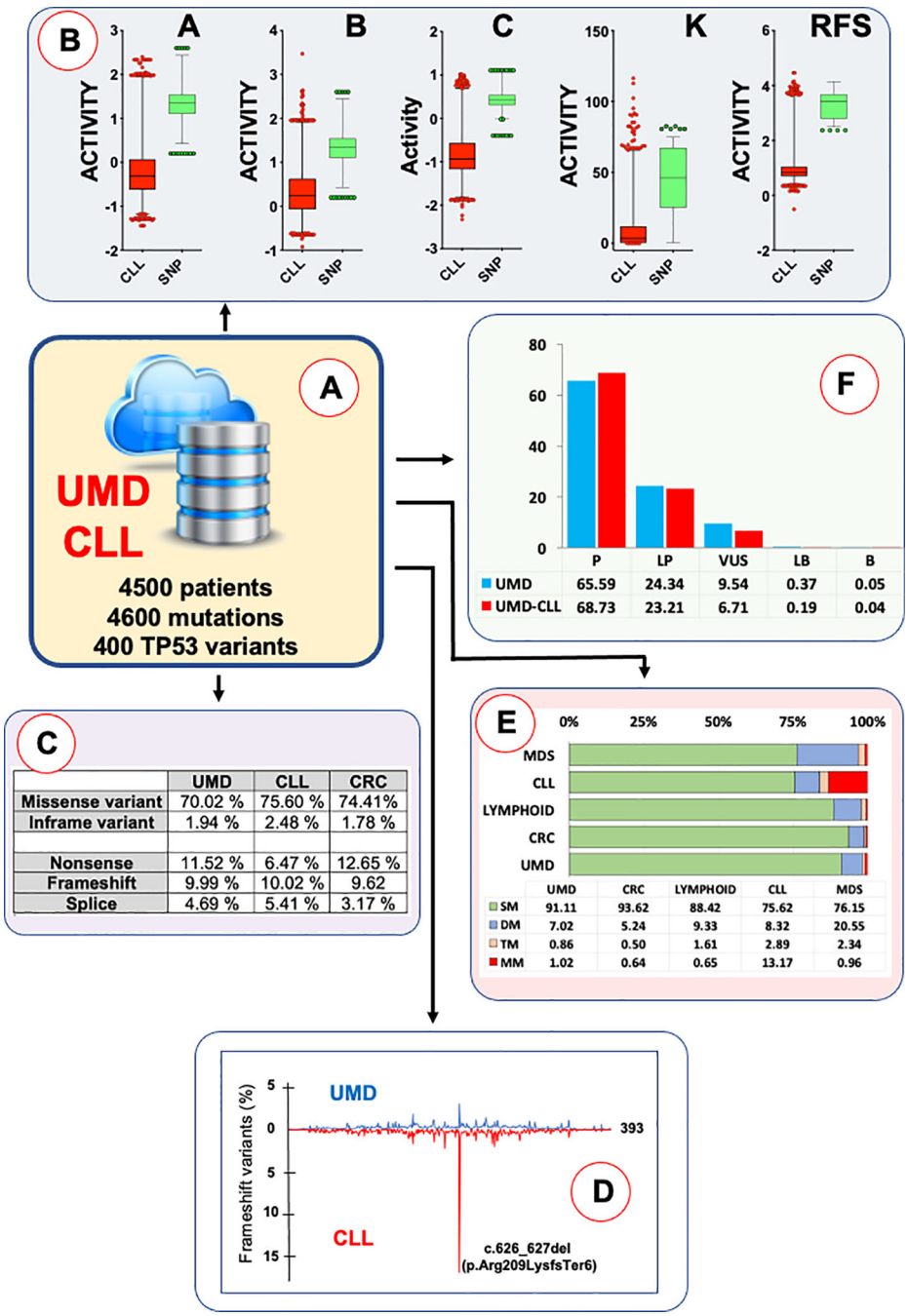

FIGURE 3 | The UMD_CLL database. **(A)** *TP53* mutations from CLL patients included in UMD_*TP53* have been manually curated to correct for study duplication. For patients analyzed *via* Sanger in the nineties and *via* NGS more recently, only the more recent data were kept in the database as the sensitivity of NGS uncovered less frequent variants. **(B)** The UMD_CLL database includes three independent sets of functional data used to assess the loss of function of more than 10,000 *TP53* variants: A, B and C, data from Giacomelli et al. in mammalian cells; RFS, data from Kotler et al. in mammalian cells; K, data from Kato et al. in yeast cells. Correlation analysis and multidimensional scaling showed excellent agreement between these three sets of data (19). Each dataset has been used to compare the *TP53* variants from UMD_CLL (red) to benign *TP53* SNPs (green). **(C)** The landscape of *TP53* variants in CLL is similar to that of other types of cancer, with 78% of tumors expressing a mutant *TP53* (missense and in-frame variants) and 22% null variants (splice, nonsense and frameshift mutations); **(D)** Analysis of the distribution of *TP53* variants in TP53 protein from CLL patients showed several unusual features, such as a frameshift mutation in codon 209. See text for more details. **(E)** At least 25% of CLL patients carry at least two pathogenic *TP53* variants, and up to 13% carry more than four. This situation is shared only with myelodysplastic syndrome, where up to 20% of patients show two *TP53* variants. As half of the CLL data in UMD_*TP53* originated from Sanger analyses, it is likely that CLL intratumor heterogeneity is underestimated. **(F)** All *TP53* variants from UMD_*TP53* have been classified according to ACMG criteria. For this purpose, all newly discovered, rare, benign SNPs misidentified as pathogenic mutations have been removed from the database.

variants requires some adjustment (48). The two main criteria used for the levels of evidence were based on population (BA1, BS1 and BS2) and functional (BS3) data, which are now fully available in UMD_*TP53*.

One of the main advantages for *TP53* over other tumor suppressor genes is the availability of a range of functional data for all possible missense mutations occurring in the coding region for the large isoform of the protein. Data from three independent large-scale saturation mutagenesis screening studies carried out in different settings (yeast or mammalian) and with different readouts (transcription, growth arrest or apoptosis) are currently available (17, 18, 49). A correlation analysis showed excellent agreement between LOF for the protein and the occurrence of these variants in different cancer types, making this criterion suitable for defining PS3 for ACMG classification (**Figure 3B**) (19). An analysis of the UMD_CLL database indicates that 91.9% of the *TP53* variants identified in CLL, whether clonal or subclonal, are classified as pathogenic and 6.7% as VUS (**Figure 3F**).

A SNAPSHOT OF *TP53* MUTATION STATUS IN CLL

TP53 status in tumors is complex as multiple mechanisms can impair *TP53* tumor suppression pathways. Furthermore, it is quite likely that cancer specificity plays an important role in this process due to the large diversity of *TP53* function and regulation among the various tissues. Although MDM2 expression is upregulated in numerous cancers, resulting in a loss of p53-dependent activities, its frequency in CLL is quite low. Other mechanisms, such as the dysregulation of the microRNA network that controls *TP53*, are also possible but their importance in CLL needs further investigation.

In contrast, CGDs have made it possible to identify the co-occurrence or mutual exclusivity of specific genetic events. In the former, alterations of certain combinations of genes tend to co-exist in a same tumor, whereas in the latter, mostly only one out of a group of genes is altered. Individual alterations targeting similar biological processes are believed to be mutually redundant, with one alteration being sufficient to deregulate the affected process. Identifying mutual exclusivity can therefore help to identify unknown functional interactions. In CLL, this type of analysis is averted by the important genetic heterogeneity of the tumors, showing multiple subclones with different genetic alterations. Because NGS gives a global picture of these events, defining whether or not they occur in the same cells is difficult. This problem will likely be resolved once sufficient single-cell genomic analyses have been performed.

As shown in **Figure 4**, *TP53* status in CLL can be very heterogeneous, as the prevalence of *TP53* abnormalities, including 17p deletion and *TP53* mutations, varies across the different phases of the disease (26). Furthermore, the subclonal heterogeneity of the tumors can sometimes be misleading. Indeed, bulk NGS analyses generate an averaged picture of a given population of cells, which may result in an underestimation of their true heterogeneity. Nevertheless, a general picture emerges from the various studies. *TP53* mutations are not the prime event in CLL. In the early phase of the disease (stage 0), *TP53* mutations appear to be either absent or infrequent, but this issue needs to be carefully reevaluated using NGS assays validated for limits of detection (LOD) ranging from 0.05% to 1% (**Figure 4**, panel 1). Furthermore, because these variants are usually not associated with a deletion of the second allele, FISH or SNP arrays are not suitable for early detection analyses (**Figure 4**, panel 2). 17p deletion and complex karyotypes occur during disease progression, leading to the conventional view of CLL with a single *TP53* mutation associated with *TP53* loss of heterozygosity (LOH) (**Figure 4**, panel 3). Targeted, high-depth, NGS of *TP53* coupled with an adequate pipeline able to reach a LOD of at least 1% has led to the discovery of multiple subclones expressing different pathogenic *TP53* variants (**Figure 4**, panel 4). Why CLL has such a propensity for *TP53* mutations is currently unknown. However, it is clear that CLL depends on signals from the microenvironment and that its cells cycle between lymphoid tissue sites such as lymph nodes and peripheral blood. It is possible that the strong proliferation signals provided by the microenvironment in lymph nodes require a loss of several anti-proliferative signals such as that provided by *TP53*.

Another genetic configuration observed in CLL is copy neutral LOH (cnLOH), with the same mutation in both alleles of a given cell (**Figure 4**, panel 5). This genetic event is attributed to mitotic recombination in tumor cells where the wild-type allele is replaced by the mutant allele leading to a large region of homozygosity that can be detected early by SNP-arrays. Inversely, this situation cannot be detected by any karyotyping analyses and could be misinterpreted as heterozygous mutation if the sequencing VAF is below 50%. The situation described in panel 6 of **Figure 4** (two different mutations in the two alleles of a single cell) is theoretically possible and often described as a potential status in CLL and other tumors. However, such a situation appears to be very uncommon and has never been formally observed in CLL.

TP53 CLINICAL CONSIDERATIONS

TP53 mutations have been described in CLL since the early 1990s (50, 51). An association between *TP53* mutations, drug resistance and poor clinical outcomes was first demonstrated in 1993 by El Rouby et al. and thereafter confirmed in further studies (52–54). In 2000, using FISH analysis for multiple chromosomal markers, Dohner et al. showed that 17p deletion, where the *TP53* gene is located, was an independent predictor of disease progression and survival (55). Using either 17p deletion or *TP53* mutation as a biomarker, subsequent studies confirmed this finding and resulted in *TP53*'s classification as a well-established prognostic marker furthermore able to provide pertinent information for establishing an appropriate course of treatment for patients.

The therapeutic approach for CLL carrying *TP53* mutations will be addressed in detail in another review in this series. There are,

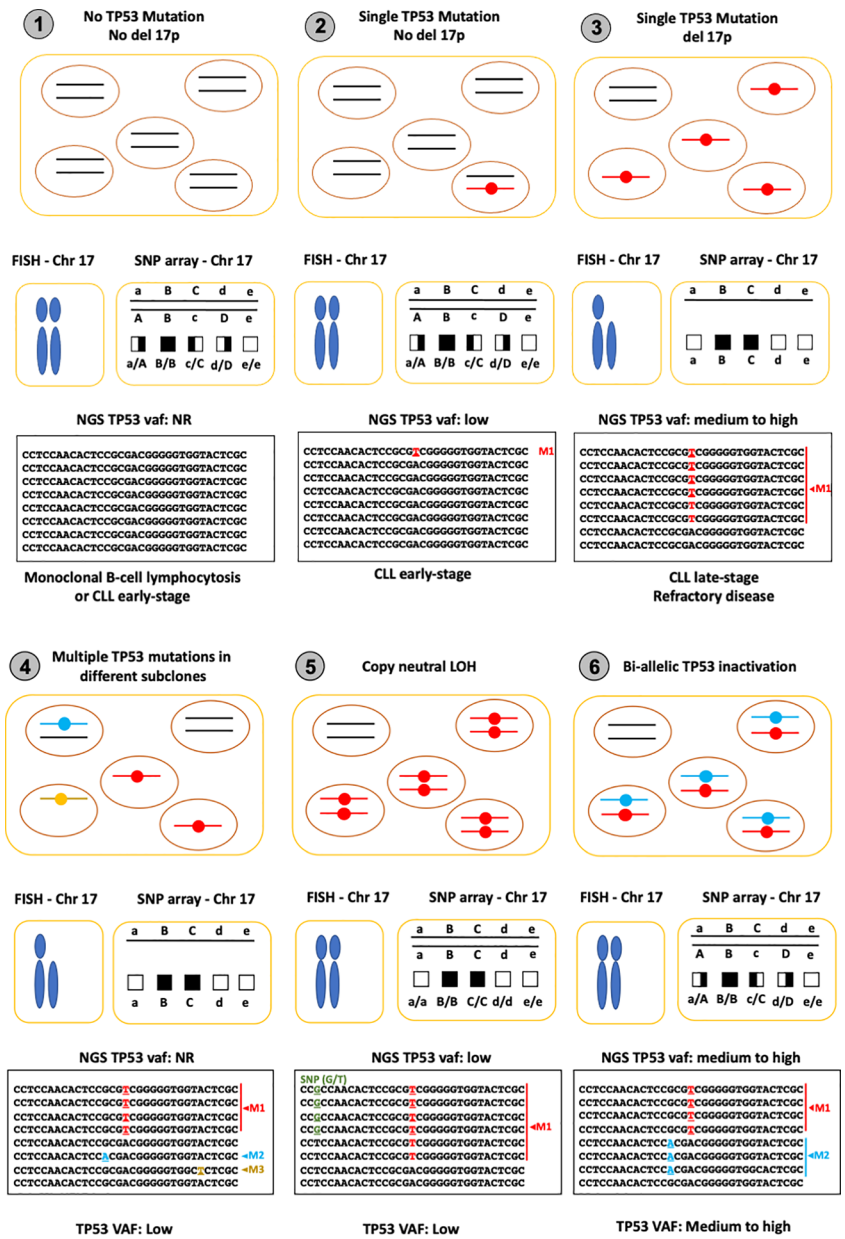

FIGURE 4 | *TP53* status in CLL patients, a snapshot. The top panel displays a schematic view of the tumor with the two *TP53* alleles. The middle panel shows cytogenetic analysis performed by FISH (left) or by SNP arrays (right). The lower panel displays an example of the read alignments from NGS. 1: No *TP53* mutation: In monoclonal B-cell lymphocytosis, *TP53* mutation and 17p deletion are very rare, leading to negative results for both FISH and genetic analysis. 2: *TP53* mutation without LOH: In early stages of CLL, the frequency of *TP53* mutation is low (less than 10%) with many cases showing no LOH. Sensitive sequencing analysis with NGS is able to identify low VAF *TP53* variants (variant M1 in the lower panel). 3: *TP53* mutation with LOH: In late-stage or relapsing disease, *TP53* mutations associated with 17p deletion can be found in 30 to 50% of CLL patients. In the majority of cases, VAF is greater than 50% due to the loss of the second allele. This situation is commonly seen in CLL. 4: Multiple *TP53* and LOH: in both early and late-stage disease, FASAY (functional analysis of separated alleles of p53 on yeast) or SMRT (single-molecule, real-time sequencing) has demonstrated a high level of intratumoral heterogeneity in CLL with the presence of multiple independent subclones expressing different pathogenic *TP53* variants (M1, M2 and M3 in the lower panel). Although 17p deletion is often observed in these patients, it is difficult to determine if subclones expressing different *TP53* variants are associated with it, and even more so if the VAF of the variant is low. 5: Copy neutral LOH: Following the initial mutational inactivation of one allele, the remaining wild-type allele is deleted concurrently with the duplication of the mutated allele, leading to copy neutral LOH (cnLOH). Detecting cnLOH is difficult and thus the frequency of the event is currently unknown. Without SNP array analysis and if the VAF of the variant is lower than 50%, this situation can be misidentified as a tumor without LOH. Tumors with VAF greater than 50% without obvious 17p deletion should be checked for cnLOH. 6: Bi-allelic mutations: Inactivation of the *TP53* gene *via* different mutations in the two alleles is possible but difficult to distinguish from intratumoral heterogeneity. Although this situation is often described as plausible in many reviews, it has never been formally identified, as only single-cell sequencing would be able to validate bi-allelic *TP53* inactivation.

however, some biological aspects and some issues related to methodological/technical details that can be discussed here.

There is no longer a place for chemo-based regimens in patients with CLLs presenting *TP53* mutations. The introduction of novel targeted agents has greatly altered the clinical course of these patients, who now benefit from responses that were never observed during the chemo(immunotherapy) era (56). That said, even with the use of novel agents, CLL remains incurable. Patients with *TP53* disruption (*TP53* mutation/17p deletion) exhibit worse clinical outcomes compared to those without it, indicating that the management of the former is still an unmet challenge (57). This is more evident for relapsed/refractory (R/R) CLL (58–62) where data on front-line therapies are still scarce because the follow-up of clinical trials at the front line is still short (63, 64). Moreover, little is known on R/R CLL response to novel agents, a setting wherein *TP53* disruption seems to be an unfavorable prognostic/predictive factor (65, 66).

Another parameter that needs to be taken into consideration is that in both clinical trials and clinical care, *TP53* disruptions are considered equal whatever their nature. There is thus no differentiation being made between patients with monoallelic or bi-allelic aberrations, despite data suggesting that the latter may exhibit more aggressive clinical courses (33, 60). Similarly, the number or type of mutations receives no consideration as a specific clinical feature either.

Moreover, in untreated CLL with *TP53* mutations, there is a subset of patients with indolent clinical courses, which suggests that other disease- and/or patient-related parameters may alter the impact of *TP53* disruption (67, 68). Also, genomic instabilities at the chromosomal and molecular level, as well as the immunogenetic features of the clonotypic B-cell receptor, namely the somatic hypermutation status of the immunoglobulin heavy variable gene, have been proposed as factors that may aggravate or alleviate the impact of *TP53* mutations (69–72).

Finally, there is a discrepancy regarding the threshold for reporting *TP53* mutations detected by NGS in the clinical setting versus the official guidelines that merits discussion. According to the latest versions of these latter, only mutations with VAF ≥10% should be reported and used for directing treatment choice (73). This conservative approach within the official guidelines is based mainly on the fact that the clinical impact of small *TP53* clones, especially those below 5%, has not been demonstrated to date in prospective clinical trials. However, diagnostic laboratories are becoming more experienced in NGS data output management, and resultantly, clones down to 2-5% are being reported in the clinical setting and, in the majority of cases, taken into consideration for clinical decision-making.

REMAINING CHALLENGES AND PERSPECTIVES

Compared to other cancer types, the clinical value of *TP53* status in CLL has always been uncontested and it is now a required biomarker for patient stratification. It is therefore essential that *TP53* analyses be performed in a standardized manner to provide consistent data across the various clinical laboratories. For this purpose, the *TP53* Network of the European Research Initiative on Chronic Lymphocytic Leukemia (ERIC) had released a first recommendation in 2012 and updated it recently to take into account the emergence of NGS (73, 74). Nevertheless, considering the quick evolution of methodologies and the discovery of the subclonal heterogeneity of *TP53* variants with low VAF clones, a new consensus must be reached for the controversial issue of the limit of detection used to report *TP53* variants in clinical laboratories. Although conventional Sanger sequencing has been widely used in the past, it is now clear that NGS-based analysis should become mandatory for the clinical detection of low VAF clones. The current situation is unclear, with several studies suggesting that patients with low VAF *TP53* clones have the same clinical prognosis as patients with high VAF ones, and other studies unable to confirm that finding. Reaching a consensus to define a robust, clinically justified LOD will be essential for improving patient stratification. Furthermore, despite their relative infrequency, it will be important to evaluate the real incidence of multiple *TP53* subclonal mutations using adequate methodologies as well as their evolution during the course of disease and with different types of treatment. Whether or not this reservoir of heterogenous oncogenic *TP53* variants is an essential component of the plasticity of CLL remains to be addressed.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Biology and Treatment of Richter Transformation

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Richter transformation (RT), defined as the development of an aggressive lymphoma on a background of chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), represents a clinical unmet need because of its dismal prognosis. An increasing body of knowledge in the field of RT is arising from the recent development of preclinical models depicting the biology underlying this aggressive disease. Consistently, new therapeutic strategies based on a genetic *rationale* are exploring actionable pathogenic pathways to improve the outcome of patients in this setting. In this review, we summarize the current understandings on RT biology and the available treatment options.

Keywords: Richter transformation, Richter syndrome, CLL, biology, DLBCL, Hodgkin lymphoma, treatment

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DEFINITION OF RICHTER TRANSFORMATION

Richter transformation (RT) is defined as the development of a high-grade lymphoma in patients with a previous or concurrent diagnosis of chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) (1).

RT was originally depicted as a 'reticular cell sarcoma' with presence of 'leukemic and tumor cells' on a lymph node biopsy from a male patient with CLL and rapid clinical deterioration by Maurice N. Richter in 1928 (2). The occurrence of secondary aggressive lymphomas on a CLL background took the definition of 'Richter transformation' in 1964, when a case series of 14 patients with CLL developing malignant reticulopathy was described by Lortholary and colleagues (3).

The estimated incidence of RT in patients with CLL/SLL previously treated with chemo/chemoimmunotherapy was reported to be 0.5–1% per year (4). Different histopathologic variants of RT have been described in the literature, ranging from the more common diffuse large B-cell lymphoma subtype (DLBCL-RT) which accounts for up to 90–95% of RT cases, to the less represented Hodgkin lymphoma subtype (HL-RT) accounting for up to 5–10% of cases (1). Few cases (<1%) of plasmablastic transformation have been also reported (5).

EPIDEMIOLOGY AND CLINICAL FEATURES

The large variability of the reported prevalence of RT (1–23%) has been related to different factors, mainly depending on the diagnostic assessment of RT (biopsy-proven or just clinically suspected), and on the setting (clinical trials involving fit patients or real-world data) from which data were derived (5–8).

Recently, the German CLL Study Group (GCLLSG) reported a 3% prevalence of RT in a cohort of 2,975 patients with CLL longitudinally monitored after their enrolment in clinical trials (9).

Data coming from the SEER database on 74,116 patients with CLL diagnosed between 2000 and 2016, depicts a 0.7% incidence of transformation, mostly emerging with nodal involvement (74%) (10). The gastrointestinal tract, the skeletal system, and the brain/CNS are the most commonly reported extra-nodal sites, being described in 25, 19, and 12% of cases, respectively. Median time to transformation is 1.8–1.9 years for DLBCL-RT (3, 11) and 4.6–7.5 years for HL-RT (12, 13), even if no significant difference according to different histotypes is reported in other datasets (10).

A higher incidence of RT has been reported for highly pretreated relapsed/refractory (R/R) CLL patients enrolled in the first clinical trials with novel agents (2–15%), while in first-line the incidence of RT is 0–4% in this treatment setting (13–21). However, these data refer to short follow-up periods and longer observation time is needed to properly evaluate the impact of chemo-free treatments on second malignancies/transformation. Similar clonal evolution patterns are described for patients experiencing transformation under novel agents or chemo-immunotherapy (CIT) (22, 23).

DIAGNOSIS

Rapid physical deterioration and/or occurrence of B symptoms (i.e., fever with no infectious background, weight loss), rapid and localized growth of lymph nodes, rise in lactate dehydrogenase (LDH) levels, and hypercalcemia, are all signs that should raise suspicion for aggressive transformation, particularly in a patient with known CLL. However, these clinical findings are specific for RT in only 50–60% of cases, the remaining ones being manifestations of histologically aggressive CLL (aCLL) or solid cancers (24).

The gold standard for RT diagnosis is histologic documentation with an open biopsy. Fine needle biopsy may not illustrate the whole lymph node structure, leading to false positive diagnoses (i.e., expanded proliferation centers may be seen in fine needle biopsies from patients with progressive or aCLL) (25).

Role of ^{18}FDG PET/CT

Since RT is often limited to one single lesion at the time of evolution, any biopsy aimed at confirming RT should be directed at the 'index' lesion (the lesion showing the most active dimensional dynamics). ^{18}FDG PET/CT may assist in the choice of whether and where to perform a biopsy (24, 26, 27). When a standard uptake value (SUV) cut-off of 5 is chosen, the high negative predictive value (97%) of the ^{18}FDG PET/CT in this setting supports a non-biopsy approach for lesions with SUV <5. Given the limited positive predictive value (53%) of ^{18}FDG PET/CT for lesions with an SUV ≥5, the biopsy should be performed at the site of the index lesion (24, 26, 27).

A higher positive predictive value (60.6%) has been described when establishing an SUV cut-off of 10, with a sustained elevated negative predictive value (99.2%) and a good correlation with overall survival (OS). Patients with lesions displaying an SUV ≥10 showed a median OS of 6.9 months, while for patients displaying lesions with an SUV <10 the reported median OS was 56.9 months (28). However, for patients with RT arising after kinase inhibitor therapy, the SUV threshold of 10 showed lower negative predictive values (50%) (29).

Morphology and Immunophenotype

Morphology of RT Subtypes

The presence of confluent sheets of large neoplastic B lymphocytes characterizes the morphology of the DLBCL-RT (4, 30). Notably, an enlargement of proliferation centers in lymph nodes can occur also in the 'aggressive' or 'accelerated' CLL (aCLL), which needs to be distinguished from the proper transformation, as it is associated with an outcome intermediate between typical CLL and classic RT (4). Morphologic discrimination of RT from aCLL is mainly based on the characteristics of B-cells nuclei and growth pattern (a nuclear size equal or larger than macrophage nuclei or >2× a normal lymphocyte and a diffuse growth pattern are more typical for RT) (31, 32).

The HL-RT subtype is characterized by the presence of Reed–Sternberg cells either in a typical background of small T cells, epithelioid histiocytes, eosinophils and plasma cells or scattered in a background of CLL cells (4, 30, 33).

Phenotype

DLBCL-RT cells express CD20, and less typically CD5 (~30% of cases), or CD23 (~15% of cases) (4, 34). PD-1 expression is described in DLBCL-RT neoplastic B-cells, while a weak expression is restricted on the paraimmunoblasts of proliferation centers of CLL samples and rarely found in *de novo* DLBCL specimens (35, 36). The positivity of transformed B-cells for PD-1 showed a 90% correlation with molecularly defined clonal relationship between CLL and DLBCL-RT. Accordingly, PD-1 expression has been proposed as a candidate surrogate for defining the clonal relationship of DLBCL-RT (35).

HL Variant

Hodgkin and Reed–Sternberg cells show a characteristic CD30$^+$/CD15$^+$/CD20$^-$ immunophenotype and are often EBV positive (4, 34).

Clonal Relationship Between RT and the Underlying CLL

The definition of clonal relationship between RT and the underlying CLL relies on the analysis of the rearrangement of IGHV-D-J genes [by PCR or next-generation sequencing (NGS) methods]. Most cases of DLBCL-RT (~80%) are clonally related to the previous CLL phase, representing true transformations (34, 37). Clonally unrelated cases represent *de novo* DLBCL arising in a patient with concomitant CLL, and are usually described on an IGHV-mutated CLL background (4). Clonal

relationship impacts meaningfully on the prognosis of patients with DLBCL-RT, with clonally related cases showing a median OS of less than 1 year. Conversely, for patients with clonally unrelated RT the reported survival is~ 65 months, similarly to *de novo* DLBCLs (6, 30).

Clonal relationship between HL-RT and the underlying CLL has been reported in only 30% of cases (30).

BIOLOGY OF RT

Genetic alterations leading to RT are progressively being described for DLBCL-RT, which displays some common characteristics with other transformed lymphomas. Less is reported on HL-RT, whose molecular background and behavior are similar to *de novo* HL.

Biology of DLBCL-RT

Somatic alterations involving genes of tumor suppression, cell cycle and proliferation pathways (i.e., mutations or disruptions of *TP53*, *NOTCH1*, *MYC*, and *CDKN2A*) are the main genetic clues of DLBCL-RT and can explain its aggressive disease kinetics and chemoresistance (30, 37, 38).

TP53 is a master regulator of the DNA-damage-response pathway, and leads to cell apoptosis if activated (i.e., as in response to the antiproliferative effect of chemotherapies). *TP53* mutations/deletions can be acquired at the time of transformation and are the most frequent genetic lesions of DLBCL-RT, being described in 60% cases (38).

MYC is involved in a transcription regulating network and is found altered in ~40% of DLBCL-RT (11, 30, 37–39).

CDKN2A is a negative regulator of cell cycle transition from G1 phase to S phase and can be deleted in 30% of RT cases (30, 38). The rapid kinetics and aggressive behaviour of RT may be explained by cell cycle deregulation linked to *CDKN2A* alterations. It has been recently demonstrated that a concomitant loss of function of *TP53* and *CDKN2A*/*CDKN2B* enables a B-cell receptor (BCR)-dependent proliferation of large pleomorphic cells with a diffuse RT-like morphology (40).

The biased usage of subset 8 configuration in the BCR has been associated to *NOTCH1* somatic mutations. This molecular setting allows for autonomous BCR signaling and a dynamic responsiveness of neoplastic B cells to auto-antigens and/or immune stimuli from the microenvironment (33, 41). The reported 5-year rate of transformation for patients with CLL and subset 8 usage is~ 70% (31).

NOTCH1 mutations represent the only validated risk factor for RT. The reported cumulative risk of developing DLBCL-RT is 45% among patients with CLL and mutated *NOTCH1*, while it is 4% for CLL with wild-type *NOTCH1* (42–44).

Mutational whole-genome sequencing (WGS) data from paired circulating CLL and RT biopsies were reported and independently confirmed by RNA expression profiling for 17 patients diagnosed with DLBCL-RT. RT was characterized by mutations in the DNA damage pathway and in poor-risk CLL drivers (45). *TRAF3* (a signaling regulator), *NF-kB*, and mitogen-activated protein kinase pathways, were reported to commonly harbor heterozygous deletions (45). *PTPN11*, a positive regulator of the MAPK–RAS–ERK signaling pathway, was overexpressed in RT samples (45). *SETD2* (showing alterations in ~30% of RT samples) and *PTPRD*, a tumor suppressor gene found silenced in many cancers *via* hypermethylation, were recurrently deregulated. Compared with the paired CLL, RT samples were characterized by increased mutational burden mainly related to some genes previously unrecorded in CLL (*BDKRB1*, *WWP1*, *TFCP2*, *SVIL*, *SLC9B1*, *RELN*, *PTK2*, *IRF2BP2*, *IL7*) (45), and whose role in RT pathogenesis needs to be clarified by functional studies. Further mutations were described in non-coding regions of immune-regulatory genes (i.e., *BTG2*, *CXCR4*, *NFATC1*, *PAX5*, *NOTCH1*, *SLC44A5*, *FCRL3*, *SELL*, *TNIP2*, and *TRIM13*), suggesting their potential role in RT pathogenesis (45). Consistently, distinct immune signatures between peripheral blood and lymph nodes from patients with RT have been depicted in another study (46). A low T-cell TCR clonality was found in peripheral blood, with a consequent high diversity of the T cell repertoire and a potentially active host immune response. RT samples were characterized by enhanced PD-L1 expression in histiocytes and PD-1 in neoplastic B cells, and also infiltration of FOXP3-positive T cells and CD163-positive macrophages. These findings depict a peculiar RT-immune microenvironment and may explain the higher response rates to immune checkpoint inhibitors (47).

According to the model proposed by Teng et al. to classify tumor microenvironments based on PD-L1 expression in tumor cells and tumor-infiltrating lymphocytes (TIL), RT may harbor a type I microenvironment ($PDL1^+$, TIL^+), reflecting an adaptive immune resistance environment, which can be the target of checkpoint inhibitors (48, 49). CLL, on the other hand, seems to be characterized by immunological ignorance defined as type II microenvironment ($PD\text{-}L1^-$, TIL^-) with poor expected response from checkpoint suppressors (47–49).

An increased *LAG3* gene expression has been reported in RT, with respect to *de novo* DLBCL and other transformed lymphomas (50). LAG3 membrane protein is expressed on both neoplastic B cells and/or TILs and is involved in the delivery of inhibitory stimuli on activated T cells. In RT, *LAG3* shows a strong positive correlation with HLA Class II and immune regulatory genes (namely, *TIGIT* and *PD-1*), with an immune microenvironment characterized by potential adaptive immune resistance when *LAG3* is overexpressed (51, 52).

Constitutive phosphorylation of AKT is higher among patients with CLL at high risk for RT transformation (i.e., CLL with *NOTCH1* mutation, aggressive CLL with *TP53* disruption) (53). In a new experimental TCL1 mouse model of CLL with a constitutively active Akt allele (Akt-C) in B cells, the development of an aggressive lymphoma and a massive splenomegaly was reported by the age of 7 months confirming the driving role of *AKT* for RT-like transformations. Akt-C mice showed a highly expressed NOTCH signaling, with an expansion of CD4 T cells expressing DLL1 (the NOTCH1 ligand present on T cells) in the microenvironment. This upregulation has been related to the *NOTCH1* activation of tumor cells, accordingly to their commitment for transformation.

Regulating the homing of immune cells, the CXCR4–CXCL12 axis is crucial for the interaction of CLL cells and microenvironment (54–57). In the Eμ-TCL1 mouse model, the introduction of a gain-of-function *Cxcr4* mutation (*Cxcr4*^{C1013G}) that hyperactivates CXCR4 signaling, led to cell cycle dysregulation *via PLK1*/*FOXM1* (58). These neoplastic cells showed a transcriptional signature similar to that of patients with RT.

The main pathways with a reported involvement in RT pathogenesis are resumed in **Table 1** and **Figures 1**, **2**.

PROGNOSIS OF RT

The DLBCL-RT prognosis is overall poor, with a reported median OS of 10 months (10). As already described, the most impactful prognostic factor is the clonal relationship between the transformed DLBCL and the underlying CLL (see section *Clonal Relationship Between RT and the Underlying CLL*).

Prognostic Scores

The RT prognostic score based on clinical and laboratory variables (Zubrod performance status >1, increased LDH levels, platelets ≤100× 10^9/L, tumor size ≥5 cm, and >2 prior lines of therapy) allows to differentiate 4 risk groups, with a median survival of 13–45 months for low risk patients (0–1 risk factors); 11–32 months for low-intermediate risk (2 risk factors); 4 months for high-intermediate risk (3 risk factors); 1–4 months for high risk patients (4–5 risk factors) (59).

Complex karyotype (CK) diagnosed on the underlying CLL has a negative impact on RT-related outcome (60). Type-2 CK

TABLE 1 | Summary of the main biomarkers involved in DLBCL-RT pathogenesis.

Biomarker (s)	Frequency	Role	Consequence	Note	Reference
Biased usage of BCR subset 8	8%	BCR signaling	Autonomous signaling and increased response to auto-antigens and immune stimuli	5-years transformation rate of patients with CLL and subset 8 usage: ~70%	(33, 41)
TP53	60%	Regulation of DNA-damage-response pathway	inactivation	Impaired apoptosis in response to the antiproliferative effect of chemotherapies due to *TP53* loss may explain the chemorefratoriness of RT	(38)
MYC	40%	Regulation of transcription network	Overexpression	Key transcription factor which regulates up to 15% of human genes, constantly involved in transformation from indolent to aggressive lymphomas	(11, 30, 37–39)
CDKN2A	30%	Regulation of cell cycle	Inactivation	Concomitant loss of function of *TP53* and *CDKN2A/B* leads to BCR-dependent proliferation of abnormal B cells	
NOTCH1	40%	NFκB activation	Activation	*NOTCH1* gene have been reported in ~10% of patients with CLL at diagnosis, mainly those with CLL of the IGHV-UM	(42–44)
AKT signaling	>50%	Driver of protein synthesis, cell survival, proliferation, and glucose metabolism	Activation, constitutive phosphorylation	AKT is activated in high-risk CLL and in >50% of patients with RT. Constitutive AKT may amplify the NOTCH1 signal or add additional signals that accelerate transformation	(53)
SETD2	30%	Histone methyltransferase that catalyses the trimethylation of lysine 36 on histone 3 (H3K36me3), epigenetic regulator of gene transcription	Inactivation	Deletions and mutations in ~7% of CLL patients requiring treatment	(45)
TRAF3	–	Signaling regulator, namely, Toll-like receptor signaling, NF-κB, and mitogen-activated protein kinase pathways	Inactivation	TRAF3 deficiency enhances survival of B cells and increases transformation risk *via* upregulation of PIM3 and c-MYC expression	(45)
PTPN11	–	Regulator of MAPK-RAS-ERK pathway	Activation	Rare CLL driver	(45)
PTPRD	–	Tumor suppressor colocated with *CDK2NA*	Inactivation	Receptor protein tyrosine phosphatase regulating cell growth	(45)
LAG3	–	Membrane protein expressed in B cells and/or TILs	Increased gene expression	Immune checkpoint gene. LAG3 protein is expressed on immune cells and in the setting of persistent antigen exposure; co-expressed with other immune checkpoints in dysfunctional T cells.	(50)
CXCR4	–	G-protein-coupled receptor regulating hematopoietic stem cell homeostasis, myelopoiesis, lymphopoiesis, and homing of immune cells toward its ligand C-X-C motif chemokine 12 (CXCL12)	Activation *via PLK1*/*FOXM1*	Involved in the migration and trafficking of malignant B cells	(58)

BCR, B-cell receptor; CLL, chronic lymphocytic leukemia; TIL, tumor infiltrating lymphocytes; RT, Richter transformation.

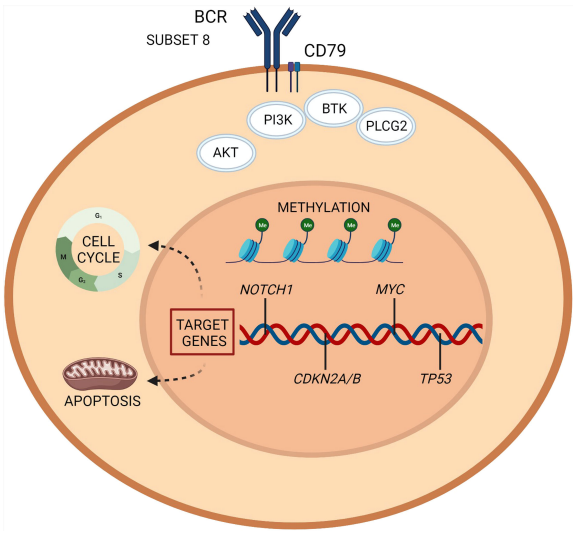

FIGURE 1 | Richter transformation: intrinsic vulnerabilities and targets for treatment. A representation of the molecular pathogenesis of Richter transformation, resulting from a number of epigenetic and genetic lesions occurring in the tumor cell population. Recurrently mutated genes affect DNA repair, B cell receptor, and chromatine modification. Created with BioRender.com.

(CK2, CK with major structural abnormalities) or high-CK (CK with >5 chromosome abnormalities), together with IGHV unmutated status, 11q deletion, *TP53* disruption and Binet stage B/C, have been identified as predictors for RT prognosis. According to the Richter syndrome scoring system, patients with high-CK and/or CK2 show a 10-year risk of developing RT of 31%; patients with unmutated IGHV/11q deletion/*TP53* disruption/>B Binet stage show a 10-year risk of 12%; while patients with mutated IGHV without CK and with wild type *TP53* display a 10-year risk of developing RT of only 3% (60).

Role of Previous Treatment

Longer survival is reported for patients with treatment-naïve CLL when compared to the relapsed/refractory setting (12 vs 7 months) (10, 61–65). RT after ibrutinib or venetoclax shows an aggressive behavior. The median OS after progression for double

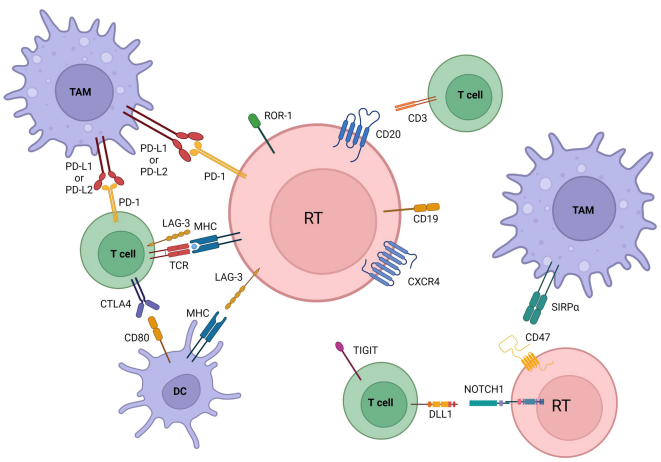

FIGURE 2 | Microenvironmental crosstalks and druggable targets in Richter transformation. Pathway activation and changes in immune checkpoints profile are also involved in transformation. Communication between the tumoral cells, dendritic cells, tumor associated macrophages (TAM), and T cells is established by direct contact, chemokine/cytokine receptors, adhesion molecules and ligand-receptor interactions. Immune inhibitory molecules (PD-L1 among others) facilitate tumor cells to evade immune-response and maintain tolerance. All of the here represented are druggable targets in RT. BCR, B cell receptor; DC, dendritic cells; TAM, tumor associate macrophage. Created with BioRender.com.

class-resistant CLL patients (i.e., CLL resistant to both BTK and BCL2 inhibitors) is 3.6 months, and this class of patients represents a clinical unmet challenge in the era of novel agents (66).

TREATMENT OF DLBCL-RT

History and comorbidities of patients developing RT drive the choice of treatment in this challenging setting. A proposed algorithm for DLBCL-RT is depicted in **Figure 3**.

Chemo-Immunotherapy

Translating treatment experience from the aggressive B-cell non-Hodgkin lymphoma setting, combinations of anti-CD20 monoclonal antibodies and polychemotherapy regimens have been indicated to treat patients with DLBCL-RT.

The historical standard regimen for DLBCL R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone) produced response rates of up to 67% (complete response, CR 7%), reaching a median progression free survival (PFS) of 10 months and a median OS of 21 months. Reported adverse events were mainly hematological (65%), while severe infections were described in 28% of patients (67). Another case series reports data on 48 patients with DLBCL-RT treated with R-CHOP with a response rate of 37% and a median OS of 35 months (9).

The combination of CHOP chemotherapy with the anti-CD20 ofatumumab (O) showed an overall response rate (ORR) of 46% (CR 27%), a median PFS of 6 months and a median OS of 11 months. Reported adverse events were infections and hematologic toxicities (thrombocytopenia, febrile neutropenia, sepsis) (68, 69).

More aggressive CIT regimens were assessed, though not achieving an improved outcome. R-EPOCH (rituximab, etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin), a regimen indicated in high grade B-cell lymphoma, reached a response rate of only 20%, a median PFS of 3 months and a median OS of 6 months (70). Shorter PFS and OS were observed in patients with disrupted *TP53* and an underlying CLL characterized by complex karyotype.

Poor median OS and response rates of 40% were reported with the hyper-CVAD regimen (fractioned cyclophosphamide, vincristine, doxorubicin, and dexamethasone), alone or in alternating combination with methotrexate and ara-C. Severe hematotoxicity, high infection rates (developed by 50% of patients) and a treatment-related mortality of nearly 20% were reported (71), even under the proper prophylaxis with granulocyte–macrophage colony stimulating factor (GM-CSF) (72).

The OFAR 1 and 2 trials explored the combination of oxaliplatin, fluradabine, ara-C and rituximab at different dosages to prevent toxicities. The ORR ranged from 39 to 50%, being characterized by a median PFS of 3 months and a median OS of 6–8 months (73, 74). The main complication was myelotoxicity, with no significant improvement in myelosuppression severity for patients enrolled in the OFAR 2 trial compared to the OFAR 1 trial (74).

Consolidation With Stem Cell Transplantation

Due to the high rate of relapses and poor OS after CIT, stem cell transplantation (SCT) has been proposed as a consolidation

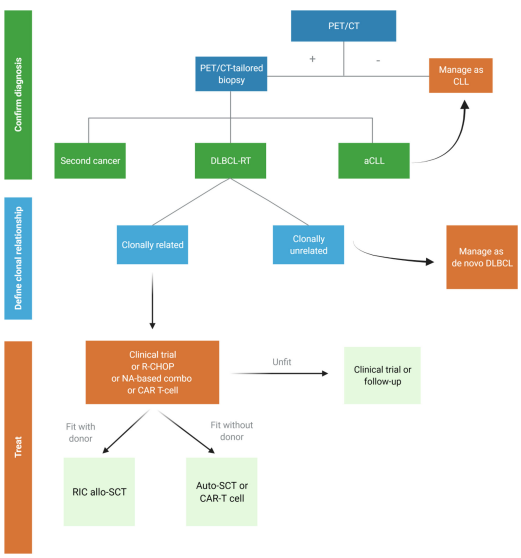

FIGURE 3 | Proposed algorithm for the management of suspected diffuse large B-cell Richter transformation (DLBCL-RT). aCLL, accelerated chronic lymphocytic leukemia; auto-SCT, autologous stem cell transplantation; DLBCL, diffuse large B-cell lymphoma; NA, novel agents; RIC allo-SCT, reduced intensity conditioning stem cell transplantation. Created with BioRender.com.

strategy for DLBCL-RT. The benefit of receiving SCT is underlined by a median survival of 5 years vs <1 year for patients not undergoing SCT, and relies on high-dose cytotoxic therapy combined to a graft-versus-leukemia effect in the case of allogeneic SCT. The latter is confirmed by a plateau in relapse-free survival curves after allogeneic SCT (75).

However, most patients (~85%) cannot access SCT, either due to their poor performance status, a refractory disease to induction treatments, and/or the lack of donor availability (75).

The Center for International Blood and Transplant Research (CIBMTR) registry study evaluated outcomes in 53 and 118 patients with DLBCL-RT treated with autologous SCT and allogeneic SCT, respectively. A 37% relapse incidence, 48% PFS, and 57% OS at 3 years was reported in the autologous SCT cohort. For patients treated with allogeneic SCT, relapse incidence, PFS, and OS at 3 years were 30, 43, and 52%, respectively. In the latter cohort, outcomes strongly correlated with the response status at SCT (3-year OS 77% for patients reaching a CR with induction therapy versus 57% for partial responses), while cytogenetic abnormalities and prior novel therapy did not show an impact on survival (76).

A single-center retrospective analysis of 23 RT patients undergoing reduced intensity conditioning (RIC-SCT) reports a 5-year PFS of 40% and OS of 58% (77). Young age (<60 years), deeper response at SCT and having received <3 previous lines of therapy positively correlated with outcomes, while cytogenetic/molecular features and exposure to novel agents did not show an impact on PFS/OS (77, 78). Total body irradiation (TBI) resulted in poorer outcomes (77).

A median OS of 17 months has been recently reported by GCLLSG for 3 patients undergoing allogeneic SCT for RT (9).

In a meta-analysis evaluating the outcome of patients with RT undergoing allogeneic SCT, the relapse rate was 28% and the non-relapse mortality 24%, showing similar rates previously reported for patients diagnosed with other lymphoproliferative diseases (78).

Overall, young and fit patients with DLBCL-RT attaining deep responses with induction treatment can benefit both from autologous SCT and RIC allogeneic SCT, while TBI-containing RIC should be considered with caution.

Novel Agents

Recent advances in the understanding of deregulated molecular pathways in RT led to investigate the efficacy of targeted agents, with promising results.

XPO1 is a nucleo-cytoplasmic transporter of tumor suppressor proteins, whose activity is often upregulated in cancers. Selinexor, a selective inhibitor of nuclear export, acts with the aim of maintaining tumor suppressors within the nucleus to preserve their activity. In DLBCL-RT selinexor produced a response rate of 33% with an acceptable toxicity profile (79). Unfortunately, the phase 2 study (NCT02138786) was closed prematurely due to enrolment hurdles.

Bruton's tyrosine kinase (BTK), a component of BCR, plays a central role in B-cell malignancies, regulating cell proliferation and survival. Ibrutinib, the first-in-class BTK inhibitor, showed activity in DLBCL-RT (80–82), with a survival benefit and a 16 months PFS (82). Responses to ibrutinib rechallenge have been reported after incidental RT diagnosis upon ibrutinib discontinuation in three patients with CLL (83). Acalabrutinib is a second generation oral BTK-inhibitor with an ORR of 40% (CR 8%) (84) and a median PFS of 3 months. The phase 1/2 BRUIN study (NCT03740529) evaluated safety and efficacy of pirtobrutinib (loxo-305), a next generation, highly selective, non-covalent BTK inhibitor in previously treated RT (85). Among 15 patients, pirtobrutinib reached a response rate of 67% (CR 13%). The 6-month PFS rate was estimated to be 52%. The median number of prior lines of system therapy was 6, with 82% of DLBCL-RT patients having received a prior BTK inhibitor, 59% a prior BCL-2 inhibitor, and 6% CAR T-cell therapy.

The reversible BTK inhibitor nemtabrutinib (previously known as ARQ531 or MK-1026) showed efficacy in *in vivo* BTK-resistant CLL/RT models (i.e., Eμ-MYC/TCL1 murine model recapitulating the disease phenotype of RT) (86, 87). Inhibitory activity of ARQ531 on the BCR pathway was reported both upstream and downstream of BTK *via* SYK, AKT, and MEK1/ERK. This effect was maintained also in presence of the C481S *BTK* resistance mutation and autoactivating *PLCγ2* mutations. Safety and activity profile of nemtabrutinib are being explored in ongoing clinical trials enrolling patients with B-cell malignancies, including RT (NCT03162536, NCT04728893) (see **Table 2**).

Patients with *TP53*/*NOTCH1*-disrupted high-risk CLL and RT display increased constitutive AKT phosphorylation (88). Some activity data has been reported with the PI3K inhibitor idelalisib in patients with RT (89), prompting further investigation of these agents in this condition.

Considering that DLBCL-RT harbors *TP53* alterations, novel treatments and combinations in this setting need to act in a *TP53*-independent way. Venetoclax inhibits BCL2 and is strongly active in high-risk CLL, acting independently from *TP53* (90). In the M12-175 (NCT01328626) phase I study, 7 patients with DLBCL-RT were treated with escalating doses of venetoclax, attaining a response rate of 43% (no CRs reported) (90). In the phase 2 study on the combination venetoclax-R-EPOCH (NCT03054896), the ORR reached 62% (42% CR with unmeasurable residual CLL in bone marrow). Median PFS and median OS were 10.1 and 19.6 months, respectively. Main adverse events were related to grade 3–4 neutropenia (65%), thrombocytopenia (50%) and febrile neutropenia (38%). No tumor lysis syndrome (TLS) occurred with daily venetoclax ramp-up after 1 lead in cycle of R-EPOCH (91).

Immune checkpoint deregulation is common in the setting of DLBCL-RT, which frequently develops upon an exhausted immune system. Immune checkpoint blockade with the monoclonal anti-PD1 antibody pembrolizumab produced 44% response rate (NCT02332980) (47). Importantly, responses were observed only in patients previously exposed to ibrutinib, with a median OS not reached (median OS of 10.7 months for the whole cohort). Preclinical studies reported synergistic antitumor effects between BTK and the PD-1/PD-L1 inhibitors (92). Ibrutinib exerts immune modulating effects through IL-2 inhibition,

TABLE 2 | Ongoing trials with targeted agents in diffuse large B-cell Richter transformation.

Interventions	Targeted pathway and/OR Antigen	Ref.
Acalabrutinib + R-CHOP	BTK	NCT03899337
Ibrutinib + DA-EPOCH-R	BTK	NCT04992377
Venetoclax + DA-EPOCH-R	BCL-2	NCT03054896
Blinatumomab after R-CHOP	CD19	NCT03931642
Polatuzumab vedotin + DA-EPOCH-R	CD79b	NCT04679012
Epcoritamab	CD3/CD20	NCT04623541
Nemtabrutinib (ARQ 531)	BTK	NCT03162536
		NCT04728893
Ibrutinib + Nivolumab	BTK + PD-1	NCT02420912
Zanubrutinib + Tislelizumab	BTK + PD-1	NCT04271956
Duvelisib + Nivolumab	PI3K + PD-1	NCT03892044
Copanlisib + Nivolumab	PI3K + PD-1	NCT03884998
Duvelisib + Venetoclax	PI3K + BCL-2	NCT03534323
Umbralisib + Ublituximab	PI3K, CK1 + CD20	NCT02535286
Obinutuzumab + Ibrutinib + Venetoclax	CD20 + BTK + BCL-2	NCT04939363
Atezolizumab + Obinutuzumab + Venetoclax	PD-L1 + CD20 + BCL-2	NCT02846623
Atezolizumab + Obinutuzumab + Venetoclax	PD-L1 + CD20 + BCL-2	NCT04082897
Ipilimumab + Ibrutinib + Nivolumab	CTLA-4 + BTK + PD-1	NCT04781855
TG-1801 + Ublituximab	CD47/CD19 + CD20	NCT04806035
ALX148 + Rituximab + Lenalidomide	CD47 + CD20	NCT05025800
VIP152	CDK9	NCT04978779
Zilovertamab vedotin (VLS101)	ROR1	NCT03833180
CD19 CAR-T cell	CD19	NCT04892277
CD19 CAR and PD-1 Knockout T Cells	CD19	NCT03298828
CAR70/IL15 NK cells	CD70	NCT05092451

deregulating T-cell proliferation and differentiation signaling. The combination of ibrutinib with nivolumab (an anti-PD1 antibody) was assessed in patients with relapsed or refractory hematological malignancies, namely, high-risk CLL/SLL, follicular lymphoma, DLBCL, and RT (93). The ORR was 65% in the DLBCL-RT cohort (10% CR), with a median duration of response of 6.9 months. A phase 2 trial is exploring the combination of the anti-PD-L1 antibody atezolizumab with venetoclax and the anti-CD20 antibody obinutuzumab in patients with untreated or R/R RT (NCT02846623). Venetoclax treatment is introduced at cycle 2, after obinutuzumab + atezolizumab lead-in. Data from this ongoing trial report an ORR of 100% (71% CR) for the first 7 patients with untreated RT enrolled, with responses achieved early after the introduction of venetoclax (94). After a median follow-up of 11.2 months, three of the complete responders underwent consolidation with allogeneic-SCT and no fatalities were reported.

Glofitamab is a T-cell-engaging bispecific antibody with a 2:1 anti-CD20/CD3 structure, that has been investigated in a phase I study enrolling patients with R/R non-Hodgkin lymphoma (*de novo* DLBCL, transformed follicular lymphoma, primary mediastinal B-cell lymphoma, mantle cell lymphoma, and RT). In this study, the reported ORR and CR rates were 48 and 33%, including 41 and 28% in patients with DLBCL (95). Cytokine release syndrome (CRS) was the most common adverse event (25% grade 3, 2% grade 4), and its incidence increased with higher doses but declined after the first administration (13% events at cycle 2, 6% at cycle 3 or later).

CD19 is a transmembrane protein found invariably on B cells (except for plasma cells) with a pivotal role in BCR signaling (96). Its sustained expression even upon tumoral transformation of B cells led to the development of CAR T-cell targeting its surface antigenic domain (97, 98). It should be noted that a proportion of patients relapsing after treatment with CD19 CAR-T cells may develop a $CD19^{-}/CD19^{dim}$ disease as a mechanism of escape (99–101). In the setting of DLBCL-RT, CD19 CAR-T cells showed response rates at 4 weeks after infusion ranging from 71 to 83% (101–103) and a 1-year OS and PFS of 86 and 59%, respectively (102). In one of these studies 8 patients with RT after chemoimmunotherapy and therapy with BTK and/or BCL2 inhibitors were enrolled (103). Patients received locally produced 1×10^6 autologous CD19 CART-cells/kg, modified with retroviral vector encoding a CAR comprising FMC63 anti-CD19 ScFv linked to a CD28 costimulatory domain, and CD3-zeta intracellular signaling domain. RT patients receiving CD19-CAR T-cells had a median age of 64 years (median age at CLL diagnosis 56 years), being previously treated with a median of 3 lines of therapy for CLL. On day 28 a complete response was reported in all the responders (71%, 5/8 patients). After median follow up of 6 months, two patients proceeded to allogeneic-SCT. CRS grade ≥3 requiring tocilizumab was described in 3/8 patients, while grade 3 central nervous system (CNS) toxicity was experienced by two patients.

Higher response rates (8/9 DLBCL-RT patients) are reported using axicabtagene ciloleucel CAR-T cell therapy (104). Of these patients, 8 were previously treated with kinase inhibitors and one patient died due to an infection. A CR was reported for 5/8 patients, while a partial response was described in 3 patients.

In another phase 1 study, four patients with RT were treated with escalating doses of autologous 19-28z/4-1BBL+ CAR T cells (NCT03085173) (105). Of the responders, 2/3 achieved CR and no severe CRS was reported.

ARI-0001 are autologous T-cells transduced with a CD137-based CAR construct targeting CD19 and developed at the Hospital Clinic of Barcelona (106). The CAR-T product ARI-0001 was administered to six patients with RT (five patients with DLBCL-RT and one patient diagnosed with plasmablastic transformation), achieving CRs in three patients sustained at 1.4, 12.5, and 26.7 months after treatment, respectively. With a median follow-up of 5.6 months, one patient had a stable disease, and two patients experienced a CD19-negative relapse despite no prior anti-CD19 therapy. The safety profiled was considered acceptable, with only one fatality reported due to the COVID pandemic in a patient not being treated.

Natural killer (NK) cells belong to the innate immune system and play a central role in immune surveillance. Their manageability relies upon the possibility to administer them without the need for full HLA matching, even when obtained from an allogeneic source (i.e., cord blood) (107). In the setting of CAR-engineering, this translates into an easier manufacture since there is no need to generate a patient-specific product. CAR-NK cells derived from cord blood and transduced with anti-CD19 CAR, interleukin-15, and inducible caspase 9 were explored in patients with $CD19^+$ lymphoid tumors including CLL/RT, with promising results (108). Interestingly, one patient with RT experienced CR from his transformed component but persistence of the CLL counterpart. No major toxic effect and/or graft-versus-host disease was reported. Despite the HLA mismatch, CAR-NK cells were found to persist at low levels after 12 months from infusion.

TREATMENT OF HL-RT

The standard of care for *de novo* HL is the regimen indicated for patients with the HL-RT (109–112), with a reported response rate of 40-60% under ABVD (Doxorubicin, Bleomycin, Vinblastine, Dacarbazine). The median OS is 4 years in this setting. Bleomycin exposure can cause a severe pulmonary toxicity, leading to investigate the omission of this agent from the standard ABVD regimen (112). Following the results coming from the setting of advanced HLs, bleomycin can be safely omitted after two cycles of ABVD if interim PET shows remission (Deauville score 1–3). Escalation to BEACOPP in fit and younger patients should be considered in case of a positive interim PET, while radiotherapy could be an option for older and unfit patients (113). Stem cell transplantation is less used for consolidation in this setting, because of the longer survival observed compared to the DLBCL variants.

FUTURE PERSPECTIVES

Diagnosis

Artificial intelligence tools can assist the diagnostic process for patients with a suspected RT. Four biomarkers have been recently identified to have consistent value for an RT-diagnosis model, according to cytologic (nuclear size and nuclear intensity) and architectural (cellular density and cell to nearest-neighbor distance) characteristics (114). This model was used to distinguish CLL from aCLL and RT cases with a good performance, and could be of support for further studies. Given the importance of distinguishing between aCLL and RT to select the correct therapeutic approach, more efforts to define a biological picture underlying the proliferation of RT cells are of outmost value in the era of targeted therapies.

PET/CT parameters SUV-related (i.e., SUV lean body mass, SUV body surface area, lesion-to-liver SUV ratio, and lesion-to-blood-pool SUV ratio) showed a correlation with DLBCL-RT diagnosis and/or OS and represent possible candidates for diagnostic biomarkers to further explore (115, 116). Moreover, novel PET radiotracers and PET–MRI are being explored in the setting of RT (117).

Biology and Treatment

CDK4/6 inhibitors (i.e., palbociclib) have been recently identified as potential agents to overcome *CDKN2A/B* dysregulation (40). Palbociclib demonstrated activity in inhibiting RT-cell proliferation and showed an *in vitro* synergistic activity when combined with the BCR-signaling directed compounds ibrutinib, idelalisib, and fostamatinib.

LAG3 is an emerging target for immune checkpoint blockade (50). Clinical trials are investigating *LAG3* inhibitors in hematological and solid cancers (NCT02061761; NCT01968109). Further assessment of *LAG3* inhibition, either alone or in combination with anti-PD-1 to enhance anti-tumor T-cell responses in RT is warranted.

Genomic data from the WGS confirm the pathogenic role of DNA damage response (DDR) pathway deregulation in RT (45). The role of DDR inhibitors such as PARP or ATR inhibitors has still to be assessed in RT.

The antibody-conjugate VLS-101 includes a humanized immunoglobulin G1 monoclonal antibody that binds ROR1, which is expressed by CLL lymphocytes to regulate chemotaxis and proliferation signaling (118, 119). VLS-101 attained complete and sustained remissions in RT patient-derived xenografts (RT-PDXs) expressing high levels of ROR1 (120). A phase 1 clinical trial of VLS-101 (NCT03833180) is enrolling patients with RT and other hematological neoplasms. Concomitantly, a phase 1 clinical trial (NCT02706392) is exploring the efficacy of anti-ROR1 CAR-T cells in patients with refractory CLL.

U-RT1, is a cell line derived from a highly proliferating RT clonally related to the underlying CLL (121). It is characterized by a complex karyotype with driver aberrations characteristic for RT such as genetic alterations of *TP53*, *CDKN2A*, and *NOTCH1*. This model represents a valuable tool for RT investigations and drug development.

Data on three newly established PDX models of RT-DLBCLs were recently published, namely, clonally-related and clonally-unrelated RT (122). These PDX models display protein expression of IRF4, TCF4, and BCL2. CRISPR knockout of *IRF4* led to reduced c-Myc levels and increased sensitivity to BET inhibitors. Co-treatment with a BET inhibitor or BET-PROTAC and ibrutinib or venetoclax showed synergistic *in vitro*

lethality in the RT-DLBCL cells. When compared to single agent, combination of BET-PROTAC and venetoclax significantly reduced tumor burden and improved survival in immune-depleted mice engrafted with clonally related RT-DLBCL.

A potential synergistic effect of PI3K and BCL2 inhibitors has been proposed, based on the crosstalk between PI3K and apoptotic pathways (123). It has been shown that the inhibition of PI3K signaling by duvelisib leads to GSK3b activation and subsequent degradation of both c-Myc and Mcl-1. This crosstalk sensitizes RT cells to BCL-2 inhibition. Drug combination trials are ongoing, also in the setting of RT-DLBCL (NCT03892044).

In the field of CARs, targeting the transmembrane protein CD37 is another potential application for patients with B-cell malignancies. CD37 is expressed in mature B cells and at lower levels also on plasma cells and dendritic cells. Indeed, CD37 CAR-T cells were found to play a cytotoxic activity *in vivo* in B-cell tumor models (124). Dual targeting has already been suggested as a method to overcome treatment resistance due to the development of specific antigen loss consequent to CAR infusion. A bispecific CD37/CD19 CAR-T product is being developed to assess safety and efficacy in preclinical B-cell tumor models. Bispecific CD19/22 CAR-T cells have been already explored in non-Hodgkin lymphomas (NCT03196830), showing promising results (ORR 79.3%, CR 34.5% with 12-month PFS and OS of 40 and 63%, respectively) (125). The employment of CD19 CAR-NK cells in B-cell malignancies is also being explored in different ongoing phase 1 trials (i.e., NCT04887012, NCT04639739, NCT04796675, and NCT05020678), and novel targets for CAR-NK cells are object of study (i.e., CAR70/IL15-transduced NK cells in NCT05092451). Efficacy of these agents needs to be assessed in the setting of RT.

A list of ongoing trials with targeted agents in RT is reported in **Table 2** (updated from clinicaltrials.gov on Feb 20, 2022).

CONCLUSIONS

Patients with CLL progressing on novel agents represent a new high-risk prognostic group with adverse outcome in case of transformation. The promising combination of CIT with the novel agent venetoclax for DLBCL-RT confirms the synergistic effect of the approaches. The availability of new preclinical models is progressively expanding our understanding of RT biology, laying the foundations for targeted treatments which might be better tolerated.

AUTHOR CONTRIBUTIONS

AC and DR wrote the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Cellular Therapy in High-Risk Relapsed/Refractory Chronic Lymphocytic Leukemia and Richter Syndrome

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Despite the development of highly effective, targeted inhibitors of B-cell proliferation and anti-apoptotic pathways in chronic lymphocytic leukemia (CLL), these treatments are not curative, and many patients will develop either intolerance or resistance to these treatments. Transformation of CLL to high-grade lymphoma—the so-called Richter syndrome (RS)—remains a highly chemoimmunotherapy-resistant disease, with the transformation occurring following targeted inhibitors for CLL treatment being particularly adverse. In light of this, cellular therapy in the form of allogenic stem cell transplantation and chimeric antigen receptor T-cell therapy continues to be explored in these entities. We reviewed the current literature assessing these treatment modalities in both high-risk CLL and RS. We also discussed their current limitations and place in treatment algorithms.

Keywords: CLL (chronic lymphocytic leukemia), CAR (chimeric antigen receptor) T-cell therapy, richter syndrome, cellular therapy, allogenic stem cell transplantation

INTRODUCTION

Substantial progress in understanding the pathobiology of chronic lymphocytic leukemia (CLL) has led to the development of drugs targeting key mechanisms of tumor proliferation and survival. Agents targeting the B-cell receptor (BCR) signaling cascade and B-cell lymphoma-2 (BCL2) expand treatment options for high-risk CLL including *TP53*-disrupted and relapsed/refractory (R/R) disease. While combination therapy can achieve deep and durable remissions, CLL remains incurable. High-grade transformation of CLL into aggressive B-cell lymphoma called Richter syndrome (RS) complicates CLL in 2%–15% (1–4). The wide range in incidence may be explained by the heterogeneous mutational status of CLL patients from different studies. In fact, specific biomarkers (e.g., NOTCH1, TP53 abnormalities, and trisomy 21) coupled with definite microenvironmental interactions associate to a higher risk of RS transformation (5, 6). Disease progression and high-grade transformation are a frequent cause of targeted therapy discontinuation in trial (3, 7) and non-trial (8–11) populations. Infrequently, RS presents *de novo* in untreated CLL patients.

Most RS cases represent transformation to a clonally related activated B-cell-type diffuse large B-cell lymphoma (DLBCL) (90%–95%), with a small proportion transforming to Hodgkin lymphoma (12). RS shares morphological characteristics with DLBCL, but its molecular profile is distinct. RS is enriched for mutations in poor-risk CLL drivers and the DNA damage response pathway (13).

Therapy for RS typically mirrors DLCBL, but outcomes are considerably worse (5, 14, 15) with median overall survival (OS) of 6–12 months (16–19). Intensification with hyper-CVXD (fractionated cyclophosphamide, vincristine, liposomal daunorubicin, dexamethasone with or without methotrexate) or OFAR (oxaliplatin, fludarabine, cytarabine, and rituximab) protocols may deliver improved responses, but responses are not sustained and OS remained <12 months (14, 20–23). Novel therapies targeting the BCR pathway continue to be explored in RS. Ibrutinib (24), acalabrutinib (25), or venetoclax monotherapy experience is reported in small series, with a short progression-free survival (PFS). Acalabrutinib plus R-CHOP is being examined in the STELLAR trial (26). Venetoclax with dose-adjusted R-EPOCH has shown promise, albeit in a selected cohort (27).

HISTORY OF ALLOGENIC STEM CELL TRANSPLANTATION IN CLL

Given the evidence for graft-versus-leukemia (GVL) effect (28–31), there is continuing interest in defining the exact role of allogenic hematopoietic stem-cell transplantation (alloSCT) in CLL. Prospective data demonstrated a promising 2- to 6-year event-free survival (EFS) and OS rates ranging 30%–70% following reduced intensity conditioning (RIC) (32–34), demonstrating curative potential for R/R CLL patients. However, owing to significant inherent risks (33), alloSCT has historically been reserved for patients with sufficiently high-risk disease in the context of conventional chemoimmunotherapy induction.

Based on the 2007 EBMT consensus paper, high risk was defined in younger/fit patients as non-response or relapse within 24 months of having achieved a response to purine-analogue-based induction or post-autologous transplantation and the presence of deletion of 17p13 [del(17p)] by fluorescence *in situ* hybridization (FISH) or TP53 mutation by sequencing (28). Based primarily on retrospective data, guidelines advocated for the early consideration of related or unrelated donor alloSCT during CLL therapy in high-risk individuals (28). Complex karyotype (CK) defined as ≥3 distinct chromosomal abnormalities, in more than one metaphase, is increasingly recognized as heralding an adverse clinical course and informs selection of patients for cellular therapies (35–38).

In the pre-novel therapy era, these recommendations represented pragmatic guidance for the management of high-risk chemoimmunotherapy refractory patients and were accordingly widely adopted. However, the subsequent introduction and demonstration of long-term efficacy and safety of targeted inhibitors in CLL has unsurprisingly resulted in a marked reduction in transplantation (34, 39). The precise role of alloSCT within the current CLL treatment paradigm remains undefined.

OUTCOMES IN DUAL TARGETED (BTK AND BCL2) INHIBITOR-EXPOSED CLL PATIENTS

With the advent of highly effective targeted inhibitors of Bruton tyrosine kinase (BTKi) (40–42) and BCL2 (43, 44) as treatment at frontline or relapse, the importance of adverse factors described in the immunochemotherapy era is challenged, e.g., 11q deletion and survival outcomes continue to markedly improve. Where access allows, most CLL patients will now cycle through time-limited venetoclax-based therapy (including potentially re-treatment) and continuous covalent BTK inhibition (cBTKi) with or without anti-CD20 monoclonal antibody. Accumulating evidence suggests that the order of such therapy is of relatively little importance with evidence of cross-resistance of drug classes lacking (45–47). Although high-risk patients are still often defined as those with IGVH-unmutated disease, TP53 mutations and/or 17p deletion, and CK, outcomes are demonstrably poor in the relatively small published patient series who develop resistance or intolerance to both major classes of targeted inhibitors, namely, cBTKi and BCL2i (11, 45, 48, 49).

A series of 17 patients who developed progressive disease (PD) after both cBTKi and BCL2i classes were recently reported (49). The cohort was heavily pre-treated with a median of four prior lines of therapy and displayed high-risk genomic features (CK in 12/12 tested, del17p/TP53 mutations in 15/17). Median time to progression on prior venetoclax was 24 months and that on prior cBTKi was 25 months. Progression following both agents was with CLL in 11 patients and RS in 6. Median OS at this juncture was only 3.6 months.

Phosphoinositide 3-kinase (PI3K) inhibition is a licensed option in this space; however, data are both limited and disappointing. Seventeen cBTKi/BCL2i-exposed patients observed an overall response rate (ORR) of 47% with a median PFS (mPFS) of only 5 months (45).

Non-covalent BTKis (ncBTKi) hold great promise in this dual-exposed space. Pirtobrutinib is a reversible ncBTKi active in C481S mutated and wild-type CLL (50, 51). Accumulating data from the BRUIN trial demonstrated an ORR of ~70% in 108 dual-exposed patients (median of five prior lines) and an mPFS of 18 months (52). Other ncBTKis such as MK1026 are in development and demonstrate efficacy in dual-exposed patient, but to date, data are less mature, and small patient numbers are reported (53). Despite clear promise, ncBTKis are not licensed to date.

The largest series describes outcomes in 125 "dual-exposed" CLL patients to cBTKi and venetoclax (54). Most common subsequent strategies included ncBTKi (n=45), cBTKi (n=43), immunochemotherapy (n=23), PI3Ki (n=24), alloSCT (n=17), chimeric antigen receptor (CAR) T-cell therapy (n=9),

venetoclax re-treatment (n=6), and others (n=44). ORR and PFS estimates were as follows: CAR T-cell therapy (85.7%; mPFS, 4 months), alloSCT (76.5%; mPFS, 11 months), ncBTKi (75.0%; mPFS, not reached), PI3Ki (40.9%; mPFS, 5 months), CIT (31.8%; mPFS, 3 months), and venetoclax re-treatment (ORR, 40%; mPFS, 14 months), demonstrating the lack of clear standard approach in this setting.

In summary, this so-called "dual-exposed" patient cohort now represents the area of greatest and rapidly growing unmet medical need in CLL (54, 55).

ALLOSCT FOR CLL IN THE TARGETED INHIBITOR ERA

Given the limited prognosis faced by multiply R/R patients in the current targeted inhibitor era, there is renewed interest in the curative potential of alloSCT. Several recent series highlight the efficacy of alloSCT in dual-exposed patients (**Table 1**).

The Dana–Farber Cancer Institute (DFCI) reported outcomes of 108 RIC alloSCT for high-risk CLL, defined as any of the following: del(17p); ≥3 prior therapies; CK (≥3 abnormalities); IGHV unmutated; R/R to fludarabine, cyclophosphamide, and rituximab (FCR) prior to targeted therapy; poor response to prior chemoimmunotherapy; and poor response to targeted therapy. Thirty patients received prior targeted inhibitors, and 93% were refractory to ≥2 agents. The median age was 60 years, median prior therapies was 4, 76% had del(17p), 46.2% had ≥5 cytogenetic abnormalities, and 78.9% were IGHV unmutated. Median time to transplant from first-line therapy was 39 months. Remission status at alloSCT was CR in 20% and partial response (PR) in 73%. The 3-year OS and PFS were 87% and 69%, respectively. The cumulative incidence of relapse and non-relapse mortality (NRM) was 24% and 7%, respectively. The hematopoietic cell transplantation-specific comorbidity index (HCT-CI) was the only baseline clinical features (including HLA matching status, number and type of prior targeted inhibitors, and adverse genetic features) associated with an increased risk of death [hazard ratio (HR), 1.4; p=0.032] on univariable analysis (56).

The above data supported by a US/European collaboration where outcomes of 65 patients treated predominantly with RIC alloSCT following exposure to ≥1 targeted therapy are reported. Most patients had adverse genetic features including *TP53* mutation (51%), del(17p) (44%), and CK (50%). Two-year OS, PFS, NRM, and relapse incidence was 81%, 63%, 13%, and 27%, respectively. Grade ≥3 graft-versus-host disease (GVHD) developed in 27%, with a day+100 cumulative incidence of moderate–severe acute GVHD of 24%. Critically, adverse genetics features, prior number/type of targeted inhibitor exposure, remission status (CR vs. PR), and transplant characteristics were not independently associated with PFS/OS (57).

The most recent small (n=35) US series also analyzed the efficacy of RIC alloSCT in high-risk CLL patients (n=35), including a subset with RS. Of the CLL cohort without RS, 85% had adverse genetic features, and 65% were in PR at alloSCT. The 5-year PFS and OS was 40% and 58%, respectively. There was no statistically significant difference between RS and non-RS patients. Outcomes were again agnostic to adverse baseline genetic characteristics and prior targeted inhibitor exposure. The key clinical features associated with an improved PFS/OS following RIC alloSCT were treatment-sensitive response and ≤3 lines of prior therapy at alloSCT. Use of total body irradiation (TBI) containing RIC regimens was associated with an inferior PFS, OS, and relapse-free survival (58).

Taken together, these retrospective series highlight the efficacy and safety of RIC alloSCT in patients with high-risk R/R CLL following targeted inhibitor exposure and provide evidence for a durable graft-versus-leukemia effect. Critically, they advocate for the early identification of eligible patients and prioritization of alloSCT in those with treatment-sensitive disease.

Within the current CLL treatment paradigm, the curative potential of alloSCT must be balanced against novel agents accessible within trials and the well-described risks, which preclude a significant proportion of patients by virtue of their age, frailty, or comorbidities. For this subset of high-risk patients, alternative novel strategies, including cellular therapies and ncBTKi should be explored.

CHIMERIC ANTIGEN RECEPTOR T CELL THERAPY IN CLL

Over the past decade, CAR T-cell therapy has revolutionized the treatment of non-Hodgkin lymphoma (NHL). Three pivotal trials in multiply R/R-aggressive NHL patients demonstrated

TABLE 1 | Allogenic stem cell transplantation for CLL and Richter syndrome.

Study group	Diagnosis	N.	OS	PFS	NRM
Kim et al. (56)	CLL	108	87% (3 years)	68% (3 years)	7% (3 years)
Roeker et al. (57)	CLL	65	81% (2 years)	63% (2 years)	13% (2 years)
Lahoud et al. (58)	CLL	35	66% (2 years)	46% (2 years)	26% (2 years)
Cwynarski et al. (59)	RS	25	36% (3 years)	27% (3 years)	26% (3 years)
Kim et al. (60)	RS	28	53% (4 years)	39% (4 years)	29% (4 years)
Lahoud et al. (58)	RS	23	74% (2 years)	65% (2 years)	13% (2 years)
Herrera et al. (61)	RS	118	52% (3 years)	43% (3 years)	27% (3 years)
Kharfan-Dabaja et al. (62)	RS	19	50% (4 years)	50% (4 years)	40% (4 years)

CLL, chronic lymphocytic leukemia; NRM, non-relapse mortality; OS, overall survival; PFS, progression-free survival; RS, Richter syndrome.

ORR rates of 52%–74% with 1-year OS rates of 48%–59% (63–65), resulting in the incorporation of this option in clinical practice (66–68). CAR T-cell therapy has been explored in CLL, first as monotherapy and recently in combination with ibrutinib (**Table 2**).

Turtle and colleague enrolled 24 R/R CLL patients in a phase I/II trial where a defined composition of autologous CD4+- and CD8+ CD19-specific CAR T cells were infused following lymphodepletion. Eighty-three percent developed cytokine release syndrome (CRS), but only 25% (n=6) required tocilizumab and corticosteroids. Thirty-three percent had concomitant neurotoxicity, with 5/8 reaching grade 3 and one fatal event (69). These data are in line with relapsed/refractory DLBCL data, where CRS and neurotoxicity were reported in 42%–92% and 21%–67% of patients, respectively (63–65, 76). Notably, the ORR was 71% with an mPFS of 12.3 months (69).

The recent TRANSCEND CLL004 study enrolled 23 R/R CLL/SLL patients to receive Lisocabtagene maraleucel (Liso-cel). ORR and CR were achieved in 82% and 45%, respectively, with 75% and 65% of patients (n=20) achieving MRD negativity in peripheral blood and bone marrow, respectively. mPFS was 18 months but was significantly longer in those who achieved MRD negativity in blood and/or marrow. CRS complicated the course in 74% (9% grade 3), and 39% had neurological immune-related toxicity (22% grade 3–4) (70).

In both studies, patients had received at least two prior lines of therapy (100% had ibrutinib; 25%–65% had venetoclax), and the majority presented high-risk features including mutated TP53 and del(17p) (69, 70).

Recent data have demonstrated the persistence of CAR T cells at more than 10 years follow-up in two CLL patients who remained in complete remission. This study suggested the presence of distinct CAR T-cell populations possibly contributing to different phases of the anti-leukemia response. In one patient, an expansion of CD8+ or CD4−CD8− Helios^{hi} γδ CAR T cells in the first months after the infusion was seen, whereas later time points showed that a predominance of CD4+ CAR T cells was observed. In addition, both phenotype and antigenic signaling pathway analysis suggest that CAR T-cell proliferation was likely maintained through ongoing antigenic signaling through the transduced CAR (77).

Multiple groups are investigating strategies to improve CAR T-cell function in CLL patients. Several studies showed that ibrutinib is capable of modulating the immune dysfunction characterizing CLL patients by increasing Th1 and Th17 subsets (78, 79) and possibly reversing the exhausted T-cell phenotype associated to the expression of PD-1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on lymphocytes (79, 80). Altogether, these findings suggest that BTKi could enhance CAR T-cell expansion and effector function.

An *in vitro* study from Fan and colleagues investigated the effect of ibrutinib on CLL patient-derived CAR T-cell production and found that the viability and expansion were increased. In addition, the CAR T-cell pool displayed a decreased expression of exhaustion markers (PD-1, TIM-3, and LAG-3) and was enriched with less-differentiated cells (81), which are thought to have the greatest capacity of engraftment and long-term persistence *in vivo* (82–84).

Following these promising data, a recent study on R/R CLL patients (n=19) investigated the effect of ibrutinib administered from 2 weeks before leukapheresis to 3 months after CAR T-cell infusion. The combined treatment showed good tolerability and efficacy with an ORR of 83% and 61% of patients achieved marrow MRD negativity by IGH sequencing. Ibrutinib appeared to mitigate the CRS severity despite equivalent CAR T-cell expansion (71).

The inhibition of PI3K signaling during manufacturing has been proposed as an alternative strategy to produce less differentiated and exhausted CAR T cells. Funk and colleagues showed that the *in vitro* addition of duvelisib (PI3Kδ/γ inhibitor) can decrease the expression of exhaustion markers; increase the number of T-stem cell memory, naive, and memory cells; and normalize the CD4/CD8 ratio (85).

Finally, Liu and colleagues conducted a phase 1/2 study using HLA-mismatched anti-CD19 CAR-NK cells derived from cord blood in 11 patients (5 CLL, 1 concomitant RS). Notably, no patients experienced CRS or neurotoxicity. At a median follow-up of 13.8 months, three CLL patients obtained a CR, which was maintained at last follow-up although with the use of post-remission therapy. Despite some limitations, this proof of concept may ultimately lead to the possibility of well-tolerated NK-based off-the-shelf product (72).

ALLOSCT FOR RICHTER SYNDROME

While the outcomes for RS are dismal with conventional chemoimmunotherapy, the role of cellular therapy remains somewhat uncertain. Published data supporting alloSCT in RS are predominantly retrospective, single-center studies and report

TABLE 2 | Chimeric antigen receptor (CAR) T cell therapy in CLL and Richter syndrome.

Study group	Diagnosis	N.	ORR	CR	CRS	Neurotoxicity
Turtle et al. (69)	CLL	19	74%	11%	95%	37%
Siddiqi et al. (70)	CLL	23	82%	45%	74%	39%
Gauthier et al. (71)	CLL	19	83%	22% (all CRi)	74%	26%
Liu et al. (72)	CLL	5	80%	40%	0	0
Kittai et al. (73)	RS	9	89%	55%	100%	33%
Benjamini et al. (74)	RS	8	71%	71%	87%	37%
Turtle et al. (69)	RS	5	60%	40%	40%	20%
Ortiz-Maldonado et al. (75)	RS	5	80%	60%	80%	0

CLL, chronic lymphocytic leukemia; CR, complete response; CRi, complete response incomplete; CRS, cytokine release syndrome; ORR, overall response rate; RS, Richter syndrome.

a selected RS cohort enriched for younger, fit, chemosensitive patients. Four of the 204 patients proceeded to alloSCT in one large single-institution publication of biopsy-proven RS (86), underlying the unmet need for effective induction therapies and the rarity of transplant-eligible RS patients.

EBMT reported on 25 RS patients who underwent alloSCT between 1997 and 2007 in the pre-targeted inhibitor era. One-third were chemo-refractory. The 3-year NRM was 26%, and OS was only 36%. The authors concluded that alloSCT is a viable therapeutic option for chemosensitive RS (59). Outcomes from the pre-BTKi era may not be applicable to contemporary practice.

Many recent reports are single center but describe similarly unsatisfactory outcomes in the targeted inhibitor era. One-year NRM ranges 24%–40% and 4-year OS at 49%–53% (60, 62). A systematic review and pooled meta-analysis of studies reporting ≥10 RS alloSCTs report an NRM of 24% and OS of 49% (87).

Two recent retrospective studies describe the experience of alloSCT for RS in the BCR inhibitor era. The 1-year NRM remains significant at 12%–23%. In both groups, one-third of the patients relapsed within 3–5 years (58, 61).

Comparable outcomes for RIC alloSCT for 35 R/R CLL and 23 RS were observed (58). All RS patients were considered for alloSCT at first remission. In univariate analysis, R/R CLL and treatment-responsive RS had comparable NRM, PFS, and OS following allograft.

Disease response status pre-alloSCT is predictive of outcome in the EBMT, Memorial Sloan Kettering and CIBMTR series (58, 59, 61). Thrombocytopenia, high LDH, and HCT-CI ≥2 identified patients at increased risk (60). While ≥3 lines of therapy were associated with adverse outcomes, there was no significant difference in outcomes between patients exposed and naive to BTKi, BCL2i, or PI3K inhibitors (58, 60, 61).

There remains a paucity of data to guide decisions regarding the source of stem cells, conditioning regimes, and GVHD prophylaxis. The MSK series observed inferior outcomes with TBI-containing conditioning and recommended against its use in this population. As most RS patients are older adults, non-myeloablative regimes using mobilized peripheral blood stem cells (PBSCs) are frequently employed.

Data on cellular therapy for Hodgkin-like transformation of CLL is even scarcer. One Hodgkin-like RS was reported in the Dana–Farber series; Hodgkin-like RS was excluded from the CIBMTR publication (60, 61).

The literature on alloSCT for RS is confounded by heterogeneous RS populations, variable approaches to alloSCT, and a rapidly changing therapeutic landscape for CLL and RS. In both high-risk CLL and RS, prior exposure to BTKi or BCL2i does not appear to confer an adverse prognosis in those receiving an alloSCT. Both NRM and relapse remain significant challenges in this population.

CHIMERIC ANTIGEN RECEPTOR T CELL THERAPY IN RICHTER SYNDROME

The promising results of CAR T-cell therapy in aggressive NHL prompted studies in RS. Data on CAR T-cells efficacy in this setting are limited and conflicting to date.

A recent retrospective study reviewed nine RS patients who were heavily pre-treated (median, four lines for CLL and/or RS). All patients had high-risk features including del(17p) (n=3), CK (n=6), and *TP53* mutation (n=2). Two patients received a BTKi as bridging before Axicabtagene ciloleucel (Axi-cel) infusion, while five other patients continued the BTKi for ≥30 days after the infusion. CRS occurred in all patients (grade ≤2, n=8; grade 4, n=1), whereas grade ≥3 neurotoxicity occurred in three patients. Five patients achieved CR, and three patients obtained a PR. One patient died of bacterial pneumonia. At a median follow-up of 6 months, only one patient had progressed, whereas all the others showed sustained responses (73).

Another cohort of eight patients with similar baseline characteristics was enrolled in a single-center phase 2 trial conducted in Israel exploring the use of CAR T cells after targeted therapies. At a median follow-up of 6 months, five patients achieved CR, while three patients progressed. Seven patients developed CRS (grade 3–4, n=3) and three developed neurotoxicity (grade 3, n=2) (74).

Heterogeneous responses were observed in the five RS patients enrolled in the study conducted by Turtle and colleagues. After CAR T-cell product JCAR017 infusion, CR was observed in two patients, PR in one patient, and PD in two other cases (69).

Interestingly, a Spanish phase I study infused ARI-0001, a novel CAR T-cell construct, to five RS patients using a fractionated dose scheme. The CRS rate was 80%, whereas neurotoxicity was not observed. One patient received only 10%–40% of the expected cell dose due to CRS. Four patients responded to treatment (CR, n=3), while one remained with SD according to iwCLL/Lugano criteria. However, MRD negativity was achieved in all patients both in peripheral blood and bone marrow (75). ARI-0001 has recently been approved by the Spanish Medicines Agency (AEMPS) for patients with R/R acute lymphoblastic leukemia (ALL) >25 years of age.

Other small studies suggested lack of response to CAR T-cell therapy or non-sustained response in the context of RS (88, 89).

Published reports examining the role of CAR T-cell therapy for RS are limited by small numbers, variable approaches to concurrent therapies, and short follow-up. Despite these restrictions, disease responses are observed at least for a minority. However, the above reports highlight manageable toxicity and promising outcomes for heavily pre-treated and high-risk patients with few therapeutic options left. Further work is needed to determine the precise role of CAR T-cell therapy in the treatment of RS.

DISCUSSION

High-risk R/R CLL—particularly patients now "dual exposed" to BTKi and BCL2i—and R/R RS remain areas of ongoing clinical need and investigation. Cellular therapy in the form of alloSCT represents an ongoing option for fit, younger CLL patients achieving disease control in these settings and has demonstrable utility in the targeted inhibitor era. While CAR T-cell therapy

provides cause for optimism, the clinical data supporting this therapeutic modality at present are limited. Ongoing investigation into improving T-cell function and further prospective clinical data are needed before this treatment becomes a *de facto* standard of care approach across a wider range of R/R CLL and RS patients. Despite this, the limited data in R/R RS are promising, and, where available, this modality could be considered in R/R RS patients who can be bridged to reinfusion with reasonable performance status and disease control.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Duplication of 8q24 in Chronic Lymphocytic Leukemia: Cytogenetic and Molecular Biologic Analysis of *MYC* Aberrations

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Chronic lymphocytic leukemia (CLL) with cytogenetics findings, such as complex karyotype and deletions of *TP53* or *ATM*, is associated with adverse clinical outcomes. Additional chromosomal abnormalities further stratify patients into groups with diverse prognoses. Gain of 8q24 is one of the abnormalities considered as prognostically unfavorable. In our study, we performed a FISH analysis in an initial cohort of 303 consecutive CLL patients and determined the frequency of +8q to be 6.3 %. Our analysis confirmed the association with *TP53/ATM* aberrations and CK, as the frequency of +8q reached 26.7 % in an extended del*TP53/ATM*+CK cohort. M-FISH analysis enabled the identification of partner chromosomes where the segment of the duplicated 8q arm was localized. More detailed mapping of the gained 8q region using the M-BAND method determined the smallest amplified region 8q23-8qter. We observed significantly shorter overall survival (OS; 9.0 years in +8q-positive vs. 10.6 years in +8q-negative; p=0.02) and detected slightly higher MYC mRNA/protein levels in +8q-positive vs. +8q-negative patients.

Keywords: chronic lymphocytic leukemia, MYC, complex karyotype, 8q24 gain, prognosis

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia among adults in the Western world, with a median age of disease presentation of about 70 years. Clinical outcomes for most CLL patients have improved remarkably in the last decade, but there is still a group of high-risk patients whose treatment remains challenging (1). In the era of chemotherapy, these patients progressed in less than two years after initial treatment (2). Independent biomarkers of adverse prognosis include unmutated immunoglobulin heavy chain variable gene (UM-IGHV), *TP53* mutation/deletion, and high complex karyotype (CK), defined as five or more cytogenetic structural/numerical aberrations (3–5). Patients bearing these negative biomarkers benefit from

treatment with specific B-cell receptor (BCR) signaling inhibitors and the BCL-2 antagonist in the first line (6).

CLL patients with CK (defined as ≥ 3 cytogenetic aberrations) constitute a heterogeneous group with variable clinical outcomes. It is necessary to study these cases in more detail to reveal subgroups with less favorable prognosis, as shown in a study by Baliakas and colleagues (3). The authors showed that trisomy of chromosomes 12 and 19 predicted an indolent course in patients with CK. On the other hand, in CK with up to four cytogenetic aberrations, the presence of *TP53* aberration predicted an aggressive disease course, similar to the sole presence of high complex karyotype (defined as ≥ 5 cytogenetic aberrations) (3).

According to a study by Leeksma and colleagues, the gain of 8q encompassing the *MYC* gene (+8q) is one of the independent factors significantly associated with shorter overall survival (OS) in CLL patients (7). In the unselected CLL population, a frequency of +8q appears to be low, between 3 – 5 % (8, 9). However, in contrast, in relapsed/refractory cases, +8q is particularly enriched (10). In the context of karyotype complexity, a frequency of +8q is significantly higher in CK than in non-CK karyotypes (11, 12) and often coincides with *TP53* or *ATM* aberrations (13, 14). Nevertheless, the contribution of +8q to adverse outcomes in patients with CK remains unclear (7, 11).

In the tested cohort, we confirmed the association of *TP53/ATM* aberrations and complex karyotype with 8q gain. Additionally, we identified the smallest duplicated 8q region and the partner chromosomes where the duplicated 8q region localizes. Shorter overall survival of +8q-positive patients supported the hypothesis that the 8q gain further contributes to the adverse prognosis of patients with *TP53/ATM* aberrations and complex karyotypes.

MATERIAL AND METHODS

Patient Cohorts

In this study, we analyzed peripheral blood samples obtained from CLL patients monitored at the University Hospital Brno, the Czech Republic. For all samples, written informed consent with their research use was obtained in accordance with the Declaration of Helsinki.

In the first part of this study, we performed an initial screening of all consecutive CLL patients examined in our laboratory in 2018 (303 patients in total). This pre-screening aimed to determine the frequency of +8q in unselected CLL population and to identify cytogenetic aberrations that coincide with +8q.

Next, a second patient cohort was selected based on the results of the pre-screening study. All CLL patients tested in our laboratory within the years 2015-2018 who met the condition of CK and del*ATM* (deleted *ATM*) and/or del*TP53* (deleted *TP53*) (90 patients in total) were enrolled for further analysis. The characteristics of the analyzed cohort are in **Table 1**.

Cytogenetic Analysis

Peripheral blood samples were treated according to the stimulation protocol for metaphase induction based on CpG-oligonucleotide DSP30 plus interleukin-2 for 72 hours before fixation and Giemsa staining (15). Karyotypes were captured at magnification 1000x and documented on LUCIA Cytogenetics software (Laboratory Imaging s.r.o, Prague, the Czech Republic). Karyotypes were evaluated according to the recommendations of the ISCN 2020 (International System for Human Cytogenomic Nomenclature). Patients' karyotypes with 1 (or more) clones with 3 (or more) abnormalities were evaluated as complex karyotypes (CK). A clone had to have at least two metaphases with the same aberration if the aberration was a chromosome gain or a structural rearrangement, and at least three metaphases if the abnormality was a loss of a chromosome (16).

Molecular Cytogenetic Analyses

For FISH analyses, the probes were hybridized according to the instructions of manufacturers. For detection of del*ATM*, del*TP53*, del(13q) and +12, the standard CLL panel was used (XL ATM/TP53, XL DLEU/LAMP/12cen; MetaSystems GmbH, Altlussheim, Germany). For detection of the *MYC*-coding sequence, probe CL 6q21/8q24 (MetaSystems) was used; this custom-mixed probe is a combination of two locus-specific probes – the 6q21 locus from the XL 6q21/6q23/6cen probe (probe length 304 kb, coordinates D6S1594 – D6S1396E; the results for the 6q21 probe are not elaborated in detail in this study) and the 8q24 locus from the XL MYC amp probe (342 kb, coordinates RH77966 to D8S490). For detection of *MYC* translocations, the break apart *MYC* probe was used (Zyto*Light* SPEC MYC Dual Color Break Apart Probe; ZytoVision GmbH, Bremerhaven, Germany). The proximal part of this probe covers the region approx. 387 kb to 856 kb centromeric of the *MYC* locus including the region of focal gains described by Edelmann and colleagues (8). Hybridization signals in at least 200 nuclei were scored on a Nikon Eclipse Niu fluorescence microscope at magnification 1000x (Nikon Instruments Europe BV, Amsterdam, Netherlands). For the *ATM*, *TP53*, and *MYC*-detecting probes, the threshold for the positivity was set to 10 % to enhance the chance to find metaphases with these aberrations for the M-FISH (multicolor fluorescence *in situ* hybridization) and M-BAND (multicolor banding) analyses. Signals were documented using LUCIA Cytogenetics software (Laboratory Imaging s.r.o, Prague, the Czech Republic).

The routine cytogenetic analysis of G-banded chromosomes allowed the identification of patients with complex karyotypes, but the sensitivity of this method is limited. Therefore, the M-FISH method was used in the cohort of selected patients for a more precise description of all karyotype changes. Subsequently, the M-BAND analysis enabled identifying the extent of the duplicated 8q region with higher accuracy. For M-FISH and M-BAND analyses, probes were hybridized according to the manufacturer's instructions (24XCyte, XCyte8; MetaSystem). The M-BAND8 probe covered chromosome 8 with different fluorochromes along the entire chromosome length. M-BAND patterns are independent of chromatin condensation and

TABLE 1 | The characteristics of the analyzed cohort of 90 patients with CK.

		Whole dataset		*MYC* pos		*MYC* neg	
Gender	F	31	34%	9	38%	22	33%
	M	59	66%	15	63%	44	67%
Subgroup	del*ATM* + CK	50	56%	7	29%	43	65%
	del*TP53* + CK	28	31%	11	46%	17	26%
	del*TP53* + del*ATM* + CK	12	13%	6	25%	6	9%
OS status	alive	47	52%	11	46%	36	55%
	dead	43	48%	13	54%	30	45%
Rai	I	28	36%	7	32%	21	38%
	II	8	10%	1	5%	7	13%
	III	6	8%	2	9%	4	7%
	IV	13	17%	6	27%	7	13%
	0	23	29%	6	27%	17	30%
Binet	A	44	56%	13	57%	31	56%
	B	15	19%	2	9%	13	24%
	C	19	24%	8	35%	11	20%
IGHV status	MU	10	12%	2	10%	8	13%
	UM	71	86%	18	90%	53	84%
	UM + MU	2	2%	0	0%	2	3%
FISH del(13q) total	Y	70	78%	18	75%	52	79%
	N	20	22%	6	25%	14	21%
FISH del(13q) monoallelic	Y	67	74%	18	75%	49	74%
	N	23	26%	6	25%	17	26%
FISH del(13q) biallelic	Y	17	19%	2	8%	15	19%
	N	73	81%	22	92%	63	81%
FISH del*ATM*	Y	62	69%	13	54%	49	74%
	N	28	31%	11	46%	17	26%
FISH del*TP53*	Y	40	44%	17	71%	23	35%
	N	50	56%	7	29%	43	65%
FISH trisomy 12	Y	5	6%	2	8%	3	5%
	N	85	94%	22	92%	63	95%
TP53	MU	45	54%	18	75%	27	45%
	UM	39	46%	6	25%	33	55%
ATM	MU	5	71%	0	–	5	71%
	UM	2	29%	0	–	2	29%
Complex karyotype (no of changes)	3 or 4	31	34%	9	38%	22	33%
	5	27	30%	14	58%	13	20%
	ND	32	36%	1	4%	31	47%
MYC	pos	24	27%	24	100%	0	0%
	neg	66	73%	0	0%	66	100%
No of treatment lines	median (range)	3 (0-10)		3 (1-10)		3 (0-9)	

F, female; M, male; CK, complex karyotype; OS, overall survival; IGHV, immunoglobulin heavy chain gene; MU, mutated; UM, unmutated; ND, not determined (the exact number of changes in the CK not determined as the detailed analysis by M-FISH method was not performed), Y, yes; N, no.

provide a resolution equivalent to the 550-band level for G-bands. The metaphases were captured using an Axio Imager Z2 microscope at magnification 630x (Zeiss, Jena, Germany) and analyzed with the NEON/ISIS software (MetaSystems).

Gene Expression Analysis Using Quantitative Real-Time PCR

As input material, B lymphocytes separated from peripheral blood using gradient centrifugation on Ficoll-Pague PLUS (GE Healthcare, Uppsala, Sweden) coupled with the RosetteSep® B Cell Enrichment Kit (StemCell Technologies Inc., Vancouver, Canada) were used. RNA was isolated with TRI Reagent (MRC, Cincinnati, USA). As positive controls, RNA samples from cell lines NALM6 and MEC-1 were used. Then, RNA was reverse transcribed into cDNA using SuperScript® II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Gene expression (*APEX1, CDK4, CDKN1A, CDKN1B, CDKN2B, DUSP1, GADD45A, NCL TERT*) was analyzed by real-time PCR on the QuantStudio™ 12K Flex system (both Thermo Fisher Scientific, Waltham, USA) using ThermoFisher Scientific TaqMan assays. The *HPRT1* and *TBP* genes were used as endogenous controls. All reactions were pipetted in triplicates. After removing outlying Ct values (i.e., the values differing from the remaining two replicates by ≥0.3 Ct; 4.6% of Ct values) to correct on the technical accuracy of the method, relative quantification using the $2^{-\Delta\Delta CT}$ method was performed.

Antibodies and Immunoblotting

Protein extracts were obtained and subjected to western blot analysis as described previously (17). For MYC and β-actin immunodetection, the following specific primary antibodies were used: MYC (D84C12), β-Actin (13E5) (both Cell Signaling Technology, Danvers, Massachusetts, USA). Secondary antibody: anti-rabbit (7074; Cell Signaling

Technology). Chemiluminescence was detected with Clarity™ Western ECL Blotting Substrate (Bio-Rad, Hercules, USA). Signals were quantified with ImageJ Software (www.imagej.net) and referred to the respective controls, i.e., β-actin levels in individual samples. Protein extracts from cell lines NALM6 and MEC-1 were used as positive controls.

Statistical Analysis

All statistical analyses were performed in freeware R. For statistical comparison of mRNA and protein levels between groups, we used the Kruskal-Wallis ANOVA and the Mann-Whitney test. A logrank test was applied to evaluate differences in survival of distinct groups of patients. The Kaplan-Meier curves were used for visualization of survival in patient groups. Patients after bone marrow transplantation (n=3) were excluded from the OS analysis. The level of significance was set at alpha=0.05.

RESULTS

MYC Aberrations Are Associated With del*ATM*, del*TP53*, and Complex Karyotypes

In the pre-screening, consecutive samples from 303 CLL patients (96 treatment-naïve, 194 treated, 13 follow-up loss) were analyzed for DNA copy number changes in our laboratory in 2018 by using the standard diagnostic FISH panel to detect del*ATM*, del*TP53*, del(13q), +12) and by the 6q21/8q24 MetaSystems probes. In this unselected group of patients, the frequency of 8q24 gains covering the *MYC*-coding sequence reached only 6.3 % (19/303). Within the subgroups defined by the most common CLL-related recurrent cytogenetic aberrations, the cases with *MYC* gain reached the following frequencies – del*ATM*: 10.0 % (8/80), del*TP53*: 14.8 % (4/27), del(13q): 5 % (10/201), +12: 0 % (0/30), del*ATM*+del*TP53*: 16.6 % (1/6), and patients negative for the standard CLL-FISH probe panel: 4.2 % (2/48) (**Figure 1A**).

G-banding karyotype analysis (available for 283/303 patients; 93.4% of the cohort) revealed a significant association of *MYC* gain with CK (defined by the presence of ≥3 numerical or structural abnormalities in the same clone). There were 82.3% (233/283) patients without CK, among them 3% (7/233) with *MYC* gain. On the other hand, in the group of patients with CK (17.7% of patients; 50/283), the *MYC* gain was detected in 20 % (10/50; p<0.0001) of cases (**Figure 1B**).

Based on the pre-screening results, we aimed to enrich the cohort with *MYC* aberrations with additional cases tested in our laboratory during the years 2015-2018. Thus, we searched for those meeting the condition of complex karyotype (clone/s with 3 or more cytogenetic aberrations) together with del*ATM*, del*TP53*, or both. The resulting cohort consisted of 90 patients, who had CK together with del*TP53* (28/90, i.e., 31.1 %) or with del*ATM* (50/90, i.e., 55.6 %) or with del*ATM*+del*TP53* (12/90, i.e., 13.3 %) (**Figure 1C**). Basic clinical, cytogenetic, and molecular biologic (IGHV, *TP53*, and *ATM* mutations) characteristics of these 90 patients in the context of *MYC* aberrations are summarized in **Table 1**.

All 90 additional samples from CLL patients were examined for *MYC* aberrations using FISH i) with the probe covering the *MYC* coding sequence and ii) with the *MYC* break-apart probe. The former probe confirmed a gain of one or more *MYC* gene copies. The latter probe surrounding the common break sites was used to identify breaks in proximity to the *MYC* regulatory regions, i.e., the translocation. Representative FISH results for both probes are shown in **Figure 2** (**Figures 2A, B**). In 24/90 patients (26.7%), *MYC* aberration was detected. *MYC* gain was the predominant change observed in 21/24 (87.5 %) cases as a sole aberration and in 2/24 (8.3 %) cases, it combined with *MYC* translocation. One additional copy of the *MYC* gene (+8q24) was the most common aberration (16/24, i.e., 66.7 %), followed by the combination of two clones with one or two additional *MYC* copies (3/24, i.e., 12.5 %). In one case, two additional *MYC* copies were detected (1/24, i.e., 4.2 %). Interestingly, an extrachromosomal amplification of *MYC* signal (double minutes; dmins) was observed in one patient – this is a rare finding in CLL. The *MYC* translocation was detected in three cases, either as a sole aberration (1 case) or combined with *MYC* gain (2 cases). We also observed that the clone with *MYC* aberration was either smaller or of a similar size as the del*ATM*/del*TP53* clone in most cases (23/24 patients; 95.8 %). All detected types of *MYC* aberrations and the size of clones (% of nuclei) are summarized in **Table 2**.

The *MYC* aberrations identified using the FISH method reached the following frequencies among these CK subgroups – del*ATM*: 14 % (7/50), del*TP53*: 39.3 % (11/28), del*ATM* +del*TP53*: 50 % (6/12). These results were similar to the initial cohort, with the frequency of *MYC* aberrations increasing in del*ATM* -> del*TP53* -> del*ATM*+del*TP53* subgroups. There were no significant differences between the *MYC*-positive and *MYC*-negative groups of patients regarding IGHV mutation status and *ATM* deletion. The *TP53* aberration was detected significantly more frequently (p>0.004) in the *MYC*-positive group: 83.3% (20/24) than in the *MYC*-negative group: 48.5 % (32/66). All but four patients in this cohort were treated previously.

The Amplified 8q Regions Translocate to Random Chromosomes Within the CK Subgroup

To identify partner chromosomes where the duplicated 8q region was localized, the M-FISH method was performed in 19 of 24 samples with an *MYC* aberration, (in the remaining 5 cases, an insufficient number of metaphases was obtained or no more material was available). In one patient (no. 9), the subclone with *MYC* aberration was not detected in metaphases. The ISCN notation for *MYC*-aberrant clones is summarized in **Table 2**. A representative M-FISH karyotype is shown in **Figure 2** (**Figure 2C**). Our results revealed that the site of integration of the gained 8q region containing the *MYC* gene is random and that there is no recurrent chromosomal partner (**Figure 1D**). Most often, the translocation of the duplicated 8q region was detected on chromosome 4 (5/18 cases; 28%); nevertheless, the localization of

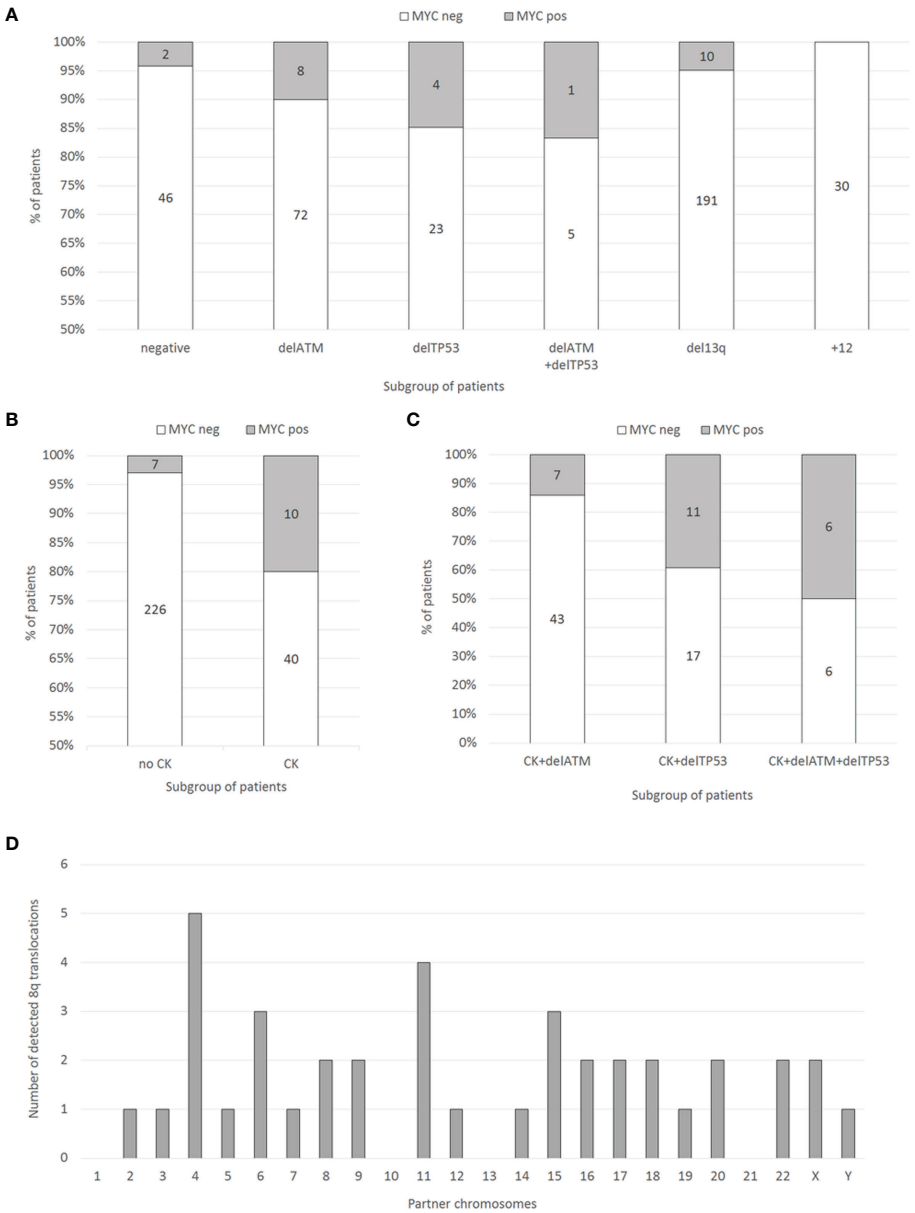

FIGURE 1 | **(A)** Distribution of *MYC* gains in the groups of patients with recurrent aberrations (del*ATM*, del*TP53*, del(13q), +12, or negative) determined by FISH in 303 consecutive CLL patient samples. Numbers within columns represent absolute numbers of patients. **(B)** Distribution of *MYC* gains (determined by FISH) in the groups of patients with or without CK (determined by conventional chromosome banding) in 283 consecutive CLL patient samples. Numbers within columns represent absolute numbers of patients. **(C)** Distribution of *MYC* aberrations (determined by FISH) in 90 patient samples selected for the presence of CK (determined by conventional chromosome banding) together with either del*TP53* or del*ATM*, or both (determined by FISH). Numbers within columns represent absolute numbers of patients. **(D)** The frequency and localization of the duplicated 8q region on individual chromosomes identified by the M-FISH method.

the break site differed among the patients; thus, no recurrent target site on chromosome 4 was involved. In 9/18 patients (50 %), the duplicated 8q region translocated to different chromosomal partners in several individual clones (**Table 2**; break sites highlighted in bold). In one patient (no.11), translocation in the *MYC* regulatory region 8q24.21 (as determined using the break-apart probe) without any copy-number change in *MYC*-coding sequences was identified as t(8;22)(q24;q?12). In another patient (no. 18) with both 8q24 gain and translocation detected using the FISH method in interphase nuclei, a minor clone with 8q24.21 translocation was not detected in metaphases.

To identify the smallest duplicated region and the sites of breaks, the M-BAND8 analysis was performed in 18 of 24 patient samples with *MYC* aberration (in 5 cases, an insufficient number of

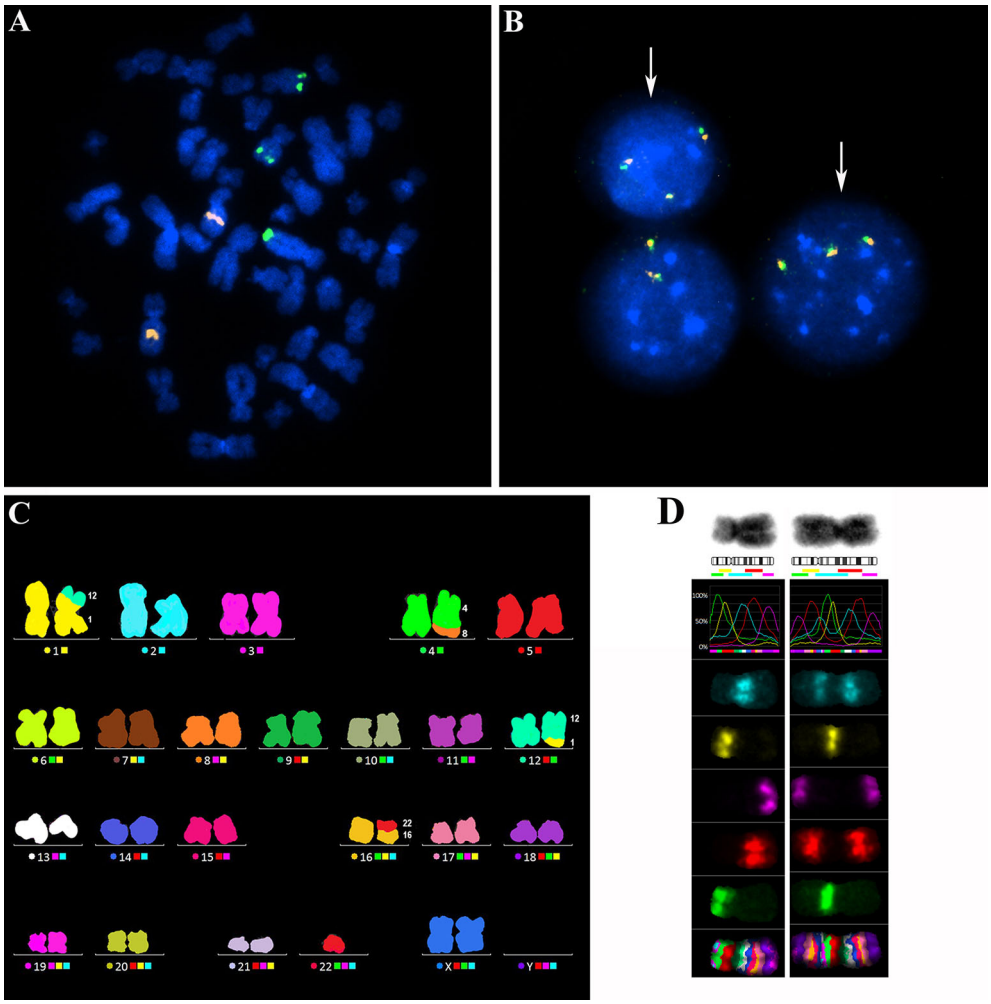

FIGURE 2 | **(A)** A representative metaphasis with three copies of the *MYC* gene visualized by the FISH method. The probe CLL 6q21/8q24 (MetaSystems), covering the *MYC* coding region 8q24 (green signals) and a control region 6q21 (orange signals), was used. **(B)** Representative nuclei with three copies of the *MYC* gene (white arrows), visualized by the FISH method using the *MYC* break-apart probe (ZytoVision). This probe surrounds the common break sites for *MYC* gene translocations. The orange-green fusion signals indicate that the break site is not in proximity to the *MYC* regulatory regions. **(C)** A representative metaphasis hybridized with the M-FISH probe 24XCyte (MetaSystems) for FISH analysis of the whole karyotype. In this metaphasis, one balanced translocation t(1;12) and two unbalanced translocations dic(16;22) and der(4)t(4;8) with 8q gain were detected. **(D)** Analysis of the extent of 8q gain using the M-BAND8 probe XCyte8 (MetaSystems). Normal chromosome 8 (on the left) and a derivative chromosome 8 (on the right) with the duplicated 8q13-8qter region on an 8p-arm.

metaphases was obtained, or no more material was available, in the remaining case no. 9, the subclone with *MYC* aberration was not detected in metaphases). A representative M-BAND8 analysis is shown in **Figure 2** (**Figure 2D**). The results of M-FISH together with the M-BAND8 analysis are summarized in **Table 2**. As shown in **Figure 3**, the duplicated region varied from 8q13-8qter to 8q23-8qter, the latter being determined as the smallest duplicated region in our hands (**Figure 3**).

Expression of MYC mRNA and Protein Is Slightly Increased in +8q Samples

Next, we determined the mRNA level of *MYC* and its downstream genes (*APEX1, CDK4, CDKN1A, CDKN1B, CDKN2B, DUSP1, GADD45A, NCL, TERT*) in +8q-positive patients and two control groups, i.e., 10 patients negative in both cytogenetic and molecular-cytogenetic analyses (i.e., 46,XX or 46,XY with CLL-FISH negativity; negative control group) and randomly selected 9 patients with CK and del*ATM*/del*TP53* but without the +8q aberration (+8q-negative CK group). As positive controls, cell lines MEC-1 and NALM6 with a high level of MYC expression were used. No significant difference in the expression of downstream genes was observed between the +8q-positive and +8q-negative groups (**Supplementary Figure 1**). The median values of *MYC* mRNA relative level determined by the $2^{-\Delta CT}$ method were 109.442 in negative controls, 134.9 in +8q-negative CK controls, while 172.969 in +8q-positive CK samples

TABLE 2 | Cytogenetic analysis of *MYC* aberrations in 24 *MYC*-positive patients with CK.

Case No	Gender	del*ATM*/ del*TP53*/*MYC* aberration clone size (%)	FISH: type of *MYC* aberration	CK (no of changes)	M-FISH/M-BAND: ISCN notation of clone(s) with *MYC* aberration
1	F	0/88/70	dmins	≥5	–
2	M	0/64/39	3xMYC	≥5	–
3	F	0/97/13	3xMYC	3-4	46,XX,der(3)t(3;8)(q?27;**q22.3**),i(17)(q11.2),der(22)t(X;22)(q?13;q13.1)[5]
4	M	0/87/76	3xMYC	≥5	44,XY,?inv(3)(p21.2q?27),der(3)t(3;14)(p?21.3);?,del(6)(p21.1p?24),-8,der(10)t(3;10)(?;q?24.3),der(14)t(10;14)(q?;q)?ins(14;17)(q?23);?, der(15)t(8;15)(**q22.1**;q)?,der(15)?del(15)(p12)del(15)(q?13),?dic(17;20)(p?11.2;p?11.2),der(18)t(15;18)(q?;p)?ins(15;8)(q?;**q22.1**)[cp5]
5	M	0/91/89	3xMYC	3-4	45,XY,der(6)t(6;17)(q23.1;q?21.3),dic(14;18)(p?11.1;p?11.2),der(20)t(8;20)(**q13**;q?13.3)[12]
6	M	0/86/74	3xMYC/ 4xMYC	3-4	45,XY,dic(13;17)(p?13;p?11.2),der(22)t(8;22)(**q13**;q11.2),der(Y)t(Y;8)(q11.2;**q13**)[8]/45,XY,dic(13;17)(p?13;p?11.2),der(17)t(8;17)(**q13**;q21.3)[2]/45,XY,der(11)t(8;11)(**q21.2**;q?13.5),dic(13;17)(p?13;p?11.2)[1]/45,XY,-3,dic(13;17)(p?13;p?11.2),der(18)t(8;18)(**q13**;q21.1)[1]/45,XY,der(2)t(2;8)(q32.1;**q22.3**),dic(13;17)(p?13;p?11.2)[1]
7	F	0/13/15	3xMYC	≥5	76-87,XXXX,-5,-6,der(6)t(6;8)(p?21;**q21.2**),-8,-9,der(9)t(9;15)(?p?;q)?,-10,-13,dic(13;17)(p?11.2;q?11.2),der(14)t(1;14)(?;q?31)x2,-15,+16,der(17)t(17;22)(?p11.2);?,+20,+20,-22[cp11]
8	F	0/57/53	3xMYC	≥5	40,XX,-4,-5,der(6),-7,-8,der(12)t(X;12),-15,?i(17q),dic(19;22),der(20)(20pter->?q12::8q24.3->8**q13**::8p11.2->8pter)[2]
9	M	0/56/12	3xMYC/ 4xMYC	≥5	–
10	F	0/74/81	3xMYC	3-4	46,XX,der(12)t(8;12)(**q23**;q)?,der(17)t(17;18)(p?12);?,t(11;15)(q?13.1;q?24)[19]
11	M	0/75/27	translocation	3-4	46,XY,t(8;22)(**q24**;q?12),der(17)t(2;17)(?;p11.2),t(17;21)(q23.1,q22.1)[4]
12	M	17/78/13	4xMYC	≥5	46,XY,der(11)t(8;11)(**q22.1**;q14.1),der(11)t(8;11)(**q22.3**;q22.2),del(17)(p?11.2)[3]/47,X,dic(Y;17)(?p11.2;?p11.2),del(8)(q)?,der(15)invins(15;8)(q?;**q21.3**)[2]/44,X,-Y,?dic(8;14),del(17)(p?11.2)[1]/43,Y,der(X)ins(X;8)(?q13);?,-9,dic(10;15)(?q23.1;p11.2),dic(17;20)(p11.2;?q12)[1]
13	M	89/16/67	3xMYC/ 4xMYC/ translocation	≥5	46,XY,del(1)(q?25.1),t(5;10)(q?22;q21.1),t(8;9)(**q24**;q34),t(11;13)(p?11.2;q?14.3),der(17)t(17;17)(q?11.2;q?12)[8]
14	M	89/30/46	3xMYC/ 4xMYC	≥5	–
15	F	42/48/27	3xMYC	≥5	–
16	M	87/87/56	3xMYC	≥5	46,XY,der(5)t(5;8)(p14;**q13**),?i(17)(q12),der(20)t(2;20)(?;?q12)[15]/45,XY,-8,dup(8)(?q)?,del(17)(p12),der(20)t(2;20)(?;?q12)[4]/43,XY,-7,-8,?dic(8;18)(p11.2;p11.2),del(11)(q?22),?i(17)(q12),der(20)t(2;20)(?;?q12)[3]
17	M	4/20/46	3xMYC	3-4	46,XY,?del(1)(?q24q32.2),der(3)t(3;10)(?p25);?,der(15)t(8;15)(?;p?13)[5]/45,Y,der(X)t(X;8)(q?23;**q21**),?dic(13;14)(p11.2;p11.2),der(21)t(13;21)(p13);?[2]/46,XY,?del(1)(p22.1p?35),der(6)t(6;7)(q?21);?,der(16)t(8;16)(**q21**;q?22)[1]/45,XY,der(8)t(4;8)(?;q)?,dic(13;21)(p11.2;p11.2),der(22)t(13;22)(?;p13)[1]
18	M	74/0/84	3xMYC/ 4xMYC/ translocation	3-4	46,XY,del(1)(?q21),der(4)t(4;8)(p?14;**q22.3**),der(6)(8qter->**8q23**::6p?21->6q21::6q27->6qter)[3]/46,XY,del(1)(?q21),?del(2)(q?21),der(7)t(7;8)(q?22;**q21.3**)[2]
19	M	73/0/20	3xMYC	ND	–
20	M	95/0/90	3xMYC	3-4	46,XY,der(4)(4qter->q16::4p14->q12::8**q22.1**->8qter),der(8)(8pter->8p23::8p11.2->8q11.2::4q12->4qter),der(11)t(8;11)(**q13**;q14.1)[24]/46,XY,der(11)t(8;11)(**q13**;q14.1)[2]
21	F	96/0/85	3xMYC	3-4	46,XX,t(5;11)(q31.3;q13.3),t(6;10)(p21.1;p11.2),der(22)t(8;22)(**q22.1**;q12.3)[8]/46,XX,t(5;11)(q31.3;q13.3),der(8)(8qter->8**q21.3**::8p23.2->8qter)[3] +der(11)t(8;11)(**q22.1**;q)?[3]
22	F	96/0/94	3xMYC	≥5	45,XX,der(9)t(8;9)(**q22.1**;q?32),del(11)(q11.3),-17,der(19)t(17;19)(q?;q13.3)[10]
23	M	92/0/67	3xMYC	≥5	46,XY,der(4)t(4;8)(p?14;**q22.1**),del(11)(q14)[3]/45,XY,der(6)t(6;8)(q?21;**q22.1**),der(8)t(8;21)(p)?;?,del(11)(q14),-19[2]/45,X,-Y,t(2;7)(q?24.3;q?32),?der(4)t(4;12)(p)?;?,der(17)t(8;17)(**q23**;q?21.3)[1]/44,XY,der(4)t(4;9)(p14);?,der(9)t(8;9)(**q23**;p?13),del(11)(q14),-16,der(17)t(17;21)(?p)?;?,-21[1]
24	F	97/0/41	3xMYC	≥5	45,XX,t(1;12)(p33;q23),der(4)t(4;8)(q33;**q23**),del(11)(q14q)?,?dic(16;22)(?p11.2;p12)[5]/45,XX,t(1;12)(p33;q23),del(11)(q14q)?,del(13)(q14q)?,der(16)t(8;16)(**q23**;q?13)[3] +der(8)(8qter->8**q22.1**::8p23.2->8qter)[2]

F, female; M, male; dmins, double minutes; CK, complex karyotype; M-FISH, multicolor FISH method; M-BAND, multicolor banding method. ND, not determined (the exact number of changes in the CK not determined as the detailed analysis by the M-FISH method was not performed). ISCN, International System for Human Cytogenomic Nomenclature. The position of breaks on chromosome 8 is highlighted in bold.

(**Figure 4A**). We also compared the level of MYC protein among the tested groups using western blot immunodetection. Similar to mRNA, we observed higher levels of MYC protein in the +8q-positive CK group when compared to controls (**Figure 4B**). The median values of MYC protein relative level (after normalization to β-Actin) were 0.102 in negative controls, 0.065 in +8q-negative CK controls and 0.173 in +8q-positive CK samples.

Survival Analysis

Eighty-five patients with CK and del*ATM*/del*TP53* were included in the survival analysis (2 patients were excluded due to the follow-up loss, another 3 patients due to bone marrow transplantation). They were divided into two groups, the +8q-negative and +8q-positive groups. We observed significantly shorter median survival for OS in the +8q-positive group (9.0

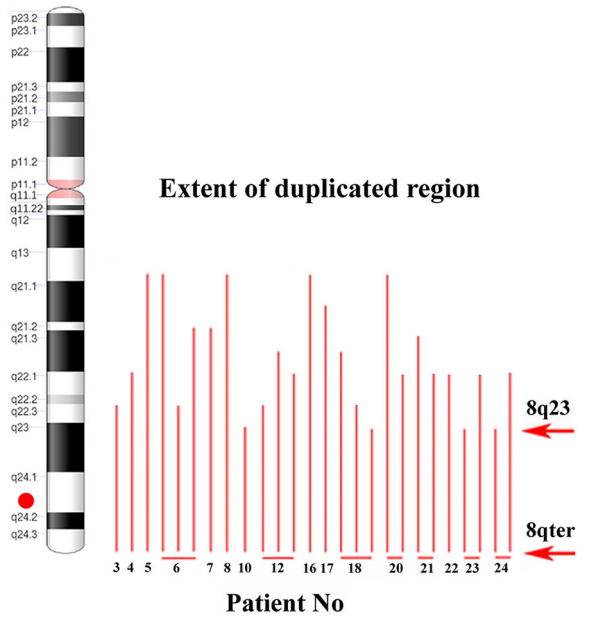

FIGURE 3 | The identification of the smallest duplicated region using the M-BAND method. An ideogram of chromosome 8 on the left side. A red dot indicates the position of the *MYC* gene. Red lines show the size of the duplicated 8q region individual patients (patient numbers under red lines; several lines in one patient indicate more than one clone with *MYC* gain). Red arrows delimit the smallest duplicated region.

years in +8q-positive vs. 10.6 years in +8q-negative; hazard ratio 2.14; p=0.02) (**Figure 5A**). No statistically significant difference was observed when comparing the time to first treatment (TTFT) between the +8q-positive vs. +8q-negative patients (**Figure 5B**). The distribution of clinico-biological features of the +8q-negative and +8q-positive groups is summarized in **Table 1**.

DISCUSSION

CLL exhibits remarkable clinical heterogeneity that often requires the employment of a variety of treatment strategies. Intrinsic (genetics, microenvironment) and extrinsic (therapy) pressures select distinct clones and subclones that can underlie relapsed/refractory disease. Cytogenetically abnormal clones are identified in about 40 – 70 % of newly diagnosed CLL cases by chromosome analysis and about 80% by FISH (18–21). Among chromosomal abnormalities, del*ATM*, del*TP53*, and complex karyotype are associated with poor clinical outcome. Our results are consistent with the findings that duplication of the 8q chromosome arm segment often coincides with both *TP53* and *ATM* aberration and complex karyotype (11–14). We also observed that the +8q clone was either smaller or of a similar size as the del*ATM*/del*TP53* clone in most cases. Furthermore, in both cohorts together, the *MYC* aberration was detected predominantly in patients treated previously (4 treatment naïve vs. 32 treated in *MYC*-positive group/95 treatment naïve vs. 215 treated in *MYC*-negative group). Similar findings were observed in the study by Landau et al. (22). Although the literature suggests that 8q aberration may precede as well as follow the del*TP53* occurrence (23), our results indicate that in most cases, the 8q aberration was gained as a later event in the disease course. Such findings are in concordance with known MYC functions. This protein acts both as a pro-proliferative and pro-apoptotic regulator (24). In cells with damaged apoptotic

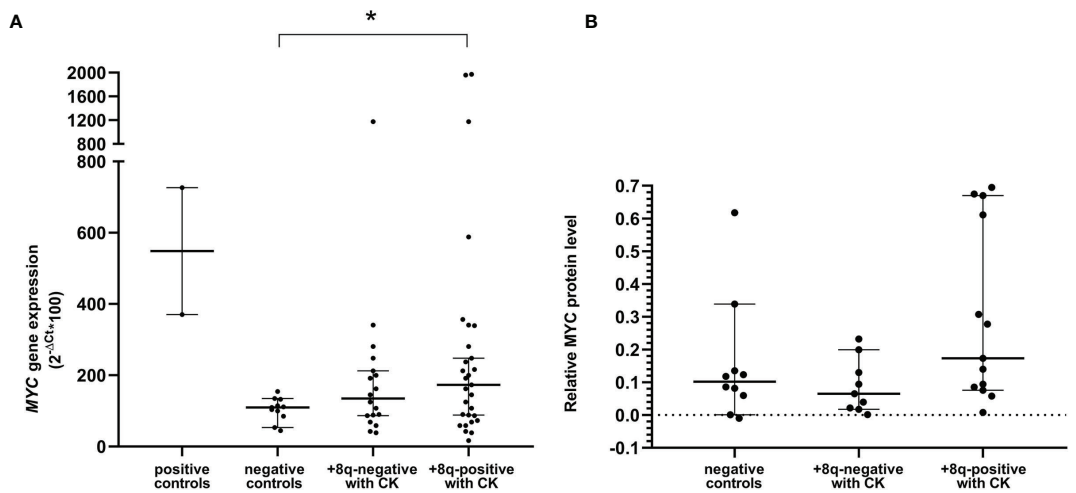

FIGURE 4 | **(A)** Quantification of *MYC* mRNA levels using quantitative real-time PCR (qRT-PCR). **(B)** Quantification of MYC protein levels using western blot immunodetection. Positive controls: cell lines NALM6 and MEC-1. Negative controls: patients negative in both the cytogenetic and molecular-cytogenetic analyses (n=10). +8q-positive with CK; patients with complex karyotype and *MYC* aberration (n=13). +8q-negative; patients with complex karyotype but without *MYC* aberration (n=9). The Mann-Whitney tests were applied to confirm a significant difference in gene expression between the groups. A statistically significant difference (p=0.05) is marked by an asterisk.

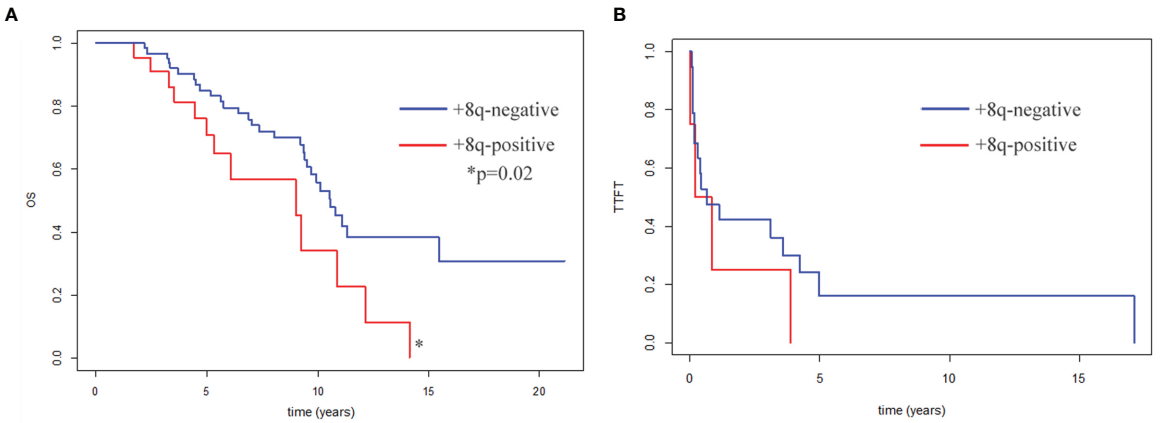

FIGURE 5 | **(A)** Kaplan–Meier curves for patients' overall survival (OS). OS of +8q-negative patients (n=63) and +8q-positive patients (n=22) was compared. Patients after bone marrow transplantation (n=3) were excluded from the OS analysis. **(B)** Kaplan–Meier plots for time to first treatment (TTFT). TTFT of +8q-negative patients (n=64) and +8q-positive patients (n=24) was compared. Differences were evaluated by a logrank test. A statistically significant difference (p=0.02) is marked by an asterisk.

signaling (*ATM/TP53* aberration), the MYC pro-proliferative effect dominates, and thus a higher level of the MYC protein can provide a selective advantage to cancer cells.

MYC expression can be deregulated by mutation, amplification, translocation, regulation of transcription, and RNA/protein stability (25). In CLL cells, the frequency of somatic mutation in the coding sequence of the *MYC* gene is scarce, reaching only 0.4 % according to the COSMIC database of somatic mutations (26). Deregulated *MYC* expression is commonly found in lymphoma due to *MYC*-coding sequence translocation to the vicinity of immunoglobulin enhancers (27). On the other hand, the +8q aberration that we describe in our group of patients presumably adds one copy of the *MYC* gene while preserving the intact regulatory and coding sequences. Edelmann and colleagues described two types of gains, broad gains covering the *MYC* locus and focal gains (<500 kb) in the super-enhancer region (8). We did not detect these focal gains in the super-enhancer region (the range of our smallest duplicated region was 8q23-8qter), although the FISH probe we used covered its locus (the proximal part of the break apart *MYC* probe). It supports the finding that only the broad +8q gains are enriched in high-risk CLL cases, while the focal gains are relatively rare (1.4 %) independently of risk groups (10).

Importantly, in CLL patients with high MYC mRNA/protein levels, a significantly shorter time to first treatment was observed, showing that *MYC* might be one of the negative prognostic factors (28). The presence of one or more additional copies of the *MYC* gene should, in theory, lead to a higher *MYC* expression. We detected slightly higher levels of MYC mRNA/protein in +8q-positive vs. +8q-negative patient samples, nevertheless, high variability and a small number of samples precluded obtaining statistically significant results. Physiologically, the *MYC* expression is strongly induced by activating stimuli in germinal centers of lymph nodes (LN) and its activation has a localized and transient nature (29). Likewise, Herishanu and colleagues showed that the MYC mRNA/protein level is high in CLL cells isolated from the LN compared to relatively low MYC mRNA/protein levels in the quiescent cells circulating in peripheral blood (30). The remarkable feature of the MYC mRNA/protein is its very short half-life (30 min/20 min) (31). Therefore, we suppose that the MYC level rapidly decreases after leaving the lymph node, and in peripheral blood, only residual mRNA/protein levels are detected. Together, this reasoning might explain why we failed in finding any correlation between the level of mRNA/protein expression and the size of the clone with *MYC* aberration. On the other hand, other mechanisms (mentioned above) deregulating the expression and especially the stability of MYC mRNA/protein can explain higher MYC levels in patients without the 8q gain.

Localization of the translocating break site exactly to 8q24 (within the *MYC*-regulatory region) usually leads to deregulation of *MYC* expression due to the proximity of strong transcription enhancers (typically immunoglobulin's; IGH, IGK, IGL) without changing the number of *MYC* coding sequences. In CLL, about two-thirds of reported cases with *MYC* translocations involved immunoglobulin partners, while in the remaining cases, less common breakpoints with an unknown effect on *MYC* expression were observed, as reviewed in the study of Fonseka and Tirado (32). *MYC* translocation, either with immunoglobulin genes or other unknown partners is one of the changes acquired in about 16 % - 37 % of CLL patients with Richter's transformation (33–36). On the other hand, translocations with a gain of 8q have not been mapped in detail yet. Here we describe that the distribution of +8q to other chromosomes is rather random, though the most common translocation partner was chromosome 4 (5/18 cases). Interestingly, we also detected the gained 8q region on the p-arm of one of the chromosomes 8 in 2/18 patients. Generally, the *MYC* gain might be challenging to detect in karyotype with the routinely used G-banding method. Without FISH analysis, this aberration often remains cryptic, especially in subclones, and without M-FISH analysis, the partner chromosomes remain largely unmapped.

Regarding the clinical impact of +8q, the genomic array-based study of the largest cohort so far (2293 cases) revealed that the 8q gain encompassing the *MYC* gene is an important factor significantly associated with shorter OS (7). The *MYC*-affected downstream pathways include the B cell receptor signaling (37), which implies a possible interference with Bruton tyrosine kinase inhibitors and conceivably challenging treatment of CLL patients with *MYC* abnormalities. Indeed, *MYC* upregulation correlated with ibrutinib resistance in mantle cell lymphoma cell lines (38). In contrast, another study (101 cases) did not prove a significant independent clinical impact of *MYC* aberrations (11). In our cohort, the *MYC* aberrations were significantly associated with *TP53* aberrations. In a retrospective study investigating 195 cases with del*TP53*, the 8q24 gain was a significant predictor of short OS in multivariate analysis (39). In concordance with these assumptions, we observed significantly shorter OS in patients with +8q in our cohort of patients. As reviewed by Nguyen-Khac, the double-hit CLL (bearing *TP53* aberration + *MYC* gain) might have an inferior outcome even within the del*TP53* group, but these results from a limited retrospective study have yet to be confirmed in larger cohorts of patients (23). In the study of Leeksma et al., *MYC* gain correlated with UM-IGHV and higher karyotype complexity, another two important factors contributing to unfavorable prognosis (7). In our cohort of patients with CK, the distribution of cases with UM-IGHV did not differ between the +8q-positive and +8q-negative groups. On the other hand, *MYC* aberration correlated with higher karyotype complexity within our dataset. *MYC* deregulation promotes an overall induction of chromosomal instability, as reviewed in several studies (40, 41). Therefore, we conclude that 8q24 gain together with del*TP53* and complex karyotype have a synergistic impact on outcome and predict a particularly poor prognosis. Larger studies are warranted to fully understand the role of *MYC* in the context of other negative biomarkers and its impact on the outcome of high-risk CLL patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EO and MBOH performed the FISH, M-FISH and M-BAND analysis, PŠ performed the cytogenetic analysis. KZ with KP (RNA) and MBOU with PČ (proteins) carried out the expression experiments. LR performed the statistical calculations. AP, MO and MD were responsible for the clinical data. EO wrote the manuscript with support from MJ and KP. MJ supervised the project, contributed to the interpretation of the results and to the final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.859618/full#supplementary-material

Supplementary | Quantification of mRNA levels of MYC-downstream genes using quantitative real-time PCR (qRT-PCR). Positive controls; cell lines NALM6 and MEC-1. Negative controls; patients negative in both the cytogenetic and molecular-cytogenetic analyses (n=10). +8q-positive with CK; patients with complex karyotype and MYC aberration (n=13). +8q-negative; patients with complex karyotype but without MYC aberration (n=9). No significant difference among individual groups was detected (the pairwise U-test, Bonferroni corrected, was used to calculate the p-value). The median values of relative expression are stated down in the table.

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BRIEF RESEARCH REPORT
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TP53 Mutations Identified Using NGS Comprise the Overwhelming Majority of *TP53* Disruptions in CLL: Results From a Multicentre Study

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Limited data exists to show the correlation of (tumour protein 53) *TP53* mutation detected by Next generation sequencing (NGS) and the presence/absence of deletions of 17p13 detected by FISH. The study which is the largest series to date includes 2332 CLL patients referred for analysis of del(17p) by FISH and *TP53* mutations by NGS before treatment. Using a 10% variant allele frequency (VAF) threshold, cases were segregated into high burden mutations (≥10%) and low burden mutations (<10%). *TP53* aberrations (17p [del (17p)] and/or *TP53* mutation) were detected in 320/2332 patients (13.7%). Using NGS analysis, 429 *TP53* mutations were identified in 303 patients (13%). Of these 238 (79%) and 65 (21%) were cases with high burden and low burden mutations respectively. In our cohort, 2012 cases did not demonstrate a *TP53* aberration (86.3%). A total of 159 cases showed *TP53* mutations in the absence of del(17p) (49/159 with low burden *TP53* mutations) and 144 cases had both *TP53* mutation and del(17p) (16/144 with low burden mutations). Only 17/2332 (0.7%) cases demonstrated del(17p) with no *TP53* mutation. Validated NGS protocols should be used in clinical decision making to avoid missing low-burden *TP53* mutations and can detect the vast majority of *TP53* aberrations.

Keywords: chronic lymphocytic leukaemia, p53, deletion 17p, prognosis, next generation sequencing

INTRODUCTION

Deletion of chromosome 17p [del(17p)] and *TP53* mutation (*TP53* mut) referred to as *TP53* aberrations can be found in 8%–10% of previously untreated chronic lymphocytic leukaemia (CLL) patients and in up to 30%–40% of relapsed/refractory cases. *TP53* aberrations represent the most relevant risk factors for both progression free and overall survival following chemoimmunotherapy (1, 2). The introduction of small molecule inhibitors has led to enhanced response rates in patients with *TP53* aberrations (3–5). Therefore, the identification of *TP53* aberrations is essential for determining treatment decisions in CLL (6, 7). Historical data using Sanger sequencing suggests that approximately 80% of patients with del (17p) also carry a mutation in the second allele (8). A subset of patients also exhibits *TP53* mut without del(17p) (8).

The assessment of del(17p) is routinely performed by Fluorescence *in situ* hybridization (FISH). The cut-off for a positive result varies within laboratories with the threshold >20% of cells with del(17p) deemed to be a clinically relevant clone (9). However, it is recognized that a subset of patients with del(17p) have stable disease without the need for treatment (10).

Sanger sequencing is widely used for *TP53* mutational analysis, however it may misclassify cases of *TP53* mutations as wildtype when variants with allelic frequencies below the detection limit of Sanger sequencing are present. Recent studies using next generation sequencing (NGS) have shown that *TP53* mutations can be present at low clonal abundance in tumour cell populations, termed low-burden and have in certain studies the same detrimental effect on disease course (11–13). Therefore updated guidelines from the *TP53* network of ERIC (European Research Initiative on CLL- www.ericcll.org) suggest a threshold of 10% allelic burden for reporting mutations detected by NGS segregating these into high burden (≥10 variant allele frequency (VAF)) and low burden (<10% VAF) mutation (6). In the literature, contradictory results exist regarding the biological relevance of low burden mutations in CLL. This in part may be due to various sequencing strategies.

Therefore, the aim of this study, which is the largest cohort to date, was to investigate the presence of low and high burden *TP53* mutations in a "real-world" cohort of 2332 CLL cases using sensitive NGS and to correlate results with FISH data.

METHODS

Pretreatment peripheral blood samples from 2332 CLL patients referred for analysis of del(17p13) by FISH and *TP53* mutations by NGS were available for the present study diagnosed between 2015-2019. A retrospective audit of *TP53* status was undertaken. Best practice in the UK follows established guidelines, meaning that *TP53* testing is recommended prior to each line of treatment but not at diagnosis. As participants are part of Specialized HaemOnc Diagnostics services, requests for *TP53* testing in newly diagnosed patients would automatically be rejected.

The study was conducted according to the Declaration of Helsinki. Patients were diagnosed according to iwCLL guidelines (14). In all cases, analysis was performed on DNA obtained from >50% tumour cells. FISH analysis for del(17p13) was performed using Vysis Probes with a 10% cut-off for a positive result. *TP53* mutation screening was performed by NGS with a panel covering exons 2-11 as previously described (15) or by an Illumina amplicon-based strategy. Briefly, the amplicon-based panel was a bespoke assay and amplicon libraries for are generated by Reverse Complement PCR (RC-PCR) technology. The technique permits both the amplification and the ability to append sequences or functional domains of choice independently to either end of the generated amplicons in a single closed tube reaction. Primers for the *TP53* assay were designed in house and sequencing was performed on the Miseq using Illumina chemistry. Raw data was aligned using GATK. Indels are realigned using GeminiMulti indel realigner and Pisces is used for variant calling (both Illumina). Normally analyses with read depths below 5000 are failed.

A VAF cut-off of 1% was used to exclude false positive variants within the cohorts. Pathogenicity assessment of all variants was performed according to ERIC guidelines (6).

RESULTS

Altogether 2332 patients entering first line treatment were included in this study with *TP53* aberrations detected in 320/2332 patients (**Figures 1A, B**). Using NGS analysis, 429 *TP53* mutations were identified in 303 patients (13%). More than one *TP53* mutation was detected in 76 patients (2-8 mutations per patient, **Supplementary Table 1**). When considering all 429 *TP53* mutations in the cohort the VAF ranged from 1-97%; mean 28%. Using the 10% VAF threshold, cases were segregated into high burden mutations (≥10%) and low burden mutations (<10%). The high and low burden separation was based on the VAF of the most prevalent *TP53* mutation. 271 (63%) were classified as high burden mutations (VAF range: 10-97%; mean 42%). 158 were classified as low burden mutations (VAF range: 1-9%; mean 5%) (**Figure 2**).

This translated into 238 patients classified as high burden cases and 65 identified as low burden cases (**Supplementary Table 1**). The needle plot graphs demonstrated no differences in *TP53* coding mutations between high and low burden cases (**Figures 3A, B**). The mutation profile revealed that the majority of mutations were missense mutations followed by frameshift, splicing and nonsense mutations and is in keeping with previous reports (**Figure 3C**) (16, 17). No significant difference within mutation type existed between the low and high burden groups (P=0.5). The amino acid most frequently mutated were at positions 175, 209, 234, 248 and 273 indicating the classical hot spot mutations in CLL. Codons 175, 209, 234, 248 and 273 represented 110/429 (25%) mutations in the total cohort and showed similar allocation in low and high burden case (**Figures 3A, B**).

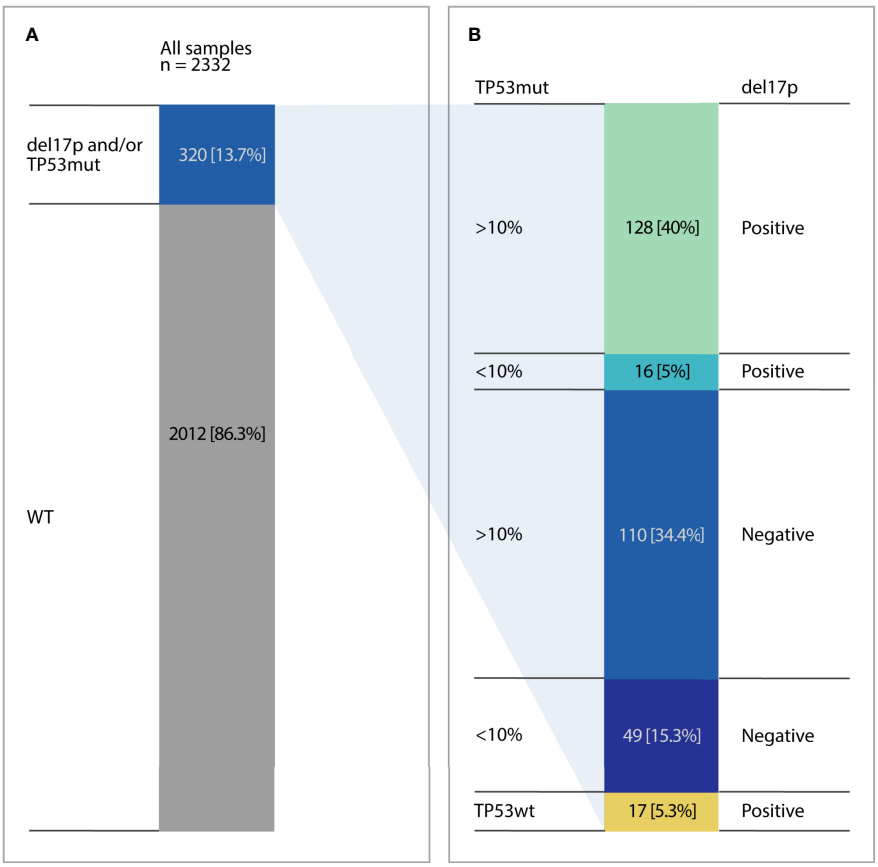

FIGURE 1 | *TP53* aberrations in the analyzed cohort. Composition of *TP53* defects.

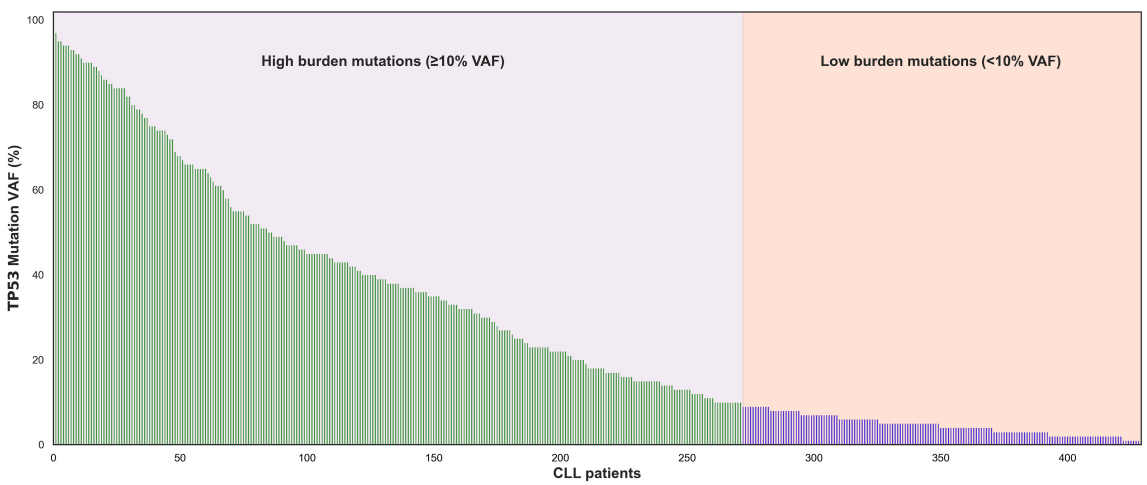

FIGURE 2 | Molecular Profile of *TP53* mutations in the cohort. Using a cutoff of 10% VAF 271 *TP53* mutations (228 patients) had high burden mutations and 158 *TP53* mutations (65 patients) had low burden mutations.

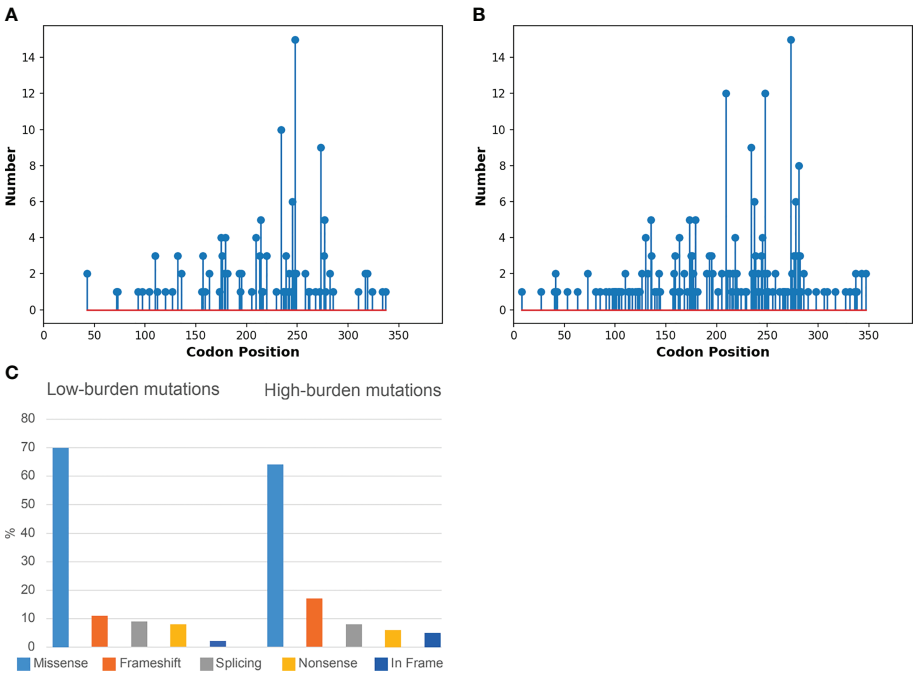

FIGURE 3 | Molecular Profile of *TP53* mutations in low and high burden cohorts. **(A)** Needle plot graph of low burden *TP53* mutations along the *TP53* coding sequence. **(B)** Needle plot graph of high burden *TP53* mutations along the *TP53* coding sequence. **(C)** Bar chart of mutations effect on the p53 protein in terms of amino acid changes in the low and high burden context.

Combining FISH data on del(17p) with *TP53* mutation data in our cohort, 2012 cases did not demonstrate a *TP53* aberration (86.3%). However, 17 cases demonstrated del(17p) only (0.7%). Average del(17p) was 40% (range 10-91%) in del(17p) only cases and was significantly higher in del(17p)/*TP53* mut cases (55% (range 10-100%: $p<0.05$). One hundred and fifty-nine patients (159) were *TP53* mutated only cases (49/159 with low burden TP53 mutations) and 144 cases with both del(17p) and *TP53* mutation (16/144 with low burden mutations, **Figure 1B**).

DISCUSSION

In this study, which is the largest study to date assessing *TP53* aberrations for both del(17p) and *TP53* mutation by NGS in cases of treatment naïve CLL. Using NGS analysis, 429 *TP53* mutations were identified in 303 patients (13%). Current guidelines from the *TP53* network of ERIC suggest a threshold of 10% allelic burden for reporting mutations detected by NGS (6). An acknowledgement is made in reference to cases with 5-10% VAF. In this study we employed a threshold of 10% VAF separating the cohort into high and low burden subgroups. High burden mutations were evident in 10.2% (238 cases) and low burden mutations in 2.8% (65 cases). This figure is lower than that reported in other studies and is likely due to the threshold of 1% used in this study (12, 13). Even with this threshold, 49 cases [*TP53* mut/del (17p) wt] in this cohort would have been misclassified as *TP53* proficient cases. This is an important observation given the recent publication that clearly demonstrates a shorter survival in cases with VAFs of 5-10% (13). This study again questions the threshold of 10% VAF and the impact this has in the misclassification of *TP53* aberrations.

Whilst most tumour suppressors are inactivated by frameshift or nonsense mutations, the most frequent mode of inactivation of *TP53* in CLL is by missense mutations which is a unique phenomenon. The mutation profile of the cohort did not differ when separated into high and low burden mutations. The vast majority of mutations were missense and no significant differences were observed between the low and high burden cohorts (**Figures 3A–C**). Unique to CLL is the presence of a specific hot spot variant leading to premature termination [p.(R209Kfs*6)]. This specific variant was demonstrated both in low and high burden cases highlighting the similar mutation profile between the cohorts (**Figures 3A, B**). The majority of *TP53* mutations are located within the DNA binding domain of the gene and hot spot mutations are frequently observed in CLL. This study showed an enrichment of mutations in codons 175, 209, 234, 248 and 273 representing (25%) of all mutations in the total cohort. A similar pattern was evident in both low and high burden subgroups confirming the disease specific *TP53* mutation profile in CLL (**Figures 3A, B**) (16).

Combining FISH data on del(17p) with *TP53* mutation data in our cohort, 2012 cases did not demonstrate a *TP53* aberration (86.3%) whereas *TP53* aberrations were detected in 13.7% of patients. This is in keeping with recent data from independent

groups that utilized various NGS strategies and bioinformatics pipelines (11, 13, 18).

In this study we have demonstrated the existence of del(17p) in the absence of a *TP53* mutation in 17/2332 (0.7%) which is in keeping with the literature (1, 13). The average del(17p) clone was 40% with a range of 10-91% (**Supplementary Table 1**) with 8/17 cases having a del(17p) clone less than 25%. Patients in population based cohorts are still routinely screened for del(17p) by FISH, whilst testing for *TP53* mutations can vary substantially by institution. This is despite very clear guidelines to the contrary (6, 14). Screening for only del(17p) in our study would have missed 50% of the alterations in the cohort (159/320). The relevance of FISH only based studies in the era of NGS is questionable as only a minority of p53 deficient cases are missed by NGS. In this series 0.7% of p53 deficient cases were missed by NGS of which 8 cases had a del(17p) clone size of less than 25%. Also recent data showing low-frequency del(17p) sub clones (<25% of CLL cells) in the absence of a *TP53* mutation has been demonstrated to mirror that of cases with no del(17p) in the chemoimmunotherapy setting (12, 19). In the study by Do et al. 15/20 (75%) patients demonstrated a low frequency subclone of del(17p) (<25)). This is a well recognized phenomena in the literature with subset of patients with low frequency del(17p) clones having enhanced progression free survival (10). This subgroup of patients is enriched with a mutated IGHV gene and relatively few copy number alterations. The study by Do et al. represents a surprisingly high percentage of low level del (17p) not previously described and likely reflects the genomic composition of the elderly trial cohort in the study. In the current study, we demonstrated 28/144 (19%) cases where del(17p) <25% with 17 cases demonstrating a high burden (≥10%) mutations and 11 cases with low burden mutations (**Figure 1B**). Unfortunately clinical data was not available in this study to ascertain the IGHV status in the cohort of del(17p) subclones.

TP53 aberrations are still relevant in the era of novel therapies. Long term survival outcomes remain inferior in cohorts of patients with *TP53* aberrations (20, 21). This is likely attributable to the role of p53 in the maintenance of genomic stability. It is well recognized that mutations in *TP53* occur early in the disease progression proceeding the genomic instability generated by chromosomal abnormalities.

This has been further addresses in a recent study demonstrating that patients treated with single-agent ibrutinib carrying only a single *TP53* hit have a superior long term response while multi-hit *TP53* is associated with a shorter progression free and overall survival (22). In this scenario single hit CLL can be classified by the presence of either del (17p) or *TP53* mut. Multi hit CLL arises when either del(17p) and *TP53* mut occur together or when greater than one *TP53* mutation is found. Whilst this is of interest it has yet to be verified in larger cohorts or indeed in separate treatment regimens. In our current study 55% (176/320) were single hit with 45% (144/320) of cases demonstrating a multi hit CLL. In this study ≥2 *TP53* mutations were detected in 76 patients with the majority of cases in the *TP53* mut/del(17p) wt cohort (46/76) with the remaining 30 cases in the *TP53* mut/no del(17p) cohort. This reinforces the need to redefine a VAF threshold to aid in the selection of *TP53* mutated patients benefiting from targeted treatments.

In conclusion, in the largest series to date we have demonstrated the presence of low and high burden *TP53* mutations in a series of CLL cases. The use of NGS prevents cases being misclassified as normal TP53 due to its enhanced sensitivity. In the investigation of *TP53* aberrations, NGS is an important strategy for patient management in this setting.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Using the NHS Health Research Authority decision tools this study classes as clinical audit (i.e., no randomization of patients, no alteration to standard clinical care and informs practice in our setting) and therefore formal research ethics is not required.

AUTHOR CONTRIBUTIONS

MAC, AS and PT conceived the project. DW, LC, DC, PS, MM and DC performed data analysis. DD, SL, EE, AH, MRC, DO'S, JQ, PM, JS, KM, FF, SI, NC, AS and PT provided clinical input. All authors read and agreed with the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.909615/full#supplementary-material

Supplementary Table 1 | *TP53* mutations from mutated cohort.

Supplementary Table 2 | Sequencing metrics from Amplicon and Capture technologies.

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NOTCH1 Signalling: A key pathway for the development of high-risk chronic lymphocytic leukaemia

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NOTCH1 is a cell surface receptor that releases its intracellular domain as transcription factor upon activation. With the advent of next-generation sequencing, the *NOTCH1* gene was found recurrently mutated in chronic lymphocytic leukaemia (CLL). Here, virtually all *NOTCH1* mutations affect the protein's PEST-domain and impair inactivation and degradation of the released transcription factor, thus increasing NOTCH1 signalling strength. Besides sequence alterations directly affecting the *NOTCH1* gene, multiple other genomic and non-genomic alterations have by now been identified in CLL cells that could promote an abnormally strong NOTCH1 signalling strength. This renders NOTCH1 one of the key signalling pathways in CLL pathophysiology. The frequency of genomic alterations affecting NOTCH1 signalling is rising over the CLL disease course culminating in the observation that besides *TP53* loss, 8q gain and *CDKN2A/B* loss, *NOTCH1* mutation is a hallmark genomic alteration associated with transformation of CLL into an aggressive lymphoma (Richter transformation). Both findings associate de-regulated NOTCH1 signalling with the development of high-risk CLL. This narrative review provides data on the role of *NOTCH1* mutation for CLL development and progression, discusses the impact of *NOTCH1* mutation on treatment response, gives insight into potential modes of NOTCH1 pathway activation and regulation, summarises alterations that have been discussed to contribute to a de-regulation of NOTCH1 signalling in CLL cells and provides a perspective on how to assess NOTCH1 signalling in CLL samples.

KEYWORDS

CLL (chronic lymphocytic leukemia), NOTCH1, high-risk, review, mutation

Introduction

The Notch family consists of four protein paralogs (NOTCH1-4) that are single-pass transmembrane receptors involved in cell fate decisions and cell differentiation by releasing a transcription factor upon receptor activation (1). De-regulated NOTCH signalling is frequently associated with malignant transformation of haematologic and

solid cancers (2). Gain-of-function mutations of the *NOTCH1* paralog were first discovered in T-cell acute lymphoblastic leukaemia (T-ALL) with a frequency of 56% in a cohort of 96 samples taken at diagnosis (3). With regards to other haematologic malignancies, de-regulated NOTCH signalling was also discovered in B-cell lymphomas *via* mutations in the *NOTCH1* and *NOTCH2* genes. In chronic lymphocytic leukaemia (CLL), recurrent *NOTCH1* mutations were observed at all disease stages, whereas mutations in the paralogs *NOTCH2*, *NOTCH3* and *NOTCH4* were rare events with frequencies <1% (*NOTCH2* in 0.9%; *NOTCH3* in 0.7%; *NOTCH4* in 0.6%) (4).

In B-cell lymphoma, *NOTCH1* mutations are almost always located in exon 34 and affect the protein's PEST-domain responsible for inactivation and degradation of the NOTCH1 intracellular domain (NICD1), which is released as transcription factor after NOTCH1 activation (5). This is different to findings made in T-ALL, where mutations are often located in the NOTCH1 heterodimerization domain (HD-domain). While PEST-domain *NOTCH1* mutations prolong transcription factor activity, mutations in the HD-domain disrupt the receptor's autoinhibitory conformation and lead to a stronger dysregulation of signalling strength than PEST-domain mutations (increase by factor 1.5 to 2 for PEST-domain mutations, by factor 3 to 9 for HD-domain mutations and by factor 20 to 40 when both mutation types affect the same NOTCH1 allele) (3).

In contrast to HD-domain mutations, PEST-domain mutations can only exert pathogenic effects after NOTCH1 activation and NICD1 release. This at least partly depends on ligand-binding, which inflicts shear forces opening the receptor's autoinhibitory domain and making a cleavage site accessible for the metalloenzymes ADAM10 and ADAM17. ADAM-mediated cleavage generates an intermediate cleavage-product termed NEXT (NOTCH1 extracellular truncation), which is ultimately cleaved by the gamma-secretase complex releasing NICD1 (6–8).

Within the PEST-domain, a hotspot mutation could be identified accounting for >90% of *NOTCH1* mutant CLL cases. It represents a deletion of two nucleotides in the 2514 proline codon leading to a premature stop-codon in the fourth altered codon (c.7541_7542delCT, p.P2514Rfs*4). The other exon 34 mutations represent more proximal stop codons so that loss of the C-terminal amino acid sequence is a common characteristic of all PEST-domain mutations (at least 39 amino acids plus sequence alteration of the 3 preceding amino acids, Figures 1A, B) (5, 9).

NOTCH1 mutation frequency in CLL

Screening CLL samples for *NOTCH1* mutations within prospective clinical trial cohorts by exon 34 targeted next-generation sequencing revealed an enrichment for *NOTCH1* mutations over the disease course. In monoclonal B-cell lymphocytosis (MBL, defined by <5000 CLL-phenotypic cells per µl peripheral blood) (10), *NOTCH1* mutations were found in 11% of cases compared to a frequency of 13% observed in early stage CLL (Binet A) not requiring treatment (frequencies in MBL and early stage CLL did not significantly differ when assessed within the same clinical trial, NCT00917450; p=0.6046 as inferred by Fisher's exact test) (10). In unselected CLL patients needing first-line treatment, frequencies ranged from 17% to 23% (11, 12), in relapsed/refractory (R/R) CLL patients from 24% to 29% (13, 14). Sequencing studies outside of clinical trial cohorts and/or using less sensitive sequencing techniques revealed somewhat lower frequencies (9, 15–22). In Richter transformation (RT) comprising progression of CLL into aggressive lymphoma, *NOTCH1* mutation was identified as hallmark genomic alteration next to *TP53* alteration, *CDKN2A/B* loss and *MYC* gain. *NOTCH1* mutation frequencies were 25 and 41% in a limited number of RT-cases screened (N=28 and 27) (23, 24).

In addition to coding *NOTCH1* mutations, non-coding mutations were found in the 3' untranslated region (UTR) with recurrent 139390152A>G and 139390145A>G sequence alterations (referring to the GRCh37/hg19 reference genome) (25, 26). Within the UK LRF CLL4 trial, non-coding *NOTCH1* mutations were identified in 2.4%, mutually exclusive from coding *NOTCH1* mutations found in 10.1% of patients. Both *NOTCH1* mutant patient groups had a comparable clinical outcome with inferior progression-free survival (PFS) as compared to patients with wild-type *NOTCH1*. This is in line with the biologic effect of 3'-UTR mutations leading to the loss of at least 53 terminal amino acids by creating a new acceptor site in the 3'-UTR and involving a cryptic donor site in the coding region of exon 34 or less frequently, the canonical donor site on exon 33 for aberrant splicing (Figure 1C) (25, 26).

Impact of NOTCH1 mutation on the CLL disease course

Whether *NOTCH1* mutation initiates CLL development, was addressed in a study analysing multipotent hematopoietic progenitor cells flow-sorted from the bone marrow of CLL patients for sequence variations (27–29). Accounting for sorting impurities and demonstrating multipotency of progenitor cells by enforcing myeloid colony formation, *NOTCH1* mutations were identified in progenitor cells at unexpectedly high frequencies. The same accounted for other lymphoid oncogenes such as *BRAF*, *SF3B1*, *NFKBIE* and *EGR2* (27). In line with *NOTCH1* mutation being an early event in CLL development, functional analyses using a constitutively active form of NOTCH1 induced CLL disease onset in an IgH.TEµ mouse model and had an impact on direct and indirect cell-cycle

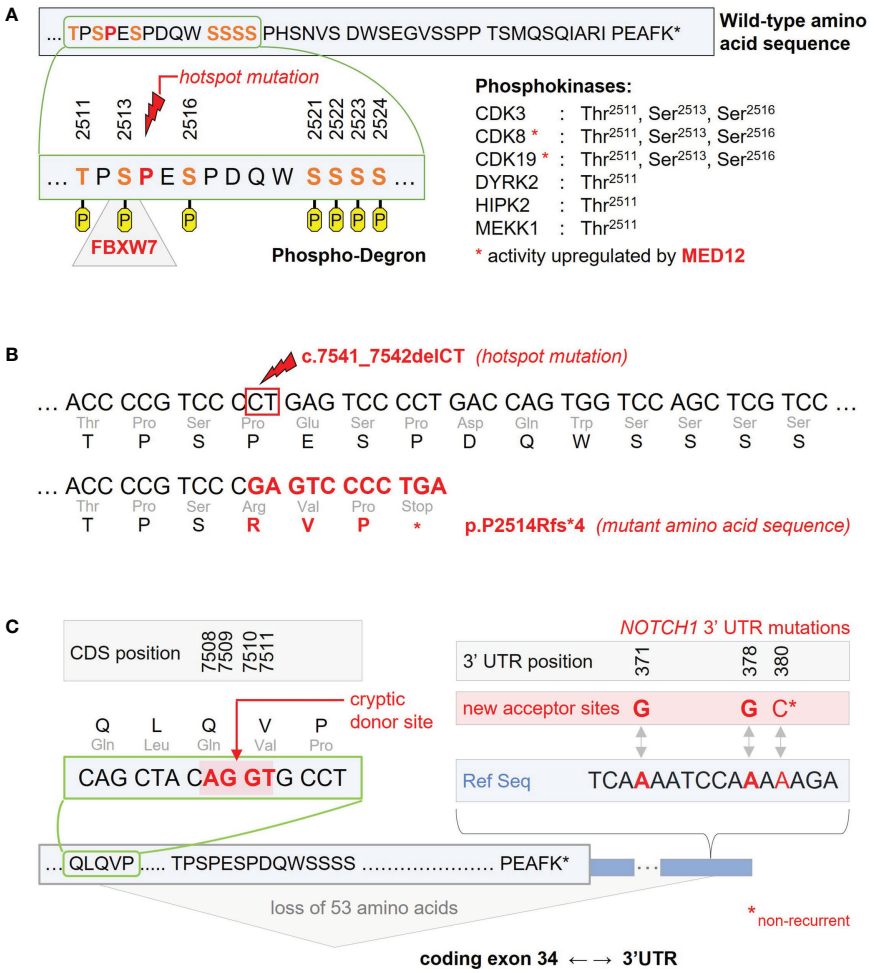

FIGURE 1
Recurrent *NOTCH1* gene mutations impairing inactivation and degradation of the NICD1 transcription factor. **(A)** C-terminal wild-type amino acid sequence encoded by *NOTCH1* exon 34. The amino acid sequence shown is part of the protein's PEST-domain and held responsible for inactivation and ubiquitination of the NICD1 transcription factor released after NOTCH1 receptor activation. According to PhosphoSitePlus, the Thr^{2511}, Ser^{2513}, Ser^{2516}, Ser^{2521}, Ser^{2522}, Ser^{2523}, and Ser^{2524} amino acid residues were identified as phosporylation sites potentially involved in NICD1 inactivation and degradation. As part of this putative phospho-degron, Ser^{2513} has been identified as binding site for the ubiquitin ligase FBXW7 recurrently affected by inactivating mutations in CLL. The FBXW7 binding site is directly adjacent to the Pro^{2514} codon harbouring the c.7541_7542delCT hotspot mutation. Phosphokinases associated with Thr^{2511}, Ser^{2513}, and Ser^{2516} phosphorylation are CDK3, CDk8 and CDK19. Activation of CDK8 and CDK19 is at least partly mediated by MED12 found to be recurrently mutated in CLL. **(B)** C-terminal nucleotide and amino acid sequences as found with the c.7541_7542delCT hotspot mutation. The resulting frameshift mutation leads to a premature stop in the fourth altered codon. **(C)** Non-coding mutations in the 3' untranslated region (3' UTR) of *NOTCH1* induce a new acceptor site for alternative splicing. Recurrent single-nucleotide variants were found in position 371 (corresponding to chr9 position 139390152 in the GRCh37/hp19 reference genome) and in position 378 of the *NOTCH1* 3'UTR (chr9 position 139390145). A non-recurrent single-nucleotide variant was described for position 380 (chr9 position 139390143). Interaction with a cryptic donor site located in the coding part of exon 34 (positions 7508-7511 of the coding sequence, CDS) leads to loss of the 53 terminal amino acids.

regulation increasing the *in-vivo* proliferation rate of lymphoid cells (30). Moreover, the NOTCH1 target gene repertoire is supposed to initiate a broad program aiming at survival and proliferation of mature B-cells by including *MYC* and other genes involved in B-cell receptor and cytokine signalling (31).

Although the results outlined above strongly support a role for *NOTCH1* mutation as driver of CLL initiation and progression, this notion was not fully backed up when analysing sequential CLL samples for dynamics in *NOTCH1* mutant cancer cell fractions (CCFs). While some studies demonstrated an increase of *NOTCH1* mutant CCFs over the disease course or a constantly high mutation burden (4, 32), other studies identified individual cases with receding or disappearing *NOTCH1* mutant clones (33). Given the limited number of sequential samples analysed for the dynamics of *NOTCH1* mutant CCFs, more work needs to be invested to fully

understand the behaviour of *NOTCH1* mutant CLL cells during periods of "watch & wait" and under the selective pressure of therapy.

Impact of NOTCH1 mutation on response to treatment

The role of *NOTCH1* mutations in conferring treatment resistance was first assessed in the setting of chemotherapy. Analyses within the UK LRF CLL4 trial comparing chlorambucil mono versus fludarabine mono versus fludarabine plus cyclophosphamide (FC) identified *NOTCH1* mutation as an independent risk factor for shorter PFS and overall survival (OS) (26, 34). However, the unfavourable impact of *NOTCH1* mutation on response to chemotherapy was not reproducible in the FC-arm of the CLL8 trial and in the chlorambucil-arm of the CLL11 trial of the German CLL Study Group (GCLLSG) (9, 35).

In treatment arms combining an anti-CD20 monoclonal antibody (mAb) with chemotherapy, presence of *NOTCH1* mutation was associated with inferior PFS. This included the FC-rituximab-arm of the GCLLSG CLL8 trial, the ofatumumab-chlorambucil-arm in the COMPLEMENT-1 trial and the obinutuzumab-chlorambucil arm in the GCLLSG CLL14 trial (9, 11, 12). With regards to type I anti-CD20 mAbs (rituximab and ofatumumab), *NOTCH1* mutation could be identified as predictive marker for no or only weak benefit from anti-CD20 mAb addition to chemotherapy (9, 11, 17), whereas results obtained in the GCLLSG CLL11 trial implied that the type II anti-CD20 mAb obinutuzumab was able to overcome this non-benefit (35).

Regarding explanations for reduced benefit from rituximab and ofatumumab, studies demonstrated lower CD20 expression on the surface of *NOTCH1* mutant CLL cells reducing the antibodies' capacity to elicit complement-dependent cytotoxicity (36, 37). This was explained by higher levels of active histone deacetylases (HDACs) in the nucleus of *NOTCH1* mutant cells due to greater disruption of RBPJ/HDAC protein complexes by increased nuclear protein levels of mutant NICD1 (36). Free HDAC1 and HDAC2 were shown to interact with the promoter of the CD20 coding gene *MS4A1* and to suppress its transcription (36, 38). However, this explanation remained contradictory since associations between low CD20 expression and *NOTCH1* mutation were not reproducible in the GCLLSG CLL8 nor in the COMPLEMENT-1 trial (9, 11). As another explanation, *in-vitro* studies on SU-DHL4 revealed strong activation of NOTCH1 signalling by rituximab, but not obinutuzumab, possibly explained by distinct intracellular signalling events that both anti-CD20 mAbs induce in the B-cell receptor (BCR) signalling cascade (Figure 2) (39). Exceedingly strong transcriptional changes induced after release of mutant NICD1 in concert with pro-survival signalling changes following rituximab binding to CD20 could thus also be responsible for a higher resistance of *NOTCH1* mutant CLL cells towards rituximab-based chemo-immunotherapy. Taken together, the reasons for reduced benefit of NOTCH1 mutant CLL from type I anti-CD20 mAbs are not sufficiently understood and warrant further research.

Modulation of NOTCH1 signalling through BCR signalling, as suggested above, was supported by *in-vitro* and *in-vivo* data obtained under BTK inhibition, since ibrutinib treatment was shown to suppress NOTCH1 signalling (39, 40). In keeping with this notion, the presence of *NOTCH1* mutation had no negative impact on response to ibrutinib treatment in the RESONATE trial (13). Suppressive effects on NOTCH1 signalling were also found for the Pi3K-inhibitor idelalisib in an *in-vitro* setting (39), whereas clinical data based on nine patients suggested poor response of *NOTCH1* mutant CLL patients to idelalisib (41).

With regards to the BCL2-inhibitor venetoclax, a pooled dataset on results from monotherapy showed that *NOTCH1* mutation was associated with a shorter duration, but not probability of response (40). In the MURANO trial combining venetoclax with rituximab, *NOTCH1* mutation had no adverse impact on PFS, but was associated with lower rates of undetectable minimal residual disease (MRD) at the end of treatment (20). The GCLLSG CLL14 trial combining venetoclax with obinutuzumab did not reveal a negative impact of *NOTCH1* mutation on PFS and MRD rates (12).

Activation of the NOTCH1 receptor

In the lymph node, CLL cells were shown to frequently express high NICD1 protein levels. Hence, presence of the NOTCH1 ligands JAG1, JAG2, DLL1, DLL3, and DLL4 expressed by microenvironmental cells in the lymph node constitutes one regulative factor for NOTCH1 activation (42, 43). In the perinodal area, CLL cells were shown to express only low levels of the NICD1 transcription factor suggesting that NOTCH1 signalling rapidly decreases once cells exit their lymph node niche (42, 43). This is in line with the relatively short half-life of few hours described for the NICD1, which allows a dynamic regulation of NOTCH1 target genes (44–46). In contrast to these findings, about 50% of CLL cases lacking a *NOTCH1* mutation present with high NOTCH1 signalling levels in virtually all peripheral CLL cells suggesting a continuous induction of NOTCH1 cleavage in the blood stream (31). This may occur by ligand-dependent mechanisms such as interaction with other CLL cells expressing JAG1 and JAG2 or interaction with ligand-expressing endothelial cells (31).

Alternatively, a ligand-independent mode of NOTCH1 activation could be responsible for continuously high signalling levels. This mode is less well understood, likely executed by ADAM17 and possibly occurring in the intracellular compartment where NOTCH1 is expressed on endosomal membranes (47, 48). Importantly, ligand-independent NOTCH1 cleavage was described to occur shortly

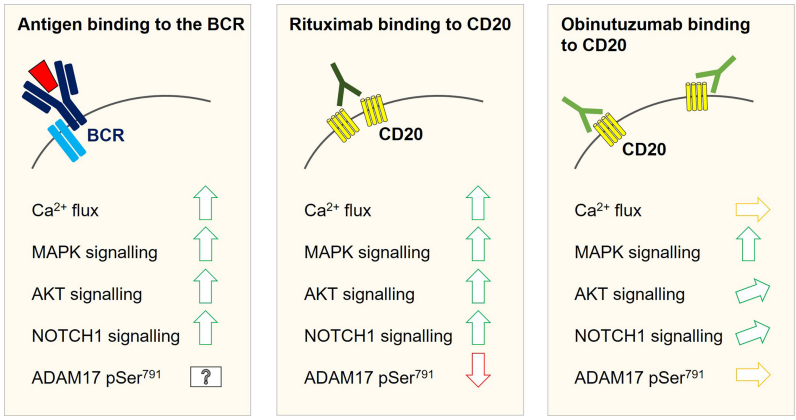

FIGURE 2
Intracellular signalling changes with a potential relevance for NOTCH1 activation as observed in SU-DHL4 cells after rituximab and obinutuzumab *in-vitro* treatment. Antigen binding to the B-cell receptor induces Ca^{2+}-flux and activates the MAPK and Pi3K/AKT signalling pathways. All three signalling events have been linked to modulation of ADAM10/ADAM17 activity. Both metalloproteases were shown to promote the first cleavage step to release the NICD1 transcription factor. Likewise, an increase in NOTCH1 signalling could be associated with an activation of the B-cell receptor signalling cascade. As well as B-cell receptor activation, rituximab binding to CD20 can induce Ca^{2+}-flux, MAKP signalling and AKT activation. In keeping with this notion, NOTCH1 signalling was shown to be inducible by rituximab treatment in SU-DHL4 cells. Phosphoproteomic studies in SU-DHL4 cells revealed a significant decrease in ADAM17 Ser^{791} phosphorylation at one hour after start of rituximab treatment. ADAM17 Ser^{791} de-phosphorylation has been associated with an increase in ADAM17 activity. Obinutuzumab binding to CD20 induces MAPK signalling, but only weak AKT activation and no measurable Ca^{2+}-flux in SU-DHL4 cells. NOTCH1 signalling was induced to a much lower degree by obinutuzumab than by rituximab treatment and ADAM17 Ser791 phosphorylation was not significantly altered.

after activation of T-cell as well as B-cell receptors (39, 49–54), a finding that associates NOTCH1 signalling with the response of T- and B-cells to antigen recognition and is interesting against the notion that high frequencies of *NOTCH1* mutation could be associated with aggressive stereotyped B-cell receptor subsets (subsets #1, #6, #8, and #59) (55).

These notions raise questions on how ligand-independent NOTCH1 cleavage could be regulated. Liquid chromatography-tandem mass spectrometry conducted on samples from SU-DHL4 cells treated with rituximab for one hour revealed significant de-phosphorylation of ADAM17 Ser^{791} as compared to untreated and obinutuzumab treated cells, which coincided with an increase in NOTCH1 signalling (39). Metalloprotease activity is modulated by phosphorylation changes on the ADAM intracellular domain and ADAM17 Ser^{791} de-phosphorylation has been associated with an increase in ADAM17 activity (56). MAPK and Pi3K/AKT signalling were shown to be up-stream of ADAM10/ADAM17 phosphorylation changes (56–58). This potentially links antigen-induced BCR signalling to NOTCH1 activation. *In-vitro* results from SU-DHL4 cells revealed that MAPK and AKT activation can also be observed after rituximab treatment, whereas obinutuzumab is a strong activator of the MAPK pathway, but only a weak activator of AKT and NOTCH1 signalling (39). MAPK and particularly Pi3K/AKT may hence constitute links between the BCR signalling pathway and NOTCH1 cleavage (Figure 2). Against this background, it is interesting that a more recent study revealed coincidence of AKT overactivation with increased NOTCH1 signalling levels in an Eμ-TCL1 CLL mouse model (59). Taken together, these findings warrant a better understanding of the regulatory processes behind NOTCH1 cleavage to fully understand the NOTCH1 activation process in CLL cells.

Regulation of NOTCH1 transcription factor activity

Upon translocation of NICD1 into the nucleus, the transcription factor gets integrated into a protein complex, termed coactivator complex. This complex encompasses the DNA-adapter protein RBPJ and chromatin modifiers to regulate expression of NOTCH1 target genes. RBPJ has a dual role in the transcriptional regulation of NOTCH1 target genes, since in the absence of NICD1 in the nucleus, it is integrated into a protein complex repressing NOTCH1 target gene transcription. Upon arrival of NICD1 in the nucleus, this repressor complex is disrupted allowing the formation of the coactivator complex formed around RBPJ (60).

Besides RBPJ, the NOTCH1 repressor complex consists of SPEN, CTBP, NCOR, CIR, possibly SNW1 and a histone deacetylase (60). Of note, *SPEN* was found recurrently mutated in CLL with a rising frequency over the disease course. An unselected CLL cohort needing first-line treatment revealed a *SPEN* mutation frequency of 1.8% (NCT00281918; 5/

278 cases), a R/R-cohort a frequency of 3.7% (NCT01392079; 4/108 cases) and an RT-cohort a frequency of 18.5% (5/27 cases) (4, 14, 23). While in the first cohort, *SPEN* mutation was not found to co-occur with *NOTCH1* mutation, this was frequently observed at more advanced disease stages (1/4 cases in the R/R-cohort, 3/5 cases in the RT-cohort), suggesting synergistic effects of both mutations (4, 14, 23). Expression of two well-established NOTCH1 target genes (*HES1* and *DTX1*) was significantly increased in *SPEN* mutant primary CLL samples, possibly explained by de-repression of NOTCH1 target genes upon disruption of the NOTCH1 repressor complex (14).

With regards to other components of the corepressor complex, the *RBPJ* and *SNW1* gene loci were found to be recurrently deleted in high-risk CLL (minimally deleted regions: del (4)(p15.1-p15.2) for *RBPJ* loss; del (14)(q24.3-q32.1) for *SNW1* loss). *RBPJ* deleted CLL samples presented with higher *DTX1* but not *HES1* expression levels rendering del (4)(p15.2) an alteration, which may be linked to de-regulated NOTCH1 signalling (14). *SNW1* deleted CLL samples revealed no evidence for increased NOTCH1 target gene transcription (14).

As a well-established NOTCH1 target gene, *MYC* is recurrently affected by chromosomal gains (14, 61, 62). Large 8q-gains encompassing the *MYC* gene locus were found with a rising frequency over the disease course (~16% in high-risk cases) next to focal gains inside a *MYC* enhancer region (14, 31, 62). The latter contained NICD1 binding sites and represented the only recurrent focal gain found in CLL (frequency ~1% in unselected CLL cases) (62). As to what extent NOTCH1 signalling drives CLL progression *via* modulation of *MYC* transcription is yet unclear.

Notably, *SF3B1* mutation as one of the most frequently altered gene in CLL could also be associated with increased NOTCH1 signalling (63). This is particularly interesting against the notion that *NOTCH1* and *SF3B1* mutations virtually show mutual exclusivity (9). The *SF3B1* gene encodes a spliceosome component and its mutation was found to induce alternative splicing of the *DVL2* gene (63). DVL2 is described as negative regulator for transcription of NOTCH1 target genes *via* binding to RPBJ, whereas the identified *DVL2* splice variant is associated with up-regulated transcription of the NOTCH1 target gene *HES1* (63, 64). If this observation is explainable *via* RBPJ's role in the NOTCH1 corepressor or activator complex, remains a subject for future investigation.

Inactivation and degradation of the NOTCH1 transcription factor

The PEST-domain is responsible for inactivation and degradation of NICD1. The inactivation process is thought to involve phosphorylation of serine residues located inside and directly adjacent to the PEST-domain fragment that gets lost by the pP2514Rfs*4 mutation (65). CDK8 and its paralog, CDK19, are two kinases that have been associated with inactivating phosphorylation of NICD1 phosphorylation sites (65, 66). In addition, the NOTCH1 Ser^{2513} residue was identified as binding site for the FBXW7 ubiquitin ligase targeting NICD1 for proteasomal degradation (Figure 1A) (67). Interaction between NICD1 and FBXW7 was particularly shown for the α-(nucleoplasmic) and γ-isoform (nucleolar) of FBXW7, but not for its β-isoform (cytoplasmic), suggesting that NICD1 interacts with FBXW7 inside the nucleus (67, 68).

Mutations in the *FBXW7* gene were found in 4% of previously untreated CLL patients (36/905). They frequently affect hotspots in the protein substrate binding domain leading to a reduced binding capacity of FBXW7 to NICD1 and other proteins. NICD1 protein levels were increased in *FBXW7*-mutant CLL cases, comparable to findings made in *NOTCH1*-mutant CLL cases (68). However, if accumulating non-ubiquitinated NICD1 retains transcription factor activity or is at least partly inactivated by phosphorylation or other signalling events needs further investigation.

Another genomic event possibly involved in NOTCH1 de-regulation is *MED12* mutation. In a meta-analysis including 1429 samples from 5 studies, these mutations were discovered in 2.9% of CLL patients. Of note, they were found to be mutually exclusive from *NOTCH1* mutation. Similar to results obtained for *FBXW7* mutation, *MED12* mutations could be associated with increased NICD1 protein amounts (69). One possible explanation for NICD1 accumulation is that MED12 has been associated with CDK8 and CDK19 kinase activation (70–72).

Discussion

The findings outlined above demonstrate that NOTCH1 de-regulation can occur at the level of NOTCH1 receptor activation, NOTCH1 target gene expression, and NICD1 inactivation. When assessing the impact of abnormally strong NOTCH1 signalling, it is hence not sufficient to only screen for *NOTCH1* gene mutations. Moreover, non-mutational NOTCH1 activation found in ~50% of CLL cells lacking a *NOTCH1* mutation implies that even an extended panel including *SPEN*, *SF3B1*, *FBXW7* and *MED12* mutations will neither be sufficient to identify all patients with abnormally strong NOTCH1 signalling. Approaches beyond the genomic level to measure NOTCH1 signalling strength are the detection of NICD1 at protein level or the assessment of a "NOTCH1 gene expression signature" as compiled by Fabbri et al. *via* an integrated analysis of gene expression profiling and NOTCH1 ChIP-Seq results (31). Importantly, the assessment of NOTCH1 signalling strength requires standardized conditions, as for example, CLL cells may not be collected in EDTA acting as strong inducer of NOTCH1 signalling due to its Ca^{2+}-chelating ability (73).

Sufficiently large CLL and RT patient cohorts have hitherto not been screened systematically for the different mechanisms underlying NOTCH1 activation. The overall impact of NOTCH1 signalling on CLL progression is hence not appropriately addressed and it is yet unclear whether NOTCH1 affecting alterations such as *FBXW7* and *SPEN* mutations have the same effects on prognosis and treatment response than *NOTCH1* mutations. Due to the relatively low frequency of these mutations even in high-risk CLL cohorts, it will be challenging to seek clarity on this question. Targeted next-generation sequencing approaches assessing all recurrent mutations associated with NOTCH1 de-regulation will have to be applied to large-scale patient cohorts and likewise, more effort has to be invested to unravel causes for de-regulated NOTCH1 signalling beyond the genomic level.

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JE conceived and wrote the manuscript and created the figures. The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Opinion: What defines high-risk CLL in the post-chemoimmunotherapy era?

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chronic lymphocytic leukemia (CLL), high-risk, TP53, definition, BTK - Bruton's tyrosine kinase, BCL2 (B-cell lymphoma 2), COVID - 19, risk factor

The definition of high-risk chronic lymphocytic leukemia (CLL) was relatively simple in the chemoimmunotherapy era, as it was defined by only one genomic marker, *TP53* alteration, along with poor responses to purine-analogue based treatment (1). While other biomarkers such as unmutated IGHV, del(11q), high ZAP70 expression and high CD38 expression were associated with inferior prognosis, *TP53* deficiency by mutation and/or del (17p) remained the only biomarker that clearly guided treatment decisions (2).

The emergence of targeted compounds has rendered chemoimmunotherapy virtually obsolete for CLL treatment, with it remaining an option only for patients with a mutated IGHV, normal *TP53* and a non-complex karyotype (3). Instead, non-chemotherapeutic targeted treatment has now become the standard of care. Approved treatment options in first- and second-line include continuous treatment with a covalent BTK inhibitor (e.g. ibrutinib, acalabrutinib) plus/minus anti-CD20 monoclonal antibody (4–9), fixed duration therapy with the BCL2 inhibitor venetoclax plus anti-CD20 monoclonal antibody (10, 11), fixed duration therapy with venetoclax plus ibrutinib (12, 13), and for *TP53* altered cases, continuous monotherapy with venetoclax (14). Moreover, clinical trials are currently evaluating triple drug regimens that combine BTK and BCL2 inhibitors with anti-CD20 treatment (15–18). Looking forward, non-covalent BTK inhibitors (e.g. pirtobrutinib and nemtabrutinib) (19, 20), BTK degraders (e.g. NX-2127) (21), and second-generation BCL2 inhibitors (e.g. Lisaftoclax) (22) are promising alternatives in clinical development, along with immunotherapeutic approaches such as CAR T-cells and bispecific antibodies.

The paradigm shift from chemoimmunotherapy to targeted therapy and the ever-increasing number of treatment options has meant that defining high-risk CLL is less straight-forward. This is mainly because BTK and BCL2 inhibitors have been demonstrated to markedly improve progression-free and overall survival (PFS and OS) in *TP53*-deficient and IGHV unmutated CLL patients (4, 5, 7, 9, 10, 14, 23–25). Limited data from clinical trials evaluating ibrutinib first-line and acalabrutinib have even raised the possibility that BTK-inhibition may overcome the adverse effects of *TP53* deficiency (5, 6, 9, 26). Although results

from a direct PFS comparison between *TP53* deficient and non-deficient cases are not available yet, data from the SEQUOIA and ALPINE trials testing the second-generation covalent BTK inhibitor zanubrutinib in first-line and in relapsed/refractory CLL further support this hypothesis (27, 28). In contrast, *TP53* alterations remained prognostic for shorter PFS in studies on ibrutinib treatment of relapsed/refractory CLL (29–31). This difference may be explained by a high prior treatment load in the relapsed/refractory population leading to a selection of adverse risk factors associated with *TP53* deficiency such as high karyotype complexity (32–34). Genomic characterization of sequential samples taken pre-ibrutinib treatment and at disease progression demonstrated that *TP53*-deficient subclones were not necessarily responsible for ibrutinib failure. For instance, several studies on the clonal dynamics of *BTK* mutation as a frequent resistance mechanism towards covalent BTK inhibitors have shown that at relapse, *BTK* mutation can evolve within a *TP53* wild-type subclone while the *TP53*-deficient subclone is eliminated or remains effectively controlled (35–37).

With regards to BCL2 inhibition, clinical trial data revealed that fixed-duration first-line treatment with venetoclax in combination with obinutuzumab could not completely overcome the adverse effects of *TP53* deficiency (10), with corresponding results after combination with ibrutinib pending. As data on continuous venetoclax first-line treatment and on venetoclax re-exposure is also currently lacking, it remains unclear as to what extent the impact of *TP53* deficiency relates to the mode of action and what relates to the treatment duration (time-limited versus continuous).

Results from PFS comparisons between CLL cases with mutated and unmutated IGHV status suggested that continuous ibrutinib and acalabrutinib monotherapy was able to abrogate the negative prognostic impact of unmutated IGHV in treatment-naïve and in relapsed/refractory CLL (4, 5, 26, 30). In treatment arms combining ibrutinib with rituximab or obinutuzumab, the PFS seems to be shorter in the IGHV unmutated than mutated subgroup, but direct comparisons are missing and results on the combination of acalabrutinib and obinutuzumab did not suggest a prognostic impact of the IGHV status (5–7, 9). With regards to venetoclax-based fixed-duration therapy, unmutated IGHV status retained prognostic significance and one can speculate that as IGHV unmutated patients achieved high response rates and MRD negativity, shorter PFS may reflect the more proliferative nature of IGHV unmutated CLL cells potentially leading to a faster re-growth of the CLL clone after end of treatment (10, 38–40).

Given the long PFS in IGHV unmutated (7-year PFS 58% in the RESONATE-2 trial) (4) and in *TP53* altered CLL cases (6-year PFS 61% in a phase II clinical trial) (23) that can already be achieved by continuous BTK inhibition in first-line, these characteristics should no longer be seen as high-risk features for treatment failure *per se*. They should rather be seen as factors associated with an increased risk for early disease progression in certain therapeutic regimens. To fully evaluate the impact of *TP53* alteration and IGHV status, longer follow-up data and more direct PFS comparisons of *TP53* altered versus non-altered and IGHV mutated versus unmutated cases are clearly required for all targeted treatment approaches. Likewise, disease and patient characteristics beyond *TP53* and IGHV must be validated or newly defined, and potentially integrated in new prognostic models, since risk scores like the CLL International Prognostic Index (CLL-IPI) and the Continuous Individualized Risk Index (CIRI)) were developed using data from patients treated by chemoimmunotherapy with re-evaluation in the context of novel agents pending (41, 42). For patients treated with ibrutinib, a four-factor scoring system involving *TP53* alterations, prior treatment, serum β2-microglobulin concentration, and lactate dehydrogenase level was developed to identify patients at increased risk of ibrutinib failure by the time of treatment initiation and relapse (43). This prognostic score is independently evaluated (44, 45), but remains to be evaluated in clinical trials testing second-generation covalent BTK inhibitors.

The absence of fully validated prospective biomarkers and generally valid risk scores stratifying treatment outcome has led to a return to a clinical definition of high-risk CLL: as being described by dual resistance towards BTK and BCL2 inhibition (46). While this approach can help to select patients for more perilous treatment strategies such as allogeneic stem cell transplantation, the obvious limitation is that this "post-hoc" definition comes too late for the patients. Hence, there remains a requirement to define biomarkers that identify high-risk disease at the time of diagnosis or first relapse.

Analyses of CLL cells resistant towards BTK or BCL2 inhibitors have identified biomarkers that predict for non-durable response to targeted treatments (33, 34, 47). Genomic instability is one example, possibly due to it facilitating the evolution of clones resistant to the selective pressure of therapy (33, 34). High levels of pro-proliferative stimuli driven by *MYC* gain, constitutive BCR-signaling and loss of cell-cycle control (e.g. by *CDKN2A*/*CDKN2B* deletion) may have similar effects on clonal evolution and drive CLL cells towards transformation (48–50). Furthermore, the immune microenvironment has been shown to play a crucial role in CLL, but it is not clear how to integrate these factors into risk stratification models (51).

Besides these non-treatment-specific risk factors, the acquisition of resistance mutations in *BTK*, *PCL2G* or *BCL2* represents an alternative mechanism of resisting the relevant inhibitor (52–56). While it is tempting to speculate that patients with these risk factors may benefit from treatment intensification with multi-agent combinations, prospective validation of this assumption is challenging as resistance mutations cannot be anticipated at the time of treatment initiation.

Therefore, the "brave new world of personalized CLL medicine" (51) remains a distant goal, with isolated analyses of putative biomarkers in individual clinical trial cohorts struggling to bring it closer. Biomarkers should be seen within the context of pathobiology and grouped for the definition of molecular CLL subtypes that will derive the most benefit from specific drug classes or treatment combinations (57). To reach that goal, collaborative initiatives as the CLL HARMONY Alliance are vital to compile patient registries that incorporate clinical trial as well as real-world data. This requires the application of complex "big data" analytical techniques including artificial intelligence and machine learning to identify the best biomarkers, to clearly define patient subgroups and to develop tailored therapeutic approaches.

Apart from this focus on the CLL cells and their biological heterogeneity, we feel that the definition of "high-risk CLL" should be broadened by including factors such as individual patient characteristics, treatment design, and the situational context of a patient's care (see Figure 1). Some of these factors were already encapsulated within former CLL treatment algorithms such as the

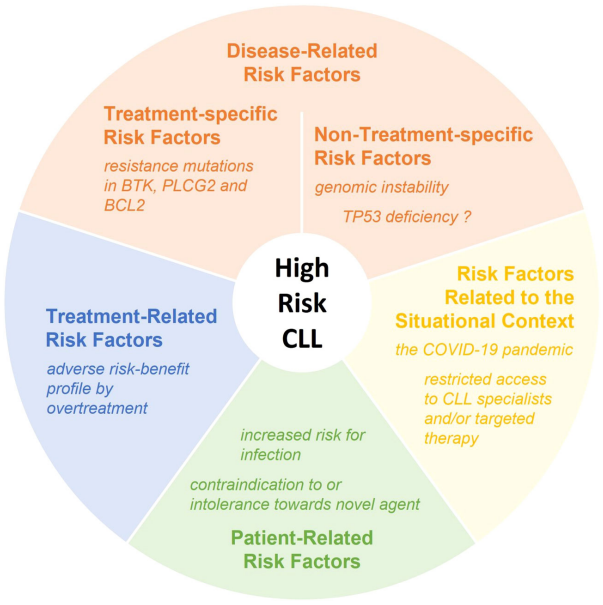

FIGURE 1
Factors increasing the risk for early CLL-related death. Examples are provided for each category of risk factors.

"go-go", "slow-go" and "no-go" three-tier "traffic light" approach developed during the chemotherapy era (58). The enhanced tolerability of targeted therapies has led to this approach becoming less important since a wider range of patients can now benefit from highly effective treatments, but on the other hand, targeted therapies have brought a new set of considerations that impact outcome.

For example, the situational context of a patient can become a risk factor when access to CLL specialists is restricted or when the health care system of a country does not permit the prescription of more expensive targeted therapies. Moreover, patients who have a contraindication to or are intolerant towards one of the novel agents lack an important treatment option, which may become critical over the course of the disease. A patient with mechanical heart valve requiring anticoagulation could hence be regarded as high-risk due to BTK inhibition contraindication, even if high-risk biological factors are lacking. Another good example is the risk from infection, which has been brought into sharp focus in the context of the COVID-19 pandemic. Infection is a major cause of morbidity and mortality for CLL patients, including those with early-stage disease (59–61). This risk can be aggravated by treatment, as for instance, both anti-CD20 monoclonal antibodies and BTK inhibitors are associated with reduced ability to respond to anti-COVID-19 vaccination (62–64). Therefore, the pandemic has shown very clearly how the situational context can change a patient's individual risk of harm from a certain treatment approach and that it remains important to balance the benefit and risks from treatment to avoid overtreatment. As an example, the benefit from addition of anti-CD20 therapy to targeted therapy must be critically evaluated particularly for BTK inhibitors, as the addition of rituximab to ibrutinib was shown to provide no clinical benefit (5, 31). Furthermore, the choice between a monotherapy, a dual therapy or a triple drug regimen must be adjusted to the patient's individual risk profile to avoid situations where the risks of serious or even fatal adverse events from treatment exceed the risks from the disease itself.

Taken together, we believe that a more holistic definition of "high-risk CLL" would be to define it simply as any patient who has an increased risk of early CLL-related death. This could be from treatment, from infection, or many other factors on top of risks from the disease itself. With this definition in mind, risk assessment would be based on a combination of "prospective" biomarkers, such as *TP53* alterations, IGHV mutation status and karyotype complexity and "retrospective" factors, such as the duration of response to, and side effects from, a particular therapy. It would hence require regular updates over the disease course as suggested by the CIRI score (42). Such perspective would encourage investigators conducting future clinical trials to focus on the elements influencing overall survival, with greater consideration of a patient's journey through multiple lines of treatment rather than just a single intervention. This would be stark contrast to the current situation where, for example, patients with a contraindication to one drug class are excluded from the relevant clinical trial. While a prospective approach would be the ideal, this will be close to impossible due to the timescales and rapid evolution of therapies. Alternatively, large-scale retrospective analyses could be employed to determine the best sequencing of drugs across multiple treatment lines for molecularly and/or risk stratified patient subgroups. Future research should therefore aim to incorporate all of the elements described above to tailor treatment towards the specific circumstances of individual patients.

Author contributions

All authors listed above have made a substantial, direct and intellectual contribution to the work, wrote the article and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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