Ultra-deep mutational landscape in chronic lymphocytic leukemia uncovers dynamics of resistance to targeted therapies

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Abstract

BTK inhibitors, Bcl-2 inhibitors, and other targeted therapies have significantly improved the outcomes of patients with chronic lymphocytic leukemia (CLL). With increased survivorship, monitoring disease and deciphering potential mechanisms of resistance to these agents are critical for devising effective treatment strategies. We used duplex sequencing, a technology that enables detection of mutations at ultra-low allelic frequencies, to identify mutations in five genes associated with drug resistance in CLL and followed their evolution in two patients who received multiple targeted therapies and ultimately developed disease progression on pirtobrutinib. In both patients we detected variants that expanded and reached significant cancer cell fractions (CCF). In patient R001, multiple known resistance mutations in both *BTK* and *PLCG2* appeared following progression on zanubrutinib (*BTK* p.L528W, p.C481S; *PLCG2* S707F, L845F, R665W, and D993H). In contrast, patient R002 developed multiple *BTK* mutations following acalabrutinib treatment, including known resistance mutations p.C481R, p.T474I and p.C481S. We found that pirtobrutinib was able to suppress, but not completely eradicate, *BTK* p.C481S mutations in both patients, but other resistance mutations such as mutations in *PLCG2* and new *BTK* mutations increased while the patients were receiving pirtobrutinib. For example, *BTK* p.L528W in patient R001 increased in frequency more than 1,000-fold (from a CCF of 0.02% to 35%), and the CCF in p.T474I in patient R002 increased from 0.03% to 4.2% (more than 100-fold). Our data illuminate the evolutionary dynamics of resistant clones over the patients' disease course and under selective pressure from different targeted treatments.

Introduction

Standard frontline therapies for chronic lymphocytic leukemia (CLL) consist of Bruton tyrosine kinase (BTK) inhibitors, such as the irreversible covalently binding agents ibrutinib, acalabrutinib, and zanubrutinib, and the Bcl-2 inhibitor venetoclax with obinutuzumab.[1] First- and second-generation BTK inhibitors irreversibly inhibit BTK activity by covalently binding cysteine residue C481 in the ATP-binding site of BTK.[2-4] Although 80-90% of CLL patients respond to targeted therapy, relapses occur. Patients given ibrutinib as a first-line treatment had a 7-year progression-free survival of 59%, while those treated in the relapsed setting had a lower 5-year progression-free survival of 40%.[5,6]

Most patients with CLL (65-85%) who progress after BTK inhibitor therapy develop resistance mutations after 24-48 months of treatment at the BTK drug binding site or activating mutations in *PLCG2*.[7-15] Therapy toxicity is the most common reason for discontinuing BTK inhibitor treatment in real-world clinical practice, followed by disease resistance and progression.[16] To overcome these limitations, non-covalently binding, third-generation BTK inhibitors were developed.[17] Pirtobrutinib (LOXO-305), a reversible selective BTK inhibitor that inhibits Y223 autophosphorylation, even in cells with the known resistance mutation *BTK* C481, has shown efficacy in ibrutinib-naïve and -resistant CLL patients.[17-19] Based on the findings of early phase I and phase II studies, pirtobrutinib was reported to be safe and effective for CLL treatment.[20]

The Bcl-2 inhibitor venetoclax, combined with an anti-CD20

monoclonal antibody, is another frontline treatment for CLL. Resistance mutations resulting from venetoclax monotherapy include those observed in *BCL2* and *BAX*.[21,22] Other targeted therapeutic agents for CLL include phosphatidyl-inositol 3-kinase inhibitors (duvelisib and idelalisib), with reported objective response rates of 85%.[23] These drugs are less utilized due to toxicity concerns and the fact that resistance mechanisms are less well known.[24]

The success of BTK inhibitors, Bcl-2 inhibitors, and other targeted therapies[3,25] has changed the paradigm of disease monitoring, and data now support that demonstration of undetectable measurable residual disease (MRD) at the end of treatment is an independent indicator of favorable prognosis.[26-28] Discovery of ultra-low levels of resistance mutations, and the ability to monitor the evolution of such clones, could be an essential element of CLL management and care during treatment with targeted therapies.[26,29]

Duplex sequencing is one of the most accurate sequencing methods currently available and has been used for MRD detection in acute myeloid leukemia, acute lymphocytic leukemia, and chronic myeloid leukemia.[30-32] In duplex sequencing, each DNA strand is tagged with molecular barcodes to enable double-strand error correction, providing unprecedented resolution for the identification of mutant variants (<1/10,000).[33-36] This high resolution could change the paradigm for the early identification of therapy resistance and MRD in hematologic malignancies but has not yet been applied to CLL.

In this work, we follow the complex clinical course of two patients with CLL who developed resistance to multiple targeted therapies and study the molecular and cytogenetic clonal evolution of their disease at unprecedented resolution. We combined duplex sequencing with longitudinal assessment to screen for mutations in five key genes responsible for resistance to targeted treatments: *BTK*, *PLCG2*, *BCL2*, *BAX*, and *TP53*. We report the detection of resistance mutations at ultra-low allelic frequencies in serial marrow and blood samples collected as the disease progressed through various therapies, with important implications for understanding and monitoring clonal dynamics in CLL.

Methods

Patients and samples

This study features two patients who received pirtobrutinib in a separate study of patients with previously treated CLL or non-Hodgkin lymphoma (NCT03740529). After discontinuing pirtobrutinib due to progression, the patients gave consent to a Fred Hutchinson Cancer Center institutional review board-approved biorepository protocol. Samples were then obtained for tumor banking and resistance mutation profiling. In addition to collecting fresh post-pirtobrutinib peripheral blood (PB) samples, archival specimens were retrieved from as many timepoints as possible from cytogenetic, molecular, histology, and flow cytometry clinical laboratories (*Online Supplementary Tables S1* and *S2*). Mononuclear cells were isolated from fresh PB samples, using Ficoll-Paque media and density gradient centrifugation. They were then suspended in fetal bovine serum with 10% dimethylsulfoxide and cryopreserved in liquid nitrogen. DNA was extracted from the cryopreserved cells using the Gentra Puregene Blood Kit (Qiagen) and was quantified using the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific). Clinical archival specimens were extracted according to Clinical Laboratory Improvement Amendments/College of American Pathologists (CLIA/CAP) standard operating procedures per laboratory of origin.

DNA duplex-sequencing library preparation

From each sample, 500 ng of DNA were prepared into libraries for duplex sequencing according to published protocols[33,35] and using commercially available kits (Twin-Strand Biosciences, Seattle, WA, USA) with a customized panel to target the coding region of *TP53* and key hotspot drug resistance mutations in *BCL2, BAX, BTK*, and *PLCG2* identified in relapsed CLL (*Online Supplementary Tables S3* and *S4*).[7-10,37] Library preparation consisted of sequential sonication, end-repair, A-tailing, ligation to duplex adapters, fragment amplification, two rounds of hybridization capture with biotinylated probes (*Online Supplementary Methods*), and library amplification. Proper library fragment size was confirmed by an Agilent 4200 High Sensitivity TapeStation. Indexed libraries were quantified using a Qubit dsDNA HS Assay Kit. Libraries were sequenced using 150 bp paired end reads on a NovaSeq Illumina platform on site or HiSeq at Genewiz (South Plainfield, NJ, USA), allocating approximately 15 million reads per sample for a target average depth of about 10,000x.

Duplex-sequencing analysis

Sequencing reads were analyzed using pipeline v2.1.2 available at *https://github.com/Kennedy-Lab-UW/Duplex-Seq-Pipeline*, which grouped them by molecular tags to produce highly accurate consensus "duplex reads" (*Online Supplementary Methods*). Variant allele frequencies (VAF) were calculated by dividing the number of mutant duplex reads by the duplex depth at the mutated position. Variants present in all samples from a given patient at a VAF >0.9 (homozygous) or between 0.4 and 0.6 (heterozygous) and had a single nucleotide polymorphism (SNP) database identifier were considered SNP (*Online Supplementary Tables S5* and *S6*). Variants present in all samples from a patient, with a SNP database identifier, and a difference in VAF across samples (maximum minus minimum VAF) >0.2, were considered SNP-loss-of-heterozygosity (SNP-LOH). All non-SNP and non-SNP-LOH variants with a depth >1,000 were considered mutations. Each sample's mutation frequency was calculated as the number of mutant positions in coding regions divided by the total number of

duplex nucleotides sequenced in coding regions. To study the clonal evolution of CLL under therapy, we converted the VAF of each mutation to its cancer cell fraction (CCF) (i.e., the fraction of cancer cells containing the mutation), which incorporates the sample's tumor burden and ploidy at the genomic location (*Online Supplementary Methods*).

Results

Patients and specimens

For our first patient (R001), we analyzed six samples (A to F): four PB samples (A, B, C, and F) and one bone marrow aspiration (BMA) sample processed in replicate (D and E) (*Online Supplementary Table S1*). The same BMA sample was archived at two different clinical laboratories (cytogenetics and molecular) and underwent two independent specimen processing, DNA extraction, and library preparation processes, thus providing a technical replicate for quality control. For our second patient (R002), we analyzed five samples (A to E): one PB sample and four BMA samples (*Online Supplementary Table S2*).

Patient R001 was diagnosed with *IGHV*-unmutated CLL with del(11q) and del(17p) at the age of 67, 6 years before beginning pirtobrutinib therapy. Prior therapies included bendamustine and rituximab, ibrutinib (discontinued at 7 months due to multiple soft tissue infections, arthralgia, and myalgia), and venetoclax. Venetoclax was discontinued after 17 months because of progressive disease (*Online Supplementary Figure S1*). Fluorescence *in situ* hybridization (FISH) and chromosomal genomic array testing (CGAT) performed analysis were performed on a PB sample (R001-A) collected at this time demonstrated del(17p) with loss of *TP53*. The patient was referred for an investigational CD19-targeted chimeric antigen receptor (CAR) T-cell therapy which was unsuccessful. She subsequently received acalabrutinib, which was discontinued after 9 months because of arthralgia and myalgia, followed by zanubrutinib which was discontinued after 9 months due to progressive disease. Sample R001-B was collected after discontinuation of acalabrutinib, and samples R001-C/D/E were collected prior to zanubrutinib discontinuation. The patient received pirtobrutinib for 4 months until she developed progressive disease. She then received duvelisib for 1.5 months (during which sample R001-F was collected) without response and subsequently died due to complications of CLL. Overall, six samples (including a technical replicate of a BMA) were available for study spanning over 3 years of follow up (Figure 1, *Online Supplementary Figure S1*, *Online Supplementary Table S1*).

Patient R002 was diagnosed with CLL in his early 60s; he initially received fludarabine, cyclophosphamide, and rituximab and achieved a durable remission. At the time of relapse, cytogenetic and molecular characterization of his CLL revealed several abnormalities such as deletions of 1q41qter-, 7pterp21-, and 9p21, gain of 3q26qter+ and 8q13qter+, and copy-neutral LOH of 9pterp13. There was no evidence of del(17p) or other alterations to *TP53*, and *IGHV* was unmutated. The patient received ibrutinib for 3 years until his disease progressed, followed by venetoclax for 2 years until further progressive disease. He subsequently received the combination of ibrutinib and venetoclax. Ibrutinib was switched to acalabrutinib due to the development of atrial fibrillation. Sample R002-A was collected after 3 months of combination therapy with ibrutinib and venetoclax. The novel agents were discontinued in favor of a CD19-directed CAR T-cell clinical trial, which was unsuccessful. Sample R002-B was collected a month after CAR T-cell infusion. The patient resumed acalabrutinib nearly 4 months after CAR T-cell infusion and experienced controlled disease for 9 months. Sample R002-C was collected at the time of progressive disease. The patient then received pirtobrutinib on a clinical trial for 2.5 months; sample R002-D (BMA) was collected at the time of progressive disease. Subsequent therapies included idelalisib (10 days, stopped due to gastrointestinal intolerance), a bispecific anti-CD20/CD3 antibody, high-dose corticosteroids, and bendamustine. The patient died from complications of CLL 2 months after stopping pirtobrutinib. A PB specimen (R002-E) was collected 2 weeks before his death. Four BMA samples were available: three before pirtobrutinib treatment and one after pirtobrutinib treatment (Figure 2, *Online Supplementary Figure S2*, *Online Supplementary Table S2*).

Testing for targeted chronic lymphocytic leukemia drug resistance mutations by duplex sequencing

Eleven samples, six from patient R001 and five from patient R002, were duplex sequenced with a panel including *BAX*, *BCL2, BTK, PLCG2,* and *TP53* (*Online Supplementary Table S3*). The average depth of sequencing across samples was 9,708x (range, 7,812x to 11,170x) (*Online Supplementary Table S7*, *Online Supplementary Figure S3A*). Both patients showed most mutations at the latest datapoint, which also corresponded to the highest percentage of disease (95%) (*Online Supplementary Table S7*, *Online Supplementary Figure S3B*). All coding mutations identified in either patient are listed in *Online Supplementary Tables S8* and *S9* and summarized by gene in *Online Supplementary Figure S3C*.

Monitoring clonal trends by cancer cell fraction derived from serial duplex data

To determine clonal dynamics during the disease course, we transformed the VAF into CCF for all mutations with more than one duplex reads in at least one of the samples analyzed, taking into consideration the percentage of disease in the sample and the estimated LOH by SNP duplex sequencing data and cytogenetics (see the Methods section). Patient R001 had no cytogenetic alterations affecting *BAX*, *PLCG2*, and *BTK*, which was confirmed by the

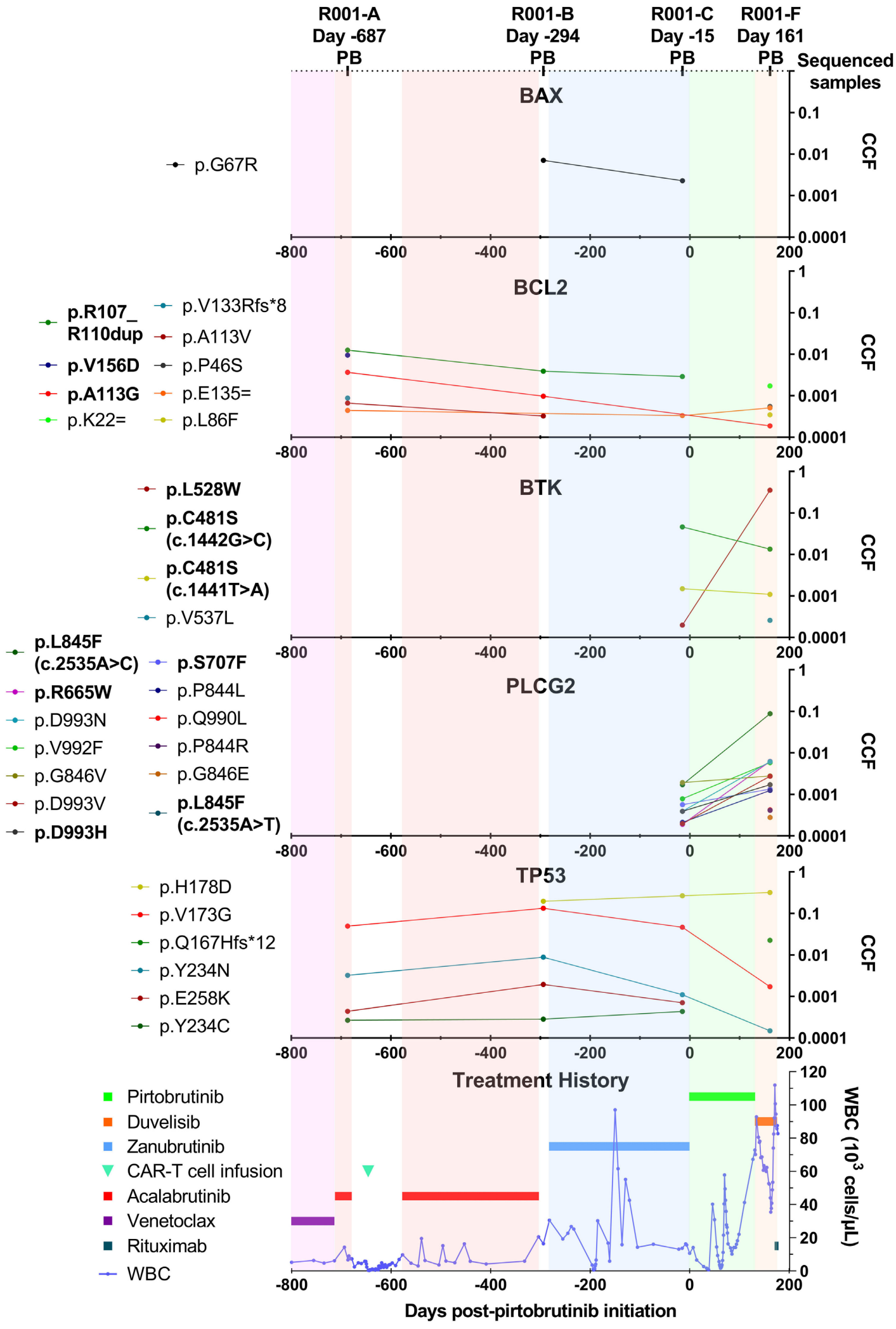

Figure 1. Patient R001 clonal evolution and treatment history. The timeline on the x-axis indicates days after initiation of pirtobrutinib treatment. Four peripheral blood samples were collected at the indicated days. The top five panels correspond to sequenced genes and include all mutations identified in at least one sample by two mutant duplex reads. The cancer cell fraction of each mutation is indicated on the y-axis. Mutations are color-coded and listed to the left of the plots, using bold font to indicate resistance mutations common in chronic lymphocytic leukemia. The bottom panel depicts the patient's treatment history and disease evolution based on white blood cell counts. Only treatments within a year prior to the first sample and that lasted more than 30 days are shown. PB: peripheral blood; CCF: cancer cell fraction; WBC: white blood cell count.

presence of heterozygous SNP at a VAF of approximately 50% in all samples (*Online Supplementary Tables S5* and *S10*). Patient R001 had two heterozygous SNP (c.1101-375G>A and c.97-6C>T) that indicated SNP-LOH in *TP53*, at a frequency ranging from 51% to 91%. This finding was corroborated by cytogenetic data including CGAT analysis, which confirmed deletion of 17p at three timepoints prior to treatment with pirtobrutinib at a frequency comparable between CGAT and duplex sequencing (*Online Supplementary Table S10*). In patient R002, with our target panel, no SNP-LOH was found to alter CCF calculations. Thus, for this male patient, a copy number of two was used for mutations located on autosomes (except for the high-VAF *BAX* mutation p.E41Gfs*33; see *Online Supplementary Methods*), and a copy number of one was used for mutations located on the X chromosome. The CCF values are indicated in *Online Supplementary Tables S11* (patient R001) and *S12* (patient R002) and were used to illustrate the evolution of mutations in serial samples over the treatment course for patient R001 (Figure 1) and patient R002 (Figure 2).

Clonal evolution in both patients prior to pirtobrutinib treatment

Both patients' first sequencing sample was collected after cessation of the Bcl-2 inhibitor venetoclax (patient R001: +29 days; patient R002: +1 day) (Figures 1 and 2, respectively). At this point, both patients had several *TP53* mutations, and no mutations in either *BTK* or *PLCG2*. Patient R001 also had multiple *BCL2* mutations (3 known resistance mutations), whereas patient R002 had multiple *BAX* mutations (1 known resistance mutation). After venetoclax, both patients went on to receive CAR T-cell therapy followed by acalabrutinib monotherapy. After cessation of acalabrutinib, patient R001 developed a low frequency *BAX* mutation and a new *TP53* mutation at the greatest CCF of all *TP53* mutations (p.H178D, CCF 19%). No mutations in *BTK* or *PLCG2* appeared after acalabrutinib treatment in this patient, but multiple known resistance mutations in *BTK* and *PLCG2* did emerge after subsequent treatment with the BTK inhibitor zanubrutinib, including *BTK* p.L528W and p.C481S as well as *PLCG2* S707F, L845F, R665W, and D993H. In contrast, patient R002 developed multiple *BTK* mutations following acalabrutinib treatment, including known resistance mutations p.C481R, p.T474I and p.C481S. Additionally, the BAX mutation E41Gfs*33, which he carried at a CCF of 9% after venetoclax cessation, increased dramatically to a CCF of 92% in less than a year after cessation of acalabrutinib.

Effect of pirtobrutinib on the clonal landscape of patient R001

After treatment with zanubrutinib, patient R001 received pirtobrutinib which suppressed minor clones with mutations in *BAX* and *BCL2*. Strikingly, even though pirtobrutinib was able to suppress the pre-existing *BTK* C481S mutation, which was present at a CCF of 5% before pirtobrutinib and decreased to a CCF of 1% after treatment, the CCF of another *BTK* mutation, L528W, increased by three orders of magnitude following pirtobrutinib treatment, from 0.02% to 35% (Figure 1). Importantly, ultra-deep duplex sequencing detected this mutation at a very low VAF in the pre-pirtobrutinib sample, demonstrating that it was pre-existing and did not arise *de novo* during pirtobrutinib treatment.

We also detected multiple *PLCG2* mutations in patient R001 who exhibited a significant CCF increase following pirtobrutinib. Most notably, the L845F mutation increased in CCF from 0.2% before pirtobrutinib to 9% after pirtobrutinib. Similarly, the *TP53* H178D mutation, which was first detected at a CCF of 2% about a year prior to pirtobrutinib initiation, steadily increased to a CCF of 32% after pirtobrutinib cessation. The *TP53* mutations occurred on the background of *TP53* LOH/deletion, which was present at 80-100% CCF (i.e., clonal, or nearly clonal) in all sequenced samples from this patient (*Online Supplementary Table S11*).

Effect of pirtobrutinib on the clonal landscape of patient R002

In patient R002, most *TP53* mutant clones were suppressed by acalabrutinib treatment and remained at low CCF during subsequent treatment with pirtobrutinib. As in patient R001, while some *BTK* clones (including a low frequency C481S mutation) were suppressed by pirtobrutinib treatment, one clone harboring a common CLL resistance mutation, p.T474I, expanded more than two orders of magnitude from a CCF of 0.03% to 4.2% (Figure 2, *Online Supplementary Table S12*). Several additional *BTK* mutations appeared following the start of pirtobrutinib treatment, including multiple CLL resistance mutations, which persisted during the subsequent idelalisib treatment. A pre-existent *PLCG2* mutant clone (p.Y648C) also expanded during pirtobrutinib treatment, and a new mutant clone harboring a common *PLCG2* CLL resistance mutation (p.L845V) emerged after the treatment. The major mutant *BAX* clone, which was detected after venetoclax treatment and expanded during therapy with acalabrutinib, persisted at a very high CCF after pirtobrutinib (CCF 90%) and expanded fully after idelalisib (CCF 100%).

Reproducibility of duplex-sequencing findings across technical replicates and different sample types

We leveraged the availability of two BMA samples from patient R001 (R001-D and R001-E), collected in a single procedure but processed independently, to test the reproducibility of low frequency mutation detection with duplex sequencing. The sequencing depth was lower for R001-D than for R001-E and fewer mutations were detected (32 *vs.* 37) (*Online Supplementary Table S7*). Twenty of those

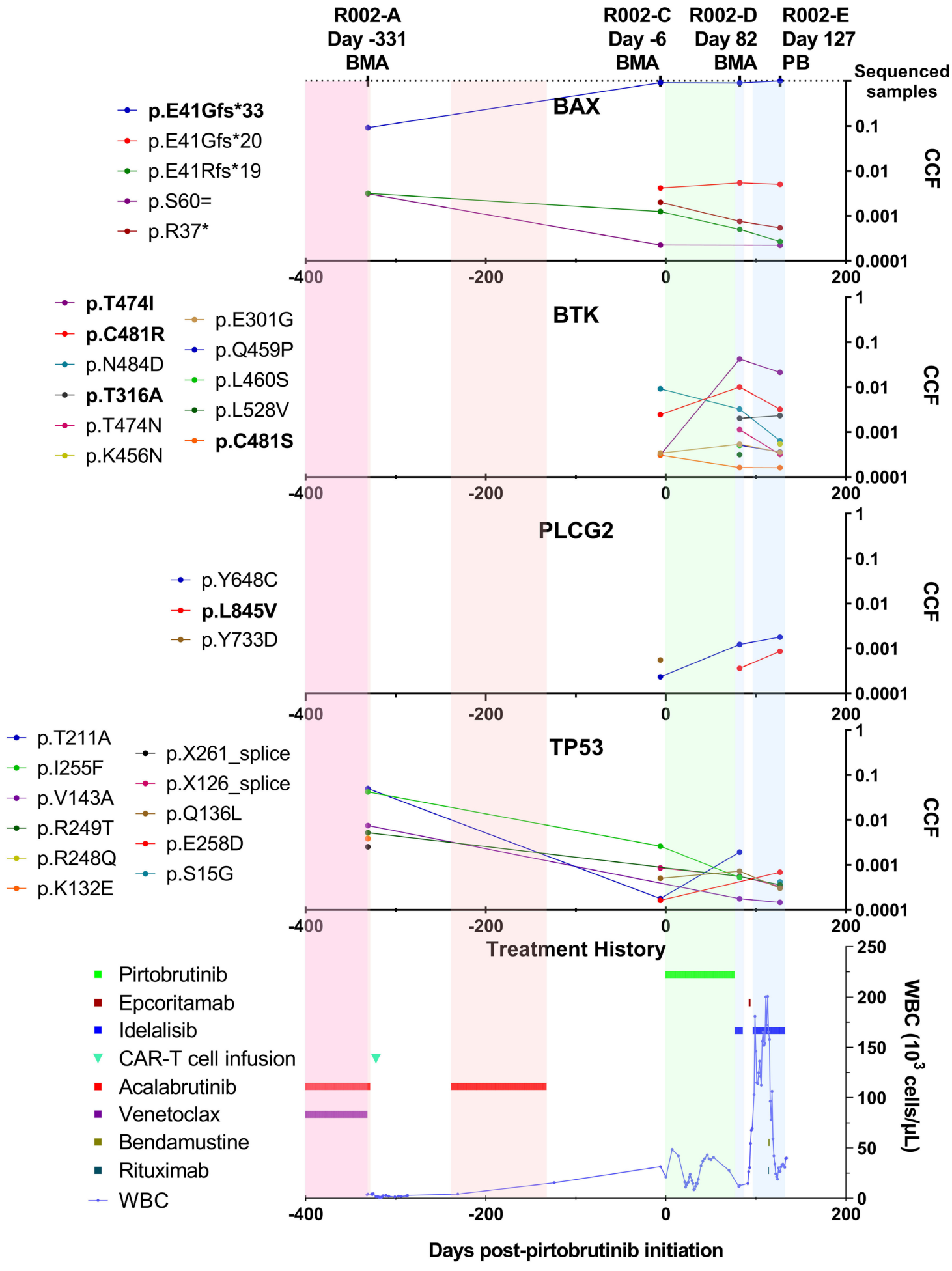

Figure 2. Patient R002 clonal evolution and treatment history. The timeline on the x-axis indicates days after initiation of pirtobrutinib treatment. Three bone marrow aspirate samples and one peripheral blood sample were collected at the indicated days. The top five panels correspond to sequenced genes and include all mutations identified in at least one sample by two mutant duplex reads. The cancer cell fraction of each mutation is indicated on the y-axis. Mutations are color-coded and listed to the left of the plots, using bold font to indicate common chronic lymphocytic leukemia resistance mutations. The bottom panel depicts the patient's treatment history and disease evolution based on white blood cell counts. Only treatments within a year prior to the first sample and that lasted more than 30 days are shown. BMA: bone marrow aspirate; PB: peripheral blood; CCF: cancer cell fraction; CAR: chimeric antigen receptor; WBC: white blood cell count.

mutations were, however, common in the two samples and showed very reproducible VAF values (Spearman r=0.89, $P<2.2x10^{-16}$) (*Online Supplementary Figure S4*). Most of the mutations that were not seen in both samples corresponded to mutations that had only one mutant read and very low VAF (<0.02%), which are likely subject to sampling error. To demonstrate the comparability between BMA and PB for mutational assessment, we compared the CCF of mutations identified in the two BMA samples with those identified in PB collected 9 days before the BMA (Figure 3). Mutations seen in BMA were also found in PB at similar CCF, confirming the suitability of both types of sample for the assessment of resistance mutations.

Orthogonal validation of duplex sequencing with clinical next-generation sequencing and earlier detection of resistance mutations

While the availability of clinical next-generation sequencing (NGS) data was limited, such sequencing did identify two mutations in patient R001 (*BTK* p.C481S, c.1442G>C and *TP53* p.H178D, c.532C>G) and one mutation in patient R002 (*BTK* p.T474I, c.1421C>T) included in the regions analyzed by

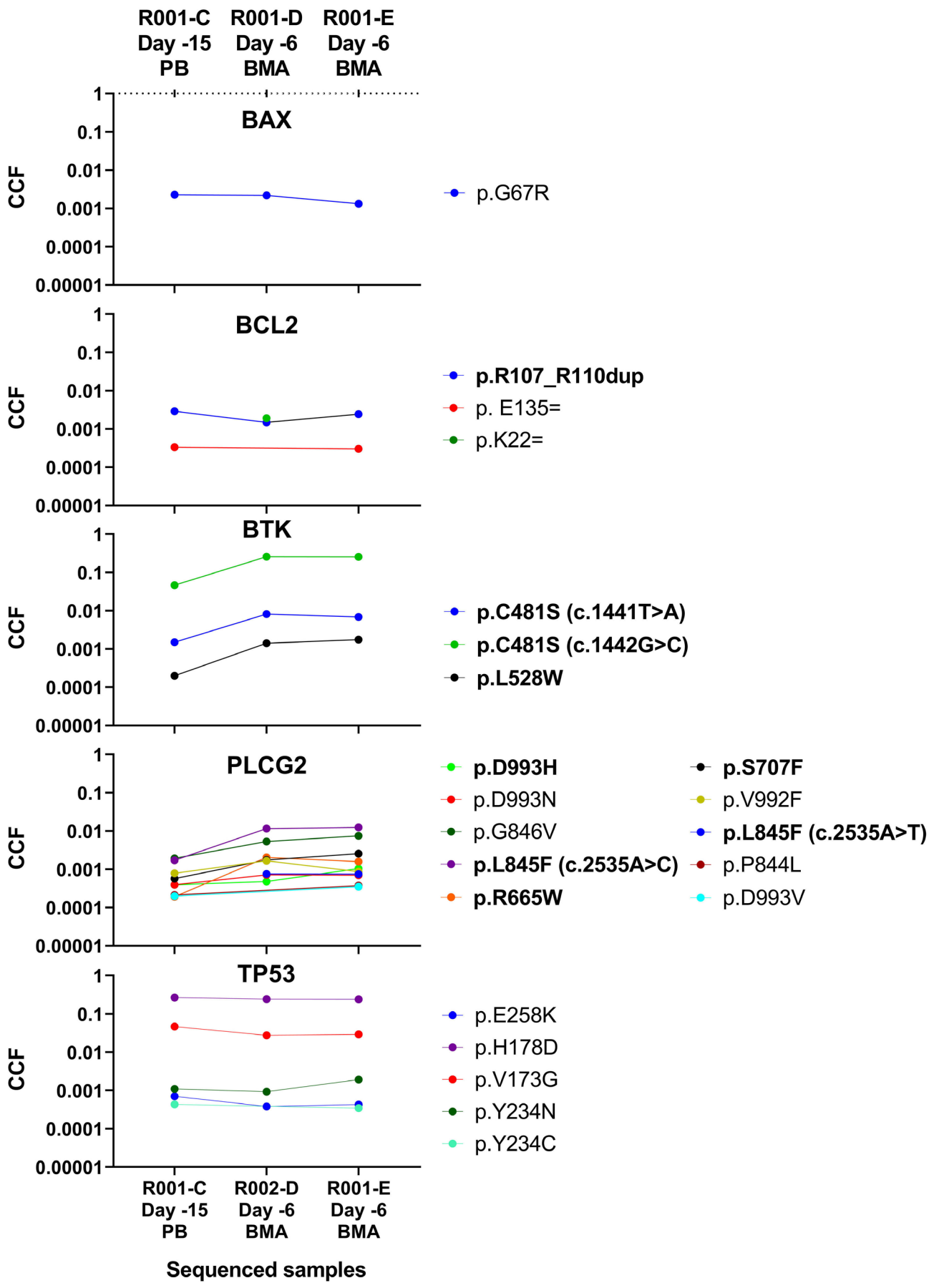

Figure 3. High sensitivity detection of chronic lymphocytic leukemia resistance mutations in bone marrow aspirate and peripheral blood collected 9 days apart. Variant allele frequency (VAF) is indicated on the y-axis. All mutations with a VAF >0.0002 are represented for each gene and sample, with known chronic lymphocytic leukemia resistance mutations in bold on the right side of each plot. The two bone marrow aspirate samples are replicates from a single procedure. CLL: chronic lymphocytic leukemia; BMA: bone marrow aspirate; PB: peripheral blood: Days: days prior to pirtobrutinib treatment.

duplex sequencing. We confirmed that the three mutations were detected by duplex sequencing in the corresponding samples at VAF very similar to those measured by NGS (*Online Supplementary Table S13*). Importantly, for patient R002, the resistance mutation *BTK* p.T474I, c.1421C>T was identified by duplex sequencing in sample R002-C, which was collected 6 days prior to the pirtobrutinib initiation, at a VAF of 0.03% (Figure 4). This VAF is about two orders of magnitude under the limit of detection of the clinical NGS method and thus was not identified by clinical NGS in the same sample. These results were corroborated by digital polymerase chain reaction (*Online Supplementary Figure S5*) and indicate that the high sensitivity of duplex sequencing is helpful to identify very low frequency pre-existing CLL resistance mutations.

Discussion

We report mutation analysis of targeted DNA duplex sequencing on serial PB and BMA samples from two patients whose CLL demonstrated clinical resistance to pirtobrutinib. Our data highlight the presence of resistance mutations that complicate pirtobrutinib therapy and demonstrate the evolutionary dynamics of resistant clones under different targeted therapeutic pressures including a Bcl-2 inhibitor, and multiple covalent and non-covalent BTK inhibitors. Our first patient had a more aggressive CLL, with primary disease demonstrating 17p and 11q deletions. Her disease was treated within 2 years of initial diagnosis with different targeted agents, including BTK inhibitors and CAR T cells, but progressed prior to enrollment in the pirtobrutinib clinical trial. In contrast, our second patient had a long-standing history of CLL (over a decade) before starting a series of targeted therapies to which the CLL developed resistance, including BTK and Bcl-2 inhibitors and CAR T-cell therapy, before enrollment in the pirtobrutinib clinical trial, which was then followed by treatment with a novel bispecific antibody. By using ultra-deep, ultra-accurate sequencing, we demonstrate that resistance mutations often exist prior to the start of therapy, at very small (CCF <0.01%) frequencies. Importantly, while other studies typically focus on a single treatment modality to identify resistance mutations through either a single snapshot in time or a comparison of the diagnostic sample to a relapse sample,[9,38] our study models itself on the designs of studies by Burger *et al.*[39] and Landau *et al.*,[40] who sequenced serial PB and BMA samples to recapitulate the trends of a growing number of clones at low allelic frequencies prior to relapse or progression, and as CLL disease responds to or persists through various targeted therapy pressures. However, our study is limited by this sequencing assay only targeting five key genes recognized in CLL resistance to targeted therapies. For example, while we observed multiple *BTK*, *BCL2*, and *TP53* mutations contract or expand differently with specific treatments, our study does not explore whether other genes co-mutate with these variants to confer a selective advantage.

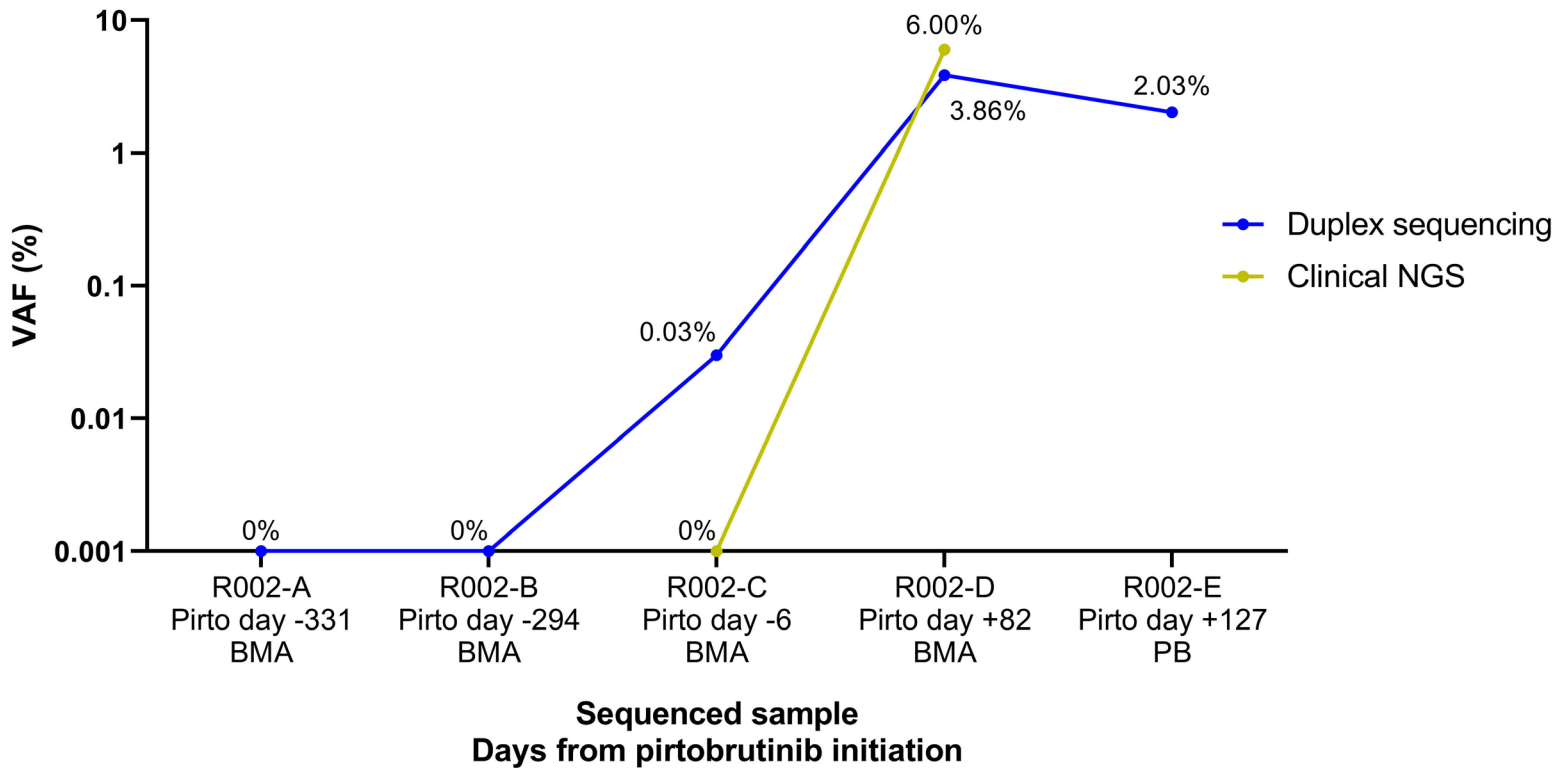

Figure 4. Detection of pre-existent *BTK* p.T474I (c.1421C>T) resistance mutation using duplex-sequencing *versus* clinical next-generation sequencing. Variant allele frequency (VAF) is indicated on the y-axis. In sample R002-C the resistance mutation *BTK* p.T474I (c.1421C>T) was identified by duplex-sequencing but not clinical next-generation sequencing and was identified with both methods in sample R002-D at a greater VAF. Samples with 0% VAF were assigned a surrogate value of 0.001% for purposes of presentation on a logarithmic scale. Clinical next-generation sequencing was not performed on samples at timepoints A, B, and E and could not be directly compared to Duplex-sequencing. NGS: next-generation sequencing; Pirto: pirtobrutinib; BMA: bone marrow aspirate; PB: peripheral blood.

Pirtobrutinib, a third-generation, non-covalent BTK inhibitor, was developed to target both wild-type CLL and CLL with *BTK* resistance mutations such as C481[20] and showed great promise with high overall response rates in initial trials.[41] However, both of our patients with post-pirtobrutinib relapse demonstrated increased counts of pathogenic variants and showed clonal expansion of resistance mutations in *PLCG2* and *BTK*, with persistent mutations in *TP53*. In particular, we found that the resistance mutation *BTK* L528W,[42] seen in both of our patients after treatment, was potentially a significant driver of pirtobrutinib resistance especially in patient R001. Both patients also carried persistent, low-level *BTK* C481 mutations during and after pirtobrutinib treatment. The observations of expanding *PLCG2* and *BTK* resistance mutations are supported by other groups who also noted resistance developing under treatment with pirtobrutinib.[43,44] Our study pioneers the use of duplex sequencing on serial CLL samples to detect clonal trends in resistance mutations at ultra-low levels, enabling the identification of allelic frequencies down to a VAF of 0.01%. This was most notably demonstrated in the case of the pre-existing resistance mutation *BTK* p.T474I, C.1421C>T, which was found at low levels (CCF 0.03%) in the pre-pirtobrutinib sample R002-C by duplex sequencing. In contrast, NGS detected 0% of this variant (*Online Supplementary Table S13*).

More recently, Naeem *et al.* reported, in primary CLL cells from patients treated with pirtobrutinib, that at progression, CLL cells showed increased BCR signaling and cell viability as well as accumulation of *TP53* mutations and second-site *BTK* mutations such as *BTK* L528W and *BTK* T474I.[44] Our study confirms these findings in longitudinally collected pirtobrutinib-resistant samples. In addition, we show that monitoring the clonal evolution of *TP53* in this manner might inform prognosis. For example, patient R001 carried a 17p deletion at a very high clonal frequency which served as a background for the emergence of a pathogenic *TP53* H178 mutant clone after acalabrutinib treatment, followed by its significant clonal expansion during pirtobrutinib-resistant disease. In concordance with others who have noted associations with shorter overall survival and alterations in *TP53*,[37] patient R001 also had a more aggressive clinical course and shorter survival than patient R002, who did not experience major *TP53* clonal expansions after pirtobrutinib relapse.

Examining the clonal evolution patterns observed in patient R002, the most remarkable finding was the expansion of *BAX* mutation p.E41Gfs*33, which was reported as a mechanism of resistance in CLL[45,46] and acute myeloid leukemia.[47] Mutations in *BAX* were previously described in CLL patients treated with venetoclax.[45,46,48,49] Interestingly, Blombery *et al.* reported that in CLL patients, loss of function *BAX* mutations occur in the myeloid compartment and are associated with clonal hematopoiesis.[45,50] In our study we could not discern with certainty the lineage of the cells harboring the mutation because the duplex sequencing was conducted with bulk sequencing. However, that this patient had a high disease burden in the three samples collected after acalabrutinib treatment, i.e., 95% (R002-C), 91% (R002-D), and 95% (R002-E) (*Online Supplementary Table S2*), and that this mutation was present with a high CCF in those samples (92.13%, 90.69% and 100%, respectively) indicates that this mutation occurred and expanded in neoplastic lymphocytes and persisted after pirtobrutinib treatment.

In summary, we present new data that illustrate the evolutionary dynamics of resistant clones in CLL and elucidate their underlying resistance mechanisms under pressure from targeted therapies. There is an important need for MRD monitoring in patients receiving targeted therapy for CLL to identify low VAF clones of resistance mutations and, as expanding clones are detected, the addition of a secondary therapy which acts via a different mechanism is recommended. We envision ultra-sensitive targeted NGS assays will become an established mechanism for monitoring resistance mutations in CLL and guide subsequent therapies based on mutational profiles.

Disclosures

CY and RAR are consultants and equity holders at TwinStrand Biosciences Inc. RAR is an equity holder at NanoString Technologies Inc. RAR is named inventor on patents owned by the University of Washington and licensed to TwinStrand Biosciences Inc.

Contributions

DWW curated clinical data, isolated and extracted raw materials, drafted, reviewed and revised the manuscript, and created figures. NDL analyzed data and drafted the manuscript. MS conceived and designed the study, identified the patients, participated in the enrollment of the patients into the study, and reviewed and revised the manuscript. EL performed experiments and contributed to data analysis and drafting the manuscript. XRT and JF performed experiments. BK analyzed data and created the figures. CU was the primary-site enrolling Principal Investigator and reviewed and revised the manuscript. AE and BT contributed samples and reviewed and revised the manuscript. MF analyzed data, reviewed clinical data, and reviewed and revised the manuscript. JR reviewed clinical data and reviewed and revised the manuscript. IB obtained funding for the study, developed data analysis methods, and drafted, reviewed and revised the manuscript. RAR conceived and designed the study, obtained funding for the study, supervised experiments, analyzed data, and drafted, reviewed and revised the manuscript. CCSY conceived and designed the study, obtained funding for the study, reviewed patients' charts and clinical data, and drafted, reviewed and revised the manuscript.

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Data-sharing statement

The Duplex-seq Pipeline is available at https://github.com/Kennedy-Lab-UW/Duplex-Seq-Pipeline. Sequencing data from this study have been submitted to the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject) under accession number (SRA number) SUB12404430.

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