

Single-cell sequencing demonstrates complex resistance landscape in CLL and MCL treated with BTK and BCL2 inhibitors

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Key Points

- Therapy resistance to both individual and sequential BTK and BCL2 inhibition is clonally complex and most commonly oligoclonal.
- Single-agent resistance mutations occur in mutually exclusive clones; resistance mutations to sequential monotherapies variably co-occur.

The genomic landscape of resistance to targeted agents (TAs) used as monotherapy in chronic lymphocytic leukemia (CLL) is complex and often heterogeneous at the patient level. To gain insight into the clonal architecture of acquired genomic resistance to Bruton tyrosine kinase (BTK) inhibitors and B-cell lymphoma 2 (BCL2) inhibitors in CLL, particularly in patients carrying multiple resistance mutations, we performed targeted single-cell DNA sequencing of 8 patients who developed progressive disease (PD) on TAs (either class). In all cases, analysis of single-cell architecture revealed mutual exclusivity between multiple resistance mutations to the same TA class, variable clonal co-occurrence of multiple mutations affecting different TAs in patients exposed to both classes, and a phenomenon of multiple independent emergences of identical nucleotide changes leading to canonical resistance mutations. We also report the first observation of established *BCL2* resistance mutations in a patient with mantle cell lymphoma (MCL) following PD on sequential monotherapy, implicating *BCL2* as a venetoclax resistance mechanism in MCL. Taken together, these data reveal the significant clonal complexity of CLL and MCL progression on TAs at the nucleotide level and confirm the presence of multiple, clonally independent, mechanisms of TA resistance within each individual disease context.

Introduction

Targeted agents (TAs), specifically Bruton tyrosine kinase (BTK) inhibitors (BTKis) or the selective B-cell lymphoma 2 (BCL2) inhibitor (BCL2i) venetoclax, are commonly used for the treatment of chronic lymphocytic leukemia (CLL). However, despite their substantial efficacy, emergent resistance is a significant cause of treatment failure. In particular, outcomes for patients with progressive disease after sequential treatment with both TA classes are poor.¹ Resistance mechanisms of the BTKi ibrutinib include *BTK* mutations at the Cys481 residue, which alter drug binding,^{2,3} and *PLCG2*-activating mutations, which effectively bypass BTK.⁴ Venetoclax resistance mechanisms include *BCL2* mutations that affect drug binding⁵⁻⁷ as well as overexpression of alternative prosurvival BCL2 family members mantle cell lymphoma 1 (MCL1)⁸ and BCLxL.⁵ We report the results of targeted single-cell DNA sequencing

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Requests for data sharing may be submitted to Ella R. Thompson (ella.thompson@petemac.org)

The full-text version of this article contains a data supplement.

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Table 1. *BTK*, *PLCG2*, and *BCL2* variants detected among 37 clinical samples

Gene (RefSeq transcript)	Nucleotide variant (HGVS)	Amino acid change (HGVS)	Total
<i>BTK</i> (NM_000061.2)	c.1442G>C	p.(Cys481Ser)	21
	c.1441T>A	p.(Cys481Ser)	9
	c.1583T>G	p.(Leu528Trp)	6
	c.1442G>T	p.(Cys481Phe)	4
	c.1442G>A	p.(Cys481Tyr)	3
	c.1421C>T	p.(Thr474Ile)	2
	c.1441T>C	p.(Cys481Arg)	1
	c.1442_1443delinsCT	p.(Cys481Ser)	1
<i>PLCG2</i> (NM_002661.3)	c.2120C>T	p.(Ser707Phe)	2
	c.2120C>A	p.(Ser707Tyr)	1
	c.2977G>C	p.(Asp993His)	1
<i>BCL2</i> (NM_000633.2)	c.302G>T	p.(Gly101Val)	12
	c.467T>A	p.(Val156Asp)	8
	c.319_330dup	p.(Arg107_Arg110dup)	7
	c.307G>T	p.(Asp103Tyr)	6
	c.309C>A	p.(Asp103Glu)	5
	c.338C>G	p.(Ala113Gly)	4
	c.308A>T	p.(Asp103Val)	2
	c.309C>G	p.(Asp103Glu)	2
	c.386G>T	p.(Arg129Leu)	2
	c.326_327insGCGCCGCTACCG	p.(Arg107_Arg110dup)	1
c.302_303delinsTT	p.(Gly101Val)	1	

(scDNAseq) analysis performed to investigate the clonal structure and evolution of resistance in patients with CLL whose disease harbors multiple resistance mutations to either a single TA or sequential TAs.

Methods

Mutation data from ~5500 samples referred for diagnostic targeted DNAseq for investigation of hematological malignancy at the Peter MacCallum Cancer Centre between 2017 and 2020 were reviewed to identify specimens with mutations in *BTK*, *PLCG2*, or *BCL2* among patients with CLL after treatment with a BTKi or venetoclax. A single case of MCL was also identified for analysis. scDNAseq libraries were prepared using a custom Tapestry panel (Mission Bio) as previously described.⁹ The data supplement provides a gene list and additional details of sequence variant and copy number analyses.

Results and discussion

Cohort of patients with CLL for scDNAseq

Thirty-seven patients with CLL with disease progression (including Richter transformation [RT]) after treatment with a TA (BTKi, n = 21; venetoclax, n = 11; sequential venetoclax/BTKi, n = 4; sequential BTKi/venetoclax, n = 1) whose disease harbored at least 1 mutation in *BCL2*, *BTK*, or *PLCG2* on bulk sequencing analysis were identified among the screening cohort (neither the total number of patients with CLL or MCL within this cohort nor their treatment history was available). Of these cases, a majority harbored

multiple different potential resistance mutations for the same TA (BTKi-exposed patients harboring multiple *BTK/PLCG2* mutations, n = 14 of 26 [median mutations per patient, 2; range, 1-5]; venetoclax-exposed patients harboring multiple *BCL2* mutations, n = 12 of 16 [median mutations per patient, 3; range, 1-7]). Mutation details are provided in Table 1.

From this cohort of 37 patients, 8 with multiple resistance mutations were identified with suitable specimens for scDNAseq analysis, including 8 samples containing CLL and 1 additional sample collected after the subsequent development of RT (from the only patient among the 8 whose disease transformed to high-grade lymphoma). All 8 patients had received prior fludarabine and alkylator therapy (supplemental Table 1). scDNAseq performance for these 9 samples is described in the data supplement.

Clone analysis revealed an overall complex architecture of resistance within these samples, as presented in Figure 1. The main observations from this analysis are described in the following paragraphs.

Multiple resistance mutations targeting the same TA class occur in mutually exclusive CLL clones

Mutual exclusivity between single-agent resistance mutations was observed within individual genes (n = 3 of 3 and 4 of 4 patients with multiple *BCL2* and *BTK* mutations, respectively), including in both patients (CLL-D and CLL-G) who harbored the recently described¹⁰ zanubrutinib resistance mutation *BTK*

Leu528Trp. Interestingly, clone analysis inferred that individual nucleotide changes arose multiple times independently in some patients (CLL-E and CLL-G), indicating further clonal complexity within these disease cell populations that cannot be detected by bulk sequencing and the possibility of additional variant-identical clones not distinguishable by targeted sequencing.

Multiple resistance mutations targeting different TA classes variably co-occur within the same CLL clones

Analysis of the clonal relationship between BTKi and venetoclax resistance mutations in patients with CLL demonstrated their clonal co-occurrence in 2 of 3 patients (CLL-G and CLL-H) with

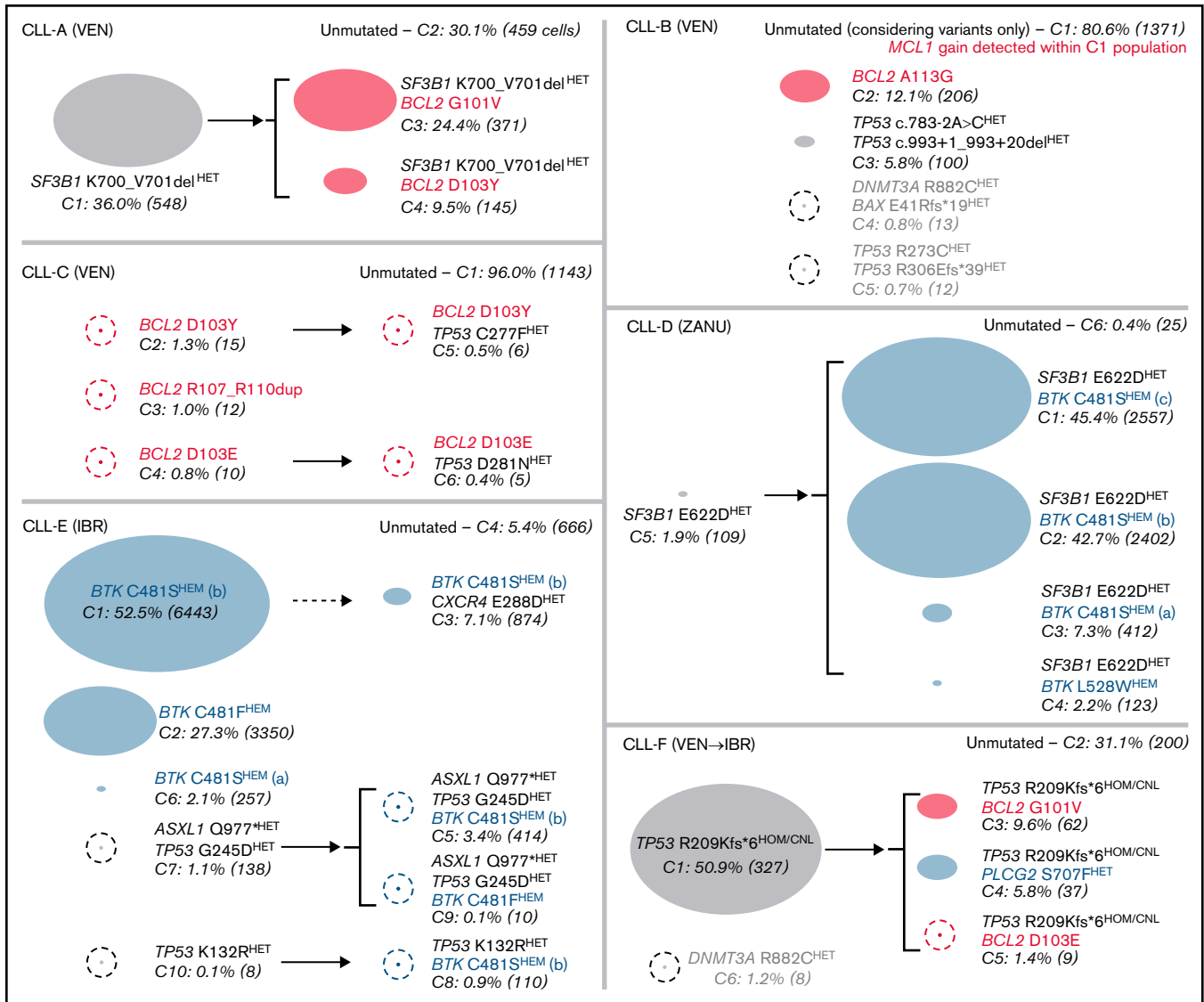


Figure 1. Clonal relationships between resistance mutations in CLL (n = 8 patients) and MCL (n = 1 patient) inferred from variant-based analysis of scDNAseq data. Clones are shown with their defining variants (ie, all detected nonsynonymous coding or splice variants, excluding germ line polymorphisms), clone size (percentage of analyzed cells), and number of cells. Clones with size <1.5% are indicated with a dashed circle. Zygosity of each variant is indicated (heterozygous [HET], homozygous [HOM], or hemizygous [HEM]), with the exception of BCL2, for which zygosity was not determined because of poor sequence quality (supplemental Figure 1). The BCL2 Val156Asp mutation detected in bulk sequencing data in patients CLL-G (at progression during venetoclax treatment) and MCL-A was not assessable on the scDNAseq panel, and MCL1 amplification was not assessable by bulk sequencing (patient CLL-G). Arrows indicate inferred clonal relationships (with the dashed arrow indicating a possible clonal relationship). Each clone was assessed for MCL1 copy number gain and TP53 copy number loss (CNL), and these are indicated when detected (supplemental Table 4; supplemental Figure 2). Clones harboring established or putative BCL2i and BTKi resistance mutations or both are indicated in red, blue, or purple, respectively. Myeloid clones (inferred by variant allele frequency analysis of samples taken at different time points containing little or no CLL disease) are indicated with gray text (patients CLL-B and CLL-F). BTK C481S mutations are followed by a suffix (a-d) to denote which nucleotide change was observed: a indicates NM_000061.2:c.1441T>A; b, NM_000061.2:c.1442G>C; c, NM_000061.2:c.1442_1443delinsCT; and d, NM_000061.2:c.1440_1441delinsGA. Targeted agent exposure for each patient is shown (venetoclax [VEN], zanubrutinib [ZANU], or ibrutinib [IBR]).

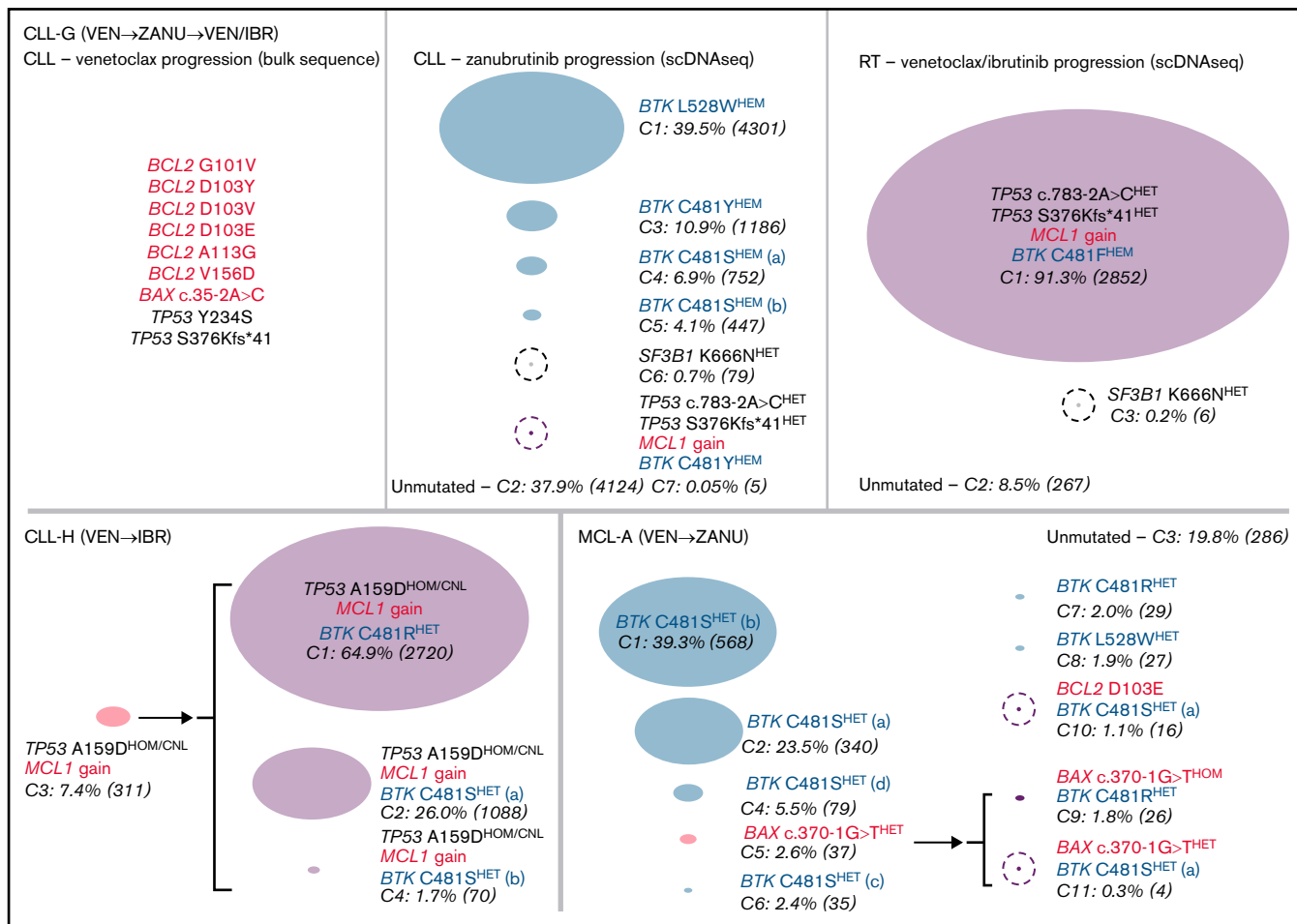


Figure 1. (continued).

progressive disease after sequential monotherapy with both drug classes. In patient CLL-F, however, BTKi and venetoclax resistance mutations were observed in different clones. Similarly, patients CLL-G and CLL-H also harbored clones with genomic evidence for only single-agent resistance despite progressive disease after sequential monotherapy with both drug classes. Although there may be other resistance mechanisms in dual TA-resistant clones that were not detected by our targeted approach, the absence of such clones could alternatively represent the potential retention of single-agent therapy sensitivity within CLL subclones.

Patient CLL-G demonstrated near-complete clonal exchange occurring in conjunction with sequential TA monotherapies, from a venetoclax-resistant population (containing 6 *BCL2* mutations and a *BAX* splice variant after single-agent venetoclax therapy detected in historical bulk sequencing data) to a predominantly *BTK*-mutated population before the emergence of a dual TA-resistant RT (with the absence of *BCL2* mutations, including *BCL2* Val156Asp, confirmed by bulk sequencing data at these latter time points). Although not established as a resistance mechanism in CLL, *BAX* mutations have previously been described in venetoclax-resistant CLL¹¹ and are associated with resistance in vitro.¹²

Resistance mutations are variably clonally related to CLL-associated mutations

We next assessed the inferred clonal hierarchy of resistance mutations relative to other CLL-associated mutations. Resistance mutations were detected subclonally to parental *TP53*, *SF3B1*, or *ASXL1* mutations in some patients (CLL-A, CLL-D, CLL-E, CLL-F, CLL-G, and CLL-H) and independently in others (CLL-E and CLL-G). In addition, in patients CLL-C and CLL-E, possible further evolution of resistant clones was observed through the development of *TP53* and *CXCR4* mutations within clones harboring acquired TA resistance mutations, consistent with continued clonal evolution within the resistant disease compartment. The significance of the novel *CXCR4* transmembrane domain missense mutation (Glu288Asp) detected in patient CLL-E is unclear, considering that resistance-associated mutations in Waldenström macroglobulinemia typically lead to truncation of the C-terminal cytoplasmic tail, resulting in pathway activation.¹³ In contrast, the Glu288 residue is predicted to be a critical site for CXCL12 binding,^{14,15} and in vitro analysis of Glu288Asp demonstrated reduced CXCL12 signaling (but not binding) in HEK-293 cells.¹⁶

Novel genomic resistance mechanisms in a dual TA-resistant MCL

Finally, to explore the applicability of these observations to other lymphoid malignancies, scDNAseq was performed on a progression sample from a female patient with relapsed/refractory MCL (MCL-A) after sequential venetoclax/zanubrutinib monotherapy in whom multiple *BTK* mutations and a *BCL2* Val156Asp mutation were detected by bulk sequencing. Substantial clonal complexity was similarly observed in MCL (Figure 1). Clone analysis revealed 9 clonally independent *BTK* mutations (including Leu528Trp, not previously reported in zanubrutinib-treated MCL), the heterozygosity of these (and of *BTK* mutations in female patient CLL-H with CLL) inferring the sufficiency of a single mutant allele to drive resistance in a diploid context (compared with male patients in whom the chromosome X-located *BTK* mutations were hemizygous). Whereas the previously detected *BCL2* Val156Asp mutation was not assessable in patient MCL-A, a second *BCL2* mutation (Asp103Glu) was detected with a coexisting *BTK* mutation, and additionally, a *BAX*-mutated clone was detected, with evidence of 2 subclonal *BTK* mutations; these represent the first descriptions of *BCL2* and *BAX* mutations (established and putative venetoclax resistance mechanisms in CLL, respectively) in venetoclax-treated MCL.

In summary, these data highlight the significant clonal complexity of CLL and MCL progression during venetoclax and BTKi treatment. Our data show that disease progression in this context is consistently oligoclonal, with separate clones harboring distinct identifiable resistance mechanisms, signifying convergent clonal evolution under therapeutic pressure. For patients carrying dual TA-class mutations, dual resistance mutations were present in the same cells in some cases, whereas they occurred in different cells in other cases. Future studies incorporating larger patient cohorts (including longitudinal sampling to study the evolution of resistance mechanisms) and additional discovery approaches (eg, single-cell whole-transcriptome sequencing) will be valuable in further characterizing this complexity. Finally, although specifically designed trials would be required to test this hypothesis, especially considering the previously reported poor overall efficacy of BTKi retreatment,¹⁷ these data may have implications for the potential utility of retreatment with previously efficacious targeted therapies in CLL cases where the genomic data were supportive (ie, demonstrating the presence of BTKi and BCL2i resistance mutations in separate, rather than dual-resistant, subclones) and may provide a rationale for the early use of disease-appropriate combination targeted therapies.

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Authorship

Contribution: P.B. conceived of the project and designed the study; T.N. and R.T. performed laboratory studies; E.R.T., T.N., Y.K., J.F.M., R.T., P.S.-H.Y., and P.B. performed data analysis; M.A.A., S.M.H., C.S.T., J.F.S., and A.W.R. provided clinical data and patient samples; D.A.W. and P.B. supervised the study; E.R.T. wrote the first version of the manuscript; and all authors reviewed the data and contributed to critical revision of the manuscript.

Conflict-of-interest disclosure: A.W.R. and M.A.A. are employees of the Walter and Eliza Hall Institute of Medical Research, which has received milestone and royalty payments related to venetoclax, and are recipients of a share of royalty-related income to the Walter and Eliza Hall Institute of Medical Research. S.M.H. has received honoraria from Gilead and nonfinancial assistance from AbbVie. C.S.T. has received honoraria and research funding from AbbVie and Janssen and honoraria from BeiGene. A.W.R. has received research funding from AbbVie, Genentech, Servier, Janssen, and BeiGene. J.F.S. receives research funding from AbbVie, Genentech, Celgene, and Janssen and is an advisory board member for and has received honoraria from AbbVie, Acerta, Celgene, Genentech, Janssen, Roche, Sunesis, and Takeda. M.A.A. has received honoraria from AbbVie, Janssen, AstraZeneca, Novartis, and CSL Behring. The remaining authors declare no competing financial interests.

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