Single-Cell Sequencing Demonstrates Complex Resistance Landscape in CLL and MCL Treated with BTK and BCL2 Inhibitors

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Abstract:
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 BTK inhibitors and BCL2 inhibitors in CLL, particularly in patients carrying multiple resistance mutations, we performed targeted single-cell DNA sequencing of eight 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.

Conflict of interest: COI declared - see note

COI notes: AWR and MAA are employees of the Walter and Eliza Hall Institute of Medical Research, which has received milestone and royalty payments related to venetoclax. AWR and MAA are recipients of a share of royalty-related income to the Walter and Eliza Hall Institute of Medical Research. SMH has received honoraria from Gilead and nonfinancial assistance from AbbVie. CST has received honoraria and research funding from AbbVie and Janssen and honoraria from BeiGene. AWR has received research funding from AbbVie, Genentech, Servier, Janssen, and BeiGene. JFS 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. MAA has received honoraria from AbbVie, Janssen, AstraZeneca, Novartis and CSL Behring. The remaining authors declare no competing financial interests.

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ABSTRACT

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 BTK inhibitors and BCL2 inhibitors in CLL, particularly in patients carrying multiple resistance mutations, we performed targeted single-cell DNA sequencing of eight 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.

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, and resistance mutations to sequential monotherapies variably co-occur
INTRODUCTION

Targeted agents (TAs), specifically BTK inhibitors (BTKi) or the selective 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 following sequential treatment with both TA classes are poor. BTKi ibrutinib resistance mechanisms include BTK mutations at the Cys481 residue altering drug binding and PLCG2 activating mutations which effectively bypass BTK. Venetoclax resistance mechanisms include BCL2 mutations that affect drug binding as well as over-expression of alternative pro-survival BCL2 family members, MCL1 and BCLxL. Here we report the results of targeted single-cell DNA sequencing (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 approximately 5,500 samples referred for diagnostic targeted DNA sequencing 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 following treatment with a BTKi or venetoclax. A single case of mantle cell lymphoma (MCL) was also identified for analysis. scDNAseq libraries were prepared using a custom Tapestri panel (Mission Bio) as previously described. See Supplementary Methods for gene list and further details of sequence variant and copy number analyses.

RESULTS AND DISCUSSION

CLL patient cohort for scDNAseq

Thirty-seven patients with CLL with disease progression (including Richter transformation [RT]) following treatment with a TA (21 BTKi, 11 venetoclax, 4 sequential venetoclax/BTKi, 1 sequential BTKi/venetoclax) whose disease harbored at least one mutation in BCL2, BTK or PLCG2 on bulk sequencing analysis were identified amongst the screening cohort (the total number of patients with CLL and MCL within this cohort and their treatment history was not available). Of these cases, the majority harbored multiple different potential resistance mutations for the same TA (14/26 BTKi-exposed patients harbored multiple BTK/PLCG2 mutations [median mutations per patient 2, range 1-5], 12/16 venetoclax-exposed patients harbored multiple BCL2 mutations [median mutations per patient 3, range 1-7]). Mutation details are provided in Table 1.

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

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 below.

**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 (3/3 and 4/4 patients with multiple BCL2 and BTK mutations, respectively), including in both patients (CLL-D, CLL-G) who harbored the recently described\textsuperscript{10} zanubrutinib resistance mutation BTK Leu528Trp. Interestingly, clone analysis inferred that individual nucleotide changes arose multiple times independently in some patients (CLL-E, 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 2/3 patients (CLL-G, CLL-H) with progressive disease following 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 following sequential monotherapy with both drug classes. Whilst there may be other resistance mechanisms in dual-TA resistant clones that have not been 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 six BCL2 mutations and a BAX splice variant following single-agent venetoclax therapy detected in historical bulk sequencing data) to a predominantly BTK-mutated population prior to 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 timepoints). Although not established as a resistance mechanism in CLL, BAX mutations have previously been described in venetoclax-resistant CLL\textsuperscript{11} and are associated with resistance \textit{in vitro}\textsuperscript{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 sub-clonally to parental TP53, SF3B1 or ASXL1 mutations in some patients (CLL-A, CLL-D, CLL-E, CLL-F, CLL-G, CLL-H) and independently in others (CLL-E, 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. In contrast, the Glu288 residue is predicted to be a critical site for CXCL12 binding and in vitro analysis of Glu288Asp demonstrated reduced CXCL12 signaling (but not binding) in HEK-293 cells.

**Novel genomic resistance mechanisms in a dual-TA resistant MCL**

Finally, to explore the applicability of these observations to other lymphoid malignancies, scDNAnseq was performed on a progression sample from a female patient with relapsed/refractory MCL (MCL-A) following 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 nine clonally independent BTK mutations (including Leu528Trp, not previously reported in zanubrutinib-treated MCL), the heterozygosity of these (and of BTK mutations in female CLL patient CLL-H) inferring the sufficiency of a single mutant allele to drive resistance in a diploid context (compared to male patients in whom the chrX-located BTK mutations were hemizygous). Whilst the previously detected BCL2 Val156Asp mutation was not assessable in patient MCL-A, a second BCL2 mutation (Asp103Glu) was detected with a co-existing BTK mutation and additionally, a BAX-mutated clone was detected with evidence of two subclonal BTK mutations, these representing 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 on venetoclax and BTKi. 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 (such as single-cell whole transcriptome sequencing) will be valuable in further characterizing this complexity. Finally, whilst specifically designed trials would be required to test this hypothesis, especially considering the previously reported poor overall efficacy of BTKi retreatment\textsuperscript{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 (i.e. demonstrating the presence of BTKi and BCL2i resistance mutations in separate, rather than dual-resistant, subclones) as well as providing a rationale for the early use of disease-appropriate combination targeted therapies.

**DATA SHARING STATEMENT**

For data sharing, please contact the corresponding author at ella.thompson@petermac.org.

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**AUTHORSHIP CONTRIBUTIONS**

PB conceived of the project and designed the study; TN and RT performed laboratory studies, ET, TN, YK, JM, RT, PSHY and PB performed data analysis; MAA, SMH, CST, JFS, AWR provided clinical data and patient samples; DAW and PB supervised the study; ERT wrote the first version of the manuscript; and all authors reviewed the data and contributed to critical revision of the manuscript.

**DISCLOSURE OF CONFLICTS OF INTEREST**

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# TABLES

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

| Gene (RefSeq transcript) | Nucleotide variant (HGVSc) | Amino acid change (HGVSp) | Total |
|--------------------------|----------------------------|--------------------------|-------|
| **BTK (NM_000061.2)**   | c.1442G>C                  | p.(Cys481Ser)            | 21    |
|                          | c.1442T>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     |
| **PLCG2 (NM_002661.3)** | c.2120C>T                  | p.(Ser707Phe)            | 2     |
|                          | c.2120C>A                  | p.(Ser707Tyr)            | 1     |
|                          | c.2977G>C                  | p.(Asp993His)            | 1     |
| **BCL2 (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_327insGCGCGCTACCG     | p.(Arg107_Arg110dup)     | 1     |
|                          | c.302 303delinsTT          | p.(Gly101Val)            | 1     |

## FIGURE LEGENDS

**Figure 1.** Clonal relationships between resistance mutations in chronic lymphocytic leukemia \( (n = 8\) patients) and mantle cell lymphoma \( (n = 1\) patient) inferred from variant-based analysis of single cell DNA sequencing data. Clones are shown with their defining variants \( (i.e. all detected non-synonymous coding or splice variants, excluding germline polymorphisms),\) clone size \( (\%\) of analyzed cells) and number of cells. Clones with size < 1.5% are indicated with a dashed circle. Zygosity of each variants is indicated \( (\text{HET} – \text{heterozygous}, \text{HOM} – \text{ homozygous}, \text{HEM} – \text{hemizygous})\) with the exception of BCL2 for which zygosity was not determined due to poor sequence quality (Supplementary Figure 1). The BCL2 Val156Asp mutation detected in bulk sequencing data in patients CLL-G (at progression on venetoclax) 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 \( (\text{CNL})\) and these are indicated when detected (Supplementary Table 4 and Supplementary Figure 2). Clones harboring established or putative BCL2i and BTKi resistance mutations or both are indicated in pink, 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 grey 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) NM_000061.2:c.1441T>A, (b) NM_000061.2:c.1442G>C, (c) NM_000061.2:c.1442_1443delinsCT, (d) NM_000061.2:c.1440_1441delinsGA). Targeted agent exposure for each patient is shown (VEN – venetoclax, ZANU – zanubrutinib, IBR – ibrutinib).
